ระบาดวิทยาเชิงโมเลกุลและวิวัฒนาการของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอและบี

นางสาวนิภาพร เทวาวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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MOLECULAR EPIDEMIOLOGY AND EVOLUTION OF

INFLUENZA A AND B VIRUSES

Miss Nipaporn Tewawong

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Science
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Thesis Title	MOLECULAR EPIDEMIOLOGY AND EVOLUTION OF INFLUENZA A AND B VIRUSES
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Field of Study	Medical Science
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นิภาพร เทวาวงค์ : ระบาดวิทยาเชิงโมเลกุลและวิวัฒนาการของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอและบี (MOLECULAR EPIDEMIOLOGY AND EVOLUTION OF INFLUENZA A AND B VIRUSES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ. ยง ภู่วรวรรณ, หน้า.

ไวรัสไข้หวัดใหญ่เป็นสาเหตุสำคัญในการเกิดโรคระบบทางเดินหายใจชนิดรุนแรง โดยทั่วโลกพบอัตราป่วยเฉลี่ย 3-5 ล้านราย อัตราตาย 250.000-500,000 ราย และเข้ารับการรักษาตัวในโรงพยาบาล 200,000 รายต่อปี การป้องกันการติดเชื้อไวรัสที่มีประสิทธิภาพที่สุดและลดความรุนแรงของโรค คือการได้รับวัคซีนไวรัสไข้หวัดใหญ่ประจำปีหรือการใช้ยาต้านไวรัสในกลุ่มนิวรามินิเดสอินฮิบิเตอร์ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาระบาดวิทยาเชิงโมเลกุลและวิวัฒนาการของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอและบีในประเทศไทยปีพ.ศ.2553-2558 โดยพัฒนาวิธี real time PCR และ melting curve analysis ในการวินิจฉัยแยกสายพันธุ์ (lineage) ของเชื้อไวรัสไข้หวัดใหญ่ชนิดบี วิธีนี้มีความไว 100 copies ต่อไมโครลิตร ตรวจตัวอย่างจากระบบทางเดินหายใจของผู้ป่วยที่ติดเชื้อไวรัสไข้หวัดใหญ่ชนิดบีในปีพ.ศ.2553-2555 พบว่าเป็นสายพันธุ์วิคตอเรียมากกว่ายามากาตะร้อยละ 83.5 และ 16.5 ตามลำดับ ปีพ.ศ.2556 ไม่พบการระบาดของสายพันธุ์วิคตอเรีย เริ่มพบการระบาดอีกครั้งในปี พ.ศ.2557 ร้อยละ 22.5 ในขณะที่พบสายพันธุ์ยามากาตะร้อยละ 77.5 และในปี พ.ศ.2558 พบสายพันธุ์วิคตอเรียมากกว่า (ร้อยละ 62.3) ศึกษาความหลากหลายระดับเชิงโมเลกุลของจีโนมไวรัสไข้หวัดใหญ่ชนิดบีโดยวิธี PCR-sequencing และการวิเคราะห์ด้วยชีวสารสนเทศศาสตร์ พบการผสมกัน (reassortment) ระหว่างยีน HA และ NA จำนวน 5 ตัวอย่าง (ร้อยละ 6.8) นอกจากนี้เมื่อศึกษาการกลายพันธุ์ของยีน HA ในไวรัสไข้หวัดใหญ่ชนิดเอ H3N2 และ เอ (H1N1)pdm09 ด้วยวิธี $P_{epitope}$ model พบว่า ไวรัสไข้หวัดใหญ่ชนิดเอ (H1N1)pdm09 มีการกลายพันธุ์น้อยกว่าชนิดเอ H3N2 และมีประสิทธิภาพของวัคซีนมากกว่า ความหลากหลายของยีน HA1 ของไวรัสไข้หวัดใหญ่ชนิดเอ H3N2 เกิดจากการระบาดของไวรัสมากกว่า 1 เคลด ในปีพ.ศ.2557-2558 ทำให้ประสิทธิภาพของวัคซีนไข้หวัดใหญ่ประจำปีนั้นลดลง และบ่งชี้ให้เห็นถึงการเปลี่ยนแปลงแอนติเจน (HA) ของไวรัสไข้หวัดใหญ่ชนิดเอ H3N2 ในช่วงเวลาดังกล่าว ในขณะที่เมื่อศึกษาวิวัฒนาการเชิงโมเลกุลของยีน NA ในไวรัสไข้หวัดใหญ่ชนิดเอและบีที่พบในประเทศไทย รวมทั้งตรวจหาตำแหน่งการกลายพันธุ์ของโปรตีน NA ที่ส่งผลต่อความไวต่อยาต้านไวรัสไข้หวัดใหญ่ในกลุ่มนิวรามินิเดสอินฮิบิเตอร์ พบว่ายีน NA ของไวรัสไข้หวัดใหญ่ชนิดเอมีการกลายพันธุ์ในระดับนิวคลีโอไทด์และวิวัฒนาการที่รวดเร็วกว่าชนิดบี แต่มีความหลากหลายของระดับยีนน้อยกว่าไวรัสไข้หวัดใหญ่ชนิดบี แสดงให้เห็นถึงความสัมพันธ์ที่แปรผกผันกันระหว่างอัตราวิวัฒนาการและความหลากหลายทางพันธุกรรมของยีน NA นอกจากนี้ยังพบการกลายพันธุ์ของโปรตีน NA ที่อาจส่งผลต่อความไวต่อยาในกลุ่มนิวรามินิเดสอินฮิบิเตอร์ บริเวณตำแหน่งที่ I222V และ S331G ของไวรัสไข้หวัดใหญ่ชนิดเอ H3N2 และตำแหน่งที่ A395T/D/V และ D342S ของไวรัสไข้หวัดใหญ่ชนิดบี ศึกษาการกลายพันธุ์ของโปรตีน NA บริเวณตำแหน่งดังกล่าวโดยการสร้างรีคอมบิแนนท์ไวรัสโดยใช้เทคนิค 7+1 reverse genetics เพื่อตรวจหาประสิทธิภาพการทำงานของเอนไซม์ NA การยับยั้งการทำงานของเอนไซม์โดยยาในกลุ่มนิวรามินิเดสอินฮิบิเตอร์ เสถียรภาพของยีน NA และประสิทธิภาพการเจริญของรีคอมบิแนนท์ไวรัสในหลอดทดลอง จากผลการวิจัยพบว่ารีคอมบิแนนท์ที่มียีน NA ที่สนใจนั้น มีความไวต่อยาในกลุ่มนิวรามินิเดสอินฮิบิเตอร์เป็นปกติ ประสิทธิภาพของเอนไซม์ NA ในรีคอมบิแนนท์ไวรัสและไวลด์ไทป์ไวรัส มีความแตกต่างกัน ไวรัส A/PR8-S331G/R NA มีค่าของ K_m และ V_{max} ที่เพิ่มขึ้น ในขณะที่ไวรัส B/Yam-D342S NA มีค่า K_m และ V_{max} ลดลง นอกจากนี้ไวรัส B/Yam-A395V และ D342S NA พบว่ามีการกลายพันธุ์ที่ตำแหน่งกรดอะมิโนดังกล่าวกลับไปเหมือนกับไวรัสสายพันธุ์ไวลด์ไทป์หลังจากมีการเพิ่มจำนวนในเซลล์เพาะเลี้ยงชนิด MDCK เป็นจำนวน 3 รอบ ซึ่งเป็นตัวบ่งชี้ว่า A395 หรือ D342 ในยีน NA มีความเหมาะสมต่อการเจริญของไวรัสในเซลล์มากกว่า V395 หรือ S342 การเปลี่ยนแปลงกรดอะมิโนของโปรตีน NA ในไวรัสไข้หวัดใหญ่ชนิดเอและบีอาจส่งผลต่อคุณสมบัติของเอนไซม์ NA และการเพิ่มจำนวนของไวรัสในเซลล์ กล่าวโดยสรุปงานวิจัยนี้แสดงให้เห็นถึงวิวัฒนาการของจีโนมไวรัสไข้หวัดใหญ่ชนิดเอและบี โดยเฉพาะอย่างยิ่งยีน HA และ NA การศึกษาวิวัฒนาการของจีโนมและการเฝ้าระวังทางระบาดวิทยาของไวรัสไข้หวัดใหญ่อย่างต่อเนื่องจะช่วยในการควบคุมและป้องกันการระบาดของเชื้อไวรัสดังกล่าวได้อย่างมีประสิทธิภาพ

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NIPAPORN TEWAWONG: MOLECULAR EPIDEMIOLOGY AND EVOLUTION OF INFLUENZA A AND B VIRUSES. ADVISOR: PROF. YONG POOVORAWAN, M.D., pp.

The influenza viruses are a major cause of the respiratory severe illness about 3 to 5 million cases and contribute to substantial over 250,000-500,000 death and 200,000 hospitalizations annually worldwide. The influenza vaccination and antiviral prophylaxis are the effective way to prevent the infection and reduce the severity of the disease burden. The aim of the study was to elucidate the epidemiology and molecular evolution of influenza A and B viruses in Thailand between 2010 and 2015. The real time PCR with melting curve analysis for differentiation the lineage specific of influenza B viruses was developed and validated. The lower detection limit was 100 copies per microliter. Using this assay, during 2010 to 2012, there were more B/Vic strains than B/Yam strains (83.5% vs. 16.5%, respectively). B/Vic strains were not detected between February 2013 and the end of 2014. In 2015, B/Yam strains were more prevalent than B/Vic strains (77.5% vs. 22.5%, respectively), while B/Vic dominated the first half of 2016 (62.3%). In addition, the whole genome of influenza B viruses was characterized using PCR-sequencing and bioinformatics analysis. The results found 5 influenza B strains (6.8%) were of mixed lineages between HA and NA genes. Moreover, this study also examined the genetic variability in the nucleotide of encoding HA1 sequences and quantify the antigenic drift of the HA1 domain protein among influenza A(H3N2) and A(H1N1)pdm09 viruses using $P_{epitope}$ model. The vaccine efficacy for A(H1N1)pdm09 (79.6–93.4%) was generally higher than that of A(H3N2). The emergence of multiple circulating strains of A(H3N2) in 2014-2015 seasons contributed to the reduced vaccine efficacy in Thailand that year. These findings further confirmed the accelerating antigenic drift of the circulating influenza A(H3N2) during this period. Finally, the study was to investigate the molecular evolution of NA gene and examine for the presence of NA substitutions associated with reduced susceptibility to NAIs among seasonal influenza A and B viruses identified in Thailand. The evolutionary patterns of the NA gene indicated a more rapid genetic drift for influenza A than influenza B virus due to higher nucleotide substitution rate, although there was more genetic diversity in influenza B than in influenza A virus. There was an inverse relationship between the evolutionary rate and genealogical diversity. The results found different NA amino acid substitutions at the same residues causing reduced inhibition of influenza A (H3N2) (I222V and S331G) and B (A395T/D/V and D342S) viruses by NAIs. The recombinant influenza viruses containing these NA mutations were generated using 7+1 reverse genetic system for examining the NA activity and thermostability, NA enzyme inhibition by NAIs, NA enzyme kinetic, genetic stability of NA, and establish influenza A(H3N2) and B virus replication kinetic *in vitro*. All of recombinant influenza A(H3N2) and B viruses carrying NA substitutions were susceptible to NAIs. There was a difference between wild type and recombinant viruses in NA activity and thermostability. The A/PR8-S331G/R NA viruses revealed increasing of K_m and V_{max} values, while B/Yam-D342S NA virus contributed to the K_m and V_{max} values were decreased. The stability of B/Yam-A395V and D342S NA were not stable after three passages in vitro, indicating A395-NA or D342-NA are more suitable in the viral fitness than V395 NA or S342. This finding suggests that NA substitutions of influenza A and B viruses may affect to NA enzyme properties and *in vitro* virus replication. In conclusion, this study demonstrated the ongoing evolution of genome of influenza A and B viruses, especially HA and NA genes. Continual monitoring of evolutionary dynamics of influenza genome and epidemiological surveillance will assist public health for effective control and prevention influenza infection.

