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Porcine Reproductive and Respiratory Syndrome Virus: Description of Persistence in Individual Pigs upon Experimental Infection†

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We studied the persistence of porcine reproductive and respiratory syndrome virus (PRRSV) in individual experimentally infected pigs, during a period of up to 150 days postinfection (dpi). The results of this study suggest that the persistence of PRRSV involves continuous viral replication but that it is not a true steady-state persistent infection. The virus eventually clears the body and seems to do it in most of the animals by 150 dpi or shortly thereafter. High genetic stability was seen for several regions of the persistent PRRSV's genome, although some consistent mutations in the genes of envelope glycoproteins and M protein were also observed.

Previous reports indicate that porcine reproductive and respiratory syndrome virus (PRRSV) can persist in pigs for long periods of time after initial infection and that persistently infected animals can shed infectious virus (4, 5, 17, 19). The mechanisms by which or the condition in which PRRSV persists in the animal host is not known. Few studies of the occurrence of in vivo persistence of PRRSV have been reported (4, 14). Likewise, the genetic stability of PRRSV during persistence is unknown. The genetic stability of ORF7 (the gene coding for the PRRSV nucleocapsid) has been studied during serial in vivo passages (10). Likewise, a segment of 537 nucleotides comprising the carboxyl-terminal end of ORF4 and the amino-terminal end of ORF5 has been studied in individual persistently infected animals (14). While the ORF7 sequence was reported to be highly stable during persistence in a group of animals (9, 10), the ectodomain of ORF5 has shown mutations that were suggested to be associated with persistence in individual hosts (14).

We experimentally infected pigs with the pathogenic PRRSV isolate 16244B (2) to study the extent of PRRSV gene expression during persistence in the natural host. We also determined the genetic stability of the persistent isolates (those recovered sequentially from an individual animal) by analyzing some selected areas of the genome. The genomic areas studied included those probably important for polymerase recognition, replication initiation, and subgenomic RNA synthesis; the coding sequence for the structural and NSP1a proteins; and the 5′ and 3′ noncoding regions (NCRs).

Ten pigs (1 to 2 months old) from an unvaccinated PRRSV-free herd were inoculated intranasally with $10^{6.0}$ 50% cell culture infectious doses of PRRSV strain 16244B per ml, delivered in volumes of 0.5 ml/nostril. Three uninfected pigs, matching the infected group in age, breed, and farm of origin, were used as controls. Different isolation rooms (biosafety level 2) were used to separate control animals from those infected with PRRSV during the 5-month period of the experiment. Animals were clinically inspected on a daily basis. Serum and tonsil biopsy samples were taken at different times postinfection (p.i.), ranging from 7 to 150 days p.i. (dpi), and used for (i) assessment of PRRSV antibody response, (ii) infectious virus isolation, and (iii) PRRSV RNA detection by reverse transcription-PCR (RT-PCR). At 150 dpi, the experiment was terminated and all of the animals were euthanatized by electrocution, exsanguinated, and necropsied. Samples from serum, lung, bronchial lymph nodes, and tonsils were collected and snap-frozen for further PRRSV isolation and viral RNA detection. Samples were assayed for infectious virus on monolayers of porcine alveolar macrophages (PAMs) and MARC-145 cells (8) or by conducting bioassay experiments (see below). The maintenance medium used for PAMs was RPMI 1640 (Sigma) supplemented with 10% fetal calf serum and gentamicin (50 μg/ml). The growth medium used for the MARC-145 cell line was Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum and gentamicin (50 μg/ml). Virus was isolated from tissue and serum samples by inoculating six-well plates containing monolayers of PAMs maintained at 37°C. If no cytopathic effect was observed in the first passage in PAMs, supernatants were used as inoculum for passages in MARC-145 cells. A total of two additional passages in MARC-145 cells were done before scoring the sample as negative for infectious PRRSV. The identity of viral isolates was confirmed by immunofluorescence with the monoclonal antibody SDOW-17 specific for the PRRSV nucleocapsid protein (13) and identified with the identification (ID) number of the animal from which the sample inoculum was originated plus the date (time p.i.) when the sample was collected. All inoculated pigs developed viremia by day 7 p.i. as indicated by the recovery of infectious PRRSV from serum samples in MARC-145 cells (Table 1). Seroconversion, determined by a commercial enzyme-linked immunosorbent assay (ELISA) (Idexx Laboratories, Portland, Maine), took place between 7 and 14 dpi, after which ELISA antibodies peaked at 28 to 56 dpi (as assessed by the commercial ELISA's sample-to-positive ratios). The antibody titers remained on a plateau after peaking and then declined but remained always above the positive threshold for the rest of the observation period (data not shown).

