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Different immunological features of two genetically distinct type 2 porcine reproductive and respiratory syndrome (PRRS) viruses

Nadeem Shabir, Amina Khatun, Won-Il Kim*

College of Veterinary Medicine, Chonbuk National University, Jeonju 561-756, Korea

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Abstract

Although it has been generally accepted that porcine reproductive and respiratory syndrome virus (PRRSV) induces weak and delayed protective immunity after infection, it is unclear that the same immunological features can be applicable to all PRRS viruses because huge genetic variation exists even among the same genotypes of PRRSV (Type 1 and 2). In the current study, two genetically distinct type 2 PRRSV strains (VR-2332 and JA142) which showed approximately 90% nucleotide homology based on ORF5 sequences were characterized by both *in vitro* and *in vivo* assessments to determine the immunological features of the viruses. For *in vitro* assessment, porcine alveolar macrophages (PAM) were infected with the viruses at 10^{-3} multiplicity of infection (MOI) and then supernatants and cells were collected separately at 36 hrs post infection to determine the relative expression levels of IL-1α, IL-12, TNF-α and INF-α/β by quantitative RT-PCR. In addition, five PRRSV-free pigs were inoculated with either of JA142 or VR2332 for *in vivo* assessment. Serum samples were collected every week until 6 weeks post challenge. The serum samples were analyzed for the levels of viremia, PRRSV nucleocapsid-specific antibody and virus neutralizing antibody. Based on those assessments, the two viruses showed different patterns of cytokine expression in PAM and immune responses in pigs after infection. These results indicate that genetically distinct PRRSV strains have different immunological features, which might be criteria for virus classification and selection of candidate virus strains for vaccine development in the future.

Key words : PRRSV, Porcine alveolar macrophages, Cytokine expression, Serum virus neutralizing antibody, ELISA

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive disorder in breeding animals and respiratory distress in all ages of pigs (Bilodeau et al, 1991; Goyal, 1993). Its emergence was first reported in the United States in 1987 (Keffaber, 1989) and subsequently reported in Europe in 1990 (Paton et al, 1991). Isolation of PRRS virus (PRRSV), the causal agent of the disease, was first reported in Europe in 1991 (Wensvoort et al, 1991) and later in North America in 1992 (Collins et al, 1992). Since then, the virus has been identified in most of pig-producing regions worldwide and has caused significant economic losses in swine industries (Dea et al, 1992; Paton et al, 1992; Murakami et al, 1994). PRRSV is a member of the family *Arteriviridae* in the order *Nidovirales* (Cavanagh, 1997) and an enveloped virion containing a single-stranded positive-sense RNA genome. PRRSV has been classified into two genetically and antigenically distinct groups, European (Type 1) and North American (Type 2) genotype (Meng et al, 1995; Murtaugh et al, 1995). Remarkable genetic and antigenic variations have been observed between the genotypes and within the

*Corresponding author: Won-Il Kim, Tel. +82-63-270-3981, Fax. +82-63-270-3780, E-mail. kwi0621@jbnu.ac.kr

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same genotype (Yang et al, 1999; Cheon and Chae, 2000; Key et al, 2001; Forsberg et al, 2002; Pesch et al, 2005; Fang et al, 2007; Zhou et al, 2009). Such genetic and antigenic variation has hampered effective prevention and control of PRRS.

