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VIROLOGY

Virology 317 (2003) 197–207

www.elsevier.com/locate/yviro

Quasispecies variation of porcine reproductive and respiratory syndrome virus during natural infection☆

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Received 11 June 2003; returned to author for revision 17 July 2003; accepted 28 July 2003

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) displays notorious genetic, antigenic, and clinical variability. Little is known, however, about the nature and extent of viral variation present within naturally infected animals. By amplifying and cloning the open reading frame 5 gene from tonsils of naturally infected swine, and by sequencing individual clones, we characterized viral diversity in nine animals from two farms. All animals harbored multiple PRRSV variants at both the nucleic and the amino acid levels. Structural variation and rates of synonymous and nonsynonymous nucleotide substitution were no different within known epitopes than elsewhere. Analysis of molecular variance indicated that differences between farms, among animals within farms, and within individual animals accounted for 92.94, 3.84, and 3.22% of the total viral genetic variability observed, respectively. PRRSV exists during natural infection as a quasispecies distribution of related genotypes. Positive natural selection for immune evasiveness does not appear to maintain this diversity.

Keywords: PRRS virus; Arteriviridae; Quasispecies; Variation; Natural selection; Evolution

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of porcine reproductive and respiratory syndrome (PRRS), an economically damaging disease of domestic swine worldwide (Wensvoort et al., 1991; Benfield et al., 1992). PRRSV was identified as a member of the family *Arteriviridae* over a decade ago (Conzelmann et al., 1993; Meulenberg et al., 1993). Despite significant subsequent research, the virus has remained difficult to control. Vaccination, in particular, varies in its efficacy and often fails to reduce transmission or to alleviate clinical signs (Dewey et al., 1999; Mavromatis et al., 1999; Mengeling et al., 1999; Dee et al., 2000; Halbur et al., 2000; Meng, 2000; Nodelijk et al., 2001). Current control strategies include such costly solutions as testing and removing positive animals or depopulating and repopulating herds (Dee and Joo, 1997; Dee et al., 1997, 2001a).

PRRSV infection ranges in severity from subclinical to lethal and affects swine at both the growth and the reproductive stages (Zimmerman et al., 1997; Mengeling et al., 2000; Nodelijk, 2002). This remarkable clinical variability poses a serious barrier to diagnostics, control, and biocontainment. Unfortunately, the root causes of such variability are poorly understood. It could result from factors operating at the level of the host, the pathogen, and/or the environment.

One likely underlying cause for the clinical variability in PRRS is the biological variability of the virus itself. European varieties of PRRSV differ substantially from North American varieties genetically and antigenically, implying a high degree of evolutionary divergence between continents (Allende et al., 1999; Nelsen et al., 1999; Forsberg et al., 2001). Within both Europe and North America, PRRSV exists as a diverse array of genotypes, displaying considerable antigenic and clinical heterogeneity but little geographically relevant population substructure (Dea et al., 1996; Halbur et al., 1996; Kapur et al., 1996; Mengeling et al.,

☆ Sequence data from this article have been deposited with the GenBank Data Library under the Accession Numbers AY318766–AY318774.

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doi:10.1016/j.virol.2003.07.009

1996; Le Gall et al., 1997; Goldberg et al., 2000; Meng, 2000; Stadejek et al., 2002; Mateu et al., 2003). PRRSV can vary genetically even within individual herds of pigs (Goldberg et al., 2000; Dee et al., 2001b). Laboratory investigations have shown that PRRSV mutates quickly in pigs (Rowland et al., 1999; Chang et al., 2002), and that multiple variants can coexist in experimentally infected animals (Chang et al., 2002).

PRRSV has a short (approximately 15 kb) single stranded, positive-sense RNA genome (Benfield et al., 1992; Conzelmann et al., 1993; Meulenberg et al., 1997). Small RNA viruses such as PRRSV are thought to exist in nature as distributions of related genotypes know as "quasispecies" (Eigen and Schuster, 1979; Domingo et al., 1996; Domingo et al., 2002). The evolutionary plasticity of such distributions could account for the notorious ability of RNA viruses to evade host immunity and to evolve novel clinical manifestations (Domingo et al., 1997). Such effects have been demonstrated for certain RNA viruses of humans, most notably human immunodeficiency virus (HIV) (Essajee et al., 2000; Paolucci et al., 2001; Nowak et al., 2002) and hepatitis C virus (HCV) (Farci and Purcell, 2000; Farci et al., 2002; Pawlotsky, 2003). Understanding whether PRRSV behaves similarly would be critical for designing rational and realistic strategies to limit its clinical effects.

We hypothesize that PRRSV exists during natural infection as a quasispecies distribution of related genotypes and that this variation could account for the inability of traditional approaches such as vaccination to control PRRS adequately. To test this hypothesis, we sampled naturally infected animals in the field and characterized viral genetic variability both within farms and within individual animals on those farms. We also hypothesize that host immunity sustains the genetic variability of PRRSV by selecting for viral variants with novel antigenic properties. To test this hypothesis, we examined whether gene regions coding for immunogenic epitopes differed from gene regions not coding for immunogenic epitopes in their genetic variation and rates of molecular selection within naturally infected animals. This article provides the first data on the nature and extent of within-animal quasispecies-level genomic variability in PRRSV during natural infection. The results are pertinent to the control of PRRS in the field, as well as to the biology and control of positive-sense RNA viruses in general.

Results

Blood and tonsil biopsies were collected from 68 pigs on two farms (Farm A and Farm B). ELISA sample:positive (S:P) ratios ranged from 0.9 to 2.6 for the 31 pigs from Farm A, and from 0.2 to 3.3 for the 37 pigs from Farm B, with seroprevalence rates (S:P ratios $\geq$ 0.4) of 100 and 81%, respectively. All biopsy samples were histologically confirmed to contain tonsillar tissue. PRRSV genomic RNAs were detected by reverse-transcription PCR in the tonsils of seven pigs from Farm A (22.6%) and two pigs from Farm B (5.4%).

The complete open reading frame 5 (ORF5) gene (603 bases) was sequenced directly in all nine PCR-positive animals (A1–A7, and B1–B2). Comparison of the ORF5 sequences generated from Farms A and B to published sequences indicated that PRRSV variants recovered from both farms were highly divergent wild-type North American viruses and not vaccine revertants. PCR products were also cloned into plasmid vectors, and individual clones were sequenced to measure within-animal viral genetic variability. Twelve clones from each sample were successfully sequenced in this manner.

Reverse-transcription, PCR, and DNA sequencing are error-prone, and associated errors can obscure patterns of RNA viral variability (Bracho et al., 1998; Malet et al., 2003). We therefore deemed it necessary to measure the influence of random errors generated during RT-PCR and/or sequencing on our results. To estimate this error empirically, we propagated a field strain of PRRSV in vitro, purified it to the level of a single clone using a method of serial limiting dilution, and measured genetic variability within this sample using protocols identical to those applied to field samples. Twelve additional sequences were generated from this limiting dilution (LD) sample, for a total of 78.39 kb of sequence data in this study.

From the data generated, three categories of sequences were defined. The first category, "modal sequences," consisted of sequences generated directly from PCR products, without cloning. These sequences represent the modal sequence of the population of molecules within an individual sample. The second category, "clone sequences," consisted of the 12 sequences of individual clones generated for each sample. The third category, "consensus sequences," consisted of the consensus of all clone sequences within a sample, defined for each nucleotide position of ORF5 as that base represented by the greatest proportion of individual clone sequences.

