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Nataya Charoenvisal

Juthatip Keawcharoen

Donruethai Sreta

Siriporn Tantawet

Suphattra Jittimanee

See next page for additional authors

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Authors

Nataya Charoenvisal, Juthatip Keawcharoen, Donruethai Sreta, Siriporn Tantawet, Suphattra Jittimanee, Jirapat Arunorat, Korakrit Poonsuk, Alongkorn Amonsin, and Roongroje Thanawongnuwech

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Original Article

Probable Pig to Duck Transmission of the Pandemic H1N1 2009 (pH1N1) and Its Reassortant in Commingling Experimental Condition

Nataya Charoenvisal[1] Juthatip Keawcharoen[2] Donruethai Sreta[3] Siriporn Tantawet[4] Suphattra Jittimanee[1] Jirapat Arunorat[1] Korakrit Poonsuk[1] Alongkorn Amonsin[5,6] Roongroje Thanawongnuwech[1,6*]

Abstract

Pandemic H1N1 2009 (pH1N1) virus is considered as a low pathogenic influenza virus, however, it rapidly spread among humans and was finally found in the swine population of 6 continents. Interspecies transmissions among different animal species are of interest. In this study, sentinel ducks were commingled with pH1N1 or pandemic H1N1 reassortment virus (rH1N1) inoculated pigs in separate groups. According to the results, both studied viruses were able to cross-species transmit to a few sentinel ducks with mild or no clinical signs. Viral shedding measured by a modified real time RT-PCR detection from the oropharyngeal and cloacal swabs were also observed in both studied viruses. Interestingly, ducks commingled with pH1N1-infected pigs showed higher number of infected ducks detected by viral shedding in cloacal swabs. This present study suggested that pH1N1 and rH1N1 were able to transmit from pigs to ducks but viral replication in ducks were limited. As a result clinical signs were not obvious and low levels of viral shedding were detected in both sentinel duck groups.

Keywords: ducks, interspecies transmission, pandemic H1N1 2009, pigs, reassortant

[1] *Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Henri-Dunant Rd., Bangkok, 10330, Thailand*
[2] *Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Henri-Dunant Rd., Bangkok, 10330, Thailand*
[3] *Faculty of Veterinary Medicine, Rajamangala University of Technology Tawan-ok, Bangpra, Chonburi, 20110, Thailand*
[4] *Department of Clinical Science and Public Health, Faculty of Veterinary Science, Mahidol University, Salaya, NakhonPathom, 73170, Thailand*
[5] *Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Henri-Dunant Rd., Bangkok, 10330, Thailand*
[6] *Center of Emerging and Re-emerging Infectious Diseases in Animals, Faculty of Veterinary Science, Chulalongkorn University, Henri-Dunant Rd., Bangkok, 10330, Thailand*
***Corresponding author: E-mail:** *Roongroje.T@chula.ac.th*

บทคัดย่อ

ความเป็นไปได้ของการติดเชื้อข้ามชนิดสัตว์จากสุกรไปเป็ดของเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 2009 (pH1N1) และไวรัสลูกผสมในสภาวะการทดลองเลี้ยงร่วมกัน

ณ ทยา เจริญวิศาล [1] จุฑาทิพย์ เขียวเจริญ [2] ดลฤทัย ศรีทะ [3] สิริพร ตัณฑเวส [4] สุภัทตรา จิตติมณี [1]
จิรภัทธ อรุโณรัตน์ [1] กรกฤต พูนสุข [1] อลงกร อมรศิลป์ [5,6] รุ่งโรจน์ ธนาวงษ์นุเวช [1,6]*

