Tilmicosin Reduces PRRSV Loads in Pigs *in vivo*

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Abstract

Porcine respiratory and reproductive syndrome virus (PRRSV) is an important pathogen having a significant economic impact on the swine industry worldwide. Tilmicosin is a new semi-synthetic macrolide antibiotic developed from tylosin B. Tilmicosin can enter pulmonary alveolar macrophages (PAMs) and inhibit the replication of PRRSV in PAMs *in vitro*. This study was conducted to evaluate the impact of tilmicosin in controlling the replication of PRRSV *in vivo*. Forty and 635 weaned piglets were randomly chosen from PRRSV-contaminated farrow-to-finish herds in Taiwan and China, respectively. The piglets were equally divided into two groups and housed in the same pen but separated into individual spaces. Tilmicosin (Tilmovet® 20% premix, 400 mg/kg) was administered after weaning for 21 days (treated group). The untreated group of piglets did not receive tilmicosin. Blood samples were collected at 4, 6, 8, 10 and 12 weeks of age to detect of the PRRSV load. At 8 and 10 weeks of age, the tilmicosin-treated piglets had a significantly lower PRRSV load than the untreated piglets ($P < 0.05$) in Taiwan. At 6, 8, 10, and 12 weeks of age, the tilmicosin-treated piglets had a significantly lower PRRSV load than the untreated piglets ($P < 0.05$) in China. These data indicates that animals treated with tilmicosin exhibited not only reduced PRRSV loads but also improved average daily weight gain during the study period.

Keywords: PRRSV, viral load, tilmicosin, *in vivo* test

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an important swine disease that causes significant economic losses in most pig-producing countries (Zimmerman et al., 2012). The causative agent, the PRRS virus (PRRSV), was identified in the early 1990s. This immunopathogenic porcine disease is similar to dengue virus (DV) and feline infectious peritonitis (FIP), which are found in humans and cats, respectively (Yoon, Wu, Zimmerman, & Platt, 1997). These diseases enhance virus infection by a process known as antibody-dependent enhancement. This process plays an important role in enhancing the opportunity for viruses to infect target cells

and enhances the severity of infections caused by PRRS (Qiao et al., 2011; Yoon, Wu, Zimmerman, Hill, & Platt, 1996; Yoon et al., 1997), DV (Halstead, 2003) and FIP diseases (Hohdatsu et al., 1998; Olsen, Corapi, Ngichabe, Baines, & Scott, 1992; Takano, Kawakami, Yamada, Satoh, & Hohdatsu, 2008).

Infection with PRRSV predominantly exists at a subclinical level as PRRSV participates as a cofactor with porcine circovirus type 2 (PCV2) in porcine respiratory disease complex (PRDC) and porcine circovirus-associated disease (PCVAD) (Chand, Trible, & Rowland, 2012). Vaccination against PCV2 infection has been reported that the average daily weight gain increased significantly and the mortality rate decreased for finishing pigs and nursery-finishing pigs (Kristensen, Baadsgaard, & Toft, 2011). Although PRRSV has been known as the causative pathogen of PRRS for more than 20 years, current management strategies mainly focus on preventing PRRSV infection using vaccination (Murtaugh & Genzow, 2011). However, the currently available modified live vaccines are not sufficient to eradicate the virus and do not provide complete immunity from infection (Murtaugh & Genzow, 2011).