In general, PRRSV induces anti-inflammatory cytokines (Suradhat et al, 2003; Charerntantanakul et al, 2006; Gimeno et al, 2011; Dwivedi et al, 2012) and weak proinflammatory cytokines (López -Fuertes et al, 2000; Thanawongnuwech et al, 2004). Thus, it stimulates delayed and weak protective immunity [i.e., serum virus neutralizing (SVN) antibody and interferon (IFN)-γ secreting T cells] which seldom appears until 3 to 4 weeks after infection (Meier et al, 2003; Faaberg et al, 2006; Vanhee et al, 2009). Passively acquired SVN antibody alone was proven to prevent viremia and reproductive failure in pigs challenged with virulent PRRSV strains (Osorio et al, 2002), suggesting the critical role of SVN antibody in the control of virus infection. Nonetheless, cell-mediated immunity (CMI) appears to be essential for the clearance of PRRSV since PRRSV has been detected in lungs and lymph nodes despite the presence of SVN antibodies in serum or bronchoalveloar lavage fluid (Wills et al, 1997; Labarque et al, 2000; Bierk et al, 2001). The mechanisms for the weak induction of protective immunity by PRRSV have been explored in previous studies. The structural and non-structural proteins (nsp) are believed to modulate host immune responses (Wang et al, 2009). It has been recently shown that nsp1 of PRRSV modulates the expression of type I interferon (Beura et al, 2010; Beura et al, 2012; Chen et al, 2010; Wang et al, 2013; Kim et al, 2010) and TNF-α (Subramaniam et al, 2012), which leads to persistent infection (Lamontagne et al, 2003). In addition, PRRSV infects and destroys porcine alveolar macrophage (PAM), which is an active producer of cytokines and leukotrienes and has important roles in inducing pro- and anti-inflammatory responses in the alveolus (Gordon and Read, 2002) and innate immune response against respiratory infections (Pribul et al, 2008).

As described above, the weak and delayed protective immunity after PRRSV infection has been reported by many previous studies, it is still unclear that the same immunological features can be applicable to all PRRS viruses because huge genetic variation exists among PRRSV strains and only a few PRRSV strains have been well characterized for their immune responses after infection. In the current study, two genetically distinct type 2 PRRS viruses (VR-2332 and JA142) which showed approximately 90% nucleotide homology based on ORF5 sequences were characterized by both *in vitro* and *in vivo* assessments to determine the immunological features of the viruses.

MATERIALS AND METHODS

Cells and viruses

PAM cells were harvested from ten, 6-week old, PRRS negative pigs as described. Pigs were euthanized and the lungs along with trachea and bronchus were aseptically collected from pigs. The lungs were lavaged thrice with 0.01 M phosphate buffer saline (PBS, pH 7.4) and the harvested wash fluid was centrifuged for 10 minutes at 1,000×g. The resulting pellet was washed three times with PBS and resuspended in 5 mL of PBS. To evaluate cell viability, the cells were diluted 100 times in PBS and mixed with 0.4% trypan blue in 1 : 1 ratio and counted by the Countess™ Automated Cell Counter (Invitrogen, Carlsbad, CA, USA). After counting, cells adjusted to 5×10^7/mL were dispensed in cryovials and saved in −80°C until used. For culturing PAM, the cells were briefly thawed in a waterbath at 37°C with gentle stirring and diluted 10 times in RPMI-1640 (Sigma Aldrich, St. Louis, USA) containing 10% FBS, 1% L-glutamine and 1% antibiotics (10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of Amphotericin B) (Gibco, Carlsbad, CA, USA) so as to maintain a final cell number of 5×10^6/mL. The cells were seeded in each well of a 6-well plate (BD Falcon, Franklin Lakes, NJ, USA) and cultured overnight. Two North American (Type 2) PRRSV strains, JA142 and VR2332, were selected for used in this study and propagated in MARC-145 cells.

Evaluation of transcriptional activation of cytokines in PAM cells

PAM cells were infected with 10^{-3} MOI of JA142 or VR2332. Cells were harvested at 36 hrs after infection and cellular RNA was extracted using GeneAll® Hybrid-R™ kit (GeneAll Biotechnology, Seoul, Korea) following the manufacturer's instructions. RNA was reverse transcribed into cDNA using high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster city, CA) and real-time polymerase chain reaction (PCR) was then performed on a 7500 Fast Real-time PCR system (Applied Biosystems) using various cytokine specific primers by following the manufacturer's instructions. Primer sequences used in this study are shown in Table 1. Ten μL of 2× Power SYBGR (Applied Biosystems), 2 μL of cDNA and 1 μL of each forward primer (10 pm/μL) and reverse primer (10 pm/μL) were used for PCR amplification. All the samples were tested in duplicate and the cycling conditions were as follows: (a) Holding for 10 min at 95°C; (b) 40 cycles of 15 s at 95°C and 1 min at 60°C and (c) Melt curve stage for 15 s at 95°C, 1 min at 60°C, 15 s at 95°C and 15 s at 60°C. Relative quantities of cytokine mRNA in infected and non-infected cells were normalized to β-actin mRNA and the amounts were determined by the $2^{-\Delta\Delta Ct}$ method.

