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RESEARCH ARTICLE

Molecular Characterization of Three Porcine Reproductive and Respiratory Syndrome Virus Isolates and Their Susceptibility to Antiviral Drugs

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most common swine pathogens that cause severe economic losses to the pig industry worldwide irrespective of the use of live or inactivated vaccines. This study aims to investigate the biological characteristics of three PRRSV isolates and their susceptibility to two antiviral drugs. Sequence analysis of the NSP2 gene classified two isolates as highly pathogenic (isolates FY and ZS) and one as classically pathogenic (isolate JX). Isolate FY grew faster than the other two isolates in MARC-145 cells; however, its RNA replication was lower than isolate ZS. By contrast, isolate JX exhibited slower growth and lower RNA replication capability. PRRSV infection suppressed the production of interferon β induced by poly (I:C). The viruses also differed in their susceptibility to antiviral drugs. Ribavirin exerted potent antiviral activity against all three viral isolates at concentrations of 7.5 and 15 μg/mL in MARC-145 cells. Acyclovir was found effective only on the classically pathogenic isolate. We suggest that ribavirin could have potential as an antiviral therapy for porcine reproductive and respiratory syndrome when vaccination is not able to provide effective protection.

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in sows and respiratory disease in piglets (Verheije *et al.*, 2003). Although the causal virus (PRRSV) strains from different countries cause similar clinical signs in pigs, they are different in virulence in infected animals and heterologous genetically and antigenically (Meng, 2000). PRRSV strains are divided into North American (NA) and European (EU) genotypes based on genetic differences in the genome structure (Nelsen *et al.*, 1999). PRRSV was first isolated in the Netherlands (Lelystad virus, LV) that belongs to the EU genotype (Wensvoort *et al.*, 1991). The first NA genotype strain VR-2332 was isolated by Collins *et al.* (1992). PRRSV is a positive-stranded RNA virus that possesses a genome size of approximately 15kb containing eight open reading frames (ORFs) (Godeny *et al.*, 1993). ORF1 encodes a polyprotein that is proteolytically cleaved into 13 mature nonstructural proteins (NSPs) assumed to be involved in viral replication and transcription. Of these NSPs, NSP2 is a multi-domain protein that contains a cysteine protease active site in the N-terminal vital to the viral life cycle (Han *et al.*, 2009).

In the summer of 2006, swine herds in China suffered from a large-scale outbreak of PRRS that caused tremendous economic losses. A highly virulent PRRSV strain with discontinued deletion of 30 amino acids in its NSP2-coding region was implicated in the outbreak (Li *et al.*, 2007; Zhou *et al.*, 2009). This highly pathogenic virus continues to evolve in multiple epicenters of China. Inactivated and modified live vaccines are not able to provide effective protection (Murtaugh and Genzow, 2011; Karuppannan *et al.*, 2012). Some studies have indicated that strain differences could affect the efficacy of current PRRS vaccines and that vaccines containing multiple strains could be more protective than single-strain vaccines (Huang and Meng, 2010; Geldhof *et al.*, 2012). Apparently, effective PRRS control has been

hampered by evolving genetic diversities among field isolates (Huang and Meng, 2010). Therefore, identification of the biological and genetic characteristics of the epidemic PRRSV strain is important.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has antiviral activity against some DNA and RNA viruses (Witkowski *et al.*, 1972). It is a pleiotropic antiviral agent with direct and indirect antiviral effects (Tam *et al.*, 2001). Acyclovir is a nucleoside analog that exerts its antiviral effects by competitive inhibition of viral DNA polymerase activity after three phosphorylation steps (Stranska *et al.*, 2005). Anti-PRRSV inhibitors are crucial at the early stages of a pandemic, especially when specific PRRSV vaccines are unavailable. However, there is paucity of information on antiviral drugs against PRRSV.

In this study, three PRRSV isolates from swine herds involved in the PPRS outbreak in China in 2006 were compared for their growth characteristics, susceptibility to antiviral drugs and inhibition of IFN-β to poly(I:C) stimulation.