In our previous study, pigs with high PRRSV loads ($> 10^{4.2}$ PRRSV genomes/µl of serum) appeared to demonstrate that high loads correlate with the presence of PRDC (Lin, Lin, Hung, Wang, & Chiou, 2013). These data indicate that reducing the PRRSV load in the serum may prevent pigs from developing clinical signs after PRRSV infection. Several studies have attempted to identify effective anti-PRRSV treatments *in vitro* and *in vivo* (Benfield, Chase, Moore, Wagner, & Zeman, 2002; Du, Yoo, Paradis, & Scherba, 2011; Han, Fan, Patel, & Zhang, 2009; Jiang, Fang, Luo, Xiao, & Chen, 2010; Karuppannan, Wu, Qiang, Chu, & Kwang, 2012; Keirstead, Lee, Yoo, Brooks, & Hayes, 2008; Kreutz & Ackermann, 1996; Li et al., 2009; Opriessnig et al., 2011; Patel et al., 2008; te Velthuis et al., 2010; van der Meer et al., 2007; Wei et al., 2011; Yang et al., 2013; Zhuge et al., 2012). However, there are no effective commercial drugs available to prevent PRRSV infection in pigs *in vivo*.

Tilmicosin is a new semi-synthetic macrolide antibiotic derived from tylosin B (Scorneaux & Shryock, 1998) and is an effective antimicrobial for Gram-positive and some Gram-negative bacteria (Brumbaugh et al., 2002; DeRosa, Veenhuizen, Bade, & Shryock, 2000; Fittipaldi et al., 2005; Shryock, Staples, & DeRosa, 2002; Thacker, Young, Erickson, & DeBey, 2001). A recent study showed that tilmicosin exhibited strong antiviral effects on PRRSV replication in cultured porcine alveolar macrophages in a dose-dependent manner (Du et al., 2011). In pigs that were experimentally infected with PRRSV and treated with tilmicosin as a feed additive, a reduction in the severity of lymph node hypertrophy, lung lesions and viremia was observed compared with untreated infected controls (Benfield et al., 2002). In addition, the use of tilmicosin model systems *in vitro* and *in vivo* demonstrated that this antibiotic has potent immune-modulatory and anti-inflammatory effects (Buret, 2010; Cao et al., 2006; Ci, Chu, Xiang, Li, & Deng, 2011).

Based on the antiviral activities of tilmicosin, we hypothesized that tilmicosin could serve as an antiviral agent against PRRSV in naturally infected pigs. Therefore, the purpose of this study was to evaluate the impact of tilmicosin in controlling the replication of PRRSV *in vivo*.

2. Method

2.1 Animal and Specimen Collection and Sample Preparation

Forty four-week-old and 635 three-week-old weaned piglets were randomly chosen from PRRSV-contaminated farrow-to-finished herds in Taiwan and China, respectively. Both pig herds have shown that most nursery pigs become infected with PRRSV during 6-8 weeks of age (data not shown). The piglets were equally and randomly divided into two groups and housed in the same pen but separated into individual spaces. Food and water were provided *ad libitum* throughout the experiment. The piglets in the treated group were fed with tilmicosin (2 mg/kg feed, Tilmovet® 20% premix, Huvepharma, Belgium) for 3 consecutive weeks starting from weaning. The untreated group of piglets, which received the same feed as the treatment group but without tilmicosin, served as the control group. All piglets were weighed at 4 and 12 weeks of age in the Taiwanese experiment. Blood samples from Taiwanese and Chinese experiment were collected at 4, 6, 8, 10 and 12 weeks of age. Serum was separated and stored at -80 °C for the subsequent determination of viral RNA.

2.2 Real-Time PCR

All of specimens from Taiwanese experiment screened using zip nucleic acids probe-based real-time PCR for PRRSV, as described by Lin et al (Lin et al., 2013). Viral RNA isolation from the Chinese specimens was performed using an RNA extraction kit (E. Z. N. A.® Blood RNA Kit, Omega Bio-Tck Inc, Guangzhou, China) according to the manufacturer's instructions. Reverse transcription (RT) and real-time PCR were performed on the Chinese samples using a one step RT kit (One Step SYBR® PrimeScript RT-PCR Kit II, TaKaRa Biotechnology, Dalian, China). The oligonucleotide primers used to quantify the PRRSV load of Chinese

samples were qPCR-F, 5'-GAT AGC ACA GCT CCA CAG AAG G -3' and qPCR-R, 5' -CAC GAA TGT CAT GTA CCC GAA G-3'. All of Taiwanese and Chinese samples were analyzed at National Pingtung University of Science and Technology, Taiwan and South China Agricultural University, China, respectively.