No two modal sequences in the study were identical at the nucleic acid level. At the amino acid sequence level, the modal sequences from Farm A represented four distinct amino acid sequences, and both modal sequences from Farm B were different. The mean pairwise difference among modal sequences from Farm A was 2.85/603 nucleotide positions and 1.43/201 amino acid positions, and the pairwise difference between the two modal sequences from Farm B was 5/603 of nucleotide positions and 4/201 amino acid positions. The mean pairwise difference among all nine modal sequences from both farms was 27.17/603 nucleotide positions and 9.94/201 amino acid positions, which is comparable to the mean pairwise difference among a larger set of 55 previously published sequences from Illinois and surrounding states (39.26/603 nucleotide positions and 15.72/201 amino acid positions; Goldberg et al., 2000),

Table 1
Descriptive statistics on quasispecies variation in PRRS virus

ID[a]	n[b]	h[c]	con=mod?[d]	clone≠con[e]	clone≠mod[f]	Positions[g]	s[h]	d[i]	Range d[j]
				Nucleic acids					
A1	12	8	Yes	7	7	603	9	1.50	0–4
A2	12	10	Yes	8	8	603	14	2.61	0–7
A3	12	11	Yes	10	10	603	13	2.44	0–4
A4	12	6	Yes	6	6	603	7	1.30	0–4
A5	12	7	No (1)	6	11	603	6	1.24	0–3
A6	12	9	Yes	8	8	603	8	1.65	0–3
A7	12	9	Yes	9	9	603	16	3.03	0–8
B1	12	9	Yes	8	8	603	17	2.97	0–8
B2	12	9	Yes	8	8	603	14	2.47	0–5
LD	12	4	Yes	3	3	603	6	1.00	0–4
				Amino acids					
A1	12	5	Yes	5	5	201	6	1.00	0–3
A2	12	8	No (1)	7	12	201	10	1.80	0–4
A3	12	7	No (1)	7	12	201	6	1.14	0–2
A4	12	5	Yes	5	5	201	5	0.97	0–3
A5	12	5	No (1)	5	10	201	4	0.91	0–3
A6	12	8	Yes	7	7	201	7	1.48	0–3
A7	12	8	Yes	9	9	201	11	2.20	0–5
B1	12	7	Yes	7	7	201	12	2.00	0–5
B2	12	8	Yes	6	6	201	8	1.47	0–4
LD	12	3	Yes	2	2	201	2	0.33	0–2

[a] ID = sample identifier.
[b] n = number of sequences.
[c] h = number of haplotypes (unique clone sequences).
[d] Con=mod? = is the consensus sequence identical to the modal sequence? (If no, then number of differing positions).
[e] Clone≠con = number of clone haplotypes that are different from the consensus sequence.
[f] Clone≠mod = number of clone haplotypes that are different from the modal sequence.
[g] Positions = number of positions (nucleotide or amino acids) per sequence.
[h] s = number of segregating sites (variable positions).
[i] d = mean pairwise differences between all clone sequences within a sample (units = nucleic or amino acid positions).
[j] Range d = range of pairwise differences between all clone sequences within a sample (units = nucleic or amino acids positions).

indicating that the viral diversity sampled in this study is geographically representative.

Examination of clone sequences revealed that all animals in the study harbored multiple variants of PRRSV at both the nucleic acid and the amino acid levels. Descriptive statistics on the genetic variation of clone sequences within individual animals are given in Table 1. Clone sequences differed by an average of 2.13/603 nucleotide positions and 1.44/201 amino acid positions within individual animals. The most divergent variants recovered from any animal differed by eight nucleic acid positions and four amino acid positions. Diversity within the LD sample was lower by all measures than that within any field sample.

In most cases, the consensus sequence for a sample was identical to the modal sequence for that same sample (Table 1). Where this was not the case, the consensus sequence differed from the modal sequence by only a single position (nucleotide or amino acid). The overall congruity of the modal and consensus sequences indicates that sequencing 12 clones per sample provided an adequate representation of overall genetic variability within each sample. Most clone sequences differed by at least one position (nucleotide or amino acid) from the consensus sequence, indicating that the consensus sequence was (paradoxically) not highly represented among individual PRRSV genomes within an animal.

The 108 clone sequences generated from field samples represented a total of 73 haplotypes (unique sequences) at the nucleic acid level and 54 haplotypes at the amino acid level. Fig. 1 depicts relatedness among the haplotypes at the nucleic acid level as a phylogenetic tree. The tree was constructed using the neighbor joining algorithm of Saitou and Nei (1987), implemented with the computer program PAUP* (Swofford, 2000). Pairwise distances between sequences were corrected for multiple substitutions using a maximum likelihood distance correction in which the transition/transversion ratio was set to 11.72, the nucleotide frequencies (A, C, G, and T, respectively) were set to 0.193, 0.260, 0.267, and 0.278, and in which the distribution of rates at variable sites was described using a γ-distribution with shape parameter $\alpha = 1.380$, with no sites assumed invariant. These parameter values were estimated from the data using an iterative likelihood ratio approach implemented using the computer program Modeltest, Version 3.06 (Posada and Crandall, 1998).

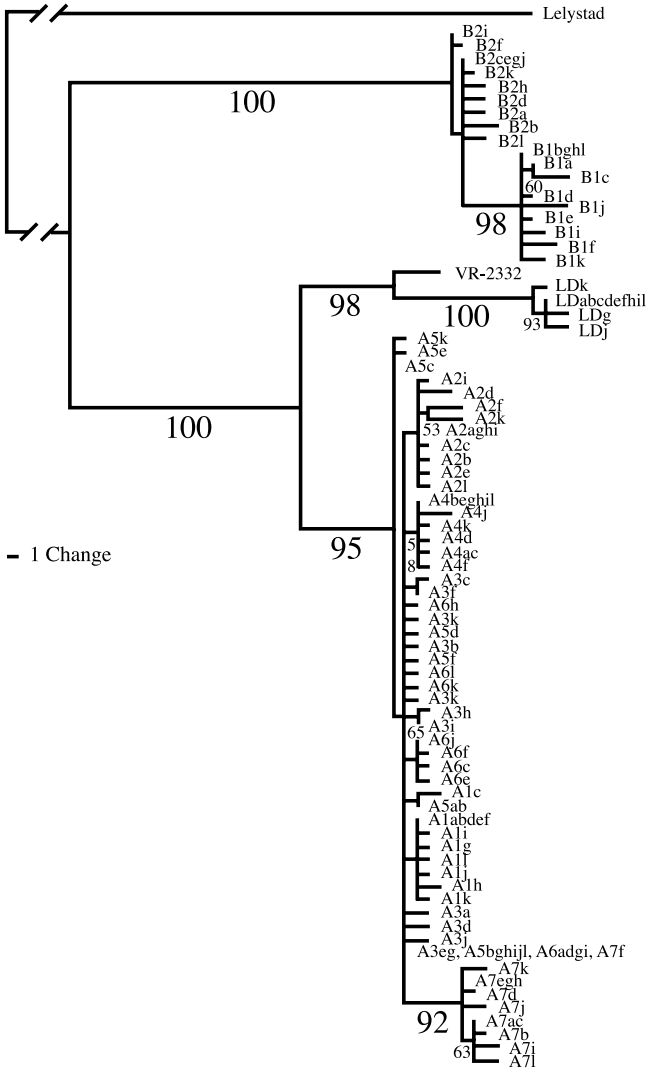

Fig. 1. Phylogenetic tree of PRRSV ORF5 sequences. The viral ORF5 gene was amplified and cloned, and individual clones were sequenced (12 clones per sample) directly from tonsil biopsies of infected swine on two farms. Taxon names refer to the farm (A or B), animal (1–7), and clone (a–l). LD refers to sequences generated from the limiting dilution experiment (see text for full explanation). VR-2332, the U.S. reference strain (GenBank Accession No. U87392) is also included for comparison. The tree is rooted using the European reference strain (Lelystad; GenBank Accession No. M96262). Numbers below branches are bootstrap values (%) obtained from 1000 bootstrap replicates of the data. Only bootstrap values ≥50% are shown.

