OCCURRENCE OF SWINE INFLUENZA VIRUS INFECTION IN SWINE WITH PORCINE RESPIRATORY DISEASE COMPLEX

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Abstract. We studied the occurrence of swine influenza virus (SIV) infection in piglets with respiratory symptoms resembling porcine respiratory disease complex (PRDC). A total of 106 samples including nasal swab and lung suspension from sick piglets were collected from 30 farms of medium size in the central and eastern parts of Thailand from August 2006 to February 2007. Samples were inoculated onto Mardin-Darby Canine Kidney (MDCK) cells and SIV infection was confirmed by immunofluorescent assay (IFA) and reverse transcriptase polymerase chain reaction (RT-PCR) specific for M gene. Of 106 samples, 3 pigs from 3 different farms were found to be SIV positive on all assays. The positive samples were further identified by RT-PCR as H3N2 subtype using specific primers for hemagglutinin (HA) and neuraminidase (NA) genes. SIV infection was found in 2.8% of swine suffering from respiratory distress suggesting SIV may not be the major pathogen for PRDC in the central and eastern Thailand. SIV was present in 3 of 30 farms (10%) indicating the prevalence of SIV in these regions is considerable. Since pigs are vulnerable to infection from both human and avian influenza viruses and interspecies transmission between humans and swine occurs sporadically, it is essential to continue surveillance and monitoring of SIV infection in the swine population.

INTRODUCTION

Swine influenza virus (SIV) is a member of the family Orthomyxoviridae. SIV is one of the causes of porcine respiratory disease complex (PRDC), the most important disease affecting the swine industry worldwide. It was first isolated in 1930 (Shope, 1931). SIV subtypes H1N1, H1N2 and H3N2 have been reported in pigs throughout of the world (Kristien *et al*, 2002). In Europe, SIV subtype H1N1 caused the first outbreak which rapidly spread in swine (Pensaert *et al*, 1981). In 1973, the human-like H3N2 viruses antigenically similar to the human pandemic "Hong Kong" virus in 1968 were found to infect swine in Europe (Brown, 2000). Subsequently, six internal protein genes of the human -like H3N2 SIV were replaced by those of the avian - like H1N1 virus. The H1N2 subtype was first diagnosed in acute respiratory disease outbreaks in swine in Great Britain in 1994 (Brown *et al*, 1995) and on the European mainland a few years later (Marozin *et al*, 2002). The European H1N2 SIV was double reassortant containing the hemagglutinin (HA) gene from human H1N1 virus, the neuraminidase (NA) gene from SIV H3N2 and the internal protein genes from avian-like swine H1N1 virus (Marozin *et al*, 2002).

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In North America, the H1N1 viruses belong to the classical swine lineage which is genetically related to the human H1N1 viruses responsible for the 1918 Spanish influenza pandemic (Reid *et al*, 1999). From 1965 to 1980 the H1N1 viruses were highly conserved both antigenically and genetically (Sheerar *et al*, 1989). H1N1 was the only subtype of SIV circulating in American swine until in 1998 when H3N2 subtype emerged and caused outbreaks in swine in the Midwest. This H3N2 SIV contained gene segments from humans, swine and avian viruses by reassortment (Zhou *et al*, 1999). In 1999, SIV of subtype H1N2 was found in sows in Indiana and thereafter outbreaks were reported widely in midwestern America (Karasin *et al*, 2002).

In Asia, SIV subtype H1N1 has been detected in several countries (Guan *et al*, 1996) such as Hong Kong (Yip, 1976), Japan (Yamane *et al*, 1978), India (Das *et al*, 1981), China, Taiwan (Shortridge and Webster, 1979), and Thailand (Kupradinun *et al*, 1991; Chutinimitkul *et al,* 2008). SIV subtype H3N2 first isolated in 1968 in Asian swine was found to be antigenically similar to the contemporary human influenza A virus subtype H3N2 (Shortridge *et al*, 1979). Subsequently, reassortant of H1N2 containing the HA gene from SIV H1N1 and the NA gene from contemporary human influenza virus subtype H3N2 was detected in Japan and China (Nerome *et al*, 1983; Xing *et al*, 1992). Recently, all three subtypes of SIV from Thailand were isolated and characterized; however, their HA and NA genes might not be related to the influenza viruses concurrently appearing in the human population (Chutinimitkul *et al,* 2008).

