JOURNAL OF VIROLOGY, May 2002, p. 4750–4763 Vol. 76, No. 10
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.10.4750–4763.2002

Evolution of Porcine Reproductive and Respiratory Syndrome Virus during Sequential Passages in Pigs

C.-C. Chang,[1] K.-J. Yoon,[1]* J. J. Zimmerman,[1] K. M. Harmon,[1] P. M. Dixon,[2] C. M. T. Dvorak,[3] and M. P. Murtaugh[3]

Department of Veterinary Diagnostic and Production Animal Medicine[1] and Department of Statistics,[2] Iowa State University, Ames, Iowa 50010, and Department of Veterinary PathoBiology, University of Minnesota, St. Paul, Minnesota 55108[3]

Received 6 November 2001/Accepted 12 February 2002

Porcine reproductive and respiratory syndrome (PRRS) viruses are recognized as possessing a high degree of genetic and antigenic variability. Viral diversity has led to questions regarding the association of virus mutation and persistent infection in the host and has raised concerns vis-à-vis protective immunity, the ability of diagnostic assays to detect novel variants, and the possible emergence of virulent strains. The purpose of this study was to describe ongoing changes in PRRS virus during replication in pigs under experimental conditions. Animals were inoculated with a plaque-cloned virus derived from VR-2332, the North American PRRS virus prototype. Three independent lines of in vivo replication were maintained for 367 days by pig-to-pig passage of virus at 60-day intervals. A total of 315 plaque-cloned viruses were recovered from 21 pigs over the 367-day observation period and compared to the original plaque-cloned virus by virus neutralization assay, monoclonal antibody analysis, and sequencing of open reading frames (ORFs) 1b (replicase), 5 (major envelope protein), and 7 (nucleocapsid) of the genome. Variants were detected by day 7 postinoculation, and multiple variants were present concurrently in every pig sampled over the observation period. Sequence analysis showed ORFs 1b and 7 to be highly conserved. In contrast, sequencing of ORF 5 disclosed 48 nucleotide variants which corresponded to 22 amino acid variants. Although no epitopic changes were detected under the conditions of this experiment, PRRS virus was shown to evolve continuously in infected pigs, with different genes of the viral genome undergoing various degrees of change.

Porcine reproductive and respiratory syndrome (PRRS) first appeared as catastrophic, uncontrollable, clinical outbreaks in swine herds in North America, Europe, and Asia in the late 1980s and early 1990s (10, 13, 26, 57). The etiology of PRRS was established in 1991 when a previously unrecognized virus was identified as the causal agent (57).

PRRS virus (PRRSV) is a member of the family *Arteriviridae*, along with equine arteritis virus, lactate dehydrogenase-elevating virus of mice, and simian hemorrhagic fever virus (9, 48). PRRSV is enveloped and has a polyadenylated, single-stranded, nonsegmented, positive-sense RNA genome 15 kb in size (7, 14, 34, 35). The genome consists of eight open reading frames (ORFs) that are expressed through the production of a nested set of subgenomic 3′-coterminal mRNAs (14, 34, 35). ORF 1, which comprises two-thirds of the genome, encodes the viral RNA-dependent RNA polymerase (14, 37). ORFs 2 to 7 are postulated to encode structural proteins, but only three proteins have been consistently identified in virions and/or lysates of virus-infected cells. These three are the 15-kDa nucleocapsid (N), 19-kDa matrix (M), and 25-kDa envelope (E) glycoproteins that are encoded by ORFs 7, 6, and 5, respectively (41, 42). Proteins encoded by ORFs 2 to 4 are designated GP2, GP3, and GP4, where GP indicates glycoprotein and the number designates the ORF from which it is derived (36, 56).

PRRSV infection is prevalent in swine-producing regions throughout the world. Infection with PRRSV produces viremia with subsequent dissemination to and viral replication in multiple organs (50, 52). The virus replicates predominantly in cells of the monocyte/macrophage lineage (47, 50, 52, 57). Clinically, infection may range from totally inapparent to causing severe disease. Generally, clinical signs involve reproductive disorders in pregnant animals and/or respiratory disease in pigs of all ages (11, 20, 21, 23, 47, 51, 63). Reproductive disease in pregnant animals is manifested as late-term abortions or premature farrowings. Affected litters have higher proportions of stillbirths and piglets born weak and increased preweaning mortality (11, 23, 63).

PRRSV produces a persistent infection despite an active immune response (1, 6, 20, 27, 60). The PRRSV carrier state was first recognized in an experiment that documented the transmission of virus from animals infected 99 days earlier to commingled sentinel pigs (66). Subsequently, Wills et al. (60) reported the isolation of virus at up to 157 days postinoculation (p.i.). Persistence is an important epidemiological feature because it provides a ready means for PRRSV to perpetuate itself through a cycle of transmission from carrier to susceptible animals. As a consequence, the elimination of PRRSV from herds is difficult, and cyclic bouts of PRRSV-associated health problems are commonplace.

The mechanism(s) by which the virus persists in the host is not known, but PRRSV isolates are characterized by a high degree of genetic and antigenic variability. Persistence in the host and viral diversity could be two manifestations of the same function. Genetic analyses have shown the existence of at least two major virus genotypes, the European and the North American, with extensive genetic variability both within and between

* Corresponding author. Mailing address: Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011. Phone: (515) 294-1083. Fax: (515) 294-6619. E-mail: kyoon@iastate.edu.

these genotypes. Nelsen et al. (40) found important differences between the prototypic North American (VR-2332) and European (Lelystad) viruses in the 5′ leader sequence and parts of ORF 1a. Marked differences were also found between European and North American isolates in some structural genes (24, 39). ORF 7 is highly conserved among North American isolates, with 95 to 100% amino acid homology, but a comparison of North American viruses and the Lelystad virus revealed only 57 to 59% amino acid homology (32, 39). ORF 6 is the most conserved gene among North American isolates, with up to 100% amino acid identity, and is the most conserved gene between North American and European isolates, with 70 to 81% identity (24, 33, 39). The amino acid sequence homology for ORF 5 varies from 88 to 97% among North American isolates and from 51 to 59% when North American viruses are compared to the Lelystad virus (4, 24, 31, 39). A comparison of the Lelystad virus with isolate ATCC VR-2332, the North American prototype virus, revealed amino acid identities of 63, 58, and 68% for ORFs 2, 3, and 4, respectively (39). Similar results were reported when the Lelystad virus was compared with U.S. isolate VR-2385 (38).

Genetic diversity is mirrored in antigenic diversity among PRRSV isolates. Antigenic variation was initially demonstrated in a comparison of European and North American isolates. Using an immunoperoxidase monolayer assay, Wensvoort et al. (58) evaluated the reactivity of porcine polyclonal antibodies raised against either the Lelystad virus or North American isolate ATCC VR-2332 with PRRSV isolates from around the world. The investigators were able to differentiate European from North American isolates on the basis of differences in immunoperoxidase monolayer assay antibody titers. That is, significantly higher antibody titers were obtained in the homologous assay system. Later studies found even greater antigenic diversity among PRRSV isolates than initially suspected (5, 15, 17, 28, 64). Yoon et al. (64) examined 22 PRRSV isolates recovered from samples collected between 1989 and 1993 in eight different U.S. states. With a panel of five monoclonal antibodies (MAbs) specific for the N protein, the 22 virus isolates fell into one of three groups based on their reactivity patterns. Yang et al. (61) expanded this study to 70 North American isolates from samples collected between 1989 and 1995 and a panel of 23 MAbs against the N protein. Their results showed five antigenic groups, with the European Lelystad virus representing an antigenic group distinct from any of the North American groups identified. Furthermore, with antibodies against discontinuous epitopes of the N and M proteins and continuous epitopes of the E protein and GP3, the 65 North American isolates in the first and second antigenic groups (I_{15} and II_{15}) were further subdivided into nine and four antigenic subgroups, respectively (62).

That field isolates of PRRSV show a remarkable degree of genetic and antigenic variability has become abundantly evident. Understanding the source and degree of virus diversity is important because of its potential impact on viral virulence, persistence in the host, protective immunity, and even on the ability to diagnose the infection. The assumption is that these changes occur during the course of in vivo replication in swine and arise, in large part, because of errors that occur during RNA replication. However, the degree and rate of mutation of PRRSV in infected pigs over time are not known. To address this question, we examined the genetic and antigenic changes that occurred in a well-characterized PRRSV isolate during the course of multiple pig passages over a period of 367 days.

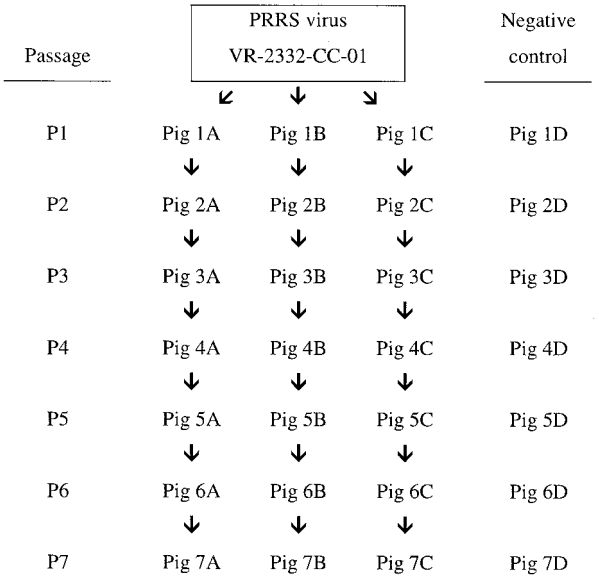

FIG. 1. Experimental design. The diagram illustrates three independent lines (A, B, and C) and seven pig passages (P1 to P7) of PRRSV. Pigs 1A, 1B, and 1C were inoculated with plaque-cloned PRRSV. Pigs in P2 to P7 were inoculated with tissue filtrates from the corresponding pig in the previous passage.

MATERIALS AND METHODS

Experimental design. The objective of the study was to characterize PRRSV mutations during prolonged in vivo replication. The experiment began by inoculating pigs free of PRRSV and virus-specific antibody with a highly homologous inoculum of a well-characterized PRRSV. Three independent lines (A, B, and C) of in vivo virus replication were established and monitored over seven animal passages (passage 1 [P1] to P7). Pigs in a fourth line (D) served both as mock-infected negative controls and as environmental sentinels (Fig. 1). Virus was periodically recovered from individual pigs in lines A, B, and C over a period of 367 days and compared genetically and antigenically to the original PRRSV.

In P1, three 14- to 21-day-old PRRSV-free pigs (1A, 1B, and 1C) were inoculated intranasally (1 ml per naris) and intramuscularly (8 ml per pig) with a 3×-plaque-cloned PRRSV designated VR-2332-CC-01 (see below) at a titer of 10^6 50% tissue culture infective doses ($TCID_{50}$) per ml. A fourth pig (1D) was inoculated with cell culture medium. Animals were housed in HEPA-filtered isolation units (Barrier Systems, Inc., Tom River, N.J.) to prevent exposure to extraneous viruses. For 60 days p.i., pigs were observed daily and serum samples were collected periodically for virological and serological assays. At 60 days p.i., the animals were euthanatized and whole blood in EDTA, samples of tissues (tonsils, lungs, spleen, and tracheobronchial and medial iliac lymph nodes), and bronchoalveolar lavage fluid were collected. For each subsequent passage after P1, four PRRSV-free pigs were randomly assigned to the two groups and inoculated as described above, except that tissue homogenate filtrate prepared from the postmortem samples collected from the pig in the previous passage was used in place of the cell culture-derived PRRSV inoculum. The use of tissue homogenate made it possible to expose animals directly to pig-adapted PRRSV and avoid any selective pressures arising from isolation and replication of the virus in cell cultures.

