LONGITUDINAL SURVEY OF SWINE INFLUENZA VIRUS INFECTION IN A PIG FARM IN THAILAND,2017-2018

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ชนกันต์ นาสำราญ : การสำรวจระยะยาวของการติดเชื้อไวรัสไข้หวัดใหญ่สุกรในฟาร์มสุกรในประเทศไทย, ปี ค.ศ. 2017-2018. (LONGITUDINAL SURVEY OF SWINE INFLUENZA VIRUS INFECTION IN A PIG FARM IN THAILAND,2017-2018) อ.ที่ปรึกษาหลัก : ศ. น.สพ. ดร.อลงกร อมรศิลป์

โรคไข้หวัดใหญ่สุกรเป็นหนึ่งในโรคสัตว์สู่คนที่สำคัญในสุกรและมีผลกระทบด้านเศรฐกิจและการสาธารณสุข เชื้อไวรัสโรคไข้หวัดใหญ่สุกรสายพันธุ์ H1N1, H1N2 และ H3N2 เป็นเชื้อไวรัสที่ทำให้เกิดโรคระบบทางเดินหายใจและสามารถพบในสุกรทั่วโลก วัตถุประสงค์ของวิทยานิพนธ์ฉบับนี้ คือ ศึกษาพลวัฒการติดเชื้อไวรัสไข้หวัดใหญ่สุกร สายพันธุ์ต่างๆในฟาร์มสุกรช่วงปี พ.ศ. 2560-2561 และตรวจติดตามความหลายหลากทางพันธุกรรมของเชื้อไวรัสไข้หวัดใหญ่สุกร โดยได้เก็บตัวอย่างโพรงจมูก (n=436) และตัวอย่างเลือด (n=436) จากสุกรอายุ 4-10 สัปดาห์ ระหว่าง มกราคม พ.ศ. 2560 ถึง พฤศจิกายน พ.ศ. 2561 จากการตรวจพิสูจน์เชื้อไวรัสไข้หวัดใหญ่ชนิดเอจากตัวอย่างโพรงจมูกด้วยวิธี real-time RT-PCR พบเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ จำนวน 38.76% (169/436) และสามารถแยกเชื้อไวรัสไข้หวัดใหญ่ จำนวน 13.07% (57/436) และเมื่อตรวจหาแอนติบอดี้ต่อเชื้อไวรัสไข้หวัดใหญ่สุกร จากตัวอย่างเลือด พบแอนติบอดี้ต่อเชื้อไวรัสไข้หวัดใหญ่สุกรชนิด H3N2, H1N1 และเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 จำนวน 37.23% (140/436), 18.35% (69/436) และ 27.13% (102/436) ตามลำดับ และได้คัดเลือกเชื้อไวรัสไข้หวัดใหญ่สุกรจำนวน 24 ตัว มาถอดรหัสพันธุกรรมทั้งหมด ด้วยวิธี Next generation sequencing จากผลการถอดรหัสพันธุกรรม พบเชื้อไวรัสไข้หวัดใหญ่สุกรสายพันธุ์ H1N1 จำนวน 18 ตัวอย่าง และ H3N2 จำนวน 6 ตัวอย่าง และจากผลการวิเคราะห์ลักษณะพันธุกรรมของเชื้อไวรัสไข้หวัดใหญ่สุกร พบ จีโนไทป์ของเชื้อไวรัสไข้หวัดใหญ่สุกร จำนวน 3 จีโนไทป์ คือ rH1N1 (pdm+2), rH1N1 (pdm+1) และ rH3N2 (pdm+2) โดยลักษณะทางพันธุกรรมของจีโนไทป์ rH1N1 (pdm+2) ประกอบด้วยยีน HA และ NA จากเชื้อไวรัสไข้หวัดใหญ่สุกร H1N1 ดั้งเดิมที่พบในประเทศไทย และ 6 ยีนจากเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ส่วนจีโนไทป์ rH1N1 (pdm+1) ประกอบด้วยยีน HA จากเชื้อไวรัสไข้หวัดใหญ่สุกร H1N1 ดั้งเดิมที่พบในประเทศไทย และ 7 ยีน จากเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 และจีโนไทป์ rH3N2 (pdm+2) ประกอบด้วยยีน HA และ NA จากเชื้อไวรัสไข้หวัดใหญ่สุกร H3N2 ดั้งเดิมที่พบในประเทษไทย และ 6 ยีน จากเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ทั้งนี้พบว่าจีโนไทป์ rH1N1 (pdm+2) และ rH3N2 (pdm+2) เคยมีรายงานในประเทศไทย แต่จีโนไทป์ rH1N1 (pdm+1) ไม่เคยมีรายงานมาก่อนในประเทศไทย นอกจากนี้จากการวิเคราะห์ลักษณะพันธุกรรม พบว่าเชื้อไวรัสไข้หวัดใหญ่สุกรมีกรดอะมิโนที่คล้ายกับเชื้อไวรัสในของคนและสัตว์ปีก จากผลการศึกษาสามารถสันนิฐานได้ว่า เชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ติดเชื้อวนเวียนในสุกรในฟาร์มและสามารถก่อให้เกิด โรคสัตว์สู่คน และ/หรือ โรคจากคนสู่สัตว์ ระหว่างคนงานในฟาร์มสุกรและสุกร ดังนั้นการตรวจติดตามเชื้อไวรัสไข้หวัดใหญ่ในสุกรและคนในฟาร์มสุกรจึงเป็นสิ่งสำคัญและควรดำเนินการอย่างต่อเนื่อง นอกจากนี้การควบคุมและการป้องกันติดเชื้อไวรัสไข้หวัดใหญ่ในสุกรและคน ควรมีการจัดการฟาร์มและการปฏิบัติตัวที่เหมาะสม เช่นการมีสุขอนามัยส่วนตัวที่ดีและการใส่อุปกรณ์ป้องกันทางการแพทย์อย่างเหมาะสม

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Chanakarn Nasamran : LONGITUDINAL SURVEY OF SWINE INFLUENZA VIRUS INFECTION IN A PIG FARM IN THAILAND,2017-2018. Advisor: Prof. ALONGKORN AMONSIN, D.V.M., Ph.D.

Swine influenza cause by influenza A virus, is the one of the most important zoonotic disease of pigs. Swine influenza virus (SIV) infection in pigs causes both economic loss in pig production and public health problem. SIVs subtypes H1N1, H1N2 and H3N2 cause respiratory disease in pigs worldwide. The objectives of this thesis were to determine the dynamic of swine influenza virus subtypes circulation in a pig farm in 2017-2018 and to monitor genetic diversity of swine influenza virus in a pig farm. During January 2017 to November 2018, the nasal swab samples (n=436) and blood samples (n=436) were collected from 4-10 weeks-old pigs from a pig farm. For influenza A virus detection, the nasal swabs were tested for influenza A virus by real-time RT-PCR. Our result showed that 38.76% (169/436) were positive and suspected for influenza A virus. Subsequently, 13.07% (57/436) could be isolated for influenza A virus. For serological test, 37.23% (140/436), 18.35% (69/436) and 27.13% (102/436) of pigs had antibodies against SIV-H3N2, SIV-H1N1 and pdmH1N1 viruses, respectively. For SIV characterization, 24 SIVs were characterized by whole genome sequencing using next-generation sequencing. Our result showed that SIVs can be subtyped as SIV-H1N1 (n=18) and SIV-H3N2 (n=6). The SIV-H1N1 can be classified into 2 genotypes,rH1N1 (pdm+2) and rH1N1 (pdm+1), and SIV-H3N2 can be classified into 1 genotype, rH3N2 (pdm+2). Genotype rH1N1 (pdm+2) contained HA and NA genes from endemic Thai SIV-H1N1, and internal genes from pandemic H1N1-2009. Genotype rH1N1 (pdm+1) contained HA gene from endemic H1N1, while NA and internal genes from pandemic H1N1-2009. Genotype rH3N2 (pdm+2) contained HA and NA genes from human-like swine from endemic Thai-SIV and the internal genes from pandemic H1N1-2009. It is noted that genotype rH1N1 (pdm+1) is the novel genotype which never been reported in Thailand. While the rH1N1 (pdm+2) and rH3N3 (pdm+2) were previously observed in Thailand. The results of genetic analysis showed that SIVs in this thesis possessed most human-like characteristics and some avian-like characteristics. Base on our result, it could be speculated that pandemic H1N1-2009 was circulating in a pig farm for a period of time, Thus, zoonotic and reverse zoonotic infection between workers and pigs in the pig farm is common. Thus, monitoring of SIVs in pigs and workers is important and should be routinely conducted. Good practice and management in pig farm including personal hygiene and personal protective equipment (PPE) should be practiced and used in the farm for prevention and control of influenza virus transmission in pigs and humans.

Field of Study:	Veterinary Public Health	Student's Signature .....
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Chanakarn Nasamran

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introduction

1.1 Importance and Rationale

Swine influenza, caused by influenza A virus, is one of the most important zoonotic diseases of pigs. Influenza A virus is an enveloped, negative sense, single-stranded RNA virus which contains eight gene segments (PB2, PB1, PA, HA, NP, NA, M and NS genes). Recently influenza A virus has been named to *Alphainfluenzavirus* (Smith et al., 2017; King et al., 2018). Influenza A virus can be classified into different subtypes based on surface proteins, hemagglutinin (HA) and neuraminidase (NA). Currently, there are 18 HA and 11 NA subtypes of influenza viruses which recent influenza subtype H18N11 was reported in bats in 2013 (Wu et al., 2014).

It has been known that there are two evolution mechanisms of influenza viruses, genetic drift and genetic shift. For genetic drift, the viral RNA polymerase is lack of proofreading activity. Thus, during viral replication, viral genomes could change or mutate (Shao et al., 2017). Due to the minor changes in viral gene especially HA and NA genes. The viruses can then escape from host immunity. For genetic shift or genetic reassortment, the host cell is infected by two or more different influenza A viruses and the viral genes are then exchange during viral replication (Webster et al., 1982; Kim et al., 2018). Pig is considered as a mixing vessel of the influenza viruses because it is susceptible to both avian and human influenza viruses. Thus, pig is a potential host to contribute suitable environment for the reassortment of influenza A virus and possibly generate novel influenza viruses (Kuntz-Simon and Madec, 2009). Due to the major changes in viral genes, the influenza viruses can then escape from host immunity and sometime more virulence causing major influenza outbreaks.

Swine influenza viruses (SIVs) cause respiratory disease in pigs worldwide. The SIV subtypes circulating in pigs are H1N1, H3N2 and H1N2. In Thailand, SIV-H1N1 and

SIV-H3N2 were first reported in the 1970s (Schultz-Cherry et al., 2013). In 2005, SIV-H1N2 was reported in pig farms (Chutinimitkul et al., 2008). Previous study reported that genetic constellation of Thai SIV-H1N1 contained HA gene from Classical swine lineage and seven genes from Eurasian swine lineage (designated as 7+1) or HA and NS genes from Classical swine lineage and six genes from Eurasian swine lineage (designated as 6+2) (Kitikoon et al., 2011; Nonthabenjawan et al., 2015). For SIV-H3N2, the genetic constellation of SIV-H3N2 showed that HA and NA genes are originated from human lineage and other internal genes are originated form Eurasian (PB1, PB2, PA and M genes) and Classical swine lineages (NP and NS genes) (Vincent et al., 2014). For SIV-H1N2, the genetic constellation of SIV-H1N2 combine Thai SIV-H3N2 and Thai SIV-H1N1. Thus, SIV-H1N2 contains NA gene from human lineage, two segments (HA and NS) from Classical swine lineage and other five internal genes (PB2, PB1, PA, NP and M) from Eurasian swine lineage (Nonthabenjawan et al., 2015). It is important to note that after the introduction of pandemic influenza H1N1-2009 (pdmH1N1-2009), most SIV viruses were reassorted with pdmH1N1-2009 which containing triple reassortment internal genes (TRIG) from pdmH1N1-2009. Many studies reported that the reassortant SIVs with pdmH1N1-2009 became predominant SIVs in pigs in Thailand (Charoenvisal et al., 2013; Dee, 2005; Hiromoto et al., 2012). Due to the novel SIV reassortants could be found in pig farms, routine genetic monitoring of SIVs in pig farms is important. In this thesis, longitudinal survey of SIV subtypes circulating in a pig farm with history of endemic swine influenza virus infection was conducted. Moreover, evidence of genetic reassortment of SIVs in the pig farm was provided.

1.2 Research questions

1. What are the dynamics of swine influenza viruses and subtypes circulating in a pig farm with history of endemic swine influenza virus infection in 2017-2018?
2. What are the evidences of genetic reassortment of swine influenza viruses isolated from a swine influenza endemic pig farm in 2017-2018?

1.3 Objectives of study

- To determine the dynamic of swine influenza virus subtypes circulating in a pig farm in 2017-2018
- To monitor the genetic diversity of swine influenza viruses isolated from a swine influenza endemic pig farm in 2017-2018

1.4 Hypothesis

Several subtypes of swine influenza viruses are circulating in a pig farm with history of endemic swine influenza virus infection. Genetic diversity and reassortment of swine influenza viruses could be observed in this pig farm.

Chapter 2

Literature Review

2.1 Influenza A virus (IVA)

Influenza virus, a single-stranded RNA virus, belongs to the family Orthomyxoviridae. Influenza virus contains eight gene segments; PB2 (polymerase basic2), PB1 (polymerase basic1), PA (polymerase acidic), HA (hemagglutinin), NP (nucleoprotein), NA (neuraminidase), M (matrix protein) and NS (non-structural protein). Influenza virus can be classified into four major types; A, B, C and D. Recently, four major types of influenza viruses are named as Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus and Deltainfluenzavirus, respectively (King et al., 2018). influenza A and B viruses cause epidemic or seasonal influenza. Influenza C virus causes mild respiratory clinical signs, while influenza D virus cause disease in cattle, but not found in human (Ferguson et al., 2016). Among 4 types, influenza A virus is the most important pathogen and reported in birds and mammals. Influenza A virus can be classified into different subtypes by HA and NA proteins. There are 18 HA and 11 NA subtypes, which H18N11 was recently reported in bats in 2013 (Wu et al., 2014)

2.2 Swine Influenza virus (SIV)

Swine influenza viruses (SIV) cause respiratory disease in pigs worldwide. The SIV subtypes circulating in pigs are H1N1, H3N2 and H1N2. Swine influenza virus can evolve in two difference mechanisms, genetic shift and genetic drift. For genetic drift, this mechanism is small mutation of genes. Genetic drift occurs continually over time during viral replication by a lack of proofreading activity in viral RNA polymerase. After influenza virus goes through genetic drift many times, the virus is not recognized by host immunity. Another mechanism is genetic shift or genetic reassortment, and this mechanism is a major change of influenza viruses. First host cells are infected by two or more influenza viruses. Then, the viral genes are exchanged or reassorted during replication process resulting in new HA and NA of influenza virus. Novel influenza

viruses can sometime infect or spread across species and/or cause major influenza outbreaks (Smith et al., 2009).

2.3 Epidemiology of swine influenza

Swine influenza is an important zoonotic disease and highly contagious viral infection in pigs. Clinical signs of swine influenza in pigs are fever, coughing, sneezing, depression, nasal or ocular discharge, eye redness or inflammation, and decrease or stop feeding. Incubation period is 1-3 days and illness duration is about 4-7 days. Economic impact from swine influenza is that the pigs decrease feeding and slow weight gain resulting in an increase in the number of days to reach market weight.

Recently, the main subtypes of swine influenza are H1N1, H1N2 and H3N2. There were many major influenza outbreaks related to the reassortment of swine influenza. For example, the first outbreak of influenza virus is Spanish flu as human H1N1 in 1918 (H1N1-1981) which later considered originated from swine origin influenza viruses. The second outbreak is Asian pandemic in 1957, H2N2 influenza viruses were generated from human H1N1 and avian H2N2. The third pandemic influenza virus is Hong Kong flu in 1968 with influenza virus subtypes H3N2. The latest outbreak is pandemic influenza H1N1 2009. Pandemic H1N1-2009 virus is the result of reassortment of human, swine and avian influenza viruses.

Swine influenza viruses are evolved from a single genotype to more diverse with more complex gene composition. The SIV-H1N1 was first isolated in North America in 1930, called “Classical swine influenza virus (C-SIV)” and closely related to Spanish flu (Vincent et al., 2008). Subsequently, C-SIV was then found distribute in North America, Europe and Asia. In 1979, avian-like SIV-H1N1 with all avian lineage genes was entered in pig population and replaced C-SIV genotype, called “Eurasian avian-like swine influenza virus (EA-SIV)” (Scholtissek et al., 1983). After Hong Kong flu pandemic outbreak in the 1970s, human-like H3N2 virus was introduced in European pig population. Thus, avian-like SIV-H1N1 and human-like SIV-H3N2 were reassorted and

contributed a new virus consist of human-like genes (H3 and N2 gene) and 6 avian-like internal genes (Campitelli et al., 1997). In the 1990s, The SIV-H3N2 with avian-like internal genes reassorted with human H1N1 viruses and contributed SIV-H1N2 consist of human gene (HA gene), human-like gene (NA gene) and avian-like internal genes (Brown et al., 1998). In the 2000s, SIV-H1N1, H1N2 and H3N2 SIV were co-circulating in Europe and Asia.

In the 1990s, human H3N2 viruses were introduced in pig population in North America resulting in the reassortment between C-SIV-H1N1 and human H3N2 called "triple-reassortants H3N2". The triple-reassortant SIV-H3N2 contain genes originated from avian-lineage (PB2 and PA gene), human-lineage (HA, NA and PB1 genes) and C-SIV lineage (NP, M and NS genes) (Zhou et al., 1999; Vincent et al., 2008). The genetic constellation of triple reassortant SIV-H3N2 is referred to as the triple-reassortant internal gene (TRIG) cassette. (Karasin et al., 2002).

2.4 Epidemiology of swine influenza in Thailand

In Thailand, SIV-H1N1 and SIV-H3N2 were first reported in the 1970s (Schultz-Cherry et al., 2013). In 2005, SIV-H1N2 was reported in pig farms in Thailand (Chutinimitkul et al., 2008). For Thai SIV-H1N1, the genetic constellation of the virus contains HA from Classical swine lineage and seven genes from Eurasian swine lineage (7+1) or HA and NS gene from Classical swine lineage and six genes from Eurasian swine lineage (6+2) (Kitikoon et al., 2011; Nonthabenjawan et al., 2015). For Thai SIV-H1N2, the genetic constellation of SIV-H1N2 contains Thai SIV-H3N2 and Thai SIV-H1N1. Thus, SIV-H1N2 contains NA gene from human lineages, two genes (HA and NS) from Classical swine lineage and other five internal genes (PB2, PB1, PA, NP and M) from Eurasian swine lineage (Nonthabenjawan et al., 2015).

In 2009, the pandemic outbreak of pandemic influenza H1N1-2009 (pdmH1N1-2009) viruses were reported. pdmH1N1-2009 virus has unique genome which the reassortment of between triple-reassortment SIVs from North American lineage and

avian-like SIV from Eurasian lineage. The genetic constellation of pdmH1N1-2009 contains with six gene segments (PB2, PB, PA, HA, NP and NS) originated from triple-reassortment of North America lineage and the M and NA genes originate from Eurasian lineage (Dawood et al., 2009). Subsequently, pdmH1N1-2009 virus was introduced into pigs and domestic animals worldwide (Howden et al., 2009; Howard et al., 2011; Lin et al., 2012). Reassortment of pdmH1N1-2009 and predominant endemic SIVs in the areas was documented in many countries. For example, in China, reassortant SIV-H1N1 (rSIV-H1N1) contains HA gene from EA-lineage, NA and internal genes (TRIG) from pdmH1N1-2009 (Vijaykrishna et al., 2010). Reassortant SIV-H3N2 (rSIV-H3N2) consists of HA and NA genes of human-like SIV-H1N1 and the internal genes of pdmH1N1-2009 (Lin et al., 2012). In USA, reassortment of pdmH1N1-2009 and other SIV subtypes contribute to new reassortant SIV-H1N1 and SIV-H3N2 (Ducatez et al., 2011b; Kitikoon et al., 2012). In Europe, reassortant SIV-H1N1 consists of NA gene from the endemic SIV and the other genes from pdmH1N1-2009. Reassortant SIV-H1N2 contains HA and NA genes from human-like SIV and the other six genes from pdmH1N1-2009 (Howard et al., 2011; Moreno et al., 2011). In Thailand, the genetic constellation of reassortant SIV-H1N1 consists of NA gene from EA-lineage and the other seven genes from pdmH1N1-2009 (Kitikoon et al., 2011). The other reassortant SIV-H1N1 contains HA gene from Classical swine lineage, NA gene from Eurasian swine lineage and the other six genes from pdmH1N1-2009 (Nonthabenjawan et al., 2015). For reassortant SIV-H3N2, HA and NA genes are originated from human-like SIV and the other six genes are from pdmH1N1-2009 (Hiromoto et al., 2012; Nonthabenjawan et al., 2015). For reassortant SIV-H1N2, the virus contains NA gene from human-like virus and the other seven genes from pdmH1N1-2009 (Nonthabenjawan et al., 2015).

2.5 Genetic characterization of swine influenza

Genetic characterization of swine influenza virus can be conducted by using gene or whole genome sequencing. To generate the nucleotide sequences, there are several sequencing techniques. For example, Sanger sequencing is a conventional sequencing technique. While next generation sequencing is a new and up to date sequencing technique.

