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The Attenuation Phenotype of a Ribavirin-Resistant Porcine Reproductive and Respiratory Syndrome Virus Is Maintained during Sequential Passages in Pigs

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ABSTRACT

In a previous study, ribavirin-resistant porcine reproductive and respiratory syndrome virus (PRRSV) mutants (RVRp13 and RVRp22) were selected, and their resistance against random mutation was shown in cultured cells. In the present study, these ribavirin-resistant mutants were evaluated in terms of their genetic and phenotypic stability during three pig-to-pig passages in comparison with modified live virus (MLV) (Ingelvac PRRS MLV). Pigs challenged with RVRp22 had significantly lower ($P < 0.05$) viral loads in sera and tissues than pigs challenged with MLV or RVRp13 at the first passage, and the attenuated replication of RVRp22 was maintained until the third passage. Viral loads in sera and tissues dramatically increased in pigs challenged with MLV or RVRp13 during the second passage. Consistently, all five sequences associated with the attenuation of virulent PRRSV in RVRp13 and MLV quickly reverted to wild-type sequences during the passages, but two attenuation sequences were maintained in RVRp22 even after the third passage. In addition, RVRp22 showed a significantly lower ($P < 0.001$) mutation frequency in nsp2, which is one of the most variable regions in the PRRSV genome, than MLV. Nine unique mutations were found in open reading frames (ORFs) 1a, 2, and 6 in the RVRp22 genome based on full-length sequence comparisons with RVRp13, VR2332 (the parental virus of RVRp13 and RVRp22), and MLV. Based on these results, it was concluded that RVRp22 showed attenuated replication in pigs; further, because of the high genetic stability of RVRp22, its attenuated phenotype was stable even after three sequential passages in pigs.

IMPORTANCE

PRRSV is a rapidly evolving RNA virus. MLV vaccines are widely used to control PRRS; however, there have been serious concerns regarding the use of MLV as a vaccine virus due to the rapid reversion to virulence during replication in pigs. As previously reported, ribavirin is an effective antiviral drug against many RNA viruses. Ribavirin-resistant mutants reemerged by escaping lethal mutagenesis when the treatment concentration was sublethal, and those mutants were genetically more stable than parental viruses. In a previous study, two ribavirin-resistant PRRSV mutants (RVRp13 and RVRp22) were selected, and their higher genetic stability was shown *in vitro*. Consequently, in the present study, both of the ribavirin-resistant mutants were evaluated in terms of their genetic and phenotypic stability *in vivo*. RVRp22 was found to exhibit higher genetic and phenotypic stability than MLV, and nine unique mutations were identified in the RVRp22 genome based on a full-length sequence comparison with the RVRp13, VR2332, and MLV genomes.

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important infectious disease in pigs worldwide, characterized by respiratory disorder in pigs of all ages and severe reproductive failures in sows (1–6). The annual loss to the U.S. swine industry associated with PRRS has been estimated to be approximately 664 million USD (7). PRRSV is a member of the *Arteriviridae* virus family, along with the equine arteritis virus (EAV), the lactate dehydrogenase-elevating virus (LDV) in mice, and the simian hemorrhagic fever virus (SHFV), under the order *Nidovirales* (8, 9). PRRSV is a small, enveloped virus, with a single-stranded, nonsegmented, positive-sense RNA genome that is approximately 15 kb in length and with a 5′ cap and a 3 polyadenylated tail (9–11). The PRRSV genome contains at least 10 open reading frames (ORFs): ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5a, ORF5, ORF6, and ORF7 (9–18). ORF1a and ORF1b cover approximately three-fourths of the viral genome and encode two large polyproteins, pp1a and pp1ab; the latter is synthesized by a −1 ribosomal frameshift in the overlapping region of ORF1a/ORF1ab (18, 19). The polyproteins, pp1a and pp1ab, are sequentially cleaved to generate 14 further nonstructural proteins (nsp's), 10 nsp's (nsp1α, nsp1β, nsp2 to nsp6, nsp7α, nsp7β, and nsp8) encoded in ORF1a and 4 nsp's (nsp9 to nsp12) encoded in ORF1b (17, 18, 20), through proteolysis regulated by viral proteases nsp1α, nsp1β, nsp2, and nsp4. Eight other short 3′-proximal ORFs (ORF2a, ORF2b, ORFs 3 to 7, and ORF5a) are translated from a nested set of six major subgenomic mRNAs: encoded proteins GP2/2a (ORF2a), E (envelope; ORF2b), GP3 (ORF3), GP4 (ORF4), GP5 (ORF5), M (membrane;

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ORF6), N (nucleocapsid; ORF7), and a newly identified protein encoded in ORF5a that overlaps the 5′ end of ORF5 (18).

PRRSV is grouped into two genotypes, European (type 1) and North American (type 2). The clinical signs of infection are similar, but the individual strains are very different in terms of virulence in infected animals (21, 22) and antigenic and genetic properties (11, 23–32). Currently, vaccination is the only way to control PRRS; it decreases the incidence of clinical disease, but it does not prevent viral infections. Enormous genetic and antigenic diversity among PRRSV isolates is a big hurdle in the development of a more genetically stable, cross-protective, and efficacious vaccine to control PRRS. Modified live virus (MLV) vaccines have been most commonly used to control PRRSV because they confer better protection against homologous virus strains than a killed vaccine or recombinant vaccines (33–36); however, there have been increasing concerns regarding the safety of using MLV vaccines because of the quick reversion to virulence during replication in pigs (33, 36–42). Due to the adverse effects of current PRRSV vaccines, continuing efforts have been made to develop a new generation of vaccines that are more genetically stable during replication in pigs and that will confer better cross-protective efficacy.

A number of mutagens (ribavirin, 5-fluorouracil, 5-azacytidine, and amiloride hydrochloride hydrate) have been successfully used in a number of previous studies as an antiviral compound against different viruses (43–57). These antiviral mutagens maintained lethal mutagenesis by increasing the mutation frequency of RNA viruses above the tolerable error threshold during replication, ultimately driving virus infections into extinction. However, mutagen-resistant mutants reemerged by escaping lethal mutagenesis when RNA viruses were sequentially passed in the sublethal concentrations of those antiviral mutagens, and those mutants were found to be genetically more stable than their parental viruses (56, 58–60). Therefore, in a previous study (61), this strategy was applied to select ribavirin-resistant PRRSV mutants (RVRp13 and RVRp22), which are genetically and phenotypically stable through acquired resistance to random mutation. Consequently, in the present study, both of these ribavirin-resistant mutant viruses were evaluated in terms of their genetic and phenotypic stability during replication in three pig-to-pig passages in comparison with a commercial MLV vaccine (Ingelvac PRRS MLV).

MATERIALS AND METHODS

Cells and viruses. MARC-145, a cell line that is highly permissive to PRRSV (62), was used for virus propagation, virus assay, and virus plaque purification assay. Porcine alveolar macrophages (PAMs), the major target cells for PRRSV, were also used for virus assay. PAMs were collected from 6-week-old PRRSV-free pigs via bronchoalveolar lavage, as described previously (63). In brief, the pigs were euthanized, and their lungs, tracheas, and bronchi were aseptically extracted. Then, the extracted lungs were flushed two to three times with a total volume of 200 to 300 ml sterilized phosphate-buffered saline (PBS) in 50-ml conical tubes. The washed cell pellet was resuspended with freezing medium (10% dimethyl sulfoxide [DMSO; Hybri-Max, Sigma-Aldrich], 20% heat-inactivated fetal bovine serum [FBS; Invitrogen, Carlsbad, CA, USA], 70% Dulbecco modified Eagle's medium [DMEM; Welgene, Inc., Daegu, South Korea]) at a final cell population of approximately 5×10^7 macrophages per ml and aliquoted and stored at −80°C until use. Both MARC-145 cells and PAMs were maintained in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, and 100× antibiotic-antimycotic solution (Anti-anti [Invitrogen]; 1× solution contains 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B [Fungizone]) (here called RPMI growth medium) at 37°C in a humidified 5% CO_2 atmosphere.

Two ribavirin-resistant mutant viruses (RVRp13 and RVRp22) were rescued through the serial passaging of VR2332 (a prototype strain of North American PRRSV) in the presence of 0.1 or 0.2 mM ribavirin, respectively, as characterized in a previous study (61). In the present study, both mutants, RVRp13 and RVRp22, were evaluated in terms of their genetic stability during replication in pigs in comparison to MLV (Ingelvac PRRS MLV; Boehringer Ingelheim, USA), a vaccine virus that was attenuated by sequential passages of VR2332 in MARC-145 cells (1, 64–66). For this purpose, each virus (RVRp13, RVRp22, and MLV) was purified by three rounds of plaque purification in MARC-145 cells to prepare a highly homologous virus inoculum, similar to a previously described method (61, 67, 68). The plaque-purified viruses were propagated again, and virus titers were measured in MARC-145 cells and stored at −80°C until use.