The phylogenetic tree in Fig. 1 demonstrates that PRRSV variants from Farm A are genetically distinct from those of Farm B. PRRSV variants from both farms are genetically distinct from either the VR-2332 vaccine variant or the LD variant. Within Farm B, clone sequences from animal B1 form a monophyletic group, distinct from clone sequences from animal B2. Within Farm A, clone sequences from animals A2 and A4 form a monophyletic group, but clone sequences from all other animals are paraphyletic.

Individual animals harbored an average of 8.7 nucleic acid haplotypes and 6.8 amino acid haplotypes per 12 clone sequences (see Table 1). The number of nucleic acid haplotypes (4) and amino acid haplotypes (3) in the LD sample was lower than that in any field sample. The structure of within-sample genetic diversity is depicted graphically in Fig. 2 as a set of "mismatch distributions," or histograms of pairwise genetic differences ("mismatches") among sequences within a sample (Rogers and Harpending, 1992). The nine field samples differ considerably in the nature and extent of genetic variation present, at both the amino acid and nucleic acid levels. The structure of genetic variation within the LD sample clearly differs from that of any field sample, with most pairs of sequences differing by zero or one mismatches.

Fig. 3 shows the distribution of structural (amino acid level) variation along the ORF5 gene within individual samples. Although field samples differ considerably in the positions at which their clone sequences vary, no universal hypervariable regions are apparent. Structural variation among clone sequences within the LD sample is restricted to two amino acid positions and is clearly lower than that within any field sample.

Previous studies have mapped the ORF5 gene product antigenically and have identified three linear epitopes (Rodriguez et al., 2001; Ostrowski et al., 2002; Plagemann et al., 2002). These are located at amino acid positions 27–30 (nonneutralizing), 37–45 (neutralizing), and 170–201 (nonneutralizing) and are depicted as thickened lines along the horizontal axis of Fig. 3. Table 2 presents data on quasi-

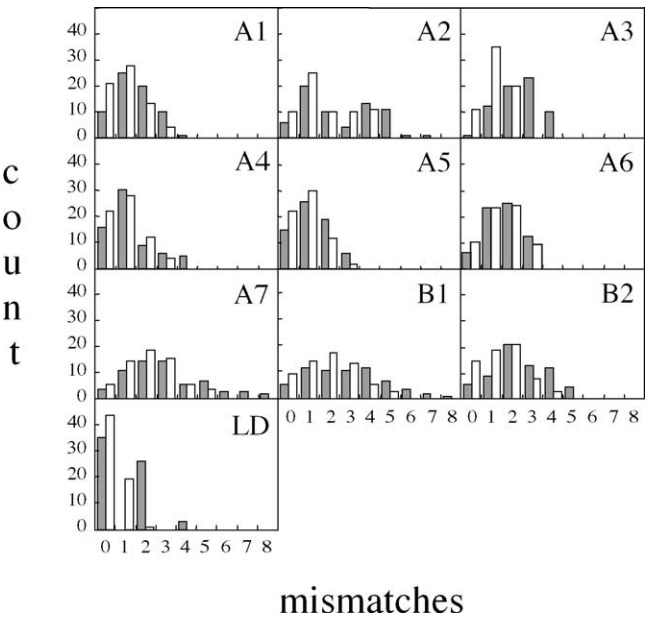

Fig. 2. Mismatch distributions of PRRSV ORF5 sequences. The ORF5 gene was amplified and cloned, and individual clones were sequenced (12 clones per sample) directly from tonsil biopsies of infected swine on two farms (A and B). Individual graphs are histograms of pairwise genetic differences (mismatches, measured as the raw number of different positions) among pairs of sequences within an animal (A1–B2) or sample (LD, the limiting dilution sample). Open bars represent nucleic acid mismatches, and shaded bars represent amino acid mismatches.

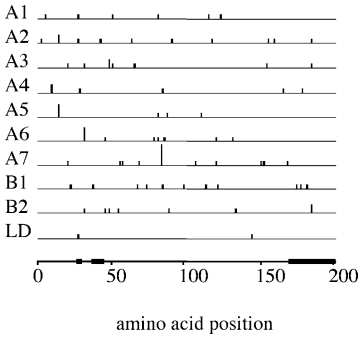

Fig. 3. Quasispecies-level structural variation of PRRSV within naturally infected animals. The ORF5 gene was amplified and cloned, and individual clones were sequenced (12 clones per sample) directly from tonsil biopsies of infected swine on two farms (A and B). The locations of variable amino acids along the ORF5 gene are represented for each animal (A1–B2) or sample (LD, the limiting dilution sample) as vertical bars. Bars are proportional in height to the number of sequences (of 12 in all cases) differing from the consensus sequence at that position. Heights of bars range from 1 to 5 amino acid differences. Thick lines along the horizontal axis indicate the positions along ORF5 of known linear epitopes.

species-level genetic variation within samples separately for epitopic regions and nonepitopic regions, as well as for the ORF5 gene as a whole. All three epitopic regions were combined in this analysis because results from the individual regions did not differ.

Genetic diversity within field samples (measured as nucleotide diversity; Nei, 1987) did not differ significantly between epitopic and nonepitopic regions (paired Student's t test; $t = 0.56$; $P = 0.59$; 8 d.f.). The strength of natural selection on different gene regions was measured as the ratio of synonymous substitutions per synonymous site to non synonymous substitutions per nonsynonymous site (d_s/d_n), averaged across all pairs of haplotypes within a sample (Nei and Gojobori, 1986). In four cases, this ratio was undefined in the epitopic regions because no nonsynonymous substitutions were observed (Table 2). Considering only the remaining five cases, no significant difference existed in d_s/d_n between the epitopic and nonepitopic regions (paired Student's t test; $t = 1.03$; $P = 0.36$; 4 d.f.). In both epitopic and nonepitopic regions, however, the average value of d_s/d_n was greater than 1, indicating a predominance of synonymous substitutions and an overall pattern of stabilizing selection within ORF5.

Table 3 presents the results of an analysis of molecular variance (AMOVA) (Excoffier et al., 1992) conducted on clone sequences from the field samples in this study using the computer program Arlequin (Schneder et al., 2000). AMOVA partitions genetic variation into hierarchical components and thus quantifies population genetic substructure. The majority of the total genetic variation present among clone sequences in this study (92.94%) is accounted for by differences between Farm A and Farm B. A smaller, but nevertheless statistically significant, proportion of the total genetic variation (3.84%) is accounted for by differences among animals within farms. Genetic diversity among clone sequences within individual animals accounts 3.22% of the total genetic variation observed and is also statistically significant.