แม้ว่าเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 2009 (pH1N1) ไม่ได้ก่อโรครุนแรงในมนุษย์ แต่เชื้อไวรัสสามารถแพร่ระบาดไปได้อย่างรวดเร็ว ถึง 6 ทวีปทั่วโลกในระยะเวลาเพียง 2 เดือน และหลังจากการแพร่ระบาดในมนุษย์ ได้มีรายงานการพบเชื้อไวรัสชนิดนี้ในสุกรในหลายๆประเทศ รวมทั้งประเทศไทยด้วย ดังนั้น การติดเชื้อไวรัสข้ามชนิดสัตว์จึงเป็นประเด็นที่น่าสนใจในขณะนี้ และเป็นที่มาของการศึกษาในครั้งนี้ ซึ่งทำการศึกษาโดยนำเป็ดมาเลี้ยงรวมกับสุกรที่ให้เชื้อไวรัส โดยสุกรในกลุ่มที่ 1 ให้เชื้อ pH1N1 และสุกรในกลุ่มที่ 2 ให้เชื้อไวรัสลูกผสมของเชื้อ pH1N1 (rH1N1) ผลการศึกษาพบว่าไวรัสทั้ง 2 ชนิดสามารถติดต่อไปยังเป็ดได้ โดยที่เป็ดไม่แสดงอาการ หรือมีอาการทางคลินิกเล็กน้อย แต่สามารถตรวจพบสารพันธุกรรมของไวรัสโดยวิธี real-time RT-PCR ได้ในระดับต่ำๆ ทั้งจากตัวอย่างที่เก็บจากช่องปากและทวารหนักของเป็ด ซึ่งพบว่าเป็ดที่เลี้ยงรวมกับสุกรในกลุ่มที่ให้เชื้อ pH1N1 มีจำนวนตัวที่ให้ผลบวกมากกว่ากลุ่ม rH1N1 และพบเชื้อจากตัวอย่างที่เก็บจากทวารหนักมากกว่าตัวอย่างจากช่องปาก การศึกษาในครั้งนี้บ่งชี้ว่า เชื้อไวรัส pH1N1 และ rH1N1 สามารถติดจากสุกรไปยังเป็ดได้ แต่ไวรัสอาจมีข้อจำกัดในการแบ่งตัวในเป็ด ดังนั้นเป็ดจึงไม่แสดงอาการ แต่เป็ดสามารถขับเชื้อออกสู่สิ่งแวดล้อมได้

คำสำคัญ: เป็ด การติดเชื้อไวรัสข้ามชนิดสัตว์ เชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 2009 สุกร เชื้อไวรัสลูกผสม

[1] *ภาควิชาพยาธิวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330*
[2] *หน่วยไวรัสวิทยา ภาควิชาจุลชีววิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330*
[3] *คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเทคโนโลยีราชมงคลตะวันออก อ. บางพระ จ. ชลบุรี 20110*
[4] *ภาควิชาเวชศาสตร์คลินิกและการสาธารณสุข คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล ต. ศาลายา อ. พุทธมณฑล จ. นครปฐม 73170*
[5] *ภาควิชาสัตวแพทย์สาธารณสุข คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330*
[6] *ศูนย์เชี่ยวชาญเฉพาะทางโรคอุบัติใหม่และโรคอุบัติซ้ำในสัตว์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330*
***ผู้รับผิดชอบบทความ E-mail:** *Roongroje.T@chula.ac.th*

Introduction

In April 2009, a pandemic H1N1 influenza A virus (pH1N1) emerged and spread worldwide. The pH1N1 is a reassortant virus of the North American triple reassortant (TRIG) swine virus and the avian-like Eurasian swine lineage (Garten et al., 2009). The pH1N1 virus efficiently transmitted back to pigs observed in many countries (Ducatez et al., 2011), including Thailand (Sreta et al., 2010) and the pH1N1 reassortant virus (rH1N1) was evidently found in pigs in 2010 in Thailand. The rH1N1 contains Neuraminidase (NA) gene of a Thai endemic swine influenza virus (SIV) and the other 7 genes were closely related to the pH1N1 virus (Kitikoon et al., 2011).