Animal study. The two ribavirin-resistant mutants (RVRp13 and RVRp22) were serially passaged in pigs three times to identify their genetic stability during pig-to-pig virus passage in comparison to that of MLV. Six 3-week-old PRRSV-negative pigs were purchased. On arrival, the pigs were randomly divided into three groups for treatment with each virus (RVRp13, RVRp22, and MLV) and kept three more days for acclimatization. Then, the pigs were confirmed by quantitative real-time reverse transcription PCR (qRT-PCR) (TaqMan chemistry) and enzyme-linked immunosorbent assay (ELISA) to be PRRSV negative. For each passage, two pigs were injected intramuscularly (2.5 ml/pig) with each virus (RVRp13, RVRp22, and MLV) at a titer of 1×10^6 50% tissue culture infective dose ($TCID_{50}$) per ml. The pigs were then observed daily for clinical signs prior to feeding, up to 28 days postchallenge (dpc). Serum was separated on 0 (before challenge), 7, 14, 21, and 28 dpc for virological and serological assays. At the end of the 28-day observation period for each passage, all pigs were euthanized for necropsy. Clinical signs were observed, and gross lung lesions were scored with the help of an expert pathologist. To evaluate the gross and microscopic lesions of the lungs, each lobe was scored for lung consolidation in percentages (21) and interstitial pneumonia, respectively, due to PRRSV infection. Scoring for microscopic lung lesions was performed as follows: 0, no lesion; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffused interstitial pneumonia; and 4, severe interstitial pneumonia. A variety of tissue samples (lungs, bronchial lymph nodes, and tonsils) were collected and stored at −80°C until laboratory processing. Tissues were also collected in 10% neutral buffered formalin for histopathological study. Viremia and residual virus loads were quantified in serum and tissues (lungs, bronchial lymph nodes, and tonsils), respectively. The virus was isolated from a tonsil of each pig and then plaque-cloned to create a highly homogenous virus inoculum. The virus prepared from the first passage (P1) was inoculated in such a way to keep two independent pig lines for each virus during two subsequent passages (P2 and P3). The animal experiment protocols were approved by the Chonbuk National University Institutional Animal Care and Use Committee (approval number 2012-0025).

Serology. A PRRSV-specific antibody (IgG) was detected using a commercially available ELISA kit (PRRS X3 Ab test; IDEXX Laboratories, AG, Berne, Switzerland) in accordance with the manufacturer's instructions. The S/P ratio (the ratio between the net optical density of test samples and the net optical density of positive controls) of the samples was ≥0.4, which was considered to be positive for the PRRSV antibody.

Serum viremia and residual virus loads in tissues. Viral RNA was extracted from serum and tissue (lung, lymph node, and tonsil) samples using a MagMAX viral RNA isolation kit (Ambion; Applied Biosystems, Life Technologies, Inc., Carlsbad, CA, USA) and a total RNA extraction kit (Hybrid-R; GeneAll, Seoul, South Korea), respectively, according to the manufacturers' instructions. Serum viremia and residual virus loads in different tissues were quantified by a real-time reverse transcription-

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TABLE 1 Sequences of the primers used for PCR amplification and sequencing

Sequenced region	Primer name	Nucleotide positions[a]	Sequence[b] (5′-3′)	Sequenced length (bp)
Frag-1	$_{p^*}$1a160F	160–178	ACTGCTTTACGGTCTCTCC	3,374
	$_{p^*}$1a3514R	3514–3533	GCATCATCACAAACTCACAC	
	1a859R	839–859	GTTCGCAATCAACTTCAACTC	
	1a712F	712–730	CCCCTTTGAGTGTGCTATG	
	1a1481R	1463–1481	GGGAGTAGTGTTTGAGGTG	
	1a1366F	1366–1383	CTCTTGTGCGACTGCTAC	
	1a2115R	2097–2115	TACAGGTCAATCTTTGCTG	
	1a2058F	2058–2075	CCCAGAACAAAACCAACC	
	1a2867R	2850–2867	ATTGCGGTGAGGACACAA	
	1a2771F	2771–2788	TGGGAAGATTTGGCTGTT	
Frag-2	$_{p^*}$1a3472F	3472–3491	GTTCCTCCCAAAAATGATAC	3,233
	$_{p^*}$1a6687R	6687–6704	ACACTCCATCGCCAACAA	
	1a4276R	4258–4276	CAGTAACCTGCCAAGAATG	
	1a4141F	4141–4158	CGCTGCTTGTGAGTTTGA	
	1a4861R	4844–4861	AAGGACGAGGTTTGTGGT	
	1a4648F	4648–4665	CAACCAAGCCGTAAAGTG	
	1a5434R	5416–5434	ACCAAGAAGCCAAAGAGAA	
	1a5288F	5288–5306	TGGTTGCTTTGTGTGTTTC	
	1a5942R	5924–5942	CGAATCCTTTTCCAATGAC	
	1a5705F	5705–5723	GGGAAAGTCAAGTGCGTAA	
Frag-3	$_{p^*}$1b6634F	6634–6653	CCTACTTGCCATCATTTTGT	2979
	$_{p^*}$1b9594R	9594–9612	ATTGGACCTGAGTTTTTCC	
	1a7123R	7106–7123	AATGGCTTGGAGGGTATG	
	1a7095F	7095–7112	GTAGCACCAAGCATACCC	
	1b7899R	7882–7899	GAGCACAACTCCACCATC	
	1b7732F	7732–7749	GGCGGCTTGGTTGTTACT	
	1b8382R	8365–8382	CTGTTTAGGGCAGTCAGG	
	1b8166F	8166–8185	GCAGAATACAAGGTTTGGAG	
	1b8954R	8937–8954	TCAGCACAGGCAAGTTCA	
	1b8809F	8809–8826	GCCCTCGGAAAGAACAAG	
Frag-4	$_{p^*}$1b9304F	9304–9322	GACCCAAAGAAGACAGCAA	2842
	$_{p^*}$1b12145R	12127–12145	AGAACTCCGTGAAAGCATC	
	1b10091R	10073–10091	CAGTATGTTTTCCCAGCAC	
	1b9962F	9962–9981	TACCAGACGGTGATTATGCT	
	1b10688R	10670–10688	GCTCTTTGCCTGTTGAGTG	
	1b10484F	10484–10504	CAGATTACAGGGACAAACTCA	
	1b11195R	11178–11195	TATGACACGACCCCAGGA	
	1b11059F	11059–11078	ACCCAGAACAATGAAAAGTG	
	1b11683R	11664–11683	ACACCGTAGAGTTGACAGGA	
	1b11529F	11529–11547	CAAGTGCTGGAAAATGATG	
Frag-5	$_{p}$Pr11661F	11661–11680	TGTTCCTGTCAACTCTACGG	3725
	$_{p}$Pr15385R	15368–15385	TAAATCTCACCCCCACAC	
	Pr12001F^{r}	12002–12023	CCACTGCCACCAGCTTGAAGTT	
	P12527R	13283–13306	GCGAACGCCTGAGAAACCAAGAGA	
	P24-2F	12713–12735	CATTCCTCCATATTTTCCTCTgT	
	P24-2R	12810–12833	TCGAAAgAAAAATTgCCCCTAACC	
	P24-3F	13255–13278	TTCTTTTCCTCgTggTTggTTTTA	
	P5F^{r}	13716–13734	CCTGAGACCATGAGGTGGG	
	p6F	14320–14339	AGAGTTGTGCTTGATGGTTC	
	p6R	14983–15000	TCTGGACTGGTTTTGCTG	
	P7F^{r}	14836–14857	TCGTGTTGGGTGGCAGAAAAGC	
	P7R^{r}	15299–15320	GCCATTCACCACACATTCTTCC	
nsp2	$_{p}$ nsp2F^{R}	1249–1268	CCTCCTCAGAATAAGGGTTG	3588
	$_{p}$ nsp2R^{R}	5120–5138	TGTCAAGGGCAGGGTAAG	
	1a 1481R^{R}	1463–1481	GGGAGTAGTGTTTGAGGTG	
	1a 1366F^{R}	1366–1383	CTCTTGTGCGACTGCTAC	

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TABLE 1 (Continued)