The frequency of recovery of infectious virus by cell culture

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TABLE 1. PRRSV isolation in cell culture or in bioassay experiments from pigs experimentally infected with the 16244B isolate[a]

Animal ID no.	Result for assay at dpi:					
	7 (cc)	56 (cc)	84		150	
			cc	ba	cc	ba
3	+	−	+	+	−	+
4	+	−	−	−	−	ND
9	+	−	−	−	−	ND
14	+	−	−	−	−	ND
17	+	−	−	−	−	ND
22	+	−	−	+	−	−
23	+	−	−	−	−	ND
27	+	−	−	+	−	−
31	+	−	−	+	−	+
32	+	+	−	+	−	−

[a] Viruses were isolated in cell culture (cc) (PAMs or MARC-145 cells) or in bioassays (ba). Day 7 samples were serum samples and were assayed only on MARC-145 cells. Day 56 and day 84 samples were tonsil biopsy and serum samples. Day 150 was the time of necropsy (tonsil, bronchial lymph node, lung, and serum samples). +, positive; −, negative; ND, not done.

assays (PAMs or MARC-145 cells) decreased with time, and PRRSV was recovered only occasionally up to 84 dpi (Table 1). In addition to cell cultures, experimental inoculation of susceptible swine (bioassay experiment) was performed with samples taken at 84 and 150 dpi. We inoculated 1- to 2-week-old piglets, obtained from an unvaccinated PRRSV-free herd, to assay for the presence of infectious PRRSV in tissue collected from the pigs at long-term-p.i. periods. Tonsil biopsy samples collected at 84 dpi from each infected animal were ground separately and mixed with a serum sample collected at the same time p.i. from the same animal. Each homogenate typically consisted of 50 to 80 mg of ground tonsil tissue and 2 ml of serum. Approximately 0.5 ml from each homogenate was stored at −80°C for viral isolation assay in cell cultures. The remaining 1.5 ml of homogenate was diluted to a volume of 8 ml with MEM, supplemented with gentamicin (50 μg/ml), and used to inoculate the piglets. Tissue samples taken at necropsy (150 dpi) from the infected animals were ground separately. Pools of ground tissue from each individual pig were mixed with serum collected at the same p.i. time point from the same animal. Typically, each homogenate consisted of 5 to 10 g each of ground lung, lung lymph node, and tonsil tissues and 4 ml of serum. MEM supplemented with gentamicin (100 μg/ml) was added in a volume of 15 ml. The suspension was then frozen and thawed and clarified by low-speed centrifugation. Supernatants were then transferred to new sterile tubes and diluted 1:5 in MEM containing 100 μg of gentamicin per ml. A volume of 12 ml from each sample suspension was used to inoculate the piglets. Bioassay experimental piglets were inoculated with 1 ml of the supernatants from tissue suspension delivered in each nostril with the remaining 10 ml of inoculum being delivered intraperitoneally. Each inoculated animal remained individually isolated during the bioassay experiments for a period of 3 weeks. The progress of the experimental inoculation was monitored by daily clinical observation and weekly serum sampling which was used for the assessment of viremia and PRRSV antibody response by ELISA. The isolates obtained through this bioassay were identified with the ID number of the animal from which the sample inoculum originated and the corresponding date of collection, followed by the letter "b." At 84 dpi, we used 10 piglets for the bioassay experiment (one for each principal animal). For the sake of economy, at 150 dpi we assessed only the samples from those animals that had yielded positive results at 84 dpi.

