Evidence that persistent porcine reproductive and respiratory syndrome virus infection in pigs does not require significant genetic change in open reading frame 5

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Summary

Objective: To assess genetic and antigenic changes in porcine reproductive and respiratory syndrome virus (PRRSV) over the course of infection in individual, persistently infected pigs.

Materials and methods: Three individually housed "principal pigs" were inoculated (Day 0) with a plaque-cloned virus (CC-01) derived from the North American PRRSV isolate, VR-2332. Homogenates of tissues collected on Day 120 were inoculated into individually housed PRRSV-naive bioassay pigs. Genetic changes in open reading frames (ORFs) 1b, 5, and 7 in plaque-cloned viruses isolated from the original inoculum and serum of principal and bioassay pigs collected 7 days post inoculation were assessed by sequencing. Antigenic changes (in GP 5 and N proteins) were assessed by monoclonal antibody analysis.

Results: One bioassay pig became infected with PRRSV. A total of 71 PRRSV plaque-cloned isolates were recovered from the virus inoculum, serum from the persistently infected principal pig, and serum from its corresponding bioassay pig. ORF 1b and ORF 7 amino-acid sequences from 30 plaque-cloned viruses isolated from the bioassay pig were identical with the virus inoculum (CC-01). Analysis revealed three ORF 5 amino-acid variants in the bioassay pig, with the dominant variant identical to one isolated from serum collected from the principal pig on Day 7. Monoclonal antibody analysis found no changes in the phenotypic epitopic profiles of the plaque-cloned isolates.

Implications: Persistent PRRSV infection does not depend on mutations in ORFs 1b, 5, or 7. These results have implications for PRRSV immunology and for efforts to control or eliminate the virus.

Keywords: swine, porcine reproductive and respiratory syndrome virus, persistence, evolution

Received: May 18, 2009
Accepted: July 7, 2009

Resumen – Evidencia de que la infección persistente del virus del síndrome reproductivo y respiratorio porcino en cerdos no requiere un cambio genético significativo en el marco de lectura abierta 5

Objetivo: Valorar los cambios antigénicos y genéticos del virus del síndrome reproductivo y respiratorio porcino (PRRSV por sus siglas en inglés) durante el curso de la infección en cerdos individuales persistentemente infectados.

Materiales y métodos: Se inocularon tres "cerdos principales" alojados individualmente (Día 0) con un virus clonado en placa (CC-01) derivado del aislado VR-2332, del PRRSV americano. Para realizar bioensayos, se inocularon homogeneizados de tejidos colectados el Día 120 a cerdos alojados individualmente y libres del PRRSV. Mediante secuenciación, se valoraron los cambios genéticos en los marcos de lectura abierta (ORFs) 1b, 5, y 7 en virus clonados en placa aislados del inoculo original y del suero de cerdos principales y de bioensayo colectados 7 días post inoculación. Se valoraron los cambios antigénicos (en las proteínas GP 5 y N) por medio del análisis de anticuerpos monoclonales.

Resultados: Un cerdo de bioensayo se infectó con el PRRSV. Un total de 71 aislados clonados en placa del PRRSV se recuperaron del inoculo del virus, del suero del cerdo principal persistentemente infectado, y del suero del cerdo de bioensayo correspondiente.

Las secuencias de los aminoácidos de los ORF 1b y ORF 7 de 30 virus clonados en placa aislados de los cerdos de bioensayo fueron idénticos al inoculo del virus (CC-01). El análisis reveló tres variantes de los aminoácidos del ORF 5 en el cerdo de bioensayo, siendo la variante dominante idéntica a un aislado del suero del cerdo principal colectado en el Día 7. El análisis de anticuerpos monoclonales no encontró cambios en los perfiles epitópicos fenotípicos de los aislados clonado en placa.

Implicaciones: La infección persistente de PRRSV no depende de las mutaciones en los ORFs 1b, 5, ó 7. Estos resultados tienen implicaciones en la inmunología del PRRSV y para los esfuerzos de control ó eliminación del virus.