Influenza A virus is a highly contagious pathogen and able to cause disease in mammalian and avian species. Influenza viruses normally require 2 specific receptors; Sialic acid (SA) α2, 3 receptors commonly found in the intestinal tract of avian species, and SA α2, 6 receptors commonly found in the respiratory tract of humans (Forrest and Webster, 2010). Wild aquatic ducks are known as the natural reservoir of the influenza A virus. Influenza A viruses in avian species are commonly divided into highly pathogenic (HPAI) and low pathogenic (LPAI) avian influenza viruses (Kim et al., 2009) and the pH1N1 and rH1N1 viruses were classified as LPAI viruses (Babiuk et al., 2010). LPAI viruses normally cause limited lesions within 2 days in the epithelial cells of respiratory and digestive tracts showing mild pneumonia and infiltration of lymphocytes and macrophages. In addition, the replication site is mainly in the large intestine since high concentration of viral shedding is found in feces up to 7 days (Van Reeth, 2007; Kim et al., 2009). Interestingly, mallard ducks are able to act as the viral reservoir transmitting the virus to domestic poultry (Keawcharoen at al., 2008). Hence, the avian-like swine virus such as Eurasian H1N1 lineage containing PB2 and PA genes of the first isolated TRIG virus was closely related to the virus isolated from duck (Brown, 2000) indicating that duck's viruses can effectively transmit either the whole virus or a few gene reassortment to pigs. It should be noted that Bao et al. (2010) challenged

pH1N1 (human isolate) in Peking ducks and found no viral RNA detection in the lung tissue nor in the epithelial cells of the digestive tract, the main replication site of LPAI. Moreover, avian viruses such as the 1918 H1N1 and pH1N1 can cross species transmission resulting in fatal diseases in humans but no or mild clinical signs in poultry, particularly in ducks (Babiuk et al., 2010, Kalthoff et al., 2010).

Commingling among domestic species is commonly seen in pig ecosystems particularly in backyard farming in most Asian countries. Interspecies transmission of influenza viruses is possible when pH1N1 is widespread in the domestic animals, particularly in pigs. The viruses used in this study were isolated from naturally infected pigs. The infectivity and pathogenesis of these viruses in sentinel ducks were elucidated.

Materials and Methods

Viruses: A/swine/Thailand/CU-RA29/2009(H1N1) (Sreta et al., 2010), a pandemic H1N1 of pig origin (pH1N1) and A/swine/Thailand/CU-SA43/2010 (H1N1) (Kitikoon et al., 2011), a novel reassortant virus of pig origin (rH1N1) were individually propagated 3 times in 9 day old embryonated chicken eggs. Allantoic fluids were collected 72 hours later and virus concentrations were calculated using 50% tissue culture infectious dose ($TCID_{50}$) in Madin-Darby canine kidney (MDCK) cells by Reed and Muench method. The virus concentration of both isolates was adjusted to10^4 $TCID_{50}$/ml and kept at -80^0C until used.

Animals: Twenty one, 6-week-old ducks (Anas platyrhynchos domestica) were placed in the animal facility biosafety level 2 with adequate appropriate nutritional food and clean water. Before starting the experiment, oropharyngeal and cloacal swabs and sera were collected from each duck. All samples were tested negative using routine RT-PCR detection for influenza A virus (M gene).

At the same time, 21 nursery pigs were divided into 3 groups. Group 1 containing 9 pigs received intratracheally inoculation with pH1N1 virus (A/swine/Thailand/CU-RA29/2009(H1N1)). Group 2 also had 9 pigs receiving intratracheally inoculation with rH1N1 ((A/swine/Thailand/CU-SA43/2010 (H1N1)). Group 3 was a negative control group containing 3 pigs receiving mock inoculation with cell culture media.

One day post inoculation (1 DPI) in pigs, sentinel ducks were divided into 3 groups similar to the pig experiment and placed into each pig room. As a result, pigs per ducks ratio are 1:1 and those animals were commingling in each separate group throughout the experiment. At 3, 5 and 13 DPI, 3 ducks from group 1 and group 2 and one duck from the control group were randomly selected for necropsy. Adequate appropriate foods and water for pigs and ducks were provided in each room. The animal usage and procedures were approved by Chulalongkorn University-Faculty of Veterinary Science Animal Care and Use committee (protocol No. 11310052).

