Original Article

Dual infection of a Thai isolate HP-PRRSV and the pdmH1N1 2009 SIV in weanling pigs

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Abstract

After the introduction of the pandemic H1N1 swine influenza virus (pdmH1N1 SIV) in Thai swine farms in 2009, a mild clinical outcome was observed and its role in porcine respiratory disease complex (PRDC) is questionable. A highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) has become endemic in Thai swine farms, recently. This study was conducted to determine the pathogenicity of the pdmH1N1 SIV when co-infected with a HP-PRRSV. Thirty-two weanling pigs from a free PRRSV, SIV, porcine circovirus type 2 (PCV-2) and *Mycoplasma* spp. commercial farm were randomly divided into 4 groups; Negative, PRRSV, the SIV and Co-infection groups. Pigs in the PRRSV and Co-infection groups were challenged intranasally with the Thai isolate HP-PRRSV at 0 day post HP-PRRSV challenge (dphc) and pigs in the SIV and Co-infection groups were inoculated intratracheally with the pdmH1N1 SIV at 6 dphc. The 1st and 2nd necropsies were at 2 and 4 days post SIV challenge (dsc). A comparison of the lung lesion scores between the PRRSV and Co-infection groups at 2 dsc showed no statistical difference, but at 4 dsc, the lung lesion scores of the Co-infection group had higher average scores than those of the PRRSV group. Similarly, the SIV viral load in the lung of the Co-infection group a showed higher viral load at 4 dsc, compared to the only SIV-infected group. The results suggest that low pathogenic pdmH1N1 SIV could enhance the lung severity when co-infected with the HP-PRRSV leading to PRDC mimicking the field scenarios.

Keywords: co-infection, highly pathogenic PRRSV, pandemic H1N1 2009, pathogenesis, swine influenza virus

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Introduction

A high anaesthetic concentration is one of the main In the swine industry, porcine respiratory disease complex (PRDC) is commonly found. This pneumonic condition is a multiple microbial interaction causing huge economic losses worldwide due to high costs of treatment, control and high losses from increasing morbidity and mortality rates (Opriessnig et al., 2011). PRDC consists of a primary pathogen capable of inducing the infection on its own and is enhanced in severity by secondary pathogens. PRRSV is the most common pathogen associated with PRDC (Cheong et al., 2017). Moreover, PRRSV is known to cause negative immunomodulatory effects by the up regulation of interleukin-10 (IL-10) as early as 5 days post infection (Suradhat and Thanawongnuwech, 2003). IL-10 can inhibit inflammatory cytokines such as tissue necrosis factor alpha (TNF-α) and interleukin 1 (IL-1), leading to the impairment of innate immunity. Then, the infected pigs can undergo immunosuppression, having low lung clearance capacity (Thanawongnuwech et al., 2000a) and get infected with a secondary infection. In 2007, highly pathogenic PRRSV (HP-PRRSV) first occurred in China (Tian et al., 2007), and spread to other countries including Thailand (Nilubol et al., 2012). In Thailand, swine respiratory problems are usually associated with viral infection especially the highly pathogenic PRRSV (HP-PRRSV), which has become a dominant circulating strain (Jantafong et al., 2015) causing more severe clinical appearance along with high morbidity and mortality rates.

In 2009, the pandemic H1N1 (pdmH1N1) SIV first occurred in the human population in Mexico and the United States (Dawood et al., 2009). This virus genome was derived from classical swine and Eurasian swine lineages and the North American H3N2 containing triple-reassortant internal gene (TRIG) from swine, human and avian lineages (Pensaert et al., 1981; Brown, 2000; Smith et al., 2009b). Clinical appearance in patients showed a high fever, dry cough, muscle pain and death due to respiratory failure, causing high morbidity and mortality rates (Smith et al., 2009a). After the outbreak in humans, this virus was first detected in Canadian pigs (Howden et al., 2009) and spread worldwide (Pasma and Joseph, 2010; Pereda et al., 2010) including Thailand (Sreta et al., 2010). Although, this virus was able to induce a lethal outcome in humans, it induced only mild clinical signs in pigs causing mild fever, coughing, sneezing and nasal discharge. In Thailand, multiple SIV lineages have been circulating in swine farms. Since this virus was first introduced into the swine population, recent surveillance data has shown pdmH1N1 and pdmH1N1 reassortant viruses were co-circulating in Thai swine farms after the introduction of pdmH1N1 (Nonthabenjawan et al., 2015) possibly due to the TRIG cassette (Vincent et al., 2008). Most importantly, the Thai isolate pdmH1N1 infected pigs showed only mild clinical signs and minimal gross lesions (Arunorat et al., 2017). In addition, SIV infection is commonly found along with other respiratory pathogens causing a major impact on the swine industry.