Sequenced region	Primer name	Nucleotide positions[a]	Sequence[b] (5′-3′)	Sequenced length (bp)
	1a 2115R^{R}	2097–2115	TACAGGTCAATCTTTGCTG	
	1a 2058F^{R}	2058–2075	CCCAGAACAAAACCAACC	
	1a 2867R^{R}	2850–2867	ATTGCGGTGAGGACACAA	
	1a 2771F^{R}	2771–2788	TGGGAAGATTTGGCTGTT	
	1a 3581R^{R}	3563–3581	CAATGGTAAGGTCGCTCTC	
	1a 3511F^{R}	3511–3529	TCCGTGTGAGTTTGTGATG	
	1a 4276R^{R}	4258–4276	CAGTAACCTGCCAAGAATG	
	1a 4141F^{R}	4141–4158	CGCTGCTTGTGAGTTTGA	
ORF2	P2F^{r}	11934–11957	AAACGGTGAGGACTGGGAGGATTA	
	P242F	12713–12735	CATTCCTCCATATTTTCCTCTgT	
	P2R^{r}	12810–12833	TCGAAAGAAAAATTGCCCCTAACC	
ORF5	$_{p}$P5F^{r}	13716–13734	CCTGAGACCATGAGGTGGG	603
	$_{p}$P5R^{r}	14457–14479	TTTAGGGCATATATCATCACTGG	
ORF6	p6F	14320–14339	AGAGTTGTGCTTGATGGTTC	
	p6R	14983–15000	TCTGGACTGGTTTTGCTG	

[a] Positions of primers in a full-length genome of VR2332 (GenBank accession number AY150564) (101).
[b] Sequences of primers. The subscript prefix p* indicates a primer used for PCR amplification and sequencing. The subscript prefix p indicates a primer used only for PCR amplification. The remaining primers were used for sequencing. Superscripts R and r indicate the reference primers used in previous studies (61, 67).

PCR (RT-PCR) using TaqMan chemistry, as described previously (69). The primer and probe sequences were as follows: forward primer, TGTC AGATTCAGGGAGRATAAGTTAC; probe, 6-FAM (6-carboxyfluorescein)-TGTGGAGTTYAGTYTGCC; and reverse primer, ATCARGCGCA CAGTRTGATGC. Virus loads in sera and tissues were quantified using a one-step quantitative real-time RT-PCR (qRT-PCR) kit (AgPath-ID one-step RT-PCR; Ambion, Applied Biosystems) in each virus-challenged pig at every passage. One-step qRT-PCR was performed in a 25-μl reaction mixture containing 5 μl of template RNA, 12.5 μl of 2× RT-PCR buffer, 0.5 μl of each forward and reverse primer (20 pmol, as a final concentration of 0.8 pmol), 0.2 μl of TaqMan probes (25 pmol, as a final concentration of 1 pmol), 0.5 μl of RNase inhibitor (40 U/μl; RiboLock, Thermo Fisher Scientific, Inc., Germany), 1 μl of 25× RT-PCR enzyme mix, and 4.8 μl of nuclease-free water. The qRT-PCR conditions were in accordance with the manufacturer's instructions (Applied Biosystems). In brief, the PCR amplification was performed on a model 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). The cycling conditions were as follows: (i) reverse transcription for 10 min at 45°C; (ii) a 10-min activation step at 95°C; and (iii) 40 cycles of 15 s at 95°C and 45 s at 60°C. Samples with a threshold cycle (C_{T}) of 35 cycles or fewer were considered positive. A standard curve previously made of known virus titers was used to calculate the amount of PRRSV in each sample by converting the C_T values to virus titers ($TCID_{50}$/ml).

Virus titration. Virus titers were measured in PAMs and MARC-145 cells at the end of each passage using a microtitration infectivity assay (70) on rescued viruses in each virus-challenged group and were compared to the titers of inoculated viruses from the previous passage. The detailed procedure of virus titration has been described in a previous study (61). At 5 to 6 days postinoculation (dpi) of the virus assay, the virus titers were measured, and the virus antigen was detected by immunofluorescence microscopy. Virus titers were calculated based on the cytopathic effect (CPE) and were expressed as $TCID_{50}$/ml (71). The virus titers of each virus in PAMs and MARC-145 cells were compared to see whether the cell preference of each virus changed during the passages in pigs.

Assessment of genetic stability of ribavirin-resistant mutant virus during replication in pig-to-pig passages. Viruses were recovered from tonsils at the end of each passage in each virus-challenged group. Genetic stability was evaluated on those rescued viruses in each virus group based on the assessment of the stability or reversion of attenuation-related amino acid residues (64). The mutation frequency was measured (in the nsp2 and ORF5 regions) for the plaque-cloned virus population in each virus-challenged group. Viral RNA was extracted from the recovered viruses and the plaque-cloned virus population in each virus group using a MagMAX viral RNA isolation kit (Ambion; Applied Biosystems, Life Technologies, Inc., Carlsbad, CA, USA) according to the manufacturer's guidelines.

Parts of ORF1a, ORF1b, ORF2, ORF5, and ORF6 were amplified from viral RNA in each virus group using the primer sets (Frag-1, Frag-4, and Frag-5) listed in Table 1 with a one-step RT-PCR kit (TaKaRa Bio, Inc., Japan) according to the manufacturer's instructions. Amplified PCR products were submitted for sequencing (Macrogen, Inc., South Korea) to determine the stability or reversion of these unique sequences, which are potentially attenuation- or virulence-related genetic determinants in the genome of PRRSV, as reported previously (64).

In addition, the mutation frequency was determined for the plaque-cloned virus population (30 clones) in each virus group between P1 and P3 in the nsp2 (nonstructural proteins) and ORF5 (structural proteins) regions, which are known to be highly variable regions in the PRRSV genome (26, 29, 72–76). The nsp2 and ORF5 genes were amplified using specific primer sets, as described previously (61) using a one-step RT-PCR kit (TaKaRa), and the PCR products were then submitted for sequencing (Macrogen). The PCR amplification and sequencing primers are shown in Table 1.

Sequence comparison of ribavirin-resistant mutant viruses. To see the sequence comparison in a full-length genome, viral RNA was extracted from both ribavirin-resistant mutants, RVRp13 and RVRp22 (61), their parental strain, VR2332, and MLV using a commercial kit (Ribo-Spin vRD; GeneAll, Seoul, South Korea) according to the manufacturer's instructions. The coding regions of the PRRSV genome were amplified separately into five fragments (Frag-1 to Frag-5) using five pairs of overlapping primer sets (Table 1) with a high-fidelity, one-step RT-PCR kit (SuperScript one-step RT-PCR for long template; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. In brief, the reaction conditions were 1 cycle of cDNA synthesis and predenaturation at 50°C for 30 min and 94°C for 2 min, followed by 35 cycles of PCR amplification at 94°C for 15 s (denaturation), 55 to 60°C for 30 s (annealing), and 68°C for 1 min/kb (extension). A final extension of 1 cycle was performed at 72°C for 5 min. All fragments of PCR products were submitted for sequencing (Macrogen). Nucleotide sequences of the coding regions in the PRRSV genome were aligned as a consensus sequence and analyzed using SeqManII and Lasergene MagAlign software (DNASTAR,

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Inc., Madison, WI, USA). The sequences of the PCR amplification and sequencing primers are listed in Table 1.

Data analysis. The significance of the variability within the virus-challenged groups was analyzed by repeated-measures analysis of variance (ANOVA) for serum viremia and anti-PRRSV antibodies. A nonparametric *t* test (Mann-Whitney *U* test) was used to compare the mutation frequencies and residual virus loads in tissues within the three virus groups as well as to determine the difference in replication efficiency of each virus between PAMs and MARC-145 cells. The nucleotide sequences were aligned and analyzed using Seqman II and Lasergene MegAlign software (DNASTAR, Inc., Madison, WI, USA), respectively. A difference was considered to be statistically significant at a P of <0.05. GraphPad Prism 5.0.2 (GraphPad Software, Inc., CA, USA) was used for making graphs, and statistical analysis was performed using SPSS Advanced Statistics 17.0 software (SPSS, Inc., Chicago, IL, USA).

Nucleotide sequence accession numbers. The nucleotide sequences of the coding regions in a full-length genome for the two ribavirin-resistant mutant viruses were deposited in the NCBI database under GenBank accession numbers KM386622 (RVRp22) and KP256233 (RVRp13).