Discussion

The results of this study demonstrate that multiple variants of PRRSV can exist simultaneously on farms, and within individual animals, during natural infection. Variants

Table 2
Quasispecies variation and selection in epitopic and nonepitopic regions of the PRRSv ORF 5 gene

ID[b]	n[c]	Epitopic regions[a]					Nonepitopic regions[a]					Whole gene[a]				
		h[d]	pos[e]	π[f]	s[g]	d_s/d_n[h]	h[d]	pos[e]	π[f]	s[g]	d_s/d_n[h]	h[d]	pos[e]	π[f]	s[g]	d_s/d_n[h]
A1	12	2	135	1.23	1	0.00	8	468	2.85	8	3.43	8	603	2.49	9	3.05
A2	12	4	135	4.94	4	1.98	7	468	4.14	10	2.58	9	603	4.32	14	3.45
A3	12	3	135	3.48	2	N/A	10	468	4.21	11	4.29	11	603	4.05	13	4.69
A4	12	3	135	3.70	3	2.23	5	468	1.72	4	3.43	6	603	2.16	7	2.27
A5	12	1	135	0.00	0	N/A	7	468	2.65	6	3.24	7	603	2.06	6	3.13
A6	12	1	135	0.00	0	N/A	9	468	3.53	8	2.69	10	603	2.74	8	2.41
A7	12	2	135	1.23	1	N/A	9	468	6.12	15	3.63	9	603	5.03	16	2.59
B1	12	5	135	8.64	7	3.27	7	468	3.85	10	2.10	9	603	4.92	17	2.48
B2	12	4	135	4.71	3	3.13	8	468	3.92	11	3.03	9	603	4.10	14	4.07
LD	12	2	135	1.23	1	0.00	4	468	1.78	5	6.86	4	603	1.66	6	5.95

[a] Epitopic regions include nucleotide positions 79–90, 109–135, and 508–603. All other positions were considered nonepitopic.
[b] ID = sample identifier (LD = limiting dilution sample).
[c] n = number of sequences.
[d] h = number of haplotypes (unique sequences).
[e] pos = number of nucleotide positions.
[f] π = nucleotide diversity, or mean proportion of pairwise nucleotide sequence differences (multiplied by 1000).
[g] s = number of segregating sites (variable positions).
[h] d_s/d_n = ratio of the proportion of synonymous substitutions per synonymous site to nonsynonymous substitutions per nonsynonymous site, averaged over all pairs of haplotypes within a sample. N/A refers to cases where $d_n = 0$, making d_s/d_n undefined.

Table 3
Analysis of molecular variance (AMOVA) of quasispecies diversity for PRRSv ORF5 sequences from two farms

Variance component	Observed partition					P[a]	ϕ-statistics
		d.f.	Sum of squares	Variance	% total variance		
Among farms	ϕ_a^2	1	1167.27	30.83	92.94	0.025	$\phi_{CT} = 0.544$
Among animals within farms	ϕ_b^2	7	114.61	1.28	3.84	<0.001	$\phi_{SC} = 0.968$
Within animals	ϕ_c^2	99	105.67	1.07	3.22	<0.001	$\phi_{ST} = 0.929$

[a] Probability of obtaining a more extreme variance component and ϕ-statistic than the observed values by chance alone, calculated by 1000 random permutations of the data according to the method of Excoffier et al. (1992), implemented using the computer program ARLEQUIN (Schneder et al., 2000).

of PRRSV existed at both the genetic (nucleic acid) and structural (amino acid) levels. Variation among modal sequences (directly sequenced PCR amplicons) from different animals on a farm was considerable, with no two animals on any farm sharing an identical modal ORF5 nucleic acid sequence. Variation within animals was also considerable; few clone sequences within any animal were identical. Individual animals varied widely in the diversity of clone sequences that they harbored.

The extent of variation observed within animals in this study cannot be explained by random errors generated during PCR or sequencing. Diversity in the LD sample was lower by all measures than was diversity in the nine field samples. Variability in the LD sample reflects the combined error associated with (1) replication of the virus from a single infectious unit in a single tissue culture well, (2) reverse transcription, (3) nested PCR, and (4) DNA sequencing. The limiting dilution experiment therefore yields an "upper end" estimate of the variability that could be attributed to random error in this study. The greater level of viral genetic diversity observed in all field samples must therefore reflect the presence of multiple viral variants within the tonsillar tissue of the animals at the time of sampling.

The nature of the viral genetic variation observed within individual animals in this study justifies the use of the descriptor quasispecies. A hallmark feature of quasispecies variation is that few, if any, individual sequences within a "mutant swarm" exactly match the average, or consensus, sequence (Domingo et al., 1996; Domingo, 2002). As Table 1 demonstrates, few clone sequences exactly matched their respective consensus or modal sequence. Wild-type PRRSV in naturally infected swine therefore exists as a quasispecies distribution of related but distinct viral variants.

We believe that quasispecies variation in PRRSV is a general phenomenon, and that its presence should be expected wherever PRRSV occurs. All nine animals examined in this study were found to harbor genetic and structural variants of PRRSV. This occurred even though the two farms sampled in this study had highly divergent North American variants of PRRSV. Quasispecies variation in PRRSV therefore appears to occur independently of viral genotype or host population.

Less clear than the existence of quasispecies variation in PRRSV is its underlying cause. Quasispecies variation within individual animals could result either from viral mutations occurring within the animal itself or from transmissions of exogenous viral variants from other animals. The phylogenetic tree shown in Fig. 1 bears on this issue. Complete monophyly of clone sequences from individual animals would be expected in the absence of between-animal transmission. However, clone sequences from only three of nine animals form monophyletic groups on this tree. Other clades on the tree consist of intermingled sequences from two or more animals. Many animals share identical haplotypes. We interpret this pattern as evidence that between-animal transmission of PRRSV does occur and that it does contribute to the quasispecies variation observed within animals. Nevertheless, many separate clone sequences from the same animal appear closely related, and distinct from clone sequences from other animals. We interpret this pattern as evidence that within-animal mutation also occurs and that it too contributes to the quasispecies variation observed within animals. In other words, our phylogenetic analysis provides evidence that both phenomena—within-animal mutation and between-animal transmission—contribute to the generation and maintenance of quasispecies diversity within individual animals.

Individual animals were not uniform in the number or diversity of distinct viral variants that they harbored. Differences among animals in these measures could reflect differences in the duration of infection or the number of horizontally transmitted exogenous viral variants to which they were exposed. If so, then quasispecies diversity within animals may be epidemiologically informative. Measures of viral diversity within animals could be used to reconstruct the timing of infection or the frequency of horizontal viral transmission.

In this study, for example, modal sequences from Farm B were approximately twice as diverse genetically as modal sequences from Farm A. Phylogenetically, clone sequences from Farm B animals tended to cluster into monophyletic groups to a greater degree than did clone sequences from Farm A animals. These results would be consistent with a higher frequency of between-animal viral transmission on Farm A than on Farm B, and/or a longer duration of infection for animals from Farm B. This interpretation is consistent with the clinical history of animals from the two farms. All animals sampled from Farm A were housed in a single, large building, whereas animals sampled from Farm B were housed in several smaller buildings. The potential for horizontal viral transmission was therefore greater for animals

from Farm A than for animals from Farm B. Also, animals from Farm A had entered the farm between 4 and 8 weeks prior to sampling, whereas animals on Farm B had been born on the farm between 7 and 11 weeks prior to sampling. Durations of infection were therefore potentially longer for animals from Farm B than for animals from Farm A.

