Investigation of influenza A virus infection in pigs from 5 reported AIV outbreak provinces in 2004

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Abstract

Three hundred and fifty nine porcine tracheal swabs and 553 serum samples collected during May - Octcber 2004 from 5 reported avian influenza virus (AIV) H5N1 outbreak provinces (Chachoegsao, Kanchanaburi, Khonkaen, Sukothai and Supanburi) were examined for virus and antibodies to influenza A viruses. The influenza virus was isolated by egg inoculation and identification by haemagglutination-inhibition (HI) test and reverse-transcriptase polymerase chain reaction. All tracheal swab samples were negative for AIV H5N1 and swine influenza virus (SIV) H3N2 but two samples were positive for SIV H1N1. All serum samples examined by serum neutralization test (SNT) were negative to AIV H5N1 but positive to SIV H1N1 (7.9%) and H3N2 (20.6%). The HI test gave higher percentage of seropositive samples than SNT for both SIV H1N1 and H3N2. The present results indicated that AIV H5N1 was not detected in pigs during this investigation period.

Keywords : investigation, influenza A viruses, pigs

Introduction

Influenza A virus can infect many species of birds and mammals, but the natural host and reservoir are believed to be free-living aquatic birds. It has been shown that migratory ducks, domestic ducks and pigs in southern China play important roles in emerges of pandemic influenza virus strains (Kida *et al.*, 1987; Yasuda *et al.*, 1991). During the past few years, several subtypes of avian influenza (AI) (A) have been reported to be capable of infecting humans (Trampuz *et al.*, 2004). In 2004-2006, H5N1 influenza A viruses caused outbreaks in 22 different Asia countries including Afghanistan, Azerbaijan, Cambodia, China, Hong Kong, Kazakhstan, India, Indonesia, Iraq, Iran, Japan, Jordan, Korea, Loas, Malaysia, Mongolia, Myanmar, Pakistan, Phillippines, Taipei China, Vietnam and Thailand (OIE 2006). The AI virus H5N1 emerged in Thailand causing severe epidemic outbreak in the poultry industry in 2004 which resulted in a major economic loss. Thus surveillance of influenza virus circulating in birds and mammals in this area is the urgent argenda. Pigs were reported to play a crucial role as mixing vessels in the emergence of new pandemic strains because they possess receptors for both avian and human influenza viruses and hence are susceptible to infection by swine, avian and human influenza viruses. In this study, we performed the virological and serological survey in order to determine the extent of AIV in pigs from the AI reported area.

Materials and methods

Samples. In 2004, 359 swine tracheal swabs from slaughter house were collected from 5 AI reported provinces (Chachoengsao, Kanchanaburi, Khonkaen, Sukothai and Supanburi). The samples were kept at 4°C in viral transport medium and processed as soon as possible.

Virus isolation and identification. The tracheal swabs were processed for virus isolation by egg inoculation using specific antibody negative, embryonated fowl eggs according to the protocol described by Office International des Epizooties (OIE) Manual, 2004. Briefly, the samples were clarified by centrifugation at 1000 g at 4°C and 0.1 - 0.2 ml of the supernatants were inoculated into the allantoic sac and amniotic cavity of at least five embryonated fowl eggs of 9 -11 days incubation. Then eggs were incubated at 35-37 °C for 4-7 days. Eggs containing dead or dying embryos and all eggs remaining at the end of the incubation period were chilled to 4°C and the allantoic and amniotic fluids were collected and tested for haemagglutination (HA) activity. The samples that gave a negative reaction were passaged into at least one further batch of embryonated fowl eggs. The HA positive samples were confirmed for the presence of influenza virus by haemagglutination inhibition (HI) test and reverse transcriptase polymerase chain reaction (RT-PCR). The HI test performed according to the OIE Manual, 2004 and the reference positive sera were kindly provided by the OIE Reference Laboratory for Highly Pathogenic Avian

Influenza, Hokkaido, Japan.