RESULTS

Serum viremia. RVRp13-challenged pigs showed the highest viremia at 7 dpc, which was observed in all three passages. However, virus titers were dramatically increased by subsequent passages. RVRp13-challenged pigs at P1 had virus titers of $10^{2.04}$ $TCID_{50}$/ml at 7 dpc, which gradually declined to $10^{1.12}$ $TCID_{50}$/ml at 28 dpc. However, at P2 and P3, the virus titers increased dramatically to $10^{3.84}$ and $10^{3.87}$ $TCID_{50}$/ml at 7 dpc and dropped to $10^{0.83}$ and $10^{0.94}$ $TCID_{50}$/ml at 28 dpc, respectively (Fig. 1). In contrast, MLV-challenged pigs showed peak viremia at 21 dpc, with virus titers of $10^{2.14}$ $TCID_{50}$/ml, which also slowly declined to $10^{1.24}$ $TCID_{50}$/ml at 28 dpc, observed at P1. Between P2 and P3, the pigs in those groups (RVRp13- and MLV-challenged pigs) showed an acute-phase of viremia at 7 dpc, with virus titers of $10^{3.71}$ and $10^{3.80}$ $TCID_{50}$/ml, respectively, which also rapidly decreased to $10^{1.44}$ and $10^{1.29}$ $TCID_{50}$/ml, respectively, at 28 dpc (Fig. 1). However, RVRp22-challenged pigs at P1 had a significantly lower level of viremia than MLV- and RVRp13-challenged pigs. Virus titers ranged from $10^{0.83}$ to $10^{1.27}$ $TCID_{50}$/ml detected between 7 and 21 dpc, which were approximately $\geq$10 times lower ($P < 0.05$) than the titers measured in RVRp13- and MLV-challenged pigs (Fig. 1). In addition, the attenuated replication of RVRp22 was maintained at P2 and P3. RVRp22-challenged pigs had virus titers of $10^{1.02}$ and $10^{1.39}$ $TCID_{50}$/ml at P2 and P3, respectively, measured at 7 dpc; these values were approximately $\geq$100 times lower ($P < 0.001$) than the titers measured in MLV- and RVRp13-challenged pigs, which reached $10^{1.44}$ and $10^{0.89}$ $TCID_{50}$/ml, respectively, at 28 dpc (Fig. 1). There was no significant difference found in the levels of viremia measured in the MLV- and RVRp13-challenged groups between P1 and P3. The viremia results indicated that the virulence of RVRp22 was significantly attenuated in pigs and that the attenuated replication was maintained during sequential passages, even after three passages.

Anti-PRRSV antibodies (IgG). The PRRSV-specific antibody (IgG) response was measured with ELISA based on the nucleocapsid (N) protein among all three virus-challenged groups. As summarized in Fig. 2, there was no significant difference found in the detectable levels of the IgG response within the three virus groups between P1 and P3, although the RVRp13 and MLV viruses showed a slightly higher S/P ratio. The pigs in each virus group slowly became seropositive at 21 dpc at P1. However, from P2 to P3, the pigs in each virus group showed a slightly faster antibody response and became seropositive at 14 dpc in comparison to the pigs at P1. The S/P ratio in each virus-challenged group increased gradually through 28 dpc (Fig. 2).

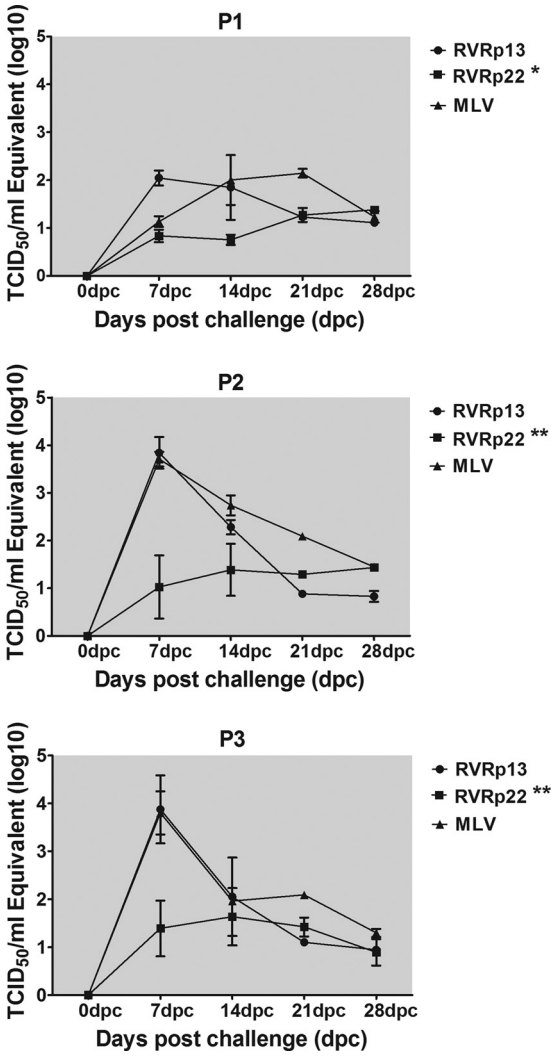

FIG 1 Detection of virus loads in pig serum in each virus-challenged group via qRT-PCR (TaqMan chemistry). Serum samples were collected from pigs in each virus group (RVRp13, RVRp22, and MLV) during pig-to-pig passages at 0 (before challenge), 7, 14, 21, and 28 dpc. Serum viremia was measured using qRT-PCR (TaqMan chemistry). The level of viremia was calculated and is expressed as $TCID_{50}$/ml equivalents based on the standard curve of the cycle threshold (C_T) number plotted against the known virus titer of VR2332. Asterisks indicate significant differences in viremia compared to that of the MLV group (*, $P < 0.05$; **$P < 0.001$).

Residual virus loads and tissue histopathology. The residual virus loads in different tissues (lungs, bronchial lymph nodes, and tonsils) were measured by qRT-PCR and are summarized in Fig. 3. Between P1 and P3, RVRp22-challenged pigs had significantly lower virus loads in different tissues than MLV- and RVRp13-challenged pigs. RVRp22-challenged pigs at P1 had virus titers of $10^{1.04}$, $10^{2.08}$, and $10^{1.91}$ $TCID_{50}$/ml in the lungs, bronchial lymph nodes, and tonsils, respectively, which were significantly lower than the virus titers measured in MLV- and RVRp13-challenged pigs ($P < 0.05$). In addition, RVRp22-challenged pigs also exhibited lower ($P < 0.05$) residual virus loads in all three different

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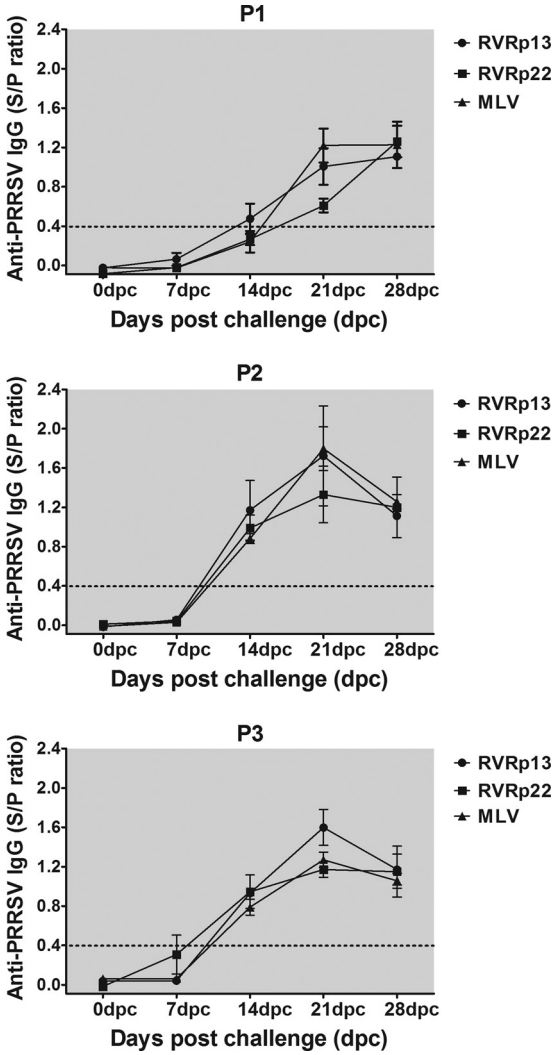

FIG 2 Kinetics of the anti-PRRSV antibody (IgG) response in serum measured by ELISA (PRRS X3 Ab test; IDEXX Laboratories) in groups challenged by each virus (RVRp13, RVRp22, and MLV). Serum samples were collected from pigs in each virus group during pig-to-pig passages (P1 to P3) at 0 (before challenge), 7, 14, 21, and 28 dpc. ELISA was performed according to the manufacturer's guidelines. The result is expressed as an S/P ratio, and an S/P ratio of samples of ≥0.4 was considered positive for PRRSV antibodies (IgG).