2.3 ELISA

Specific anti-PRRSV antibody detection was conducted on serum samples collected from the treated and untreated piglets at 4, 6, 8, 10 and 12 weeks of age in the Taiwanese experiment using the HerdChek PRRS X3 Virus Antibody test kit (IDEXX Laboratories, Inc., Westbrook, ME, USA) according to the manufacturer's instructions. An ELISA sample-to-positive (S/P) ratio less than 0.4 was considered to be negative for the presence of anti-PRRSV antibodies.

2.4 Statistical Analysis

Student's *t*-test was used to compare viral loads at different time points between the treated and untreated groups. A chi-square test was used to evaluate differences in the survival rates between the treated and untreated groups. *P* values < 0.05 and < 0.001 were considered significant and highly significant, respectively.

3. Results

3.1 Survival Rate and PRRSV Detection Rate

During the Taiwanese experimental period (from 4 to 12 weeks of age), 5 and 1 piglets died in the untreated and treated groups, respectively, whereas during the Chinese experimental period (from 3 to 12 weeks of age), 9 and 12 piglets died in the untreated and treated groups, respectively; the differences were not significant between the untreated and treated groups in both experiments (data not shown). The presence of PRRSV was tested using serum from both the untreated and treated groups. As determined by real-time PCR, 31 of the 93 samples (33.3%) from the untreated group and 30 of the 96 samples (31.3%) from the treated group were positive in Taiwan (Table 1). The correlation of the PRRSV detection rate between the untreated and treated groups was calculated. Between the two experiments that were analyzed, a positive result for PRRSV did not appear to be significantly correlated with tilmicosin treatment at each time point (Table 1).

3.2 Evaluation of PRRSV Load at Different Time Points

The evaluation of the PRRSV load at different time points revealed that the PRRSV load was significant higher ($P = 0.0003$) in the untreated group (ranging from 1.96 to 5.19 $\log_{10}$ PRRSV genomes/µl, median 3.17 $\log_{10}$) compared with the treated group in Taiwan (ranging from 1.25 to 3.73 $\log_{10}$ PRRSV genomes/µl, median 2.50 $\log_{10}$) (Table 1). Similar results were also observed in the Chinese experiment as the PRRSV load was highly significant ($P = 6.86E-07$) in the untreated group (ranging from 0.15 to 5.84 $\log_{10}$ PRRSV genomes/µl, median 2.66 $\log_{10}$) compared with the treated group (ranging from 0.86 to 3.95 $\log_{10}$ PRRSV genomes/µl, median 2.11 $\log_{10}$) (Table 1). PRRSV viremia was detected in both groups throughout the trial, except at 4 weeks of age (all negative in Taiwanese experiment). The mean PRRSV nucleic acid copy number in the treated group was lower than that in the untreated group at each time point, with significant differences observed at 8 ($P = 0.027$) and 10 ($P = 0.007$) weeks of age (Figure 1) in the Taiwanese group. Similar results were obtained in the Chinese experiment, except at 4 weeks of age. The mean PRRSV nucleic acid copy number in the treated group was lower than that in the untreated group at each time point, with significant differences observed at 6 ($P = 0.014$), 8 ($P = 0.006$), 10 ($P = 0.007$), and 12 ($P = 0.001$) weeks of age (Figure 2). The maximum and minimum copy numbers at different time points were consistently lower in the piglets treated with tilmicosin than in the untreated piglets in both studies (Figures 1 and 2).

3.3 Measurement of PRRSV Antibody in the Taiwanese Experiment

The ELISA results showed that the piglets from both groups had the lowest S/P ratios at 6 weeks of age, which subsequently increased from 8 to 12 weeks of age. The differences between the groups were not statistically significant (Figure 3).