RT-PCR, The RT-PCR was performed using the previously published protocol and primers specific for H5 and N1 (Poddar *et al.*, 2002); H1 and H3 (Lee *et al.*, 2001) and, N1 and N2 (Choi *et al.*, 2002). Briefly, viral RNA was extracted from allantoic fluid and amniotic fluid by using the viral RNA Mini kit (Qiagen, Valencia, California). RNA was retrotranscribed to amplify haemagglutinin and neuraminidase gene. Amplification were achieved in a final volume of 25 µl in a thermal cycles with the following cycling conditions: 15 minutes at 95°C of initial denaturation then 40 cycles of 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 minute follwed by a last step of extension at 72°C for 10 minutees. RT-PCR amplicons were separated on 1.5% agarose gel and visualized after ethidium bromide staining. Differentiation between subtypes was achieved by the size of amplified products in the following, 356 bp and 106 bp (H5 and N1), 467 bp and 754 bp (H1 and N1), and 722 bp and 502 bp (H3 and N2) as shown in Fig 1.

Serum samples. A total of 553 pig serum samples from slaughter house were collected from 5 reported AIV outbreak provinces during May - October, 2004 were tested for the presence of antibodies to influenza virus by serum-neutralization test (SNT) and HI test.

Virus. Influenza viruses, A/Ck/Thailand/73/2004 (H5N1) (Ck/Th/73/04), and A/Swine/Iowa/15/30 (H1N1) (Sw/IA/15/30) and A/Aichi/2/68 (H3N2) (Aichi/2/68), were obtained from our laboratory and the OIE Reference Laboratory for Highly Pathogenic Avian Influenza, Hokkaido, Japan, respectively. All of the viruses were used in the HI test and SNT.

Cells. Mardin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) supplemented with 5% bovine calf serum and used for the test of SNT of viral infectivity.

Haemagglutination inhibition (*HI*) test. Before the HI tests, all sera were pretreated with Trypin-Heat-Periodate as described in WHO Manual, 2004. The antigens used in the test were Sw/IA/15/30 (H1N1) and Aichi/2/68 (H3N2) which came directly from infected allantoic and amniotic fluid were diluted to four heamagglutination unit. The procedure was according to the OIE Manual, 2004. The HI titers equal to or greater than 1:40 were recorded as being positive (Webby, *et al.*, 2000).

Serum neutralization test. (SNT). The SNT using cell cultures were performed, according to Kida *et al.*, (1982). Briefly, serial dilution of Trypsin-Heat-Periodate serum samples were mixed with 10^{2} $TCID_{50}$ of each virus strains of Ck/Th/73/04, Sw/IA/15/30 and Aichi/2/68 and incubated at 37 °C for 1 h. The virus and serum mixture was inoculated on to confluent MDCK cell monolayers in 96-well plates and incubated 37 °C in 5% CO_2. After 1 h the inoculum was removed and 100 µl of MEM was added to each well. Cells were incubated at 35°C in 5% CO_2 for 2-3 days. The SNT titer was determined as the reciprocal of the serum dilution that caused 50% inhibition of the cytopathic effect. Serum neutralization titers equal to or greater than 1:40 were recorded as being positive (Ninomiya *et al.*, 2002.).

Results

Influenza A virus was isolated from 2 of 359 tracheal swabs by egg inoculation and identification by HA test (Table 1). Both isolates were SIV H1N1 subtype by HI test and RT-PCR. The size of the PCR products from the RT-PCR were 467 bp for H1 and 754 bp for N1 (Fig.1).

A collection of 553 pig sera from the same area was analyzed by serum neutralization test for the presence of antibodies to H5N1 (Ck/Th/73/04), H1N1 (Sw/IA/15/30) and H3N2 (Aichi/2/68). The percentages of seropositive animals were 0%, 7.9% and 20.6% against H5N1, H1N1 and H3N2, respectively (Table 2). The 553 pig sera against influenza virus H1N1 (Sw/IA/15/30) and H3N2 (Aichi/2/68) by HI test and SNT were conducted. The total percentages of seropositive animals against H1N1 and H3N2 by HI test and SNT were 14.1% and 22.1%, and for H3N2 were 7.9% and 20.6%, respectively (Table 3).