Viruses, cells, and media. The North American prototype PRRSV ATCC VR-2332 (American Type Culture Collection, Manassas, Va.) was used in the study. VR-2332 was selected because the complete genomic sequence has been published (GenBank accession no. PRU87392) (40) and the epitopic profile has been described (61, 62). The virus was propagated on MARC-145 cells, a clone of the African monkey kidney cell line MA-104 that is considered highly permissive to PRRSV (25). The cells were cultured and maintained in culture

medium composed of Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St Louis, Mo.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 50 μg of gentamicin (Sigma)/ml, and 0.25 μg of amphotericin B (Fungizone; Sigma)/ml.

To produce a highly homologous challenge virus, the stock virus was subjected to three rounds of plaquing on MARC-145 cells. Briefly, the stock virus was 10-fold serially diluted (i.e., 10^{-1} to 10^{-6}) with culture medium. Two milliliters of each dilution was inoculated into each well of a six-well plate (Corning Inc., Corning, N.Y.) containing 24-h-old confluent MARC-145 cells. Inoculated cells were incubated for 2 h at 37°C in a humidified 5% CO_2 incubator. At the end of the incubation period, the inocula were discarded and the cells were rinsed twice with culture medium and then covered with 4 ml of overlay medium composed of 0.2% agarose (FMC Bioproducts, Rockland, Maine), 10% fetal bovine serum, 50 μg of gentamicin/ml, and 0.25 μg of amphotericin B/ml in DMEM. The cells were incubated for 3 to 7 days at 37°C in a humidified 5% CO_2 atmosphere. During the incubation period, well-demarcated plaques were selected, suspended in 2 ml of culture medium, and then propagated once on MARC-145 cells. This process was repeated three times. Following the third round of plaquing, one virus clone, designated VR-2332-CC-01 (hereafter referred to as CC-01), was selected and propagated once on MARC-145 cells to obtain an amount sufficient for inoculating the pigs in P1. Surplus CC-01 was divided into aliquots and stored at −80°C for future use.

The same procedure was used to recover plaque-cloned virus isolates from pigs. That is, 15 plaque-cloned virus isolates were recovered from each of the three pigs in each of the seven passages by directly diluting the serum samples collected on day 7 p.i. with the procedure described above. The use of viremic serum samples made it possible to recover viruses directly from pigs and eliminated the requirement for virus isolation in cell cultures prior to plaque cloning. This process also served to reduce the selection effect of cell culturing on the recovery of plaque-cloned viruses. It was not expected that sequencing 15 clones from each pig and passage would identify all the unique nucleotide and amino acid sequences present in each pig. A rare sequence was unlikely to be included in the sample. However, if the true prevalence of a sequence exceeded 18%, then it was presumed with 95% or higher probability that we would be able to observe at least one clone of that sequence in our sample. Selected plaque-cloned viruses were propagated once on MARC-145 cells, divided into aliquots, and stored at −80°C until used. Fifteen plaque-cloned viruses were also obtained from the original virus inoculum to document the genetic and antigenic characteristics of CC-01, assess the variability of the virus population within the inoculum, and provide a baseline for comparisons.

Animals and animal care. Throughout the study, animals were housed and cared for in compliance with the requirements given in *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (17a).

For the study, crossbred pigs were obtained from a closed specific-pathogen-free herd known to be free of PRRSV. Pigs were weaned at 10 to 14 days of age, ear tagged, and randomly assigned to a treatment. Weaned pigs were group housed in HEPA-filtered isolation units for several days prior to exposure to virus and then individually housed in the isolation units from the time of exposure until the end of the observation period. The isolation units were equipped with internal flush mechanisms that allowed for disposal of waste products while maintaining a biosecure environment. Likewise, a sealed system permitted feeding without jeopardizing biosecurity. Contact with the external environment and other animals was eliminated to the greatest degree possible. Inoculations and sample collections were scheduled so that only one pig was removed from its isolation unit in any 24-h period. Immediately following inoculation or sample collection, the environment was cleaned and then disinfected with chlorhexidine diacetate disinfectant (Nolvasan Solution; Fort Dodge Laboratories, Fort Dodge, Iowa).

Biological samples. Blood samples were collected from all pigs on days 0, 7, 14, 21, 28, 35, and 60 p.i. with a single-use blood collection system (Vacutainer; Becton Dickinson, Franklin Lakes, N.J.). After 30 min at room temperature, the serum was harvested by centrifugation of the sample at 1,000 × *g* for 10 min. At day 60 p.i., pigs were euthanatized and necropsied. Whole blood, tonsils, tracheobronchial and medial iliac lymph nodes, spleen, lungs, and bronchoalveolar lavage fluid were collected from each animal. Whole blood samples were collected with a single-use blood collection system containing EDTA (Vacutainer) and processed immediately after sampling to obtain peripheral blood leukocytes (PBLs) as previously described (43). Briefly, whole blood in EDTA was centrifuged at 800 × *g* and 4°C for 30 min. The PBLs at the interface of the plasma and red blood cells were collected and resuspended in 3 ml of Hanks' balanced salt solution (HBSS; Sigma). The cell suspension was then layered over an equal volume of Histopaque-1077 (Sigma) and centrifuged at 1,200 × *g* and 4°C for 20 min. The PBLs at the interface of the upper medium layer and the lower erythrocyte layer were collected and then washed twice by resuspension in 10 ml of HBSS and centrifugation at 200 × *g* for 10 min. All samples were stored at −80°C.

To prepare inocula for P2 through P7, biological samples (tonsils, lymph nodes, spleen, lungs, PBLs, and lung lavage fluid) were suspended in cold HBSS at approximately 20% (wt/vol). The pool was homogenized in a Stomacher 80 (Seward, London, United Kingdom) for 2 min and then centrifuged at 4,000 × *g* for 30 min at 4°C. The supernatant was passed through a 0.2-μm-pore-size nonpyrogenic nitrocellulose membrane filter (Corning) and used to inoculate the next pig.

ELISA. A commercial enzyme-linked immunosorbent assay (ELISA) kit (IDEXX Laboratories, Inc., Westbrook, Mass.) for the detection of antibody specific for PRRSV was used by following the directions supplied by the manufacturer. According to the manufacturer, samples for which the ratio of net optical density of test sample to net optical density of positive control (S/P) is ≥0.4 are positive for antibody against PRRSV.

Virus titration. A microtitration infectivity assay was performed to assess the virus titer of the original inoculum, as well as the levels of PRRSV in serum samples collected over time from pigs in P1 through P7. Briefly, samples were 10-fold serially diluted (i.e., 10^{0} to 10^{-6}) in culture medium. One hundred microliters of each dilution was added to each of 3 wells of a 96-well microtitration plate (Corning) containing 24-h-old confluent MARC-145 cell monolayers. Inoculated cells were incubated at 37°C in a humidified 5% CO_2 incubator. Each sample was run in duplicate. The cells were monitored daily for cytopathic effects (CPE) for up to 7 days. If CPE were not evident, the cells were fixed with aqueous 80% acetone solution, dried, stained with fluorescein isothiocyanate-conjugated MAb SDOW17 (Rural Technologies, Brookings, S.Dak.), which is specific for the N protein of PRRSV, and visualized with fluorescence microscopy. The presence of PRRSV was determined based on the observation of virus-specific CPE and/or fluorescence reaction. Virus titers were determined by using the method described by Reed and Muench (49) and were expressed as $TCID_{50}$ per milliliter.

VN assay. A one-way virus neutralization (VN) assay with constant antibody and various virus concentrations was performed as described previously (62) to compare the relative susceptibilities of pig-derived plaque-cloned virus isolates to the neutralizing activity of serum and to screen for potential escape mutant viruses. A total of 105 plaque-cloned viruses were examined in the one-way VN assay, i.e., 15 clones from the CC-01 inoculum, 45 clones from P1 (15 clones from each of three pigs), and 45 clones from P7 (15 clones from each of three pigs). The P1 clones represented 7 days and the P7 clones represented 367 days of in vivo replication.

To perform the assay, virus clones were 10-fold serially diluted in DMEM supplemented with 50 μg of gentamicin/ml and 0.25 μg of amphotericin B/ml. Serum collected from each of three pigs (1A, 1B, and 1C) at the termination of P1 (day 60 p.i.) served as the source of antibody. The assay was performed in duplicate for each combination of antibody source and virus clone. Serum samples were heat inactivated at 56°C for 45 min and then diluted 1:2. Fifty microliters of each diluted virus clone was mixed with an equal volume of diluted serum. An additional 50 μl of each diluted virus clone mixed with an equal volume of cell culture medium instead of antiserum served as an untreated control. All mixtures were incubated for 1 h at 37°C, and then 100 μl of each mixture was added to 96-well plates containing 24-h-old confluent MARC-145 cell monolayers in the same manner as that described above for virus titration. Inoculated cells were incubated for up to 7 days. At the end of the 7-day incubation period, the virus titer of each clone was calculated for each plaque clone in the presence and absence of antiserum and was expressed as $TCID_{50}$ in log_{10} units. The relative susceptibility of plaque-cloned viruses was calculated with the following equation and was expressed as the VN index: $[(TCID_{50}$ without antiserum − $TCID_{50}$ with antiserum)/$TCID_{50}$ without antiserum$] \times 100$.

MAb analysis. The epitopic profile of each of 150 plaque-cloned viruses, i.e., 15 clones from the CC-01 inoculum, 45 clones from P1 (day 7 of in vivo replication), 45 clones from P2 (day 67 of in vivo replication), and 45 clones from P7 (day 367 of in vivo replication) was determined with a panel of MAbs in an indirect immunofluorescence assay (61). The panel consisted of five MAbs against the N protein and two against the E protein. The production and characterization of the MAbs have been described elsewhere (61, 62).