Field of Study: Medical Science   Student's Signature --------------------------
Academic Year: 2016   Advisor's Signature --------------------------

ACKNOWLEDGEMENTS

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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
RNA	=	Ribonucleic acid
nm	=	nanometer
mRNA	=	messenger Ribonucleic acid
kDa	=	Kilo Dalton
PB2	=	Polymerase Basic protein 2
PB1	=	Polymerase Basic protein 1
PA	=	Polymerase Acidic protein
HA	=	Hemagglutinin
NP	=	Nucleoprotein
NA	=	Neuraminidase
M	=	Matrix
NS	=	Non-structural protein
IAV	=	Influenza A virus
vRNP	=	viral Ribonucleoprotein
pH1N1	=	pandemic H1N1
pdm09	=	pandemic 2009
μl	=	Microliter
μM	=	Microgram
bp	=	Base pair
PCR	=	Polymerase Chain Reaction
RT-PCR	=	Reverse Transcriptase Polymerase Chain Reaction
cDNA	=	complementary Deoxynucleic acid
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
H_2O	=	Water
dNTP	=	Deoxynucleotide
C_t	=	Threshold cycle
T_m	=	Melting Temperature
Vic	=	Victoria

Yam	=	Yamagata
GISAID	=	Global Initiative on Sharing Avian Influenza Data
NCBI	=	National Center for Biotechnology Information
WHO	=	World Health Organization
CDC	=	Centers for Disease Control and Prevention
IRB	=	Institutional Review Board
ILI	=	Influenza Like Illness
ECDC	=	European Centre for Disease Prevention and Control
VE	=	Vaccine Efficacy
NAIs	=	Neuraminidase Inhibitors
HA titer	=	Hemagglutination titer
WT	=	Wild type
MOI	=	Multiplicity of infection
h	=	Hour
min	=	Minute
MUNANA	=	2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic aci

CHAPTER I
Introduction

The world Health Organization (WHO) revealed that the influenza viruses are a major cause of the severe illness about 3 to 5 million cases and contribute to substantial over 250,000-500,000 death and 200,000 hospitalizations annually worldwide (Thompson et al., 2003). The attack rate of influenza estimated at 20-30% among children group, while the incidence rate in adult group was found to be 5-10% (Thompson et al., 2004). In addition, the total economic burden of annual influenza epidemics using projected statistical life values amounted to 87.1 billion US dollar (Molinari et al., 2007). Some people, such as children younger than 5 years old, people 65 years and older, people with underlying disease (such as asthma, diabetes, or heart disease), and pregnant women are high risk for serious influenza complications. The examples of complication related influenza burden are sinusitis, otitis media, bronchitis, and pneumonia, which can be life-threatening and result in death (Rothberg, Haessler, & Brown, 2008). The influenza A/H3N2, A/H1N1, and B viruses have globally co-circulated in human since 1977. The influenza vaccination is the effective way to prevent the infection and reduce the severity of the disease burden. Under the selective pressure from the human immune system, antigenic epitopes of hemagglutinin (HA) continually evolved, which contributes to the emergence of new virulent strains. Thus, the seasonal influenza vaccine is reformulated annually based on the antigenicity prediction of the anticipated upcoming virus strains. The surveillance of HA gene sequencing analysis can be lead to influenza vaccine selection process when it is combined the serological antigenic analysis. Anti-influenza prophylaxis and treatments are empirical strategy to control and prevent influenza infection particularly when the vaccination was ineffective or unavailable. Nowadays, neuraminidase inhibitors (NAIs) are recommended for treating the influenza infection. However, recently antiviral influenza surveillance demonstrated that the prevalence of NAIs resistant influenza strain was increased. The information about the vaccine efficacy and antiviral influenza surveillance in Thailand

were underdetermined. Therefore, the continuing evolution of HA and neuraminidase (NA) genes of seasonal influenza A(H3N2), A(H1N1) pdm09, and influenza B will be elucidated.

Currently, the knowledge of the evolution of influenza virus is improved by the several of molecular approach such as complete genome sequencing, epidemiological modeling, and antigenic mapping (Nelson & Holmes, 2007). In addition, recent studies revealed that the evolution dynamics of influenza virus might be more complicate than was previously realized. Therefore, the integrate interplay between the phylogeny, antigenic variation (Smith et al., 2004), the selection-driven cluster jumps (Wolf, Viboud, Holmes, Koonin, & Lipman, 2006), the migration and reassortment event among multiple co-circulating lineages of the same subtype (Koelle, Cobey, Grenfell, & Pascual, 2006; Nelson et al., 2006) will provide the understanding about the evolution of influenza virus. Additionally, this study developed the real time PCR with melting curve analysis to identify the lineage-specific of influenza B and consequently to determine the match ratio between the circulating influenza B strains and vaccine strains during this time period.

Therefore, this study hypothesized as follows:

1. Identification the prevalence of influenza B lineage is crucial for optimal selection of the strain to be included in the annual vaccine.

2. The HA1 domain of influenza A(H3N2) has higher antigenic drift than A(H1N1)pdm09.

3. There are different evolutionary patterns of NA gene and NA enzymatic properties between influenza A and B viruses.

Part 1: Molecular epidemiology and evolution of influenza B virus

Research questions

1. What is the prevalence of lineage-specific influenza B circulating in Thailand?
2. How much the percentage of matching ratio between the circulating influenza B strains and vaccine B strains?
3. What is the difference of genome signatures between circulating influenza B virus in Thailand and vaccine strains?
4. What is the genetic evolutionary profile and reassortment event of influenza B virus in Thailand?

Objectives

1. To develop the real-time PCR using SYBR green I dye with melting curve analysis for identifying the lineage-specific influenza B virus from respiratory specimens.
2. To investigate the matching ratio between the circulating strains and vaccine strains of influenza B.
3. To determine the prevalence of influenza B virus infection in Thailand from individuals with influenza-like illness.
4. To characterize whole genome of influenza B viruses on the basis of phylogenetic topology and pairwise amino acid variations.

Part 2: Assessing antigenic drift of human seasonal influenza A virus

Research questions

1. What is epidemiology pattern of seasonal human influenza A virus in Thailand?

2. What is the evolution dynamics of HA gene of human seasonal influenza A viruses?

3. How antigenic drift of HA1 of influenza A viruses affect the vaccine efficacy.

Objectives

1. To examine the evolutionary patterns and epidemiological dynamics of influenza A virus circulating in Thailand highlighting in genetic and antigenic variation, rate of nucleotide substitution, natural selection, and complete profile of proteins variability.

2. To assess the influenza A antigenic drift between the vaccine strain and the circulating strain using by the number of amino acid changes in the dominant epitope ($P_{epitope}$ model)

Part 3: Evolution of the neuraminidase gene and antiviral drug susceptibility of seasonal influenza A and B viruses

Research questions

1. What is the evolution dynamics of NA genes of human seasonal influenza A and B viruses?

2. What are the patterns of NAIs resistance among influenza A and B viruses in Thailand?

3. What is the effect of NA substitutions of influenza A and B viruses that found in Thailand on the level of susceptibility by NAIs, NA enzymatic properties, and viral replication *in vitro*?

Objectives

1. To determine the evolution dynamics of NA gene of influenza A and B viruses circulating in Thailand in term of rate of nucleotide substitution, selection pressure, and relative genetic diversity.

2. To investigate the mutations of NA gene of influenza A and B viruses that are associated with reduced inhibition by NAIs

3. To generate the recombinant influenza A and B viruses carrying the interested NA substitutions using 7:1 reverse genetic systems for assessing NA activity and thermostability, NAIs susceptibility pattern, and replication efficiency of virus in MDCK cells.

Conceptual framework
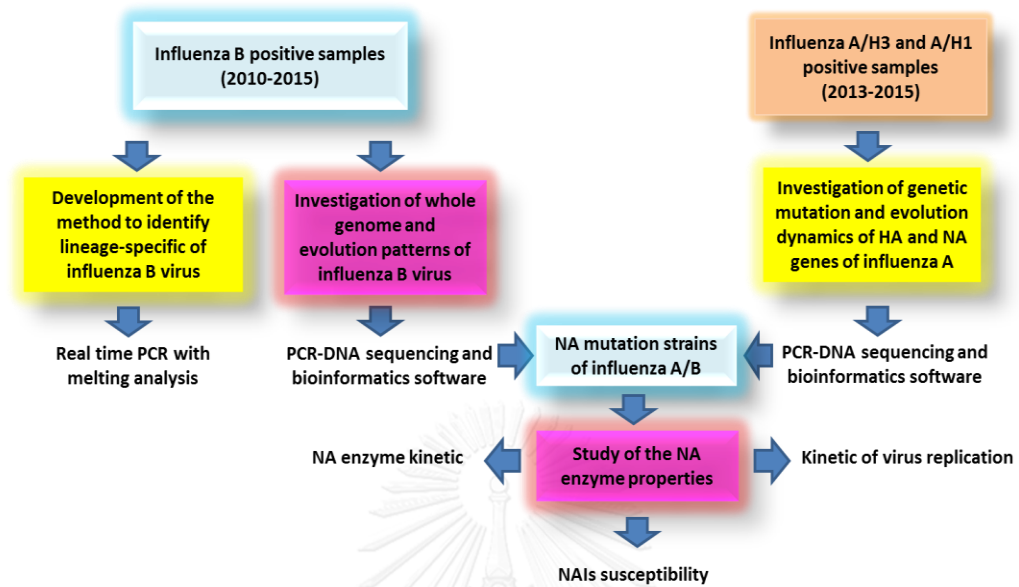
CHAPTER II
Review of related literatures

Influenza (Latin: influentia, meaning "influence"), commonly known as flu, is an infectious disease by RNA viruses, which named influenza. Influenza can be infected avian and mammals and transmitted by three main ways: direct transmission, the airborne route, and contact with body fluid secretion (saliva, respiratory secretions, feces, and blood) or with contaminated surfaces. In general, the influenza virus shedding increases 24 to 36 hours after infection, peaks on hour 48 and can persist as long as 9 days (an average duration of 5 days) (Carrat et al., 2008). The most common symptoms of flu are fever (>38 ℃), cough, headache, body ache, runny nose, and other respiratory symptoms. In children, gastrointestinal symptoms are found such as diarrhea and abdominal pain (F. M. Munoz, 2002; Richards, 2005). Influenza is enveloped RNA virus and sensitive to disinfectants and detergents. Therefore, frequently wash hand with soap or alcohol gel can be reduced the risk of infection.

Taxonomy of influenza viruses

Family *Orthomyxoviridae* (orthos, Greek for "straight"; myxa, Greek for "mucus") are negative single-stranded RNA that comprises of the six genera including Influenza virus A, Influenza virus B, Influenza virus C, Isavirus (infectious salmon anemia virus), Thogotovirus (arboviruses) and Quaranjavirus (*International Committee on Taxonomy of Viruses Index of Viruses — Orthomyxovirus* 2006). The first three genera are major cause of influenza illness in humans including the others animals. Influenza A viruses are identified from types B and C on the basis of the characteristic of nucleoprotein (NP) and matrix (M1), which are the major internal protein antigen of the viruses. The influenza A is a widest range of the host such as human, avian, equine, swine, and canine, whereas influenza B can be infected in human, seal and ferret. Influenza C is less common than the other types, which usually causes mild disease in children, including swine and canine.