In the 1970s, Sanger sequencing was described. Sanger sequencing is known as the dideoxynucleotide method or the sequencing by synthesis method. This technique uses chemically modified nucleotides call "dideoxy-nucleotide dNTPs (ddNTPs)" which inhibit nucleotide elongation. Then, DNA fragments are ended by a ddNTPs with different sizes. The fragments are separated by using electrophoresis and the bands of DNA fragments can be visualized by an imaging system. In the 2000s, new generation sequencing (NGS) is introduced and overcome Sanger sequencing. The basic principle of NGS is generating of millions of short nucleotide sequences in single run. NGS is new DNA sequencing technique, high throughout and provides large scale nucleotide sequence data. The difference between Sanger sequencing and NGS is sequencing volume. The Sanger method can sequence a single DNA fragment at a time, but NGS can sequence millions of fragments per run. Thus, NGS can generate large scale data, which can detect novel or variant genomes.

NGS has 3 major sequencing platforms consists of 1) Roche (454) sequencer, 2) Illumina genome analyzer and 3) SOLiD sequencer. Roche (454) sequencer using pyrosequencing concept which based on the detection of pyrophosphate released after new synthetic DNA strand. The DNA sequences are ligated with adaptor and amplified by emulsion PCR on the surfaces of hundreds of thousands of agarose beads. Millions of oligomers attach on surface of these beads. The beads are transferred to picotiter plate (PTP) and pyrosequencing method. Ion torrent sequencer has same principle with 454 pyrosequencing, but it does not use fluorescent labeled

nucleotides. This method based on the detection hydrogen ion released during sequencing. SOLiD sequencer (Sequencing by Oligo Ligation and Detection) uses catalyzing by DNA ligase for sequencing DNA. This platform starts from attaching adaptors to DNA fragments and fixing on beads, then amplification by emulsion PCR. These beads are transferred on glass slide and sequencing by using 8-mer with fluorescent label end are ligating to DNA fragment and release color. Illumina genome analyzer uses the principle of sequencing by synthesis. This platform starts from ligation adaptor to DNA fragments, attachment these fragments to glass flow cell for amplification by bridge PCR, and the sequencing by synthesis is applied. Surface of flow cells are attached with oligomer complementary to specific adaptor. After that, millions of short amplicons are synthesized, and large scale of nucleotide sequence are generated and analyzed.

Post-sequencing, there are two approaches for analyzing read data. If reference genome is available, the read data can be mapped to the reference genome. This method could not apply for searching or identifying novel genes or sequences, but it can provide rapid information regarding to substitutions, deletions, insertions of nucleotide sequences. On the other hand, if reference genome is not available, sequence read could be assembled by using de-novo algorithms which finding overlapping between sequence reads and leading to sequence contigs. This approach can help discovery of novel gene and small sequence variants.

Chapter 3

Materials and Methods

This thesis consists of 3 study phases including: phase 1. Sample collection from a swine influenza virus endemic pig farm, phase 2. Viral detection, isolation and serological test for swine influenza viruses and phase 3. Genetic characterization of swine influenza viruses isolated from SIV endemic pig farm. The conceptual framework of this study is shown in Figure 1.

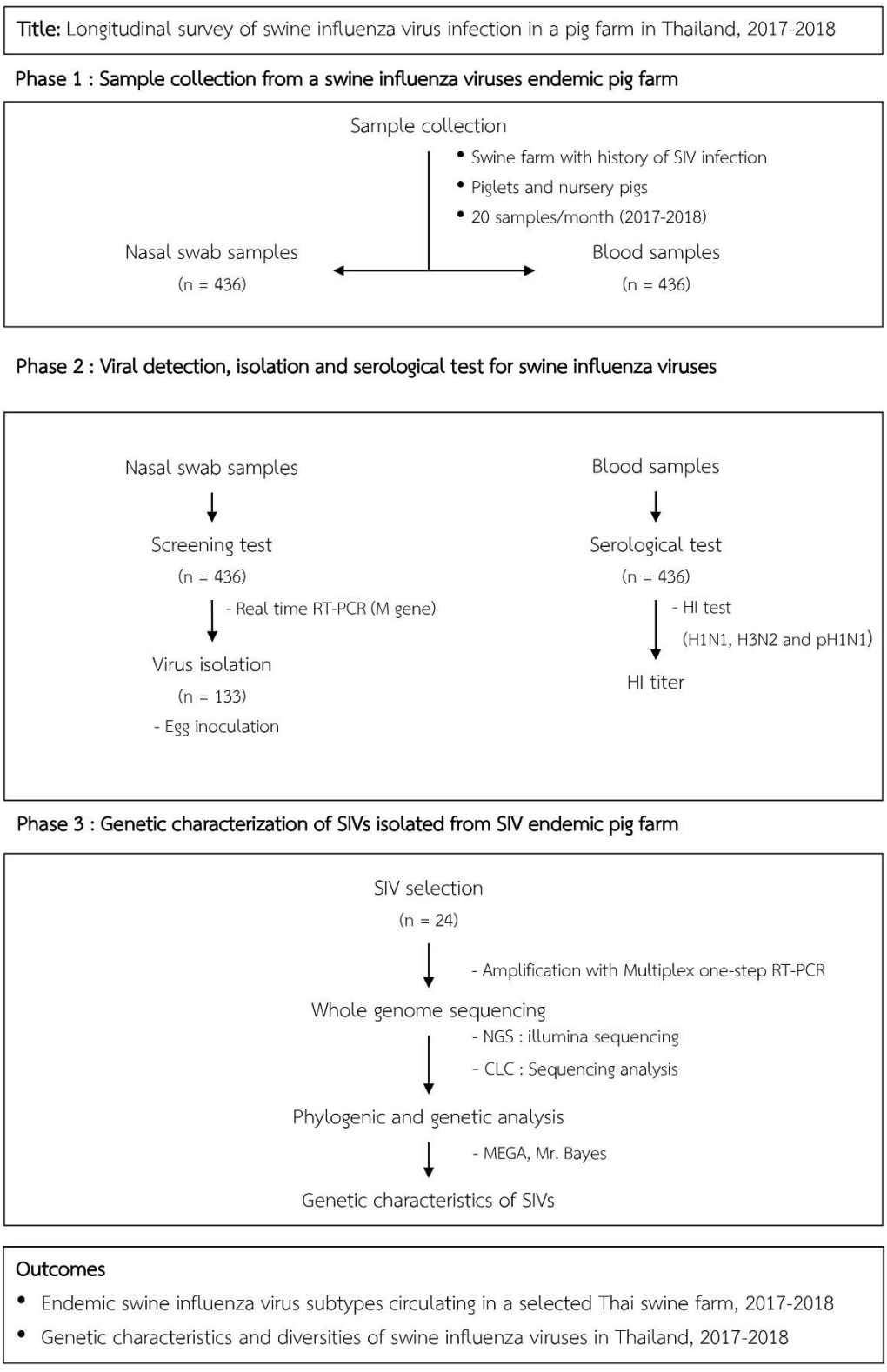


Figure 1: Conceptual framework of this study.

3.1 Phase 1. Sample collection from a swine influenza virus endemic pig farm

3.1.1 Pig farm selection

In this thesis a pig farm was selected based on a history of swine influenza virus subtype H1N1, H1N2 and H3N2 infection in the farm. The selected pig farm is located in Ratchaburi province. This pig farm is a large-scale pig farm with 1,600 sows and 2,000 piglet production per month. The farm has open-housing system with moderate biosecurity which birds, dogs and cats can access to pig housing areas. In the previous studies, this farm has been investigated for porcine respiratory and reproductive syndrome viruses (PRRSV), porcine circovirus type 2 (PCV2) and swine influenza viruses (SIV). The results from previous studies showed that 3 SIV subtypes (H1N1, H3N2 and H1N2) had been circulating in the farm (Nonthabenjawan et al., 2015).

3.1.2 Sample collection from a swine farm

Sample collection was conducted in the pig farm in Ratchaburi province during January 2017 to November 2018. The samples were collected from 436 pigs including nasal swab samples (n=436) and blood samples (n=436). The samples collection was carried out every 4 months in 2017 and every month in 2018. In each visit, the samples were collected from 30-34 pigs/visit (30-34 nasal swab and 30-34 blood samples). The samples were collected from piglets and nursery pigs (4-10-week-old) with clinical signs of swine influenza such as coughing, sneezing and nasal discharge (Table 1). The nasal samples were placed in viral transport media (MEM with 7% BSA, 100 U/ml penicillin, 100 mg/ml streptomycin and 1 mg/ml trypsin). The samples were kept on ice and transported to the laboratory within 24 hours.

Table 1: Detail of nasal swabs and blood samples collected from piglets and nursery pigs in this thesis

Year	Month	Age of pigs	Number of pigs	Number of nasal swabs	Number of blood samples
2017	**Jan-17**	4-10 weeks	30	30	30
	Apr-17	4-10 weeks	30	30	30
	Jun-17	4-10 weeks	30	30	30
	Dec-17	4-10 weeks	30	30	30
2018	**Feb-18**	4-10 weeks	31	31	31
	Mar-18	4-10 weeks	34	34	34
	Apr-18	4-10 weeks	32	32	32
	May-18	4-10 weeks	31	31	31
	Jun-18	4-10 weeks	30	30	30
	Jul-18	4-10 weeks	30	30	30
	Aug-18	4-10 weeks	31	31	31
	Sep-18	4-10 weeks	33	33	33
	Oct-18	4-10 weeks	32	32	32
	Nov-18	4-10 weeks	32	32	32
	Total		436	436	436

3.2 Phase 2. Viral detection, isolation and serological test for swine influenza viruses

3.2.1 Swine influenza virus detection

Viral RNA was extracted from all nasal swab samples (n=436) by GeneAll® GENTi™ Viral DNA/RNA Extraction Kit (GeneAll®; Lisbon, Portugal) on a Genti™ 32 (GeneAll®; Lisbon, Portugal). In brief, the sample (200 µ) was mixed with 600 µl of buffer for lysis and adjusting solution. RNA was bond with magnetic beads in optimal condition. Next step, the mixture was washing away contaminant three times. Final step, RNA was eluted with elution buffer (50 µl). Finally, Purified RNA was kept at -20 °C until use.

RNA samples were screened for influenza A virus by using one-step Real-time RT-PCR with TaqMan probe specific to Matrix gene (M gene). In this study, one-step Real-time RT-PCR was performed by using SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen™; California, USA). In detail, a 12.5 µl reaction mixture consists of 6.25 µl of 2X Reaction Mix (Invitrogen™; California, USA), 0.5 µl of each primer (10 µM), 0.5 µl of probe (2.5 µM), 0.08 µl of 50mM $MgSO_4$, 0.25 µl of SuperScript™ III RT/Platinum™ Taq Mix (Invitrogen™; California, USA), 4 µl of RNA samples and distilled water. The amplification process was performed with cycling protocol 50 °C for 15 minutes and 95 °C for 2 minutes, follow by 50 cycles of 95 °C for 15 sec and 60 °C for 30 sec. The primers and probe sequences was designed following the CDC protocol recommendation (Organization, 2009). The real-time RT-PCR reactions were performed and analyzed on a Rotor-Gene 3000 (Corbett Research; Sydney, Australia). Real-time RT-PCR result was interpreted by cycle threshold (Ct-value) of < 36 as positive, Ct-value of 36-40 as suspect, and Ct-value of > 40 as negative.

3.2.2 Swine influenza virus isolation by egg inoculation

In this study, the influenza A positive (n=82) and suspected (n=87) samples by real-time RT-PCR were subjected to influenza A virus isolation by egg inoculation following WHO recommendation (Organization, 2002). For egg inoculation, each nasal swab sample was inoculated into 3 embryonated chicken eggs (9 to 11-day-old). The eggs were clean with alcohol and betadine and punch a small hole over the air sac. Sample supernatant (100 µ) was injected into allantoic cavity. The inoculated eggs were incubated in 37 ℃ for three days. Inoculated eggs with early death within 24 hours were discharged as contamination. After 72 hours inoculation, allantoic fluid was collected and tested for hemagglutinin activity with hemagglutination test (HA test). HA test was performed by using 0.5% suspension of turkey red blood cells. In detail, first, 50 µl of PBS was filled in a 96-well V-bottom microtiter plate. 50 µl of allantoic fluid was added to the first well and serially 2-fold diluted. The results of dilutions

were range 1:2 to 1:16. Then 50 µl of 0.5% turkey erythrocyte was added to each well and incubated at room temperature 30 minutes. For HA test interpretation, wells with spreading for erythrocyte were positive. Wells with button of erythrocyte at bottom of well were negative for hemagglutinating activity. The samples with HA titer of $\geq$ 4 HA unit per 50 µl were selected and subjected for further influenza A confirmation. For virus confirmation, RNA of suspected and positive samples from 3.2.2 were extracted. RNA samples were tested for influenza A virus by using one-step Real-time RT-PCR with TaqMan probe specific to Matrix gene (M gene) (Detail see 3.2.1).

3.2.3 Serological test for swine influenza virus

Blood samples were centrifuged at 3,000 rpm for 10 minutes to separate serum. For Hemagglutination inhibitor test (HI test), the serum sample was incubated 30 minutes at 56 °C for removing heat-labile non-specific factor. Then 100 µl of serum sample and reference sample were treated to remove non-specific inhibitor and non-specific hemagglutinin.

For HI test of H3N2 virus, receptor destroying enzyme (RDE) and absorption with 50% chicken red blood was used to treat serum. In detail, 50 µl of serum was mixed with 150 µl of RDE and incubated overnight at 37 °C to remove non-specific inhibitor. For inactivating of RDE, the serum was incubated for 1 hour at 56 °C. The RDE-treated serum was mixed with 100 µl of 50% chicken erythrocyte and incubated at room temperature for 1 hour for removing non-specific hemagglutinin. After centrifuging at 2000 rpm for 10 minutes, the supernatant was free from non-specific inhibit and non-specific hemagglutinin serum.

For HI test of H1N1 and pandemic H1N1, 20% of kaolin and absorption with 50% chicken red blood was used to treat serum. In detail, 100 µl of sera were mixed with 400 µl of 20% kaolin and incubated at room temperature for 30 minutes to remove non-specific inhibitor. For sedimentation of kaolin, the kaolin-treated serum was centrifuged at 2000 rpm for 10 minutes. The serum was added with 100 µl of 50% Chicken erythrocyte and incubated at room temperature for 1 hour for removing non-specific hemagglutinin. After centrifuging at 2000 rpm for 10 minutes, the supernatant was free from non-specific inhibitor and non-specific hemagglutinin serum.

In this thesis, reference antigens were endemic H1N1 (A/Swine/Thailand/CU-CB1/06), pandemic H1N1-2009 (A/sw/Thailand/CU-RA29/2009) and endemic H3N2 (A/sw/Thailand/CU-CB8.4/2007). Reference antigen were prepared to 8 HA unit per 50 µl. First step of HI test, 50 µl of PBS was placed in a 96-well V-bottom microtiter plate. 50 µl of treated serum was mixed in the first well and serially 2-fold diluted. 50 µl of reference serum was added in each well and incubated at room temperature for 45 minutes. 100 µl of 0.5% chicken erythrocyte was added into each well. After incubating at room temperature for 1 hour, wells with button of erythrocyte of bottom were interpreted as positive and wells with spreading for erythrocyte was interpreted as negative. The samples with HI titer of $\geq$ 1:40 were interpreted as positive against specific SIVs subtypes.

3.3 Phase 3. Genetic characterization of swine influenza viruses isolated from SIV endemic pig farm

3.3.1 Next generation sequencing

In this study, 24 SIVs were selected and subjected for whole genome sequencing by next generation sequencing (NGS). The criteria of selection of SIVs for whole genome sequencing are based on time of samples collection and high virus titer

(low Ct-value). To perform NGS, first viral RNA from allantoic fluid was extracted (Detail see 3.2.1). Then eight segments of influenza A virus were amplified by using one-step RT-PCR with SuperScript® III RT-PCR system with Plantinum® Taq DNA polymerase (Invitrogen™; California, USA) and MBT12 and MBT13 primers (Zhou et al., 2009). In detail, a 50 µl reaction mixture consists of 1.6 mM of magnesium sulfate, 0.4 µM of dNTP and 0.4 µM of each primer, 2 µl of SuperScript™ III RT/Platinum™ Taq Mix (Invitrogen™; California, USA), 5 µl of RNA samples and distilled water. The amplification process was performed with cycling protocol 42 °C for 60 minutes and 94 °C for 2 minutes, and then 5 cycles of 94 °C for 30 sec, 45 °C for 30 sec and 68 °C for 3.30 minutes, followed by 35 cycles of 94 °C of 30 secs, 57 °C for 30 secs and 68 °C for 3.30 minutes. PCR products were confirmed by appearing multiple bands in electrophoresis in 1.5% agarose gel. Then, products were purified by NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL, Düren, Germany) followed manufacturer's instruction. Purified PCR products were quantified by NanoDrop™ (ThermoFisher Scientific, Waltham, Massachusetts, USA) and submitted to Novogene co. LTD for illumine Hiseq PE150 (Illumina Corporation, San Diego, California, USA) by using NEBNext® Multiplex Oligos for Illumina® (New England BioLabs, Ipswich, Massachusetts, USA) to generate sequencing libraries. Each nucleotide sequences contained 150 base pairs. To validate and determine nucleotide sequences of eight gene segments of influenza A virus, the following steps were conducted:

a) Each nucleotide sequence data (reads) was trim-reads to remove adaptor. Then nucleotide sequences were assembly with de-novo assembly method by CLC genomics workbench software Version 11.0.1. (QIAGEN®; Hilden, Germany)
b) Results from de-novo assembly (contigs) were compared with sequence database by BLAST at https://blast.ncbi.nlm.nih.gov.
c) References of influenza viruses were selected from BLAST results
d) Trimmed sequences were used for mapping reads to references and set results of c) as references.

e) The whole genome sequences were extracted to FASTA format (.fast) by CLC genomics workbench software.

3.3.2 Phylogenetic analysis of whole genome sequences of swine influenza viruses

The nucleotide sequences of each gene of the SIVs from this study were compared with the reference SIVs. The reference nucleotide sequences of swine influenza virus (n~50) were obtained from Influenza Research Database (http://www.fludb.org). For HA1 gene, the reference viruses include the SIVs of Classical swine lineage (1A), human seasonal lineage (1B) and Eurasian avian-like lineage (1C). It is noted that the Classical swine lineage (1A) can be further divided into 5 sub-clusters: alpha (1A.1), beta (1A.2), gamma1 (1A.3.3.3). gamma 2 (1A.3.2) and pandemic 2009 (1A.3.3.2) (Anderson et al., 2016). For HA3 gene, the reference viruses include the SIVs of North American (Clade I, II, III and IV), human seasonal lineage and Eurasian swine lineage. The Clade IV of SIVs can be further divided into 6 sub-clusters: A-F. For NA1 gene, the reference viruses include the SIVs of Classical swine lineage, human seasonal lineage, North American Triple reassortant and Eurasian avian-like lineage. For NA2 gene, the reference viruses include the SIVs of North American and Eurasian swine lineages. The North American lineage can be further divided into 2 sub-groups base on year: 1998 from earlier H3N2 introduction and 2002 from 2000s human seasonal H1 introduction. For other internal genes such as NA1 gene, internal genes, the reference viruses include the SIVs of Classical swine lineage, human seasonal lineage, North American triple reassortant and Eurasian avian-like lineage.

The nucleotide sequences of each gene of the viruses (both references viruses and viruses form this study) were aligned with Muscle program v3.6 (Edgar, 2004) in MEGA v7.0 software (Kumar et al., 2016). The flanking regions at 5’ and 3’ end were

trimmed to present start and stop codon. In total, trimmed sequences of PB2, PB1, PA, HA, NP, NA, M and NS genes were 2280, 2274, 2151, 1701, 1497, 1410, 982, 828 base pairs, respectively. Then, the phylogenic trees of each gene were generated by applying neighbor-joining method with Kimura-2 parameter model and 1000 replication of bootstrap. Substitution rates among sites were set in uniform rate and gabs in the sequences were treated in pairwise deletion. Nucleotide sequences of 8 gene segments of swine influenza virus were submitted to the GenBank database.

3.3.3 Genetic constellation analysis of swine influenza viruses

From phylogenetic analysis, lineages or clusters of each gene of the virus were assigned based on the comparison to reference viruses. After lineages or clusters of gene are assigned, the combination of eight lineages or clusters were analyzed and assigned as genetic constellation of swine influenza virus in Thailand. The analysis of genetic constellation of the virus can be used to identify reassortment and genetic diversity of the viruses.

Chapter 4

Results

4.1 Sample collection from a swine farm

We conducted the sample collection at a swine farm in Ratchaburi province. The swine farm had a history of endemic SIVs infection in the past five years. During 2017-2018, there were 14 visits for sample collection (every 3 months in 2017 and every month in 2018). In each visit, we collected nasal swab (n≃30) and blood samples (n≃30) from piglets and nursery pigs with influenza-like symptoms. In total, nasal swab (n=436) and blood sample (n=436) were collected from 436 pigs (Table 1).

4.2 Detection, isolation and serological test for swine influenza viruses

4.2.1 Detection and isolation of swine influenza virus in a pig farm

During January 2017- November 2018, 436 nasal swab samples were collected form piglets and nursery pigs in a pig farm. All samples were then tested for influenza virus by using real-time RT-PCR. Our result showed that 18.81% (82/436) and 19.95% (87/436) of samples were positive and suspected for IAV. In 2017, SIVs could be detected all year round. In detail, 26.67% (8/30) of samples was IAV positive in January 2017, 16.67% (5/30) in April 2017, 33.33% (10/30) in June 2017 and 26.67% (8/30) in December 2017. In 2018, SIVs could be detected in every month of 2018, except March 2018. In detail, highest percentage of IAV positivity was in April 2018 with 40.63% (13/32), followed by May 2018 (32.26%) and August 2018 (19.35%) (Table 2 and Figure 2).

Subsequently, 169 IAV positive and suspected samples were subjected to virus isolation by egg inoculation. Our result showed that 57 influenza viruses (13.07%) could be isolated (Table 2). Unfortunately. 112 out of 169 samples could not be isolated for influenza viruses.