To prepare PRRSV-infected cell monolayers, 24-h-old confluent MARC-145 cells in 96-well plates were inoculated with optimally diluted virus. Procedures for control wells were identical except that the cells were not inoculated with virus. At 24, 48, or 72 h, plates were fixed by immersion in aqueous 80% acetone solution for 10 min, air dried, and stored at −20°C until used. MAbs used in the assay were optimally diluted with 0.01 M phosphate-buffered saline (PBS, pH 7.2). Individual wells received 40 μl of each MAb dilution, after which plates

TABLE 1. Primers used for PCR and sequencing

Primer	Sequence	Location (nucleotides)[a]
8100	5′-CTGACTGCCCTAAACAGCTGAC-3′	8346–8367
VRBP2	5′-CAGATGTTCAACCCACCAGT-3′	9259–9239
RESP1BP.1	5′-CATCGCACTAGCCCACCGAGCAGTG-3′	8713–8737
P5F	5′-CCTGAGACCATGAGGTGGG-3′	13696–13714
P5R	5′-TTTAGGGCATATATCATCACTGG-3′	14459–14437
PS1	5′-AGTAGCATCTACGCGGTCTGTGCC-3′	14093–14116
PS1R	5′-CACAGACCGCGTAGATGCTACT-3′	14114–14093
P7F	5′-TCGTGTTGGGTGGCAGAAAAGC-3′	14816–14837
P7R	5′-GCCATTCACCACACATTCTTCC-3′	15300–15279

[a] From GenBank accession no. PRU87392 (40).

were incubated at 37°C for 45 min in a humid environment. After the MAb solution was discarded, plates were washed three times with PBS. To visualize the presence of antigen-antibody complexes, each well received 40 μl of 1:100-diluted goat anti-mouse immunoglobulin G conjugated to fluorescein isothiocyanate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), after which plates were incubated for 45 min in a humid environment at 37°C. Plates were then washed three times with PBS and air dried. The reactivity of each MAb with each virus clone was evaluated by using fluorescence microscopy.

RT-PCR and sequencing. A total of 150 plaque-cloned viruses were sequenced for ORFs 1b and 7, i.e., 15 clones from the CC-01 inoculum, 45 clones from P1 (day 7 of in vivo replication), 45 clones from P2 (day 67 of in vivo replication), and 45 clones from P7 (day 367 of in vivo replication). A total of 330 virus clones were sequenced for ORF 5, including 15 clones from the CC-01 inoculum and 45 plaque-cloned viruses collected from each of the seven passages ($n = 315$). Fifty plaque-cloned viruses from the CC-01 inoculum were included in each sequencing reaction to assess the homogeneity of the inoculum and any random mutation(s) which could be introduced during reverse transcription (RT)-PCRs or sequencing reactions.

Viral RNA for RT-PCR amplification and sequencing was extracted from each plaque-cloned virus with a QIAamp viral RNA minikit (Qiagen Inc., Valencia, Calif.) by following the protocols recommended by the manufacturer. Viral RNA was collected and stored at −80°C until used.

The RT-PCR of ORF 1b was performed with 5 μl of extracted RNA by using a Qiagen OneStep RT-PCR kit according to the manufacturer's protocol and primers 8100 and VRBP2 (Table 1). RT was performed for 30 min at 50°C. Reverse transcriptase was inactivated and *Taq* polymerase was activated by raising the temperature to 95°C for 15 min. PCR amplification was achieved with 35 cycles of annealing at 57°C for 30 s, extension at 72°C for 45 s, and denaturation at 94°C for 45 s. Products were polished by incubation at 72°C for 7 min.

For ORF 5 RT-PCR, 10 μl of extracted RNA template was added to a tube containing 50 μl of 2× reaction mixture (0.4 mM each dNTP and 2.4 nM $MgSO_4$), 2 μl of RT-*Taq* mixture, and 34 μl of RNase-free H_2O (SuperScript one-step RT-PCR with Platinum *Taq*; Life Technologies, Grand Island, N.Y.); 2 μl of Prime RNase inhibitor (Brinkmann, Westburg, N.Y.); 1 μl (5 pmol) of primer P5F; and 1 μl (5 pmol) of primer P5R (Table 1). The RT-PCR was performed with a thermocycler (GeneAmp PCR system 2400; PE Biosystems, Foster City, Calif.) at 50°C for 30 min and 92°C for 2 min; then, 35 cycles of denaturation at 92°C for 15 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s followed. After the last cycle, the extension period was maintained at 72°C for another 2 min, and then all final products were stored at 4°C until used. To confirm the positive reaction, 2 μl of the final products was separated by electrophoresis in a 1% agarose gel (Amresco Inc., Solon, Ohio) containing 0.1% ethidium bromide in Tris-borate-EDTA buffer. The positive products were visualized and photographed under UV light, and the remaining products were stored at 4°C until used.

The ORF 7 RT-PCR procedure was identical to that described above for ORF 5 except for the use of a pair of sense (P7F) and antisense (P7R) primers (Table 1), designed as previously reported (12), and 58°C instead of 50°C for the annealing process.

In every round of RT-PCR, RNA extracted from reference PRRSV was used as a positive control and mock-infected cell culture fluid was used as a negative control. The final RT-PCR products were purified with either QIAquick or 96-well PCR purification kits (Qiagen) by following the procedures recommended by the manufacturer. The final purified product was collected and stored at 4°C until sequenced.

Sequencing reactions for ORF 1b were carried out at the University of Minnesota Advanced Genetic Analysis Center (St. Paul) by using 4 μl of purified PCR product and 3.2 pmol each of primers RESP1BP.1 and VRBP2 (Table 1). Sequencing of ORF 5 and ORF 7 was performed at the Iowa State University Nucleic Acid Facility (Ames) by using the appropriate amount of purified PCR product (30 μg/μl) and 5 pmol of each primer. Primers P5F, P5R, PS1, and PS1R were used for ORF 5 and primers P7F and P7R were used for ORF 7 (Table 1). For all three ORFs, pairs of primers were primed from the positions before and after the target sequences. However, for ORF 5, PS1 and PS1R were also used to increase sequence accuracy and were primed from the middle of ORF 5 in both directions.

Analysis of data. ELISA S/P values were analyzed by fitting a regression model to longitudinal data (16). An appropriate model for the time course in each pig was chosen by using plots of the results and observations of antibody responses. A simple model that fit the data from day 7 through day 60 was ELISA S/P = $\alpha + \beta \log(\text{day}) + \varepsilon$. Differences among lines and generations were tested separately for each parameter of the model.

Viremia data from days 7 to 35 were analyzed by analysis of variance and by longitudinal regression methods. Data for days 0 and 60 were excluded because no virus was detected at those times. All analyses were done with the average of two replicate determinations of $\log_{10}$ $TCID_{50}$ for each pig and day. The analysis of variance was done with a split-plot analysis to account for the repeated measures across days. However, because there was a significant three-way interaction among passage, lines (A, B, and C), and day p.i., longitudinal data analysis (16) was used to find a simpler model for the time course of viremia in each pig. A quadratic regression equation, $\log_{10}$ $TCID_{50} = \alpha + \beta\,\text{day} + \gamma\,\text{day}^2 + \varepsilon$, was fit to data from each pig. In the model, α was an intercept that was the same for all groups, β was the slope of the line, ε was the error associated with each observation, and γ was the quadratic coefficient. The day of peak viremia (D_{peak}) was estimated for each pig from the quadratic regression coefficients as follows: $D_{peak} = -\beta/2\gamma$. Differences among lines and generations were tested separately for each parameter.

Data from the VN assay were summarized by using the mean VN index for each combination of isolate and P1 antiserum. An escape mutant was considered to be a virus clone with a significantly smaller mean VN index.

Sequence alignment, comparisons, and phylogenetic analyses were carried out by using computer software from DNASTAR Inc., Madison, Wis. Nucleotide sequences of VR-2332 (40) and the RespPRRS vaccine strain (GenBank accession no. AF159149) (2) were obtained and included in the sequence analyses for comparative purposes. Like the RespPRRS vaccine strain, CC-01 is derived from VR-2332. Nucleotide mutation rates and amino acid sequence changes over the course of the experiment were compared to those of CC-01. The mutation rates were calculated as the proportion of substitutions and were expressed as mean percent change separately for ORFs 1b, 5, and 7 per plaque-cloned virus. Phylogenetic trees representing amino acid variations in the E protein (ORF 5) and the polymerase (ORF 1b), GP5, and the N protein combined were computed by using the Hein method with the PAM250 weight table (22).

RESULTS

Clinical observations, viremia, and antibody responses. HEPA-filtered housing units, necessary to protect pigs from exposure to extraneous viruses, barred the investigators from contact with animals, except as required for sample collection. For that reason, clinical observations were based on percepti-

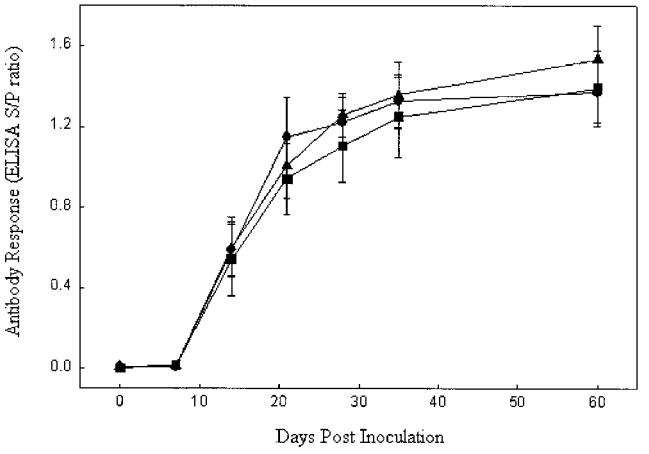

FIG. 2. Antibody response to PRRSV infection. Values represent means and standard deviations of ELISA S/P ratios in line A (●), line B (▲), and line C (■) over all passages (P1 to P7).

ble changes in behavior, rather than direct examination. Following inoculation with cell culture-derived virus or tissue homogenate filtrates from inoculated animals, all pigs in the experimental group exhibited mild to moderate lethargy and anorexia, occasionally with dyspnea, beginning on day 2 and occasionally lasting up to day 10 p.i. All pigs appeared clinically normal thereafter. No remarkable or consistent difference in the severity of disease was observed between passages.

ELISA-detectable antibody responses and PRRS viremia in successive passages (P2 to P7) showed that inoculation of pigs with tissue homogenate filtrates resulted in transmission of the infection. All pigs in lines A, B, and C showed similar humoral immune responses, as measured by the ELISA (Fig. 2). No statistically significant differences were found in the ELISA responses among lines A, B, and C for any sampling time.

All pigs in lines A, B, and C became viremic following inoculation (Fig. 3). The data for individual pigs were fit to a quadratic equation, and the day of peak viremia was estimated

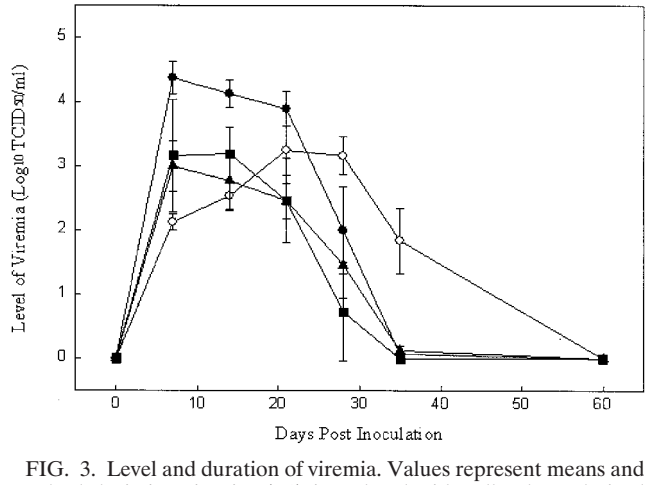

FIG. 3. Level and duration of viremia. Values represent means and standard deviations in pigs (P1) inoculated with cell culture-derived PRRSV (○) and pigs (P2 to P7) inoculated with pig-passaged virus in line A (●), line B (▲), and line C (■).