MATERIALS AND METHODS

Virus isolation: MARC-145 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and incubated at 37°C and 5%CO_2. Clinical samples, including lungs, lymph nodes and kidneys, were collected from dead pigs with late-term abortions (sows) or with acute respiratory disease (growing pigs) during the PRRS outbreak of 2006 to 2007 (Tian *et al.*, 2007). Tissue samples were homogenized with sterile phosphate buffered saline (PBS), freeze-thawed three times, and spun at 5000×*g* for 20 min at 4°C. Supernatants were passed through a 0.22μm filter and transferred to MARC-145 cells for virus isolation. Virus isolates were identified by immunofluorescence assay (IFA) using PRRSV NSP2-specific monoclonal antibodies prepared in our laboratory.

Sequence analysis: For NSP2 gene sequencing, viral RNA was extracted from PRRSV present in the culture supernatants (Hu *et al.*, 2009). The complete NSP2 gene was amplified using the primers listed in Table 1. The partial NSP2 amino acid sequences and those retrieved from the GenBank database (Fig. 1) were multiple-aligned using MegAlign 7.1.0 (DNASTAR Lasergene software package).

50% viral tissue culture infective dose ($TCID_{50}$) and growth kinetics assay: The $TCID_{50}$ of the virus was determined by cytopathic effects on MARC-145 cells and calculated by the Reed–Muench method. For growth kinetics assay, MARC-145 cells were infected with the fourth generation PRRSV at 100 $TCID_{50}$. At 12, 24, 48, 72 and 96 h post-infection (hpi), cell and cell culture supernatants were collected and frozen at –80°C for further analysis. The viral growth kinetic assays were performed in triplicate. Three independent titrations were determined by $TCID_{50}$ per milliliter, and the mean value was used to determine the viral growth curve.

Quantitative polymerase chain reaction (qPCR) : To quantitate PRRSV RNA replication levels, total RNA was isolated from the samples. Quantitative PCR was performed using SYBR Green I dye (Takara Biotech Co., Ltd., China). The target mRNA in the samples was quantified by comparison with a standard curve derived from dilutions of plasmids containing PRRSV subgenomic ORF7 (10^0 to 10^8 copies). The internal control (β-actin gene) was quantified using the same procedures as above for normalized quantitation of viral RNA (Gao *et al.*, 2008). The PCR primers are shown in Table 1.

Table 1: The primers used are this study.

Gene	Primer	Sequence (5'to 3')
NSP2	Forward	GTTGAGCCCAATACGTCACCA
	Reverse	CTCCAGCCAAGATACAGTCTGC
IFN-β	Forward	TAAGCAGCTGCAGCAGTTCCAGAAG
(real time PCR)	Reverse	GTCTCATTCCAGCCAGTGCT
β-actin	Forward	CGTGCGTGACATCAAAGAGAAG
(real time PCR)	Reverse	CGTTGCCAATAGTGATGACCTG
ORF7	Forward	ATGGCCAGCCAGTCAATC
	Reverse	TCAGTCGCTAGAGGAAAATGG

Chemicals: Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and acyclovir (2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6*H*-purin-6-one) were purchased from Xinchang Pharmaceutical Factory, Zhejiang, China. Stock solutions were prepared by dissolving the drugs in PBS at pH 7.4 and diluted to the required concentrations in the culture medium.

Cell viability assay: A colorimetric assay for cell viability was used to determine the doses of the antivirus drugs. About 1.5×10^4 MARC-145 cells per well were seeded into 96-well culture plates and allowed to adhere overnight. The medium was removed and various concentrations of antiviral drugs in DMEM were added to the wells. After 72 h, the culture medium was removed and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (Beyotime, Beijing, China) was added to the wells according to the manufacturer's instructions. After 4 h of incubation, formazan generated in the cells was dissolved in 100 μL of formazan solution per well. Absorbance was measured at 490nm using a microplate reader SpectraMax^{M2} (Molecular Device, USA). The drug concentration that decreased the cell viability by 50% was defined as the 50% inhibitory concentration (IC_{50}). Each experiment was performed in triplicate.

Viral susceptibility to antiviral drugs: MARC-145 cells (1.5×10^5 per well) were cultured in DMEM in a 24-well plate for 24 h. The culture medium was then removed and the cells were pretreated with different concentrations of ribavirin or acyclovir for 1 h before viral inoculation at 100 $TCID_{50}$. The drug-containing media were reintroduced into corresponding wells after viral inoculation. Antiviral effect was evaluated at 48 hpi. Cells were collected and total RNA was isolated to quantitate viral RNA replication. Alternatively, the culture supernatants were collected at different time points for $TCID_{50}$ titration.