Table 1. Virus isolation and typing from swine tracheal swabs by egg inoculation, haemagglutination (HA) test, haemagglutination inhibition (HI) test and reverse - transcriptase polymerase chain reaction (RT-PCR)

Provinces	Virus isolation	HI test and RT-PCR		
	and HA test	AIV(H5N1)	SIV (H1N1)	SIV (H3N2)
Chachoengsao	0/75	0/75	0/75	0/75
Kanchanaburi	0/62	0/62	0/62	0/62
Khonkaen	0/20	0/20	0/20	0/20
Sukothai	0/44	0/44	0/44	0/44
Supanburi	2/158	0/158	2/158	0/158
Total	**2/359**	**0/359**	**2/359**	**0/359**

Table 2. Serological survey of 553 pigs sera against H5N1 (Ck/Th/73/04), H1N1 (Sw/IA/15/30) and H3N2 (Aichi/2/68) by serum neutralization test

Provinces	No. seropositive animals/total		
	H5N1	H1N1	H3N2
Chachoengsao	0/48	2/48	17/48
Karnchanaburi	0/165	32/165	8/165
Khonkaen	0/100	10/100	54/100
Sukothai	0/80	0/80	4/80
Supanburi	0/160	0/160	31/160
Total	**0/553 (0)[a]**	**44/553 (7.9)**	**114/553 (20.6)**

[a] = % positive

Table 3. Comparison of antibody detection from 553 pig sera against H1N1 (Sw/IA/15/30) and H3N2 (Aichi/2/68) influenza virus by haemagglutination inhibition assay and serum neutralization test

Provinces	No. seropositive animals/total			
	HI test		SNT	
	H1N1	H3N2	H1N1	H3N2
Chachoengsao	18/48	29/48	2/48	17/48
Karnchanaburi	33/165	26/165	32/165	8/165
Khonkaen	4/100	1/100	10/100	54/100
Sukothai	0/80	4/80	0/80	4/80
Supanburi	23/160	62/160	0/160	31/160
Total (% positive)	**78/553 (14.1)**	**122/553 (22.1)**	**44/553 (7.9)**	**114/553 (20.6)**

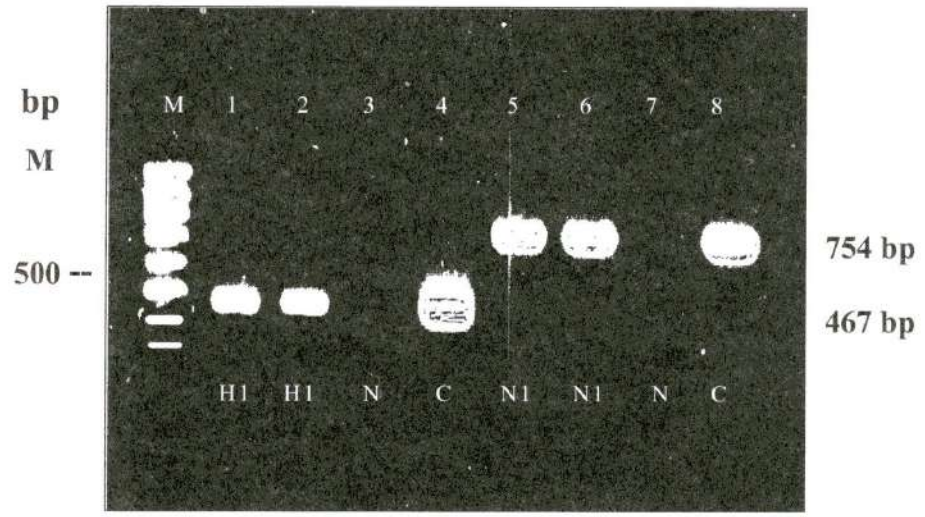

Fig. 1 PCR products of SIV H1 and N1, analysed using 1.5% agarose gel electrophoresis and ethidium bromide staining. Lane M: 100 bp ladder ; lane 1,5: A/Sw/Supanburi 1/04 (H1), (N1); lane 2,6: A/Sw/ Supanburi 2/04 (H1), (N1); lane 3, 7: negative control (H1), (N1); lane 4, 8: positive control of (H1), (N1), respectively