According to PRRSV immunosuppression, several studies regarding co-infection of PRRSV with other agents show that PRRSV mostly enhanced the disease severity (Carvalho et al., 1997; Thacker et al., 1999; Thanawongnuwech et al., 2004). Previous co-infection of PRRSV and swine influenza virus showed an increase in post-weaning mortality (Alvarez et al., 2015), decreased SIV vaccine efficacy (Kitikoon et al., 2009) and also increased clinical appearance and viral shedding during the acute phase of SIV. Notably, previous different strains of SIV could induce clinical signs and lesions but the Thai isolate pdmH1N1 showed mild clinical signs. In this study, we conducted an experiment based on the immunosuppressive ability of PRRSV to enhance the disease severity of pdmH1N1 by challenging experimental pigs with the Thai HP-PRRSV isolate followed by the pdmH1N1 of swine origin. The objective of this study was to evaluate the clinical appearance, pathological and virological studies of the co-infection model if SIV lesion could be enhanced by HP-PRRSV infection.

Materials and Methods

***Viruses and cells*:** A Thai isolate pdmH1N1 SIV (A/swine/Thailand/CU-PL65/2010(H1N1)) and a Thai isolate HP-PRRSV (HP-PRRSV/10PL01) were provided by Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL, Bangkok, Thailand). SIV was propagated in Madin Darby Canine Kidney (MDCK) cells (ATCC, USA) and titrated to $10^{5.5}$ tissue culture infectious dose ($TCID_{50}$/ml). The viral antigen was detected using immunoperoxidase assay. Anti-Influenza A nucleoprotein monoclonal antibodies (HB654404), a rabbit anti-mouse IgG conjugated horseradish peroxidase (Dako Cytomation, USA) and aminoethyle carbazole (AEC) substrate were used. HP-PRRSV was propagated in MARC-145 cells and titrated to 10^2 $TCID_{50}$/ml following a previous study (Sirisereewan et al., 2017). All inoculums were stored at -80° C until used.

***Animals*:** Thirty-two, three-week-old, crossbred pigs from a commercial farm in the central part of Thailand which had negative results against porcine circovirus type 2 and *Mycoplasma* spp. antigens and antibodies were obtained. All pigs tested negative for influenza A virus and HP-PRRSV by real time PCR and antibodies tested by a commercial ELISA kit (IDEXX PRRS X3 Ab test, USA) for PRRSV screening and a Multispecies Kit (ID Screen® Influenza A antibody competition multi-species; IDvet, Louis Pasteur-Grabel, France) for SIV screening.

***Experimental design*:** All the pigs were divided randomly into 4 groups. The Co-infection (n=9) and HP-PRRSV-inoculated groups (n=9) were inoculated intranasally with 2 ml of 10^2 $TCID_{50}$/ml HP-PRRSV (10PL01) on day 0. The pigs in the SIV positive control group (n=9) and negative control group (n=5) were mocked using minimum essential media (MEM). In both the SIV and Co-infection groups, pigs were anesthetized using 0.08 ml/kg of zolazepam and tiletamine (Zoletil®, Virbac, Thailand) intramuscularly

and inoculated intratracheally with 2 ml of $10^{5.5}$ $TCID_{50}$/ml (A/Swine/Thailand/CU-PL65/2010 (H1N1)) at 6 days post HP-PRRSV challenge (dphc), whereas, the negative group was inoculated with MEM with the same procedure. All pigs were housed at the BSL-2 animal facility at Chulalongkorn University Laboratory Animal Center (CULAC) with free access to feed and water. Nasal swab samples were collected at -2, 6, 7, 8, 9 and 10 dphc and kept in a transporting medium containing 5% BSA, 300 U/ml penicillin, 300 µg/ml streptomycin and 1 µg/ml trypsin. The animal use protocols were conducted under the approval of Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (Animal Use Protocol No. 1673032).