Résumé – Mise en évidence que l'infection persistante par le virus du syndrome reproducteur et respiratoire porcin ne requiert pas de changement génétique significatif dans le cadre de lecture ouvert 5

Objectif: Évaluer les changements génétiques et antigéniques du virus du syndrome

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This article is available online at http://www.aasv.org/shap.html.

Chang CC, Yoon KJ, Zimmerman JJ. Evidence that persistent porcine reproductive and respiratory syndrome virus infection in pigs does not require significant genetic change in open reading frame 5. *J Swine Health Prod.* 2009;17(6):318–324.

reproducteur et respiratoire porcin (PRRSV) au cours de la période d'infection chez des porcs infectés de manière persistante.

Matériels et méthodes: Trois "porcs principaux" logés individuellement ont été inoculés (Jour 0) avec un virus cloné par plaque (CC-01) dérivé de l'isolat nord-américain du PRRSV, VR-2332. Des homogénats de tissus prélevés au Jour 120 ont été administrés dans le cadre d'un bio-essai à des porcs naïfs pour le PRRSV et logés individuellement. Les changements génétiques dans les cadres de lecture ouverts (ORFs) 1b, 5, et 7 dans des virus clonés par plaque isolés de l'inoculum original et du sérum prélevé 7 jours post-inoculation provenant des porcs principaux et des porcs du bio-essai ont été vérifiés par séquençage. Les changements antigéniques (dans les protéines GP 5 et N) ont été évalués par analyse à l'aide d'anticorps monoclonaux.

Résultats: Un des porcs du bio-essai est devenu infecté avec le PRRSV. Un total de 71 isolats de PRRSV clonés par plaque a été obtenu à partir de l'inoculum viral, du sérum du porc principal infecté de manière persistante, et du sérum du porc associé dans le bio-essai. Les séquences en acides aminés des ORF 1b et ORF 7 de 30 virus clonés par plaque isolés du porc du bio-essai étaient identiques à l'inoculum viral (CC-01). Les analyses ont révélé trois variantes en acides aminés de l'ORF 5 dans le porc du bio-essai, avec la variante dominante identique à une variante isolée du sérum prélevé du porc principal au Jour 7. L'analyse à l'aide des anticorps monoclonaux n'a pas permis de trouver de changement dans les profils phénotypiques des épitopes des isolats clonés par plaque.

Implications: L'infection persistante par le PRRSV ne dépend pas de mutations dans les ORFs 1b, 5, ou 7. Ces résultats ont des implications dans l'immunologie du PRRSV et les efforts pour limiter ou éliminer le virus.

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family *Arteriviridae* in the order *Nidovirales,*[1] is the cause of significant economic disease losses in commercial swine populations. The virion is enveloped and has a polyadenylated, single-stranded, nonsegmented, positive-sense RNA genome of 15 kilobases.[2-5] The genome consists of nine open reading frames (ORFs) encoding the replication-associated proteins (ORFs 1a and 1b) and seven structural proteins (ORFs 2 to 7).[3,5-7] ORF 7 encodes the antigenically abundant nucleocapsid protein (N), ORF 6 the matrix protein (M), and ORFs 5 to 2 the four glycosylated proteins and one non-glycosylated protein, ie, the major envelope glycoprotein (GP 5), minor envelope-associated glycoproteins 4 to 2 (GP 4 to 2a), and GP 2b (E), respectively.[5,8,9] Among them, GP 5 is considered a major inducer of neutralizing antibody.[10-12]

Genetic and antigenic analyses have revealed two distinct PRRSV groups in swine populations, ie, the European (Type 1) and the North American (Type 2), with marked genetic and antigenic differences between the two genotypes,[7,13-19] as well as among viruses within each genotype. PRRS virus is an RNA virus, and the absence of an adequate proof-reading mechanism explains the diversity seen among field isolates and the emergence of new PRRSV variants and mutants in pig populations, such as the isolates recovered in association with severe clinical outbreaks in the mid-eastern region of China.[20-22] Analysis of PRRSV field strains has shown that ORF 5 is the most diverse gene.[13,14,23] Under experimental conditions, ORF 5 was also more prone to mutational changes during replication in pigs than ORF 1b or ORF 7;[24] thus, the experimental work provided data to explain the basis of the genetic and antigenic diversity of PRRSV observed in the field.