Clinical signs of swine infected with SIV alone appear to be mild. It has high morbidity with mortality in less than 5% (Kida *et al*, 1994). The primary clinical signs include cough, nasal and ocular discharge, body temperature up to or more than 40.5ºC, anorexia and dyspnea. The clinical signs improve within 10-14 days.

Single pathogen infection causes a mild, temporary disease but infection with a variety of pathogens usually causes severe or chronic disease (Halbur, 1996). PRDC is a combination of several infectious agents, including porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine respiratory coronavirus (PRCV), porcine circovirus type 2 (PCV2), *Mycoplasma hyopneumoniae, Bordetella bronchiseptica*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis* and *Haemophilus parasuis* (Thacker, 2001). The disease occurs in Europe, America and Asia (Thacker, 2001; Kim *et al*, 2002; Segalés *et al*, 2004; Van Gucht *et al*, 2004). In Thailand, clinical signs resemble PRDC usually found in pigs at 5-8 weeks of age.

Most of the previous epidemiological studies were done in healthy pigs or pigs infected with SIV alone. SIV is considered a causative agent of PRDC. The present study is evaluated the presence of SIV in PRDC in piglets. We also determined the subtypes of SIV circulating in the swine population in central and eastern Thailand.

MATERIALS AND METHODS

Sample collection

A total of 30 farms having animals with signs and symptoms of PRDC were included in this study. The signs and symptoms were fever (40 - 41ºC) and respiratory distress in piglets. Production losses had to be >5% to be included in this study. Three to 5 piglets age of 5-8 weeks with clinical signs of PRDC were collected randomly from each farm. The pigs were brought to Kasetsart University Diagnosis Laboratory, Faculty of Veterinary Medicine, Kamphaeng Saen Campus, Thailand. Nasal swabs and apical lung lobes were collected at necropsy. The nasal swabs were

soaked in viral transport media and the lung tissue were kept at -70ºC until used.

Virus isolation

Mardin-Darby Canine Kidney (MDCK) cells were grown in minimum essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum and 4 mM L-glutamine. The 10% lung suspension and nasal swabs were inoculated onto a monolayer of MDCK cells maintained in MEM with 2 µg/ml of trypsin (Sigma) and 0.3% bovine serum albumin fraction IV (Invitrogen). The cytopathic effect (CPE) was observed each day. The supernatant from the inoculated cells was subsequently cultivated two more times to assure that the samples contained no virus. The infected cells were examined for viral infection by immunofluorescent assay (IFA).

Immunofluorescent assay (IFA)

MDCK cells were inoculated with supernatant from infected cells. The inoculated cells were incubated at 37ºC with 5% CO_2 for 48 hours before being fixed with absolute methanol. The cells were then washed three times with PBST (PBS pH 7.2+0.05% tween 20) and incubated with monoclonal antibody (MAb) specific to the nucleoprotein (NP) for influenza A virus (kindly provided by Dr Prem S Paul, University of Nebraska, USA) for 1 hour at 37ºC. The cells were washed three times with PBST prior to being stained with fluorescein isothiocyanate (FITC) conjugated anti-mouse immunoglobulin G (IgG). The green fluorescent signal was then detected by a fluorescent microscope.

Quantal assay

Viruses were serially diluted in media containing MEM, 2 µg/ml trypsin (Sigma) and 0.3% bovine serum albumin fraction IV (Invitrogen). Fifty microliters of each dilution of virus were inoculated onto a monolayer of MDCK cells grown in 96 well plates at 37ºC with 5% CO_2 for 1 hour. Five replicates were included for each isolate. After inoculation, cells were incubated for 48 hours and the CPE was observed and recorded as either have infection or not. The titer of the virus was calculated from the reciprocal of the highest dilution that yielded 50% infection using Reed and Muench's method.