TABLE 2. Estimated day of peak viremia by line and passage

Passage	Day p.i. at which peak viremia occurred in line:		
	A	B	C
1	18.8	23.4	20.9
2	4.1	8.7	6.2
3	6.4	10.9	8.5
4	6.9	11.4	9.0
5	4.3	8.9	6.4
6	9.0	13.5	11.1
7	8.2	12.7	10.3

for each passage and line (Table 2). Statistical analyses showed that the responses were not the same among all lines or passages. Comparisons of the slopes of lines A, B, and C showed a significant difference in the viremia pattern between lines A and B ($P = 0.0057$) but no difference between lines A and C ($P = 0.19$) or lines B and C ($P = 0.13$). Specific comparisons between passages also showed a highly significant difference ($P < 0.0001$) between the slope of the line for P1 and the mean slope for P2 through P7. A higher level of PRRS viremia quickly developed in pigs that received tissue homogenate from P1, whereas viremia at a lower level but for a much longer duration was observed in pigs inoculated with CC-01. This result suggested an adaptive difference between cell culture-derived PRRSV and pig-passaged PRRSV.

All pigs in line D, i.e., negative controls and environmental sentinels, remained free of PRRSV and virus-specific antibody throughout the experiment. These results provided evidence that biosecurity procedures effectively prevented the inadvertent transmission of PRRSV among pigs.

Genetic characterization of the CC-01 inoculum. Partial ORF 1b sequences (435 bases), complete ORF 5 nucleotide sequences (603 bases), and complete ORF 7 nucleotide sequences (372 bases) were obtained for the 15 plaque-cloned viruses derived from the CC-01 inoculum. The ORF 1b, 5, and 7 sequences of all 15 clones were identical during repeated sequencing attempts. This result demonstrated the homogeneity of the original virus population, proved that no random mutation was introduced during PCR and sequencing, and provided a baseline for comparing and contrasting virus clones recovered in later passages.

Relative to VR-2332, the ORF 5 nucleotide sequence of CC-01 differed at three positions: 38 (G to A), 252 (C to T), and 451 (A to G). These changes resulted in amino acid substitutions at two residues: 13 (arginine to glutamine) and 151 (arginine to glycine). Compared to the RespPRRS vaccine virus, the ORF 5 sequence of CC-01 had one synonymous nucleotide change at position 252 (C to T). In contrast, the ORF 1b nucleotide sequence of CC-01 was identical to those of VR-2332 and the RespPRRS vaccine virus. The ORF 7 nucleotide sequences of VR-2332 and the RespPRRS vaccine virus were identical, but CC-01 differed in two synonymous mutations at positions 30 (G to T) and 345 (C to T).

Assessment of genetic changes. (i) Sequence analysis of PRRSV ORF 5. Complete ORF 5 nucleotide sequences were obtained for 315 swine-derived virus clones (15 clones per pig, three pigs per passage, and seven passages). These clones represented 7, 67, 127, 187, 247, 307, and 367 days of in vivo

TABLE 3. Chronological appearance of 48 5NVs within three independent lines and over seven pig passages

Genotype	No. of plaque-cloned viruses identical to each genotype within the following passage and line[a]:																				
	P1			P2			P3			P4			P5			P6			P7		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
CC-01[b]	5	12	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-01	7	—	5	10	—	13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-02	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-03	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-04	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-05	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-06	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-07	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-08	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-09	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-10	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-11	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-12	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-13	—	—	—	—	9	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-14	—	—	—	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-15	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-16	—	—	—	—	—	—	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-17	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-18	—	—	—	—	—	—	—	13	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-19	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-20	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
5NV-21	—	—	—	—	—	—	—	—	8	—	—	—	—	—	—	—	—	—	—	—	—
5NV-22	—	—	—	—	—	—	—	—	7	—	—	—	—	—	—	—	—	—	—	—	—
5NV-23	—	—	—	—	—	—	—	—	—	10	—	—	13	—	—	14	—	—	10	—	—
5NV-24	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
5NV-25	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
5NV-26	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
5NV-27	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
5NV-28	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
5NV-29	—	—	—	—	—	—	—	—	—	—	12	—	—	—	—	—	—	—	—	—	—
5NV-30	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
5NV-31	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
5NV-32	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
5NV-33	—	—	—	—	—	—	—	—	—	—	—	13	—	—	—	—	—	—	—	—	—
5NV-34	—	—	—	—	—	—	—	—	—	—	—	2	—	—	15	—	—	—	—	—	—
5NV-35	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
5NV-36	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
5NV-37	—	—	—	—	—	—	—	—	—	—	—	—	—	15	—	—	14	—	—	13	—
5NV-38	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—
5NV-39	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—
5NV-40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	14	—	—	—
5NV-41	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—
5NV-42	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	—
5NV-43	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
5NV-44	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
5NV-45	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—
5NV-46	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—
5NV-47	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	14
5NV-48	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1

[a] Within passages, A, B, and C each represent one pig from which 15 plaque-cloned viruses were collected. —, no identical virus was found.
[b] CC-01 is the original virus inoculum in P1.

replication in P1 through P7, respectively. As shown in Table 3, three or more ORF 5 nucleotide variants (5NVs) were detected in every pig beginning as early as day 7 p.i. Six 5NVs were recovered from one pig. Among the 315 virus clones, 291 differed from CC-01 in the ORF 5 nucleotide sequence. In fact, no clone with 100% homology to the CC-01 ORF 5 sequence was detected after P1. In total, 48 5NVs, each containing from one to eight nucleotide substitutions, were observed. No nucleotide insertions or deletions were detected.

Nucleotide mutations producing amino acid substitutions were observed in 289 of the 315 clones (Table 4). The amino acid sequences of the remaining 26 virus clones were identical to that of CC-01. No virus clone with the original CC-01 amino acid sequence was detected after P1. A total of 22 ORF 5 amino acid variants (5AVs) were observed, with as many as five 5AVs being detected in a single pig.

At both the nucleotide and the amino acid levels, ORF 5 continued to mutate over the course of the 367-day observation period. The majority of variants were transient, i.e., appeared and then disappeared from later passages. The most

TABLE 4. Chronological appearance of 22 5AVs within three independent lines and over seven pig passages

Phenotype	No. of plaque-cloned viruses identical to each phenotype within the following passage and line[a]:																				
	P1			P2			P3			P4			P5			P6			P7		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
CC-01[b]	5	14	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5AV-01	8	—	5	13	—	14	15	—	—	14	—	—	14	—	—	15	—	—	10	—	—
5AV-02	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5AV-03	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5AV-04	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5AV-05	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5AV-06	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5AV-07	—	—	—	—	9	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5AV-08	—	—	—	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5AV-09	—	—	—	—	—	—	—	14	—	—	1	—	—	—	—	—	—	—	—	—	—
5AV-10	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
5AV-11	—	—	—	—	—	—	—	—	15	—	—	—	—	—	—	—	—	—	—	—	—
5AV-12	—	—	—	—	—	—	—	—	—	1	—	2	—	—	15	—	—	—	—	—	—
5AV-13	—	—	—	—	—	—	—	—	—	—	12	—	—	15	—	—	15	—	—	13	—
5AV-14	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—
5AV-15	—	—	—	—	—	—	—	—	—	—	—	13	—	—	—	—	—	—	—	—	—
5AV-16	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
5AV-17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15	—	—	—
5AV-18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—
5AV-19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
5AV-20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—
5AV-21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—
5AV-22	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15

[a] See Table 3, footnote *a*.
[b] See Table 3, footnote *b*.

notable exception to this trend was 5AV-01, which was detected in line A at 7, 67, 127, 187, 247, 307, and 367 days of passage. No variant "skipped" a passage(s), i.e., appeared, went undetected in a subsequent passage(s), and appeared in a later passage(s).

The ORF 5 sequences of CC-01, VR-2332, the RespPRRS vaccine virus, and the 22 amino acid variants were aligned and compared (Fig. 4). Among the 22 variants, no specific "hot spots" were identified, except at position 151 (glycine to arginine). Most amino acid substitutions due to nucleotide changes were observed in amino acid residues located between residues 10 and 34. Eleven of the 22 5AVs had an amino acid substitution at either residue 33 (asparagine to serine) or residue 34 (asparatic acid to asparagine or serine), which is part of the ectodomain of GP5. An amino acid substitution at residue 33 abolished a postulated variable N-linked glycosylation site.

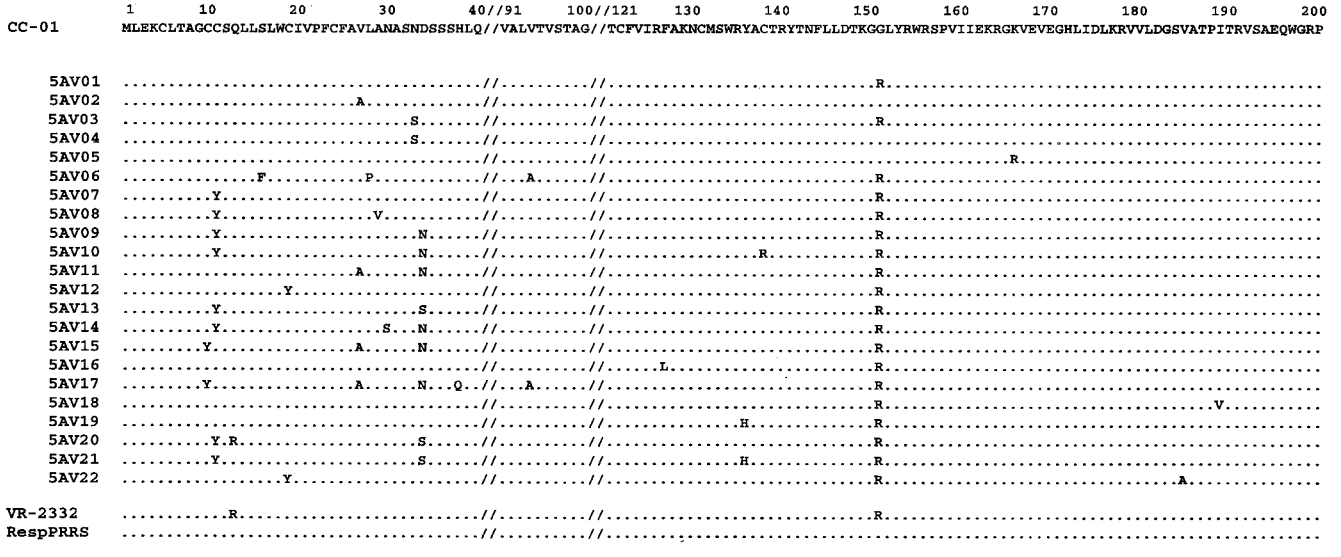

FIG. 4. GP5 amino acid sequence comparisons of CC-01, 22 amino acid variants, VR-2332, and the RespPRRS vaccine virus. Dots indicate bases identical to those in CC-01, and letters indicate amino acid substitutions. Positions 41 to 90 and 101 to 120 have been truncated.