3.4 Evaluation of Average Daily Weight in the Taiwanese Experiment

To assess the effects of tilmicosin administration on the average daily weight gain in both groups, all piglets were weighed at 4 and 12 weeks of age. The average daily weight gain was 0.45 ± 0.13 kg and 0.48 ± 0.08 kg for the untreated and treated groups, respectively (Table 2). The average daily weight gain of the piglets in the treated group was slightly higher than that in the untreated group but was not statistically different between the two groups.

Table 1. Number and descriptions of the PRRSV load results in serum collected from untreated and treated group in Taiwan and China

	Taiwan		China	
	Untreated	Treated	Untreated	Treated
Number of tested samples	93	96	70	75
Number of positive pigs (%)	31 (33.3)	30 (31.3)	64 (91.4)	73 (97.3)
Mean ± SD [1]	3.38 ± 1.01	2.58 ± 0.60**	3.01 ± 1.27	2.12 ± 0.67**
Median [1]	3.17	2.50	2.66	2.11
Range [1]	1.96 to 5.19	1.25 to 3.73	0.15 to 5.84	0.86 to 3.95

Note. [1] Log_{10} copies number per microliter in serum; ** Highly significant difference between untreated and treated group.

Table 2. Average daily weight gain (ADWG) and mean body weights of pigs in both groups at 4 and 12 weeks of age.

Age (weeks)	Group [1]		P value
	Untreated	Treated	
4	7.99 ± 0.72 (20) [2]	7.79 ± 0.73 (20)	0.397
12	33.19 ± 7.75 (15)	34.92 ± 4.64 (19)	0.424
ADWG	0.45 ± 0.13 (15)	0.48 ± 0.08 (19)	0.347

Note. [1] Data shown in this table are presented as kilograms; [2] Data are shown as the mean ± SD (numbers).

4. Discussion

The currently available modified live vaccines are inadequate to eradicate the virus and do not provide complete immunity from infection (Murtaugh & Genzow, 2011). Therefore, successful supplementary strategies should include antiviral drugs and immune-modulation therapies (Benfield et al., 2002; Du et al., 2011; Han et al., 2009; Jiang et al., 2010; Karuppannan et al., 2012; Keirstead et al., 2008; Kreutz & Ackermann, 1996; Li et al., 2009; Opriessnig et al., 2011; Patel et al., 2008; te Velthuis et al., 2010; van der Meer et al., 2007; Wei et al., 2011; Yang et al., 2013; Zhuge et al., 2012). When evaluating the antiviral effect of tilmicosin against PRRSV, our data suggested that tilmicosin would be effective in reducing the PRRSV loads *in vivo*. Tilmicosin is a new semi-synthetic macrolide antibiotic developed from tylosin with potent immune-modulation and anti-inflammatory effects (Buret, 2010; Cao et al., 2006; Ci et al., 2011). Tilmicosin has been developed for veterinary use as a premix feed formulation for swine and is considered efficacious against several porcine respiratory pathogens (DeRosa et al., 2000; Fittipaldi et al., 2005; Shryock et al., 2002; Thacker et al., 2001). Recently, Du et al. (2011) showed that tilmicosin exhibited strong antiviral effects on PRRSV replication in cultured porcine alveolar macrophages in a dose-dependent manner. Benfield et al. (2002) reported that tilmicosin reduced the severity of lymph node hypertrophy, lung lesions and viremia in experimentally PRRSV-infected pigs. This study is the first to demonstrate that tilmicosin reduces the PRRSV load in the serum of naturally infected pigs in Taiwan and China.

PRRSV infection predominantly exists at a subclinical level, with PRRSV participating as a cofactor in PRDC and PCVAD (Chand et al., 2012). Whether tilmicosin can also suppress PCV2 replication *in vivo* remains unknown. The antiviral effect of tilmicosin against PCV2 in the same piglets was examined, and no significant difference between the untreated and treated groups for PCV2 loads was observed (data not shown). This result may indicate that the tilmicosin-induced reduction in the PRRSV load was irrelevant to the PCV2 load in the serum. The mechanism underlying the anti-PRRSV effects of tilmicosin involves an increase in the pH of lysosomes and endosomes and a possible alteration of ion-channel activity on the viral membrane (Du et al., 2011). In contrast, acidification inhibitors enhance PCV2 infection (Misinzo, Delputte, & Nauwynck, 2008). Therefore, tilmicosin was reasonably effective at inhibiting the production of infectious PRRSV but not PCV2.