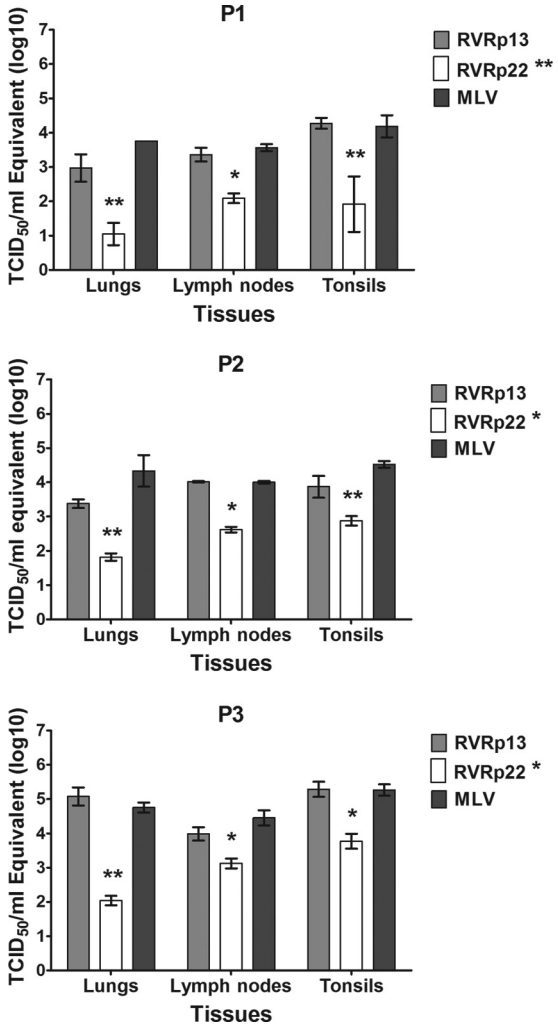

FIG 3 Detection of residual virus loads in different tissues in groups challenged with each virus (RVRp13, RVRp22, and MLV) via qRT-PCR (TaqMan chemistry). Different tissues (lungs, bronchial lymph nodes, and tonsils) were collected from pigs in each virus-challenged group during the necropsy time at the end of each passage. The residual virus loads were then measured, and virus titers in different tissues were calculated and expressed as virus titer in $TCID_{50}$/ml equivalents based on the standard curve of the cycle threshold (C_T) number plotted against the known virus titer of VR2332. Asterisks indicate significant differences in residual virus loads in tissues compared to those of the MLV group (*, $P < 0.05$; **$P < 0.001$).

tissues than MLV- and RVRp13-challenged pigs between P2 and P3 (Fig. 3). No significant differences were found in the residual virus loads in the different tissues among the MLV- and RVRp13-challenged groups between P1 and P3. Therefore, we concluded that the RVRp22 virus showed lower residual virus loads in a variety of tissues than MLV and RVRp13 after challenge, and this virus was maintained even after three passages. Based on the histopathological assessments, RVRp22-challenged pigs had lower levels of gross and microscopic lesions in the lungs than MLV- and RVRp13-challenged pigs, although there were no significant differences observed in the three virus groups between P1 and P3 (data not shown).

Evaluation of *in vitro* growth characteristics of ribavirin-resistant mutants after isolation from pigs at the end of each passage. The growth characteristics of each challenged virus (RVRp13, RVRp22, and MLV) were evaluated in PAMs and MARC-145 cells before passages in pigs, along with their parental virus VR2332, to determine their preferences between the two cell types, as summarized in Fig. 4A and Table 2. The parental virus, VR2332, was able to replicate to equivalent levels in both PAMs and MARC-145 cells, with titers of $10^{7.25}$ $TCID_{50}$/ml and $10^{7.12}$ $TCID_{50}$/ml, respectively. The replication of MLV was significantly lower than that of VR2332 in PAMs, with virus titers of $10^{2.0}$ $TCID_{50}$/ml. However, MLV replicated efficiently in MARC-145 cells, with higher titers of $10^{7.12}$ $TCID_{50}$/ml, similar to the virus titers produced by VR2332 in the same cells. Similarly, both ribavirin-resistant mutants, RVRp13 and RVRp22, showed poor replication efficiency in PAMs, as did MLV, although they were able to replicate efficiently in MARC-145 cells (Fig. 4A and Table 2).

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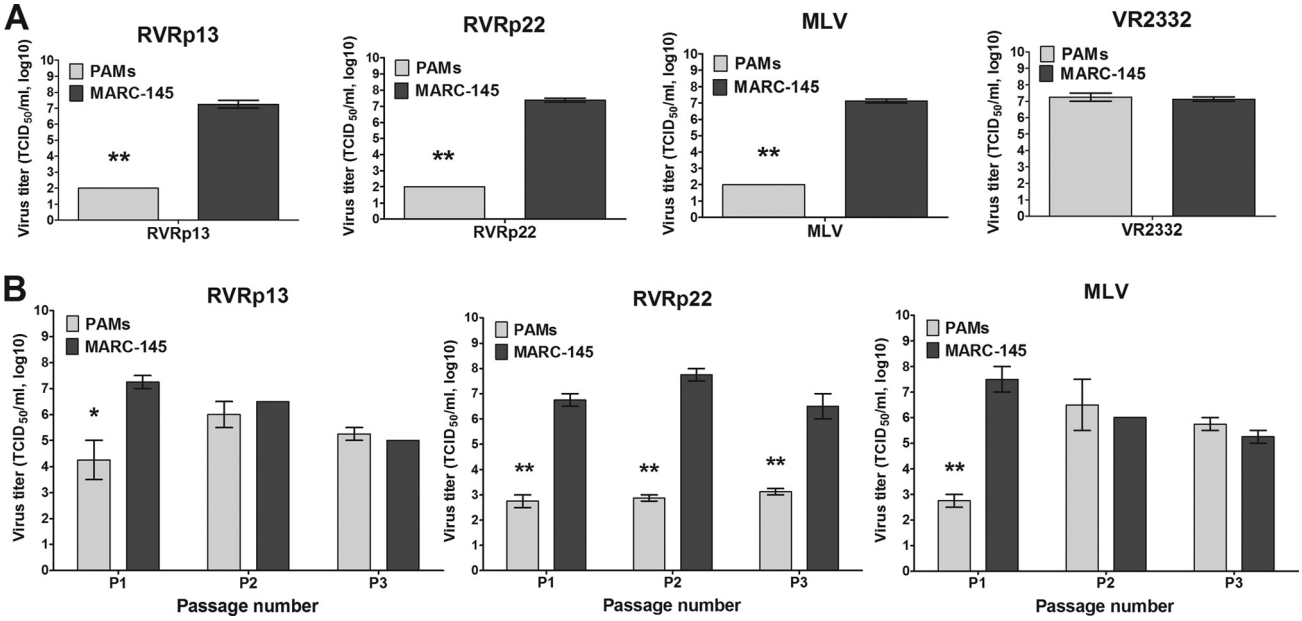

FIG 4 Evaluation of *in vitro* growth characteristics of ribavirin-resistant mutants (RVRp13 and RVRp22) and MLV before challenge of pigs and after recovery of each virus from pigs at the end of each passage. The *in vitro* growth characteristics of each challenged virus were evaluated in PAMs and MARC-145 cells in comparison with those of VR2332 both before and after challenge of pigs to determine how their cell preferences were altered during replication in pig passages. (A) Before challenge of the pigs, each challenged virus was inoculated into PAMs and MARC-145 cells to observe the cell preferences in comparison to those of VR2332. (B) At the end of each passage, the viruses recovered from the tissue (tonsils) of challenged pigs in each virus group were inoculated into PAMs and MARC-145 cells. The production of the progeny virus was measured by a microtitration infectivity assay, and virus titers were calculated and expressed as $TCID_{50}$/ml (log10). Asterisks indicate significant differences in virus titers measured in both PAMs and MARC-145 cells in each virus group (*, $P < 0.05$; **$P < 0.001$).

Viruses were rescued at the end of each passage, and their growth characteristics were evaluated in both PAMs and MARC-145 cells to determine how cell preferences were altered during replication in the pig-to-pig passages. As a result, the rescued viruses from the RVRp22-challenged group at P1, P2, and P3 were replicated in PAMs, with mean virus titers of $10^{2.75}$, $10^{2.87}$, and $10^{3.12}$ $TCID_{50}$/ml, respectively, which were significantly lower ($P < 0.001$) than the respective virus titers in MARC-145 cells (Fig. 4B and Table 2). Similarly, the recovered viruses from the RVRp13- and MLV-challenged groups at P1 replicated in PAM cells with mean virus titers of $10^{4.25}$ and $10^{2.75}$ $TCID_{50}$/ml, respectively, which were also significantly lower ($P < 0.05$) than the respective virus titers measured in MARC-145 cells. However, between P2 and P3, viruses rescued from the RVRp13- and MLV-challenged groups were able to replicate equally well in both PAMs and MARC-145 cells, suggesting that the attenuated phenotype of the RVRp22 virus was maintained even after a third passage in pigs, whereas RVRp13 and MLV lost their attenuated phenotypic characteristics after only a single passage in pigs (Fig. 4B and Table 2).