PRRSV was isolated in cell cultures from a tonsil biopsy sample of pig 32 at 56 dpi and from a homogenate of tonsil and serum samples from pig 3 at 84 dpi. Interestingly, 5 out of 10 animals were persistently infected at 84 dpi, and from those, two animals remained persistently infected up to 150 dpi, as indicated by the development of viremia and seroconversion in the bioassay piglets (Table 1). We did not quantitate the infectious virus per gram of sample. However, the difficulty in isolating infectious PRRSV by conventional methods and the strict requirement of bioassay inoculation in order to demonstrate PRRSV infectivity in later stages of the observation period (approximately 3 to 5 months p.i.) would suggest that the amount of infectious virus was decreasing progressively throughout the experiment.

To study the occurrence of viral gene expression, a strand-specific RT was performed to reverse transcribe the negative form of the viral RNA that occurs during replication, using the primer 5′-GACTGCTTTACGGTCTCTC-3′ (11), which anneals to the replicative (minus) RNA form at the 3′ end of the leader. PCR and seminested PCR, with 35 cycles of amplification each, were carried out on the strand-specific RT products. For the PCR, the following oligonucleotides were used: 5′-TGCCGCACGGTTTCATCCGATTG-3′ (forward primer, nucleotide [nt] 14728 to 14750), outer reverse primer 5′-GGT GATGCTGTGACGCGGATCAG-3′ (nt 15228 to 15250), and inner reverse primer 5′-GCAAGTCCCAGCGCCTTGATT-3′ (nt 15138 to 15158). The end product of the seminested PCR was 430 nt long corresponding to the last 170 nt from ORF6 and the first 270 nt of ORF7. A PCR for the constitutive β-actin gene of swine was performed on the tissue samples collected at necropsy as control for the RNA extraction and RT reactions using previously published primers (16). All RT reactions were performed using SuperScript II reverse transcriptase (Gibco BRL) following the manufacturer's instructions. The PRRSV-negative RNA form was detected at 28 dpi in tonsil samples from 8 out of 10 infected animals (Table 2), thus suggesting the occurrence of active viral gene expression in the tissues under study. There was a significant decrease in the number of positive animals by day 119 p.i. compared to the more acute-phase samples at 28 dpi. Only pig 31 showed evi-

TABLE 2. PRRSV-negative strand-specific RT-PCR amplification in tissue samples of pigs experimentally infected with PRRSV isolate 16244B[a]

Animal ID no.	Result for dpi:		
	28	119	150
3	−	−	−
4	+	−	−
9	+	−	−
14	+	−	−
17	−	−	−
22	+	−	−
23	+	−	−
27	+	−	−
31	+	+	−
32	+	−	−
43	−	−	−
47	−	−	−
50	−	−	−

[a] Animals 43, 47, and 50 are uninfected controls. RT-PCR was performed on RNA from tonsil samples (days 28 and 119) and from tonsil, lung, lymph node, and serum samples (day 150). +, positive; −, negative.

TABLE 3. Predicted amino acid substitutions in Nsp1α and ORF2 to ORF7 from persistent PRRSV isolates recovered from pigs infected with PRRSV strain 16244B

Genome region	Amino acid substitution from PRRSV isolate:												
	3-7	3-84	3-84b	3-150b	22-7	22-84b	27-7	27-84b	31-7	31-84b	31-150b	32-7	32-84b
Nsp1α						$A_{114}T$		$A_{114}T$		$I_{81}V$			$A_{114}T$
ORF2						$Q_{189}R$ $A_{223}T$		$Q_{189}R$ $A_{223}T$		$Q_{189}R$ $L_{203}P$			$Q_{189}R$ $A_{223}T$
ORF3		$I_{215}T$	$I_{215}T$	$I_{215}T$ $T_{64}A$		$S_{15}N$		$S_{15}N$		$H_{79}Y$ $S_{106}G$ $Q_{151}R$	$I_{215}T$		$S_{15}N$
ORF4		$E_{35}K$	$E_{35}K$	$E_{35}K$ $M_{157}T$		$A_{45}V$		$A_{45}V$		$S_{51}G$ $F_{118}L$	$E_{35}K$ $V_{127}E$		$A_{45}V$
ORF5		$N_{58}D$ $Y_{141}H$	$N_{58}D$	$N_{58}D$ $Y_{141}H$		$D_{61}N$		$D_{61}N$		$D_{61}N$	$N_{58}D$ $Y_{141}H$		$D_{61}N$
ORF6		$K_{70}E$		$K_{70}E$						$T_{63}M$	$K_{70}E$		
ORF7													