Reverse transcriptase polymerase chain reaction (RT-PCR) and SIV subtyping

Viral RNA was extracted from the supernatant of inoculated cells using Trizol® (Invitrogen). Then, cDNA was synthesized from the RNA template using specific primers and reserve transcriptase enzyme (Superscript III; Invitrogen) according to the manufacturer's instructions. Briefly, 5 µl of SIV RNA was supplemented in a total reaction volume of 20 µl containing 1XRT buffer (3.75 mM KCl, 2.5 mM Tris , pH 8.3), 10 mM dNTPs, 10 mM specific primers, 0.2 M DTT, 50 U superscript III (Invitrogen) and 20 U of RNase inhibitor. After denaturation of the RNA at 70ºC for 10 minutes, cDNA was synthesized at 42ºC for 50 minutes, followed by incubation at 70ºC for 15 minutes. The polymerase chain reaction (PCR) was performed using primers specific to SIV. These specific primers were divided into 3 sets (Table 1): the first set for the detection of M gene from samples which CPE was suspected, the second set for the determination of the HA (H1, H3) subtype, and the third set for the identification of the NA (N1, N2) subtype. The sequences of the oligonucleotides are shown in Table 1. RNA isolated from A/SW/TH/KU7.2/04 (H3N2) was used as a positive control for each PCR. The PCR reaction mixture contained 2 µl of cDNA template, 2 mM $MgCl_2$, 5 mM dNTPs, 5 mM of each primer and 1 U of *Taq* polymerase (Invitrogen) in a PCR buffer (5 mM KCl, 1 mM Tris-HCl, pH 8.3). The reaction mixture was then incubated at 94ºC for 3 minutes for DNA separation followed by 35 cycles of 94ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 1 minute. The DNA extension was continued at 72ºC for 7 minutes. The amplified product was

Table 1
The sequences of oligonucleotide primers used for amplification of SIV genes.

Primers	Sequence (5′-3′)	Positions	SN subtypes	References
MF	CCGTCAGGCCCCCTCAAAGC	70-89	-	Ellis and Zambon, 2001
MR	AGGCGATCAAGAATCCACAA	830-849		
H1F	GGG ACA TCT TAC CCA GGA GAT	345-365	H1	Choi *et al*, 2002
H1R	GCA TTG TAT GTC CAA ATA TCC A	1351-1330		
H3F	TAT GCC TGG TTT TCG CTC AA	61-80	H3	Choi *et al*, 2002
H3R	TTC GGG ATT ACA GTT TGT TG	724-705		
N1F	GGT TCC AAA GGA GAC ATT TTT G	324-346	N1	Choi *et al*, 2002
N1R	CTA TCC AAA CAC CAT TGC CAT A	1078-1057		
N2F	TGC GAT CCT GAC AAG TGT TAT C	364-385	N2	Choi *et al*, 2002
N2R	CAG ACA CAT CTG ACA CCA GGA T	866-845		

Table 2
Results of SIV isolation from nasal swabs and lung suspension.

Samples	Passage 1		Passage 2		Passage 3	
	CPE	IFA	CPE	IFA	CPE	IFA
Positive samples						
Nasal swab	2	2	2	2	2	2
Lung	1	1	1	1	1	1
Negative samples						
Nasal swabs	96	104	103	104	103	104
Lung	99	105	105	105	105	105
[a]Suspected samples	14	-	1	-	1	-

[a]Suspected samples is samples demonstrating pathological changes in cells but where IFA is negative.

visualized by standard gel electrophoresis on 1.2% agarose gel.