Quantitative PCR for virus titration

The virus titers in the supernatant and PAM cell lysates were measured by a real-time reverse transcription-polymerase chain reaction (RT-PCR) using TaqMan® chemistry. The primer and probe sequences designed are:

Forward primer: 5'-TGTCAGATTCAGGGAGRATAA-GTTAC-3';
Probe: 5'-FAM-TTTTGCACCACMGCCAGCCC-BHQ-3';
Reverse primer: 5'-ATCARGCGCACAGTRTGATGC-3'

Viral RNA was extracted from supernatant or cells using GeneAll® viral RNA extraction kit (GeneAll Biotechnology) or GeneAll® Hybrid-R™ kit (GeneAll Biotechnology), respectively, following the manufacturer's instructions. Viral quantification was carried out by real-time RT-PCR using AgPath-ID™ One-Step RT-PCR Kit (Ambion, Austin, TX, USA) in a 25 μL reaction volume using 5 μL of extracted template. The final concentration of each primer or probe was 0.8 or 1 pmol, respectively. The PCR amplification was performed as follows: (a) reverse transcription for 10 min at 45°C; (b) a 10 min activation step at 95°C; and (c) 40 cycles of 15 s at 95°C and 45 s at 60°C. Samples with a threshold cycle (Ct) of 35 cycles or less were considered positive. A set of serially diluted virus samples with known titers was used to calculate the amount of PRRSV in each sample by converting Ct value to virus titer ($TCID_{50}$/mL).

Animal study

A total of 15, three-week-old PRRSV-free pigs were randomly divided into 3 groups and housed separately. After acclimated for 3 days, 5 pigs in each group were challenged with VR2332, JA142 (2 mL of 10^3 $TCID_{50}$/mL per pig) or sham inoculum (RPMI-1640) and housed for 6 weeks. All pigs were bled at 0, 7, 14, 21, 28, 35 and 42 days post challenge (dpc). Sera were separated immediately after bleeding and stored in a −80°C freezer until used.

Table 1. The primer information for real-time RT-PCR used to measure the mRNA expression levels of various cytokines

Genes	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	*Accession/ Reference
β-Actin	GCGGGACATCAAGGAGAAG	AGGAAGGAGGGCTGGAAGAG	U07786
IL-1α	GTGCTCAAAACGAAGACGAACC	CATATTGCCATGCTTTTCCCAGAA	Duvigneau et al. (2005)
IL-12	TCAGGGACATCATCAAACCA	GAACACCAAACATCAGGGAAA	NM214013
TNF-α	TTATTCAGGAGGGCGAGGT	AGCAAAAGGAGGCACAGAGG	NM214022
IFN-α	TCTCATGCACCAGAGCCA	CCTGGACCACAGAAGGGA	Loving et al. (2006)
IFN-β	AGTGCATCCTCCAAATCGCT	GCTCATGGAAAGAGCTGTGGT	de Los Santos et al. (2006)

ELISA

All collected sera were tested by a commercial ELISA kit (Anigen PRRS Ab ELISA 4.0, Bionote, Hwasung, Korea) as per the manufacturer's instructions to determine IgG antibody specific for nucleocapsid protein of PRRSV (both type 1 and type 2).

Virus neutralization assay

Fluorescent focus neutralization (FFN) assay was performed to assess the titer of SVN antibodies in the sera as previously described (Kim and Yoon, 2008). The serum samples were first heat inactivated at 56°C for 45 min and then 2-fold serially diluted with RPMI-1640 medium growth medium. One hundred μL of each diluted serum were mixed with an equal volume of PRRSV (VR2332 or JA142) at 10^3 fluorescent focus forming unit per mL (FFU/mL). The mixtures were incubated at 37°C for 1 hr in a humidified atmosphere with 5% CO_2 and then transferred into MARC-145 cell monolayers prepared in 96-well plates (Corning Inc., Corning, NY, USA) and incubated for another 1 hr at 37°C in a humidified atmosphere with 5% CO_2 supply. After the removal of the inoculum, cells were replenished with 200 μL of fresh RPMI-1640 growth medium per well and further incubated at 37°C for 20 hr. Afterwards, cells were fixed with ice cold 80% acetone aqueous solution for 5 min. The acetone was removed and cells were air dried for 30 min. Then, cells were reacted with 1 : 10000 diluted PRRSV nucleocapsid specific monoclonal antibody SDOW-17 (Rural Technologies, Brookings, SD, USA) and stained with 1 : 250 diluted fluorescein isothiocyanate (FITC) labeled goat an-