***Clinical observation*:** Clinical signs were monitored daily including appetite, rectal temperature, depression score and clinical respiratory scores (nasal discharge, cough, sneezing and respiratory distress). Fever was set at a rectal temperature ≥ 40 °C.

***Pathological study*:** Since the SIV intratracheal inoculation showed a short viral nasal shedding duration (approximately 3 days) (De Vleeschauwer et al., 2009), consequently, seventeen and fifteen pigs were necropsied at 2 and 4 dsc, respectively. Seventeen pigs (2 pigs from the negative group and 5 pigs from the other 3 groups) were randomly selected for euthanasia using an overdose of pentobarbital sodium (Nembutal®, Ceva, Thailand) at 2 days post SIV challenge (dsc) and fifteen pigs at 4 dsc. The lung lesion score was examined as previously described (Halbur et al., 2000). All tissue samples were fixed in 10% formalin, processed and embedded in paraffin for histopathological evaluation. Microscopic lung lesions were characterized by necrotic bronchitis and bronchiolitis, along with interstitial pneumonia and lymphocytic peribronchiolar cuffing (Landolt et al., 2003; Kitikoon et al., 2006).

Modified real time RT-PCR (rtRT-PCR) for PRRSV detection: Viral RNA from serum was extracted using the Nucleospin® RNA virus (Machery-nagel, Duren, Germany). The Taqman® probe-based real-time RT-PCR was used for the quantification of PRRSV RNA (Egli et al., 2001). Reverse primer USalignEU-R (5′ AAAT|GGCTTCTC|GG|TTTT 3′) and forward primer USalignEU-F (5′ TCA|CTGTGCCAG|TGCTGG 3′) and US-PRRSV-specific probe FAM_US_rev (5′ FAM-TCCCGGTCCCTTGCCTCTGGA-TAMRA 3′) were used.

Amplification was done in a 25 µl reaction containing SuperScript™ III One-Step RT-PCR kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 1x Reaction Mix, 0.4 mM of each primer and probe, 0.5 µl of SuperScript® III RT/Platinum Taq Mix and 0.5 µl of viral RNA.

***Modified real time RT-PCR (rtRT-PCR) for swine influenza virus detection*:** Viral RNA from nasal swab samples and lung tissues were extracted using the same protocol as the PRRSV test. The modified real time RT-PCR (rtRT-PCR) assays were done on Corbett Rotor-Gene™ 6000 (Qiagen) using the SuperScript™ III Platinum® One-Step Qualitative RT-PCR System (Invitrogen, Carlabad, California, USA) according to the manufacturer's instructions. Primers and probes were used based on a previous study (Spackman et al., 2002). Amplification of a portion of the M gene (forward primer (MF3; 5′TGATCTTCTTGAAAA TTTGCAG3′) and M-64 probe (FAM-TTGTGGATTCTTGATCGTAMRA) was done. This portion is a conserve region of M gene, used for SIV genomic copy detection. A positive CT value >40 was considered as a negative result (Payungporn et al., 2006). The positive control for rtRT-PCR was used from the positive sample based on viral isolation and titration in the MDCK cell line from the previous study (Sreta et al., 2009).

***Statistical analysis*:** All data was analyzed using analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All statistical analyses were performed in GraphPad Prism for Windows (GraphPad Software Incorporated. San Diego, CA, USA).

Results

***Mean rectal temperature*:** After HP-PRRSV challenge, 2 pigs from the HP-PRRSV-inoculated group and 1 pig from the Co-infection group displayed fever (40-40.3 °C). At 6 dphc, most pigs from both groups (8/9) had a fever. After the SIV challenge, no pigs from the SIV-infected group showed fever throughout the experiment. The mean rectal temperature of the PRRSV-infected groups tended to decrease. Only 2/9 pigs had fever at 6 dphc and had a normal rectal temperature until the necropsy date, whereas, the remaining pigs from the Co-infection group still showed fever until the end of the experiment (Figure 1).