Genome structure and protein function

Influenza A and B viruses have a similar structure, while influenza C is more different. The influenza A and B usually found round but can be filamentous. The virion is a small spherical shape size about 80-120 nm in diameter and they have a lipid bilayer envelope. The influenza A and B have 13 kilo bases minus single stranded RNA genome and they consist of unique eight segmented RNA: polymerase basic-1 (PB1), PB2, polymerase acidic (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural protein (NS). Each of segments is associated with the trimeric viral RNA polymerase (PB2, PB1, and PA) and coated with multiple nucleoproteins (NPs) to form the vRNPs. Whereas the surface of the lipid envelope is spiked with multiple copies of HA, NA and a small number of M2, the M1 molecules keep vRNPs attached to the inner layer (Fig 1a).

The IAV proteins have increased from 10 to 16 proteins (Muramoto, Noda, Kawakami, Akkina, & Kawaoka, 2013). As for IAV genome, the distinct mechanisms have evolved to increase the coding capacity, including alternative splicing (NEP, M2), +1 ribosomal frame shift (PA-X) or alternative ATG in-frame initiation (PB1-F2, PB1-N40, PA-N155, and PA-N182). In contrast, influenza B genome can be encoded 11 proteins using many processes of expression, including ribosomal termination-reinitiation (BM2), alternative ATG initiation (NB), and alternative splicing (NEP). The genome segments, encoded proteins and functions of influenza A and B viruses are summarized in table 1. This table was modified from El Zowalaty and colleagues study (El Zowalaty, Bustin, Husseiny, & Ashour, 2013).

Table 1. *The genome segments and encoded proteins of influenza A and B viruses (Chakrabarti & Pasricha, 2013; El Zowalaty et al., 2013; Hayashi, MacDonald, & Takimoto, 2015; Muramoto et al., 2013; Wise et al., 2009)*

RNA segment number	RNA segment (mRNA) length in nucleotides	Gene description	Encoded protein	Location and features	Protein length (amino acids)	Approximate number of molecules per virion	Protein functions
1	2341 (2320)	PB1	Polymerase basic 1, 87 kDa	Virion interior, infected cell nuclei	757	30-60	Heterotrimeric P complex related with NP and virion RNA, RNA transcription and RNA polymerase sub unit, initiation of transcription, RNA transcriptase, RNA elongation and endonuclease activity
		PB1-F2 (only FluA)	~10.5 kDa	Infected cell	Varying size depend on subtypes	30-60	Pro-apoptotic agent, regulate polymerase activity, deregulation of innate immune response, exacerbate viral pathogenicity, increased secondary bacterial infection
		PB1-N40	82 kDa	Infected cell	718		Interact with the other subunits of the polymerase complex,
2	2341 (2320)	PB2	Polymerase basic 2, 96 kDa	Virion interior, infected cell nuclei	759	30-60	Polymerase subunit: RNA transcriptase, host cell mRNA cap recognition and virulence
3	2233 (2211)	PA		Virion interior, infected cell nuclei	716	30-60	Polymerase subunit: RNA transcriptase and protease

RNA segment number	RNA segment (mRNA) length in nucleotides	Gene description	Encoded protein	Location and features	Protein length (amino acids)	Approximate number of molecules per virion	Protein functions
		PA-X	~27 kDa	Virion interior, infected cell nuclei	191		Promote viral growth, Inhibit the host innate and acquire immune response
		PA-N155	62 kDa		500		Support a role for viral replication cycle
		PA-N182	60 kDa		473		Support a role for viral replication cycle
4	1778 (1757)	HA	Hemagglutinin, 220 kDa homotrimer	Globular head domain (HA1) and : Virion envelope	329	500	Virus binding to sialic acid-containing receptors on host cell and major antigenic determinant
				Stem (HA2): transmembrane cytoplasmic tail	221	500	Penetration of virus genome into host cell cytoplasm by fusion of virus and host cell membrane
5	1565 (1540)	NP	Nucleoprotein, 55 kDa	Virion interior, integrated with P complex and viral RNA	498	1000	Interacts with RNAs and polymerase proteins as a major component of the nucleocapsid, RNA synthesis and RNA nuclear import regulation, role in maturation and packaging of virus
6	1413 (1392)	NA	Neuraminidase,	Virion	454	100	Surface glycoprotein: antigenic

RNA segment number	RNA segment (mRNA) length in nucleotides	Gene description	Encoded protein	Location and features	Protein length (amino acids)	Approximate number of molecules per virion	Protein functions
		NB (only FluB)	11 kDa, helix	Transmembrane	100	100	Putative viral proton channel. May play a role in virus entry.
7	1027 (1005)	*M1*	M1, 28 kDa	Beneath lipid bilayer of virion envelope	252	3000	Role in replication and virus assembly, modulating nuclear transport of vRNP between nucleus and cell surface in early and late infection, mediates association of RNP with HA and NA at the cell membrane, and promoting virion maturation
		M2 (only FluA)	M2, 15 kDa homotetramer	Virion envelope, infected cell surface; Extracellular, transmembrane, cytoplasmic tail	97	3000	Membrane cation channel activity, virus uncoating and assembly. Infected cell: to protect pH-sensitive HA from raises pH of Golgi. Virion: to dissociate vRNP from M1 by permit acidification of virus interior during passage through endosomal pathway
		BM2 (only FluB)					Capture the M1-vRNP complex at the virion budding site during virus assembly.

RNA segment number	RNA segment length in nucleotides (mRNA)	Gene description	Encoded protein	Location and features	Protein length (amino acids)	Approximate number of molecules per virion	Protein functions
8	890 (868)	NS1	NS1, 25 kDa dimer	Infected cell nuclei	230		Viral interferon antagonist protein, down-regulating dsRNA-induced antiviral response
							Regulation of host gene expression, inhibiting host mRNA translation
							Regulation viral pre-mRNA splicing and translation and viral polymerase activity
		NS2/NEP	NS2, 14 kDa	Cytoplasm of infected cells	121		Associated with core components of virion
							Mediates nuclear export of vRNPs

Replication cycle of influenza virus (Das, Aramini, Ma, Krug, & Arnold, 2010)

The viral surface glycoprotein HA specific binding to the host cell receptors (surface sialic acid), and the virus is entered to the host cell by endocytosis. The low pH in the endosome triggers the two major processes for vRNPs releasing: (I) conformational change in the HA protein that contributes to fusion of the viral and endosomal membranes and (II) the flow of protons into the virus via the M2 ion channel that lead to dissociating the vRNPs from M1 matrix proteins. Nucleoprotein of the virus has nuclear localization sequences (NLSs), when the M1 molecules are dissociated that result in vRNPs are transported into the host nucleus (Figure 1b). Subsequently, the PB2 subunit binds the 5′ cap of host pre-mRNAs, and the endonuclease domain in PA subunit cleaves the pre-mRNA 10–13 nucleotides downstream from the cap. The viral mRNA transcription is initiated from the cleaved 3′ end of the capped RNA segment. This process is called 'cap snatching' (Figure 1c). The viral mRNAs are transported to cytoplasm and viral proteins are translated. The HA, NA, and M2 are glycosylated in the Goigi body and transported to the host cell membrane (Fig 1d). The 3′-end processing of host mRNAs are inhibited by viral NS1 protein that lead to decreases of interferon-β synthesis (Figure 1e). The viral RNA replication in three steps: (−) vRNA → (+) cRNA → (−) vRNA. The nucleoprotein molecules are deposited on the cRNA and vRNA during RNA synthesis and subsequently transported to the cytoplasm (Figure 1f). Nuclear localization of M1 and NS2 proteins are important for the transportation of vRNPs from the nucleus to cytoplasm. The vRNPs-M1 complex is interacted with M1 proteins, cell membrane and then budded out. The NA of new particle viruses cleaves the sialic acid residues of cell membrane and releases the virus from the host cell (Figure 1g).
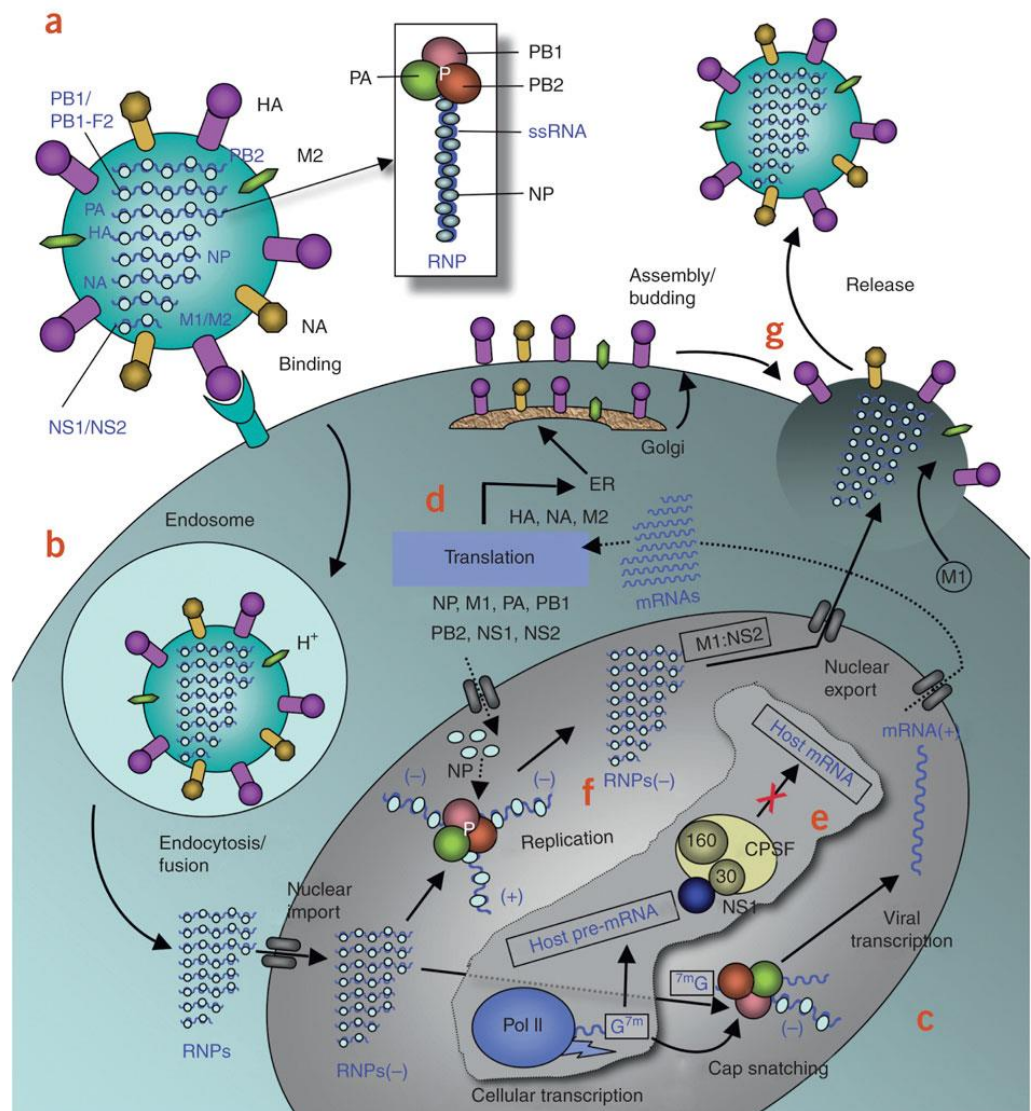
Figure 1. *Influenza A proteins structure and replication cycle (Das et al., 2010).*

Nomenclature

According to WHO recommendation, the system of influenza nomenclature consists of five parts: (I) a description of the antigenic type of the virus based on the antigenic specificity of the M and NP antigen, (II) the host of origin, (III) geographical origin, (IV) strain number, and (V) year of isolation e.g., A/Texas/1/77 (H3N2), B/England/5/66, C/Paris/1/67 ("A revision of the system of nomenclature for influenza viruses: a WHO memorandum," 1980).