Table 2: Influenza A virus detection and isolation from swine samples in this thesis

Month	Species	Number of nasal swab samples	IAV positive (% positive)	IAV suspected (% suspected)	Total IAV isolated viruses
Jan-17	Swine	30	8/30 (26.67%)	0/30 (0%)	7/30 (23.33%)
Apr-17	Swine	30	5/30 (16.67%)	0/30 (0%)	4/30 (13.33%)
Jun-17	Swine	30	10/30 (33.33%)	7/30 (23.33%)	11/30 (36.67%)
Dec-17	Swine	30	8/30 (26.67%)	10/30 (33.33%)	3/30 (10%)
Feb-18	Swine	31	1/31 (3.23%)	3/31 (9.68%)	0/31 (0%)
Mar-18	Swine	34	0/34 (0%)	3/34 (8.82%)	0/34 (0%)
Apr-18	Swine	32	13/32 (40.63%)	5/32 (15.63%)	14/32 (43.75%)
May-18	Swine	31	10/31 (32.26%)	11/31 (35.48%)	2/31 (6.45%)
Jun-18	Swine	30	3/30 (10%)	8/30 (26.67%)	2/30 (6.67%)
Jul-18	Swine	30	4/30 (13.33%)	9/30 (30%)	1/30 (3.33%)
Aug-18	Swine	31	6/31 (19.35%)	7/31 (22.58%)	1/31 (3.23%)
Sep-18	Swine	33	3/33 (9.09%)	9/33 (27.27%)	4/33 (12.12%)
Oct-18	Swine	32	5/32 (15.63%)	10/32 (31.25%)	5/32 (15.63%)
Nov-18	Swine	32	6/32 (18.75%)	5/32 (15.63%)	3/32 (9.38%)
Total		436	82/436 (18.81%)	87/436 (19.95%)	57/436 (13.07%)

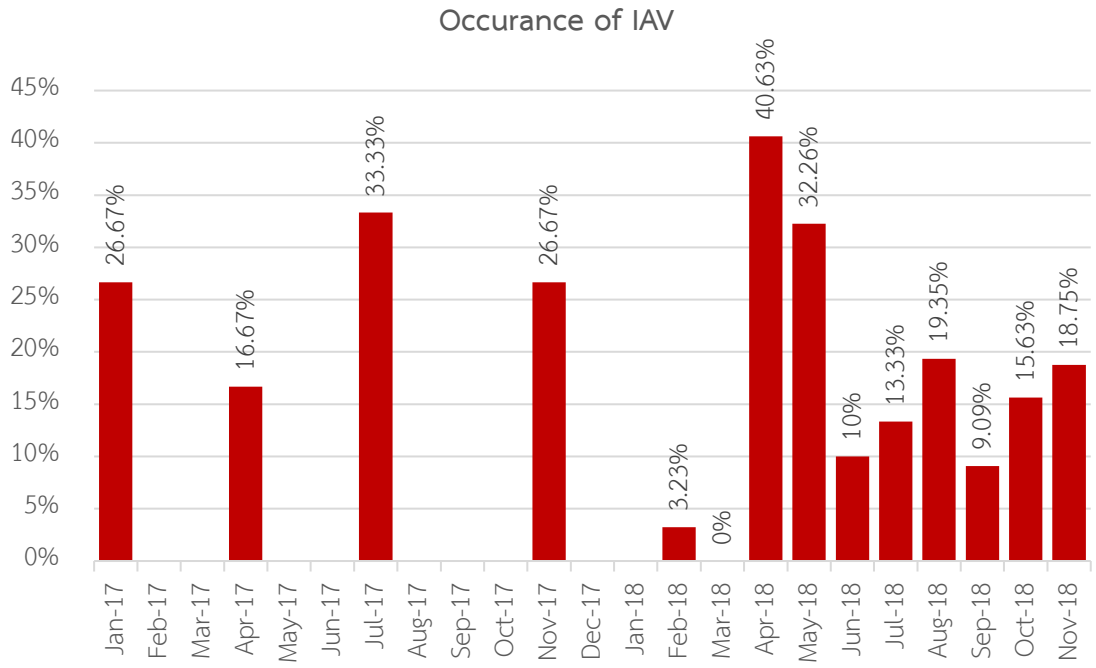


Figure 2: Occurrence of influenza A virus in a pig farm, by month.

4.2.2 Serological test of swine influenza virus

The serum samples were tested for antibody against influenza subtype H3N2, H1N1 and pdmH1N1. Our results showed that 32.11% (140/436), 15.83% (69/436) and 23.39% (102/436) of serum samples had antibody against SIV-H3N2, SIV-H1N1 and pdmH1N1 viruses, respectively. In detail, the HI titer against SIV-H3N2 (endemic Thai SIV) in 2017 was highest in April 2017 (73.33%), followed by July 2017 (63.33%), November 2017 (43.33%) and lowest in January 2017 (23.33%). In 2018, HI titer against SIV-H3N2 was highest in September 2018 (51.52%) followed by July 2018 (43.33%). For SIV-H1N1, antibody titer against SIV-H1N1 in 2017 was highest in November 2017 (16.67%), followed by April 2017 (10.00%) and lowest in January and July (6.67%). In 2018, HI titer against SIV-H1N1 was highest in August 2018 with 29.03%, followed by April 2018 with 21.88%. For pdmH1N1, in 2017, HI titer against pH1N1 was highest in November 2017 (50.00%), followed by April 2017 with 13.33% and July 2017 with 3.33% while no HI titer against pH1N1 in January 2017. In 2018, HI

titer against pdmH1N1 was highest in July 2018 (63.33%), followed by August 2018 (48.39%), May 2018 (38.71%) and March 2018 (38.24%) (Figure 3).

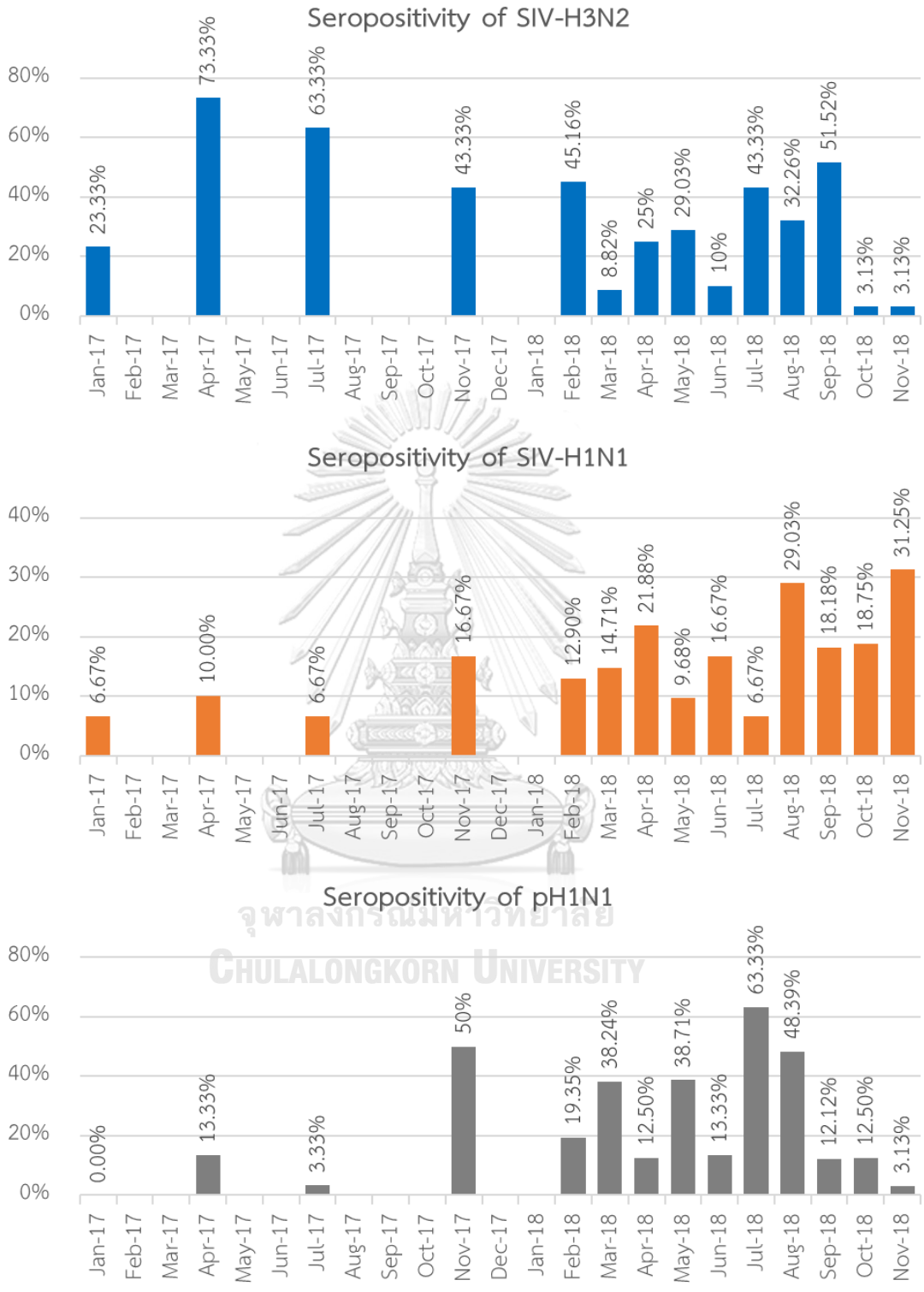


Figure 3: Percentage of HI positive samples (HI titer) against SIV-H3N2, SIV-H1N1 and pandemicH1N1-2009, by month.

4.3 Genetic characterization of swine influenza virus

24 out of 57 swine influenza viruses were selected for whole genome sequencing. The SIVs were selected based on time of sample collection (month) and viral titer. Whole genome sequencing was conducted by Next Generation Sequencing (NGS). The nucleotide sequences from NGS of each virus were validated and analyzed by CLC 11.0.1 software. In this study, the total reads of nucleotides sequences available from NGS were ranging from 6,975,414 to 13,798,830 reads. Number of sequences read mapped to references for each gene of the SIVs are provided in Table 3.

To identify subtype of swine influenza viruses, nucleotide sequences of HA and NA genes were compared with nucleotide sequences in the NCBI database by using BLAST program. Our result showed that 24 SIVs could be identified as SIV subtype H1N1 (n=18) and H3N2 (n=6) (Table 4). For genetic characterization, nucleotide sequences of each gene of 24 SIVs were subjected to phylogenetic analysis with those of reference influenza viruses. From phylogenetic trees, the lineages or sub-lineages of each gene of the viruses could be identified.

Table 3: Numbers of sequence reads mapped to references

Virus	Total read	No. reads mapped to references									
		PB2	PB1	PA	HA1	HA3	NP	NA1	NA2	M	NS
S28 NRU3732	11,800,150	383,255 (3.25%)	163,608 (1.39%)	227,688 (1.93%)	897,346 (7.6%)	-	1,502,299 (12.73%)	1,239,907 (10.51%)	-	2,912,389 (24.68%)	4,422,744 (37.48%)
S29 NRU 3743	9,216,236	238,420 (2.59%)	57,087 (0.62%)	184,843 (2.01%)	670,250 (7.27%)	-	1,253,720 (13.6%)	734,860 (7.97%)	-	2,418,452 (26.24%)	3,581,735 (38.86%)
S1 NRU3759	6,975414	11,391 (0.16%)	18,668 (0.27%)	8,473 (0.12%)	148,328 (2.13%)	-	703,063 (10.08%)	470,601 (6.75%)	-	2,294,024 (32.89%)	3,279,992 (47.02%)
S2 NRU3764	8,406,294	1,795 (0.02%)	15,791 (0.19%)	2,325 (0.03%)	198,720 (2.34%)	-	592,001 (6.97%)	656,071 (7.72%)	-	2,258,046 (26.58%)	4,603,594 (54.18%)
S3 NRU3770	8,370,724	7,365 (0.09%)	77,765 (0.93%)	5,769 (0.07%)	59,801 (0.71%)	-	454,551 (5.43%)	323,394 (3.86%)	-	2,654,210 (31.71%)	4,727,755 (56.48%)
S5 NRU3802	7,297,926	6,419 (0.09%)	75,783 (1.04%)	15,799 (0.22%)	624,323 (8.55%)	-	1,022,790 (14.01%)	761,272 (10.43%)	-	2,062,973 (28.27%)	2,657,581 (36.42%)
S8 NRU3793	10,603,702	38 (0%)	10,059 (0.09%)	21 (0%)	15,254 (0.14%)	-	81,356 (0.77%)	82,266 (0.78%)	-	467,696 (4.41%)	1,566,000 (14.77%)
S9 NRU3796	9,538,026	1,720 (0.02%)	53,351 (0.56%)	4,557 (0.05%)	558,114 (5.85%)	-	1,453,528 (15.24%)	741,784 (7.78%)	-	2,775,637 (29.1%)	3,897,298 (40.86%)
S10 NRU3803	10,060,504	17,733 (0.18%)	96,513 (0.96%)	25,065 (0.25%)	642,776 (6.39%)	-	1,352,127 (13.44%)	915,892 (9.1%)	-	2,781,248 (27.65%)	4,169,507 (41.44%)

Virus	Total read	No. reads mapped to references									
		PB2	PB1	PA	HA1	HA3	NP	NA1	NA2	M	NS
S14 SF21299	11,008,970	56,883 (0.52%)	64,223 (0.58%)	4,188 (0.04%)	12,991 (0.12%)	-	31,602 (0.29%)	124,168 (1.13%)	-	3,652,591 (33.18%)	6,883,400 (62.53%)
S15 SF21304	10,825,492	102,712 (0.95%)	311,677 (2.88%)	10,295 (0.1%)	112,897 (1.04%)	-	286,545 (2.65%)	301,726 (2.79%)	-	3,934,350 (36.34%)	5,661,139 (52.29%)
S19 SF21626	12,995,458	84,378 (0.65%)	114,117 (0.88%)	105,711 (0.81%)	685,430 (5.27%)	-	950,275 (7.31%)	744,667 (5.73%)	-	3,685,825 (28.36%)	6,584,924 (50.67%)
S23 SF21630	7,055,830	390,080 (5.53%)	314,266 (4.45%)	395,934 (5.61%)	954,409 (13.53%)	-	1,033,207 (14.64%)	699,933 (9.92%)	-	1,386,820 (19.65%)	1,845,150 (26.15%)
S20 SF21970	12,653,686	321,690 (2.54%)	165,460 (1.31%)	421,818 (3.33%)	697,746 (5.51%)	-	1,421,371 (11.23%)	1,036,922 (8.19%)	-	3,469,922 (27.42%)	5,083,232 (40.17%)
S21 SF22117	13,798,830	188,447 (1.37%)	194,360 (1.41%)	194,433 (1.41%)	1,025,494 (7.43%)	-	1,506,589 (10.92%)	1,106,226 (8.02%)	-	4,096,669 (29.69%)	5,428,672 (39.34%)
S24 SF22300	8,180,372	358,823 (4.39%)	226,691 (2.77%)	449,774 (5.5%)	827,278 (10.11%)	-	1,248,595 (15.26%)	752,798 (9.2%)	-	1,904,327 (23.28%)	2,345,787 (28.68%)
S26 SF22351	8,341,522	182,764 (2.19%)	89,384 (1.07%)	124,019 (1.49%)	843,764 (10.12%)	-	767,865 (9.21%)	737,142 (8.84%)	-	2,362,619 (28.32%)	3,188,270 (38.22%)
S27 SF22630	12,240,852	65,165 (0.53%)	86,039 (0.7%)	69,856 (0.57%)	588,400 (4.81%)	-	993,579 (8.12%)	657,231 (5.37%)	-	4,009,079 (32.75%)	5,742,078 (46.91%)

Virus	Total read	No. reads mapped to references									
		PB2	PB1	PA	HA1	HA3	NP	NA1	NA2	M	NS
S4 NRU3794	7,325,262	53,035 (0.72%)	44,157 (0.6%)	8,397 (0.11%)	-	235,074 (3.21%)	398,045 (5.43%)	-	254,287 (3.47%)	2,652,738 (36.21%)	3,629,549 (49.55%)
S6 NRU3816	8,219,616	382,693 (4.66%)	108,786 (1.32%)	54,931 (0.67%)	-	128,091 (1.56%)	613,552 (7.46%)	-	262,290 (3.19%)	2,502,358 (30.44%)	4,100,720 (49.89%)
S7 NRU3790	10,701,972	113,156 (1.06%)	147,985 (1.38%)	31,662 (0.3%)	-	383,464 (3.58%)	1,231,817 (11.51%)	-	683,608 (6.39%)	3,451,149 (32.25%)	4,591,547 (42.9%)
S12 SF20218	10,561,484	21,919 (0.21%)	56,960 (0.54%)	3,178 (0.03%)	-	13,695 (0.13%)	130,852 (1.24%)	-	176,640 (1.67%)	4,390,602 (41.57%)	5,699,981 (53.97%)
S13 SF20226	9,415,910	24,822 (0.26%)	110,966 (1.18%)	22,775 (0.24%)	-	317,810 (3.38%)	827,424 (8.79%)	-	485,069 (5.15%)	3,423,532 (36.36%)	4,142,051 (43.99%)
S25 SF22337	7,450,092	156,145 (2.1%)	93,419 (1.25%)	179,350 (2.41%)	-	694,139 (9.32%)	1,164,232 (15.63%)	-	634,513 (8.52%)	1,977,125 (26.54%)	2,490,977 (33.44%)

Table 4: Description of 24 SIVs characterized by whole genome sequencing in this thesis.

Virus	Subtype	Year	Host	Age	GenBank No.
S28 NRU3732	rH1N1 (pdm+2)	Jan-17	Swine	4 weeks	MT378014-21
S29 NRU 3743	rH1N1(pdm+2)	Jan-17	Swine	6 weeks	MT378022-29
S1 NRU3759	rH1N1 (pdm+2)	Apr-17	Swine	4 weeks	MT378030-37
S2 NRU3764	rH1N1 (pdm+2)	Apr-17	Swine	6 weeks	MT378038-45
S3 NRU3770	rH1N1 (pdm+2)	Apr-17	Swine	6 weeks	MT378046-53
S5 NRU3802	rH1N1 (pdm+1)	Jun-17	Swine	6 weeks	MT378086-93
S8 NRU3793	rH1N1 (pdm+2)	Jun-17	Swine	4 weeks	MT378062-69
S9 NRU3798	rH1N1 (pdm+1)	Jun-17	Swine	4 weeks	MT378078-85
S10 NRU3803	rH1N1 (pdm+1)	Jun-17	Swine	6 weeks	MT378094-101
S14 SF21299	rH1N1 (pdm+1)	Apr-18	Swine	6 weeks	MT377918-25
S15 SF21304	rH1N1 (pdm+1)	Apr-18	Swine	10 weeks	MT377942-49
S19 SF21626	rH1N1 (pdm+1)	Jun-18	Swine	6 weeks	MT377950-57
S23 SF21630	rH1N1 (pdm+1)	Jun18	Swine	8 weeks	MT377958-65
S20 SF21970	rH1N1 (pdm+1)	Jul-18	Swine	6 weeks	MT377966-73
S21 SF22117	rH1N1 (pdm+1)	Aug-18	Swine	4 weeks	MT377974-81
S24 SF22300	rH1N1 (pdm+1)	Sep-18	Swine	10 weeks	MT377982-89
S26 SF22351	rH1N1 (pdm+1)	Oct-18	Swine	9 weeks	MT377998-8005
S27 SF22630	rH1N1 (pdm+1)	Nov-18	Swine	4 weeks	MT378006-13
S4 NRU3794	rH3N2 (pdm+2)	Jun-17	Swine	4 weeks	MT378070-77
S6 NRU3816	rH3N2 (pdm+2)	Jun-17	Swine	10 weeks	MT378102-09
S7 NRU3790	rH3N2 (pdm+2)	Jun-17	Swine	4 weeks	MT378054-61
S12 SF20218	rH3N2 (pdm+2)	Nov-17	Swine	8 weeks	MT377926-33
S13 SF20226	rH3N2 (pdm+2)	Nov-17	Swine	12 weeks	MT377934-41
S25 SF22337	rH3N2 (pdm+2	Oct-18	Swine	6 weeks	MT377990-97

rH1N1 (pdm+2): H1N1 SIVs genotypes contained HA gene from classical swine lineage, NA gene from Eurasian avian-like lineage and internal genes from pandemic 2009-like

rH1N1 (pdm+1): H1N1 SIVs genotypes contained HA gene from classical swine lineage and other genes from pandemic 2009-like

rH3N2 (pdm+2): H3N2 SIVs genotypes contained HA and NA genes from human-like swine lineage and internal genes from pandemic 2009-like

Phylogenetic analysis of HA1

In this study, 18 SIV-H1N1 were included in the phylogenetic analysis. To genetically characterize the HA1 gene of the SIV-H1N1, phylogenetic tree was constructed by comparing 18 SIV-H1N1 in this thesis to 125 references viruses. The reference of HA1 gene included 3 major lineages including Classical swine lineage (CS; 1A), Human seasonal linage (Hu; 1B), Eurasian avian-like swine lineage (EA; 1C), and 5 sub-clusters from Classical swine lineage (1A): alpha (1A.1), beta (1A.2), gamma1 (1A.3.3.3), gamma2 (1A.3.2) and pH1N1 (1A.3.3.2). Phylogenetic tree of HA1 gene showed that all SIV-H1N1s in this thesis were clustered with alpha sub-lineage (1A.1.2) of the classical swine lineage (Figure 4). It should be noted that the alpha sub-lineage (1A.1.2) is common linage of HA1 gene of endemic swine influenza viruses in Thailand.