Clinical measurement and sampling: Clinical signs of ducks were observed at 2-8, 11 and 13 DPI. Oropharyngeal and cloacal swabs from all remaining ducks were collected at 2-8, 11 and 13 DPI. Nasal swabs were also collected from all remaining pigs on 1-7, 10 and 12 DPI. All collected swabs were tested for the presence of the influenza virus by a modified real time Reverse Transcriptase-Polymerase Chain Reaction (real time RT-PCR) and virus isolation (described below). Sera from ducks and pigs collected at necropsy were tested for influenza A virus antibody. Lung, pancreas and caecum of ducks and lung and bronchial lymph node of pigs were collected during necropsy for quantitative detection of viral RNA by the real time RT-PCR and viral isolation. Selected organs including brain, pancreas, liver, jejunum, caecum, spleen and kidney were fixed in 10% buffer formalin for histopathology study.

Viral detection by modified real time Reverse Transcriptase-Polymerase Chain Reaction (real time RT-PCR): Total RNA was extracted from collected swabs, lungs, pancreas and caecum using a commercial kit (NucleoSpin Extract Viral RNA Kit, Macherey-Nagel, Germany). A modified real time reverse transcriptase-polymerase chain reaction (real time RT-PCR) was performed using Superscript III platinum one-step quantitative RT-PCR system (Invitrogen, USA). Primers were specific to the Matrix (M) gene. Forward primer (MF3; 5′ TGATCTTCTTGAAAATTTGCAG 3′), reward primer (MR1+; 5′ CCGTAGMAGGCCCTCTTTTCA 3′) and M-probe (FAM-TTGTGGATTCTTGATCG-MGB) were used in this study as previously described (Payungporn et al., 2006). Briefly, the cycling conditions started at 48^0C for 45 min, then 95^0C for 10 min and followed by 40 cycles including denaturation (94^0C for 15 sec), annealing (55^0C for 30 sec) and extension (72^0C for 40 sec). Positive samples showed CT value over 40.

Meanwhile, homogenated tissue samples were filtrated and inoculated onto the monolayer of MDCK cells using a ten-fold serial dilution manner. The inoculated cell cultures were incubated for 72 hours. The virus was identified using anti-influenza A nucleoprotein monoclonal antibody as a primary antibody and rabbit anti-mouse IgG conjugated horseradish peroxidase as a secondary antibody (Dako Cytomation, Carpinteria, California). Then, the color was developed using a chromogen aminoethyl carbazole substrate (Sigma, St. Louis, Missouri) to identify the virus antigen in the nuclease of the infected cells (Sreta et al., 2009).

Pathological examination: Formalin-fixed tissues were embedded in paraffin and processed routinely. Briefly, sections were cut approximately 4-6 µm thick for histopathological and immunohistochemistry (IHC) staining for the influenza A virus antigen detection. The IHC staining was performed using a labeled streptavidin-biotin (LSAB) method. Primary antibody using anti-influenza A (H5N1) nucleoprotein monoclonal mouse antibodies (EVS238, B.V. European Veterinary Laboratory, the Netherlands) and secondary antibody using

biotinylated rabbit anti-mouse IgG antibody and envision polymer (Envision Polymer DAKO®, Denmark) were concurrently performed with a negative control slide. A positive control slide was also included using the SIV-infected lung section from the previous experiment (Sreta et al., 2009). The sections were developed with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin.

Serological test: All sera were tested for influenza A virus antibody by commercial ELISA (Avian Influenza virus antibody test kit, IDEXX Laboratories, USA). Concurrently, sera were randomly selected for hemagglutination inhibition (HI) test (1 sample/ group/necropsy day). Since the hemagglutinin (HA) genes of pH1N1 and rH1N1 are closely related, pH1N1 virus (A/swine/Thailand/CU-RA29/2009 (H1N1) was used as the representative antigen in the assay. Samples with HI titers ≥ 40 were considered as previously exposed to the specific tested antigen.

Results

Clinical examination: Sentinel ducks in the pH1N1 group did not show any clinical signs during 2-4 DPI, only ocular and nasal discharges were observed at 5 DPI. All 3 remaining ducks in the pH1N1 group showed conjunctivitis between 6-13 DPI. Ducks in the control and the rH1N1 groups did not show any obvious clinical signs throughout the experiment. It should be noted that the inoculated pigs in both groups showed obvious clinical signs of SIV infection including nasal discharge, coughing and sneezing.