PRRS virus produces a chronic, persistent infection in pigs, ie, virus replicates in infected individuals (carrier animals) for several months.[24] A previous study[24] suggested that on-going genetic change during in vivo replication might be a mechanism by which PRRSV could persist in the host, with multiple evolving variants present in infected pigs. However, the question remains as to whether molecular evolution and PRRSV persistence are interlinked, ie, whether persistence relies on the ability of the virus to evolve and escape the host's immune system. Furthermore, the role of immune pressure on the rate or extent of virus evolution is still unresolved. Therefore, we conducted a study to examine the molecular changes in PRRSV after an extensive period of replication (120 days) in a persistently infected pig.

Materials and methods

All animal care and handling was approved by Iowa State University Committee on Animal Care and conducted according to federal guidelines.

Experimental design

The objective of the study was to characterize genotypic changes in PRRSV over 120 days of replication in pigs. Four principal pigs were used: three pigs were inoculated (Day 0) with a well-characterized PRRSV isolate[24] designated CC-01, and the fourth pig served both as mock-infected negative control and environmental sentinel. Briefly, the three principle pigs were inoculated intranasally (1 mL per naris) and intramuscularly (8 mL per pig) with a 3× plaque-cloned PRRSV VR-2332 (CC-01) at a titer of 10^6 median tissue-culture infectious doses ($TCID_{50}$) per mL. The remaining pig (negative control) was mock-inoculated with cell-culture medium. At Day 120 post inoculation, the three infected principal pigs were euthanized. Tissue homogenates from specimens collected from the principal pigs were individually prepared and inoculated intramuscularly into three 4-week old PRRSV-free bioassay pigs (Day 0 for bioassay pigs). The use of bioassays for recovery of virus from persistently infected pigs has been previously described.[24] Recovery of virus from tissue homogenates using bioassay pigs avoids selective pressures from cell cultures on viral quasi-species generated during continuous pig passages. Thereafter, using a method previously described,[24] virus plaque-clones were recovered from the inoculum (CC-01), from serum collected at Day 7 from principal pigs, and from serum collected from one bioassay pig 7 days post inoculation. Thus, virus clones represented 0, 7, and 127 days of virus replication in pigs. Genetic mutation and antigenic variation were assessed by comparing pig-passaged virus to the original PRRSV inoculum.

Virus, cells, and media

The virus, cells, media, and plaque-cloning procedure used in the study have been previously described in detail.[24] The inoculum virus (CC-01) was derived from the North American prototype PRRSV ATCC VR-2332 by three rounds of plaque cloning, thereby producing a highly homologous challenge virus.

The 30 plaque-cloned viruses recovered from the original inoculum (CC-01) were used to provide the baseline of comparison for genetic and antigenic analyses. The same plaque-cloning procedure was also used to attempt to recover 30 virus clones directly from Day 7 serum of each pig.

Thirty clones cannot capture the complete genetic diversity within the inoculum, but this number provided at least 95% confidence of detecting a variant if its prevalence was greater than 18% in the viral population.[24] All plaque-cloned viruses were propagated once in MARC-145 cells maintained in Dulbecco's modified Eagle's medium (DMEM), aliquoted, and stored at -80°C until used.