***Clinical signs*:** After HP-PRRSV challenge, pigs in the PRRSV (5/9) and Co-infection groups (4/9) showed mild depression and respiratory distress at 2 dphc and all pigs in both HP-PRRSV-inoculated groups showed clinical signs at 5 dphc. One day after the SIV challenge (dsc), pigs in the SIV (6/9) and Co-infection groups (7/9) showed sneezing with no coughing. All pigs in the PRRSV and Co-infection groups showed moderate to severe respiratory distress with no statistically significant differences ($p>0.05$).

***Lung lesion score and histopathological findings*:** Gross examination at 2 and 4 dsc demonstrated that all gross lung lesions from the Negative and only SIV-infected groups showed grossly looking normal lung lesions, whereas, lungs from the PRRSV and Co-infection groups showed firm and failed to collapse with no significant differences ($p>0.05$) at 2 dsc. However, lung lesion scores were more severe in the co-infection pigs than those of the only PRRSV-infected group ($p<0.05$) at 4 dsc (Figure 2). Microscopically, no lungs from the Negative group showed any significant findings. Lungs from the only SIV-infected pigs, showed mild to moderate bronchointerstitial pneumonia**.** Only the PRRSV-infected lung sections

showed severe interstitial pneumonia. However, co-infected lung lesions showed severe bronchointerstitial pneumonia (Figure 3) and bronchiolar epithelium showed epithelium necrosis with lymphocytic peribronchiolar cuffing, along with mononuclear cells infiltration in the alveolar septum (Figure 3a).

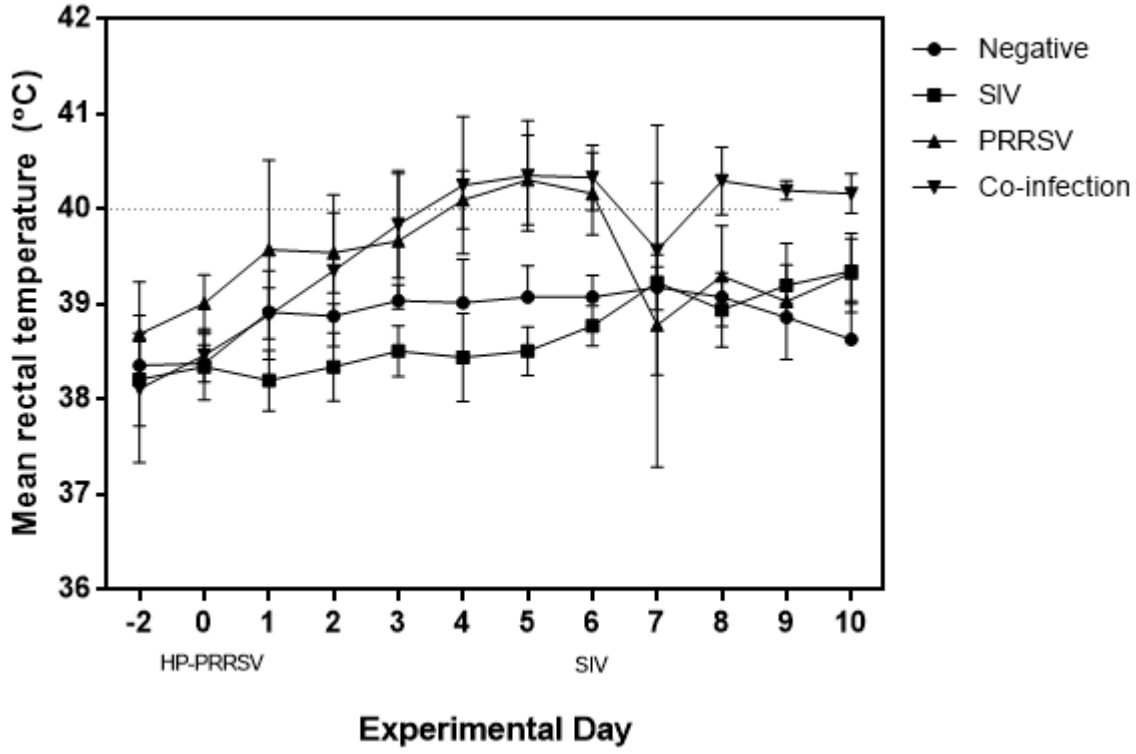

Figure 1 Mean rectal temperature of pigs.