Reservoirs of influenza viruses in the nature

The influenza A viruses infects a variety of animals, including domestic poultry, swine, equine, sea mammals and humans. The influenza A are divided into subtypes base on the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Currently, there are 18 different HA subtypes (H1 trough H18) and 11 distinct NA subtypes (N1 to N11) (Steinhauer & Skehel, 2002; Tong et al., 2012; Tong et al., 2013). The receptor binding efficiency of HA is specific to linkage of oligosaccharide of the receptor and it depends on the type of host sialic acid (Rogers & Paulson, 1983). Aquatic birds are natural reservoir host for influenza of all HA and NA subtypes (Webster, 1998), except H17 and H18 that was found in bats. The phylogenic studies of influenza A viruses demonstrated that species-specific lineages of viral genes and showed the evidences of interspecies transmission, which depends on the animal species (Webster, Bean, Gorman, Chambers, & Kawaoka, 1992) (Table 2). The H1, H2, and H3 are known to infect humans, while other subtypes such as H5, H6, H7 and H9 have the potential to cause human pandemics (Yen & Webster, 2009).

As for the influenza B viruses, they are genetically and antigenically characterized into two distinct lineages, B/Victoria/2/87-like and B/Yamagata/16/88-like (Kanegae et al., 1990a). Nowadays, Victoria and Yamagata lineages have continually co-circulated in many regions of the world (Rota et al., 1990).

Table 2. *Host range restriction of influenza A viruses*

HA subtype	Reservoir host	NA subtype	Reservoir host
H1	Avian, swine, human	N1	Avian, swine, canine, human
H2	Avian, human	N2	Avian, swine, human
H3	Avian, swine, equine, canine, human	N3	Avian, human
H4	Avian	N4	Avian
H5	Avian, feline (tiger / cat), human	N5	Avian
H6	Avian	N6	Avian
H7	Avian, equine, human	N7	Avian, swine, equine, human
H8	Avian	N8	Avian, equine, canine
H9	Avian, swine, human	N9	Avian
H10	Avian	N10	Bat
H11	Avian	N11	Bat
H12	Avian		
H13	Avian		
H14	Avian		
H15	Avian		
H16	Avian		
H17	Bat		
H18	Bat		

Evolution of influenza virus

The eight gene segments of influenza A and B continually evolve especially the two surface glycoproteins: HA and NA genes. The accumulation molecular changes of influenza genome contribute to genetic and antigenic variability. The influenza evolution can occur by different mechanisms including (I) point mutations

(antigenic drift), (ii) gene reassortment (genetic shift), (iii) defective-interfering particles, and (iv) RNA recombination (Webster et al., 1992). Genetic mutation, insertion, deletion and substitution are one of the most crucial mechanisms for antigenic drift, which produce genetic and antigenic variation in influenza viruses. The main reason of genetic mutation in RNA viruses is the lacking of proofreading among RNA polymerases that leads to RNA replication errors on the 1 in 10^4 bases (Holland et al., 1982; Steinhauer & Holland, 1987). This range approximately correspondents to the fidelity of the polymerase used in replication. RNA viruses (which use RNA-dependent RNA polymerases; RdRps) mutate faster than retroviruses (with RNA-dependent DNA polymerases (RdDps) or reverse transcriptases (Rts)), which mutate faster than DNA viruses (with DNA polymerases) (Duffy, Shackelton, & Holmes, 2008). Each round of RNA viral replication contributes to a mixed population with many variants, some of which have potentially beneficial mutations that can become dominant under the right selective conditions. The number of genetic errors (by genetic drift) can be referred mutation rate that accumulate per unit time. Mutation rate can be difficult to examine because it requires the details of viral replication (Drake, 2007). Estimating rates of nucleotide substitution by computational biology is alternative way. The rate of nucleotide substitution is defined as the number of fixed mutation changes (by natural selection or genetic drift) per nucleotide site, per unit time (commonly years) (Duffy et al., 2008). The Bayesian Markov chain Monte Carlo (MCMC) coalescent framework, as manifest in the BEAST (Bayesian evolutionary analysis by sampling trees) package are commonly used to estimate the rate of nucleotide substitution (Drummond & Rambaut, 2007).

The segmented genome of influenza viruses facilitates reassortment between isolates (intra- or inter-subtypes) that co-infection the same host cells. This mechanism is called "antigenic shift". Reassortment rapidly produces genome diversity and it occurs among influenza A in the nature. Reassortment among HA and NA subtypes was appeared in the human pandemic of pandemics of 1957 (H2N2 subtype) and 1968 (H3N2 sub-type), which also acquired a new basic polymerase 1 (PB1) segment (S. E. Lindstrom, Cox, & Klimov, 2004). Since the seasonal influenza (A/H1 and A/H3 viruses have been co-circulated over 40 years and extend evidence

of mixed infection (Ghedin et al., 2009), reassortment among them is rare (Xu et al., 2002). As for the influenza B virus, the previous studies on the evolution profiles of NP, M, and NS genes demonstrated that have multiple lineages and frequent reassortment among co-circulating viruses contribute to protein variability (S. E. Lindstrom et al., 1999). Early studies, reassortment is commonly detected through incongruences in phylogenetic relationships among the different segments of a viral genome. Nowadays, the several methods of applied phylogenetic have been estimated past reassortment of viral lineages, including the multi-dimensional scaling of tree distances (Vijaykrishna et al., 2011) or the coalescent-based Bayesian phylogenetics that infer and compare the time of most recent common ancestor (TMRCA) of each segment to infer possible reassortment (Rambaut et al., 2008).

The defective-interfering particles mechanism can affect evolution by reducing the yields of non-defective particles and modifying pathogenicity (Steinhauer & Holland, 1987). Another mechanism is intramolecular recombination in segmented RNA. This process is rare, previous studies revealed one instance of insertion of cellular mRNA sequences into the HA gene with acquisition of virulence (Khatchikian, Orlich, & Rott, 1989).

Epidemic and pandemic influenza

The antigenic drift of influenza viruses slowly emerge an increasing variety of strains until one evolves that can infect people who are immune to the pre-existing strains. The new variant strains replace the older strains in human population at the rapid sweep, causing influenza epidemic (Wolf et al., 2006). Some new strains may be similar to the older stains, thus some person will still immune against them. In contrast, the reassortment influenza viruses possess completely new antigens, thereby antigenic shift such as the reassortment between avian and human strains (H5N1, H7N9) or swine and human strains (H1N1, H1N2). All people will be susceptible to new antigens and the novel influenza will rapidly and uncontrollably spread, causing influenza pandemic (Parrish & Kawaoka, 2005).

Influenza pandemic viruses have been isolated since 1957, but the details of known subtypes or genetic composition of the before 1918 remain unclear. The

genetic relationship between human and swine of all influenza pandemic viruses during 1918 to 2009 are showed in figure 2 (Taubenberger & Kash, 2010).
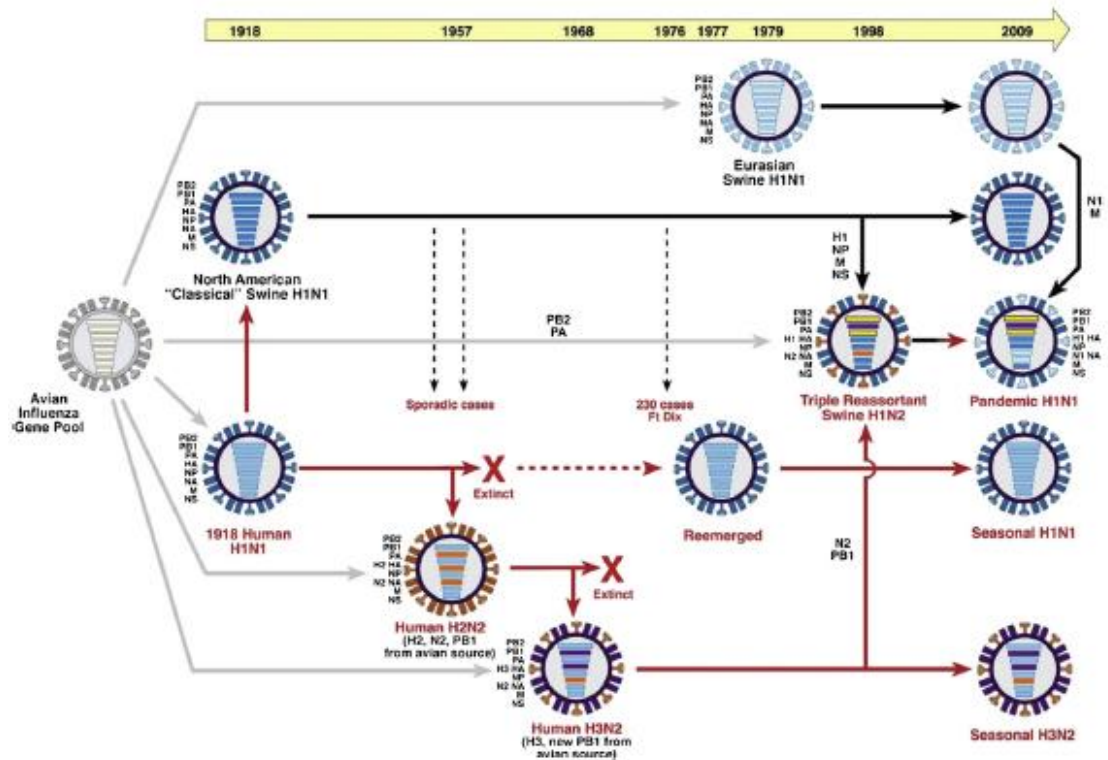
Figure 2. *The genetic relationship between human and swine influenza viruses, 1918 – 2009 (Taubenberger & Kash, 2010).*

The "Spanish flu pandemic" is the most well-known and high mortality rate during in 1918 to 1919. The influenza A(H1N1) is caused of this outbreak and approximately killed human 50 to 100 million persons (Patterson & Pyle, 1991). The large number of death was caused by highly infection rate more than 50% and more complicated from secondary bacterial pneumonia. In addition, the 1918 flu can be induced cytokine storms that contributes to the patients have extreme severity symptoms (Patterson & Pyle, 1991). Later flu pandemics were not so devastating. They included the 1957 Asian Flu (type A, H2N2 strain) and the 1968 Hong Kong Flu (type A, H3N2 strain) Russian flu (type A, H1N1 strain) and pandemic 2009 flu (type A, H1N1 strain), but even these smaller outbreaks killed millions of people.

Antibiotic were available to treat secondary bacterial infections and helped reducing the mortality rate compared to previous pandemic Spanish Flu of 1918.

Influenza activity and vaccination

Influenza A and B vaccine were created in the 1940s. These vaccines were produced in embryonated chicken eggs and consisted of crude purified whole virus inactivated with formalin and phenylmercuric nitrate, which is called whole virus inactivated vaccines (Francis, Salk, Pearson, & Brown, 1945). This vaccine was reformulated every 2 years by WHO recommendation because of antigenic drift (Payne, 1953). Since 1968 the trivalent inactivated vaccines (TIVs) was developed (Allison, Glezen, Taber, Paredes, & Webster, 1977). WHO makes recommendation for two vaccine formulation every year: northern and southern hemisphere because the influenza activity peaks different period and epidemic season. In addition, live attenuated influenza vaccine has been available in United States in 2003 and this vaccine is administered by nasal spray (Jin & Subbarao, 2015). In Thailand, the trivalent influenza vaccine (TIV) is being used, which consists of inactivated virus with HA from types A/pH1N1 and A/H3N2, and one type B that best match the predicted circulating strains. From 2010-2012, Thailand's government purchased 8.18 million doses of influenza vaccine which are provided free of charge to eight high-risk groups: healthcare personnel, poultry cullers, persons aged $\geq$ 65 years, persons with chronic disease, pregnant women, obese persons, mentally disabled persons, and children aged 6 months to 2 years. From this periods, influenza vaccine coverages were 0.1% - 20% among high-risk population in Thailand (Owusu et al., 2015).