RESULTS

The prevalence of SIV in piglets with clinical signs of PRDC

To isolate the SIV, 106 samples from lung suspensions and nasal swabs were inoculated onto MDCK cells. Three of 106 samples produced CPE typical for SIV infection during the first passage (Table 2). The samples which contained no CPE typical for SIV infection during the first passage were subsequently cultivated for two additional passages to confirm that the samples were negative. No samples negative for CPE on the first passage became positive for CPE on the second or third passages. Fourteen samples demonstrated pathological changes (suspected CPE) but were negative when analyzed by IFA.

The CPE suspected samples on cell culture appeared as one spot of cell death among normally growing cells. The area of dead cells expanded slowly over the next couple days. In positive samples, SIV infected cells had

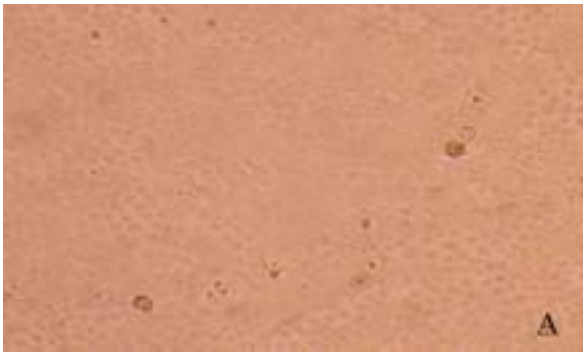

Fig 1–Results of SIV isolation using MDCK cells; mock infected cells (A) show normal cell growth. Infected cells (B) contain CPE typical for SIV infection.

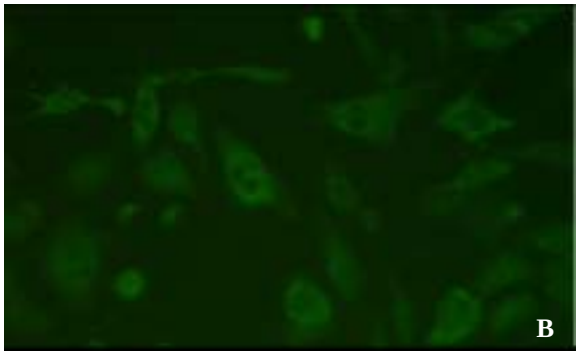

Fig 2–IFA demonstrating specific reactions between SIV and MAb to NP. MDCK cells infected with an SIV negative sample (A) and positive sample (B).

growth inhibition, where the groups of cells became rounding up (Fig 1) and detached from the anchoring surface. There was complete cell lysis and detachment from the flask by 48-72 hours post-inoculation.

Supernatant from cells inoculated with each sample, including CPE positive, negative and suspected specimens, were evaluated for the presence of NP antigen of influenza A virus by IFA. SIV infected cells reacted with MAb to NP appeared as fluorescent green (Fig 2). Three of 106 samples were positive for both viral isolation and on IFA (Table 2). Two of the samples were nasal swabs and another sample was lung suspension. Each sample was collected from a different pig on different farms. Thus, 3 of 30 farms or 10% of farms were positive for the presence of SIV.

RT-PCR analysis for M gene and SIV subtyping

Supernatant from cells demonstrating pathological changes, including CPE positive and suspected samples during the first passage, were further analyzed by RT-PCR using primers specific to the M gene of influenza A virus. The result of RT-PCR confirmed that the three IFA positive samples contained the 780 bp PCR products specific for the M gene of influenza A virus (Fig 3A). However, the 14 suspected, IFA negative samples yielded negative results on RT-PCR specific for the M gene (Fig 3B).