We do not find evidence of any effect of positive natural selection for immune evasiveness in maintaining quasispecies variation within the animals sampled. Structural variation in ORF5 appeared to be randomly distributed across the length of the gene (Fig. 3). Variation was not concentrated in any of the known linear epitopes of ORF5. Relative rates of synonymous and nonsynonymous nucleic acid substitution did not differ between epitopic and nonepitopic regions of ORF5. In the animals sampled, therefore, immune selection pressure does not appear to drive the diversification of PRRSV by selecting for variants that can evade neutralization. On the contrary, the fact that d_s/d_n ratios were uniformly greater than 1 suggests that the overall pattern of evolution of the PRRSV quasispecies within animals is dominated by stabilizing selection (Nei, 1987). We conclude that, in the animals sampled in this study, quasispecies diversity is generated by random viral mutation and is limited by stabilizing selection.

We cannot rule out the possibility that the evolution of other immunogenic regions of ORF5 may be driven by positive selection. Epitopes important for cell-mediated immunity may, for example, behave in such a fashion. Furthermore, we did not consider in this study the immune status of the particular animals sampled. Future studies quantifying the relationship between host immunity and quasispecies variation in PRRSV are clearly warranted.

The results of this study and their associated interpretations are limited to the ORF5 gene. We chose to examine this gene because of its high variability and known immunogenicity. Other loci within the PRRSV genome would almost certainly display different (probably lower) levels of quasispecies variability (Chang et al., 2002). The molecular evolution of other loci could also be driven by positive selection in ways that ORF5 does not appear to be. Studies examining the nature and extent of quasispecies variation in genes coding for the other structural proteins of PRRSV are also warranted.

A further limitation of this study is that our techniques did not allow us to determine which viral clone sequences represented infectious viral particles, and which did not. We chose to amplify viral RNA directly from tissue to maximize viral detection (Wills et al., 2003) and to avoid biases associated with virus isolation. Virus isolation could, in and of itself, select for a nonrepresentative subset of viral variants (those adaptable to growth in tissue culture), thus leading to systematic underestimations of quasispecies diversity. Nevertheless, none of the 120 clone sequences generated in this study were found to contain premature stop codons, despite significant numbers of nonsynonymous substitutions throughout. This observation, and the fact that PRRSV can freely recombine, makes it likely that the sequences we have examined are relevant to the replication and transmission of the virus.

Our analysis of molecular variance demonstrated that differences among clone sequences within animals accounted for only 3.22% of the total genetic variation observed. This level of variation was nevertheless comparable to that observed among animals within farms (3.84%). This finding was surprising, because it implies that an adequate estimate of the genetic variability of PRRSV on a farm could, in theory, be made by sampling viral variants within a single animal from that farm. This may hold true, however, only for populations in which animals can freely exchange viral variants through horizontal transmission.

The amount of genetic variation within individual animals in this study was, in an absolute sense, small. This amount of variation was statistically significant, however, and we argue that it is also biologically significant. The variation observed in PRRSV at all geographic levels (from interanimal to intercontinental) must originate from small mutations occurring within individual animals. In an ultimate sense, therefore, the quasispecies variation observed within individual animals in this study is of greater biological significance for understanding the genesis of variability in PRRS than is variation at any other level.

In other RNA viral systems, the nature and extent of quasispecies variation is a reliable indicator of the course of clinical disease (Essajee et al., 2000; Farci and Purcell, 2000; Farci et al., 2000; Paolucci et al., 2001). Studies examining the relationship between quasispecies variation in PRRSV and the course of clinical disease would be particularly interesting. So too would be studies elucidating the factors that determine how and why animals differ in the diversity of PRRSV variants that they harbor, particularly as such differences pertain to the duration of infection and the epidemiology of horizontal transmission on farms.

Finally, this study has implications for the control of PRRS in the field. If, as we have demonstrated, every animal on a farm harbors genetically and structurally divergent viral variants, then every animal must be considered a risk for the emergence of viruses with novel clinical and/or antigenic properties. Limiting contact of infectious animals within and between herds should, in this light, be considered of paramount importance to the control and biocontainment of PRRS. Any effective strategy for managing PRRSV (or other similar RNA viruses) must embrace the inherent variation of the pathogen, understand its root causes and consequences, and offer rational solutions accordingly.

Materials and methods

Field sampling

Clinical samples were collected in 2002 from swine on two farms in Illinois with histories of long-term (over 3

years), chronic reproductive losses attributable to PRRS. Farm A was a 2400-sow, single-site, breed-to-wean facility. Farm B was an 800-sow, single-site, farrow-to-finish facility. Biosecurity measures were in place on both farms to guard against the introduction of PRRSV from exogenous sources.

A cross-section of growing female replacement breeding swine was sampled on each farm (31 individuals on Farm A and 37 individuals on Farm B). Swine were between 4 and 30 weeks of age and weighed between approximately 10 and 150 kg. Swine on Farm A had been introduced into a single large building between 4 and 8 weeks prior to sampling from a PRRS-negative source herd. Swine on Farm B had been born on the farm and were sampled between 7 and 11 weeks of age. Swine were sampled evenly with respect to size and age and randomly with respect to their physical locations within buildings and pens.

Two clinical samples were collected from each individual animal: blood and a tonsil biopsy. Animals were restrained for sample collection in pens using hand-held cable snares. Blood was collected into sterile 5-ml vacuum blood collection tubes using standard venipuncture techniques with a 20-G, 1.5-in needle. Tonsil biopsies were collected using a novel method involving local analgesia and individually sterilized punch biopsy instruments. This technique was chosen because of the predilection for PRRSV to persist in tonsillar tissue (Beyer et al., 2000; Christopher-Hennings et al., 2001; Horter et al., 2002; Wills et al., 2003) and is fully described elsewhere (Lowe et al., 2003).

Serum was separated from blood via centrifugation and submitted for PRRSV testing using a commercial ELISA kit (HerdCheck Elisa Kit, Idexx, Inc.) at the University of Illinois Veterinary Diagnostic Laboratory. Tonsil biopsies were placed in RNAlater stabilizing buffer (Ambion, Inc.) in the field. Sections of tonsil tissue were subsequently fixed in 10% neutral buffered formalin for histologic confirmation.

In vitro methods

A previously characterized field isolate of PRRSV (GenBank Accession No. AF176446; Goldberg et al., 2000) was chosen for an empirical estimation of the contribution of reverse-transcription PCR and sequencing related error to the measurement of quasispecies variability in this study. Tenfold serial dilutions of this viral isolate were inoculated onto 12-well rows of 96-well tissue culture plates containing confluent monolayers of MARC-145 cells in Eagle's minimum essential medium with Earl's salts, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 50 μg/ml gentamycin sulfate, and incubated at 37°C in the presence of 5% CO_2 for 5 days or until maximum cytopathic effect was observed. When a single positive well was observed in the row containing the highest dilution, the supernatant from this well was harvested, diluted serially again, and inoculated onto another 96-well plate as described above. This process was repeated three times in succession, to ensure that the final supernatant contained viral particles derived from only a single infectious unit. Genetic variability was measured in this limiting dilution sample as described below for clinical samples. This measure provided an "upper-end estimate" of error in this study, in that it reflected the combined errors associated with growth of the isolate in tissue culture, reverse transcription, nested PCR, and DNA sequencing.