Antiviral drugs treatments

Persons at higher risk for influenza complications who are recommended for antiviral treatment in example children aged younger than 2 years, elderly aged 65 years and older, and persons with underlying disease or immunocompromised host. The secondary pneumonia is major complication of influenza infection that contributes to severe symptoms, causing of death. The three classes of antiviral drugs used against influenza are neuraminidase inhibitors (oseltamivir, zanamivir, and peramivir), M2 protein inhibitors (adamantane derivatives), and guanosine (ribonucleic) analog (ribavirin). The antiviral agent for influenza A and B infection

and indications was shown in **table 3** (Glezen, 2008; Hata, Akashi-Ueda, Takamatsu, & Matsumura, 2014). The recent season, there continues to be high levels of resistance (>99%) to adamantanes among influenza A (H3N2) and influenza A (H1N1) pdm09 ("2009 H1N1") viruses. Therefore, adamantane derivatives not recommend for influenza A treatment. Antiviral drugs can lessen symptoms and shorten the time you are sick by 1 or 2 days. Moreover, the drugs also can prevent serious flu-related complications (like pneumonia).

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Table 3 The antiviral agent for influenza A and B infection and indications.

Drug	Trade name - Manufacturer	Formulation	Dosage (aged ≥ 12 years)		Effectiveness	Percentage of side effects	Common side effects
			Treatment	Prevention			
Oseltamivir	Tamiflu, Roach	75 mg capsule	1 capsule twice daily/5 days	1 capsule/day	Influenza A and B	5 - 10	Nausea, vomiting
Zanamivir	Relenza, GlaxoSmithKline	5 mg per inhalation	2 inhalations twice daily/5 days	2 inhalations /day	Influenza A and B	<1	Brochospam
Peramivir	Rapivab, BioCryst Pharmaceuticals	200 mg per 20 mL	600 mg IV single dose/5-10 days (≥ 18 years)	Not applicable	Influenza A(H1N1) pdm09	0.5 - 11	Diarrhea, vomiting, nausea, and neutrophil count deceased
Amantadine	Symmetral, Endo Pharmaceuticals	100 mg tablet	1 tablet twice daily/3-5 days	1 tablet/day	Influenza A	0-70	Central nervous system effects (seizures in patients seizure disorder, insomnia, anorexia
Rimantadine	Flumadine, Forest laboratories	100 mg tablet	1 tablet twice daily/5 days	1 tablet/day	Influenza A	0-3	Nausea, insomnia
Ribavirin	Virazole	60 mg/ml in reservoir	Aerosol for 2 hr every 8 hr for 5 days or as indicated	Not applicable	Influenza A and B	-	-

Table 3. The antiviral agent for influenza A and B infection and indications

The first objective of this thesis was to develop the rapid method to identify the lineage-specific influenza B viruses and characterize the epidemiology and whole genome of influenza B viruses. The second aim of study was to investigate the interplay between epidemiology, phylogeny, natural selection and mutation rate of HA gene and assess the antigenic drift among seasonal influenza A viruses in Thailand. The third purposive was to investigate the molecular evolution of NA gene and examine for the presence of NA substitutions associated with reduced susceptibility to NAIs among seasonal influenza A and B viruses identified in Thailand. This study helped to better understand virus evolution and epidemiology dynamics for providing surveillance data of antiviral resistant influenza strain and guidance the selection of antiviral drugs for clinical treatment including preparedness for possible future pandemic outbreaks of influenza.

This thesis divided into three parts as follows;
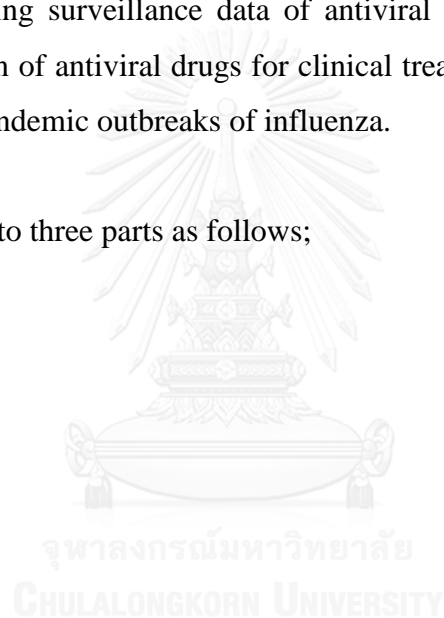
CHAPTER III

DEVELOPMENT OF REAL-TIME PCR WITH MELTING CURVE ANALYSIS FOR IDENTIFYING THE LINEAGES SPECIFIC OF INFLUENZA B VIRUS

(Part 3.1)

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CHAPTER III

Part 3.1: Lineage-specific detection of influenza B virus using real-time polymerase chain reaction with melting curve analysis

Summary

Influenza B viruses comprise two lineages, Victoria (B/Vic) and Yamagata (B/Yam), which co-circulate globally. The surveillance data on influenza B virus lineages in many countries often underestimate the true prevalence due to the lack of a rapid, accurate, and cost-effective method for virus detection. We have developed a real-time PCR with melting curve analysis for lineage-specific differential detection of influenza B virus. By amplifying a region of the hemagglutinin gene using real-time PCR with SYBR Green I dye, B/Vic and B/Yam could be differentiated based on their melting temperature peaks. This method was efficient (B/Vic = 93.2 %; B/Yam 97.7 %), sensitive (B/Vic, 94.6 %; B/Yam, 96.3 %), and specific (B/Vic, 97.7 %; B/Yam, 97.1 %). The lower detection limit was 102 copies per microliter. The assay was evaluated using 756 respiratory specimens that were positive for influenza B virus, obtained between 2010 and 2015. The incidence of influenza B virus was approximately 18.9 % of all influenza cases, and the percentage was highest among children aged 6–17 years (7.57 %). The overall percentage of mismatched influenza B vaccine was 21.1 %. Our findings suggest that real-time PCR with melting curve analysis can provide a rapid, simple, and sensitive lineage-specific influenza B virus screening method to facilitate influenza surveillance.

Introduction

Influenza viruses, predominantly influenza A (H1N1), influenza A (H3N2), and influenza B viruses, cause respiratory illness and have circulated worldwide (Fiore et al., 2010). Influenza B virus is characterized antigenically and genetically into two distinct lineages, B/Victoria/2/87-like (B/Vic) and B/Yamagata/16/88-like (B/Yam) strains, based on the viral hemagglutinin (HA) gene (Kanegae et al., 1990a; Rota et al., 1990). In the past 15 years, both lineages have co-circulated within the same year at varying levels in any given region (Ambrose & Levin, 2012). A surveillance report from 2010–2014 indicated that influenza B virus was responsible for 38 % (range 20 - 55 %) of all influenza-positive samples in Thailand (Center). Although the prevalence of influenza B virus is lower than that of influenza A virus, epidemiological studies have demonstrated that influenza B virus is capable of causing severe infection and complications (Chi et al., 2008; Gutierrez-Pizarraya et al., 2012). The trivalent influenza vaccine (TIV) used in Thailand includes inactivated influenza viruses with HA of types A/H1N1 pdm09 and A/H3N2, and one type B that best matches the predicted annual circulating strains (Owusu et al., 2015). However, recent studies in the United States, Europe, and China showed that as many as 45 – 58 % of the influenza B virus lineages in circulation did not match the chosen vaccine strain (Ambrose & Levin, 2012; Toback et al., 2012; Zhao et al., 2015).

Historically, virus propagation in tissue culture or embryonated eggs and hemagglutinin inhibition assay were used to distinguish specific lineages of influenza virus. These approaches are time-consuming, labor intensive and insufficiently sensitive. Molecular assays such as reverse transcription PCR (RT-PCR) and DNA sequencing have proved more reliable for determining the lineage of influenza B virus strains (Arvia, Corcioli, Pierucci, & Azzi, 2014; WHO, 2015). Presently, real-time RT-PCR with a specific probe can be used for rapid determination of the lineage of an influenza B virus, but the required TaqMan probe increases the cost per sample (Biere, Bauer, & Schweiger, 2010; Nakauchi et al., 2014; Zhang et al., 2012). Alternatively, real-time PCR can be combined with melting curve analysis, which exploits the melting temperature (T_m) of the double-stranded DNA in the PCR product depending on its GC content, length and heterozygosity. Intercalating indicator dyes

such as SYBR Green I fluoresce in the presence of double-stranded DNA (Reed, Kent, & Wittwer, 2007). Other saturating dyes such as LC Green, ResoLight, EvaGreen, Chromofy and SYTO 9 have also been used successfully in high-resolution melting analsis to differentiate between sequences that differ by even a single nucleotide. Real-time PCR with melting curve analysis has been used to accurately identify subtypes of influenza A virus HA (Lin et al., 2008) and neuraminidase (Varillas et al., 2011). Moreover, this method was effectively applied to discriminate between high- and low-pathogenic H5 avian influenza viruses (Payungporn et al., 2006), to detect HA antigenic variants of influenza B virus, and to differentiate specific viral lineages (T. Nakagawa, Higashi, & Nakagawa, 2008; Wong et al., 2014).

The HA gene of the influenza B/Vic lineage has been observed to contain three adenosine (AAA) insertions at nucleotide positions 538-540, while B/Yam strains do not have this insertion. This allows amplified PCR products of different lengths to be distinguished by their characteristic shifts in the Tm. Here, we customized a real-time PCR with melting curve analysis to specifically and accurately identify the lineage of influenza B virus in 756 clinical specimens. Evaluation of this assay enabled us to assess the prevalence of influenza B virus infection among Thais with influenza-like illness from 2010 to 2015 and consequently to determine the match ratio between the circulating influenza B strains and vaccine strains during this time period.

Materials and methods

Ethical considerations

All specimens were acquired with permission from the Director of King Chulalongkorn Memorial Hospital and were stored as anonymous samples. The study protocol was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (IRB No. 337/57), and the requirement for consent was waived because the samples were anonymous.

Primer design

Full-length HA gene sequences from influenza B virus were obtained from Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). Alignment of 500 HA nucleotide sequences of B/Vic and B/Yam lineages was done using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Candidate primer sequences were chosen from among the conserved nucleotide regions flanking the triple adenosine (AAA) present (in B/Vic) or absent (in B/Yam) in the HA sequence. The sequence AAA has been present in B/Vic strains since 1988 (Arvia et al., 2014). Primer design was done using Oligos software (Ruslan Kalendar, Institute of Biotechnology, University of Helsinki, Finland). The amplified region on the HA gene was 102 bp and 99 bp in length for B/Vic and B/Yam, respectively. All primers are listed in Table 4.

Viral RNA extraction and reverse transcription

Viral RNA was extracted using a commercial viral nucleic acid extraction kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Full-length cDNA was synthesized using an ImProm-II reverse transcription system (Promega, Madison, WI) and 1 μM FluB primer (5′-AGCAGAAGCA-3′) according to a published protocol (Berton, Naeve, & Webster, 1984).

Table 4 Oligonucleotide primers used to differentiate between Victoria and Yamagata influenza B virus lineages

Method	Primer Name	Nucleotide Sequences (5'-3')	Nucleotide position [a]	Influenza lineage	PCR product (bp)	Reference
HA1-PCR (outer primer)	HA-F5'	AGCAGAAGCAGAGCATTTTCTAA	1 - 23	Victoria	1142	24
	HA-R1142	GCAATCATTCCTTCCCATCCTCC	1120 - 1142			
	HA-F5'	AGCAGAAGCAGAGCATTTTCTAA	1 - 23	Yamagata	1139	
	HA-R1142	GCAATCATTCCTTCCCATCCTCC	1117 - 1139			
Multiplex PCR (inner primer)	Bvf224	ACATACCCTCGGCAAGAGTTTC	224 - 245	Victoria	284	11
	Bvr507	TGCTGTTTTGTTGTTGTCGTTTT	485 - 507			
	BYf226	ACACCTTCTGCGAAAGCTTCA	226 - 246	Yamagata	388	
	BYr613	CATAGAGGTTCTTCATTTGGGTTT	590 - 613			
Real-time PCR-Melting analysis	HA-F500	TCTTCGCAACAATGGCTTGGGC	500 - 521	Victoria	102	Present study
	HA-R601	CTTCTTCTTCTGYACAAATGTATGG	577 - 601			
	HA-F500	TCTTCGCAACAATGGCTTGGGC	500 - 521	Yamagata	99	
	HA-R601	CTTCTTCTTCTGYACAAATGTATGG	574 - 598			

[a] Nucleotide position starting from the first ATG codon of the HA gene was based on the B/Brisbane/60/2008 (CY115151) for Victoria lineage and the B/Florida/4/2006 (CY033876) for Yamagata lineage.