Discussion

This report is the first serological surveillance of avian influenza (AI) in pigs in Thailand. Pigs appear to have a relatively weak species-specific barrier against infection by avian as well as human influenza A viruses (Scholtissek *et al.*, 1993; Webster *et al.*, 1992). Avian influenza viruses were introduced into pigs in Asia and Europe (Scholtissek *et al.*, 1983; Kida *et al.*, 1994; Guan *et al.*, 1996), and genetic reassortment between human-like and avian-like or swine viruses occurred in pigs (Castrucci *et al.*, 1993; Shu *et al.*, 1994). Seropositive pigs against AI were reported in China (Ninomiya *et al.*, 2002; Li *et al.*, 2003), It might be due to the complexity of pigs flock, therefore, some AIV had over come the interspecies barrier and spread into pigs, and owing to special ecology, geography, whether and/or habit of human lifeway in that country. Subtypes that are most frequently identified in pigs include H1N1, H1N2 and H3N2. Other subtypes that have been identified in pigs include H1N7, H3N1, H4N6 and H9N2 (OIE Manual, 2004). The HI test has been widely used to detect antibody to the HA in animal and human sera. Ninomiya, *et al.* (2002) demonstrated the advantage for using SNT for detecting antibodies to avian influenza viruses in mammalian sera. The antibodies to avian influenza viruses can be detected by the SNT but not by the HI (Kida *et al.*, 1982, 1994; Lu *et al.*, 1982; Yoden *et al.*, 1982). The experiment also showed that both HI test and SNT gave the titer when react with the homologous subtype.

In the present study, neither AIV H5N1 nor antibody against AIV H5N1 were detected from tracheal swabs and sera of swine, respectively. The HI test against swine H1N1 and H3N2 influenza virus gave higher titer than SNT, confirming the observation made by Ninomiya *et al.* (2002). Results indicated

that the HI test against swine influenza virus is relatively sensitive test as the HA protein is quite antigenic and stimulates high circulating antibody concentrations (Janke, 2000).

This study emphasized the need for large scale surveillance of pig populations for more information on whether AIV H5N1 or novel subtype are potential candidates as pandemic strains.

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การสำรวจการติดเชื้อไวรัสไข้หวัดใหญ่ไทป์ A ในสุกร จาก 5 จังหวัด ที่มีรายงานการระบาดของโรคไข้หวัดนกในปี 2004

สุจิรา ปาจริยานนท์

สถาบันสุขภาพสัตว์แห่งชาติ เกษตรกลาง จตุจักร กรุงเทพฯ

บทคัดย่อ

ทำการสำรวจการติดเชื้อไวรัสไข้หวัดใหญ่ไทป์ A ในสุกรระหว่างเดือน พฤษภาคม ถึง ตุลาคม 2547 โดยเก็บตัวอย่างป้ายหลอดลม และซีรัม จำนวน 359 และ 553 ตัวอย่าง ตามลำดับ จากสุกรใน 5 พื้นที่ที่ตรวจพบการติดเชื้อไวรัสไข้หวัดนก H5N1 (ฉะเชิงเทรา กาญจนบุรี ขอนแก่น สุโขทัย และ สุพรรณบุรี) ผลการแยกเชื้อไวรัสจากตัวอย่างป้ายหลอดลมด้วยวิธีฉีดเข้าไข่ไก่ฟัก และตรวจด้วยวิธี HA ทำการ subtyping ตัวอย่างที่ให้ผล HA บวกด้วยวิธี HI และ RT-PCR ไม่พบเชื้อไวรัสไข้หวัดนก H5N1 และเชื้อไวรัส ไข้หวัดใหญ่ในสุกร H3N2 จากตัวอย่างทั้งหมด แต่มี 2 ตัวอย่างตรวจพบเชื้อไวรัส ไข้หวัดใหญ่ในสุกร H1N1 ผลการตรวจทางซีรัมด้วยวิธี serum neutralization test (SNT) ไม่พบ แอนติบอดีต่อเชื้อไวรัสไข้หวัดนก H5N1 แต่พบแอนติบอดีต่อเชื้อไวรัสไข้หวัดใหญ่ในสุกร H1N1 และ H3N2 7.9% และ 20.6% ตามลำดับ การตรวจซีรัมด้วยวิธี haemagalutination inhibition (HI) และ SNT ต่อเชื้อ H1N1 และ H3N2 พบว่าวิธี HI ให้ผลบวกสูงกว่าวิธี SNT ผลการศึกษาบ่งชี้ว่าตรวจไม่พบ การติดเชื้อไวรัสไข้หวัดนก H5N1 ในสุกรในช่วงที่ทำการสำรวจนี้

คำสำคัญ : investigation, influenza A viruses, สุกร