The three samples positive for M gene

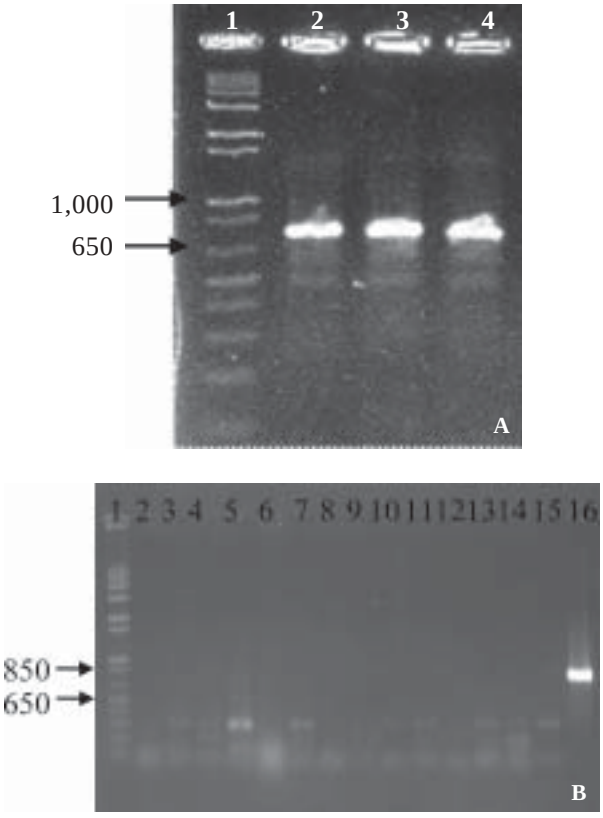

Fig 3–Results of RT-PCR using specific primers for M gene. The PCR products were electrophoresed on 1.2% gel. PCR products specific to the M gene of influenza A virus are 780 bp in length. A) Lane 1 contains DNA marker. Lanes 2, 3 and 4 contain DNA from sample nos. 21, 45 and 58, respectively. B) Lane 1 is a DNA marker. Lanes 2-15 contain DNA from suspected samples (+/-samples). Lane 16 represents a positive control.

were then subjected to subtyping by RT-PCR using primers specific for HA (H1, H3) and NA (N1, N2). The sizes of PCR products representing H1, H3, N1 and N2 DNA fragments are 1,006, 663, 754 and 502 bp, respectively. All the SIV samples were H3N2 subtype (Fig 4).

Titers of the first and third passages for SIV

The samples with typical CPE for SIV on first passage also had typical CPE in the second and third passages. The concentration of SIV in each passage was determined by quantal assay. The concentration of the virus was measured as the virus titer which was the reciprocal of the highest dilution causing 50% infection on the tissue culture units ($TCID_{50}$/ml). The results showed the concentration of virus in the first and the third passages were similar (Table 3). However, sample number 58 was not tested because there was not enough inoculum of the first passage.

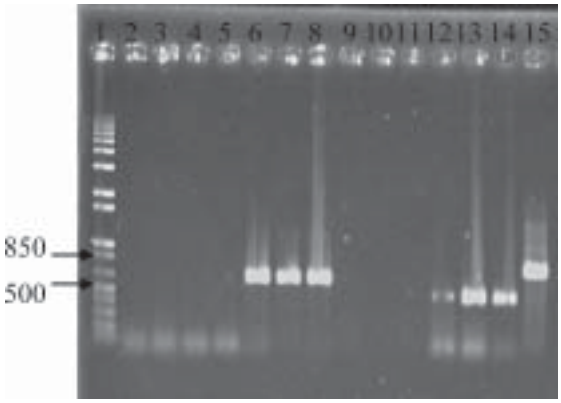

Fig 4–Lane 1 is a DNA marker; lane 2 is a negative control and; lanes 3 to 14 are SIV positive samples. Lanes 3, 4 and 5 are PCR products from SIV positive samples amplified by H1 specific primers. Lanes 6, 7 and 8 contain PCR product of 663 bp specific for H3. Lanes 9, 10 and 11 are SIV positive samples amplified using primers specific for N1. Lanes 12, 13 and 14 are N2 positive samples and contain PCR product of 502 bp and lane 15 is a positive control for the M gene.