Phylogenetic analysis of NA1

In this study, 18 SIV-H1N1 were included in the phylogenetic analysis. To genetically characterize the NA1 gene of the SIV-H1N1, phylogenetic tree was constructed by comparing 18 SIV-H1N1 in this thesis to 70 references viruses. The reference of NA1 gene included 4 major lineages including Classical swine lineage (CS), Eurasian avian-like lineage (EA), Human seasonal linage (Hu) and pandemicH1N1-2009 (pdm09). Phylogenetic analysis result of NA1 gene showed that NA1 genes of 6 SIV-H1N1s were clustered with Eurasian avian-like swine lineage and 10 SIV-H1N1s were clustered with pandemic2009-H1N1 (Figure 5). It should be noted that the Eurasian avian-like swine lineage is a common lineage of NA1 gene of endemic swine influenza viruses in Thailand.

Phylogenetic analysis of HA3

In this study, 6 SIV-H3N2 were included in the phylogenetic analysis. To genetically characterize the HA3 gene of the SIV-H3N2, phylogenetic tree was constructed by comparing 6 SIV-H3N2 in this thesis to 142 references viruses. The reference of HA3 gene included 2 major lineages; North American swine lineage and

Eurasian swine lineage. The north American can be classified into 4 clades (clade I, clade II, clade III and clade IV). Phylogenetic analysis result of HA3 gene showed that HA3 genes of all SIV-H3N2s in this thesis were clustered with Clade II of North America swine lineage, sub-cluster human seasonal-like. it is noted that sub-cluster human seasonal-like lineage is a common cluster of endemic SIV-H3N2 in Thailand (Chutinimitkul et al., 2008) (Figure 6).

Phylogenetic analysis of NA2

In this study, 6 SIV-H3N2 were included in the phylogenetic analysis. To genetically characterize the NA2 gene, phylogenetic tree was constructed by comparing 6 SIV-H3N2 in this thesis to 82 references viruses. The reference of NA2 gene included 2 major lineages; North American swine lineage and Eurasian swine lineage. North American swine lineage diversify to 2 sub-clusters; N2-1998 and N2-2002 lineage which are originated from human seasonal influenza. Phylogenetic analysis result of NA2 gene showed that NA2 genes of all SIV-H3N2s were clustered into sub-cluster human seasonal-like lineage which common cluster of endemic Thai SIVs (Figure 7).

Phylogenetic analysis of PB2

In this study, to genetically characterize the PB2 gene of the SIV-H1N1 and SIV-H3N2, phylogenetic tree was constructed by comparing 18 SIV-H1N1 and 6 SIV-H3N2 to 66 references viruses. The reference of PB2 gene included Classical swine lineage (CS), Eurasian avian-like lineage (EA), North America triple reassortant (TR), human seasonal (HU) and pandemic H1N1-2009 (pdm09). Phylogenetic analysis of PB2 gene showed that PB2 genes of all SIV-H1N1s and SIV-H3N2s were clustered with pandemic H1N1-2009 indicating SIVs in this thesis acquired internal gene PB2 (backbone) from pH1N1-2009 (Figure 8).

Phylogenetic analysis of PB1

In this study, to genetically characterize the PB1 gene of the SIV-H1N1 and SIV-H3N2, phylogenetic tree was constructed by comparing 18 SIV-H1N1 and 6 SIV-H3N2 to 84 references viruses. The reference of PB1 gene included Classical swine lineage (CS), Eurasian avian-like lineage (EA), North America triple reassortant (TR), human seasonal (HU) and pandemic H1N1-2009 (pdm09). Phylogenetic analysis of PB1 gene showed that PB1 genes of all SIV-H1N1s and SIV-H3N2s were clustered with pandemic H1N1-2009 indicating SIVs in this thesis acquired internal gene PB1 (backbone) from pH1N1-2009 (Figure 9).

Phylogenetic analysis of PA

In this study, to genetically characterize the PA gene of the SIV-H1N1 and SIV-H3N2, phylogenetic tree was constructed by comparing 18 SIV-H1N1 and 6 SIV-H3N2 to 80 references viruses. The reference of PA gene included Classical swine lineage (CS), Eurasian avian-like lineage (EA), North America triple reassortant (TR), human seasonal (HU) and pandemic H1N1-2009 (pdm09). Phylogenetic analysis of PA gene showed that PA genes of all SIV-H1N1s and SIV-H3N2s were clustered with pandemic H1N1-2009 indicating SIVs in this thesis acquired internal gene PA (backbone) from pH1N1-2009 (Figure 10).

Phylogenetic analysis of NP

In this study, to genetically characterize the NP gene of the SIV-H1N1 and SIV-H3N2, phylogenetic tree was constructed by comparing 18 SIV-H1N1 and 6 SIV-H3N2 to 63 references viruses. The reference of NP gene included Classical swine lineage (CS), Eurasian avian-like lineage (EA), North America triple reassortant (TR), human seasonal (HU) and pandemic H1N1-2009 (pdm09). Phylogenetic analysis of NP gene showed that NP genes of all SIV-H1N1s and SIV-H3N2s were clustered with pandemic H1N1-2009 indicating SIVs in this thesis acquired internal gene NP (backbone) from pH1N1-2009 (Figure 11).

Phylogenetic analysis of M

In this study, to genetically characterize the M gene of the SIV-H1N1 and SIV-H3N2, phylogenetic tree was constructed by comparing 18 SIV-H1N1 and 6 SIV-H3N2 to 78 references viruses. The reference of M gene included Classical swine lineage (CS), Eurasian avian-like lineage (EA), North America triple reassortant (TR), human seasonal (HU) and pandemic H1N1-2009 (pdm09). Phylogenetic analysis of M gene showed that M genes of all SIV-H1N1s and SIV-H3N2s were clustered with pandemic H1N1-2009 indicating SIVs in this thesis acquired internal gene M (backbone) from pH1N1-2009 (Figure 12).

Phylogenetic analysis of NS

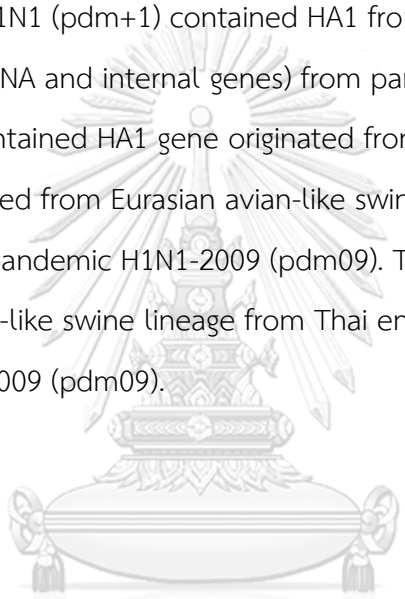
In this study, to genetically characterize the NS gene of the SIV-H1N1 and SIV-H3N2, phylogenetic tree was constructed by comparing 18 SIV-H1N1 and 6 SIV-H3N2 to 67 references viruses. The reference of NS gene included Classical swine lineage (CS), Eurasian avian-like lineage (EA), North America triple reassortant (TR), human seasonal (HU) and pandemic H1N1-2009 (pdm09). Phylogenetic analysis of NS gene showed that NS genes of all SIV-H1N1s and SIV-H3N2s were clustered with pandemic H1N1-2009 indicating SIVs in this thesis acquired internal gene NS (backbone) from pH1N1-2009 (Figure 13).

4.4 Genetic constellation analysis of swine influenza viruses

In this study, genetic constellations of swine influenza viruses were analyzed based on whole genome sequences of the viruses. To identify genetic constellation of the virus, the lineages or clusters of each gene of the virus were assigned based on the comparison to the reference viruses. After lineages or clusters of each gene were assigned, the combinations of eight lineages or clusters were analyzed and assigned as genetic constellation of swine influenza virus. In this thesis, of 24 SIVs, we observed 3 genotypes (genetic constellation) of SIVs (Table 5 and 6, Figure 14 and 15) including

- Reassort SIV-H1N1; the reassortment between pdmH1N1-2009 backbone (7 genes) and classical swine lineage (HA gene) [rH1N1 (pdm+1)]
- Reassort SIV-H1N1; the reassortment between pdmH1N1-2009 backbone (6 genes), classical swine lineage (HA gene) and Eurasian avian-like swine lineage (NA genes) [rH1N1 (pdm+2)]
- Reassort SIV-H3N2; the reassortment between pdmH1N1-2009 backbone (6 genes) and 2 human-like swine lineages (HA and NA genes) [rH3N2 (pdm+2)]

In detail, the rH1N1 (pdm+1) contained HA1 from classical swine lineage (CS) and the other 7 genes (NA and internal genes) from pandemic H1N1-2009 (pdm09). The rH1N1 (pdm+2) contained HA1 gene originated from classical swine lineage (CS), while NA1 gene originated from Eurasian avian-like swine lineage (EA). The 6 internal genes originated from pandemic H1N1-2009 (pdm09). The rH3N2 contained HA3 and NA2 genes from human-like swine lineage from Thai endemic SIVs and internal genes from pandemic H1N1-2009 (pdm09).



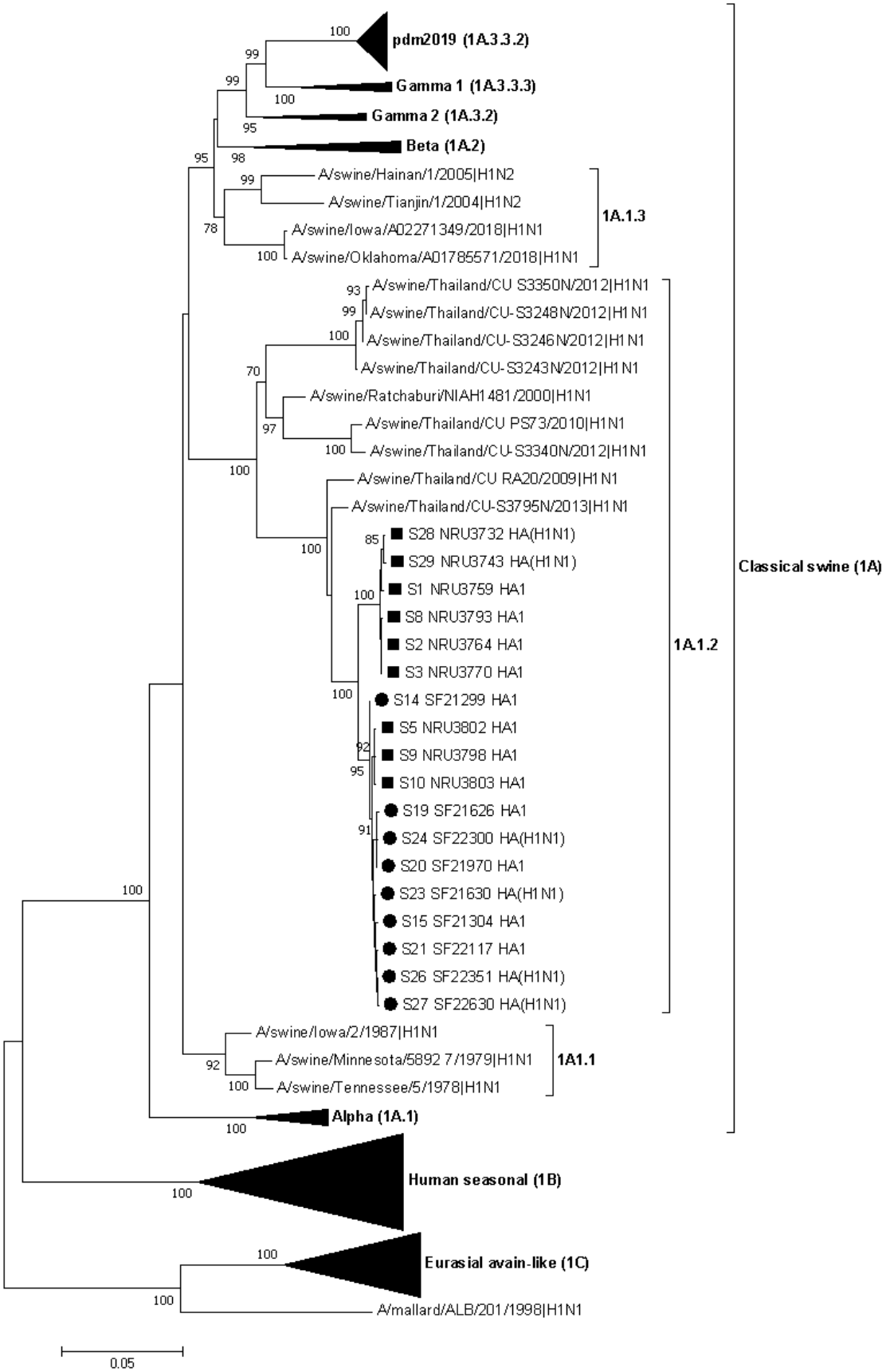
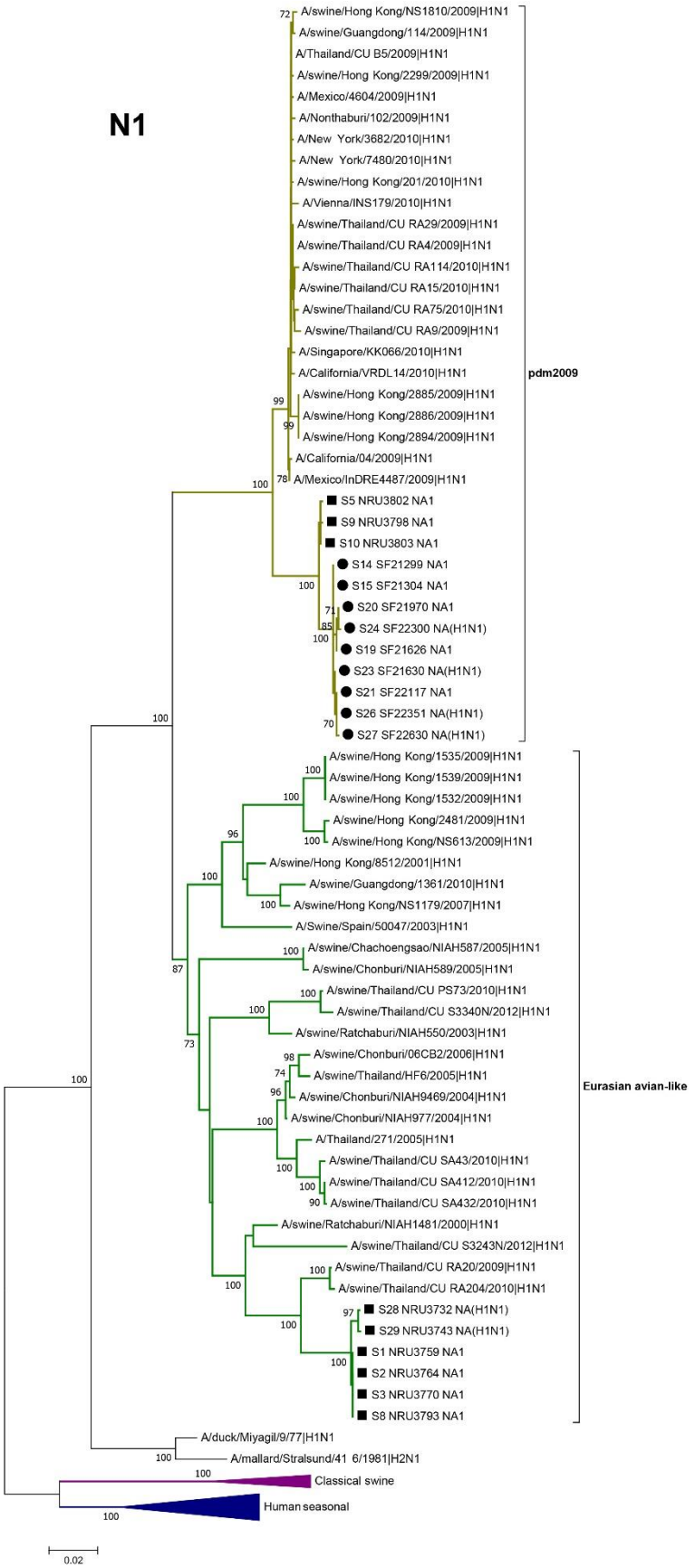

pdm2019 (1A.3.3.2)
Gamma 1 (1A.3.3.3)
Gamma 2 (1A.3.2)
Beta (1A.2)
A/swine/Hainan/1/2005|H1N2
A/swine/Tianjin/1/2004|H1N2
A/swine/Iowa/A02271349/2018|H1N1
A/swine/Oklahoma/A01785571/2018|H1N1
1A.1.3
A/swine/Thailand/CU S3350N/2012|H1N1
A/swine/Thailand/CU-S3248N/2012|H1N1
A/swine/Thailand/CU-S3246N/2012|H1N1
A/swine/Thailand/CU-S3243N/2012|H1N1
A/swine/Ratchaburi/NIAH1481/2000|H1N1
A/swine/Thailand/CU PS73/2010|H1N1
A/swine/Thailand/CU-S3340N/2012|H1N1
A/swine/Thailand/CU RA20/2009|H1N1
A/swine/Thailand/CU-S3795N/2013|H1N1
S28 NRU3732 HA(H1N1)
S29 NRU3743 HA(H1N1)
S1 NRU3759 HA1
S8 NRU3793 HA1
S2 NRU3764 HA1
S3 NRU3770 HA1
S14 SF21299 HA1
S5 NRU3802 HA1
S9 NRU3798 HA1
S10 NRU3803 HA1
S19 SF21626 HA1
S24 SF22300 HA(H1N1)
S20 SF21970 HA1
S23 SF21630 HA(H1N1)
S15 SF21304 HA1
S21 SF22117 HA1
S26 SF22351 HA(H1N1)
S27 SF22630 HA(H1N1)
1A.1.2
A/swine/Iowa/2/1987|H1N1
A/swine/Minnesota/5892 7/1979|H1N1
A/swine/Tennessee/5/1978|H1N1
1A1.1
Alpha (1A.1)
Classical swine (1A)
Human seasonal (1B)
Eurasial avain-like (1C)
A/mallard/ALB/201/1998|H1N1
0.05

Figure 4: Phylogenetic tree of HA1 gene of the SIV-H1N1. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1,000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIV-H1N1 isolated in 2017 and 2018, respectively.


N1
A/swine/Hong Kong/NS1810/2009|H1N1
A/swine/Guangdong/114/2009|H1N1
A/Thailand/CU B5/2009|H1N1
A/swine/Hong Kong/2299/2009|H1N1
A/Mexico/4604/2009|H1N1
A/Nonthaburi/102/2009|H1N1
A/New York/3682/2010|H1N1
A/New York/7480/2010|H1N1
A/swine/Hong Kong/201/2010|H1N1
A/Vienna/INS179/2010|H1N1
A/swine/Thailand/CU RA29/2009|H1N1
A/swine/Thailand/CU RA4/2009|H1N1
A/swine/Thailand/CU RA114/2010|H1N1
A/swine/Thailand/CU RA15/2010|H1N1
A/swine/Thailand/CU RA75/2010|H1N1
A/swine/Thailand/CU RA9/2009|H1N1
A/Singapore/KK066/2010|H1N1
A/California/VRDL14/2010|H1N1
A/swine/Hong Kong/2885/2009|H1N1
A/swine/Hong Kong/2886/2009|H1N1
A/swine/Hong Kong/2894/2009|H1N1
A/California/04/2009|H1N1
A/Mexico/InDRE4487/2009|H1N1
S5 NRU3802 NA1
S9 NRU3798 NA1
S10 NRU3803 NA1
S14 SF21299 NA1
S15 SF21304 NA1
S20 SF21970 NA1
S24 SF22300 NA(H1N1)
S19 SF21626 NA1
S23 SF21630 NA(H1N1)
S21 SF22117 NA1
S26 SF22351 NA(H1N1)
S27 SF22630 NA(H1N1)
pdm2009
A/swine/Hong Kong/1535/2009|H1N1
A/swine/Hong Kong/1539/2009|H1N1
A/swine/Hong Kong/1532/2009|H1N1
A/swine/Hong Kong/2481/2009|H1N1
A/swine/Hong Kong/NS613/2009|H1N1
A/swine/Hong Kong/8512/2001|H1N1
A/swine/Guangdong/1361/2010|H1N1
A/swine/Hong Kong/NS1179/2007|H1N1
A/Swine/Spain/50047/2003|H1N1
A/swine/Chachoengsao/NIAH587/2005|H1N1
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A/swine/Thailand/CU PS73/2010|H1N1
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A/swine/Ratchaburi/NIAH550/2003|H1N1
A/swine/Chonburi/06CB2/2006|H1N1
A/swine/Thailand/HF6/2005|H1N1
A/swine/Chonburi/NIAH9469/2004|H1N1
A/swine/Chonburi/NIAH977/2004|H1N1
A/Thailand/271/2005|H1N1
A/swine/Thailand/CU SA43/2010|H1N1
A/swine/Thailand/CU SA412/2010|H1N1
A/swine/Thailand/CU SA432/2010|H1N1
A/swine/Ratchaburi/NIAH1481/2000|H1N1
A/swine/Thailand/CU S3243N/2012|H1N1
A/swine/Thailand/CU RA20/2009|H1N1
A/swine/Thailand/CU RA204/2010|H1N1
S28 NRU3732 NA(H1N1)
S29 NRU3743 NA(H1N1)
S1 NRU3759 NA1
S2 NRU3764 NA1
S3 NRU3770 NA1
S8 NRU3793 NA1
Eurasian avian-like
A/duck/Miyagil/9/77|H1N1
A/mallard/Stralsund/41 6/1981|H2N1
Classical swine
Human seasonal
0.02

Figure 5: Phylogenetic tree of NA1 gene of the SIV-H1N1. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1,000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIV-H1N1 isolated in 2017 and 2018, respectively. Difference lineages were indicated by difference colors. Yellow, green, purple and blue line indicates pandemic2009-H1N1, Eurasian avian-like swine lineage, classical swine lineage and human seasonal lineage, respectively.