RNA isolation and RT-PCR

Tonsil biopsy sections were homogenized using a rotor-stator homogenizer, and total RNA was extracted from homogenates using a commercial kit (RNeasy Kit, Qiagen, Inc). RNA was extracted from cell-culture supernatants using a commercial kit (Viral RNA Mini Kit, Qiagen, Inc.).

The viral gene chosen for study was ORF5. The ORF5 gene has been well characterized among field isolates and has become the "industry standard" for measuring genetic variability in PRRSV. ORF5 varies within and between farms and mutates rapidly during serial passage in pigs (Kapur et al., 1996; Rowland et al., 1999; Goldberg et al., 2000; Dee et al., 2001b; Chang et al., 2002). The ORF5 gene product is highly antigenic, and highly antigenically variable among field isolates (Dea et al., 1996, 2000; Pirzadeh and Dea, 1998; Pirzadeh et al., 1998; Yoon et al., 1997). The ORF5 protein contains known, mapped neutralizing and nonneutralizing epitopes (Pirzadeh and Dea, 1997; Weiland et al., 1999; Yang et al., 2000; Rodriguez et al., 2001; Ostrowski et al., 2002).

ORF5 was amplified from extracted RNA using a novel "heminested" RT-PCR, incorporating an internal forward primer that overlaps partially with the external forward primer (the two reverse primers do not overlap). Primers 4FEd (5′-ATGTGCGRYTGCTYCATTTCATGACACC-3′), 4FId (5′-TCATGACACCTGARRCCATGAGGTGGGC-3′), 6REd (5′-AGGTGCARAAGCCCYAGCAGTCGGCCGC-3′), and 6RId (5′-GCCAADAKCACCTTTTSTGGAGSCGTGC-3′) were designed to anneal to conserved genomic regions in ORFs 4 and 6 and to amplify a 746-bp region containing the complete North American ORF5 gene sequence. Incorporation of degenerate bases maximized the ability of the PCR to amplify genetically divergent PRRSV variants. The external primer set (4FEd and 6REd) successfully amplifies ORF5 in both the European and the North American varieties of PRRSV, and the internal primer set (4FId and 6RId) is selective for North American PRRSV, but amplifies even the most genetically divergent isolates within the North American subtype. Preliminary data (not shown) indicate that this PCR is as sensitive as commercially available diagnostic PCRs for PRRSV. Nevertheless, we acknowledge that limitations to the sensitivity of this PCR exist. Reliance on this PCR for identification of positive animals

in this study may have led to underrepresentations of animals with low viral loads and/or long durations of infection.

RT-PCR was performed in a "one-tube system" (RobusT I RT-PCR Kit; Finnzymes, OY), which incorporates a proofreading DNA polymerase to reduce PCR-associated error (Malet et al., 2003). Reactions contained 1× RobusT Reaction Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 $\mu g/\mu l$ bovine serum albumin, 2.5 units AMV reverse transcriptase, 0.5 units DyNAzyme EXT DNA polymerase, 20 units RNAse Out RNA inhibitor (Invitrogen, Inc.), 5 pmol of primers 4FEd and 6REd, and 10–20 ng of template RNA in a 25 μl volume. Reaction conditions consisted of an initial reverse transcription step at 42°C for 30 min, then an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation (94°C for 30 s, annealing (65°C for 30 s, and extension (72°C for 1 min), and a final extension step at 72°C for 4 min. Second-round PCRs contained 50 mM Tris–HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTPs, 0.8 $\mu g/\mu l$ bovine serum albumin, 0.5 units DyNAzyme EXT DNA polymerase, 5 pmol of primers 4FId and 6RId, and 5 μl of the first-round reaction in a 25 μl volume. Reaction conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation (94°C for 30 s, annealing (65°C for 30 s), and extension (72°C for 1 min), and a final extension step at 72°C for 4 min. Appropriate negative control reactions were performed at all stages to detect potential cross-contamination.

PCR products were visualized by gel electrophoresis in 1% agarose stained with ethidium bromide under ultraviolet illumination. Amplicons were excised from gels using sterile razor blades and were purified from the agarose matrix using a commercial kit (Zymoclean Gel DNA Recovery Kit, Zymo Research, Inc.).

Cloning and plasmid purification

To measure within-animal genetic variation in PRRSV, PCR amplicons were cloned into plasmid vectors and individual inserts were sequenced. Because PCR products were blunt-ended (a proofreading DNA polymerase was used to minimize PCR errors; Malet et al., 2003), amplicons were first subjected to an "A-tailing" procedure in which adenine overhangs were added to blunt 5′ ends to facilitate TA cloning. A-tailing was performed in a 10 μl reaction volume containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dATP, 5 units Taq DNA polymerase, and 5 μl of gel-purified PCR amplicon, incubated at 70°C for 25 min. A-tailed PCR products were cloned into plasmid vectors for subsequent transformation of electroporated DH5α *Escherichia coli* cells, using a commercial kit (pGEM-T Easy Vector System, Promega, Inc.), plated onto LB agar plates containing ampicillin, IPTG, and X-Gal, and incubated at 37°C for 16–24 h. Controls were performed at all stages of cloning and transformation to ensure the efficiency of transformation and to detect potential cross-contamination.

Twelve bacterial transformant colonies were randomly selected from each sample for plasmid purification and sequencing. Selected colonies were grown in LB broth for 8 h and were subjected to plasmid isolation and purification using a high-throughput commercial kit (QIAprep 96 Turbo Kit, Qiagen, Inc.).

Sequence determination and analyses

All PCR products were directly sequenced on ABI 377 automated fluorescent DNA sequencers (Applied Biosystems, Inc.), located at the University of Illinois W.M. Keck Center for Comparative and Functional Genomics, with primers 4FId and 6RId. Amplicons were sequenced in both directions until all ambiguous bases were resolved. Purified plasmids from all clones were sequenced at the same facility, using standard M13 forward and reverse sequencing primers. Plasmid inserts were also sequenced in both directions until all ambiguous bases were resolved.

All sequences were hand-edited and aligned with respect to reference sequences using the computer program Clustal X (Jeanmougin et al., 1998). All alignments were checked manually. Phylogenetic analyses were performed using the computer programs PAUP*, version 4 (Swofford, 2000), MacClade, version 4.0 (Maddison and Maddison, 2000), and Modeltest, version 3.06 (Posada and Crandall, 1998). Population genetic analyses were performed using the programs MEGA2, version 3.0 (Kumar et al., 2000), Arlequin, version 2.000 (Schneder et al., 2000), and Mismatch, version 2b (Rogers, 1995). Tests of molecular selection (synonymous and nonsynonymous substitution rate tests) were conducted according to the method of Nei and Gojobori (1986), using the computer program SNAP (hiv-web.lanl.gov; Korber, 2000). Statistical analyses were performed using the computer program SAS, version 8.2 (SAS Institute, Inc.). Associations were considered statistically significant at the $\alpha = 0.05$ level.