DISCUSSION

This study aimed to determine the subtypes of SIV circulating in Thai swine and relationship between SIV and PRDC. One hundred six samples were collected from 30 PRDC affected swine farms in central and eastern Thailand. Nasal swabs and lung suspensions from each piglet were collected for

Table 3
Comparison of titers with the first and third passages for SIV infection in sample numbers 21 and 45.

	Sample no. 21		Sample no. 45	
	1st Passage	3rd Passage	1st Passage	3rd Passage
(TCID50/ml) titer	$2x10^9$	$2x10^9$	$2x10^{10}$	$2x10^{10}$

viral isolation. We found three samples from three different farms that were positive for SIV as confirmed by IFA and RT-PCR. All of them were H3N2 subtype. Only 2.8% of piglets with PRDC had SIV isolated (3 of 106). These results suggest that SIV may not be the major pathogen for PRDC in Thailand. However, 10% (3 of 30) of PRDC affected farms were SIV positive. Three to 5 pigs were obtained from each farm and one pig each from 3 farms were positive for SIV. This is similar to a survey in Korea of 105 pigs with PRDC using in *situ* hybridization and immunohistochemistry (Kim *et al*, 2002) in which, 13% of PRDC affected pigs were infected with SIV; SIV was not a major cause of PRDC. In our study, PRRSV and PCV2 nucleic acids were detected in 50.9% (54/106) and 21.7% (23/106), respectively. Therefore, PRRSV and PCV2 are two major pathogens producing PRDC in central and eastern Thailand.

SIV causes an acute infection in which the viruses are rapidly eliminated from the upper respiratory tract of pigs. In an experimental inoculation of pigs with SIV suggested that lesions of pneumonia recovered and the virus was not detected in the bronchioles or alveoli by Day 7 of infection (Thacker, 2001). Experimental co-infection with SIV and *M. hyopeumoniae* showed that dual infection induced more severe respiratory disease than single infection but the period of sickness was similar in both groups (Thacker *et al*, 2001). The number of SIV positive pigs in our study may be lower than reality since SIV appears in the upper respiratory tract for only a short time. Most of the samples were collected from piglets suffering from PRDC for a period of time which may have exceeded the interval for SIV detection. Some of the piglets in this study may have been infected with SIV but the samples were collected after the viral shedding period. In previous reports, SIV was isolated from 2 of 359 and 5 of 500 nasal swabs collected from healthy pigs in Thailand (Parchariyanon *et al*, 2004) and southern China (Yu *et al*, 2007), respectively. The prevalence of SIV found in the PRDC affected pigs of 2.8% is similar to that reported by others and the isolation rate is normal for SIV surveillance.

SIV outbreaks have been reported sporadically in central and eastern Thailand where most swine production plants are located. SIV H3N2, first isolated from Thai swine in 1978, is antigenically related to human H3N2 virus (Nerome *et al*, 1983). SIV subtype H1N1 was found in swine in eastern Thailand in 1988 (Kupradinun *et al*, 1991). Recently, both subtypes of SIV, H3N2 and H1N1 were detected serologically (Parchariyanon *et al*, 2004) in 0.5% of healthy pigs from five provinces in central Thailand. The HA and NA genes of 5 isolates of H1N1, 1 isolate of H1N2 and 7 isolates of H3N2 from similar regions of Thailand were characterized genetically (Chutinimitkul *et al*, 2008). In our study, SIV subtype H3N2 was isolated from pigs with PRDC confirming H3N2 SIV is still circulating in swine in Thailand.

Although SIV may not be a major pathogen causing PRDC, it has a great impact on public health. Cells lining the respiratory tract of swine contain equal numbers of receptors specific for avian and human influenza viruses (Ito *et al*, 1998). Reassortment among avian, human and swine influenza viruses occurs naturally since pigs are susceptible to swine, avian and human viruses. Pigs have become mixing vessels to generate new reassortants that may cause new outbreaks in both swine and humans. Thus, it is crucial to have continual surveillance and monitoring for the occurrence of SIV and the emergence of novel strains.

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