In a previous study, $10^{4.2}$ PRRSV genomes/µl in serum was proposed as the threshold for the presence of PRDC in pigs (Lin et al., 2013). Treatment with tilmicosin reduced PRRSV viremia in the piglets at each time point compared with the untreated group. Interestingly, none of piglets in the treated group were found to have loads as high as $10^{4.2}$ PRRSV genomes/µl in the serum (Figures 1 and 2). These data indicate that tilmicosin may

prevent the presence of PRDC in pigs naturally infected with PRRSV. Moreover, the survival rates and average daily weight gains showed no significant differences between the two groups in the present study, which may be due to the study period having lasted from 4 to 12 weeks of age.

The serology data indicated that the piglets from both groups showed no differences in the S/P ratios at each time point. Despite the reduced PRRSV load in the piglets treated with tilmicosin, these piglets were still infected with PRRSV based on the ELISA data. In addition, the tilmicosin treatment did not have any adverse effects on piglet growth. Therefore, we speculate that tilmicosin could be used clinically to reduce the PRRSV load in farms with a high prevalence of PRRSV. The further combined use of tilmicosin with the vaccination model could be considered the best method for the prevention and control of PRRSV infections.

In conclusion, we demonstrated that tilmicosin alone could significantly reduce viremia in infected animals and consequently improve average daily weight gain in PRRSV-infected pigs.
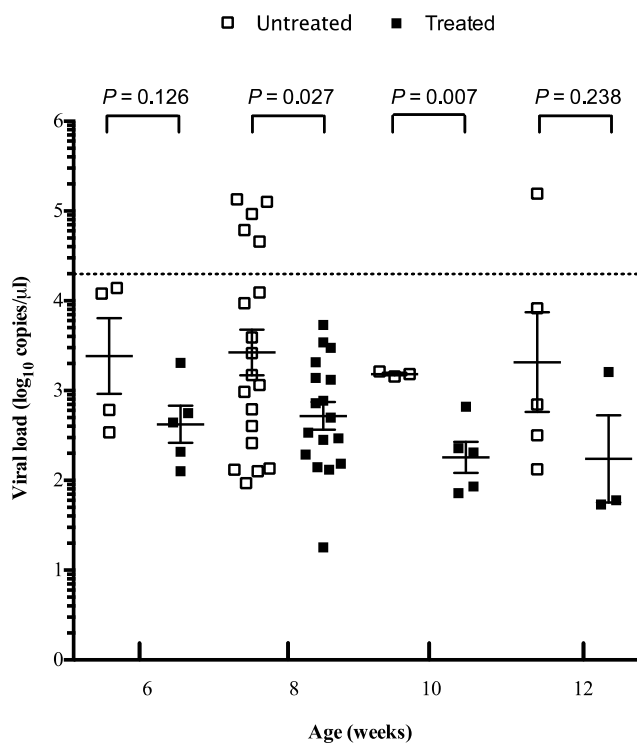