Acknowledgments

Many thanks to F. Zuckermann for helpful comments and discussions, and to M. Banerjee for assistance with histopathology. This work was supported by the Illinois Department of Agriculture and by the Illinois Council on Food and Agricultural Research (Project 02I-030-3).

References

Allende, R., Lewis, T.L., Lu, Z., Rock, D.L., Kutish, G.F., Ali, A., Doster, A.R., Osorio, F.A., 1999. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J. Gen. Virol. 80, 307–315.

Benfield, D.A., Nelson, E., Collins, J.E., Harris, L., Goyal, S.M., Robison, D., Christianson, W.T., Morrison, R.B., Gorcyca, D., Chladek, D., 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Invest. 4, 127–133.

Beyer, J., Fichtner, D., Schirrmeier, H., Polster, U., Weiland, E., Wege, H., 2000. Porcine reproductive and respiratory syndrome virus (PRRSV): kinetics of infection in lymphatic organs and lung. Zentralbl. Veterinarmed. [B] 47, 9–25.

Bracho, M.A., Moya, A., Barrio, E., 1998. Contribution of Taq polymerase-induced errors to the estimation of RNA virus diversity. J. Gen. Virol. 79, 2921–2928.

Chang, C.C., Yoon, K.J., Zimmerman, J.J., Harmon, K.M., Dixon, P.M., Dvorak, C.M., Murtaugh, M.P., 2002. Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. J. Virol. 76, 4750–4763.

Christopher-Hennings, J., Holler, L.D., Benfield, D.A., Nelson, E.A., 2001. Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. J. Vet. Diagn. Invest. 13, 133–142.

Conzelmann, K.K., Visser, N., Van Woensel, P., Thiel, H.J., 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology 193, 329–339.

Dea, S., Gagnon, C.A., Mardassi, H., Milane, G., 1996. Antigenic variability among North American and European strains of porcine reproductive and respiratory syndrome virus as defined by monoclonal antibodies to the matrix protein. J. Clin. Microbiol. 34, 1488–1493.

Dea, S., Gagnon, C.A., Mardassi, H., Pirzadeh, B., Rogan, D., 2000. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Arch. Virol. 145, 659–688.

Dee, S.A., Bierk, M.D., Deen, J., Molitor, T.W., 2001a. An evaluation of test and removal for the elimination of porcine reproductive and respiratory syndrome virus from 5 swine farms. Can. J. Vet. Res. 65, 22–27.

Dee, S.A., Joo, H.S., 1997. Strategies to control PRRS: a summary of field and research experiences. Vet. Microbiol. 55, 347–353.

Dee, S.A., Joo, H.S., Park, B.K., Molitor, T.W., Bruna, G., 2000. Attempted elimination of porcine reproductive and respiratory syndrome virus from a seedstock farm by vaccination of the breeding herd and nursery depopulation. Vet. Rec. 142, 569–572.

Dee, S.A., Joo, H.S., Polson, D.D., Park, B.K., Pijoan, C., Molitor, T.W., Collins, J.E., King, V., 1997. Evaluation of the effects of nursery depopulation on the persistence of porcine reproductive and respiratory syndrome virus and the productivity of 34 farms. Vet. Record 140, 247–248.

Dee, S.A., Torremorell, M., Rossow, K., Otake, S., Mahlum, C., Faaberg, K.S., 2001b. Identification of genetically diverse sequences (ORF 5) of porcine reproductive and respiratory syndrome virus in a swine herd. Proceedings of the 2001 Allen D. Leman Swine Conference University of Minnesota Veterinary Outreach Program, Minneapolis, Minnesota.

Dewey, C.E., Wilson, S., Buck, P., Leyenaar, J.K., 1999. The reproductive performance of sows after PRRS vaccination depends on stage of gestation. Prev. Vet. Med. 40, 233–241.

Domingo, E., 2002. Quasispecies theory in virology. J. Virol. 76, 463–465.

Domingo, E., Escarmis, C., Sevilla, N., Moya, A., Elena, S.F., Quer, J., Novella, I.S., Holland, J., 1996. Basic concepts in RNA virus evolution. FASEB J. 10, 859–864.

Domingo, E., Menendez-Arias, L., Quinones-Mateu, M.E., Holguin, A., Gutierrez-Rivas, M., Martinez, M.A., Quer, J., Novella, I.S., Holland, J.J., 1997. Viral quasispecies and the problem of vaccine-escape and drug-resistant mutants. Prog. Drug Res. 48, 99–128.

Eigen, M., Schuster, P., 1979. The Hypercycle. A Principle of Natural Self-Organization. Springer, Berlin.

Essajee, S.M., Pollack, H., Rochford, G., Oransky, I., Krasinski, K., Borkowsky, W., 2000. Early changes in quasispecies repertoire in HIV-infected infants: correlation with disease progression. AIDS Res. Hum. Retroviruses 16, 1949–1957.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.

Farci, P., Purcell, R.H., 2000. Clinical significance of hepatitis C virus genotypes and quasispecies. Semin. Liver Dis. 20, 103–126.

Farci, P., Shimoda, A., Coiana, A., Diaz, G., Peddis, G., Melpolder, J.C., Strazzera, A., Chien, D.Y., Munoz, S.J., Balestrieri, A., Purcell, R.H., Alter, H.J., 2000. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science 288, 339–344.

Farci, P., Strazzera, R., Alter, H.J., Farci, S., Degioannis, D., Coiana, A., Peddis, G., Usai, F., Serra, G., Chessa, L., Diaz, G., Balestrieri, A., Purcell, R.H., 2002. Early changes in hepatitis C viral quasispecies during interferon therapy predict the therapeutic outcome. Proc. Natl. Acad. Sci. USA 99, 3081–3086.

Forsberg, R., Oleksiewicz, M.B., Petersen, A.M., Hein, J., Botner, A., Storgaard, T., 2001. A molecular clock dates the common ancestor of European-type porcine reproductive and respiratory syndrome virus at more than 10 years before the emergence of disease. Virology 289, 174–179.

Goldberg, T.L., Hahn, E.C., Weigel, R.M., Scherba, G., 2000. Genetic, geographical and temporal variation of porcine reproductive and respiratory syndrome virus in Illinois. J. Gen. Virol. 81, 171–179.

Halbur, P., Thanawongnuwech, R., Brown, G., Kinyon, J., Roth, J., Thacker, E., Thacker, B., 2000. Efficacy of antimicrobial treatments and vaccination regimens for control of porcine reproductive and respiratory syndrome virus and Streptococcus suis coinfection of nursery pigs. J. Clin. Microbiol. 38, 1156–1160.

Halbur, P.G., Paul, P.S., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1996. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. J. Vet. Diagn. Invest. 8, 11–20.

Horter, D.C., Pogranichniy, R.M., Chang, C.C., Evans, R.B., Yoon, K.J., Zimmerman, J.J., 2002. Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. Vet. Microbiol. 86, 213–228.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G., Gibson, T.J., 1998. Multiple sequence alignment with Clustal X. Trends Biochem. Sci. 23, 403–405.

Kapur, V., Elam, M.R., Pawlovich, T.M., Murtaugh, M.P., 1996. Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J. Gen. Virol. 77, 1271–1276.

Korber, B., 2000. HIV signature and sequence variation analysis, in: Rodrigo, A.G., Learn, G.H. (Eds.), Computational Analysis of HIV Molecular Sequences, Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 55–72.