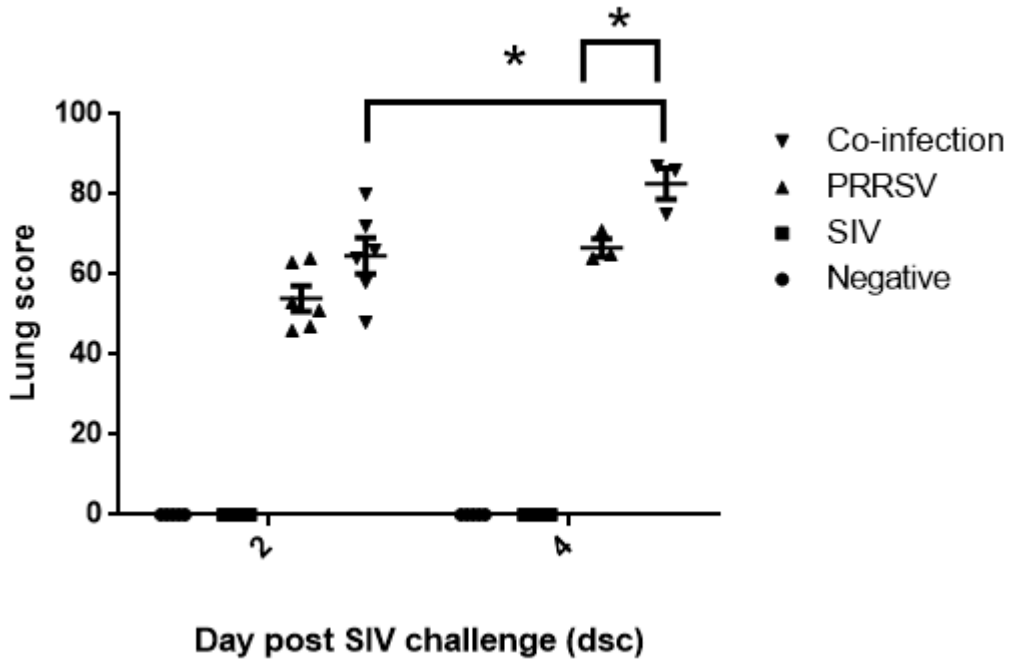

Figure 2 Lung lesion score after SIV challenge (2 and 4 dsc) (The asterisks indicate significant differences between groups ($p < 0.05$).

***SIV shedding in nasal swabs and lung tissues*:** During 1-3 dsc, no SIV shedding samples were positive in either SIV or Co-infection groups. However, at 4 dsc, only SIV-infected group had 75% positive pigs (3/4), whereas, none of the Co-infection pigs showed a positive result from the nasal swabs. At 2 dsc, the SIV RNA quantification from lung homogenate from the SIV (4/5) and Co-infection (3/5) groups showed no significant difference ($p>0.05$), whereas, at 4 dsc, all remaining lung tissues had significant SIV loading with a higher detection in the Co-infection group than those of the SIV group ($p<0.05$) (Figure 4).