Table 4. *Oligonucleotide primers used to differentiate between Victoria and Yamagata influenza B virus lineages*

Real-time PCR with melting curve analysis

One microliter of undiluted cDNA sample was amplified in a 11.5-μl reaction containing 6.25 μl of 2X Maxima SYBR Green I/ROX qPCR Master Mix (Thermo Fisher Scientific Inc., Foster City, CA), 0.1 μM each of the forward primer HA-F500 and the reverse primer HA-R601 and nuclease-free water in a final volume of 12.5 μl. The reaction conditions were established for the LightCycler Nano (Roche Diagnostics, Mannheim, Germany) as follows: pre-incubation at 50 °C for 2 minutes, 95 °C for 10 minutes, and 45 cycles of amplification at 95 °C for 10 seconds, 60 °C for 60 seconds and 72 °C for 15 seconds. Data acquisition of the fluorescent signal was performed at the end of each cycle. Post-amplification melting curve analysis was performed at 60 °C for 20 seconds, 95 °C for 20 seconds, and ramping from 60–95 °C with fluorescence data acquisition in 0.1 °C increments.

Nested-multiplex PCR

Lineage-specific conventional one-step RT-PCR for influenza B virus was performed according to the WHO protocol [11] with minor modifications. The first PCR was carried out in 10 μl of 5 PRIME MasterMix (5 PRIME, Hamburg, Germany), 0.25 mM MgCl2, 0.5 μM outer primers, 2 μl of cDNA template and nuclease-free water to a final volume of 25 μl. Amplification was performed in a thermal cycler (Eppendorf AG, Hamburg, Germany) under the following conditions: initial denaturation at 94 °C for 3 minutes, 40 cycles of denaturation for 30 seconds at 94 °C, 30 seconds of primer annealing at 55 °C, 90 seconds of extension at 72 °C, and final extension for 7 minutes at 72 °C. For the multiplex PCR, 2 μl from the first amplification served as template for a subsequent reaction containing 0.25 μM of each inner primer. The PCR conditions were initial denaturation for 3 minutes at 94 °C, 40 cycles at 94 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 1 minute, and a final extension for 10 minutes at 72 °C. The expected amplification product sizes were 284 bp (B/Vic) and 388 bp (B/Yam). Amplicons were subjected to 2 % agarose gel electrophoresis, stained with ethidium bromide, and visualized using an ultraviolet transilluminator.

Analytical sensitivity and specificity of the assays

The analytical sensitivity was determined by evaluating the viral load in terms of cDNA copy number using plasmid templates. The HA1 genes (nucleotides 1-1142) from B/Vic (B/Thailand/CU-B11603/2015) and B/Yam (B/Thailand/CU-B11155/2014 and B/Thailand/CU- B11572/2015) were amplified by RT-PCR using primers HA-F5' and HA-R1142. Products were cloned into pGEM-T Easy Vector (Promega, Madison, WI) according to the manufacturer's instructions and verified by sequencing (First BASE Laboratories, Seri Kembangan, Malaysia). The resulting plasmids (pVic and pYam) were quantified by NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE) and serially diluted tenfold (from 109 to 100 copies per microliter).

The analytical specificity of both real-time PCR with melting curve analysis and nested-multiplex PCR were evaluated for possible nonspecific amplification using nucleic acid templates from 50 other respiratory viruses, including influenza A virus subtypes H3N2 (N = 15) and pH1N1 (N = 10), coxsackievirus A (N = 7) and B (N = 1), poliovirus serotypes 1 and 3 (N = 2), echovirus (N = 4), human rhinovirus types A, B and C (N = 3), respiratory syncytial viruses types A and B (N = 2), enterovirus D68 (N = 2), parainfluenzavirus types 1, 3 and 4b (N = 3), and adenovirus (N = 1).

Screening of clinical specimens

From 2010 to 2015, 19,859 respiratory specimens were obtained from patients with influenza-like illness (ILI). These individuals had high fever (≥38 °C) and respiratory tract symptoms. The specimens included nasal swabs, nasopharyngeal swabs/aspirates, throat swabs, lung biopsy samples, and broncho-alveolar lavage fluids. Viral RNA was extracted and tested for influenza A and B viruses by one-step multiplex real-time PCR (Berton et al., 1984). Some epidemiological and sequence data have been reported previously (Prachayangprecha, Vichaiwattana, Korkong, Felber, & Poovorawan, 2015; Suwannakarn et al., 2008; Tewawong, Suwannakarn, et al., 2015). A total of 756 samples tested positive for influenza B virus and were subjected to lineage testing using both real-time PCR with melting curve analysis and

nested multiplex PCR. Age data available from 16,955 patients were used in the calculation of the relative prevalence of influenza B virus among age groups.

Diagnostic performance of the assays

We defined the diagnostic sensitivity as the ability to correctly identify samples containing influenza virus, and the specificity as the ability to accurately eliminate influenza-negative samples. Positive predictive value (PPV) was defined as the percentage of infected individuals who tested positive, while negative predictive value (NPV) referred to the percentage of uninfected individuals who tested negative (Lalkhen & McCluskey, 2008). The sensitivity of the nested-multiplex PCR and real-time PCR was assessed using 135 influenza B virus isolates. The full-length HA genes of 48 of these samples were sequenced previously (Tewawong, Suwannakarn, et al., 2015). HA sequences from an additional 87 samples were obtained by PCR amplification, agarose gel purification using an Expin Combo GP kit (GeneAll Biotechnology, Seoul, Korea), and sequencing (First BASE Laboratories, Seri Kembangan, Malaysia). In addition, 50 respiratory samples were used to test the analytical specificity as true negatives for the estimation of the specificity of the B/Vic and B/Yam PCRs. Positive samples for B/Yam were used as true negatives for the estimation of the specificity of the B/Vic PCR and vice versa. Sequence data were submitted to GenBank under the accession numbers KT725638 to KT725724. All HA gene sequences were subjected to BLAST analysis along with reference strains from the GISAID EpiFlu database.

Statistical analysis

The combined diagnostic sensitivity, specificity, PPV, and NPV of the two assays were calculated with their 95 % confidence intervals (CIs) using 185 specimens (80 B/Yamagata and 55 B/Victoria lineages identified by sequencing and 50 other respiratory samples). Data analysis was done using the Statistical Package for Social Sciences version 22.0 (SPSS Inc., Chicago, IL). The chi-square test was used to compare the diagnostic performance testing indicators and to analyze the age-group-specific prevalence of influenza B virus in ILI cases. All data were considered statistically significant at a P-value ≤ 0.05.

Phylogenetic analysis

Sequence alignment of the HA1 coding region and the construction of a phylogenetic tree were done using the neighbor-joining method and Kimura's two-parameter distance model implemented in MEGA 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The bootstrap consensus tree was inferred from 1,000 bootstrap replicates. Bootstrap values >70 % are shown.

Results

Assay design

The real-time PCR with melting analysis could differentiate between B/Vic and B/Yam influenza B virus HA genes because of the absence of three consecutive adenosines in the HA sequences of B/Yam strains (represented by clade 2 strain B/Thailand/CU-B11155/2014 and clade 3 strain B/Thailand/CU-B11572/2015) compared to those of B/Vic (represented by clade 1 strain B/Thailand/CU-B11603/2015 and clade 5 strain B/Thailand/CU-B4585/2011) (Figure 3a). This B/Yam-specific deletion resulted in an approximately 1 °C difference in the melting temperatures (Tm) of the amplicons generated by B/Vic, B/Yam2, and B/Yam3 (Figure 3b). This characteristic was consistently reproducible in triplicate experiments (Table 5).

(a)

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B/Thailand/CU-B11603/2015 (VIC1)    500 TCTTCGAAACAATGGCTTGGGCCGTCCCAAAAAACGACAAAAACAAAACAG 550
B/Thailand/CU-B4585/2011 (VIC5)         .T....C...........................................
B/Thailand/CU-B11155/2014 (Yam2)        .T....C...............T........GG..A...~~~.....AT.
B/Thailand/CU-B11572/2015 (Yam3)        .T..T.C...............T........GG..A..T~~~.....AT.


B/Thailand/CU-B11603/2015 (VIC1)    551 CAACAAATCCATTAACAATAGAAATACCATACATTTGTACAGAAGGAGAAG 601
B/Thailand/CU-B4585/2011 (VIC5)         ........................G..........................
B/Thailand/CU-B11155/2014 (Yam2)        ....G..C.........G....G..............G.............
B/Thailand/CU-B11572/2015 (Yam3)        ....G..C...CA....G.G...G.....................G....
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pYam: y = -3.3792x + 41.655, R² = 0.9977
pVic: y = -3.492x + 43.148, R² = 0.9969

Figure 3. Analysis of the influenza B virus HA genes from viruses of the B/Victoria and B/Yamagata lineages. (a) Sequence alignments of the HA genes from nucleotide positions 500 to 601. Identical nucleotides are shown as dots; missing nucleotides are represented by a tilde. The sequences of the primers HA-F500 and HA-R601 are underlined. (b) Melting curves and peaks of the amplified PCR products characteristic of each lineage. B/Vic, B/Thailand/CU-B11603/2015, representing the Victoria lineage; B/Yam2, clade 2 strain B/Thailand/CU-B11155/2014; B/Yam3, clade 3 strain B/Thailand/CU-B11572/2015. (c) Linear regression of the HA gene amplification. The copy numbers of input HA1-encoding plasmid template of either B/Vic (square) or B/Yam (triangle) were plotted against the threshold cycle (Ct) values. The mean cycle threshold was derived from duplicates of the real-time PCR standard curve. Correlation coefficient, pVic = 0.996 and pYam = 0.997; slope, pVic = -3.492 and pYam = -3.3792; intercept, pVic = 43.15 and pYam = 41.66

Table 5. *Melting temperatures (Tm) of HA amplicons from different strains of influenza B virus*

Viral strains	T_m
B/Thailand/CU-B11603/2015	79.26
(Victoria)	79.36
	79.35
Mean	79.32
B/Thailand/CU-B11155/2014	80.28
(Yamagata clade 2)	80.34
	80.33
Mean	80.32
B/Thailand/CU-B11572/2015	81.26
(Yamagata clade 3)	81.26
	81.21
Mean	81.24

Evaluating the analytical sensitivity and specificity

To determine whether our real-time PCR with melting analysis or the nested-multiplex PCR yielded nonspecific cross-amplification of non-influenza B viruses, we tested 50 samples known to contain other common respiratory viruses and observed no detectable amplification in the real-time PCR. There was also a lack of nonspecific amplification using the nested-multiplex PCR with the B/Vic and B/Yam primer set. Real-time PCR with melting analysis of tenfold serial dilution of plasmids containing B/Vic and B/Yam HA1 gene demonstrated a linear detection range from 10^9 to 10^2 genome equivalent per reaction. The correlation (R^2) was 0.997 and the slopes were -3.49 (pVic) and -3.38 (pYam) (Figure 3c). The real-time PCR was relatively robust and efficient in amplifying B/Vic (93.19 %) and B/Yam (97.65 %), yielding signals as low as 10^2 copies/μl from the HA1 cDNA template. For the nested-multiplex PCR, the lower detection limit was 10^3 copies/μl for the both of pVic and pYam.