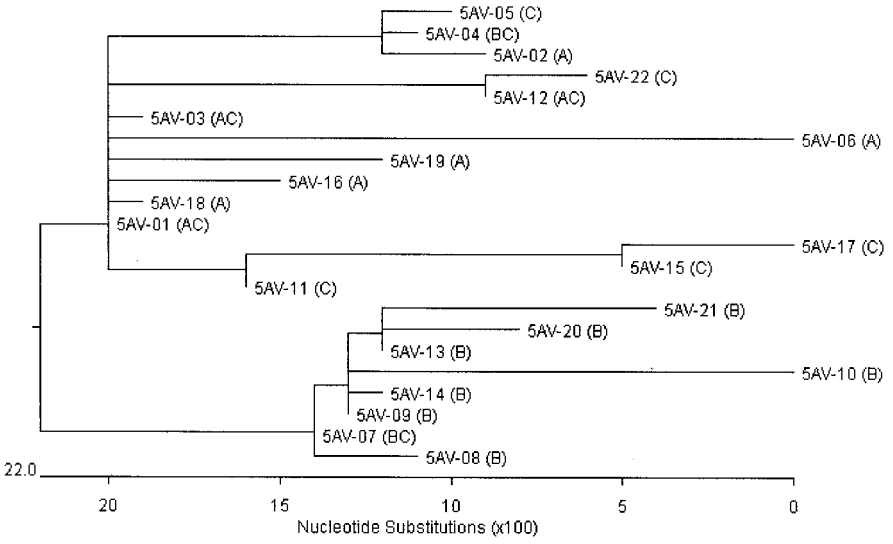

FIG. 5. Phylogenetic relationship of 22 PRRSV 5AVs detected during 367 days of in vivo replication. Letters in parentheses represent the lines (A, B, and C) of pig passages in which the variant was recovered. Lines represent the distances of sequence divergence from the ancestor (CC-01).

Phylogenetic analysis (Fig. 5) revealed two distinct branches of evolution among the 22 variants, with 8 of 9 variants from line B on one branch and 13 of 14 variants from lines A and C on the other branch.

(ii) Sequence analysis of PRRSV ORFs 1b and 7. ORF 1b (polymerase domain) and ORF 7 (N protein) sequencing was attempted with a total of 135 plaque-cloned viruses, i.e., 15 clones from each of three pigs in P1 (day 7 of in vivo replication), P2 (day 67 of in vivo replication), and P7 (day 367 of in vivo replication). Partial sequences (435 bases) of ORF 1b were obtained for 149 clones, including 15 clones from CC-01.

TABLE 5. Appearance of 16 1NVs within three independent lines and in three pig passages

Genotype	No. of plaque-cloned viruses identical to each genotype within the following passage and line[a]:								
	P1			P2			P7		
	A	B	C	A[b]	B	C	A	B	C
CC-01[c]	14	15	15	2	13	6	—	—	—
1NV-01	1	—	—	—	—	—	—	—	—
1NV-02	—	—	—	9	—	—	—	—	—
1NV-03	—	—	—	1	—	—	—	—	—
1NV-04	—	—	—	2	—	—	—	—	—
1NV-05	—	—	—	—	1	—	—	—	—
1NV-06	—	—	—	—	1	—	—	—	—
1NV-07	—	—	—	—	—	6	—	—	—
1NV-08	—	—	—	—	—	1	—	—	—
1NV-09	—	—	—	—	—	2	—	—	—
1NV-10	—	—	—	—	—	—	6	—	—
1NV-11	—	—	—	—	—	—	3	—	—
1NV-12	—	—	—	—	—	—	5	—	—
1NV-13	—	—	—	—	—	—	1	—	—
1NV-14	—	—	—	—	—	—	—	14	—
1NV-15	—	—	—	—	—	—	—	1	—
1NV-16	—	—	—	—	—	—	—	—	15

[a] See Table 3, footnote *a*.
[b] Sequencing was successful for 14 of the 15 clones.
[c] See Table 3, footnote *b*.

ORF 1b sequencing was not successful for one clone from line A in P2. ORF 7 nucleotide sequences were obtained for all 135 plaque-cloned viruses.

Relative to CC-01, a total of 16 ORF 1b nucleotide variants (1NVs) (Table 5) were observed, each containing from one to four nucleotide changes. In P1, only one variant was detected among 45 virus clones, but all 45 virus clones from P7 were variants. All nucleotide changes were randomly distributed, and only one ORF 1b amino acid variant (1AV) was found (Table 6). Specifically, the mutation at position 207 (T to C) in one virus clone recovered from line A in P7 resulted in an amino acid substitution (valine to alanine). The remaining nucleotide mutations were synonymous.

ORF 7 nucleotide sequencing revealed no changes in 107 of the 135 pig-derived virus clones compared to CC-01. Nine ORF 7 nucleotide variants (7NVs) were detected among the 28 remaining clones (Table 7), with each 7NV containing one or two nucleotide changes at various positions. Analysis of the sequences revealed that amino acid substitutions corresponding to nucleotide mutations in 8 of the 28 clones resulted in three ORF 7 amino acid variants (7AVs) (Table 8). Amino acid substitutions in the three amino acid variants occurred at residue 15 (asparagine to lysine), 128 (glutamine to arginine),

TABLE 6. Appearance of one 1AV within three independent lines and in three pig passages

Phenotype	No. of plaque-cloned viruses identical to each phenotype within the following passage and line[a]:								
	P1			P2			P7		
	A	B	C	A[b]	B	C	A	B	C
CC-01[c]	15	15	15	14	15	15	14	15	15
1AV-01	—	—	—	—	—	—	1	—	—

[a] See Table 3, footnote *a*.
[b] Sequencing was successful for 14 of the 15 clones.
[c] See Table 3, footnote *b*.

TABLE 7. Appearance of nine 7NVs within three independent lines and in three pig passages

Genotype	No. of plaque-cloned viruses identical to each genotype in the following passage and line[a]:								
	P1			P2			P7		
	A	B	C	A	B	C	A	B	C
CC-01[b]	15	15	13	13	14	13	9	15	—
7NV-01	—	—	1	—	—	—	—	—	—
7NV-02	—	—	1	—	—	—	—	—	—
7NV-03	—	—	—	1	—	—	—	—	—
7NV-04	—	—	—	1	—	—	—	—	—
7NV-05	—	—	—	—	1	—	—	—	—
7NV-06	—	—	—	—	—	1	—	—	—
7NV-07	—	—	—	—	—	1	—	—	—
7NV-08	—	—	—	—	—	—	6	—	—
7NV-09	—	—	—	—	—	—	—	—	15

[a] See Table 3, footnote *a*.
[b] See Table 3, footnote *b*.

or 239 (lysine to arginine). The disappearance of 7AV-01 and 7AV-02 from subsequent passages suggests that these variants underwent negative selection.

(iii) Collective sequence analysis. The genetic variation and evolution of CC-01 during animal passages were further characterized by combining the sequence data for ORFs 1b, 5, and 7. Among the 45 virus clones recovered from P1, 44 from P2, and 45 from P7, a total of 40 combination nucleotide variants (CNVs) and 19 combination amino acid variants (CAVs) were identified (Tables 9 and 10). Thirteen CNVs and 9 CAVs were detected among the 45 virus clones recovered at P7, i.e., after a total of 367 days of in vivo replication. Most variants underwent negative selection and disappeared from succeeding passages, but CAV-01 was detected in each line A pig in P1, P2, and P7. Phylogenetic analysis (Fig. 6) showed that these CAVs followed two distinct evolutionary directions. Similar to what was observed for ORF 5, of the 19 CAVs, 5 of 6 variants from line B were on one branch, while 12 of 13 variants from lines A and C were on the other.

(iv) Rates and types of mutations. All sequences were compared to the CC-01 sequence, and the mutation rates for ORFs 1b, 5, and 7 were calculated (Fig. 7). A very gradual increase in nucleotide changes occurred in ORF 1b (435 bases) of PRRSV over seven animal passages (Fig. 7, top). At the termination of the study, the mean percentage of nucleotide changes among 45 plaque-cloned viruses of P7 was 0.67% compared to the data for CC-01. However, most of the nucleotide changes were synonymous mutations, i.e., no amino acid substitutions. The ORF 7 gene (372 bases) of PRRSV was also conserved during serial animal passages (Fig. 7, bottom). In comparison to the data for CC-01, the mean percentages of changes among virus clones were 0.01, 0.03, and 0.13% at the nucleotide level and 0.02, 0.02, and 0.11% at the amino acid level for P1, P2, and P7, respectively.

The ORF 5 gene (603 bases) of PRRSV showed higher rates of change during animal passages (Fig. 7, middle). The mean percentages of changes in nucleotides were 0.09, 0.29, 0.53, 0.69, 0.61, 0.89, and 0.70% for P1 through P7, respectively. Most of the nucleotide changes in ORF 5 were nonsynonymous mutations, resulting in substitutions of amino acid resi-

TABLE 8. Appearance of three 7AVs within three independent lines and in three pig passages

Genotype	No. of plaque-cloned viruses identical to each genotype in the following passage and line[a]:								
	P1			P2			P7		
	A	B	C	A	B	C	A	B	C
CC-01[b]	15	15	14	15	15	14	9	15	15
7AV-01	—	—	1	—	—	—	—	—	—
7AV-02	—	—	—	—	—	1	—	—	—
7AV-03	—	—	—	—	—	—	6	—	—

[a] See Table 3, footnote *a*.
[b] See Table 3, footnote *b*.

TABLE 9. Appearance of 40 CNVs within three independent lines and in three pig passages

Genotype	No. of plaque-cloned viruses identical to each genotype in the following passage and line[a]:								
	P1			P2			P7		
	A	B	C	A[b]	B	C	A	B	C
CC-01[c]	5	12	6	—	—	—	—	—	—
CNV-01	6	—	4	1	—	5	—	—	—
CNV-02	1	—	—	—	—	—	—	—	—
CNV-03	1	—	1	—	—	—	—	—	—
CNV-04	1	—	—	—	—	—	—	—	—
CNV-05	1	—	—	—	—	—	—	—	—
CNV-06	—	1	—	—	—	—	—	—	—
CNV-07	—	1	1	—	—	—	—	—	—
CNV-08	—	1	—	—	—	—	—	—	—
CNV-09	—	—	1	—	—	—	—	—	—
CNV-10	—	—	1	—	—	—	—	—	—
CNV-11	—	—	1	—	—	—	—	—	—
CNV-12	—	—	—	8	—	—	—	—	—
CNV-13	—	—	—	1	—	—	—	—	—
CNV-14	—	—	—	1	—	—	—	—	—
CNV-15	—	—	—	1	—	—	—	—	—
CNV-16	—	—	—	1	—	—	—	—	—
CNV-17	—	—	—	1	—	—	—	—	—
CNV-18	—	—	—	—	6	—	—	—	—
CNV-19	—	—	—	—	6	1	—	—	—
CNV-20	—	—	—	—	1	—	—	—	—
CNV-21	—	—	—	—	1	—	—	—	—
CNV-22	—	—	—	—	1	—	—	—	—
CNV-23	—	—	—	—	—	5	—	—	—
CNV-24	—	—	—	—	—	1	—	—	—
CNV-25	—	—	—	—	—	1	—	—	—
CNV-26	—	—	—	—	—	1	—	—	—
CNV-27	—	—	—	—	—	1	—	—	—
CNV-28	—	—	—	—	—	—	4	—	—
CNV-29	—	—	—	—	—	—	1	—	—
CNV-30	—	—	—	—	—	—	3	—	—
CNV-31	—	—	—	—	—	—	1	—	—
CNV-32	—	—	—	—	—	—	1	—	—
CNV-33	—	—	—	—	—	—	1	—	—
CNV-34	—	—	—	—	—	—	4	—	—
CNV-35	—	—	—	—	—	—	—	3	—
CNV-36	—	—	—	—	—	—	—	10	—
CNV-37	—	—	—	—	—	—	—	1	—
CNV-38	—	—	—	—	—	—	—	1	—
CNV-39	—	—	—	—	—	—	—	—	14
CNV-40	—	—	—	—	—	—	—	—	1

[a] See Table 3, footnote *a*.
[b] One clone was excluded from the analysis due to failure in sequencing ORF 1b.
[c] See Table 3, footnote *b*.