Quantitation of IFN-β mRNA: MARC-145 cells were seeded in 24-well plates, grown in DMEM containing 8% fetal bovine serum (FBS) at 37 °C in 5% CO_2 to a density of about 1.5×10^5 cells/well, and then infected with

Fig. 1: Alignment of partial amino acid sequences of NSP2 of PRRSV isolates and reference strains. The shadows indicate the deleted amino acids.

different PRRSV isolates at 100 $TCID_{50}$ for 36 h. Finally, the cells were transfected with 0.5 μg/mL poly(I:C) (InvivoGen) for 10 h (Kim *et al*., 2010). Total RNA was extracted and cDNA was synthesized with oligo (dT_{15}) (Takara Biotech Co., Ltd., China) and Moloney Murine leukemia virus reverse transcriptase (Promega). qPCR was performed in triplicate using SYBR Green I dye on a Bio-Rad iQ5 system using primers shown in Table 1. Results for the target gene were presented after normalization to β-actin. The $2^{-\Delta\Delta CT}$ method was used to quantify the relative transcript levels shown as relative fold changes in comparison with the mock-treated control (Livak and Schmittgen, 2001).

Statistical analysis: Two-tailed Student's *t*-test was performed using Microsoft Excel software. P values < 0.05 as statistically significant, P values < 0.01 as of marked significance.

RESULTS

Sequence analysis of the partial NSP2 gene of three PRRSV isolates: Typical cytopathic effects could be observed 3 dpi. The putative viral isolates were plaque-purified thrice to ensure the isolation of a single virus and confirmed by IFA with specific monoclonal antibody against PRRSV NSP2 and by sequencing. Three clinical viral isolates JX, FY and ZS were obtained. These isolates genotypically belonged to the North American type according to the NSP2 sequences (GenBank accession no: JQ798255, JQ798256 and JQ798257 for isolates JX, FY and ZS respectively). The NSP2 of isolate ZS exhibited 96.6% homology with the highly pathogenic PRRSV strain JXA1 (Tian *et al.*, 2007), higher than the two other viruses (JX at 89.9% and FY at 90.1%). Multiple amino acid sequence comparisons showed that isolates FY and ZS had discontinuous deletion of 30 aa (located at aa 481 and aa 533 to 561) in their NSP2 regions, similar to the highly pathogenic PRRSV strain JXA1. Isolates ZS showed two more deletions located at aa 592 and aa 593. By contrast, isolate JX had its NSP2 region identical to that of an earlier Chinese isolate CH1a (An *et al.*, 2005) (Fig. 1).

Titer and growth of three viral isolates JX, FY and ZS: The $TCID_{50}$ of isolates JX, FY and ZS were $10^{7.6}$, $10^{8.3}$ and $10^{8.6}$ per mL, respectively. The titer of three isolates peaked at 72 hpi with the isolate FY having slightly higher titer, as compared with JX (P<0.05), and ZS (P<0.05) (Fig. 2A). The RNA copy numbers of isolate ZS peaked 48 hpi, earlier and higher than that of the two other viral isolates, as compared with JX (P<0.001) and FY (P<0.001) (Fig. 2B).

PRRSV infection significantly suppressed transcription of IFN-β gene in response to poly(I:C) stimulation: To determine if PRRSV infection leads to general inhibition of IFN-β expression, we examined IFN-β transcription activity in PRRSV-infected MARC-145 cells upon poly (I:C) stimulation. Fig. 3 indicates that poly (I:C)-induced IFN-β gene transcription was significantly inhibited by all three PRRSV isolates (P<0.01).