Evaluating the diagnostic sensitivity and specificity

To determine the ability for the assays to correctly differentiate influenza B virus lineages, 55 B/Vic and 80 B/Yam samples previously identified by sequencing and 50 other respiratory samples were evaluated by real-time PCR with melting analysis. Signals were detected in all specimens as B/Vic or B/Yam lineage viruses with sensitivities and specificities >94 % (Table 6), while none of the samples tested positive for both lineages. The melting temperature for each lineage was distinct with the mean (and cutoff temperatures) of 79.58 °C (77.80 – 80.15 °C), 80.33 °C (80.16 – 80.77 °C), and 81.11 °C (80.78 – 81.83 °C) for the B/Vic, B/Yam2, and Yam3, respectively (Figure 4). The diagnostic sensitivity and positive predictive value of the real-time PCR with melting analysis were significantly higher than those of nested multiplex PCR (96.3 % and 94.6 %, respectively) (Table 6).

Table 6. *Diagnostic performance of real-time PCR with melting analysis and nested multiplex PCR assays in lineage-specific identification of influenza B virus*

Parameters	Estimated % (95% CI) for B/Victoria (N=55)			Estimated % (95% CI) for B/Yamagata (N=80)		
	Real-time with melting analysis	Nested Multiplex PCR	*P*-value	Real-time with melting analysis	Nested Multiplex PCR	*P*-value
Sensitivity	94.6 (84.9 - 98.9)	92.7 (82.4 - 97.9)		96.3 (89.4 - 99.2)	91.3 (82.8 - 96.4)	
(No. TP)	(52)	(51)	0.616	(77)	(73)	0.046 [a]
Specificity	97.7 (93.4 - 99.5)	94.6 (89.2 - 97.8)		97.1 (91.8- 99.4)	96.2 (90.5 - 98.9)	
(No. TN)	(127)	(123)	0.121	(102)	(101)	0.631
PPV	94.6 (84.9 - 98.9)	87.9 (76.7 - 95.0)		96.3 (89.4 - 99.2)	94.8 (87.2 - 98.5)	
(No. FP)	(3)	(7)	0.022 [a]	(3)	(4)	0.484
NPV	97.7 (93.4 - 99.5)	96.9 (92.1 - 99.1)		97.1 (91.8- 99.4)	93.5 (87.1 - 97.3)	
(No. FN)	(3)	(4)	0.635	(3)	(7)	0.102

PPV, positive predictive value; NPV, negative predictive value; No., number; TP, true positive; TN, true negative; FP, false positive; FN, false negative

We also included fifty of other respiratory samples used to test analytical specificity as true negative for the estimation of the diagnostic specificity of both B/Vic and B/Yam.

[a] Indicate significance ($P \leq 0.05$).
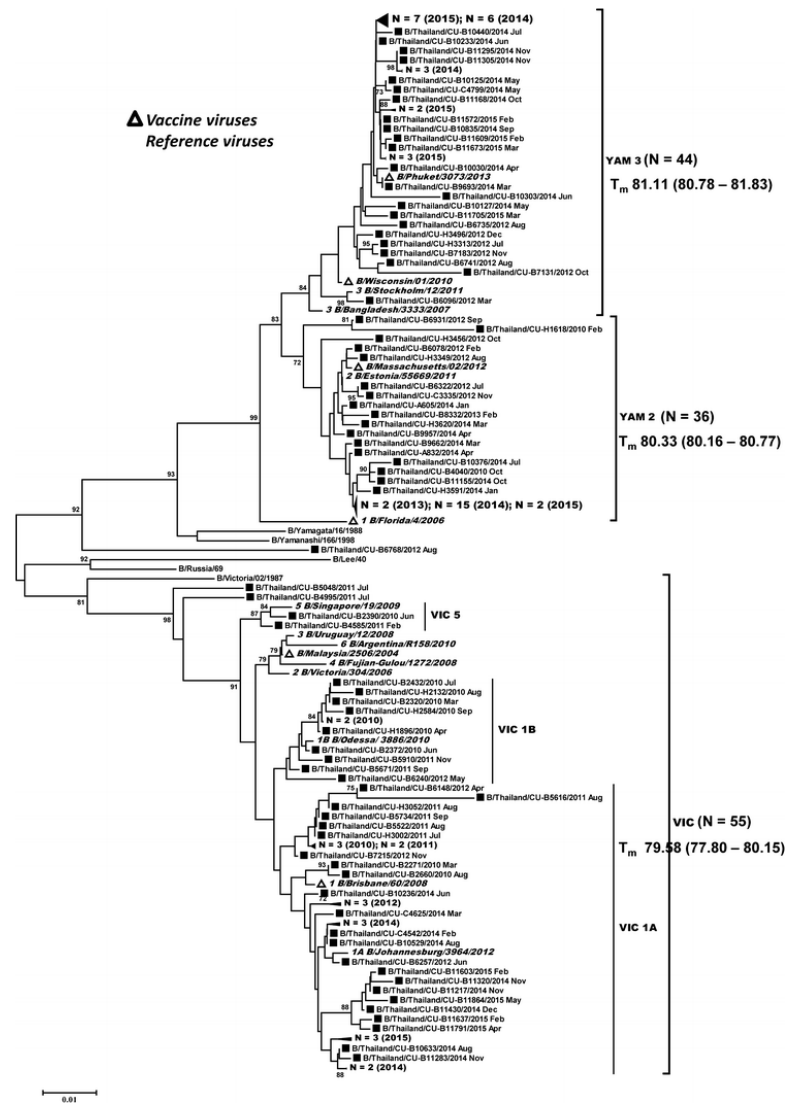
Figure 4. *Phylogenetic analysis of the HA1 nucleotide sequences of influenza B virus. Sequences from 135 strains isolated in Thailand between 2010 and 2015 (squares) were compared to the reference strains (italicized and bolded) of known clades reported by the WHO Influenza Center London, and the WHO recommended southern hemisphere vaccine strains (triangles). The phylogenetic tree was generated by the neighbor-joining method and Kimura 2-parameter model with 1,000 bootstrap replicates implemented in MEGA (version 6.06). Branch values >70 are indicated at the nodes. The scale bar represents approximately 1 % nucleotide difference between close relatives. Melting temperatures (Tm) for Victoria and Yamagata clades 2 and 3 obtained by real-time PCR with melting analysis are indicated [Mean (Max − Min)].*

Screening of clinical specimens

To further assess whether the real-time PCR with melting analysis and the nested multiplex PCR assays could reliably resolve influenza B lineages, 756 influenza-B-virus-positive respiratory samples previously tested by one-step real-time PCR from 2010 to 2015 were screened. In all, there were 138 (118 B/Vic and 17 B/Yam, 3 untypable) in 2010, 57 (55 B/Vic and 2 B/Yam) in 2011, 229 (112 B/Vic and 112 B/Yam, 5 untypable) in 2012, 12 (12 B/Yam) in 2013, 249 (15 B/Vic and 233 B/Yam, 1 untypable) in 2014, and 71 (11 B/Vic and 59 B/Yam, 1 untypable) in 2015. Performing both assays in parallel showed that specific lineages could be assigned to 98.7 % of the samples. In particular, one specimen tested positive for both A/H3N2 and influenza B virus, which was subsequently identified as B/Yam by real-time PCR. Ten influenza-B-virus-positive specimens yielded discordant results, as the HA1 gene could not be amplified and sequenced, and were therefore designated as untypable. Furthermore, real-time PCR with melting analysis was capable of differentiating different clades specific to B/Yam. Among the 426 B/Yam-confirmed samples, melting analysis revealed that 279 (65 %) were B/Yam clade 2 and 147 (35 %) were B/Yam clade 3.

Prevalence of influenza B cases in different age groups

From the 16,995 ILI samples with patient age information, 4.03 % of the samples tested positive for influenza B (Table 7). Stratification by age group showed that influenza B prevalence was significantly higher among children aged 6-17 years ($p < 0.0001$). Influenza B comprised 18.8 % of the total influenza cases (the others being A/pH1N1 and A/H3N2) and was highest among children 6-17 years (28.38 %; $p < 0.0001$) when compared to other age groups.

Table 7 *Number and percentage of influenza cases by age and viral type*

Age group	Total no. of ILI cases	No. of cases by age and viral type (%)			Total no. of Flu cases	No. of cases by age and viral type (%)		
		influenza B	influenza A/pH1N1	influenza A/H3N2		influenza B	influenza A/pH1N1	influenza A/H3N2
< 2 years	2517	25 (0.99)	100 (3.97)	70 (2.78)	195	25 (12.82)	100 (51.28)	70 (35.90)
2 - 5 years	3590	87 (2.42)	262 (7.30)	240 (6.69)	589	87 (14.77)	262 (44.48)	240 (40.75)
6 - 17 years	3631	275 (7.57) [a]	388 (10.69)	306 (8.43)	969	275 (28.38)	388 (40.04)	306 (31.58)
18 - 64 years	6398	282 (4.41)	753 (11.77)	735 (11.49)	1770	282 (15.93)	753 (42.54)	735 (41.35)
≥ 65 years	819	15 (1.83)	21 (2.56)	84 (10.26)	120	15 (12.50)	21 (17.50)	84 (70.00)
All groups	16955	684 (4.03)	1524 (8.99)	1435 (8.46)	3643	684 (18.78)	1524 (41.83)	1435 (39.39)

[a] Indicate significance ($P < 0.0001$).

Table 7. *Number and percentage of influenza cases by age and viral type*

Evaluating the match between the circulating influenza B virus and the vaccine strains