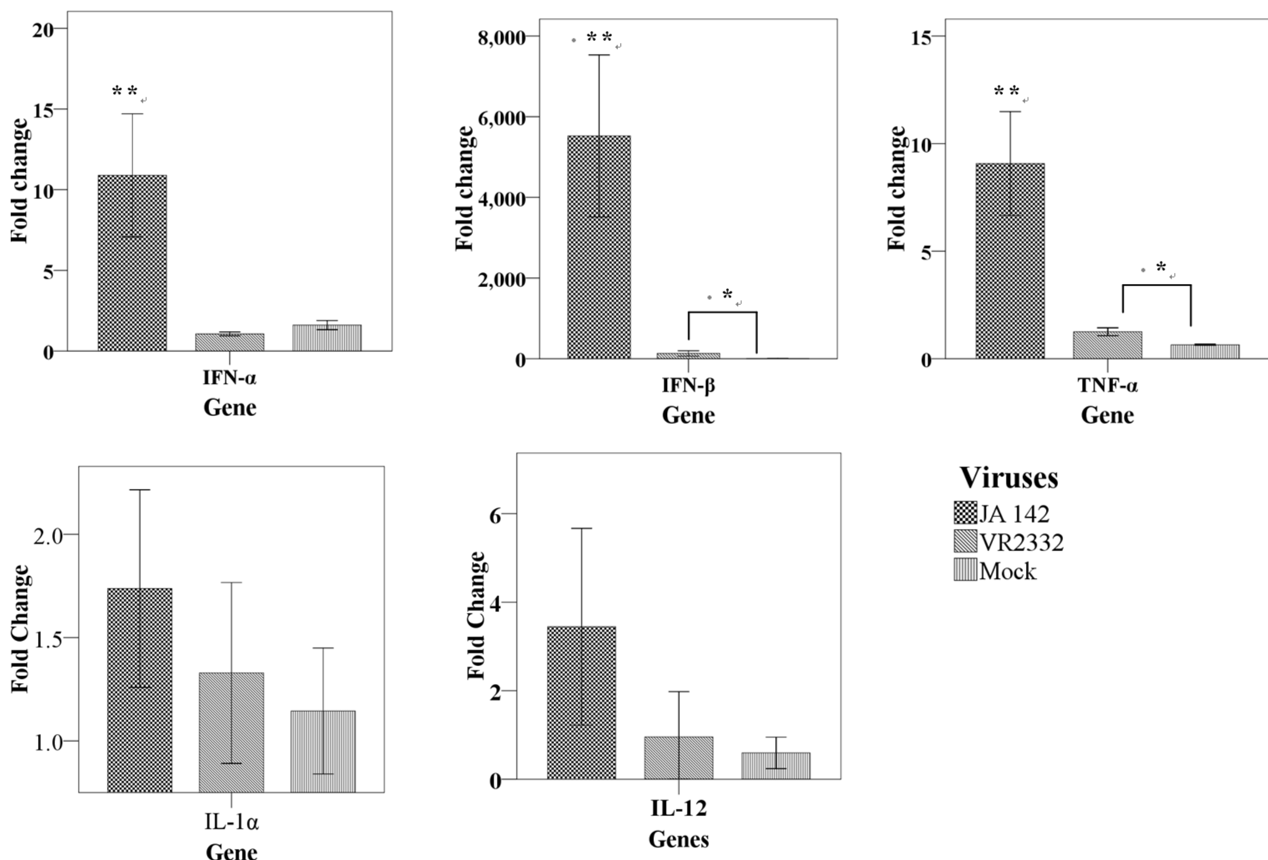

Fig. 1. Transcriptional expression of IL-1α, IL-12, TNF-α and INF-α/β in naïve PAM infected with either JA142 or VR2332. The relative expression was determined by quantitative RT-PCR. The values represent the relative expression of each cytokine normalized with β-actin. Asterisks indicate the significant differences in the cytokine expression induced by each virus as compared with mock infection (* indicates $P \leq 0.05$, ** indicates $P \leq 0.01$).