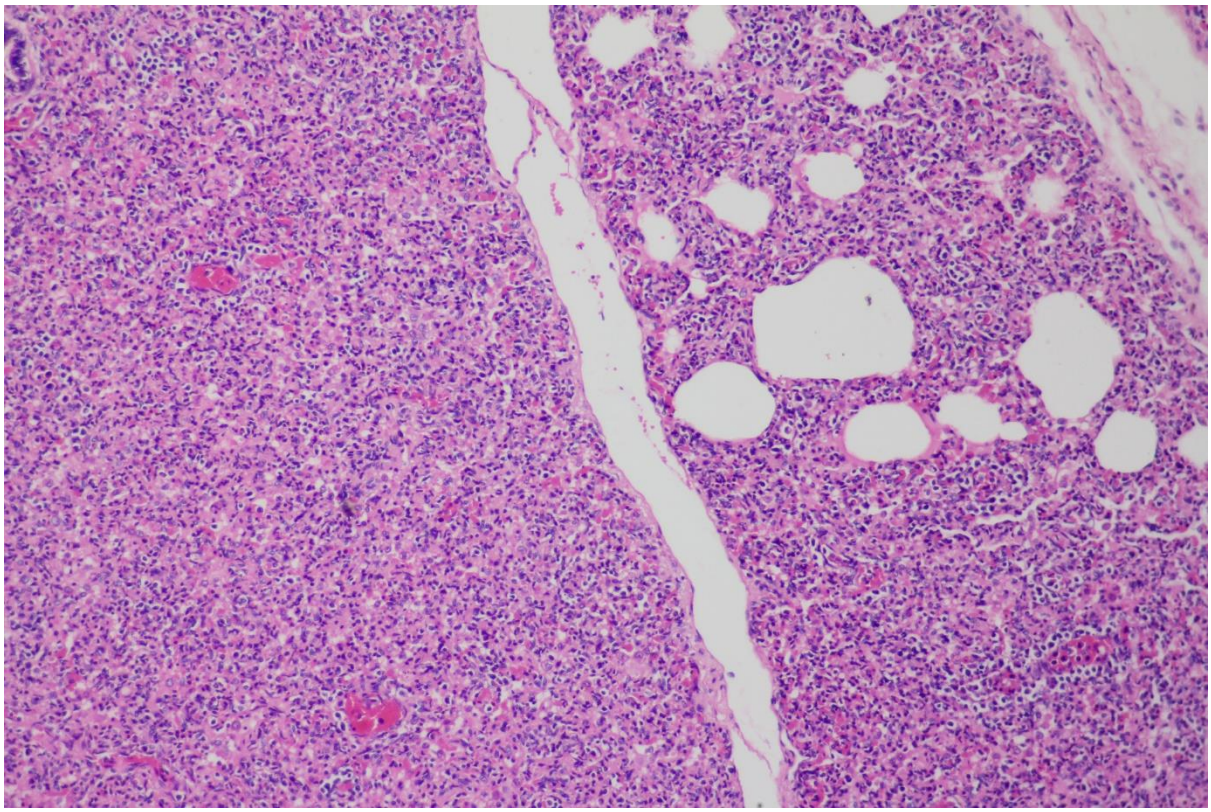

Figure 3 Lung histopathology of the Co-infection group (10x). At 4 dsc, the left alveoli are filled with monocyte infiltration with exudate and cell debris. The bronchiolar epithelium shows necrosis and lymphocytic peribronchiolar cuffing.