TABLE 2 *In vitro* growth characteristics of ribavirin-resistant mutant viruses (RVR13 and RVRp22), MLV, and VR2332 in PAMs and MARC-145 cells

Virus and pig-to-pig passage no.	Mean titer[a] ($TCID_{50}$ /ml, log_{10}) in:		PM difference[b]
	PAMs	MARC-145 cells	
VR2332	7.25	7.12	−0.13
RVRp13	2**	7.25	−5.25
P1	4.25**	7.25	−3.00
P2	6.0	6.5	−0.50
P3	5.25	5.0	0.25
RVRp22	2**	7.37	−5.37
P1	2.75**	6.75	−4.00
P2	2.87**	7.75	−4.88
P3	3.12**	6.5	−3.38
MLV	2**	7.12	−5.12
P1	2.75**	7.50	−4.75
P2	6.50	6.00	0.50
P3	5.75	5.25	0.50

[a] Asterisks represent significant differences in virus titers in PAM relative to MARC-145 cells. **, $P < 0.001$.

[b] PM difference, difference in virus titers between results for PAMs and MARC-145 cells as determined by subtracting the geometric mean titer (log_{10}) of the virus in MARC-145 cells from the geometric mean titer (log_{10}) of the virus in PAMs. This value was used to define the cell preference of the viruses.

Estimation of mutation frequency. The mutation frequency for each virus group was measured in the most variable regions (nsp2 and ORF5) of the PRRSV genome, as summarized in Fig. 5 and Table 3. In the nsp2 region, the plaque-cloned virus populations (30 virus clones from each group) that were recovered from the RVRp13- and RVRp22-challenged groups exhibited significantly lower mutation frequencies than those measured in the MLV-challenged group. The populations in the MLV-challenged group had a total of 769 nucleotide mutations, which caused a total of 537 amino acid substitutions. However, the RVRp13-challenged group exhibited a total of 380 ($P < 0.001$) nucleotide mutations, which caused a total of 215 ($P < 0.001$) amino acid changes. Similarly, the RVRp22-challenged group had a total of 490 ($P < 0.001$) nucleotide mutations, which resulted in a total of 285 ($P < 0.001$) amino acid substitutions. In contrast, the popu-

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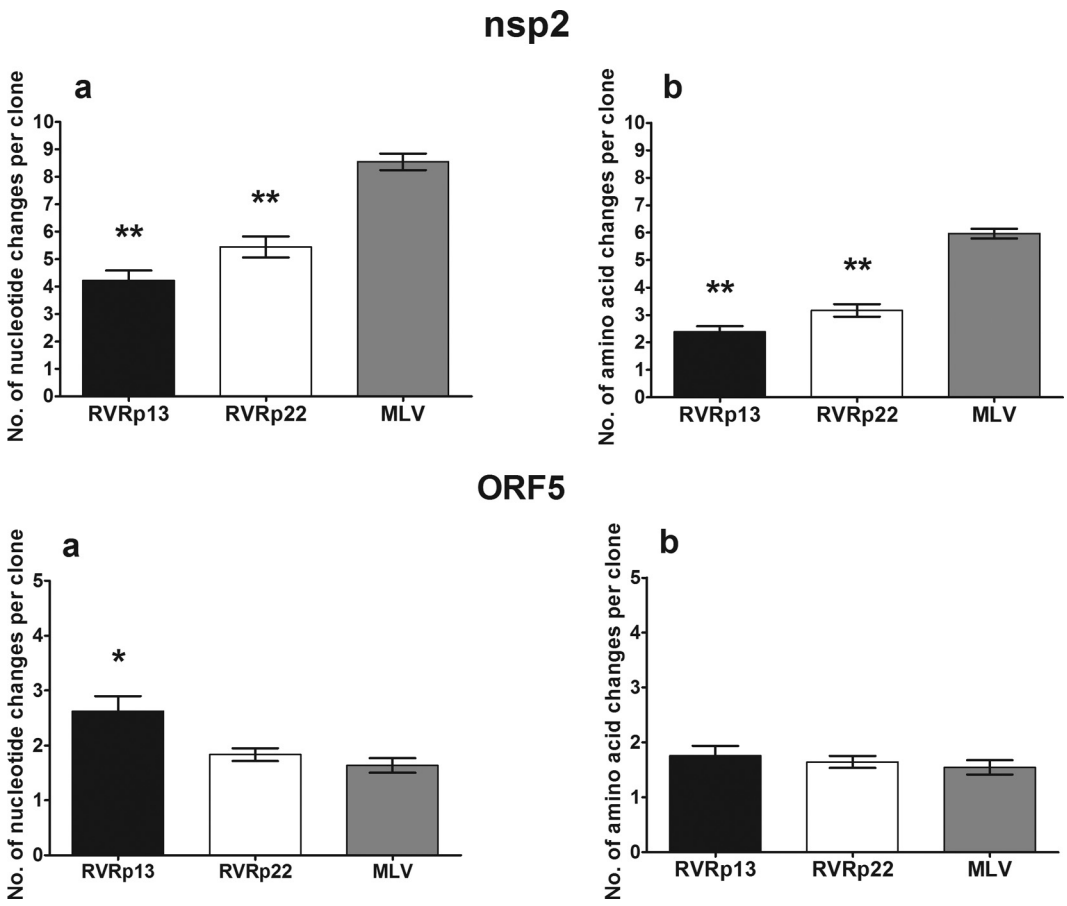

FIG 5 Estimation of mutation frequency. At the end of each passage, the mutation frequency was measured in a plaque-purified virus clone (30 clones in each group) for each virus (RVRp13, RVRp22, and MLV) group relative to the virus inoculated in the previous passage. The mutation frequency was measured in the most variable regions (nsp2 and ORF5) of the PRRSV genome, and the mutation frequency was calculated between P1 and P3 in each respective group. The mutation frequency was expressed as the number of nucleotide or amino acid changes per clone. (a) Nucleotide mutations; (b) amino acid mutations. Asterisks indicate significant differences in mutation rates compared to that of the MLV group (*, $P < 0.05$; **$P < 0.001$).

lation of the RVRp13-challenged group had a higher mutation frequency than the MLV-challenged group in the ORF5 region. However, the RVRp22-challenged group exhibited a rate of mutation similar to that of the MLV-challenged group in the same region and lower than that of the RVRp13-challenged group. The RVRp13-challenged group had a total of 236 ($P < 0.05$) nucleotide mutations that caused a total of 158 amino acid substitutions in the ORF5 region. In contrast, the MLV-challenged group had 147 nucleotide mutations, which caused 139 amino acid changes, and the RVRp22-challenged group exhibited a total of 165 nucleotide mutations, which resulted in a total of 148 amino acid substitutions in the same region (Table 3).

Assessment of the reversion of attenuation-related amino acid residues in the PRRSV genome. Based on previous studies, five marker sequences potentially linked to attenuation are present in ORF1a (331^F), ORF1b (946^H), ORF2 (10^F), ORF5 (151^G), and ORF6 (16^E) of the MLV genome (39, 64, 67). As summarized in Table 4, RVRp22 acquired all of the above-mentioned sequences found in each respective region, and RVRp13 had four of these five sequences during the previous selection procedure in MARC-145 with ribavirin (61) Therefore, in the present study, the stability of the attenuation sequences present in each challenged virus was assessed during replication in pig-to-pig passages. In the RVRp13 and MLV viruses, all amino acid residues that were postulated to be encoded by attenuation-related genetic markers in their genomes reverted to wild type after the first passage in pigs. However, two of these sequences in the RVRp22 genome, ORF2 (10^F) and ORF6 (16^E), were unchanged even after the third passage (Table 4).