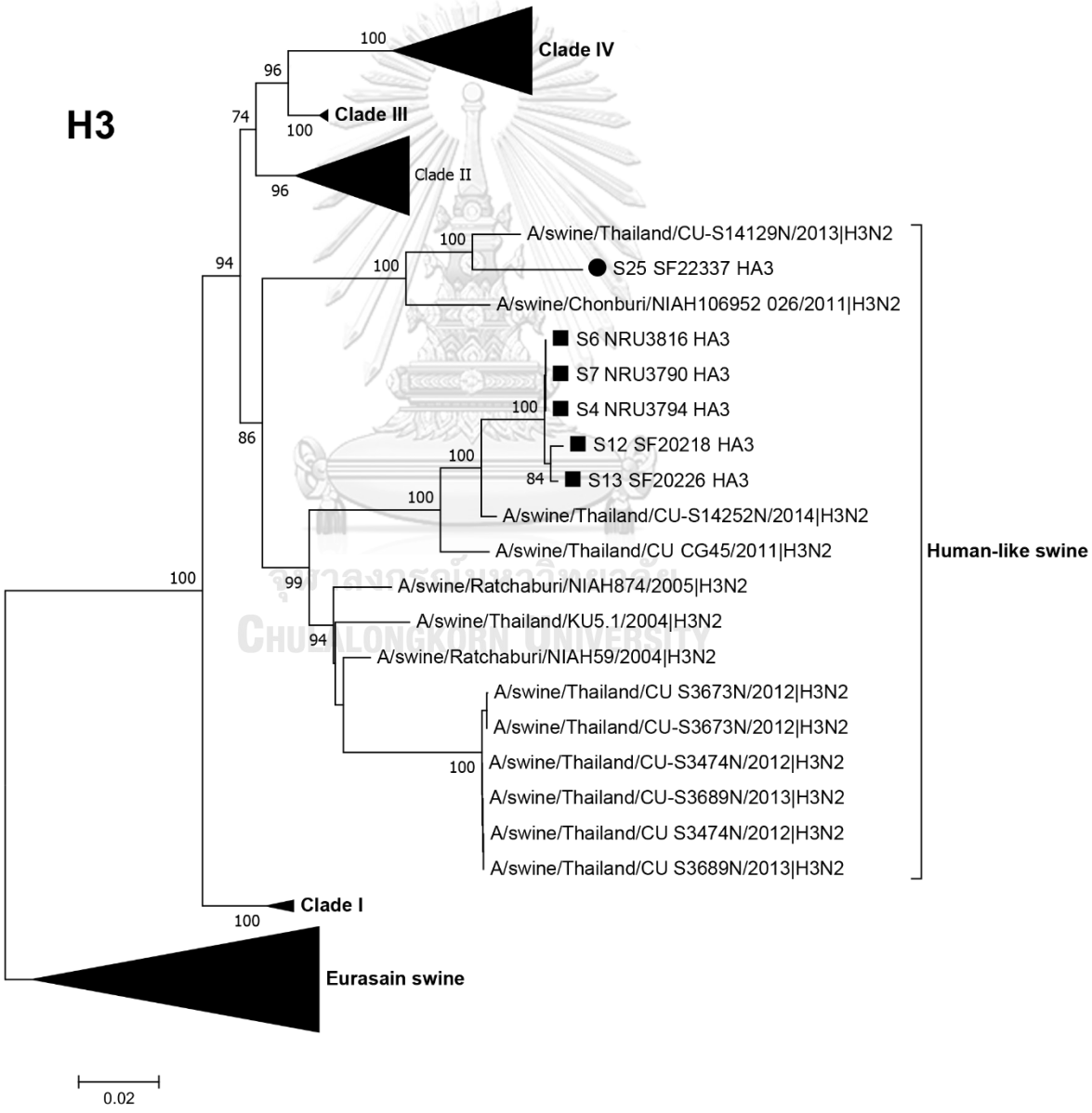

Figure 6*:* Phylogenetic tree of HA3 gene of the SIV-H3N2. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1,000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIV-H3N2 isolated in 2017 and 2018, respectively.

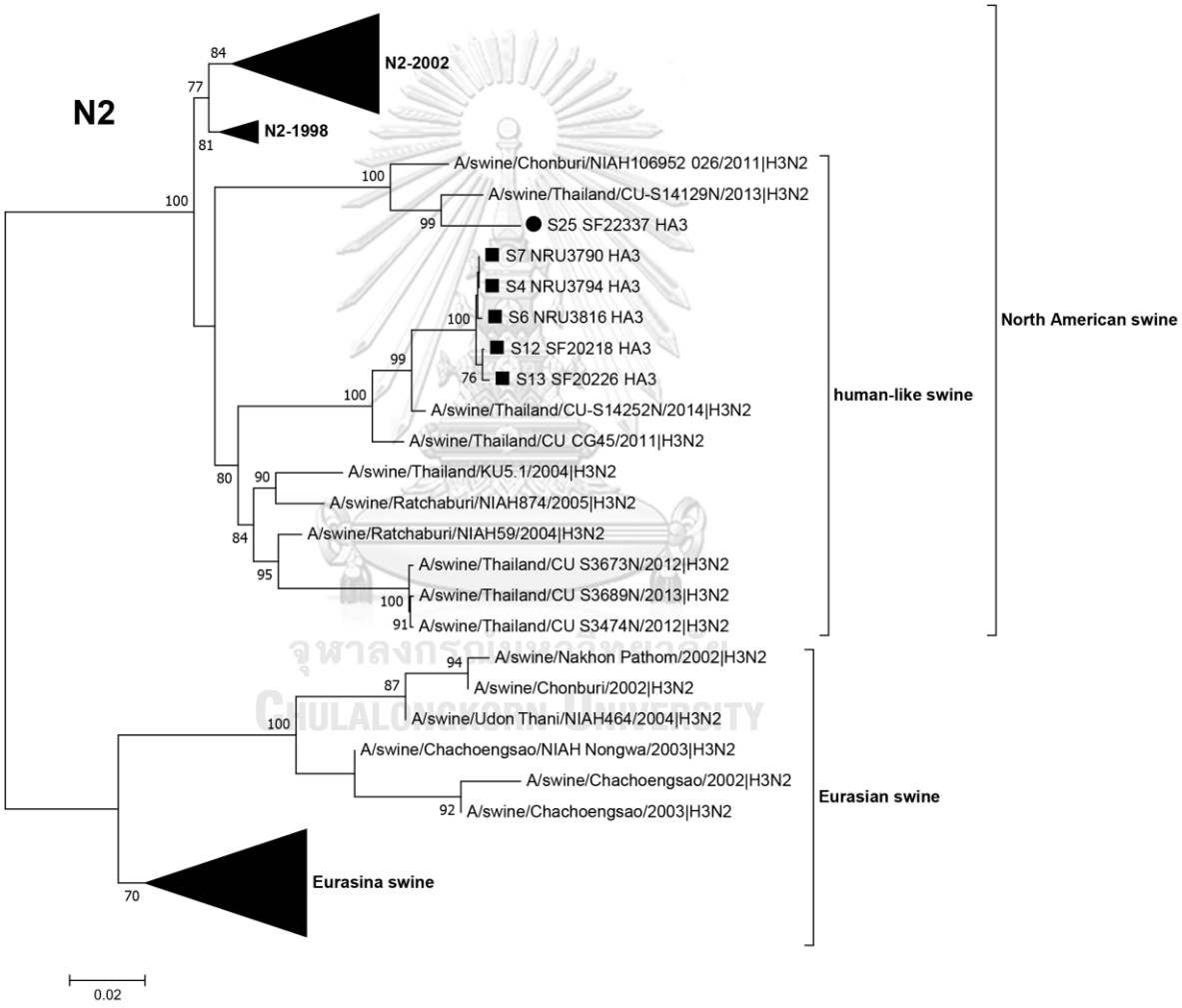

Figure 7: Phylogenetic tree of NA2 gene of the SIV-H3N2. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1,000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIV-H3N2 isolated in 2017 and 2018, respectively.

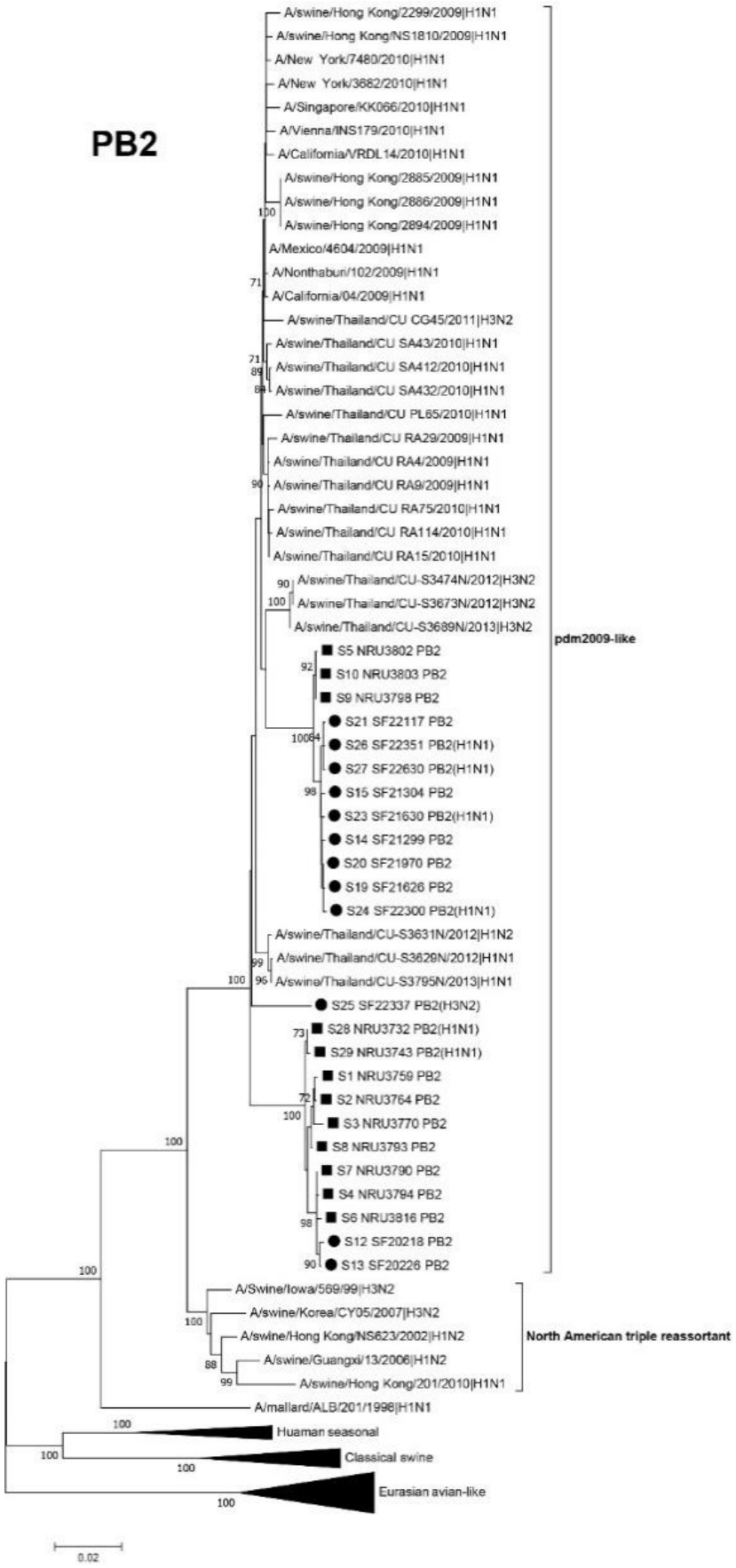
PB2
A/swine/Hong Kong/2299/2009|H1N1
A/swine/Hong Kong/NS1810/2009|H1N1
A/New York/7480/2010|H1N1
A/New York/3682/2010|H1N1
A/Singapore/KK066/2010|H1N1
A/Vienna/INS179/2010|H1N1
A/California/VRDL14/2010|H1N1
A/swine/Hong Kong/2885/2009|H1N1
A/swine/Hong Kong/2886/2009|H1N1
A/swine/Hong Kong/2894/2009|H1N1
A/Mexico/4604/2009|H1N1
A/Nonthaburi/102/2009|H1N1
A/California/04/2009|H1N1
A/swine/Thailand/CU CG45/2011|H3N2
A/swine/Thailand/CU SA43/2010|H1N1
A/swine/Thailand/CU SA412/2010|H1N1
A/swine/Thailand/CU SA432/2010|H1N1
A/swine/Thailand/CU PL65/2010|H1N1
A/swine/Thailand/CU RA29/2009|H1N1
A/swine/Thailand/CU RA4/2009|H1N1
A/swine/Thailand/CU RA9/2009|H1N1
A/swine/Thailand/CU RA75/2010|H1N1
A/swine/Thailand/CU RA114/2010|H1N1
A/swine/Thailand/CU RA15/2010|H1N1
A/swine/Thailand/CU-S3474N/2012|H3N2
A/swine/Thailand/CU-S3673N/2012|H3N2
A/swine/Thailand/CU-S3689N/2013|H3N2
pdm2009-like
S5 NRU3802 PB2
S10 NRU3803 PB2
S9 NRU3798 PB2
S21 SF22117 PB2
S26 SF22351 PB2(H1N1)
S27 SF22630 PB2(H1N1)
S15 SF21304 PB2
S23 SF21630 PB2(H1N1)
S14 SF21299 PB2
S20 SF21970 PB2
S19 SF21626 PB2
S24 SF22300 PB2(H1N1)
A/swine/Thailand/CU-S3631N/2012|H1N2
A/swine/Thailand/CU-S3629N/2012|H1N1
A/swine/Thailand/CU-S3795N/2013|H1N1
S25 SF22337 PB2(H3N2)
S28 NRU3732 PB2(H1N1)
S29 NRU3743 PB2(H1N1)
S1 NRU3759 PB2
S2 NRU3764 PB2
S3 NRU3770 PB2
S8 NRU3793 PB2
S7 NRU3790 PB2
S4 NRU3794 PB2
S6 NRU3816 PB2
S12 SF20218 PB2
S13 SF20226 PB2
A/Swine/Iowa/569/99|H3N2
A/swine/Korea/CY05/2007|H3N2
A/swine/Hong Kong/NS623/2002|H1N2
A/swine/Guangxi/13/2006|H1N2
A/swine/Hong Kong/201/2010|H1N1
North American triple reassortant
A/mallard/ALB/201/1998|H1N1
Huaman seasonal
Classical swine
Eurasian avian-like
0.02

Figure 8: Phylogenetic tree of PB2 gene of the SIV-H1N1 and SIV-H3N2. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1,000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIVs isolated in 2017 and 2018, respectively.

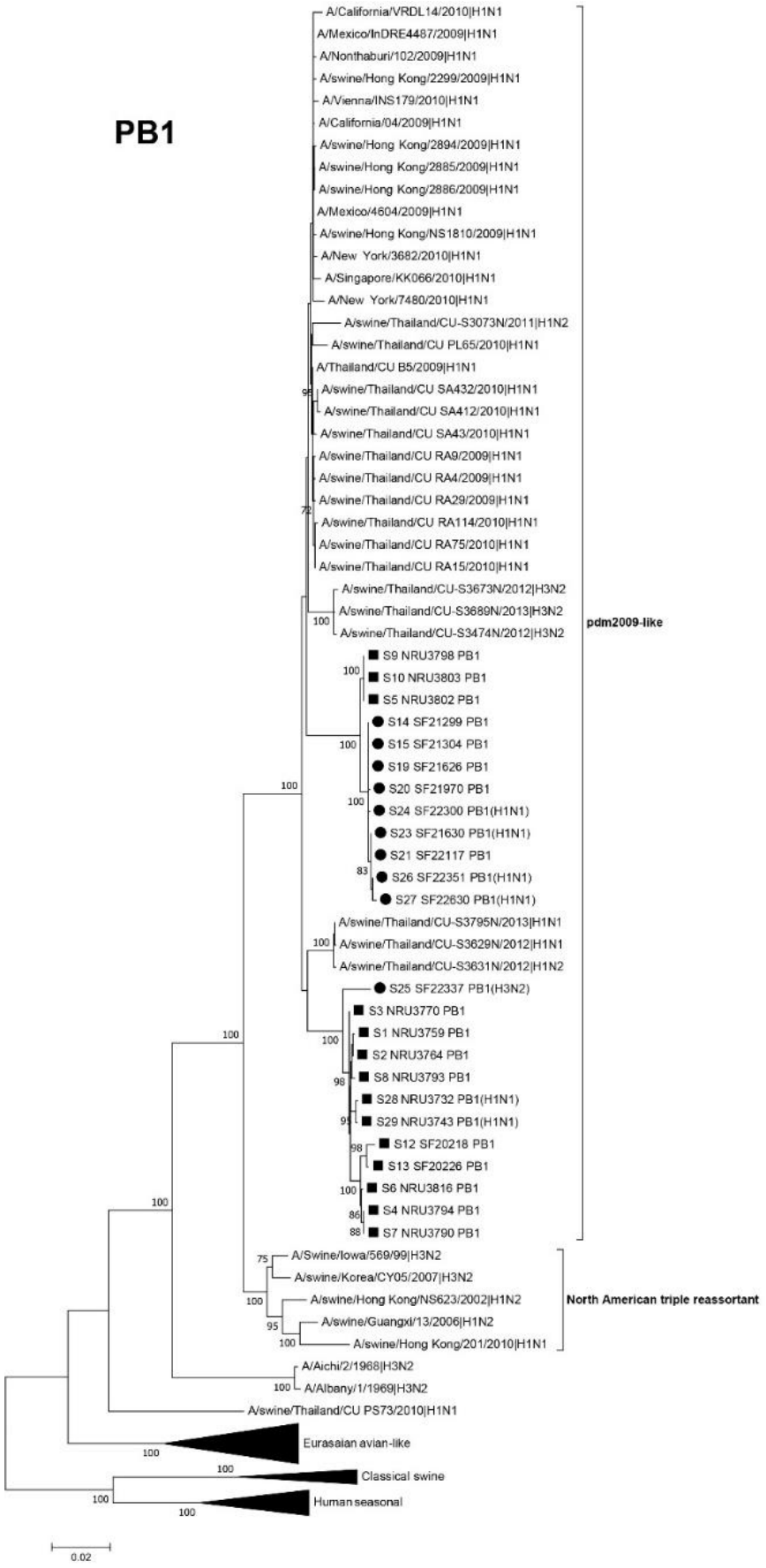
PB1
A/California/VRDL14/2010|H1N1
A/Mexico/InDRE4487/2009|H1N1
A/Nonthaburi/102/2009|H1N1
A/swine/Hong Kong/2299/2009|H1N1
A/Vienna/INS179/2010|H1N1
A/California/04/2009|H1N1
A/swine/Hong Kong/2894/2009|H1N1
A/swine/Hong Kong/2885/2009|H1N1
A/swine/Hong Kong/2886/2009|H1N1
A/Mexico/4604/2009|H1N1
A/swine/Hong Kong/NS1810/2009|H1N1
A/New York/3682/2010|H1N1
A/Singapore/KK066/2010|H1N1
A/New York/7480/2010|H1N1
A/swine/Thailand/CU-S3073N/2011|H1N2
A/swine/Thailand/CU PL65/2010|H1N1
A/Thailand/CU B5/2009|H1N1
A/swine/Thailand/CU SA432/2010|H1N1
A/swine/Thailand/CU SA412/2010|H1N1
A/swine/Thailand/CU SA43/2010|H1N1
A/swine/Thailand/CU RA9/2009|H1N1
A/swine/Thailand/CU RA4/2009|H1N1
A/swine/Thailand/CU RA29/2009|H1N1
A/swine/Thailand/CU RA114/2010|H1N1
A/swine/Thailand/CU RA75/2010|H1N1
A/swine/Thailand/CU RA15/2010|H1N1
A/swine/Thailand/CU-S3673N/2012|H3N2
A/swine/Thailand/CU-S3689N/2013|H3N2
A/swine/Thailand/CU-S3474N/2012|H3N2
pdm2009-like
S9 NRU3798 PB1
S10 NRU3803 PB1
S5 NRU3802 PB1
S14 SF21299 PB1
S15 SF21304 PB1
S19 SF21626 PB1
S20 SF21970 PB1
S24 SF22300 PB1(H1N1)
S23 SF21630 PB1(H1N1)
S21 SF22117 PB1
S26 SF22351 PB1(H1N1)
S27 SF22830 PB1(H1N1)
A/swine/Thailand/CU-S3795N/2013|H1N1
A/swine/Thailand/CU-S3629N/2012|H1N1
A/swine/Thailand/CU-S3631N/2012|H1N2
S25 SF22337 PB1(H3N2)
S3 NRU3770 PB1
S1 NRU3759 PB1
S2 NRU3764 PB1
S8 NRU3793 PB1
S28 NRU3732 PB1(H1N1)
S29 NRU3743 PB1(H1N1)
S12 SF20218 PB1
S13 SF20226 PB1
S6 NRU3816 PB1
S4 NRU3794 PB1
S7 NRU3790 PB1
A/Swine/Iowa/569/99|H3N2
A/swine/Korea/CY05/2007|H3N2
A/swine/Hong Kong/NS623/2002|H1N2
A/swine/Guangxi/13/2006|H1N2
A/swine/Hong Kong/201/2010|H1N1
North American triple reassortant
A/Aichi/2/1968|H3N2
A/Albany/1/1969|H3N2
A/swine/Thailand/CU PS73/2010|H1N1
Eurasaian avian-like
Classical swine
Human seasonal
0.02

Figure 9: Phylogenetic tree of PB1 gene of the SIV-H1N1 and SIV-H3N2. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIVs isolated in 2017 and 2018, respectively.