TABLE 10. Appearance of 19 CAVs within three independent lines and in three pig passages

Phenotype	No. of plaque-cloned viruses identical to each phenotype in the following passage and line[a]:								
	P1			P2			P7		
	A	B	C	A[b]	B	C	A	B	C
CC-01[c]	6	14	7	—	—	—	—	—	—
CAV-01	7	—	4	12	—	13	5	—	—
CAV-02	1	—	—	—	—	—	—	—	—
CAV-03	1	—	1	—	—	—	—	—	—
CAV-04	—	1	1	—	—	—	—	—	—
CAV-05	—	—	1	—	—	—	—	—	—
CAV-06	—	—	1	—	—	—	—	—	—
CAV-07	—	—	—	2	—	—	—	—	—
CAV-08	—	—	—	—	6	—	—	—	—
CAV-09	—	—	—	—	9	—	—	—	—
CAV-10	—	—	—	—	—	1	—	—	—
CAV-11	—	—	—	—	—	1	—	—	—
CAV-12	—	—	—	—	—	—	4	—	—
CAV-13	—	—	—	—	—	—	1	—	—
CAV-14	—	—	—	—	—	—	4	—	—
CAV-15	—	—	—	—	—	—	1	—	—
CAV-16	—	—	—	—	—	—	—	13	—
CAV-17	—	—	—	—	—	—	—	1	—
CAV-18	—	—	—	—	—	—	—	1	—
CAV-19	—	—	—	—	—	—	—	—	15

[a] See Table 3, footnote *a*.
[b] One clone was excluded from the analysis due to failure in sequencing ORF 1b.
[c] See Table 3, footnote *b*.

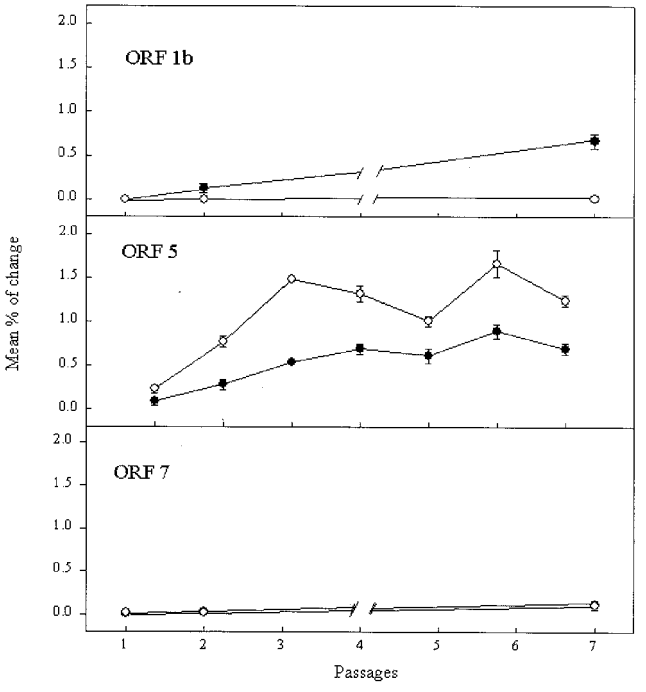

FIG. 7. Mutation rates for PRRSV ORFs 1b, 5, and 7 over seven animal passages (367 days of in vivo replication). The mutation rates were calculated as the proportions of substitutions in each plaque-cloned virus and are represented as the mean percent change on the nucleotide (●) and amino acid (○) levels. Each symbol represents the mean of mutation rates for 45 cloned isolates, except for 44 cloned viruses in P2 for ORF 1b. Error bars are the standard errors of the mean.

dues. For deduced amino acids, rates of change in ORF 5 were 0.24, 0.77, 1.49, 1.32, 1.01, 1.67, and 1.24% for P1 through P7, respectively.

The types of mutations occurring in ORFs 1b, 5, and 7 during animal passages are summarized in Tables 11 and 12. Overall, transitions (G 171 A or T 171 C) were more common than transversions (GA 171 TC). The numbers of transitions per plaque-cloned virus during passage were consistent with the mutation rates of the nucleotide sequences in the corresponding genes, since transversions occurred only in a few isolates. While ORFs 1b and 7 were relatively conserved during serial animal passages, ORF 5 showed stable increases in substitutions per clone from 0.58 to 3.87 for P1 through P7.

Assessment of antigenic changes. Five MAbs specific for the N protein and two specific for the E protein were used to detect changes in the epitopic profile of CC-01 during virus evolution in animal passages. No abolition of reactivity with

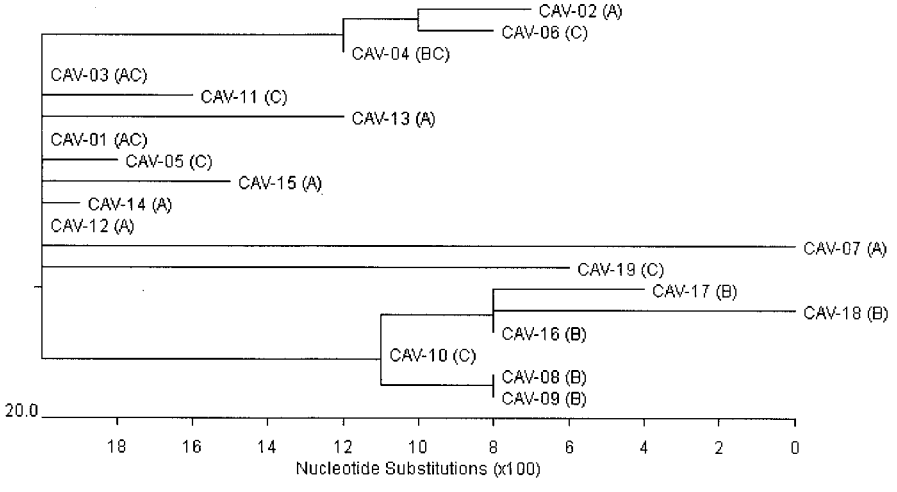

FIG. 6. Phylogenetic relationship of 19 PRRSV CAVs. Letters (A, B, and C) represent the lines of pig passages in which the variant was detected during 367 days of in vivo replication. Lines represent the distances of sequence divergence from the ancestor (CC-01).

TABLE 11. Mean number of nucleotide substitutions per plaque-cloned virus in ORF 5 (603 bases) of PRRSV over seven animal passages

Type	Mean (SEM) no. of nucleotide substitutions in the following passage[a]:						
	P1	P2	P3	P4	P5	P6	P7
Transition	0.58 (0.11)	1.69 (0.13)	3.24 (0.06)	3.91 (0.10)	3.38 (0.15)	4.73 (0.14)	3.87 (0.11)
Transversion	0 (0)	0.02 (0.02)	0 (0)	0 (0)	0.33 (0.07)	0.67 (0.07)	0.33 (0.33)

[a] A total of 45 plaque-cloned viruses were collected from each passage.

each of these MAbs was observed in any of each of the 45 plaque-cloned virus isolates collected from P1, P2, and P7, demonstrating no correlation between observed amino acid substitutions and epitopic changes. Likewise, a one-way VN assay did not reveal the presence of plaque-cloned viruses significantly resistant to the neutralizing activity of antisera collected at day 60 p.i. in P1, suggesting that no escape mutant was generated during seven animal passages under the conditions of this experiment.

DISCUSSION

The purpose of this study was to describe the evolution of PRRSV over time. Genetic and antigenic changes in a well-characterized PRRSV isolate were documented during the course of multiple pig passages over a period of 367 days under experimental conditions.

Several strengths of the experimental design are worth noting. First, a plaque-cloned virus inoculum, i.e., a uniform virus population, rather than a whole virus isolate composed of multiple variants (quasispecies) was used to initiate the infection in P1. This strategy provided a definitive ancestor against which to compare and contrast descendant isolates. Second, in P2 to P7, the use of tissue homogenates to pass virus directly from pig to pig circumvented the selection pressures arising from the in vitro isolation and propagation of viruses. Third, the 60-day passage allowed the immune system to respond and exert selective pressure on the virus population. Fourth, the use of HEPA-filtered housing systems and adherence to strict biosecurity procedures made it possible to maintain virus evolution in three independent lines (replicates) of pig passages. Fifth, the recovery of 15 plaque-cloned isolates directly from serum made it possible to randomly sample the population of PRRSV variants circulating in each pig and characterize their distribution.

For the purposes of data interpretation, it should be recognized that the sample size of 15 clones did not identify all the unique nucleotide and protein sequences present. Specifically, if the prevalence of a variant in the population of viruses circulating in a pig exceeded 18%, the probability of recovering at least one clone in the sample was greater than 0.95. As a corollary, the proportion of any sequence among the 15 clones was only an estimate of the prevalence in the pig. The exact prevalence values should not be overinterpreted because there is a moderate amount of uncertainty associated with these estimates. For example, if a sequence was observed in 12 of the 15 clones (prevalence of 80%), the 95% confidence interval for that prevalence estimate would be 52 to 95%.

Successful transmission of PRRSV via tissue samples collected at 60 days p.i. provided additional evidence that PRRSV infection persists in swine. However, it was observed that cell-adapted virus behaved much differently in pigs than pig-adapted virus. That is, inoculation of pigs in P1 with a cell-adapted clone derived from ATCC VR-2332 produced a pattern of viremia strikingly different, both in virus titer and in duration of viremia, from that seen with such inoculation of pigs in P2 to P7 (Fig. 3 and Table 2). These data are important because they suggest that the results of experiments may differ significantly depending on whether cell-adapted or pig-adapted viruses are used. Although no genetic basis for this biological difference was discovered, it is recommended that future studies with direct application to the field use PRRSV with a minimum amount of cell culture adaptation.