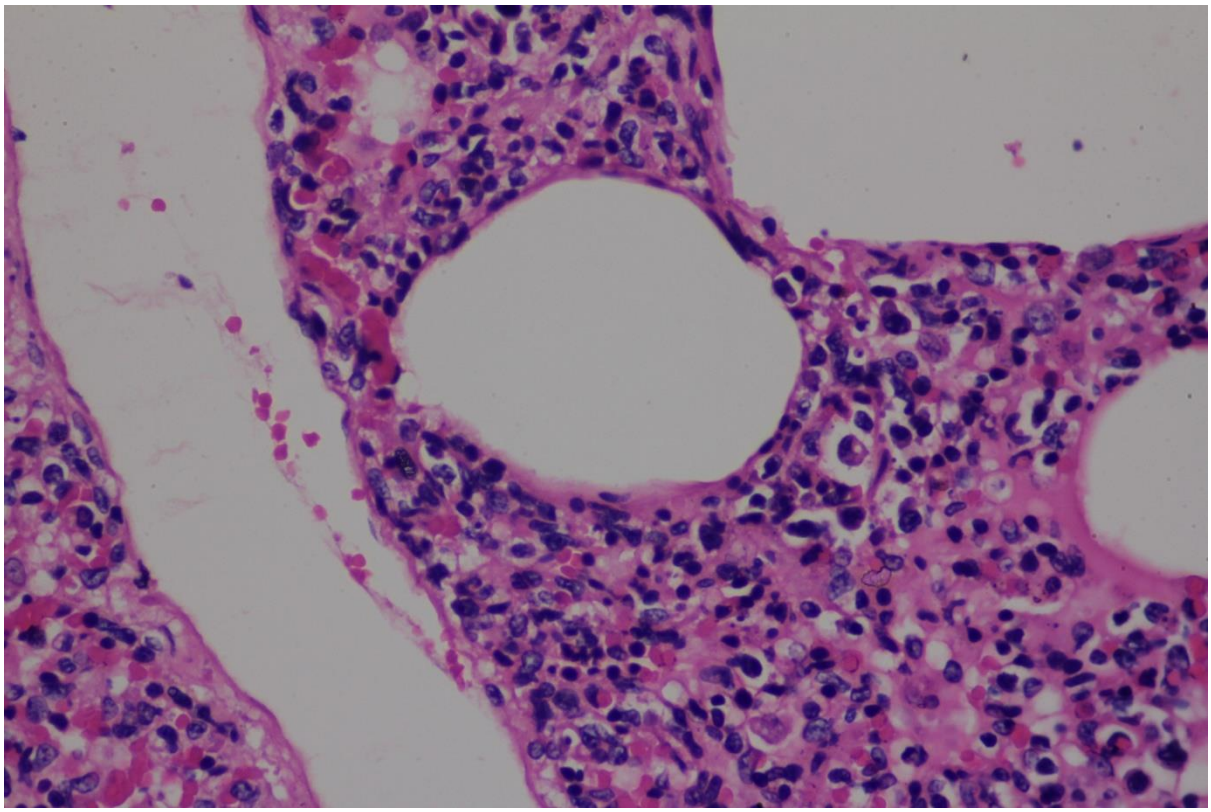

Figure 3a Lung histopathology of the Co-infection group (40x). At 4 dsc, the section shows bronchiolar epithelial necrosis (necrotizing bronchiolitis) (arrowhead) with lymphocytic peribronchiolar cuffing, along with mononuclear cells infiltration in the alveolar septum.

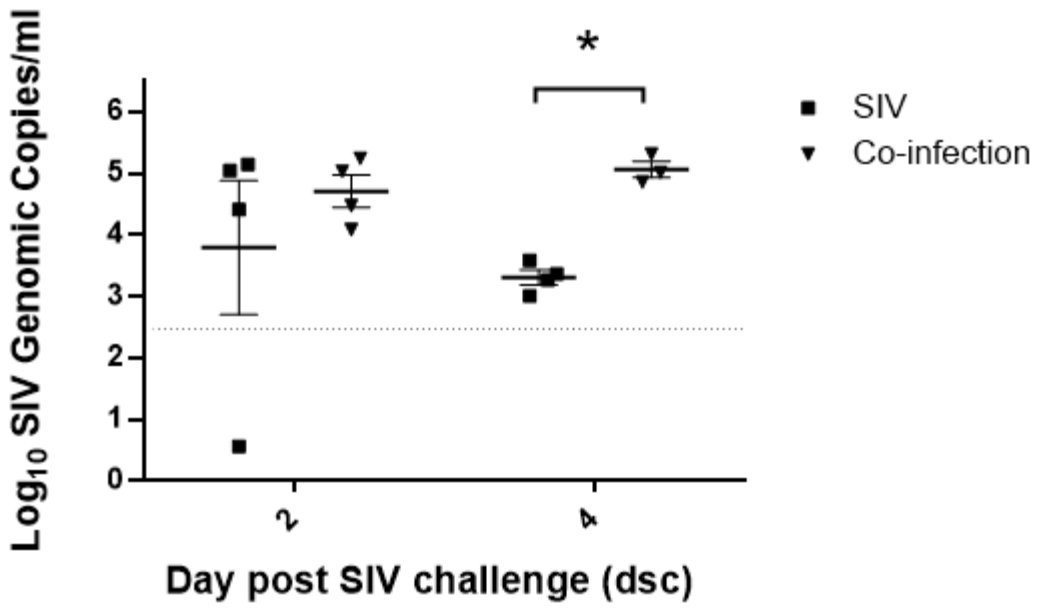

Figure 4 SIV genomic copies per microliter (log10) in lung samples after SIV challenge (2 and 4 dsc). The asterisk indicates significant difference between groups ($p < 0.05$).

Discussion

Based on the study objective, we confirmed the hypothesis that the Thai isolate HP-PRRSV could enhance the pathogenicity of the Thai pdmH1N1 SIV. It should be noted that this is the first co-infection report between HP-PRRSV and pdmH1N1 SIV in weanling pigs.