dence of persistent PRRSV gene expression by minus-strand-specific RT-PCR in the tonsil tissue (Table 2). We were unable to detect the negative form of PRRSV RNA at 150 dpi (Table 2). However, using random hexamers in the RT reaction, PRRSV total RNA was still detected in tonsil tissue from one of the persistently infected pigs (animal 31 [data not shown]) for which virological evidence of persistent PRRSV was provided in the bioassay experiment also (Table 1). The low level of detection of PRRSV RNA (minus) during the late-p.i. period could be a reflection of the lower frequency of this template in the replicative process, which made it undetectable in most of the animals by the technique we used.

Evidence for persistence of PRRSV infection in individual animals has been reported previously (5, 17, 19). In this report, we provide evidence that the virus persists in a replicating, infectious state in a continuously decreasing percentage of the convalescent animals. Together, the data from viral isolation and negative RNA RT-PCR indicate that PRRSV persists through a "smoldering" type of infection in which the virus replicates at low levels over time.

To measure the genetic stability of the PRRSV during persistence, we analyzed sequences from viruses recovered at 7 dpi (samples 3-7, 22-7, 27-7, 31-7, and 32-7), at 84 dpi (samples 3-84, 3-84b, 22-84b, 27-84b, 31-84b, and 32-84b), and at 150 dpi (samples 3-150b and 31-150b). Random hexamers were used to reverse-transcribe total RNA extracted from monolayers of MARC-145 cells infected with the above-mentioned PRRSV isolates recovered during the experiment. The resulting cDNA was used as a template in the PCR to amplify segments of the PRRSV genome from nt 25 at the 5′ NCR to nt 675 in ORF1a, the complete sequence of ORF2 to ORF7, and the 3′ NCR (all of them based on our published sequence of PRRSV 16244B [GenBank accession no. AF046869]). RT-PCR products and cloned cDNA inserts were sequenced by the dideoxynucleotide chain-termination method (15). Sequencing reactions were prepared with the BigDye Terminator Cycle Sequencing kit (Perkin-Elmer). Sequences were determined with an ABI PRISM 377 automated DNA sequencer and an ABI PRISM 3700 DNA analyzer. The ABI sequence software version 3.3 was used for lane tracking and trace extraction. Further cDNA sequencing, genome assembling, and computer analysis were done as previously described (2). A total of 4.2 kb was sequenced and analyzed for each virus isolate. The PRRSV 5′ NCR and the 3′ NCR were extremely stable during long-term persistence, suggesting that virus persistence is not a consequence of mutations in regions most likely involved in polymerase recognition or transcription initiation. The frequency of nucleotide substitutions at 7 dpi varied from 0 to 0.6/1,000 nt. By day 84 p.i., the frequency of nucleotide substitutions rose, ranging from 2.1 to 5/1,000 nt (data not shown), which is similar to that observed for the S gene of the coronavirus mouse hepatitis virus (1). At day 150 of persistence, the frequency of nucleotide substitutions varied between 3.8 and 3.6 in the two samples analyzed (3-150b and 31-150b) (data not shown), which is still similar to that reported elsewhere for the persistence models of the coronavirus mouse hepatitis virus (1). An observed feature of the nucleotide substitutions during persistence was that the mutations were almost entirely transitions. The observed bias in PRRSV persistence was higher than that reported for other virus models (1, 18) and may very well be a consequence of the PRRSV replication in a naive piglet during the bioassay experiment.