To date, no specific genetic markers have been linked to either the attenuation or the virulence of PRRSV. However, it is interesting to note the consistent substitution of a glycine residue with arginine at position 151 of ORF 5 in all virus clones after just one passage in pigs (Fig. 4). This change represents a reversion to the amino acid residue of the parental strain, VR-2332, a field isolate. Both PRRSV CC-01 and the RespPRRS vaccine virus were derived from VR-2332 through cell culture passage and, for the ORFs sequenced, shared 100% amino acid homology. Reversion of the amino acid residue at position 151 of ORF 5 has been reported for the RespPRRS vaccine virus and VR-2332 (2, 3, 40, 54, 59). Cumulatively, these results lead us to speculate that the glycine residue at position 151 of ORF 5 may be a marker for cell

TABLE 12. Mean number of nucleotide substitution per plaque-cloned virus in ORF 1b (435 bases) and ORF 7 (372 bases) of PRRSV in three animal passages

Type	Mean (SEM) no. of nucleotide substitutions in the following ORF and passage[a]:					
	ORF 1b			ORF 7		
	P1	P2[b]	P7	P1	P2	P7
Transition	0.02 (0.02)	0.57 (0.08)	2.58 (0.19)	0.33 (0.07)	0.59 (0.04)	0.47 (0.08)
Transversion	0 (0)	0 (0)	0.33 (0.07)	0.02 (0.02)	0.02 (0.02)	0 (0)

[a] A total of 45 plaque-cloned viruses were collected from each passage.
[b] A total of 44 plaque-cloned viruses were used in the assessment due to failure in sequencing 1 of the 45 clones.

culture adaptation. Whether this specific change affects the virulence or in vivo replication of PRRSV remains to be determined.

Virus evolution in the three independent lines of pig passages was independent, i.e., highly random. Although numerous genotypes were identified, with few exceptions, a single variant was dominant in each pig at each passage. Unexpectedly, the dominant variant differed from one pig to the next, even within the same line. The minor variants within a population were often different from the dominant form by more than one mutation. These observations suggested that a "purifying selection" may have occurred. This was especially true for line C, where after seven passages and a total of 367 days of in vivo replication, there was essentially only one kind of virus, based on ORF 5. No evidence was obtained for radiation, for an increase in diversity, or for the establishment of a new, dominant mutation in the population. Rather, it appeared that a single genotype gained a clear competitive advantage and became the dominant form within a specific pig or passage.

The results of this study demonstrated that PRRSV evolves continuously in infected pigs, with different genes of the viral genome undergoing various degrees of change. As anticipated for a housekeeping gene, PRRSV ORF 1b, which encodes the RNA polymerase domain, was highly conserved. ORF 7 (N protein) was also conserved during animal passages relative to ORF 5 (E protein), suggesting that the internal structural protein is genetically more stable than the external structural proteins. This scenario may be due to the immune pressures to which the external structural proteins are exposed. The relatively low rates of mutation observed in ORFs 1b and 7 are consistent with field observations (4, 18, 55).

Relative to ORFs 1b and 7, ORF 5 mutated at a much higher rate; numerous genotypes (i.e., quasispecies) were identified, with mutiple genotypes being present concurrently in all pigs. Based on an analysis of algorithms for hydrophobicity and hydrophilicity and surface probability, six potential antigenic sites were identified on the surface of GP5 of both North American and European isolates (4, 45). Sequence analyses of field isolates of PRRSV have revealed that variable regions are located among amino acid residues near either the N terminus (3 to 39) or the C terminus (164 to 200), whereas the most conserved regions (II to IV) are located between amino acid residues near 40 to 163 (4, 45). In agreement with previous reports, our study found a high degree of amino acid substitutions between residues 10 and 34, a region which contains the signal peptide (1 to 31) and one variable N-linked glycosylation site, whereas no or a few inconsistent changes were observed in amino acid residues 40 through 160. Many ORF 5 variants had amino acid substitutions at residue 33 (asparagine to arginine) or 34 (aspartic acid to asparagine or arginine). Since amino acids at positions 33 and 34 are known to be part of the ectodomain of GP5, any change in this region may affect the N-linked glycosylation or the conformation of GP5, which is important for interactions with the M protein (19, 24, 29, 30, 32, 45, 44, 46, 53). Theoretically, such a change could result in the loss of an epitope or an alteration in the immune response, but no such changes were found in this study.

The appearance of ORF 5 quasispecies during in vivo replication suggests a potential role for mutation in viral persistence. However, the overall mutation rate for ORF 5 was lower than expected on the basis of the degree of diversity seen in field isolates (24). The low rate of mutation in ORF 5 suggests that ongoing mutation is not likely to be the mechanism by which PRRSV eludes the immune system and persists in pigs.

It is believed that North American and European PRRSV isolates were derived from a common lactate dehydrogenase-elevating virus-like ancestor (40). Based on the relatively low rate of mutation observed in this study and the 25 and 36% amino acid divergences for ORFs 1b and 7 between strain VR-2332 and the Lelystad virus (39, 40), we conclude that these viruses diverged in the very distant past and experienced independent evolutionary events. Given that the earliest evidence places the introduction of PRRSV into domestic swine in 1979 (8), the slow rate of mutation documented in this study suggests that the extensive antigenic and genetic diversity observed in PRRSV field isolates cannot be fully explained simply as a function of mutations. Alternate hypotheses to explain the variability observed in field isolates would include recombination, as shown among field isolates (24) or in vitro (65), and possible ongoing evolution in alternate host species (67). At present, the factors and conditions contributing to the diversity of PRRSV remain to be established.

ACKNOWLEDGMENTS

We are grateful to Kenneth Platt for kindly providing MAbs. We also thank Roman Porganichniy for assistance in laboratory work.

This study was supported in part by funding from Check-Off dollars on behalf of the National Pork Board, an Iowa Healthy Livestock Initiative research grant, and the Iowa Livestock Health Advisory Council.

REFERENCES

1. **Albina, E., F. Madec, R. Cariolet, and J. Torrison.** 1994. Immune response and persistence of the porcine reproductive and respiratory syndrome virus in infected pigs and farm units. Vet. Rec. **134:**567–573.
2. **Allende, R., G. F. Kutish, W. Laegreid, Z. Lu, T. L. Lewis, D. L. Rock, J. Friesen, J. A. Galeota, A. R. Doster, and F. A. Osorio.** 2000. Mutations in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype. Arch. Virol. **145:**1149–1161.
3. **Allende, R., W. W. Laegreid, G. F. Kutish, J. A. Galeota, R. W. Wills, and F. A. Osorio.** 2000. Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. J. Virol. **74:**10834–10837.
4. **Andreyev, V. G., R. D. Wesley, W. L. Mengeling, A. C. Vorwald, and K. M. Lager.** 1997. Genetic variation and phylogenetic relationships of 22 porcine reproductive and respiratory syndrome virus (PRRS virus) field strains based on sequence analysis of open reading frame 5. Arch. Virol. **142:**993–1001.
5. **Bautista, E. M., S. M. Goyal, and J. E. Collins, J. E.** 1993. Serologic survey for Lelystad and VR-2332 strains of porcine respiratory and reproductive system syndrome (PRRS) virus in US swine herds. J. Vet. Diagn. Investig. **5:**612–615.
6. **Benfield, D. A., J. Christopher-Hennings, E. A. Nelson, R. R. R. Rowland, J. K. Nelson, C. C. L. Chase, K. D. Rossow, and J. E. Collins.** 1997. Persistent fetal infection of porcine reproductive and respiratory syndrome (PRRS) virus. Proc. Am. Assoc. Swine Pract. **1997:**455–458.
7. **Benfield, D. A., E. Nelson, J. E. Collins, L. Harris, S. M. Goyal, D. Robison, W. T. Christianson, R. B. Morrison, D. Gorcyca, and D. Chladek.** 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Investig. **4:**127–133.
8. **Carman, S., S. E. Sanford, and S. Dea.** 1995. Assessment of seropositivity to porcine reproductive and respiratory syndrome (PRRS) virus in swine herds in Ontario–-1978 to 1982. Can. Vet. J. **36:**776–777.
9. **Cavanaugh, D.** 1997. *Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*. Arch. Virol. **142:**629–633.
10. **Chang, C. C., W. B. Chung, M. W. Lin, C. N. Weng, P. C. Yang, Y. T. Chiu, W. F. Chang, and R. M. Chu.** 1993. Porcine reproductive and respiratory syndrome (PRRS) in Taiwan. I. Viral isolation. J. Chin. Soc. Vet. Sci. **19:**268–276.
11. **Christianson, W. T., J. E. Collins, D. A. Benfield, L. Harris, D. E. Gorcyca, D. W. Chladek, R. B. Morrison, and H. S. Joo.** 1992. Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. Am. J. Vet. Res. **53:**485–488.

12. **Christopher-Hennings, J., E. A. Nelson, J. K. Nelson, R. J. Hines, S. L. Swenson, H. T. Hill, J. J. Zimmerman, J. B. Katz, M. J. Yaeger, C. C. L. Chase, and D. A. Benfield.** 1995. Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. J. Clin. Microbiol. **33:** 1730–1734.
13. **Collins, J. E., D. A. Benfield, W. T. Christianson, L. Harris, J. C. Hennings, D. P. Shaw, S. M. Goyal, S. McCullough, R. B. Morrison, H. S. Joo, D. Gorcyca, and D. Chladek.** 1992. Isolation of swine infertility and respiratory syndrome virus (isolate TCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J. Vet. Diagn. Investig. **4:**117–126.
14. **Conzelmann, K. K., N. Visser, P. Van Woensel, and H. J. Thiel.** 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology **193:**329–339.
15. **Dea, S., C. A. Gagnon, H. Mardassi, and G. Milane.** 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.
16. **Diggle, P. J., K.-Y. Liang, and S. Zeger.** 1994. Analysis of longitudinal data. Oxford University Press, New York, N.Y.
17. **Drew, T. W., J. J. M. Meulenberg, J. J. Sands, and D. J. Paton.** 1995. Production, characterization and reactivity of monoclonal antibodies to porcine reproductive and respiratory syndrome virus. J. Gen. Virol. **76:**1361–1369.

17a. **Federation of Animal Science Societies.** 1999. Guide for the care and use of agricultural animals in agricultural research and teaching, 1st ed. Federation of Animal Science Societies, Savoy, Ill.