Animals and animal care

Eight 14- to 17-day-old crossbred pigs were obtained from a PRRSV-free herd: four principal pigs and four bioassay pigs. All animals were individually housed in HEPA-filtered isolation units (Barrier Systems, Inc, Tom River, New Jersey) to prevent infection by extraneous viruses. Pigs were kept in the HEPA-filtered isolators for 60 days post inoculation. Then, to provide sufficient space, they were housed individually in a biosecurity level-2 facility for an additional 60 days. The animal care, biological sampling, and biosafety procedures used in this study have been described elsewhere.[24]

Biological samples, bioassay, and plaque-cloning

Collection and processing of blood and tissue samples were performed as previously described.[24] Blood samples were collected on Days 0, 7, 14, 21, 28, 35, 60, 90, and 120 from the principal pigs and Days 0, 7, 14, and 21 from the bioassay pigs. Whole blood and tissues, including tonsil, tracheobronchial and medial iliac lymph nodes, spleen, lung, and bronchoalveolar lavage fluid, were collected from each principal pig at Day 120. Tissue homogenates from these specimens were prepared for inoculation into bioassay pigs. All sera and tissue samples were stored at -80°C until used.

Enzyme-linked immunosorbent assay (ELISA)

To monitor the antibody response specific for PRRSV, ELISA was performed using a commercial kit (HerdChek PRRS 2XR; Idexx Laboratories, Inc, Westbrook, Maine) following the procedure recommended by the manufacturer. Samples for which the ratio of net optical density of test sample to that of positive control (ie, S:P ratio) was ≥ 0.4 were considered positive for antibody against PRRSV.

Monoclonal antibody analysis

The epitopic profiles of plaque-cloned viruses recovered from the PRRSV inoculum (CC-01) and from serum samples from one principal pig (7 days of in vivo replication) and its corresponding bioassay pig (127 days of in vivo replication) were determined using a panel of monoclonal antibodies (MAbs) in an indirect immunofluorescence assay.[24] The panel consisted of five MAbs against the N protein and two against the GP 5 protein. Production and characterization of the MAbs has been described elsewhere.[12,18]

Sequencing

ORFs 1b, 5, and 7 were sequenced for all virus clones, as previously described.[24] A total of 30 plaque-cloned viruses from CC-01 were also sequenced to assess the homogeneity of the original inoculum. Sequence alignment and comparisons were performed using computer software (Lasergene; DNASTAR Inc, Madison, Wisconsin). Nucleotide sequences of isolate VR-2332 (GenBank access no. PRU87392) and Resp-PRRS vaccine virus (Boehringer Ingelheim Vetmedica, Inc, St Joseph, Missouri; GenBank access no. AF159149) were obtained and included in the sequence analyses for comparative purposes.[8,25] Nucleotide mutation rates and amino-acid sequence changes observed in virus clones recovered over the course of the experiment were compared to those of the inoculum (CC-01). Mutation rate was calculated as the proportion of substitution and was expressed as mean percent change separately for ORFs 1b, 5, and 7 per plaque-cloned virus.

Results

Clinical response to inoculation in principal and bioassay pigs

The environmental sentinel pig remained free of PRRSV-specific antibody throughout the experiment, indicating that biosecurity procedures effectively prevented transmission of PRRS viruses among pigs during the study. Because the animal-housing design precluded contact with pigs, clinical observations in PRRSV-inoculated pigs were limited to apparent changes in behavior, rather than direct physical examination. Mild to moderate lethargy was observed in the three principal pigs from Day 2 to 8. Pigs appeared clinically normal thereafter. No visible clinical signs were observed in the bioassay pigs throughout the observation period.

Confirmation of PRRSV infection in principal and bioassay pigs was based on the positive results of direct PRRSV plaque-cloning and ELISA-detectable antibody responses at Days 7 and 21, respectively. All three principal pigs were viremic and seropositive. Tissue homogenates from two of the three principal pigs failed to produce infection in bioassay pigs after three attempts. PRRS virus is highly infectious via intramuscular exposure;[26] hence, this result indicated that two of the principal pigs cleared virus from their tissues before the end of the 120-day observation period.