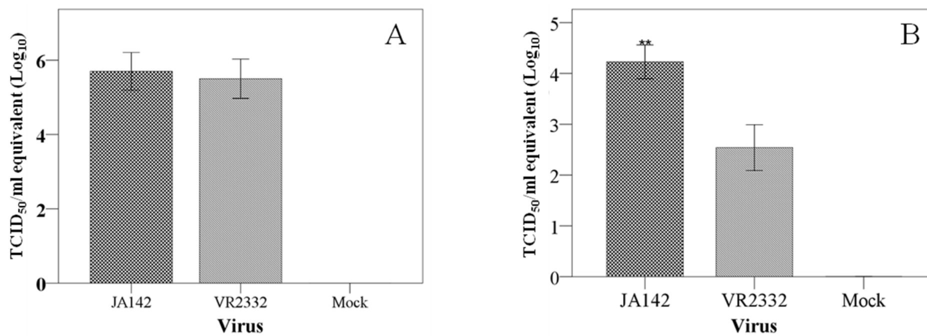

Fig. 2. Virus titers of PAM cell lysates (A) and supernatants (B) at 36 hrs after infection with either JA142 or VR2332. The virus titers were quantified by real-time RT-PCR. Asterisks indicate the significant difference in virus titers as compared with VR2332 (* indicates $P \leq 0.05$, ** indicates $P \leq 0.01$).

ti-mouse IgG (H+L) (KPL, Gaithersburg, MD, USA). Before observation under an invert fluorescent microscope, plates were washed three times with PBS and then number of virus-specific fluorescent foci in each well was counted. SVN antibody titer was expressed as reciprocal of the highest dilution in which 90% or higher reduction in the number of FFU was observed.

Data Analysis

Statistical analysis was done by SPSS Advanced Statistics 17.0 (SPSS Inc., Chicago, USA). The cytokine induction by each virus in PAM was compared with that by VR2332 using Man Whitney U test. The repeated measurements of viremia and PRRSV nucleocapsid-specific antibody levels in the challenged pigs were analyzed using repeated measures ANOVA to determine the overall difference and pairwise comparisons between groups.

RESULTS

Different PRRSV strains displayed various levels of transcriptional activation of cytokines

PAM cells were infected with JA142 or VR2332 and the transcriptional activation of cytokines, viz. TNF-α, IFN-α, IFN-β was measured by real-time PCR. JA142 induced a significantly higher mRNA expression of IFN-α, IFN-β and TNF-α (Fig. 1) compared with mock or VR2332 though the mRNA accumulation of TNF-α and IFN-β induced by VR2332 was also significantly higher compared with mock. Other cytokines, viz. IL-1α and IL-12 did not display any significant differences in their levels induced by either of viruses.

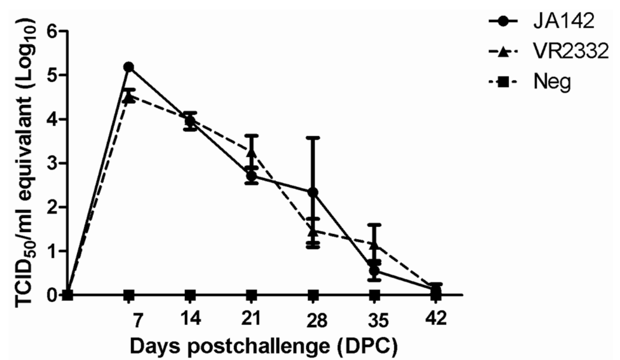

Fig. 3. The levels of viremia in sera collected from pigs challenged with JA142, VR2332 or sham inoculum. Three-week-old pigs were challenged with VR2332, JA142, or sham inoculums at 0 dpc and bled to measure viremia levels at indicated time points.