Identification of genetic determinants responsible for the higher genetic stability of RVRp22. In comparison with VR2332, the two ribavirin-resistant mutant viruses (RVRp13 and RVRp22) acquired synonymous and nonsynonymous mutations throughout the coding regions in their full-length genomes (Table 5). RVRp22 acquired a total of 38 nucleotide mutations throughout the coding regions. Among 38 mutations, 23 were nonsynonymous, namely, 2 mutations in nsp1β, 8 mutations in nsp2, 1 mutation each in nsp5, nsp7β, nsp10, and nsp11, 2 mutations in ORF2, 3 mutations in ORF3, and 2 mutations each in ORF5 and ORF6. Among the 23 nonsynonymous mutations, a total of 14 were also found in the MLV genome, whereas 5 amino acid changes encoded in ORF1a (331^F), ORF1b (946^H), ORF2 (10^F), ORF5 (151^G), and ORF6 (16^E) were potentially associated with attenuation-related genetic determinants in the PRRSV genome, as described in previous studies (39, 64, 67). The remaining 9 amino acid changes encoded in the RVRp22 genome are unique

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TABLE 3 Mutation frequencies in nsp2 and the ORF5 regions of ribavirin-resistant mutant viruses (RVR13 and RVRp22) after three passages in pigs[a]

Sequenced region	Parameter	Value for:		
		RVRp13 group	RVRp22 group	MLV group
nsp2	Total no. of clones sequenced	90	90	90
	Total no. of nt sequenced (3,599 nt/clone)	322,920	322,920	322,920
	Total no. of nt mutations	380	490	769
	Mutation rate/10^3 nt	1.17**	1.51**	2.38
	Total no. of aa sequenced (1,196 aa/clone)	107,640	107,640	107,640
	Total no. of aa mutations	215	285	537
	Mutation rate/10^3 aa	1.99**	2.64**	4.99
ORF5	Total no. of clones sequenced	90	90	90
	Total no. of nt sequenced (603 nt/clone)	54,270	54,270	54,270
	Total no. of nt mutations	236	165	147
	Mutation rate/10^3 nt	4.35*	3.04	2.71
	Total no. of aa sequenced (201 aa/clone)	18,090	18,090	18,090
	Total no. of aa mutations	158	148	139
	Mutation rate/10^3 aa	8.73	8.18	7.68

[a] The numbers of nucleotide and amino acid mutations were measured by sequencing 30 plaque-purified virus clones in each virus group at the end of each passage. The mutation frequency was calculated to compare the sequences with each challenge virus (RVRp13, RVRp22, and MLV) from the previous passage. The mutation frequency was expressed as an average value between passages (P1 to P3) in each group. nt: nucleotide, aa: amino acid. Asterisks represent significant differences in mutation frequency compared with the MLV group: *, $P < 0.05$, **$P < 0.001$.

mutations, i.e., 6 mutations in nsp2 (465^S, 788^L, 1019^E, 1186^V, 1248^H, and 1375^F) and 1 mutation each in nsp7β (2400^T), ORF2 (204^N), and ORF6 (36^A), which are different from the sequences found in the VR2332, MLV, and RVRp13 genomes (Table 5).

DISCUSSION

In the present study, two ribavirin-resistant mutants, RVRp13 and RVRp22 (61), were evaluated in terms of their genetic and phenotypic stability during replication in pig-to-pig passages in comparison to MLV (Ingelvac PRRS MLV) vaccine virus. RVRp22 was selected after 22 sequential passages in MARC-145 cells that demonstrated attenuated replication in pigs. Moreover, RVRp22 demonstrated significantly lower ($P < 0.001$) viral loads in sera and tissues than even MLV, which had been passaged over 100 times in MARC-145 cells to increase attenuation. In fact, the defective replication of ribavirin-resistant mutants in animals has been reported in other studies (57, 77–81). The attenuated replication of RVRp22 was maintained through the third passage, whereas viral loads in sera and tissues increased dramatically in pigs challenged with MLV or RVRp13 after the second passage. Consistent with this biological stability, RVRp22 showed higher genetic stability than MLV or RVRp13 when the mutation frequency of the nsp2 gene, one of the most variable regions in the PRRSV genome, was determined after three passages in pigs (Fig. 5 and Table 3).

TABLE 4 Reversion of the amino acid residues postulated as virulence attenuation-related sequences in the PRRSV genome during sequential replication in pig-to-pig passages in two independent pig lines[a]

Genomic region (no. of encoded aa)	Amino acid residue in:				Pig passage	Amino acid residue observed at respective site after passage in pigs[b]					
						RVRp13 group		RVRp22 group		MLV group	
	VR2332	RVRp13	RVRp22	MLV		Line 1	Line 2	Line 1	Line 2	Line 1	Line 2
ORF1a (331)	S	S	F	F	P1	S	S	F	F	S	S
					P2	S	S	F	F	S	S
					P3	S	S	S	S	S	S
ORF1b (946)	Y	H	H	H	P1	H	Y	H	Y	Y	Y
					P2	Y	Y	Y	Y	Y	Y
					P3	Y	Y	Y	Y	Y	Y
ORF2 (10)	L	F	F	F	P1	F	F	F	F	F	F
					P2	F	L	F	F	L	L
					P3	F	L	F	F	L	L
ORF5 (151)	R	G	G	G	P1	R	R	G	G	I	I
					P2	R	R	G	G	R	R
					P3	R	R	R	G	R	R
ORF6 (16)	Q	E	E	E	P1	G	G	E	E	Q	E
					P2	G	Q	E	E	Q	Q
					P3	Q	Q	E	E	Q	Q

[a] GenBank accession numbers for indicated viruses: AY150564 (VR2332) (101), KM386622 (RVRp22), KP256233 (RVRp13), AF159149 (MLV) (64).
[b] Two independent pig lines, lines 1 and 2, were observed.

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TABLE 5 Full-length genome sequence comparison of ribavirin-resistant mutant viruses (RVRp13 and RVRp22), their parental strain (VR2332), and MLV[a]

Genomic region	Functional protein	nt position in genome	aa position	VR2332		RVRp13		RVRp22		MLV	
				nt sequence	Encoded amino acid	nt sequence	Encoded amino acid	nt sequence	Encoded amino acid	nt sequence	Encoded amino acid
ORF1a	nsp1β[b]	785	199	GTC	V	GTC	V	ATC	I	ATC	I
		839	217	GAG	E	AAG	K	GAG	E	GAG	E
		1182	331	TCC	S	TCC	S	TTC	F	TTC	F
	nsp2	1583	465	CCA	P	CCA	P	TCA	S	CCA	P
		2193	668	TCC	S	TCC	S	TTC	F	TTC	F
		2236	682	ACC	T	ACC	T	ACT	T	ACC	T
		2553	788	TCG	S	TCG	S	TTG	L	TCG	S
		3041	951	GAC	D	GAC	D	AAC	N	AAC	N
		3245	1019	AAA	K	AAA	K	GAA	E	AAA	K
		3747	1186	GCT	A	GCT	A	GTT	V	GCT	A
		3932	1248	TAC	Y	TAC	Y	CAC	H	TAC	Y
		4313	1375	CTT	L	CTT	L	TTT	F	CTT	L
		4883	1565	CTG	L	CTG	L	TTG	L	CTG	L
	nsp5	6346	2052	CCA	P	CCA	P	CCT	P	CCT	P
		6674, 6675	2162	CCG	P	TTG	L	CTG	L	CTG	L
	nsp7α	7176	2329	CCG	P	CTG	L	CCG	P	CCG	P
	nsp7β	7381	2397	GTC	V	GTT	V	GTC	V	GTC	V
		7389	2400	AAC	N	AAC	N	ACC	T	AAC	N
		7504	2438	TCC	S	TCT	S	TCC	S	TCC	S
		7555	2455	GTC	V	GTT	V	GTT	V	GTT	V
	nsp8+nsp9	7588	2466	CAG	Q	CAA	Q	CAG	Q	CAG	Q
ORF1b	nsp9	9075	459	ACC	T	ACC	T	ACT	T	ACC	T
		9102	468	CAG	Q	CAG	Q	CAA	Q	CAG	Q
		9162	488	GAC	D	GAT	D	GAC	D	GAC	D
		9378	560	GTC	V	GTT	V	GTC	V	GTC	V
		9618	640	GAG	E	GAA	E	GAG	E	GAG	E
	nsp10	10143	815	CAG	Q	CAA	Q	CAG	Q	CAG	Q
		10338	880	AGT	S	AGC	S	AGT	S	AGT	S
		10461	921	CAG	Q	CAA	Q	CAG	Q	CAG	Q
		10527	943	CGT	R	CGC	R	CGT	R	CGT	R
		10534	946	TAC	Y	CAC	H	CAC	H	CAC	H
		10554	952	AGG	R	AGA	R	AGG	R	AGG	R
		10896	1066	GAC	D	GAT	D	GAT	D	GAT	D
	nsp11	11184	1162	GGG	G	GGG	G	GGA	G	GGG	G
		11230	1178	GTG	V	TTG	L	TTG	L	TTG	L
		11397	1233	CCG	P	CCG	P	CCA	P	CCA	P
		11448	1250	GGG	G	GGA	G	GGA	G	GGA	G
		11575	1293	GTC	V	ATC	I	GTC	V	GTC	V
	nsp12	11748	1350	GGG	G	GGA	G	GGG	G	GGG	G
ORF2	GP2	24	8	GCC	A	GCC	A	GCT	A	GCC	A
		30	10	TTG	L	TTT	F	TTT	F	TTT	F
		183	61	GTA	V	GTG	V	GTG	V	GTG	V
		231	77	GCC	A	GCT	A	GCC	A	GCC	A
		396	132	AGC	S	AGT	S	AGC	S	AGC	S
		451	151	CTA	L	TTA	L	CTA	L	CTA	L
		503	168	ATG	M	ACG	T	ATG	M	ATG	M
		528	176	GGG	G	GGT	G	GGT	G	GGT	G
		570	190	GTG	V	GTA	V	GTG	V	GTG	V
		610	204	CAT	H	CAT	H	AAT	N	CAT	H
ORF3	GP3	190	64	ACA	T	GCA	A	GCA	A	GCA	A
		248	83	GGG	G	GAG	E	GAG	E	GAG	E
		255	85	GAC	D	GAT	D	GAT	D	GAT	D
		280	94	ATA	I	GTA	V	GTA	V	GTA	V
		375	125	GAG	E	GAA	E	GAG	E	GAG	E
		519	173	GTC	V	GTT	V	GTC	V	GTC	V
		644	215	ATA	I	ACA	T	ATA	I	ATA	I