The results of the predicted protein sequence analyses are summarized in Table 3 as translated amino acid sequences compared to the parental PRRSV 16244B strain. No changes were observed in isolates recovered at 7 dpi. However, at 84 and 150 dpi, changes were observed in all the samples analyzed. The average nonsynonymous/synonymous mutation ratios for individual genes at 84 and 150 dpi indicate that selective pressure for amino acid change is likely exerted in genes coding for the major viral glycoproteins during establishment of persistence (Table 4). In fact, ORF3, ORF4, and ORF5 were the more variable genes during persistence, while ORF2 changed only moderately (Tables 3 and 4). Nsp1α and ORF6 were substantially less variable than the glycoproteins, and the ORF7 predicted protein remained unchanged during persistence (Table 4). Similar observations were reported for ORF2 to ORF7 of other arteriviruses (7). The high degree of conservation observed for N protein from persistent PRRSV isolates agrees with data reported for other arteriviruses (7). This protein as well as the 5′ NCR and 3′ NCR have a potential role in genome packaging during virion assembly or in the complex

TABLE 4. Average nucleotide substitution rate in ORF2 to ORF7 of PRRSV persistent isolates

Genome region	Ratio of nonsynonymous substitutions per site/synonymous substitutions per site at dpi:	
	84[a]	150[b]
Nsp1α	1.330	0/0.002[c]
ORF2	0.800	0/0.001[c]
ORF3	1.146	0.730
ORF4	1.964	0.790
ORF5	2.515	1.000
ORF6	0.090	0.002/0[d]
ORF7	0/0.001[c]	0/0.001[c]

[a] Averages of five animals (3-84b, 22-84b, 27-84b, 31-84b, and 32-84b).
[b] Averages of two animals (3-150b and 31-150b).
[c] All nucleotide changes were synonymous.
[d] All nucleotide changes were nonsynomous.

transcription process (6); thus, structural restrictions are imposed on nucleotides in this region of PRRSV, as indicated by our results.

Although antigenic variation of gp3 has been associated with variation of PRRSV through serial passage in swine (9), the mechanism of variant selection during persistence is not clear. High glycosylation and variability of PRRSV gp3 suggest that this protein is involved in virus-host cell interactions (9, 12). Our data indicate that strong selective pressure is likely exerted on the PRRSV gp3 during establishment of and throughout persistence in the natural host. Regarding gp5, two out of three changes observed were located in the ectodomain region of the protein. Changes in the PRRSV gp5 ectodomain have been associated with selection of a viral subpopulation through quasispecies evolution during persistence (14). Although we identified mutations in the ectodomain of the glycoprotein, we did not observe the mutation at position 34 that appeared to be the hallmark of the quasispecies identified by Rowland et al. (14) during persistence. Ours and theirs are not necessarily contrasting results, because Rowland et al. also reported that, when they repeated the experiments using a different strain of PRRSV, they again observed the emergence of a new subpopulation of PRRSV RNA, characterized by a mutation in a different position of the ectodomain (14).

Recently, PRRSV M protein has been reported to be a major target of cell-mediated immunity (3). The M protein is a structural protein with three transmembrane domains, and structural requirements likely restrict amino acid alteration in the protein. Surprisingly, we identified two amino acid mutations in this protein (Table 3). Although we did not study the cell-mediated response of the animals in these experiments and very little is known in general about the topic, our results could suggest that mutations in M protein determine a delay in the process of viral clearance from the body.

The results of this study suggest that PRRSV persists through a "smoldering" type of infection in which the virus replicates at low levels over time. The virus eventually clears the body and seems to do it in the majority of the animals by 150 dpi or shortly thereafter. High genetic stability was observed in both NCRs and in the nucleocapsid protein during persistence. However, genes coding for the glycoproteins and the M protein of PRRSV had mutations that could be associated with putative changes which may be involved in maintenance of persistence.

Nucleotide sequence accession number. The PRRSV sequences reported in this paper have been deposited in GenBank under accession no. AF299404 through AF299417.

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