Our results demonstrated that the Thai HP-PRRSV isolate (10^2 $TCID_{50}$/ml (2ml)) could induce the clinical disease on its own, although a previous report used 10^4 $TCID_{50}$/ml (Sirisereewan et al., 2017), representing the high pathogenicity of this strain. All HP-PRRSV inoculated pigs exhibited viremia at 2 dphc by qRT-PCR (data not shown) and showed moderate to severe respiratory distress and depression. After the pdmH1N1 SIV inoculation, a few SIV-infected pigs only had nasal discharge and did not show obvious clinical signs. Although, previous pdmH1N1 SIV studies showed that this virus was able to induce mild clinical signs with cranioventral pneumonic lesions in different viral concentration ranging from $10^{4.5}$-$10^{5.5}$ $TCID_{50}$/ml (Kitikoon et al., 2009; Henningson et al., 2015; Takemae et al., 2018). In this study, the SIV-infected pigs showed no lesions (9/9). Co-infection pigs showed fever during 1-4 dsc when compared to the SIV only-infected pigs. This might be due to increased cytokines production during the acute stage of SIV infection such as IL-6 (Barbe et al., 2010), inducing a febrile response in the HP-PRRSV-induced immunocompromised pigs in this experiment. According to the low nasal shedding result, this might be due to the intratracheal inoculation, by passing the upper respiratory tract (URT), which is the natural route of infection (Hemmink et al., 2016). However, in this study, we used the intratracheal inoculation due to its consistency and reproducibility of the course of lung infection (Winkler and Cheville, 1986). Unlike the intratracheal route, the intranasal route is the easiest method but it might show variable efficacy in the inoculation process since pigs might swallow the inoculum (Janke, 2013).

Interestingly, a comparison of the lung lesion scores between the PRRSV and Co-infection groups at 2 dsc showed no statistical differences, but at 4 dsc, the lung lesion score of the Co-infection group had higher average scores than that of the PRRSV only-infected group. Similarly, the SIV lung viral loading of the Co-infection group showed a higher viral load at 4 dsc comparing to the SIV only-infected group. Exact mechanisms of the co-infection model between these two studied viruses were not clearly clarified due to differences in the time interval, the order of infection or virulent strain usage. However, PRRSV has tropism via pulmonary alveolar macrophages (PAMs) (Li et al., 2012), which could interfere with SIV clearance effectively, whereas, if SIV infection firstly occurred prior PRRSV infection, it might induce phagocytic cells infiltration (Van Reeth et al., 2002) mainly pulmonary alveolar macrophages which are main replicating target cells of PRRSV, leading to prolonged PRRSV infection. Additionally, IL-10 up regulation could inhibit monocyte/macrophage functions also leading to poor clearance of the virus (Thanawongnuwech et al., 2000b; Moore et al., 2001). In addition, HP-PRRSV did not induce interleukin-1 receptor antagonist (IL-1Ra) compared to type II PRRSV during the acute phase of infection (Nedumpun et al., 2017). IL-1Ra plays an essential role in anti-inflammatory cytokines induction (Arend et al., 1998), leading to excessive cytokines production. Thus, inflammatory cytokines (IL-1, IL-6 and TNF-α) are also induced by PAMs and increase during SIV co-infection possibly due to PAMs not undergoing apoptosis (Seo et al., 2004). The results showed the potentiation for the co-infection of HP-PRRSV and pdmH1N1 SIV.

Importantly, PRDC is associated with significant production loss due to poor growth performance, higher medication and vaccination costs (Fablet et al., 2012). Thus, in Thailand, HP-PRRSV has

predominantly circulated in many swine farms causing devastating outcome especially high morbidity and mortality rates. On the other side, despite zoonotic potential, pdmH1N1 SIVs have low pathogenicity and get less attention in Thailand. This study suggests that pdmH1N1 SIV disease severity could be enhanced by HP-PRRSV infection compared to the single infection. Therefore, routine surveillance and diagnosis of SIVs should be done in order to reduce production loss and zoonotic prevention and control.

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Compliance with ethical standards: The study protocol was approved by the Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (IACUC #1673032).

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