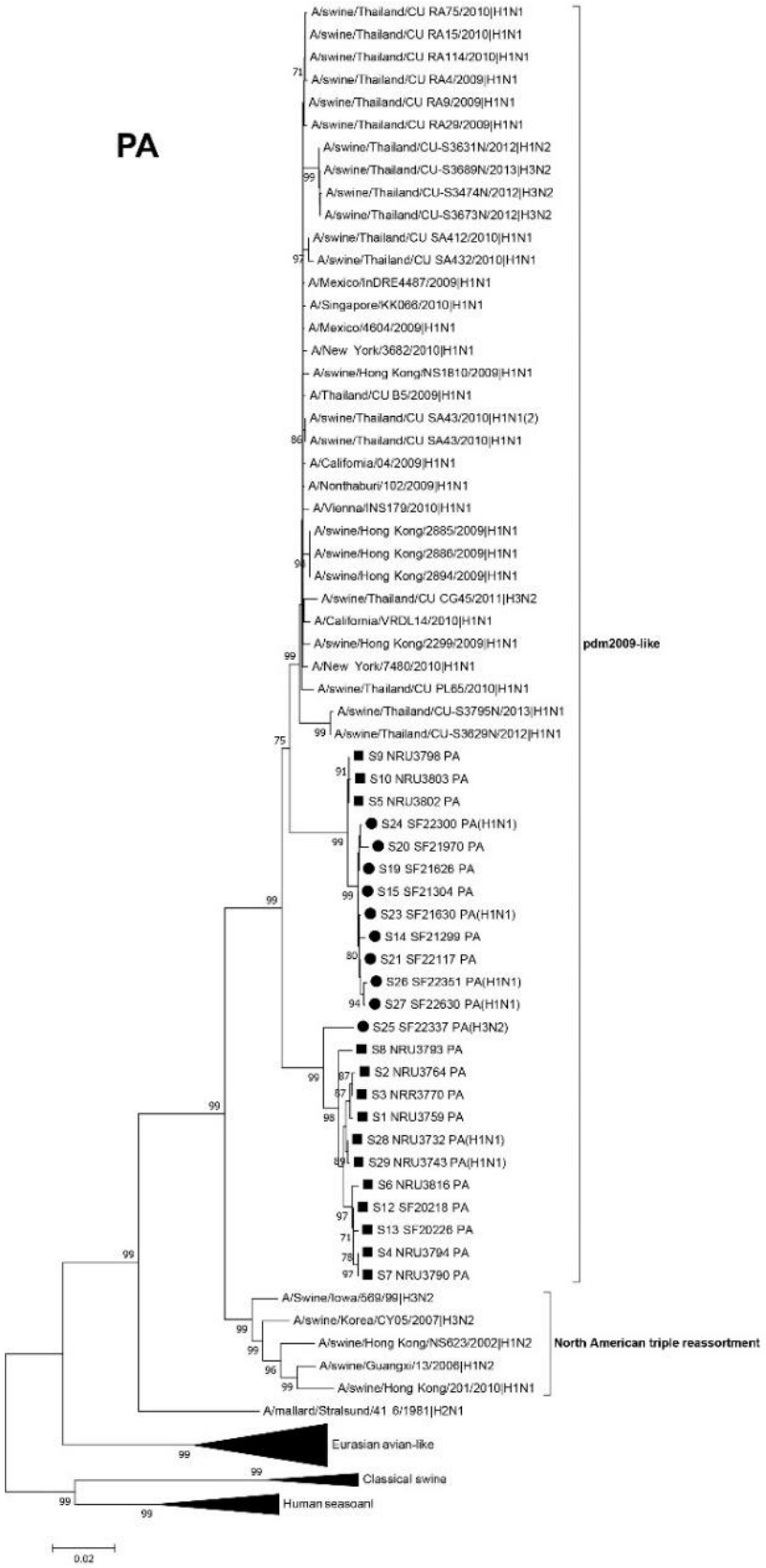
PA
A/swine/Thailand/CU RA75/2010|H1N1
A/swine/Thailand/CU RA15/2010|H1N1
A/swine/Thailand/CU RA114/2010|H1N1
A/swine/Thailand/CU RA4/2009|H1N1
A/swine/Thailand/CU RA9/2009|H1N1
A/swine/Thailand/CU RA29/2009|H1N1
A/swine/Thailand/CU-S3631N/2012|H1N2
A/swine/Thailand/CU-S3689N/2013|H3N2
A/swine/Thailand/CU-S3474N/2012|H3N2
A/swine/Thailand/CU-S3673N/2012|H3N2
A/swine/Thailand/CU SA412/2010|H1N1
A/swine/Thailand/CU SA432/2010|H1N1
A/Mexico/InDRE4487/2009|H1N1
A/Singapore/KK066/2010|H1N1
A/Mexico/4604/2009|H1N1
A/New York/3682/2010|H1N1
A/swine/Hong Kong/NS1B10/2009|H1N1
A/Thailand/CU B5/2009|H1N1
A/swine/Thailand/CU SA43/2010|H1N1(2)
A/swine/Thailand/CU SA43/2010|H1N1
A/California/04/2009|H1N1
A/Nonthaburi/102/2009|H1N1
A/Vienna/INS179/2010|H1N1
A/swine/Hong Kong/2885/2009|H1N1
A/swine/Hong Kong/2886/2009|H1N1
A/swine/Hong Kong/2894/2009|H1N1
A/swine/Thailand/CU CG45/2011|H3N2
A/California/VRDL14/2010|H1N1
A/swine/Hong Kong/2299/2009|H1N1
A/New York/7480/2010|H1N1
A/swine/Thailand/CU PL65/2010|H1N1
A/swine/Thailand/CU-S3795N/2013|H1N1
A/swine/Thailand/CU-S3629N/2012|H1N1
S9 NRU3798 PA
S10 NRU3803 PA
S5 NRU3802 PA
S24 SF22300 PA(H1N1)
S20 SF21970 PA
S19 SF21626 PA
S15 SF21304 PA
S23 SF21630 PA(H1N1)
S14 SF21299 PA
S21 SF22117 PA
S26 SF22351 PA(H1N1)
S27 SF22630 PA(H1N1)
S25 SF22337 PA(H3N2)
S8 NRU3793 PA
S2 NRU3764 PA
S3 NRR3770 PA
S1 NRU3759 PA
S28 NRU3732 PA(H1N1)
S29 NRU3743 PA(H1N1)
S6 NRU3816 PA
S12 SF20218 PA
S13 SF20226 PA
S4 NRU3794 PA
S7 NRU3790 PA
pdm2009-like
A/Swine/Iowa/569/99|H3N2
A/swine/Korea/CY05/2007|H3N2
A/swine/Hong Kong/NS623/2002|H1N2
A/swine/Guangxi/13/2008|H1N2
A/swine/Hong Kong/201/2010|H1N1
North American triple reassortment
A/mallard/Stralsund/41 6/1981|H2N1
Eurasian avian-like
Classical swine
Human seasoanl
0.02

Figure 10: Phylogenetic tree of PA gene of the SIV-H1N1 and SIV-H3N2. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1,000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIVs isolated in 2017 and 2018, respectively.

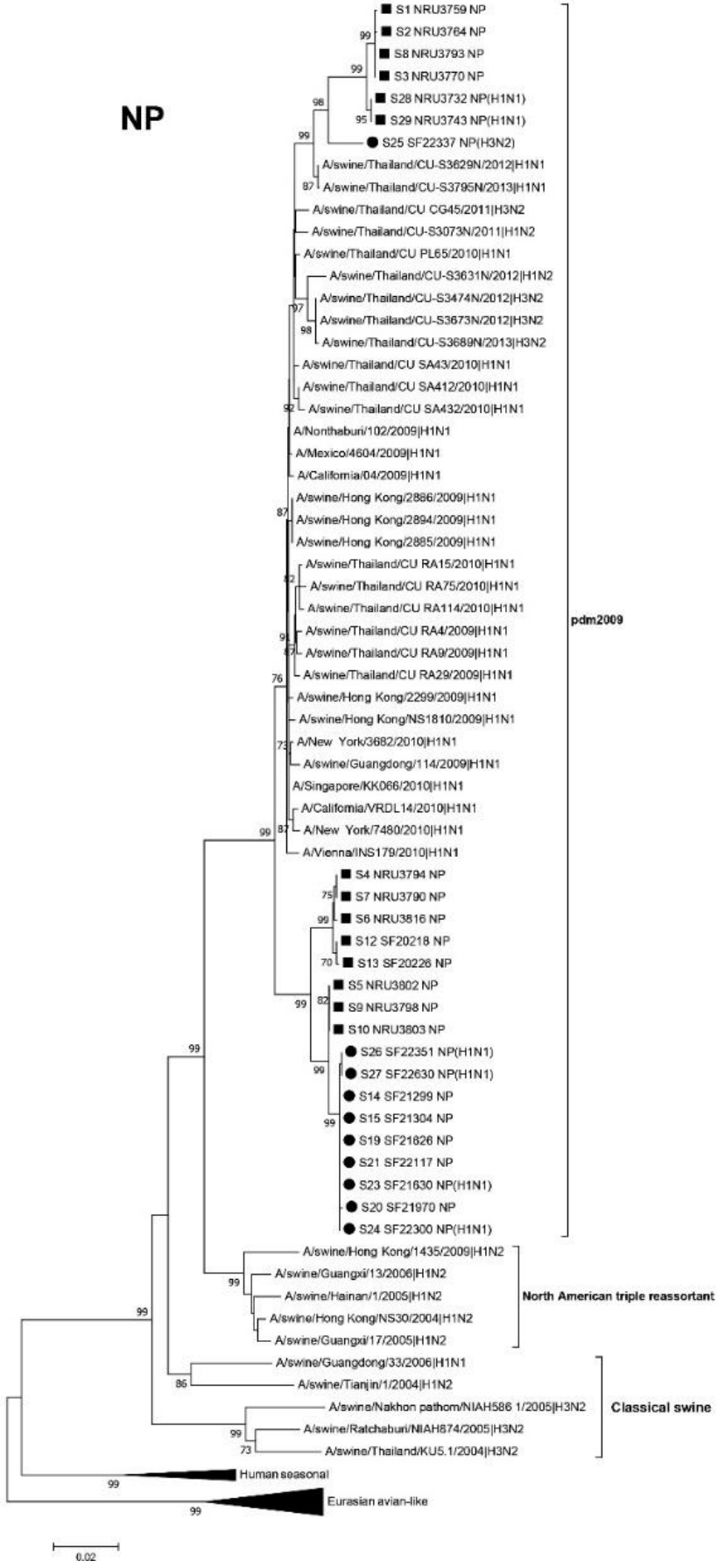
NP
S1 NRU3759 NP
S2 NRU3764 NP
S8 NRU3793 NP
S3 NRU3770 NP
S28 NRU3732 NP(H1N1)
S29 NRU3743 NP(H1N1)
S25 SF22337 NP(H3N2)
A/swine/Thailand/CU-S3629N/2012|H1N1
A/swine/Thailand/CU-S3795N/2013|H1N1
A/swine/Thailand/CU CG45/2011|H3N2
A/swine/Thailand/CU-S3073N/2011|H1N2
A/swine/Thailand/CU PL65/2010|H1N1
A/swine/Thailand/CU-S3631N/2012|H1N2
A/swine/Thailand/CU-S3474N/2012|H3N2
A/swine/Thailand/CU-S3673N/2012|H3N2
A/swine/Thailand/CU-S3689N/2013|H3N2
A/swine/Thailand/CU SA43/2010|H1N1
A/swine/Thailand/CU SA412/2010|H1N1
A/swine/Thailand/CU SA432/2010|H1N1
A/Nonthaburi/102/2009|H1N1
A/Mexico/4604/2009|H1N1
A/California/04/2009|H1N1
A/swine/Hong Kong/2886/2009|H1N1
A/swine/Hong Kong/2894/2009|H1N1
A/swine/Hong Kong/2885/2009|H1N1
A/swine/Thailand/CU RA15/2010|H1N1
A/swine/Thailand/CU RA75/2010|H1N1
A/swine/Thailand/CU RA114/2010|H1N1
A/swine/Thailand/CU RA4/2009|H1N1
A/swine/Thailand/CU RA9/2009|H1N1
A/swine/Thailand/CU RA29/2009|H1N1
A/swine/Hong Kong/2299/2009|H1N1
A/swine/Hong Kong/NS1810/2009|H1N1
A/New York/3682/2010|H1N1
A/swine/Guangdong/114/2009|H1N1
A/Singapore/KK066/2010|H1N1
A/California/VRDL14/2010|H1N1
A/New York/7480/2010|H1N1
A/Vienna/INS179/2010|H1N1
S4 NRU3794 NP
S7 NRU3790 NP
S6 NRU3816 NP
S12 SF20218 NP
S13 SF20226 NP
S5 NRU3802 NP
S9 NRU3798 NP
S10 NRU3803 NP
S26 SF22351 NP(H1N1)
S27 SF22630 NP(H1N1)
S14 SF21299 NP
S15 SF21304 NP
S19 SF21626 NP
S21 SF22117 NP
S23 SF21630 NP(H1N1)
S20 SF21970 NP
S24 SF22300 NP(H1N1)
pdm2009
A/swine/Hong Kong/1435/2009|H1N2
A/swine/Guangxi/13/2006|H1N2
A/swine/Hainan/1/2005|H1N2
A/swine/Hong Kong/NS30/2004|H1N2
A/swine/Guangxi/17/2005|H1N2
North American triple reassortant
A/swine/Guangdong/33/2006|H1N1
A/swine/Tianjin/1/2004|H1N2
A/swine/Nakhon pathom/NIAH586 1/2005|H3N2
A/swine/Ratchaburi/NIAH874/2005|H3N2
A/swine/Thailand/KU5.1/2004|H3N2
Classical swine
Human seasonal
Eurasian avian-like
0.02

Figure 11: Phylogenetic tree of NP gene of the SIV-H1N1 and SIV-H3N2. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1,000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIVs isolated in 2017 and 2018, respectively.

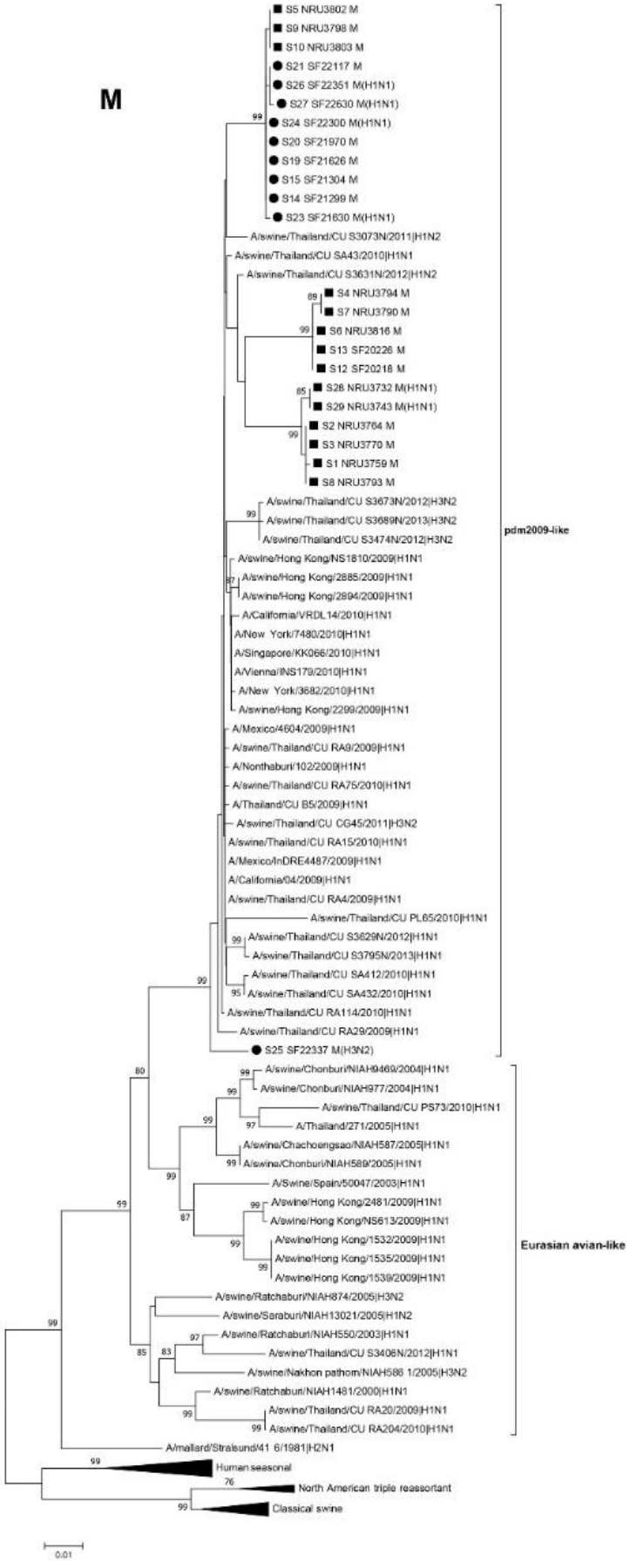
M
S5 NRU3802 M
S9 NRU3798 M
S10 NRU3803 M
S21 SF22117 M
S26 SF22351 M(H1N1)
S27 SF22630 M(H1N1)
S24 SF22300 M(H1N1)
S20 SF21970 M
S19 SF21626 M
S15 SF21304 M
S14 SF21299 M
S23 SF21630 M(H1N1)
A/swine/Thailand/CU S3073N/2011|H1N2
A/swine/Thailand/CU SA43/2010|H1N1
A/swine/Thailand/CU S3631N/2012|H1N2
S4 NRU3794 M
S7 NRU3790 M
S6 NRU3816 M
S13 SF20226 M
S12 SF20218 M
S28 NRU3732 M(H1N1)
S29 NRU3743 M(H1N1)
S2 NRU3764 M
S3 NRU3770 M
S1 NRU3759 M
S8 NRU3793 M
A/swine/Thailand/CU S3673N/2012|H3N2
A/swine/Thailand/CU S3689N/2013|H3N2
A/swine/Thailand/CU S3474N/2012|H3N2
pdm2009-like
A/swine/Hong Kong/NS1810/2009|H1N1
A/swine/Hong Kong/2885/2009|H1N1
A/swine/Hong Kong/2894/2009|H1N1
A/California/VRDL14/2010|H1N1
A/New York/7480/2010|H1N1
A/Singapore/KK066/2010|H1N1
A/Vienna/INS179/2010|H1N1
A/New York/3682/2010|H1N1
A/swine/Hong Kong/2299/2009|H1N1
A/Mexico/4604/2009|H1N1
A/swine/Thailand/CU RA9/2009|H1N1
A/Nonthaburi/102/2009|H1N1
A/swine/Thailand/CU RA75/2010|H1N1
A/Thailand/CU B5/2009|H1N1
A/swine/Thailand/CU CG45/2011|H3N2
A/swine/Thailand/CU RA15/2010|H1N1
A/Mexico/InDRE4487/2009|H1N1
A/California/04/2009|H1N1
A/swine/Thailand/CU RA4/2009|H1N1
A/swine/Thailand/CU PL65/2010|H1N1
A/swine/Thailand/CU S3629N/2012|H1N1
A/swine/Thailand/CU S3795N/2013|H1N1
A/swine/Thailand/CU SA412/2010|H1N1
A/swine/Thailand/CU SA432/2010|H1N1
A/swine/Thailand/CU RA114/2010|H1N1
A/swine/Thailand/CU RA29/2009|H1N1
S25 SF22337 M(H3N2)
A/swine/Chonburi/NIAH9469/2004|H1N1
A/swine/Chonburi/NIAH977/2004|H1N1
A/swine/Thailand/CU PS73/2010|H1N1
A/Thailand/271/2005|H1N1
A/swine/Chachoengsao/NIAH587/2005|H1N1
A/swine/Chonburi/NIAH589/2005|H1N1
A/Swine/Spain/50047/2003|H1N1
A/swine/Hong Kong/2481/2009|H1N1
A/swine/Hong Kong/NS613/2009|H1N1
A/swine/Hong Kong/1532/2009|H1N1
A/swine/Hong Kong/1535/2009|H1N1
A/swine/Hong Kong/1539/2009|H1N1
Eurasian avian-like
A/swine/Ratchaburi/NIAH874/2005|H3N2
A/swine/Saraburi/NIAH13021/2005|H1N2
A/swine/Ratchaburi/NIAH550/2003|H1N1
A/swine/Thailand/CU S3408N/2012|H1N1
A/swine/Nakhon pathom/NIAH586 1/2005|H3N2
A/swine/Ratchaburi/NIAH1481/2000|H1N1
A/swine/Thailand/CU RA20/2009|H1N1
A/swine/Thailand/CU RA204/2010|H1N1
A/mallard/Stralsund/41 6/1981|H2N1
Human seasonal
North American triple reassortant
Classical swine
0.01

Figure 12: Phylogenetic tree of M gene of the SIV-H1N1 and SIV-H3N2. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1,000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIVs isolated in 2017 and 2018, respectively.