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TABLE 5 (Continued)

Genomic region	Functional protein	nt position in genome	aa position	VR2332		RVRp13		RVRp22		MLV	
				nt sequence	Encoded amino acid	nt sequence	Encoded amino acid	nt sequence	Encoded amino acid	nt sequence	Encoded amino acid
ORF4	GP4	99	33	GAT	D	GAC	D	GAT	D	GAT	D
		228	76	CCC	P	CCT	P	CCC	P	CCC	P
		412	138	GTC	V	ATC	I	GTC	V	GTC	V
ORF5	GP5	38	13	CGA	R	CAA	Q	CAA	Q	CAA	Q
		98	33	AAC	N	AGC	S	AAC	N	AAC	N
		252	84	ACC	T	ACT	T	ACT	T	ACC	T
		451	151	AGA	R	GGA	G	GGA	G	GGA	G
		491	164	AGG	R	AAG	K	AGG	R	AGG	R
ORF6	M	46	16	CAA	Q	GAA	E	GAA	E	GAA	E
		99	33	GCC	A	GCC	A	GCT	A	GCC	A
		107	36	GTG	V	GTG	V	GCG	A	GTG	V
ORF7	N	60	20	AAT	N	AAC	N	AAT	N	AAT	N
		63	21	CAG	Q	CAA	Q	CAG	Q	CAG	Q
		355	119	GCA	A	ACA	T	GCA	A	GCA	A

[a] GenBank accession numbers for indicated viruses: AY150564 (VR2332) (101), KM386622 (RVRp22), KP256233 (RVRp13), and AF159149 (MLV) (64). nt, nucleotide; aa, amino acid.

[b] The cleavage position for each nonstructural protein (nsp) is based on a previous report (102).

In pigs, RVRp22 better maintained sequences related to virulence attenuation during the passages. RVRp22 has five amino acid mutations encoded in ORF1a (331^{F}), ORF1b (946^{H}), ORF2 (10^{F}), ORF5 (151^{G}), and ORF6 (16^{E}), whereas RVRp13 has four amino acid mutations (ORF1b [946^{H}], ORF2 [10^{F}], ORF5 [151^{G}], and ORF6 [16^{E}]) identical to the sequences in MLV. Those sequences have previously been reported to be associated with the virulence attenuation of MLV (39, 64, 66), and they quickly reverted to wild-type sequences during replication in pigs (39, 41, 65, 67, 82). Consistently, all attenuation sequences found in the RVRp13 and MLV genomes quickly reverted to wild-type sequences just after the first passage in pigs; however, two sequences in the RVRp22 genome, located in ORF2 (10^{F}) and ORF6 (16^{E}), were stable even after the third passage in pigs (Table 4), suggesting that the attenuation sequences in ORF2 and ORF6 are the main sequences involved in viral replication and virulence in pigs (16, 67, 83). Therefore, we concluded that RVRp22 has higher genetic and phenotypic stability than MLV and RVRp13 during replication in pigs.

Many studies (39, 84, 85) have reported that a number of wild-type PRRS viruses, including the VR2332 strain, were able to grow equally well in PAMs and MARC-145 cells; attenuated PRRS vaccine viruses, however, lost their replication ability partially or fully in PAMs, although they replicated efficiently in MARC-145 cells. RVRp13, RVRp22, and MLV showed poor replication in PAMs, reaching much lower titers than those measured in MARC-145 cells, whereas VR2332 (the parental virus of RVRp13 and RVRp22) replicated equally well in the two cell types (Fig. 4A and Table 2). Subsequently, the viruses were rescued from the pigs in each virus group at the end of each passage and were evaluated in terms of their growth efficiency in both cell types to determine how their cell preferences were altered after passages in pigs. As a result, the viruses rescued from the RVRp13 and MLV groups after the second and third passages replicated equally well in PAM and MARC-145 cells as did wild-type PRRSV (VR2332) (84). However, after the first passage, the rescued viruses from both groups replicated better in MARC-145 cells than in PAMs (Fig. 4B and Table 2), which is characteristic of an attenuated virus (84), indicating that RVRp13 and MLV lost their attenuation characteristics after the first passage in pigs. This result coincides with the reversion of the attenuation sequences to virulence and the increased virulence of RVRp13 or MLV in pigs between P2 and P3 (Table 2 and Fig. 1). In contrast, even after the third passage, the recovered viruses from the RVRp22-challenged groups still replicated better in MARC-145 cells than in PAMs, as the original RVRp22 did before the passage in pigs or as is characteristic of an attenuated vaccine virus (84), indicating that the attenuation of RVRp22 was maintained after three sequential passages in pigs.

As the RVRp22 mutant had higher genetic and phenotypic stability than RVRp13 or MLV, the full-length sequences of RVRp22 were compared with those of VR2332, RVRp13, and MLV (Table 5). The RVRp22 genome encoded nine unique amino acid mutations; seven mutations were encoded in ORF1a (nsp2 [465^{S}, 788^{L}, 1019^{E}, 1186^{V}, 1248^{H}, and 1375^{F}], nsp7β [2400^{T}]), and one mutation each was encoded in ORF2 (204^{N}) and ORF6 (36^{A}). The nsp2 locus is the most variable region among nonstructural proteins encoded in the PRRSV genome, and it consists of four functional regions (17, 72, 73): a papain-like protease domain (PLP2) (20) near the N terminus; a central hypervariable (HV) region with strain-specific insertions or deletions in both type 1 and type 2 PRRSV (86, 87); a putative transmembrane (TM)-hydrophobic region (86, 87); and a conserved C-terminal domain (86, 88, 89). Among the nine unique amino acid mutations found in RVRp22, six unique mutations are located in the nsp2 region, and one mutation (465^{S}) is located in the PLP2 proteinase (within the *trans*-cleavage activity site), a putative cysteine protease core domain (90) that is critical for virus replication (72) and that is highly conserved, not only in PRRSV strains but also among the members of the *Arteriviridae* family (90–94). Three mutations (788^{L}, 1019^{E}, and 1186^{V}) were found in the HV region (17, 72) of nsp2. Although the HV region of nsp2 may play a very limited role in viral replication by itself, it might be interrelated

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with the PLP2 domain, which is critically involved in nsp2/3 cleavage activity (72, 88, 93). The remaining two mutations (1248^{H} and 1375^{F}) are located in the putative TM region of nsp2, which is also a highly conserved domain among all PRRSV strains (17, 73). The TM domain is essential for PRRS virus replication, as reported previously (17, 72), and the hydrophobic domains are reported to be responsible for targeting the nonstructural proteins for intercellular membranes to form viral replication complexes for EAV (95, 96).

One mutation (2400^{T}) was also found in another nonstructural protein, nsp7β, which is a highly conserved protein that has critical roles in PRRSV RNA synthesis and translation (97, 98) and assembly of the replicase protein (99). Two additional mutations were found in ORF2 and ORF6, which encode two structural proteins. One mutation (204^{N}) is located in ORF2, which encodes the GP2a/2b protein in the PRRSV genome, which is essential for virus replication (83). The remaining mutation (36^{A}) is located in ORF6, which encodes the M (membrane) protein—an unglycosylated protein that is the most conserved structural protein in arteriviruses, including PRRSV (9, 74, 100)—which plays a key role in virus assembly and budding (16). Based on the information given above, the unique amino acid mutations encoded in ORFs 1a, 2, and 6 in the RVRp22 genome might be responsible for the lower levels of viral replication and virulence and the increased stability of RVRp22 during passaging in pigs, although the functional roles of these nine unique sequences remain to be elucidated.

In conclusion, the present study demonstrates that RVRp22, a ribavirin-resistant PRRSV mutant, has an attenuation phenotype in pigs and that it retains this attenuation phenotype and related sequences longer than a cell-adapted attenuated PRRS vaccine virus during sequential passages in pigs.

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