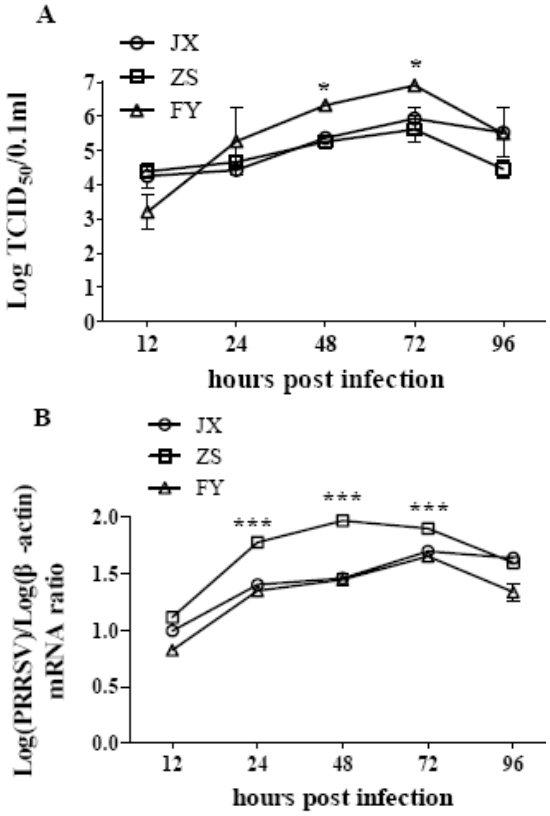

Fig. 2: Growth kinetics of three PRRSV isolates JX, ZS and FY. MARC-145 cells were infected with different virus at a 100$TCID_{50}$. At the indicated time points, cells and culture supernatants were collected for $TCID_{50}$ or RNA copy number analysis. (A) Growth kinetics was generated by virus titers plotted against time post-infection. (B) PRRSV RNA replication level was quantified by real-time RT-PCR analysis using total RNA from MARC-145 cells and cell culture supernatants. Data represent means±SD of three experiments, each in triplicate (*P<0.05; ***P<0.001).

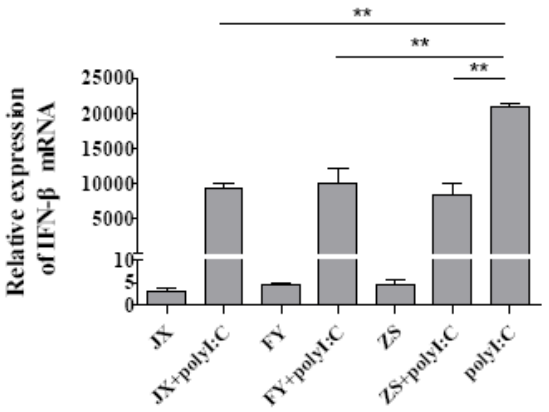

Fig. 3: Effects of PRRSV infection on IFN-β gene expression in MARC-145 cells treated with poly (I:C). MARC-145 cells were infected with PRRSV at 0.1 multiplicity of infection. At 36 h post-infection, poly (I:C) was transfected for 10 hs, and total cellular RNA was extracted. Expression of IFN-*β* was measured by RT-qPCR. Data represent means±SD of three experiments, each in triplicate (**P<0.01 when compared between PRRSV-infected/poly(I:C)-treated cells and poly(I:C) control).

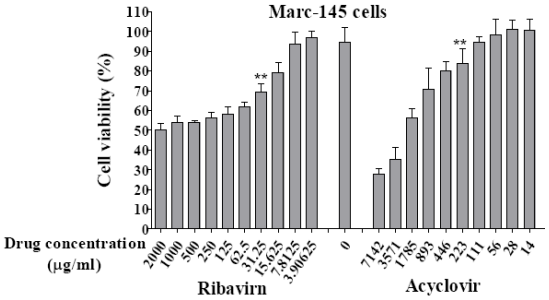

Fig. 4: Effect of ribavirin and acyclovir on viability of MARC-145 cells. Cell viability was determined by MTT assay after treatment with different levels of ribavirin or acyclovir for 48 hs. Percentage of relative cell viability represents means±SD of three experiments, each in triplicate (**P<0.01 at and above this concentration).