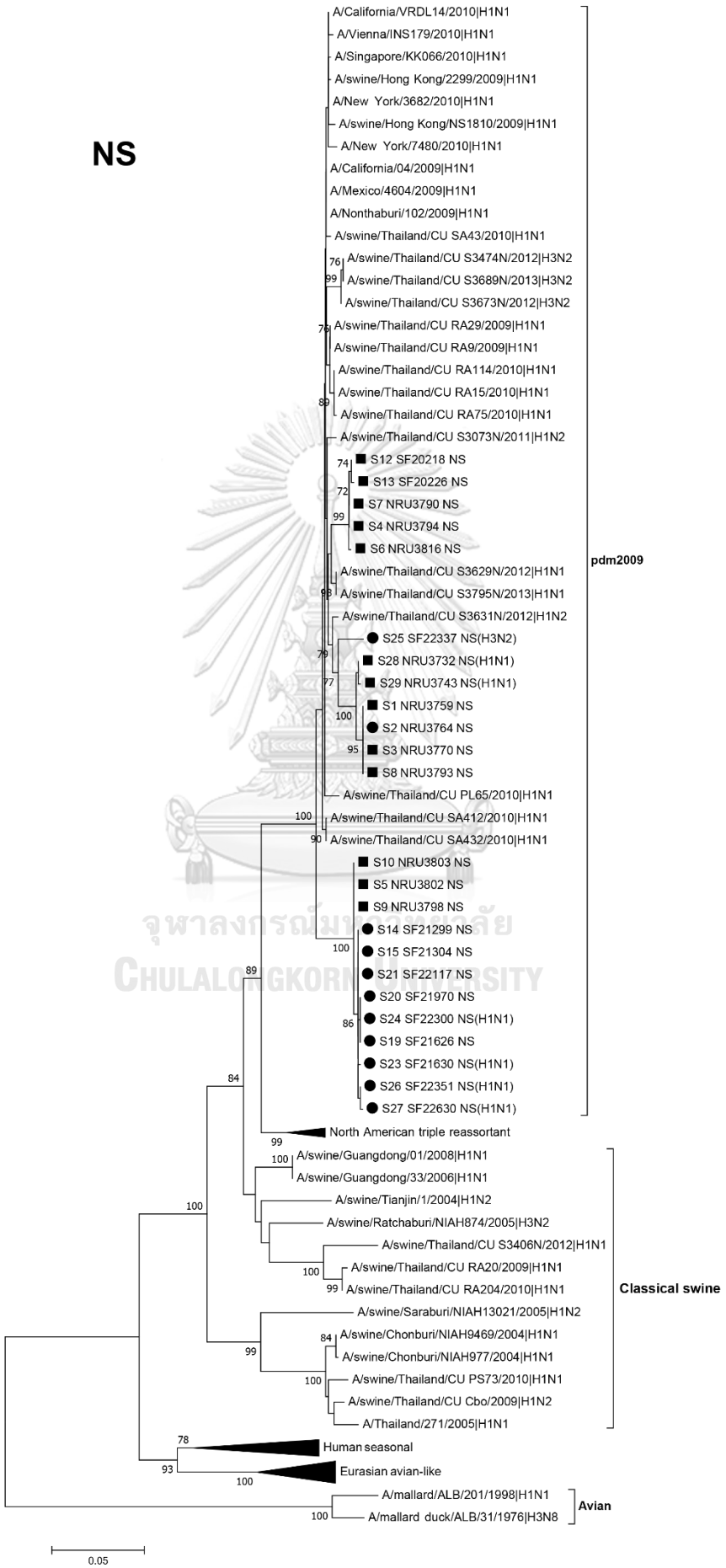
NS
A/California/VRDL14/2010|H1N1
A/Vienna/INS179/2010|H1N1
A/Singapore/KK066/2010|H1N1
A/swine/Hong Kong/2299/2009|H1N1
A/New York/3682/2010|H1N1
A/swine/Hong Kong/NS1810/2009|H1N1
A/New York/7480/2010|H1N1
A/California/04/2009|H1N1
A/Mexico/4604/2009|H1N1
A/Nonthaburi/102/2009|H1N1
A/swine/Thailand/CU SA43/2010|H1N1
A/swine/Thailand/CU S3474N/2012|H3N2
A/swine/Thailand/CU S3689N/2013|H3N2
A/swine/Thailand/CU S3673N/2012|H3N2
A/swine/Thailand/CU RA29/2009|H1N1
A/swine/Thailand/CU RA9/2009|H1N1
A/swine/Thailand/CU RA114/2010|H1N1
A/swine/Thailand/CU RA15/2010|H1N1
A/swine/Thailand/CU RA75/2010|H1N1
A/swine/Thailand/CU S3073N/2011|H1N2
S12 SF20218 NS
S13 SF20226 NS
S7 NRU3790 NS
S4 NRU3794 NS
S6 NRU3816 NS
A/swine/Thailand/CU S3629N/2012|H1N1
A/swine/Thailand/CU S3795N/2013|H1N1
A/swine/Thailand/CU S3631N/2012|H1N2
S25 SF22337 NS(H3N2)
S28 NRU3732 NS(H1N1)
S29 NRU3743 NS(H1N1)
S1 NRU3759 NS
S2 NRU3764 NS
S3 NRU3770 NS
S8 NRU3793 NS
A/swine/Thailand/CU PL65/2010|H1N1
A/swine/Thailand/CU SA412/2010|H1N1
A/swine/Thailand/CU SA432/2010|H1N1
S10 NRU3803 NS
S5 NRU3802 NS
S9 NRU3798 NS
S14 SF21299 NS
S15 SF21304 NS
S21 SF22117 NS
S20 SF21970 NS
S24 SF22300 NS(H1N1)
S19 SF21626 NS
S23 SF21630 NS(H1N1)
S26 SF22351 NS(H1N1)
S27 SF22630 NS(H1N1)
pdm2009
North American triple reassortant
A/swine/Guangdong/01/2008|H1N1
A/swine/Guangdong/33/2006|H1N1
A/swine/Tianjin/1/2004|H1N2
A/swine/Ratchaburi/NIAH874/2005|H3N2
A/swine/Thailand/CU S3406N/2012|H1N1
A/swine/Thailand/CU RA20/2009|H1N1
A/swine/Thailand/CU RA204/2010|H1N1
A/swine/Saraburi/NIAH13021/2005|H1N2
A/swine/Chonburi/NIAH9469/2004|H1N1
A/swine/Chonburi/NIAH977/2004|H1N1
A/swine/Thailand/CU PS73/2010|H1N1
A/swine/Thailand/CU Cbo/2009|H1N2
A/Thailand/271/2005|H1N1
Classical swine
Human seasonal
Eurasian avian-like
A/mallard/ALB/201/1998|H1N1
A/mallard duck/ALB/31/1976|H3N8
Avian
0.05

Figure 13: Phylogenetic tree of NS gene of the SIV-H1N1 and SIV-H3N2. The phylogenetic tree was generated by the neighbor-joining method using MEGA v7.0 software. Bootstrap analysis was analyzed with 1,000 replication and bootstrap of ≥70% are shown on branches. Black squares and circles indicated SIVs isolated in 2017 and 2018, respectively.

Table 5: Gene constellation of Thai H1N1 swine influenza viruses in this thesis

Isolate	Genotype	Date	Gene segments							
			PB2	PB1	PA	HA	NP	NA	M	NS
NIAH587	H1N1(7+1)	2005	E	E	E	C	E	E	E	E
A/swine/Thailand/CU-PL65/2010	pdm	2010	pdm	pdm	pdm	pdm	pdm	pdm	pdm	pdm
A/swine/Thailand/CU-SA43/2010	rH1N1(7+1)	2010	pdm	pdm	pdm	pdm	pdm	E	pdm	pdm
A/swine/Thailand/ CU-S3340N/2012	H1N1(6+2)	2012	E	E	E	C	E	E	E	C
A/swine/Thailand/ CU-S3629N/2012	rH1N1(pdm+2)	2013	pdm	pdm	pdm	C	pdm	E	pdm	pdm
S28	rH1N1(pdm+2)	Jan-17	pdm	pdm	pdm	C	pdm	E	pdm	pdm
S29	rH1N1(pdm+2)	Jan-17	pdm	pdm	pdm	C	pdm	E	pdm	pdm
S1	rH1N1(pdm+2)	Apr-17	pdm	pdm	pdm	C	pdm	E	pdm	pdm
S2	rH1N1(pdm+2)	Apr-17	pdm	pdm	pdm	C	pdm	E	pdm	pdm
S3	rH1N1(pdm+2)	Apr-17	pdm	pdm	pdm	C	pdm	E	pdm	pdm
S5	rH1N1(pdm+1)	Jul-17	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S8	rH1N1(pdm+2)	Jul-17	pdm	pdm	pdm	C	pdm	E	pdm	pdm
S9	rH1N1(pdm+1)	Jul-17	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S10	rH1N1(pdm+1)	Jul-17	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S14	rH1N1(pdm+1)	Apr-18	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S15	rH1N1(pdm+1)	Apr-18	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S19	rH1N1(pdm+1)	Jun-18	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S23	rH1N1(pdm+1)	Jun-18	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S20	rH1N1(pdm+1)	Jul-18	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S21	rH1N1(pdm+1)	Aug-18	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S24	rH1N1(pdm+1)	Sep-18	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S26	rH1N1(pdm+1)	Oct-18	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm
S27	rH1N1(pdm+1)	Nov-18	pdm	pdm	pdm	C	pdm	pdm	pdm	pdm

pdm: pandemic H1N1 2009-like, C: Classical swine lineage, E: Eurasian avian-like swine lineage

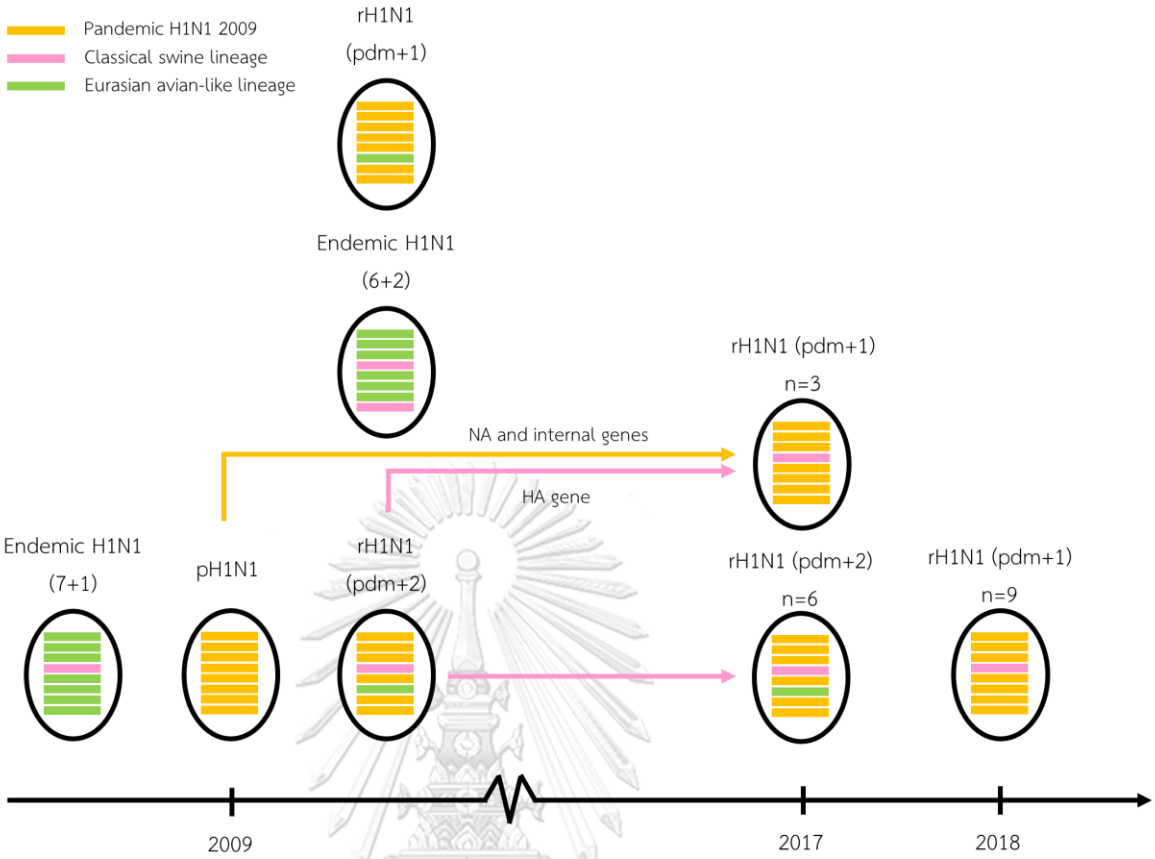

Figure 14: schematic presentation of genetic constellation of SIV-H1N1s in this thesis and endemic Thai SIVs from previous study. Oval represent the virus with 8 genes segment. First line to eighth line indicate PB2, PB1, PA, HA, NP, NA, M and NS gene, respectively. Yellow line indicated pandemic H1N1 2009 lineage (pdm09). Pink line indicates classical swine lineage (CS) and green line indicate Eurasian avian-like swine lineage (EA).

Table 6: Gene constellation of Thai H3N2 swine influenza viruses in this Thesis

Isolate	Genotype	year	Gene segment							
			PB2	PB1	PA	HA	NP	NA	M	NS
A/swine/Thailand/KU5.1/2004	eH3N2	2004	E	E	E	Hu	C	Hu	E	C
A/swine/Thailand/CU-S3673N/2012	rH3N2(pdm+2)	2013	pdm	pdm	pdm	Hu	pdm	Hu	pdm	pdm
S4	rH3N2(pdm+2)	Jul-17	pdm	pdm	pdm	Hu	pdm	Hu	pdm	pdm
S6	rH3N2(pdm+2)	Jul-17	pdm	pdm	pdm	Hu	pdm	Hu	pdm	pdm
S7	rH3N2(pdm+2)	Jul-17	pdm	pdm	pdm	Hu	pdm	Hu	pdm	pdm
S12	rH3N2(pdm+2)	Dec-17	pdm	pdm	pdm	Hu	pdm	Hu	pdm	pdm
S13	rH3N2(pdm+2)	Dec-17	pdm	pdm	pdm	Hu	pdm	Hu	pdm	pdm
S25	rH3N2(pdm+2)	Oct-18	pdm	pdm	pdm	Hu	pdm	Hu	pdm	pdm

pdm: pandemic H1N1 2009-like, C: classical swine lineage, E: Eurasian avian-like swine lineage, Hu: human-like swine

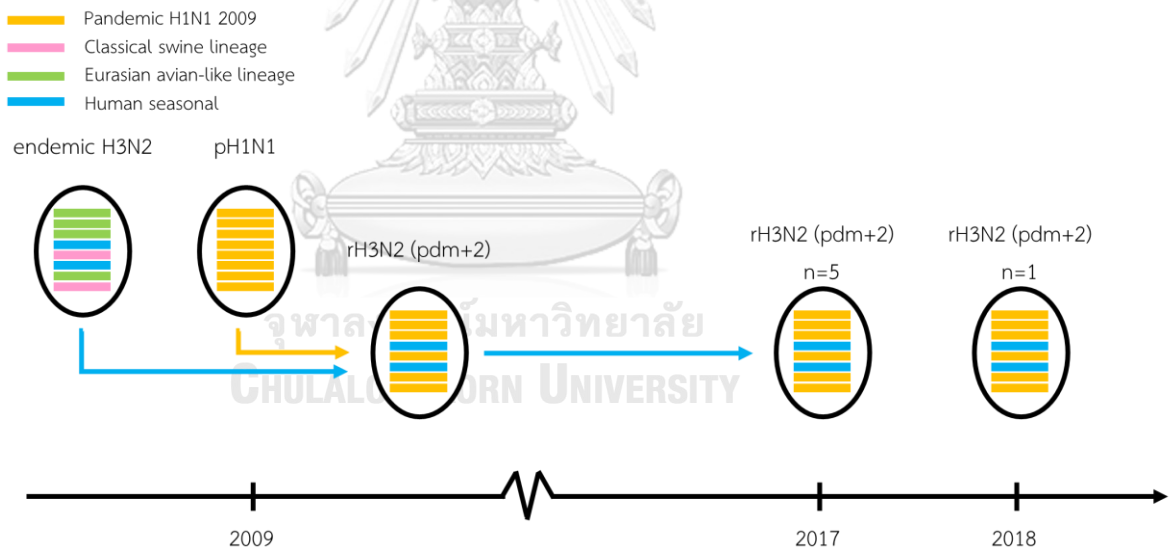

Figure 15: Schematic presentation of genetic constellation of SIV-H3N2s in this thesis and endemic Thai SIVs from previous study. Oval represent the virus with 8 genes. First line to eighth line indicate PB2, PB1, PA, HA, NP, NA, M and NS gene, respectively. Grey line indicated pandemic H1N1 2009 lineage (pdm09). Pink line indicates classical swine lineage (CS). Green line indicates Eurasian avian-like swine lineage (EA) and blue line indicates human-like swine lineage.

4.5 Genetic analysis of swine influenza viruses

For HA1 genetic analysis, analysis of 5 antigenic sites of HA1 (Sa, Sb, Ca1, Ca2 and Cb) showed that most SIVs in this thesis contain identical amino acids at antigenic sites. For Genetic analysis of receptor binding sites (HA-190, 225, 226 and 228) (Connor et al., 1994), 17 out of 18 SIVs posed 190D suggesting preferential binding to human receptor (2,6 sialic acid receptor). Expect one virus (S19) contain E190 suggesting the virus prefer to bind avian receptor (2,3 sialic acid receptor) (Vines et al., 1998) (Table 7).

For HA3 genetic analysis, analysis of 5 antigenic site of HA3 (A, B, C, D, and E) showed that antigenic sites A (140-146, 156-161) and B (189-199) contained amino acids substitution different from vaccine viruses. For receptor binding sites (HA3-190, 225, 226 and 228), all SIVs in this study posed I226 and S228 suggesting preferential binding to human receptor (Vines et al., 1998; Gao et al., 2009) (Table 8).

For genetic analysis of some internal genes (PB2, M and NS genes), all SIVs in this study pose glutamic acid (E) at position 627 in PB2 (E627) which correlated with less virulence of the viruses. While all SIVs contain aspartic acid (D) at position 701 in PB2 (N701D) and position 92 in NS (E92D), which corelate to the increasing virulence of the viruses (Hatta et al., 2001; Gao et al., 2009; Steel et al., 2009) (Table 9). For genetic analysis of antiviral resistance in M gene at position M-31, all SIVs in this study pose Asparagine (N) at position 31 (31N) suggesting the viral resistance to Amantadine (Saito et al., 2003) (Table 9). On the other hand, genetic analysis of antiviral resistance (Oseltamivir) in NA gene was performed (NA1-119, 275, 293, and 295 and NA2-146, 219, and 272). Our result showed that all SIVs in this study are sensitive to Oseltamivir antiviral drug (For NA1; E119, H275, R293 and N295 and NA2; N146, S219 and A272) (Table 10)

Table 7: Genetic analysis of the HA1 gene of Thai swine influenza viruses in this thesis

Viruses	Subtype	Amino acid determinants of HA1 gene													
		Antigenic site										Receptor binding site				HA clavage site
		Sa			Sb	Ca1			Ca2		Cb					
		128-129	156-160	162-167	187-198	169-173	206-208	238-240	140-145	224-225	78-83	190	225	226	228	325-333
A/California/04/2009	pdmH1N1	PN	KKGNS	PKLSKS	TSADQQSLYQNA	INDKG	GSS	EPG	PHAGAK	RD	SLSTAS	D	D	Q	G	PSIQSRGLF
A/swine/Thailand/ CU-S3340N/2012	eH1N1 (6+2)	PN	KKGNS	PKLSKS	TSTDQQSLYQNA	VNNKK	SSS	EPG	HYAGAN	RD	LLFKAS	D	D	Q	G	PSIQSRGLF
A/swine/Thailand/ CU-3795N/2013	rH1N1 (6+2)	PN	KKENS	PKISKS	TSNDQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LLFNAS	D	D	Q	G	PSIQSRGLF
A/swine/Thailand/ CU-SA43/2010	rH1N1 (7+1)	PN	KKGNS	PKLSKS	TSADQQSLYQNA	INDKG	GSS	EPG	PHAGAK	RD	SLSTAS	D	D	R	G	PSIQSRGLF
A/swine/Thailand/ NIAH107037_29/2011	pdm2009	PN	KKGNL	PKLSKS	TSADQQSLYQNA	INDKG	GTS	EPG	PHAGAK	RD	SLSTAS	D	D	R	G	PSIQSRGLF
A/swine/Thailand/ NIAH587/2005	eH1N1 (7+1)	PN	KKGNS	PKLSKS	TNTDQQSLYQNA	VNNKK	GSS	EPG	PYAGAN	RG	LLFAIN	D	G	Q	G	PSIQSRGLF
A/Michigan/45/2015	Vaccine strain	PN	KKGNS	PKLNQS	TTADQQSLYQNA	INDKG	GTS	EPG	PHAGAK	RD	SLSTAS	D	D	Q	G	PSIQSRGLF
S1	rH1N1 (pdm+2)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNKG	SSS	KPE	PYAGAN	RN	SLFNAS	D	N	Q	G	PSIQSRGLF
S2	rH1N1 (pdm+2)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNKG	SSS	KPE	PYAGAN	RD	SLFNAS	D	D	Q	G	PSIQSRGLF
S3	rH1N1 (pdm+2)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNKG	SSS	KPE	PYAGAN	RD	SLFNAS	D	D	Q	G	PSIQSRGLF
S5	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RD	LLFNAS	D	D	Q	G	PSIQSRGLF
S8	rH1N1 (pdm+2)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNKG	SSS	KPE	PYAGAK	RD	SLFNAS	D	D	Q	G	PSIQSRGLF
S9	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RD	LLFNAS	D	D	Q	G	PSIQSRGLF
S10	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RD	LLFNAS	D	D	Q	G	PSIQSRGLF

Viruses	Subtype	Amino acid determinants of HA1 gene														
		Antigenic site										Receptor binding site				HA clavage site
		Sa			Sb	Ca1			Ca2		Cb					
		128-129	156-160	162-167	187-198	169-173	206-208	238-240	140-145	224-225	78-83	190	225	226	228	325-333
S14	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAK	RD	LLFNAS	D	D	Q	G	PSIQSRGLF
S15	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RD	LLFNAS	D	D	Q	G	PSIQSRGLF
S19	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNEQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RD	LLFNAS	E	D	Q	G	PSIQSRGLF
S20	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RD	LLFNAS	D	D	Q	G	PSIQSRGLF
S21	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RD	LLFNAS	D	D	Q	G	PSIQSRGLF
S23	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RG	LLFNAS	D	G	Q	G	PSIQSRGLF
S24	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RN	LLFNAS	D	N	Q	G	PSIQSRGLF
S26	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RD	LLFNAS	D	D	Q	G	PSIQSRGLF
S27	rH1N1 (pdm+1)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNRG	SSS	KPE	PYAGAN	RG	LLFNAS	D	G	Q	G	PSIQSRGLF
S28	rH1N1 (pdm+2)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNKG	SSS	KPE	PYAGAN	RG	SLFNAS	D	G	Q	G	PSIQSRGLF
S29	rH1N1 (pdm+2)	PN	KKENS	PKLSKS	TSNDQQVLYQNA	FNNKG	SSS	KPE	PYAGAN	RD	SLFNAS	D	D	Q	G	PSIQSRGLF