A total of 71 plaque-cloned isolates were collected and analyzed, ie, 30 clones from the original inoculum (CC-01), 11 clones from serum collected on Day 7 from one principal pig, and 30 clones from serum collected on Day 7 from the corresponding bioassay pig. Viruses were not collected from the two principal pigs that did not produce infection in bioassay pigs.

Assessment of genetic changes

Homogeneity of the CC-01 inoculum. Nucleotide sequences from part of ORF 1b (435 bases) and all of ORF 5 (603 bases) and ORF 7 (372 bases) were identical for all 30 clones derived from the inoculum, confirming the homogeneity of the inoculum. This also provided evidence that random errors were not introduced during reverse transcriptase-polymerase chain reaction and sequencing.

Sequence analysis of PRRSV ORFs 1b, 5, and 7. Complete nucleotide sequences for ORFs 1b, 5, and 7 were obtained for 41 swine-derived virus clones, ie, 11 from the persistently-infected principal pig and 30 from the corresponding bioassay pig (Table 1). These clones represented early adaptation and then persistence of the virus in the pigs, ie, after 7 and 127 days of in vivo replication in principal and bioassay pigs, respectively.

Sequencing of virus clones for ORF 5 showed the presence of the CC-01 genotype plus five additional ORF-5 nucleotide variants on Day 7 (principle pig). At 127 days of in vivo replication (Day 7 for the bioassay pig), one of these five variants remained and three additional variants had appeared (Table 1). Each variant showed one to two nucleotide substitutions with no inserted or deleted nucleotides detected. These variations resulted in four amino-acid variants (AVs) at Day 7 and three at Day 127 (Table 1). Among the five AVs, no specific "hot spots" were identified except at position 151 (glycine to arginine; Table 2). Most amino-acid substitutions due to nucleotide substitutions were randomly distributed. Notably, one variant (5NV-02, 5AV-02) detected at Day 7 persisted and formed the majority variant in the virus population at Day 127 (Table 1).

Table 1: Genetic analysis (ORFs 1b, 5, 7) of 41 plaque-cloned PRRS viruses from one principal pig (11 clones) and its corresponding bioassay pig (30 clones) following 7 and 127 days of in vivo virus replication, respectively*

	Nucleotide variants (NVs)			Amino-acid variants (AVs)	
	Principal pig	Bioassay pig		Principal pig	Bioassay pig
ORF 1b			**ORF 1b**		
CC-01 genotype†	10	29	CC-01 genotype†	10	30
1NV-01	1	0	1AV-01	1	0
1NV-02	0	1			
ORF 5			**ORF 5**		
CC-01 genotype†	3	0	CC-01 genotype†	4	0
5NV-01	1	0	5AV-01	2	0
5NV-02	3	27	5AV-02	4	28
5NV-03	2	0	5AV-03	1	0
5NV-04	1	0	5AV-04	0	1
5NV-05	1	0	5AV-05	0	1
5NV-06	0	1			
5NV-07	0	1			
5NV-08	0	1			
ORF 7			**ORF 5**		
CC-01 genotype†	11	29	CC-01 genotype†	11	30
7NV-01	0	1			

* Principal pig was inoculated intranasally and intramuscularly with a PRRS virus isolate (ATCC VR-2332, designated CC-01) at 14-17 days of age (Day 0), and was euthanized Day 120. Tissue homogenates were similarly inoculated into a bioassay pig 14–17 days old. ORFs 1b, 5, and 7 were sequenced for all virus clones recovered from the serum of both pigs 7 days post inoculation, representing 7 and 127 days of replication in pigs.

† Identical to CC-01 (original inoculum).

ORF = open reading frame; PRRS = porcine reproductive and respiratory syndrome.

Sequencing showed that ORFs 1b and 7 were highly stable, even after 127 days of virus replication in pigs (Table 1). Comparisons of partial sequences (435 bases) of ORF 1b and complete sequences of ORF 7 (372 bases) revealed one ORF 1b NV, which corresponded to one AV, in one of 11 viral clones collected from the principal pig (Table 1). There was also one NV in the bioassay pig due to synonymous mutation. Results of ORF 7 sequencing were similar to those of ORF1b sequencing. Only one ORF 7 NV in one of the 30 clones was recovered from the bioassay pig after 127 days of pig replication, but its deduced amino-acid sequence was unchanged from the original CC-01 sequence (Table 1).