18. **Goldberg, T. L., E. C. Hahn, R. M. Weigel, and G. Scherba.** 2000. Genetic, geographical and temporal variation of porcine reproductive and respiratory syndrome virus in Illinois. J. Gen. Virol. **81:**171–179.
19. **Gonin, P., B. Pirzadeh, C. A. Gagnon, and S. Dea.** 1999. Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. J. Vet. Diagn. Investig. **11:**20–26.
20. **Halbur, P. G., P. S. Paul, M. L. Frey, J. Landgraf, K. Eernisse, X. J. Meng, J. J. Andrews, M. A. Lum, and J. A. Rathje.** 1996. Comparison of the antigen distribution of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. **33:**159–170.
21. **Halbur, P. G., P. S. Paul, X. J. Meng, M. A. Lum, J. J. Andrews, and J. A. Rathje.** 1996. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRS virus) isolates in a five-week-old cesarean-derived colostrum-deprived pig model. J. Vet. Diagn. Investig. **8:**11–20.
22. **Hein, J.** 1989. A tree reconstruction method that is economical in the number of pairwise comparisons used. Mol. Biol. Evol. **6:**669–684.
23. **Hopper, S. A., M. E. White, and N. Twiddy.** 1992. An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. Vet. Rec. **131:**140–144.
24. **Kapur, V., M. R. Elam, T. M. Pawlovich, and M. P. Murtaugh.** 1996. Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J. Gen. Virol. **77:**1271–1276.
25. **Kim, H. S., J. Kwang, I. J. Yoon, H. S. Joo, and M. L. Frey.** 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogenous subpopulation of MA-104 cells. Arch. Virol. **133:**477–483.
26. **Kuwahara, H., T. Nunoya, M. Tajima, A. Kato, and T. Samejima.** 1994. An outbreak of porcine reproductive and respiratory syndrome in Japan. J. Vet. Med. Sci. **56:**901–909.
27. **Larochelle, R., H. Mardassi, S. Dea, and R. Magar.** 1996. Detection of porcine reproductive and respiratory syndrome virus in cell cultures and formalin-fixed tissues by in situ hybridization using a digoxigenin-labeled probe. J. Vet. Diagn. Investig. **8:**3–10.
28. **Le Gall, A., E. Albina, R. Magar, and J. P. Gauthier.** 1997. Antigenic variability of porcine reproductive and respiratory syndrome (PRRS) virus isolates. Influence of virus passage in pig. Vet. Res. **28:**247–257.
29. **Mardassi, H., S. Mounir, and S. Dea.** 1995. Molecular analysis of the ORFs 3 to 7 of porcine reproductive and respiratory syndrome virus, Quebec reference strain. Arch. Virol. **140:**1405–1418.
30. **Mardassi, H., S. Mounir, and S. Dea.** 1995. Structural gene analysis of a Quebec reference strain or porcine reproductive and respiratory syndrome virus (PRRSV). Adv. Exp. Med. Biol. **380:**277–281.
31. **Meng, X. J., P. S. Paul, and P. G. Halbur.** 1994. Molecular cloning and nucleotide sequencing of the 3′-terminal genomic RNA of the porcine reproductive and respiratory syndrome virus. J. Gen. Virol. **75:**1795–1801.
32. **Meng, X. J., P. S. Paul, P. G. Halbur, and M. A. Lum.** 1995. Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRS virus): implication for the existence of two genotypes of PRRS virus in the USA and Europe. Arch. Virol. **140:**745–755.
33. **Meng, X. J., P. S. Paul, P. G. Halbur, and I. Morozov.** 1995. Sequence comparison of open reading frames 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. **76:**3181–3188.
34. **Meulenberg, J. J., E. J. de Meijer, and R. J. Moormann.** 1993. Subgenomic RNAs of Lelystad virus contain a conserved leader body junction sequence. J. Gen. Virol. **74:**1697–1701.
35. **Meulenberg, J. J., M. M. Hulst, E. J. de Meijer, P. L. M. J. Moonen, A. den Besten, E. P. Kluyver, G. Wensvoort, and R. J. Moormann.** 1994. Lelystad virus belongs to a new virus family, comprising lactate dehydrogenase elevating virus, equine arteritis virus, and simian hemorrhagic fever virus. Arch. Virol. Suppl. **9:**441–448.
36. **Meulenberg, J. J. M., A. den Besten, E. de Kluyver, R. J. M. Moormann, and G. Wensvoort.** 1995. Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. Virology **206:**155–163.
37. **Meulenberg, J. J. M., M. M. Hulst, E. J. de Meuer, P. L. M. J. Moonen, A. den Besten, E. P. de Kluyver, G. Wensvoort, and R. J. M. Moormann.** 1993. Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology **192:**62–72.
38. **Morozov, I., X. J. Meng, and P. S. Paul.** 1995. Sequence analysis of open reading frames (ORFs) 2 to 4 of a US isolate of porcine reproductive and respiratory syndrome virus. Arch. Virol. **140:**1313–1319.
39. **Murtaugh, M. P., M. R. Elam, and L. T. Kakach.** 1995. Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. Arch. Virol. **140:**1451–1460.
40. **Nelsen, C. J., M. P. Murtaugh, and K. S. Faaberg.** 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. **73:**270–280.
41. **Nelson, E. A., J. Christopher-Hennings, and D. A. Benfield.** 1994. Serum immune responses to the proteins of porcine reproductive and respiratory syndrome (PRRS) virus. J. Vet. Diagn. Investig. **6:**410–415.
42. **Nelson, E. A., J. Christopher-Hennings, T. Drew, G. Wensvoort, J. E. Collins, and D. A. Benfield.** 1993. Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J. Clin. Microbiol. **31:**3184–3189.
43. **Pang, V. F., C. C. Chang, and K. W. Lau.** 1994. Application of MTT colormetric assay to measure cell proliferation and viability for swine lymphocytes and macrophages. J. Chin. Soc. Vet. Sci. **20:**17–26.
44. **Pirzadeh, B., and S. Dea.** 1998. Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. **79:**989–999.
45. **Pirzadeh, B., C. A. Gagnon, and S. Dea.** 1998. Genomic and antigenic variations of porcine reproductive and respiratory syndrome virus major envelope GP5 glycoprotein. Can. J. Vet. Res. **62:**170–177.
46. **Pirzadeh, B., and S. Dea.** 1997. Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants. J. Gen. Virol. **78:**1867–1873.
47. **Pol, J. M., J. E. van Dijk, G. Wensvoort, and C. Terpstra.** 1991. Pathological, ultrastructural, and immunohistochemical changes caused by Lelystad virus in experimentally induced infections of mystery swine disease (synonym: porcine epidemic abortion and respiratory syndrome (PEARS)). Vet. Q. **13:**137–143.
48. **Pringle, C. R.** 1996. Virus taxonomy 1996—a bulletin from the Xth International Congress of Virology in Jerusalem. Arch. Virol. **141:**2251–2256.
49. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
50. **Rossow, K. D., D. A. Benfield, S. M. Goyal, E. A. Nelson, J. Christopher-Hennings, and J. E. Collins.** 1996. Chronological immunohistochemical detection and localization of porcine reproductive and respiratory syndrome virus in gnotobiotic pigs. Vet. Pathol. **33:**551–556.
51. **Rossow, K. D., E. M. Bautista, S. M. Goyal, T. W. Molitor, M. P. Murtaugh, R. B. Morrison, D. A. Benfield, and J. E. Collins.** 1994. Experimental porcine reproductive and respiratory syndrome virus infection in one-, four-, and 10-week-old pigs. J. Vet. Diagn. Investig. **6:**3–12.
52. **Rossow, K. D., J. E. Collins, S. M. Goyal, E. A. Nelson, J. Christopher-Hennings, and D. A. Benfield.** 1995. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. Vet. Pathol. **32:**361–373.
53. **Rowland, R. R., M. Steffen, T. Ackerman, and D. A. Benfield.** 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.
54. **Storgaard, T., M. Oleksiewicz, and A. Botner.** 1999. Examination of the selective pressures on a live PRRS vaccine virus. Arch. Virol. **144:**2389–2401.
55. **Suarez, P., R. Zardoya, M. J. Martin, C. Prieto, J. Dopazo, A. Solana, and J. M. Castro.** 1996. Phylogenetic relationships of european strains of porcine reproductive and respiratory syndrome virus (PRRSV) inferred from DNA sequences of putative ORF-5 and ORF-7 genes. Virus Res. **42:**159–165.
56. **Van Nieuwstadt, A. P., J. J. Meulenberg, A. van Essen Zanbergen, A. Petersen den Besten, R. J. Bende, R. J. Moormann, and G. Wensvoort.** 1996. Proteins encoded by open reading frames 3 and 4 of the genome of Lelystad virus (*Arteriviridae*) are structural proteins of the virion. J. Virol. **70:**4767–4772.
57. **Wensvoort, G., C. Terpstra, J. M. A. Pol, E. A. ter Laak, M. Bloemraad, E. P. de Kluyver, C. Kragten, L. van Buiten, A. den Besten, F. Wagenaar, J. M. Broekhuijsen, P. L. J. M. Moonen, T. Zetstra, E. A. de Boer, H. J. Tibben,**

M. F. de Jong, P. van't Veld, G. J. R. Groenland, J. A. van Gennep, M. T. H. Voets, J. H. M. Verheijden, and J. Braamskamp. 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. **13:**121–130.

58. **Wensvoort, G., E. P. de Kluyver, E. A. Luijtze, A. den Besten, L. Harris, J. E. Collins, W. T. Christianson, and D. Chladek.** 1992. Antigenic comparison of Lelystad virus and swine infertility and respiratory syndrome (SIRS) virus. J. Vet. Diagn. Investig. **4:**134–138.
59. **Wesley, R. D., W. L. Mengeling, K. M. Lager, A. C. Vorwald, and M. B. Roof.** 1999. Evidence for divergence of restriction fragment length polymorphism patterns following in vivo replication of porcine reproductive and respiratory syndrome virus. Am. J. Vet. Res. **60:**463–467.
60. **Wills, R. W., J. J. Zimmerman, K.-J. Yoon, S. L. Swenson, M. J. McGinley, H. T. Hill, K. B. Platt, J. Christopher-Hennings, and E. A. Nelson.** 1997. Porcine reproductive and respiratory syndrome virus: a persistent infection. Vet. Microbiol. **55:**231–240.
61. **Yang, L., K. J. Yoon, Y. Li, J. H. Lee, J. J. Zimmerman, M. L. Frey, K. M. Harmon, and K. B. Platt.** 1999. Antigenic and genetic variations of the 15 kD nucleocapsid protein of porcine reproductive and respiratory syndrome virus isolates. Arch. Virol. **144:**525–546.
62. **Yang, L., M. L. Frey, K. J. Yoon, J. J. Zimmerman, and K. B. Platt.** 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.
63. **Yoon, I. J., H. S. Joo, W. T. Christianson, H. S. Kim, J. E. Collins, J. H. Carlson, and S. A. Dee.** 1992. Isolation of a cytopathic virus from weak pigs on farms with a history of swine infertility and respiratory syndrome. J. Vet. Diagn. Investig. **4:**139–143.
64. **Yoon, K.-J., J. J. Zimmerman, S. L. Swenson, M. J. McGinley, K. A. Eernisse, A. Brevik, L. L. Rhinehart, M. L. Frey, H. T. Hill, and K. B. Platt.** 1995. Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. J. Vet. Diagn. Investig. **7:**305–312.
65. **Yuan, S., C. J. Nelsen, M. P. Murtaugh, B. J. Schmitt, and K. S. Faaberg.** 1999. Recombination between North American strains of porcine reproductive and respiratory syndrome virus. Virus Res. **61:**87–98.
66. **Zimmerman, J., T. Sanderson, K. A. Eernisse, H. T. Hill, and M. L. Frey.** 1992. Transmission of SIRS virus in convalescent animals to commingled penmates under experimental conditions. Am. Assoc. Swine Pract. Newsl. **4:**25.
67. **Zimmerman, J. J., K.-J. Yoon, E. C. Pirtle, R. W. Wills, T. J. Sanderson, and M. J. McGinley.** 1997. Studies of porcine reproductive and respiratory syndrome (PRRS) virus infection in avian species. Vet. Microbiol. **55:**329–336.