*amino acid positions are based on H3 numbering

Table 8: Genetic analysis of the HA3 gene of Thai swine influenza viruses in this thesis

Viruses	Subtype	Amino acid determinants of HA3 gene										
		Antigenic site							Receptor binding site			
		A	B		C	D	E		190	225	226	228
		140-146	156-161	189-199	277-282	205-221	171-175	243-249				
A/swine/Thailand/PB486/2009	eH3N2	KRGSVKS	KLDYKY	NDQTNLYVQAS	CNSGCI	STKRSQQTVIPSIGSRP	NDKFD	LLINSTG	D	G	I	S
A/swine/Thailand/ CU-S3795N/2013	rH3N2 (pdm+2)	KRGYVNS	QSGHKY	SDQTSLYVQAS	CNSEC	STKRSQQTVIPNIGSRP	NEKFD	LLINSTG	D	G	I	S
A/swine/Thailand/ CU-S14252N/2014	rH3N2 (pdm+2)	KRGYVNS	KLDYKY	SDQTNLYVQAS	CNSEC	STKRSQQTVIPNIGFRP	NDKFD	LLINSTG	D	G	I	S
A/Kansas/14/2017	Vaccine strain	IRRSNNS	HLNFKY	KDQIFLYAQPS	CKSECI	STKRSQQAVIPNIGSRP	NEQFD	LLITSTG	D	N	I	S
S4	rH3N2 (pdm+2)	KRGSVKS	QLNYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSTG	D	G	I	S
S6	rH3N2 (pdm+2)	KRGSVKS	QLNYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSTG	D	G	I	S
S7	rH3N2 (pdm+2)	KRGSVKS	QLNYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSTG	D	G	I	S
S13	rH3N2 (pdm+2)	KRGSVKS	QLNYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSTG	D	G	I	S
S12	rH3N2 (pdm+2)	KRGSVKS	QLNYKY	SDQTNLYVQAS	CKSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSKG	D	G	I	S
S25	rH3N2 (pdm+2)	KRGYVNS	HSGHKY	SDQTSLYVQTS	CNSECV	STQRSQQTVIPNIGSRP	NEKFD	LLINSTG	D	G	I	S

*amino acid positions are based on H3 numbering

Table 9: Genetic analysis of amino acid substitutions on PB2, M and NS genes

Viruses	Subtype	PB2		NS	M2
		E627K	N701D	E92D	S31N
S1	rH1N1 (pdm+2)	E	D	D	N
S2	rH1N1(pdm+2)	E	D	D	N
S3	rH1N1 (pdm+2)	E	D	D	N
S4	rH3N2 (pdm+2)	E	D	D	N
S5	rH1N1 (pdm+1)	E	D	D	N
S6	rH3N2 (pdm+2)	E	D	D	N
S7	rH3N2 (pdm+2)	E	D	D	N
S8	rH1N1 (pdm+2)	E	D	D	N
S9	rH1N1 (pdm+1)	E	D	D	N
S10	rH1N1 (pdm+1)	E	D	D	N
S12	rH3N2 (pdm+2)	E	D	D	N
S13	rH3N2 (pdm+2)	E	D	D	N
S14	rH1N1 (pdm+1)	E	D	D	N
S15	rH1N1 (pdm+1)	E	D	D	N
S19	rH1N1 (pdm+1)	E	D	D	N
S20	rH1N1 (pdm+1)	E	D	D	N
S21	rH1N1 (pdm+1)	E	D	D	N
S23	rH1N1 (pdm+1)	E	D	D	N
S24	rH1N1 (pdm+1)	E	D	D	N
S25	rH3N2 (pdm+2)	E	D	D	N
S26	rH1N1 (pdm+1)	E	D	D	N
S27	rH1N1 (pdm+1)	E	D	D	N
S28	rH1N1 (pdm+2)	E	D	D	N
S29	rH1N1 (pdm+2)	E	D	D	N

Table 10: Genetic analysis of amino acid substitution related to oseltamivir resistances on NA gene

Viruses	Subtype	NA1				NA2			
		E119V	H275Y	R293K	N295S	N146K	S219T	A272V	245-248 deletion
S1	rH1N1 (pdm+2)	E	H	R	N				
S2	rH1N1(pdm+2)	E	H	R	N				
S3	rH1N1 (pdm+2)	E	H	R	N				
S4	rH3N2 (pdm+2)					N	S	A	No
S5	rH1N1 (pdm+1)	E	H	R	N				
S6	rH3N2 (pdm+2)					N	S	A	No
S7	rH3N2 (pdm+2)					N	S	A	No
S8	rH1N1 (pdm+2)	E	H	R	N				
S9	rH1N1 (pdm+1)	E	H	R	N				
S10	rH1N1 (pdm+1)	E	H	R	N				
S12	rH3N2 (pdm+2)					N	S	A	No
S13	rH3N2 (pdm+2)					N	S	A	No
S14	rH1N1 (pdm+1)	E	H	R	N				
S15	rH1N1 (pdm+1)	E	H	R	N				
S19	rH1N1 (pdm+1)	E	H	R	N				
S20	rH1N1 (pdm+1)	E	H	R	N				
S21	rH1N1 (pdm+1)	E	H	R	N				
S23	rH1N1 (pdm+1)	E	H	R	N				
S24	rH1N1 (pdm+1)	E	H	R	N				
S25	rH3N2 (pdm+2)					N	S	A	No
S26	rH1N1 (pdm+1)	E	H	R	N				
S27	rH1N1 (pdm+1)	E	H	R	N				
S28	rH1N1 (pdm+2)	E	H	R	N				
S29	rH1N1 (pdm+2)	E	H	R	N				

Table 11: Pairwise comparison of HA1 gene of SIVs from this thesis with reference SIVs

Viruses	Subtype	% Similarity					
		A/California/04/2009	/Michigan/45/2015	A/swine/Thailand/NIAH107037_29/2011	A/swine/Thailand/CU_SA43/2010	A/swine/Thailand/CU-S3340N/2012	A/swine/Thailand/CU-S3795N/2013
		pdm H1N1	vaccine strain	pdm H1N1	rH1N1 (pdm)	eH1N1 (CS)	rH1N1 (CS)
S1	rH1N1 (pdm+2)	86.5	86.0	86.3	86.4	90.8	97.3
S2	rH1N1 (pdm+2)	86.6	86.1	86.4	86.4	90.8	97.4
S3	rH1N1 (pdm+2)	86.6	86.1	86.4	86.4	90.8	97.4
S5	rH1N1 (pdm+1)	86.8	86.1	86.5	86.5	90.9	97.7
S8	rH1N1 (pdm+2)	86.6	86.1	86.4	86.5	90.8	97.3
S9	rH1N1 (pdm+1)	86.8	86.1	86.5	86.5	90.9	97.7
S10	rH1N1 (pdm+1)	86.8	86.1	86.5	86.5	90.9	97.7
S14	rH1N1 (pdm+1)	86.8	86.2	86.6	86.5	90.9	97.5
S15	rH1N1 (pdm+1)	86.7	86.1	86.5	86.4	90.9	97.5
S19	rH1N1 (pdm+1)	86.7	86.1	86.5	86.4	90.6	97.3
S20	rH1N1 (pdm+1)	86.8	86.2	86.6	86.5	90.8	97.4
S21	rH1N1 (pdm+1)	86.8	86.1	86.5	86.5	90.9	97.5
S23	rH1N1 (pdm+1)	86.8	86.1	86.5	86.5	90.9	97.5
S24	rH1N1 (pdm+1)	86.8	86.1	86.5	86.5	90.7	97.3
S26	rH1N1 (pdm+1)	86.8	86.2	86.5	86.5	90.8	97.5
S27	rH1N1 (pdm+1)	86.8	86.1	86.5	86.5	90.8	97.4
S28	rH1N1 (pdm+2)	86.5	85.9	86.2	86.3	90.7	97.3
S29	rH1N1 (pdm+2)	86.5	85.9	86.2	86.3	90.7	97.3

Table 12: Pairwise comparison of HA3 gene of SIVs from this thesis with reference SIVs

Viruses	subtype	% Similarity			
		A/Kansas/14/2017	A/swine/Thailand/PB486/2009	A/swine/Thailand/CU-S14252N/2014	A/swine/Thailand/CU-S14129N/2013
		Vaccine strain	eH3N2	rH3N2	rH3N2
S4	rH3N2 (pdm+2)	87.3	90.8	98.1	89.7
S6	rH3N2 (pdm+2)	87.3	90.8	98.1	89.7
S7	rH3N2 (pdm+2)	87.3	90.8	98.1	89.7
S12	rH3N2 (pdm+2)	87.1	90.5	97.6	89.2
S13	rH3N2 (pdm+2)	87.0	90.6	97.8	89.4
S25	rH3N2 (pdm+2)	87.6	89.2	88.9	96.4

Result of pairwise comparison of nucleotide sequences of HA1 gene of the SIVs by using BLAST search in the NCBI database showed that HA1 gene of SIVs from this thesis was closely related to A/swine/Thailand/CU-S3795N/2013 (% nucleotide identities was 97.3-97.7%). However, Thai SIVs has less nucleotide identities to A/California/04/2009 (pandemic-H1N1-2009) with 86.5-86.8%, A/Michigan/45/2015 (vaccine strain) with 85.9-86.2%, A/swine/Thailand/NIAH107037_29/2011 (pandemic-H1N1-2009 in Thai swine) with 86.2-86.6%, A/swine/Thailand/CU_SA43/2010 (rH1N1 with HA originated from pandemic-H1N1-2009) with 86.3-86.5% and

A/swine/Thailand/CU-S3340N/2012 (eH1N1 in this pig farm) with 90.6-90.9% (Table 11).

For HA3 gene, pairwise comparison of nucleotide sequences of HA3 gene of the SIVs by using BLAST search in the NCBI database showed that five SIVs (S4, S6, S7, S12 and S13) have nucleotide identities similar to A/swine/Thailand/CU-S14252N/2014 (97.6-98.1%). While one SIVs (S25) has HA3 similar to similar A/swine/Thailand/CU-S14129N/2013 (96.4%). However, Thai SIVs from this thesis has less nucleotide identities to A/Kansas/14/2017 with 87.0-87.6%, A/swine/Thailand/PB486/2009 (eH3N2) with 89.2-90.8 (Table 12).

Chapter 5

Discussion

5.1 A longitudinal survey of swine influenza in a pig farm

We conducted a longitudinal study of swine influenza in a pig farm during January 2017 until November 2018. The pig farm is located in Ratchaburi province where considered as high-density pig production area in Thailand. The pig farm size approximately 4000 pigs with 43 building as farm office, 32 pig housing and 10 worker housing. The pig farm management type is a large-scale pig farm with open-house system. The farm contains approximately 1,600 sow and produce 2,000 piglets per month. The previous study on SIVs in this pig farm in 2015 showed that this pig farm had been infected with SIVs and estimated prevalence of SIVs was 6.66% (Nonthabenjawan et al., 2015). Comparing to previous study in 2015, the SIVs prevalence in this study was 18.81% (during January 2017 – November 2018) which was higher than previous report in 2015. The possible explanation is that this thesis was more focusing on target sample collection on weaning pigs and piglets (4-10 weeks-old), thus higher SIVs positivity was observed.

5.2 Swine influenza viruses in a pig farm

5.2.1 Swine influenza viruses circulating in a pig farm

Longitudinal survey of SIV in a pig farm during January 2017 to November 2018 identified 2 subtypes of SIVs (SIV-H1N1 and SIV-H3N2). Comparing to the previous study during 2000-2014, 3 SIV subtypes were identified which SIV-H1N2 could not identified in this thesis. it is noted that SIV-H1N2 has lower prevalence than SIV-H1N1 and SIV-H3N2 (Choi et al., 2002; Nonthabenjawan et al., 2015). However, only 24 out of 54 SIVs were selected and characterized, thus there is a possibility that SIV-H1N2 may not be selected or probably disappear from population in this pig farm.

5.2.2 Antibody against swine influenza virus in a pig farm

Antibody against H1N1, H3N2 and pdmH1N1 was observed in this pig farm. It is noted that pig pose antibody against pdmH1N1, even though the pdmH1N1 viruses could not be identified in this pig farm. From previous study, is has been proved that no cross-reaction between antibody against H1-SIV and H1 pandemic viruses (Sreta et al., 2012). Thus, the HI titer result suggested that pigs in this farm were exposed to H1N1, H3N2 and pdmH1N1.

Thai Meteorological Department mention winter season was started form November until February, summer was between March to May and rainy season was started from June until October (ThaiMeteorologicalDepartment, 2018). In this study, 2018, seasonal pattern was observed in occurrence of influenza A virus. there were 3 peaks of swine influenza in summer, rainy and winter season. For seropositivity of SIV-H3N2, in 2018, 2 peaks of SIV-H3N2 were observed in late summer (May) and rainy (September). For SIV-H1N1, in 2018, seropositivity had peak every season in middle summer (April), middle rainy (August) and winter (November). For pH1N1, in 2018, 2 cycles of pandemic H1N1 were presented in summer, and rainy (July). There were reports that prevalence of SIVs is low in summer and high in both rainy and winter (Agrawal et al., 2010), but we found that prevalence of SIVs was higher in summer. Follow Meteorological Department, in April 2018, temperature was lower than normal range and amount of rain was increase. (ThaiMeteorologicalDepartment, 2018). Moreover, there were some reported that were some reports that peak of SIVs were observed in summer (Kelly et al., 2013; Poljak et al., 2014). Thus, Swine influenza viruses were exposed to this farm every season including summer, rainy and winter.

5.3 Genetic characteristics of swine influenza virus isolated from a pig farm

Phylogenetic analysis of HA1 gene, there are 3 major lineages; Classical swine lineage (CS; 1A) (5 sub-clusters;α (1A.1), β(1A.2), γ1 (1A.3.3.3), γ2 (1A.3.2) and pdm09 (1A.3.3.2), human seasonal (Hu; 1B) and Eurasian avian-like swine lineage (EA; 1C) (Anderson et al., 2016). In detail, alpha sub-lineage could be classified to sub-cluster; 1A1.1, 1A.1.2 and 1A.1.3. Thai SIVs from this thesis and previous studies (Charoenvisal et al., 2013; Nonthabenjawan et al., 2015), as well as endemic SIVs are classified in 1A.1.2. Our result suggested that HA1 gene of Thai SIVs clustered in the distinct lineage (1A.1.2).

Phylogenic analysis of NA1 gene, SIVs in this thesis were closely related to either Eurasian avian-like swine lineage viruses (endemic NA1 gene) or pandemic H1N1 2009 (pdm09). This result suggested that pdmH1N1-2009 was circulating in this pig farm for a period of time and then reassorted with other endemic SIVs to generated novel genotypes of swine influenza viruses.

Phylogenic analysis of HA3 and NA2 gene, HA3 and NA2 genes were originated from human seasonal that introduced in pig during 1990s (Takemae et al., 2008). After that HA3 and NA2 genes were circulating in Thai pig population. HA3 and NA2 genes from pigs in this thesis were clustered/ grouped with human-like swine lineage.

From the BLAST result, all HA1 genes of SIVs from this thesis were similar A/swine/Thailand/CU-S3795N/2013 (percentage of identity was 97.41-97.76%). Comparison with all HA1 genes of SIVs in this thesis and vaccine strain (A/Michigan/45/2015), percentages of identity were 85.8-86.1%. For HA3 gene, five HA3 genes (S4, S6, S7, S12 and S13) of SIVs from this thesis were similar to A/swine/Thailand/CU-S14252N/2014 (percentage of identity were 97.59-98.12%). Another HA3 gene (S25) were similar to A/swine/Thailand/CU-S14129N/2013 (percentage of identity were 96.35%). Comparison with all HA3 genes of SIV from this

thesis and vaccine strain, percentage of identity were 86.9-86.4%. Previous findings show that vaccine for against human influenza could not fully protect rH3N2-SIV (Arunorat et al., 2017).

Follow antigenic site analysis, SIVs or Reassortment viruses in this thesis posed human-like characteristics, thus the viruses could infect and/or replicate in mammal host including human. Transmission of swine influenza to human was previously reported (Gray et al., 2007; Krueger and Gray, 2013; Bowman et al., 2017; Deng et al., 2020). Thus, transmission and infection of swine influenza to human should be concerned.

5.4 Genetic constellation of swine influenza viruses

In this thesis, there were 3 genotypes of SIVs; rH1N1 (pdm+1), rH1N1 (pdm+2) and rH3N2 (pdm+2). In Thailand at least 7 genotypes have been reported including eH1N1 (6+2), eH1N1 (7+1) rH1N1 (pdm+1), rH1N1 (pdm+2), rH1N2 (pdm+2), rH3N2 (pdm+2) and pdmH1N1 (Takemae et al., 2008; Charoenvisal et al., 2013; Nonthabenjawan et al., 2015). The genotypes rH1N1 (pdm+2) and rH3N2 (pdm+2) which identified in this study have been previously reported in Thailand indicating these genotypes are continuous circulating in a pig farm for several years. In contrast, novel genotype rH1N1 (pdm+1) has never been reported in this pig farm. Our result supported the reassortment or multiple-reassortment between eH1N1 (6+2) or rH1N1 (pdm+2) and pandemic H1N1. Reassortment of swine influenza virus have been reported worldwide such as in China (Lam et al., 2011; Cai et al., 2018), Europe (Zell et al., 2013) and America (Ducatez et al., 2011a; Rajão et al., 2017; Nelson et al., 2019). Our thesis supported and added evidences that pig play important role as mixing vessel for influenza virus.

Since all genotypes of Thai SIVs in this thesis contain internal genes (PB2, PB1, PA, NP, M and NS) from pandemic H1N1 2009. We suggested SIVs are fitness with those internal genes (Liang et al., 2014; Watson et al., 2015; Cao et al., 2019). It could be speculated that pandemic H1N1 2009 was circulating in a pig farm for a period of time. Thus, reverse zoonotic infection from workers to pigs in the pig farm is common. Based on our finding, monitoring of influenza virus in pigs and workers is important and should be routinely conducted. The management in pig farm is important for example personal hygiene and personal protective equipment (PPE) should be practiced and used in the farm for prevention and control of influenza virus transmission in pigs and human.

Conclusion

Swine influenza is one of the important zoonotic diseases. Swine influenza causes both pig production loss and public health problem. This thesis was aimed to survey the status of swine influenza virus infection and virus subtypes circulating in a pig farm as well as to monitor genetic diversity of swine influenza viruses isolated from a pig farm. During January 2017 to November 2018, 436 nasal swab samples were collected and tested for influenza A virus. Our result showed that 38.76% (169/436) of the samples were positive and suspected for influenza A virus by using real-time RT-PCR. Then 13.07% (57/436) of the samples could be isolated for influenza A virus. In this thesis, 24 SIVs were characterized by whole genome sequencing using next-generation sequencing. Moreover, 436 blood samples were also collected and testes for influenza antibodies. The HI test result showed that pigs had antibodies against H1N1, H3N2 and pdmH1N1 indicating exposure of the viruses (endemic Thai H1N1, endemic Thai H3N2 and pandemic H1N1 2009) in this pig farm.

The swine influenza viruses could be subtyped as SIV-H1N1 (n=16) and SIV-H3N2 (n=8). The SIV-H1N1 can be classified into 2 genotypes and SIV-H3N2 can be classified into 1 genotype. For SIV-H1N1, genotype rH1N1 (pdm+2) contained HA and NA genes from endemic Thai SIV-H1N1, and the internal genes from pandemic H1N1-2009. Another genotype rH1N1 (pdm+1) which is the novel genotype or genetic constellation of SIVs ever reported in Thailand. The rH1N1 (pdm+1) contained HA gene from endemic H1N1 and the NA and internal genes from pandemic H1N1-2009. For SIV-H3N2, genotype rH3N2 (pdm+2) was previously observed in Thailand. The genotype rH3N2 (pdm+2) contained HA and NA genes from human-like swine and the internal genes from pandemic H1N1-2009. The results of genetic analysis showed that SIVs in this thesis posed most human-like characteristics and some avian-like characteristics.

Base on the result of this thesis, the recommendations to reduce the risk and transmission of swine influenza virus in a pig farm or other pig farms are as follow:

- Pig
    - Close housing system (if possible) should be used
    - Separate equipment for each pig housing should be used
    - Separate workers of each pig houses or cleaning washing between pig houses should be practiced.
    - Monitor piglets and weaning pigs should be performed, since piglets and weaning pigs (high risk population) are susceptible to swine influenza due to decreasing of maternal antibody and stress condition.
    - SIV testing and quarantine for new pigs e.g. new gilts should be routinely tested.
- Human
    - Personal hygiene and personal protective equipment should be practiced /used to reduce SIV transmission.
    - Influenza A virus vaccine (Flu vaccine) for human should be yearly administered for workers and farm staffs.
- Farm management
    - Biosecurity should be conducted to reduce the risk of SIV transmission.
    - Surveillance and monitoring genetic diversity of swine influenza viruses in pig farms should be routinely conducted.

Future studies for prevention and control of swine influenza in the pig farm should be focusing on the following:

- Investigating novel genotypes of swine influenza viruses in Thailand to monitor potential virulence viruses in human.

- Studying efficacy of human vaccine against swine influenza virus which can be used for human protection on swine influenza infection.
- Developing new vaccine against human and swine influenza for prevention and control of influenza pigs and human.
- Developing rapid diagnosis kit for using in the field.

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