Collective sequence analysis. The 41 sequences of ORFs 1b, 5 and 7 were collectively jointed, aligned, and then compared to those of CC-01 to further characterize the genetic evolution of the virus during long-term replication in pigs. In total, 11 collective nucleotide variants and six collective amino-acid variants were identified in the principal and bioassay pigs (Table 3). Only one variant present at Day 7 in the principal pig was still detected in the bioassay pig, ie, most variants underwent negative selection, but this variant (CNV-03, CAV-03) persisted and formed the majority variant in the virus population after 127 days of in vivo replication.

Types and rates of mutation. Positions and types of mutation in ORFs 1b, 5, and 7, as well as the mean number of nucleotide substitutions, were determined by comparing each plaque-cloned virus to CC-01 (Tables 2 and 4). ORF 5 underwent more changes during replication in pigs than ORFs 1b and 7, ie, the mutation rate in ORF 5 was 0.166% at Day 7 and 0.182% at Day 127 at the nucleotide level. Substitution rates in the deduced amino-acid sequence were 0.364% at Day 7 and 0.533% after 127 days of in vivo replication (Table 5).

Assessment of antigenic changes

A panel of five MAbs specific for nucleocapsid and two specific for envelope proteins was used to detect possible changes in the epitopic profile of virus variants recovered from pigs. However, the reactivity of these MAbs to all 30 CC-01-derived clones and 41 swine-derived clones was identical. Thus, the observed amino-acid substitutions did not affect the epitopic profile represented by the panel of MAbs used in the study.

Discussion

The purpose of this experiment was to assess genetic and antigenic changes in PRRSV in individual, persistently infected pigs over the course of 120 days. We had previously reported successful pig-to-pig passage of PRRSV via tissue homogenate inoculation for ≥ 7 passes when the pig-passage interval was 60 days.[24] Variants recovered and analyzed over the course of the 60-day experiment revealed on-going

virus mutation and evolving genetic variation. We extended the pig-to-pig passage from 60 to 120 days in order to evaluate the effect of long-term immune pressure on PRRSV genetic and antigenic variation during replication in individual pigs.

In agreement with previous reports,[13,14,23,24,27-32] genetic changes occurred more frequently in ORF 5 than in ORFs 1b and 7, at both the nucleotide and amino-acid levels. Suggestive of random mutation, most nucleotide substitutions in ORFs 1b, 5, and 7 occurred at positions other than those reported earlier.[24] The degeneracy of collective variants at the nucleotide and amino-acid levels was most compatible with changes occurring in ORF 5. This observation is consistent with the concept that changes in ORF 1b and 7 were due to third-base degeneracy, whereas changes in ORF 5 nucleotides occurred most commonly in the first and second nucleotides of the codons, resulting in the substitution of amino-acid residues, ie, nonsynonymous mutations. A transversion substitution, ie, the change between purine and pyrimidine bases, was also observed at nucleotide position 443 of ORF 5 in one of 30 clones at Day 127.

Since ORF 5 encodes the major envelope protein,[13,30] the appearance of mutations in ORF 5, ie, the presence of multiple ORF 5 variants circulating concurrently within individual pigs, raises concerns regarding antigenic shift, virus-host interaction, and protective immunity. Six potential antigenic sites have been identified in the envelopes

Table 2: Mutations in ORFs 1b, 5, and 7 in 41 plaque-cloned PRRS viruses from one principal pig (11 clones) and its corresponding bioassay pig (30 clones) following 7 and 127 days of in vivo virus replication, respectively*