Virus detection: Both groups of inoculated pigs showed viral detection in the nasal swabs as early as 1 DPI and could be detected in low levels until 12 DPI (data not shown). In addition, the rH1N1-inoculated group showed higher number of pigs shedding the virus based on the nasal swab results (data not shown). In the experimental ducks, virus in the pH1N1 group was detected by the real time RT-PCR from the oropharyngeal swabs at 3 DPI (1 of 9) and cloacal swabs at 2 (1 of 9), 3 (2 of 9), 11 (1 of 3) and 13 (1 of 3) DPI. Based on the viral isolation results in the pH1N1 group, only one oropharyngeal swab and one cloacal swab showed positive results with low levels of virus concentration at 4 DPI (Table 1). In the rH1N1 group, 1 oropharyngeal and 1 cloacal swab at 2 DPI were tested positive and the virus isolation of the rH1N1 group yielded negative results (Table 2). No viral detection was found in the negative control ducks throughout the experiment. Low levels of viral genetic material was detected only in the lung of one necropsied duck in the pH1N1 group by the real time RT-PCR at 3 DPI (data not shown).

Pathological examination: In both infected groups of pigs, typical lesions of SIV infection characterized by multifocal consolidated lung lesions with a checker-board lung pattern at the cranioventral area were observed. The viral antigens were detected by IHC only in the lung and bronchial lymph node of the rH1N1 infected pigs (data not shown). However, sentinel ducks in both inoculated pig groups as well as in the control group showed only mild interstitial pneumonia and mild airsacculitis. None of the duck tissues were tested positive by IHC technique.

Serological examination: At the beginning of the experiment, all duck sera were tested negative for influenza A virus antibody using commercial ELISA. Sera collected from necropsied ducks at 3, 5 and 13 DPI were also tested negative for influenza A antibody using a commercial ELISA. Similarly, all duck sera were tested negative for the pH1N1 virus using HI test.

Table 1 Viral detection measured from oropharyngeal and cloacal swabs of sentinel ducks in the pH1N1 group using modified real-time RT-PCR and viral isolation

Duck No.	**Virus detection**																			
	1 DPI		2 DPI		3 DPI		4 DPI		5 DPI		6 DPI		7 DPI		8 DPI		11 DPI		13 DPI	
Oropharyngeal swab	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI
1	-	-	-	-	-	-	-	-	-	+	N									
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	N									
4	-	-	-	-	-	-	N													
5	-	-	-	-	+	-	N													
6	-	-	-	-	-	-	N													
7	-	-	-	-	-	-	-	-	-	-	N									
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cloacal swab																				
1	-	-	-	-	-	-	-	+	-	+	N									
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
3	-	-	-	-	-	-	-	-	-	-	N									
4	-	-	-	-	+	-	N													
5	-	-	-	-	-	-	N													
6	-	-	+	-	-	-	N													
7	-	-	-	-	-	-	-	-	-	-	N									
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

DPI: day post inoculation, rt: real time RT-PCR (+: Ct values < 40, - : Ct values ≥ 40), VI: viral isolation using MDCK , N: necropsy.

Table 2 Viral detection measured from oropharyngeal and cloacal swabs of sentinel ducks in rH1N1 group using modified real time RT-PCR and viral isolation

Duck No.	Virus detection																			
	1 DPI		2 DPI		3 DPI		4 DPI		5 DPI		6 DPI		7 DPI		8 DPI		11 DPI		13 DPI	
Oropharyngeal swab	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI
1	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	N									
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	N													
5	-	-	-	-	-	-	N													
6	-	-	-	-	-	-	N													
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	N									
9	-	-	-	-	-	-	-	-	-	-	N									
Cloacal swab																				
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	N									
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	N													
5	-	-	-	-	-	-	N													
6	-	-	+	-	-	-	N													
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	N									
9	-	-	-	-	-	-	-	-	-	-	N									

DPI: day post inoculation, rt: real time RT-PCR (+: Ct values < 40, - : Ct values ≥ 40), VI: viral isolation using MDCK, N: necropsy.