Viability of MACR-145 cells treated with ribavirin or acyclovir: Drugs that inhibit cell growth may interfere with observation of antiviral activity (Wu *et al*., 2004) and should be assessed on the viability of MARC-145 cells for determination of appropriate concentrations to be used for antiviral assays. The concentrations of ribavirin and acyclovir that inhibited cell growth by 50% (IC_{50}) were 125 and 893 μg/mL respectively. Ribavirin significantly decreased (P<0.01) MARC-145 cells viability at concentrations above 31.25 μg/mL, and acyclovir significantly decreased (P<0.01) MARC-145 cells viability at levels above 223 μg/mL. The marginal concentrations of ribavirin and acyclovir that did not affect MARC-145 cell viability should be lower than 15.6 and 111 μg/mL, respectively (Fig. 4).

Susceptibility of PRRSV isolates to ribavirin and acyclovir: Ribavirin significantly decreased the RNA transcription levels of all three viral isolates at concentrations of 7.5 and 15 μg/mL in MARC-145 cells (Fig. 5A). By contrast, acyclovir did not show inhibition of RNA transcription of two viral isolates (ZS and FY) at concentrations of 75 and 150μg/mL (Fig. 5B). Treatment with 10μg/mL ribavirin could decrease the virus titer by

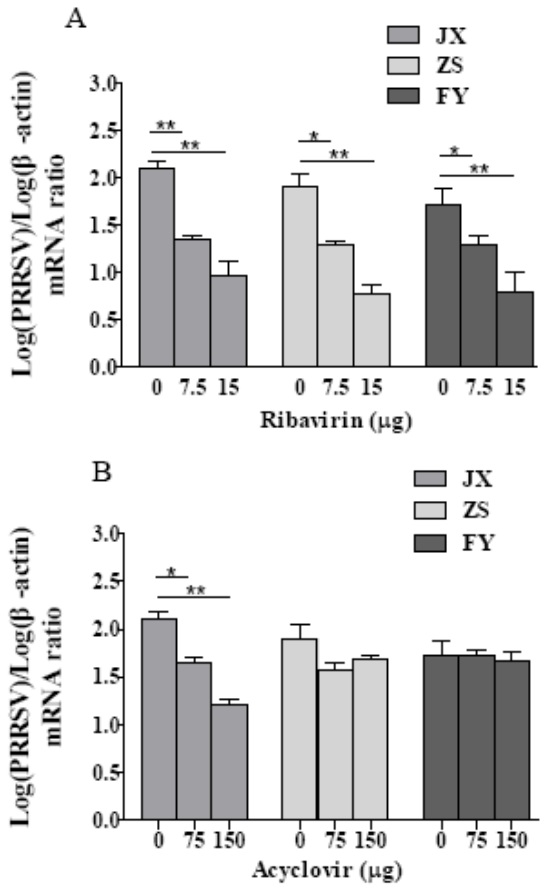

Fig. 5: Antiviral activity of ribavirin and acyclovir on PRRSV in MARC-145 cells shown as changes of viral RNA normalized to β-actin gene. Two-fold serial dilutions of ribavirin and acyclovir were prepared in DMEM and added to the MARC-145 cells for 1 h. The cells were infected with PRRSV at 100 $TCID_{50}$. Viral RNA copies were determined by real-time RT-PCR at 48h post-infection. Data represent means±SD of three experiments, each in triplicate (*P<0.05; **P<0.01).

$10^{2.96}$ (JX), $10^{1.2}$ (FY) and $10^{1.65}$ (ZS) 48 hpi. Ribavirin did not seem to have lasting antiviral effect upon further treatment to 72 hpi, particularly for isolate ZS. Treatment with 150μg/mL acyclovir did not show apparent antiviral activity on isolates ZS and FY although it did show inhibition on isolate JX (Fig. 6).

DISCUSSION

Porcine reproductive and respiratory syndrome has caused severe economic losses to the swine industry worldwide (Huang and Meng, 2010). The outbreak that occurred in 2006 was caused by a highly virulent PRRSV strain with discontinuous deletion of 30aa in its NSP2-coding region (Tian *et al.*, 2007). The low fidelity of viral RNA polymerase results in high antigenic heterogeneity. We found that isolates ZS and FY had discontinuous deletion of 30aa in NSP2 and belonged to highly pathogenic strains (Tian *et al.,* 2007), while isolate JX was similar to classical strain of North American type. This indicates that both highly pathogenic and classical strains might circulate in the epidemic areas. Occurrence of 30aa deletion may not be related to virulence of the highly pathogenic PRRSV that emerged in China (Zhou *et*