	ORF 1b (435 bases)		ORF 5 (603 bases)								ORF 7 (372 bases)
Nucleotide position	216	421	98	133	292	443	451	452	462	472	102
Nucleotide mutation†	A (G)	C (T)	A (G)	T (C)	A (G)	C (A)	G (A)	G (A)	T (C)	T (C)	C (T)
Amino-acid position	73	141	33	45	98	148	151	152	154	158	34
Amino-acid mutation‡	No	D (N)	N (S)	No	T (A)	T (N)	G (R)	G (R)	No	No	No
Recovered from principal pig	0	1	3	0	0	0	4	1	1	2	0
Recovered from bioassay pig	1	0	0	1	1	1	30	0	0	0	1

* Pigs inoculated and viruses sequenced as described in Table 1. Epitopic profiles of plaque-cloned viruses recovered from serum samples from the principal and bioassay pigs were determined using five MAbs against the N protein and two against the GP 5 protein in an indirect immunofluorescence assay.[24]

† A = adenine; C = cytosine; G = guanine; T = thymine.

‡ A = alanine; D = aspartic acid; G = glycine; N = asparagine; R = arginine; S = serine; T = threonine.

ORF = open reading frame; PRRS = porcine reproductive and respiratory syndrome; MAb = monoclonal antibody.

Table 3: Appearance of 11 collective nucleotide variants (CNV) and six collective amino-acid variants (CAV) of PRRSV from one principal pig and its corresponding bioassay pig following 7 and 127 days of in vivo virus replication, respectively*

CNVs	Principal pig	Bioassay pig	CAVs	Principal pig	Bioassay pig
CC-01 genotype†	3	0	CC-01 genotype†	4	0
CNV-01	1	0	CAV-01	1	0
CNV-02	1	0	CAV-02	2	0
CNV-03	2	25	CAV-03	3	28
CNV-04	2	0	CAV-04	1	0
CNV-05	1	0	CAV-05	0	1
CNV-06	1	0	CAV-06	0	1
CNV-07	0	1			
CNV-08	0	1			
CNV-09	0	1			
CNV-10	0	1			
CNV-11	0	1			

* Pigs inoculated, viruses sequenced, and epitopic profiles determined as described in Tables 1 and 2.

† Identical to PRRSV inoculum (CC-01) described in Table 1.

PRRSV = porcine reproductive and respiratory syndrome virus.

Table 4: Mean number (standard error of the mean) of nucleotide substitutions in 41 plaque-cloned PRRS viruses from one principal pig (11 clones) and its corresponding bioassay pig (30 clones) following 7 and 127 days of in vivo virus replication, respectively*

	ORF 1b		ORF 5		ORF 7	
Type of substitution†	**Principal pig**	**Bioassay pig**	**Principal pig**	**Bioassay pig**	**Principal pig**	**Bioassay pig**
Transition	0.09 (0.30)	0.03 (0.18)	1.09 (0.77)	1.07 (0.25)	0 (0)	0.03 (0.18)
Transversion	0 (0)	0 (0)	0 (0)	0.03 (0.18)	0 (0)	0 (0)

* Pigs inoculated and viruses sequenced as described in Table 1. Mean number of nucleotide substitutions were calculated comparing the CC-01 genotype to the recovered plaque-cloned viruses.

† Transition: substitution of one purine (adenine or guanine) for another purine, or a pyrimidine (cytosine, thymine or uracil) with another pyrimidine; transversion: substitution of a purine with a pyrimidine or vice versa.

PRRS = porcine reproductive and respiratory syndrome.

Table 5: Frequency of mutations in 41 plaque-cloned PRRS viruses from one principal pig (11 clones) and its corresponding bioassay pig (30 clones) following 7 and 127 days of in vivo virus replication, respectively*

ORF*	**Principal pig**		**Bioassay pig**	
	Nucleotide	**Amino acid**	**Nucleotide**	**Amino acid**
ORF 1b	2.09×10^{-4}	6.27×10^{-4}	7.67×10^{-5}	0
ORF 5	1.66×10^{-3}	3.64×10^{-3}	1.82×10^{-3}	5.33×10^{-3}
ORF 7	0	0	8.96×10^{-5}	0

* Pigs inoculated, viruses sequenced, and epitopic profiles determined as described in Tables 1 and 2. Frequency of mutations was calculated as the proportion of substitutions per plaque-cloned virus and was represented as mean percent change from the PRRS virus inoculum (CC-01) genotype at the nucleotide and amino-acid levels.