Figure 1. PRRSV load in the serum samples from both the untreated and treated groups in Taiwan. The dashed line indicates the threshold for the presence of clinical signs in PRRSV-infected pigs. The long horizontal lines represent the mean concentrations for each group. The error bars show the SD. Unpaired, 2-tailed Student's *t*-tests were used to compare the PRRSV load between the treated and untreated groups at each time point. *P* values < 0.05 and < 0.001 were considered significant and highly significant, respectively
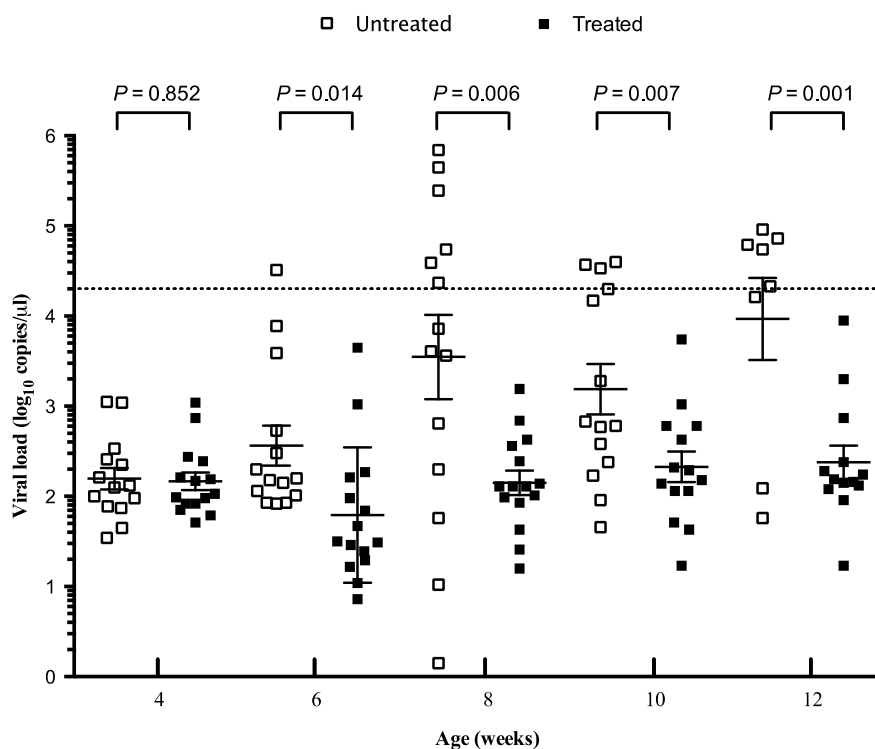
Figure 2. PRRSV load in the serum samples from both the untreated and treated groups in China. The dashed line indicates the threshold for the presence of clinical signs in PRRSV-infected pigs. The long horizontal lines represent the mean concentrations for each group. The error bars show the SD. Unpaired, 2-tailed Student's t-tests were used to compare the PRRSV load between the treated and untreated groups at each time point. P values < 0.05 and < 0.001 were considered significant and highly significant, respectively
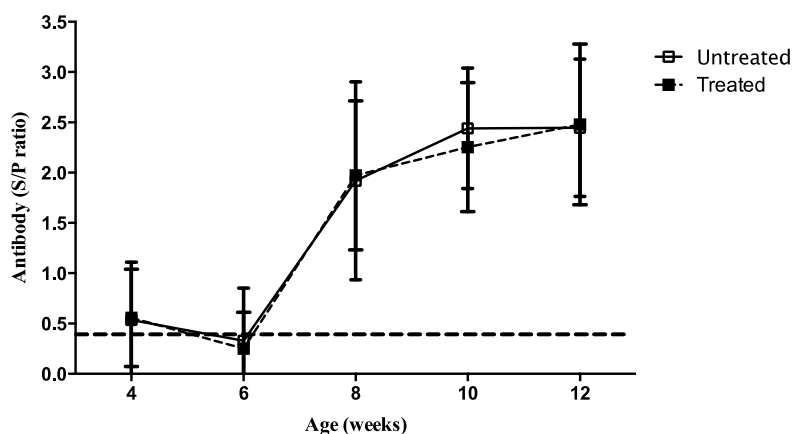

Figure 3. Kinetics of the humoral immune responses based on ELISA in both the untreated and treated groups. The results are expressed as the S/P ratio average. The dashed line indicates a S/P ratio < 0.4, the threshold below which the samples were considered negative. The error bars show the SD

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