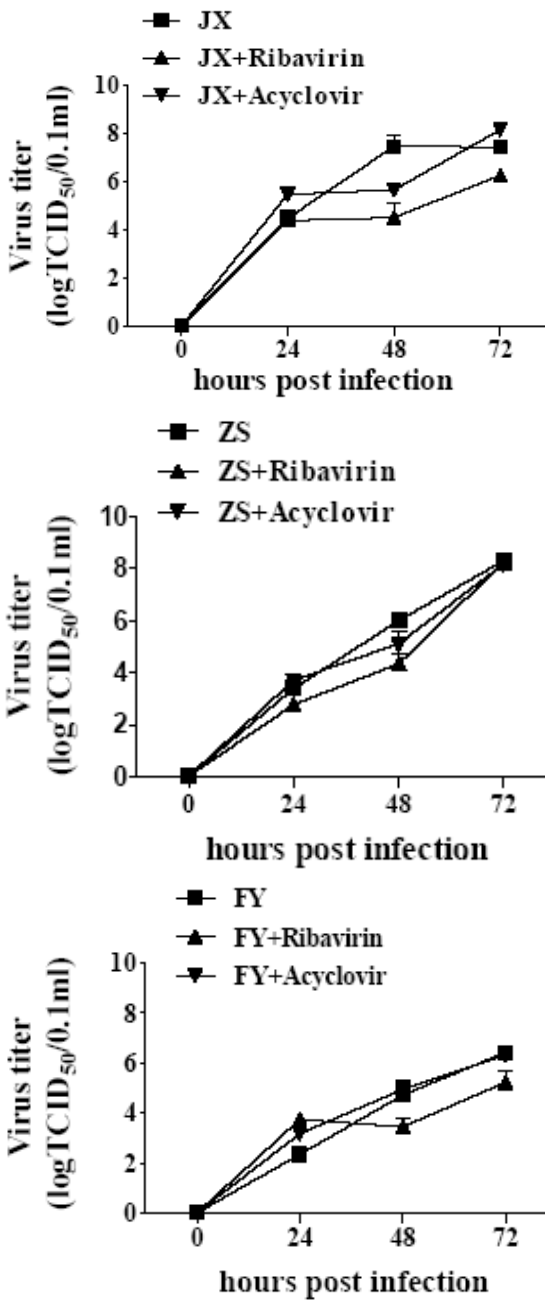

Fig. 6: The antiviral activity of ribavirin and acyclovir on PRRSV shown as changes of viral titers at different time points**.** MARC-145 cells were pretreated with ribavirin (10 μg/mL) and acyclovir (150 μg/mL) for 1 h, and then were infected with PRRSV at 100 $TCID_{50}$. Viral particles in the culture supernatants were titrated at different time points. Data represent means±SD of three experiments, each in triplicate.

al., 2009). Some differences in growth characteristics were observed between the highly pathogenic and classically pathogenic isolates *in vitro*. However, all three isolates suppressed the production of IFN-β in response to poly (I:C) induction, similar to other PRRSVs (Kim *et al.*, 2010; Li *et al.*, 2010).

Anti-PRRSV drugs could be important at the early stages of an endemic, especially when specific PRRSV vaccines are unavailable or not effective. Ribavirin and acyclovir have antiviral effects on a variety of DNA and RNA viruses (Stranska *et al.*, 2005; Tam *et al.*, 2001). However, their effects on PRRSV replication remain unknown. Here we show that ribavirin has potent antiviral activity against all three viruses at concentrations of 7.5 and 15μg/mL in MARC-145 cells, similar to findings in other *Nidovirales* (Kim and Lee, 2012). Acyclovir seems to be effective only against classically pathogenic PRRSV. Therefore, our work suggests that ribavirin could be resorted of as an antiviral drug when PRRSV is endemic without effective vaccines.

Conclusion: Of the three PRRSV isolates, two shared discontinuous deletion of 30 amino acids (aa) in NSP2 similar to the highly pathogenic type. All three isolates were highly or moderately susceptible to ribavirin, but only the classical pathogenic isolate is acyclovir-sensitive.

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