Discussion

In this experiment, pigs and ducks were commingling in close contact and might share the same water basins. Although appropriate foods for ducks and pigs were provided in separate bowls, the animals randomly consumed foods in all bowls. Mimicking the field situation in backyard farming, ducks, chickens and birds freely share the same environment and might have direct contact among those animals. The poultry not only consume leftover feed but might also contaminate the pigs from their excretions or vice versa. As a result, cross-transmission of influenza virus from pigs to ducks or vice versa could happen in the mimicking pigs to ducks interfacing environment.

As expected, the sentinel ducks did not show obvious clinical signs or significant lesions. However, viral RNA was detected in the oropharyngeal and cloacal swabs of ducks in both inoculated pig groups. In addition, pH1N1 virus could be isolated from sentinel ducks in MDCK cells with low titers implying that both studied viruses could infect, replicate, and probably transmit from pigs to ducks when commingling. Previously, ducks were demonstrated to be insusceptible to the human pH1N1 infection and no viral RNA was detected from swabs or lungs (Swayne et al., 2009; Bao et al., 2010). It should be noted that the 1918 H1N1 influenza virus could replicate in ducks with low levels of virus titers (Babiuk et al., 2010).

Based on the present study, the pH1N1 virus obtained from pigs was able to infect and replicate in the duck intestine better than the respiratory tract. This result is well correlated with the evidence that the LPAI viruses shed via fecal-oral route and persist in duck population. As a result, all subtypes of the viruses, especially LPAI, can be isolated from duck feces (Kim et al., 2009). It should be noted that the viral RNA was also found in the duck lung tissue of the pH1N1 group in this study. However, viral antigen could not be detected in all collected tissues of both inoculated groups. The results suggested that both pH1N1 and rH1N1 viruses had limited replication in sentinel ducks possibly due to the lack of SA α2, 6 receptors in ducks (Matrosovich et al., 2008). The HA genes of both pH1N1 and rH1N1 are similarly compatible with the SA α2, 6 receptors and able to infect pigs. In addition, the differences of body temperature in avian and mammals may affect the replication process (Forrest and Webster, 2010). As a result, both studied viruses had possibly limited replication in the sentinel ducks.

In the sentinel ducks, viral detection in the pH1N1 group yielded higher numbers of positive ducks than those of the rH1N1 group. In contrast, viral detection in the parallel infected pigs was observed more in the rH1N1-infected pigs. Since the NA gene of pH1N1 and rH1N1 were 89% homology and the other 7 genes had at least 99% homology, the difference of the NA gene might cause the variation in infectivity and virus shedding in the sentinel ducks. It should be noted that NA gene is responsible for releasing progeny viral particles from the infected cells (Suzuki, 2005). The NA gene of the studied rH1N1 obtained from the local swine influenza virus must be well adapted in the Thai pig population but might have the limitation on the release of new progeny virus in ducks.

Unfortunately, all collected sera showed negative results on influenza A antibody suggesting the limited infection and replication of the studied viruses. In contrast, antibody against HPAI (H5N1) could be detected as early as 4 DPI and gradually

increased by 14 DPI in experimental ducks (Jeong et al., 2009).

In conclusion, the results suggested that pH1N1 and rH1N1 isolated from pigs could probably be transmitted to the sentinel ducks when commingling in close contact with the infected pigs. However, the studied viruses had limited replication in ducks. The pH1N1 isolated from pigs appeared to have the potential for pig to duck transmission. Interspecies transmission may influence the antigenic drift and shift resulting in the emergence of a novel influenza virus. Each novel virus may adapt in the specific hosts before emerging and causing severe diseases and may transmit to another host or vice versa and so on. Since backyard farming, commonly seen in most Asian countries, creates a perfect interspecies transmission together with closely human-animal interface scenario, influenza A virus surveillance, monitoring, and pathogenesis studies are necessary.

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