Kumar, S., Tamura, K., Nei, M., 2000. MEGA2, version 3.0. Department of Biology, Arizona State Univ., Phoeniz, AZ.

Le Gall, A., Albina, E., Magar, R., Gauthier, J.P., 1997. Antigenic variability of porcine reproductive and respiratory syndrome (PRRS) virus isolates. Influence of virus passage in pig. Vet. Res. 28, 247–257.

Lowe, J.F., Firkins, L.D., Goldberg, T.L., 2003. A novel biopsy technique for the collection of ante mortem tonsil samples in swine. J. Swine Health Prod. 11, 229–232.

Maddison, D.R., Maddison, W.P., 2000. MacClade 4: analysis of phylogeny and character evolution. Sinauer Associates, Sunderland, MA.

Malet, I., Belnard, M., Agut, H., Cahour, A., 2003. From RNA to quasispecies: a DNA polymerase with proofreading activity is highly recommended for accurate assessment of viral diversity. J. Virol. Methods 109, 169–170.

Mateu, E., Martin, M., Vidal, D., 2003. Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. J. Gen. Virol. 84, 529–534.

Mavromatis, I., Kritas, S.K., Alexopoulos, C., Tsinas, A., Kyriakis, S.C., 1999. Field evaluation of a live vaccine against porcine reproductive and respiratory syndrome in fattening pigs. Zentralbl. Veterinarmed. [B] 46, 603–612.

Meng, X.J., 2000. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74, 309–329.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1999. Safety and efficacy of vaccination of pregnant gilts against porcine reproductive and respiratory syndrome. Am. J. Vet. Res. 60, 796–801.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., 2000. The effect of porcine parvovirus and porcine reproductive and respiratory syndrome virus on porcine reproductive performance. An. Reprod. Sci. 60-61, 199–210.

Mengeling, W.L., Vorwald, A.C., Lager, K.M., Brockmeier, S.L., 1996. Comparison among strains of porcine reproductive and respiratory syndrome virus for their ability to cause reproductive failure. Am. J. Vet. Res. 57, 834–839.

Meulenberg, J.J., Petersen den Besten, A., de Kluyver, E., van Nieuwstadt, A., Wensvoort, G., Moormann, R.J., 1997. Molecular characterization of Lelystad virus. Vet. Microbiol. 55, 197–202.

Meulenberg, J.J.M., Hulst, M.M., De Meijer, E.J., Den Besten, P.L.J.M., De Kluyver, E.P., Wensvoort, G., Moormann, R.J.M., 1993. Lelystad virus, the causitive agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192, 62–72.

Nei, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Nei, M., Gojobori, T., 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3, 418–426.

Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. 73, 270–280.

Nodelijk, G., 2002. Porcine reproductive and respiratory syndrome (PRRS) with special reference to clinical aspects and diagnosis. A review. Vet. Q. 24, 95–100.

Nodelijk, G., de Jong, M.C., van Leengoed, L.A., Wensvoort, G., Pol, J.M., Steverink, P.J., Verheijden, J.H., 2001. A quantitative assessment of the effectiveness of PRRSV vaccination in pigs under experimental conditions. Vaccine 19, 3636–3644.

Nowak, P., Karlsson, A.C., Naver, L., Bohlin, A.B., Piasek, A., Sonnerborg, A., 2002. The selection and evolution of viral quasispecies in HIV-1 infected children. HIV Med. 3, 1–11.

Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76, 4241–4250.

Paolucci, S., Baldanti, F., Campanini, G., Zavattoni, M., Cattaneo, E., Dossena, L., Gerna, G., 2001. Analysis of HIV drug-resistant quasispecies in plasma, peripheral blood mononuclear cells and viral isolates from treatment-naive and HAART patients. J. Med. Virol. 65, 207–217.

Pawlotsky, J.M., 2003. Hepatitis C virus genetic variability: pathogenic and clinical implications. Clin. Liver Dis. 7, 45–66.

Pirzadeh, B., Dea, S., 1997. Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants. J. Gen. Virol. 78, 1867–1873.

Pirzadeh, B., Dea, S., 1998. Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 79, 989–999.

Pirzadeh, B., Gagnon, C.A., Dea, S., 1998. Genomic and antigenic variations of porcine reproductive and respiratory syndrome virus major envelope GP5 glycoprotein. Can. J. Vet. Res. 62, 170–177.

Plagemann, P.G., Rowland, R.R., Faaberg, K.S., 2002. The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch. Virol. 147, 2327–2347.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Rodriguez, M.J., Sarraseca, J., Fominaya, J., Cortes, E., Sanz, A., Casal, J.I., 2001. Identification of an immunodominant epitope in the C terminus of glycoprotein 5 of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 82, 995–999.

Rogers, A.R., 1995. MISMATCH: computer programs for analysis of mismatch distributions, version 2b. Univ. of Utah, Salt Lake City, UT.

Rogers, A.R., Harpending, H., 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. 9, 552–569.

Rowland, R.R., Steffen, M., Ackerman, T., Benfield, D.A., 1999. The evolution of porcine reproductive and respiratory syndrome virus: quasispecies and emergence of a virus subpopulation during infection of pigs with VR-2332. Virology 259, 262–266.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Schneder, S., Roessli, D., Excoffier, L., 2000. Arlequin: a software for population genetics data analysis, version 2.000. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Stadejek, T., Stankevicius, A., Storgaard, T., Oleksiewicz, M.B., Belak, S., Drew, T.W., Pejsak, Z., 2002. Identification of radically different variants of porcine reproductive and respiratory syndrome virus in Eastern Europe: towards a common ancestor for European and American viruses. J. Gen. Virol. 83, 1861–1873.

Swofford, D., 2000. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), version 4. Sinauer Associates, Sunderland, MA.

Weiland, E., Wieczorek-Krohmer, M., Kohl, D., Conzelmann, K.K., Weiland, F., 1999. Monoclonal antibodies to the GP5 of porcine reproductive and respiratory syndrome virus are more effective in virus neutralization than monoclonal antibodies to the GP4. Vet. Microbiol. 66, 171–186.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J.M., Moonen, P.L.J.M., Zetstra, T., de Boer, E.A., Tibben, H.J., de Jong, M.F., van't Veld, P., Groenland, G.J.R., van Genep, J.A., Voets, M.T.H., Verheijden, J.H.M., Braamskamp, J., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Quarterly 13, 121–130.

Wills, R.W., Doste, A.R., Galeota, J.A., Sur, J.H., Osorio, F.A., 2003. Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. J. Clin. Microbiol. 48, 58–62.

Yang, L., Frey, M.L., Yoon, K.J., Zimmerman, J.J., Platt, K.B., 2000. Categorization of North American porcine reproductive and respiratory syndrome viruses: epitopic profiles of the N, M, GP5 and GP3 proteins and susceptibility to neutralization. Arch. Virol. 145, 1599–1619.

Yoon, K.J., Wu, L.L., Zimmerman, J.J., Platt, K.B., 1997. Field isolates of porcine reproductive and respiratory syndrome virus (PRRSV) vary in their susceptibility to antibody dependent enhancement (ADE) of infection. Vet. Microbiol. 55, 277–287.

Zimmerman, J.J., Yoon, K.J., Wills, R.W., Swenson, S.L., 1997. General overview of PRRSV: a perspective from the United States. Vet. Microbiol. 55, 187–196.