Viral titers in the supernatants and PAM cell lysates

Viral titers detected from PAM cell lysates (Fig. 2) did not show any significant differences between JA142 and VR2332 infected cells, however, the viral titers in the supernatants (Fig. 2) were significantly higher in JA142-infected cells when compared with VR2332-infected cells.

Viremia levels after challenge with JA142 or VR2332

Viremia was detected in all of the sera collected from the challenged pigs at 7 dpc (Fig. 3) when most viruses reached their highest titer. The mean levels of viremia in the pigs challenged with VR2332 or JA142 were 4.6 or 5.2 log_{10} $TCID_{50}$/mL, respectively. At 42 dpc, the last

bleeding time point, viremia was not detected in most of the challenged pigs. There were no significant differences in viremia levels between the groups challenged with JA142 or VR2332.

PRRSV nucleocapsid-specific antibody responses

Pigs seroconverted to PRRSV at 2 weeks after the exposure to PRRSV and remained seropositive until the end of the study (Fig. 4). The negative control group remained seronegative until the end of the study. No significant difference was observed between the groups challenged with JA142 or VR2332.

The induction of SVN antibodies after challenge

Levels of SVN antibodies in collected serum samples were determined by FFN. At 42 dpc (the last bleeding time point), only homologous SVN antibody was induced and no significant cross-neutralizing antibody was induced either by VR2332 or JA142 (Fig. 5). However, JA142 induces significantly higher levels of homologous SVN antibodies (1∶4 to 1∶32) as compared with VR2332 (<1∶4).

Fig. 4. The levels of PRRSV nucleocapsid-specific antibody in sera collected from pigs challenged with JA142, VR2332 or sham inoculum. The cut-off for positive is 0.4 S/P ratios.

DISCUSSION

The aberrant immune response by PRRSV infection has been one of the major hurdles in understanding the immunobiology of PRRS. Although it is known that PRRSV primarily replicates in PAM (Benfield et al, 1992; Wensvoort et al, 1992), local and systemic immune responses mediated by PAM infected with various PRRSV strains remains largely unexplored. In the present study, the immunological features of two genetically distinct type 2 PRRSV strains were characterized by *in vitro* and *in vivo* assessments. Even though the two strains showed approximately 90% nucleotide homology based on ORF5 sequences, the transcriptional activation of various cytokines induced by these two PRRSV strains varied significantly; VR2332 induced significantly lower mRNA expression of INF-α/β and TNF-α than JA142 in PAM though it induced still higher levels of IFN-β and TNF-α expression compared with mock controls. In special, VR2332 failed to induce a significant expression of INF-α, an important type I interferon and it might indicate that weak protective immune responses induced by VR2332 could be resulted from poor type I interferon expression after VR2332

Fig. 5. The levels of serum virus neutralizing (SVN) antibody against JA142 (A) and VR2332 (B) in sera collected at 42 dpc from pigs challenged JA142, VR2332 or sham inoculum. Mean of each group was indicated as a horizontal bar.

infection. The differential cytokine expression levels induced by JA142 and VR2332 is in agreement with a recently published report demonstrating that various PRRSV isolates induce different cytokine-expression profiles on antigen presenting cells (Gimeno et al, 2011).

In animal study, there were no differences in viremia and nucleocapsid-specific antibody levels detected from the pigs infected with either JA142 or VR2332 until 42 dpc, suggesting that JA142 and VR2332 replicate equally well and produce similar levels of non-virus neutralizing antibody (i.e., nucleocapsid-specific antibody) upon infection. However, the pigs infected with JA142 produced significantly higher levels of SVN antibody at 42 dpc as compared with those infected with VR2332. Because JA142 induced also higher levels of pro-inflammatory cytokine than VR2332 in PAM after infection, it was speculated that different expression levels of pro-inflammatory cytokines in PAM could play an important role in the production of SVN antibodies and protective immune responses.

The present study gives an insight into the differential immunological features of two PRRSV strains though genetic determinants and mechanisms for the modulation of host immune response by PRRSV should be explored further in the future. In addition, the current study could be useful in classifying PRRSV based on immunological characteristics and selecting candidate strains for vaccine development against PRRSV.

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