PRRS = porcine reproductive and respiratory syndrome.

of North American and European PRRSV isolates.[13,30] Moreover, analysis of PRRSV field isolates have shown that the variable regions are located near the N terminus (3-39) or C terminus (160-200), whereas the most conserved regions are centrally located.[13,30] It is believed that any change immediately before, within, or after conserved regions could alter the integrity and biological nature of the antigenic sites. These observations are relevant, because three of 11 clones collected at Day 7 from the principal pig demonstrated the substitution of a serine for an asparagine at position 33. The residues 33-35 constitute one of three glycosylation sites in PRRSV isolate VR-2332. Since this region also interacts with the neighboring M protein,[14,15,27,29-31,33-35] the loss or addition of a glycosylation site not only could result in a loss of an epitope, but could also alter the phenotypic pattern of the immune response.[10,36,37] Evidence to support this phenomenon has been widely described for lentiviruses.[36,37] For example, it was shown that a spontaneous point mutation in the V3 loop of human immunodeficiency virus type 1 resulted in the loss of a neutralization epitope.[36] Likewise, the substitution of a single amino acid in hypervariable region 5 of the envelope protein of feline immunodeficiency virus allowed it to escape from virus neutralization.[37]

The mutation rates and the mean numbers of nucleotide substitutions per plaque-cloned virus in ORFs 1b, 5, and 7 observed over 127 days of pig passage were not substantially different from those observed over 60 days of pig passage,[24] ie, the allowance for additional time and immune pressure did not accelerate the rate of virus change. The appearance of ORF 5 variants during in vivo replication suggests a potential role for mutation in viral persistence, but antigenic analyses based on a panel of anti-ORF 5 and 7 MAbs also did not detect the emergence of antigenically distinct mutants during the period studied. Furthermore, one of the collective variants observed in the study (CAV-03) was noteworthy for the fact that it was detected both early and late in the infection; indeed, it was the dominant variant at Day 127. The fact that this variant evolved early in the infection, persisted, and dominated the virus population at Day 127 suggests that on-going PRRSV mutation is selective but independent of host responses.

Overall, the cumulative genetic and antigenic results in this study suggest that PRRSV is able to maintain productive replication within persistently infected pigs through mechanisms that do not require significant viral genetic change, ie, virus genetic change is not a mechanism to evade the pig's immune response. Potential limitations of the study include the fact that entire PRRSV genomes were not sequenced, and a single, highly homologous PRRSV was used in a small number of pigs. However, two experiments (Chang et al[24] and the current study) were performed using the identical highly homologous PRRSV inoculum and identical methods. The two experiments differed only in the length of in vivo replication. Given the longer period of in vivo replication in the current study, it would be logical to expect greater diversity than reported in the earlier experiment if continuous mutation were a requirement for persistence, but this was not the case. From the perspective of hypothesis-driven research, the observation in even one pig that persistence was not dependent on mutation is sufficient to raise the question

of whether PRRSV mutation is a collateral effect of RNA replicative infidelity or serves a functional role in PRRSV "survival," either within the individual host or in the population. Regardless, it seems that future research should focus on understanding the viral mechanisms involved in persistence in the expectation that such knowledge would lead to more efficacious interventions.

Implications

- Persistent PRRSV infection over a period of 120 days without significant viral genetic or antigenic changes suggests that the virus does not mutate in response to immune pressure.
- Persistent PRRSV infection is not the result of on-going PRRSV evolution.

Acknowledgements

This study was supported in part by funding from National Pork Producers Council (Des Moines, Iowa) and the Iowa Livestock Health Advisory Council.

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