Between 2010 and 2015, influenza-B-positive rates varied seasonally from 14 % to 26 % (Figure 5a). On average, influenza B virus was responsible for 18.9 % of all annual influenza cases. We calculated the match ratio of the annual influenza B virus strains (B/Vic and B/Yam) found to be in circulation and the respective vaccine strain for that year based on the total of 746 B/Vic and B/Yam strains (excluding 10 that were untypable). The result showed that in 2010 and 2011, the frequency of B/Vic lineage was exceptionally high compared to the B/Yam lineage (3.51-12.59 %) (Figure 5b). In 2012, there were as many infections caused by B/Yam as B/Vic, a consequence of the fact that the B/Yam-containing TIV did not likely offer much protection for B/Vic that year. This was reflected by the significant detection (44 %) of the influenza-positive samples in 2012. By 2013, B/Yam completely replaced B/Vic and remained the dominant circulating lineage in 2014 (94 %) and 2015 (84 %).
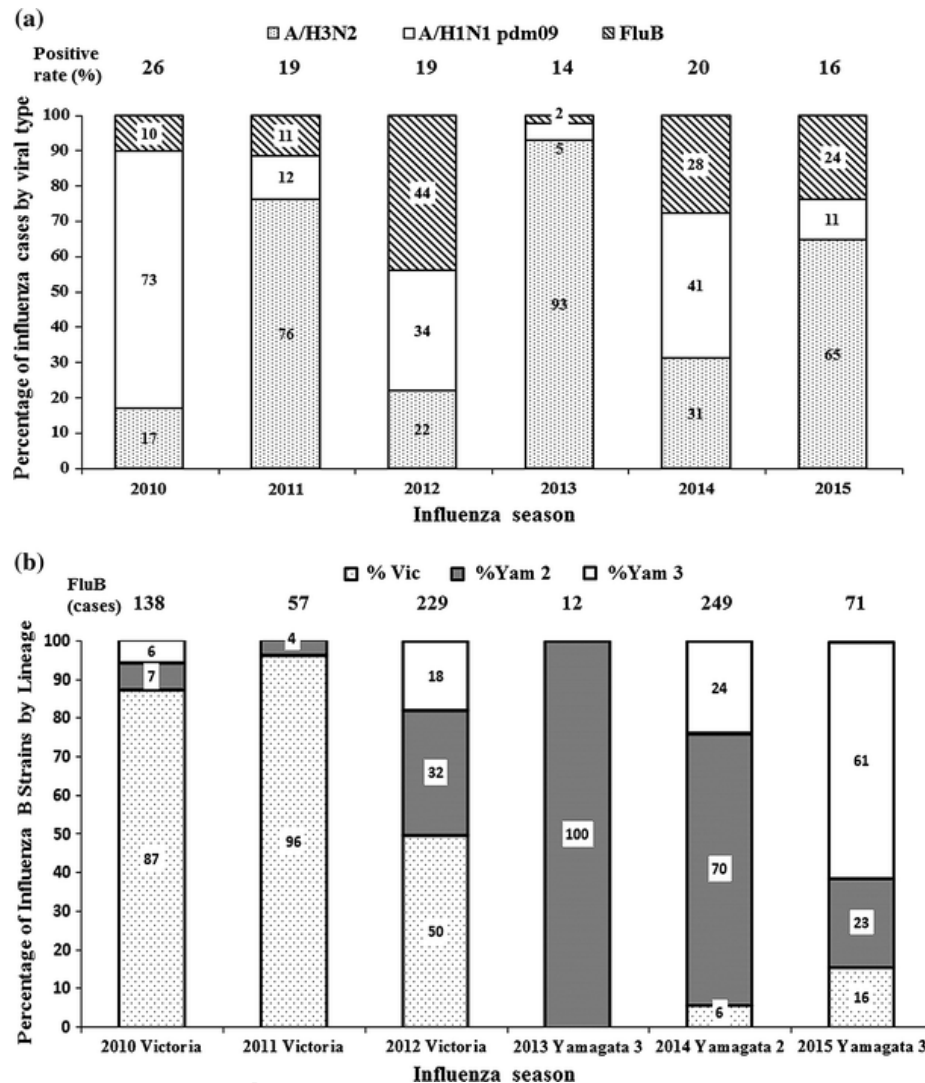
Figure 5. *Yearly influenza B virus detection between 2010 and 2015 (a) The percentage of samples testing positive for influenza virus each year are indicated above the bar graphs. For the influenza-virus-positive samples, the numbers in the bar graphs represent the percentage of A/H3N2 (stippled), A/H1N1pdm09 (white), and influenza B (hatched). (b) The total number of influenza B cases examined each year is indicated above each bar graph. The number in the bar graph denotes the percentage identified as Victoria (stippled), Yamagata clade 2 (gray), or Yamagata clade 3 (white). For each year, the influenza B virus lineage in the trivalent vaccine is indicated under the bar graph.*

Discussion

Rapid diagnosis of influenza virus infection in individuals with ILI has advanced considerably in improving viral detection. Many options are available, including several variations of real-time RT-PCR as well as commercially available assays with their respective limitations. For example, there is a rapid PCR assay that can detect influenza A and B viruses in less than 20 minutes, but it is relatively expensive and does not differentiate influenza B lineages (Binnicker, Espy, Irish, & Vetter, 2015). Here, we developed a simple real-time PCR with melting curve analysis, which allowed accurate detection of influenza B virus while providing a lineage-specific melting curve profile to differentiate B/Vic and B/Yam lineages. This assay obviated the need for a sequence-specific fluorescent probe or multiplexing of primers. Multiple specimens could be processed in a few hours at a relatively low cost per sample. We found that the assay was more sensitive and specific than the nested multiplex PCR and could reliably detect both the B/Vic and B/Yam lineages, and it could also distinguish between clades 2 and 3 of B/Yam.

Since the HA gene of influenza virus continually accumulates genetic variations, even a single-nucleotide mutation can result in an amino acid substitution sufficient to alter the antigenicity of the virus (N. Nakagawa, Kubota, Maeda, Nakagawa, & Okuno, 2000). Despite the relatively slow mutation rate for influenza B compared to influenza A virus, naturally occurring antigenic variants do occur as a result of immunological pressure (N. Nakagawa, Kubota, & Okuno, 2005), and new clades are associated with reassortments such as a combination of HA and NA segments (Chen & Holmes, 2008). Consequently, conventional real-time PCR assays using hybridization or TaqMan probes cannot detect some genetic variants and require new primers specific to the lineage or clade to accurately identify influenza B virus. Thus, the real-time PCR with melting analysis can offer an additional screening method useful for the early detection of HA genetic variants of influenza B virus before the actual nucleotide changes in the HA sequences are determined by sequencing (Lin et al., 2008; T. Nakagawa et al., 2008). In addition, identification of the influenza B virus lineage is possible in the presence of co-infection with other respiratory viruses. However, a low amount of viral RNA or the presence of an

atypical strain of influenza B virus in the specimen might limit this real-time PCR with melting analysis (as was the case with one specimen).

Our influenza surveillance data showed that 18 % of the influenza-related illnesses were caused by influenza B virus, consistent with similar findings of influenza B activity in Europe and the United States between 1994 and 2011 (Paul Glezen, Schmier, Kuehn, Ryan, & Oxford, 2013). The highest infection rate occurred among school children aged 6-17 years old, and it has been proposed that schoolchildren may be the main population responsible for the spread of influenza B virus in the community (Paul Glezen et al., 2013; Zhao et al., 2015). Vaccination efforts in Thailand between 2010 and 2012 focus on high-risk groups, including healthcare personnel, poultry cullers, and people aged ≥ 65 years, people with chronic disease, pregnant women, obese people, mentally disabled people, and children aged 6 months to 2 years (Owusu et al., 2015). Although the influenza vaccine coverage was 0.1 %-20 % in the high-risk population during this time, it resulted in a relatively lower prevalence of influenza cases (except A/H3N2) among < 2 years and ≥ 65 years groups. However, the morbidity and mortality rates for influenza during 2010-2015 seasons were high (about 100.76 and 0.06 per 100,000 people, respectively) (Center). Our data suggest that vaccination of vulnerable schoolchildren who are 6-17 years old and not yet included in the national vaccination program should be considered, as it may decrease the overall influenza rates, improve school attendance (Pannaraj et al., 2014), and reduce the rate of exposure of susceptible individuals in the community, especially the elderly and immunocompromised individuals (Gaglani, 2014).

The trivalent influenza vaccine contains only one influenza B formulation, which provides limited cross-protection against the other lineage (RB, 2007). We found seasonal variation in the match ratio between circulating influenza B strains and the vaccine B strain, which might explain the reduced efficacy of TIV (Dolin, 2013). From 2010 to 2015, there was an overall 21 % mismatch between the circulating influenza B virus strain and the vaccine strain. The most significant mismatch occurred in the 2012 season, when the influenza B virus component of the southern hemisphere influenza vaccine was of the B/Vic lineage but 50 % of the circulating strains were of the B/Yam lineage. In 2013, the influenza B virus component of the

southern hemisphere TIV was of B/Yam clade 3 (B/Wisconsin/1/2010-like virus), and 100 % of the circulating B/Yam strains detected belonged to clade 2. A better-matched influenza B virus component in the vaccines of 2014 (B/Yam clade 2 [B/Massachusetts/2/2012-like virus]) and in 2015 (B/Yam clade 3 [B/Phuket/3073/2013-like virus]) resulted in only 26-27 % of the circulating B/Yam lineage being opposite of the strain in the vaccine. Between 2014 and 2015, the B/Vic lineage increased from 6 % to 16 %, and this is expected to further increase in 2016. The varying pattern of dominance of the two influenza B lineages appears to be driven by oscillating population immunity. One lineage remains dominant until sufficient herd immunity develops, and this results in an increased frequency of the other lineage in the following season (N. Nakagawa et al., 2000). As long as the trivalent influenza vaccine remains in use, the ability to determine the prevalence of influenza B virus lineages in circulation will assist in the optimal selection of the strain to be included in the annual vaccine.

A quadrivalent influenza vaccine (QIV) has been shown to reduce the severity of disease and provide immunity to both lineages of influenza B virus (Ambrose, Yi, Walker, & Connor, 2008). However, the current cost of the QIV is significantly higher than that of the TIV, although the QIV may substantially reduce the healthcare cost to the community and third-party payers (Lee, Bartsch, & Willig, 2012). Evaluating the cost-effectiveness of the QIV used in a developing country such as Thailand will be required to formulate a sound public health policy. Finally, alternating the inclusion of B/Yam and B/Vic strains in the annual vaccine from year to year should also be considered (R, 2009; S. A. Richard, Viboud, & Miller, 2010).

CHAPTER III

MOLECULAR EPIDEMIOLOGY AND PHYLOGENETIC ANALYSES OF INFLUENZA B VIRUS IN THAILAND DURING 2010 to 2014

(Part 3.2)

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CHAPTER III

Part 3.2 Molecular Epidemiology and Phylogenetic Analyses of Influenza B Virus in Thailand during 2010 to 2014

Summary

Influenza B virus remains a major contributor to the seasonal influenza outbreak and its prevalence has increased worldwide. We investigated the epidemiology and analyzed the full genome sequences of influenza B virus strains in Thailand between 2010 and 2014. Samples from the upper respiratory tract were collected from patients diagnosed with influenza like-illness. All samples were screened for influenza A/B viruses by one-step multiplex real-time RT-PCR. The whole genome of 53 influenza B isolates were amplified, sequenced, and analyzed. From 14,418 respiratory samples collected during 2010 to 2014, a total of 3,050 tested positive for influenza virus. Approximately 3.27% (471/14,418) were influenza B virus samples. Fifty three isolates of influenza B virus were randomly chosen for detailed whole genome analysis. Phylogenetic analysis of the HA gene showed clusters in Victoria clades 1A, 1B, 3, 5 and Yamagata clades 2 and 3. Both B/Victoria and B/Yamagata lineages were found to co-circulate during this time. The NA sequences of all isolates belonged to lineage II and consisted of viruses from both HA Victoria and Yamagata lineages, reflecting possible reassortment of the HA and NA genes. No significant changes were seen in the NA protein. The phylogenetic trees generated through the analysis of the PB1 and PB2 genes closely resembled that of the HA gene, while trees generated from the analysis of the PA, NP, and M genes showed similar topology. The NS gene exhibited the pattern of genetic reassortment distinct from those of the PA, NP or M genes. Thus, antigenic drift and genetic reassortment among the influenza B virus strains were observed in the isolates examined. Our findings indicate that the co-circulation of two distinct lineages of influenza B viruses and the limitation of cross-protection of the current vaccine formulation provide support for quadrivalent influenza vaccine in this region.

Introduction

Influenza virus belongs to the *Orthomyxoviridae* family of enveloped, segmented negative-stranded RNA viruses. Influenza A and B viruses are major causes of respiratory infection in human and contribute to increasing morbidity and mortality globally (Sullivan, Monto, & Longini, 1993). Influenza A virus infects humans, swine, birds, and horses, whereas influenza B virus infects humans and seals (Osterhaus, 2000). There are 18 subtypes of influenza A virus, of which H1, H2, and H3 are known to infect humans, while other subtypes such as H5, H6, H7 and H9 have the potential to cause human pandemics (Yen & Webster, 2009). In contrast, influenza B virus has no subtypes. The first isolated strain of influenza B virus was B/Lee/40 (Krystal, Elliott, Benz, Young, & Palese, 1982). Since 1983, influenza B viruses evolved antigenically and genetically into two major lineages: B/Victoria/2/87-like and B/Yamagata/16/88-like (Kanegae et al., 1990b). Currently, Victoria and Yamagata lineages have continually co-circulated in many regions of the world (Rota et al., 1990). Although the trivalent seasonal influenza vaccines include one strain of influenza B virus, evidence suggests that the current vaccines can be improved by including both lineages (Ambrose & Levin, 2012). The genome of influenza B consists of eight segments: polymerase basic-1 (PB1), PB2, polymerase acidic (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural protein (NS). Binding of the virus to its cellular receptors, terminal sialic acids of glycoproteins and glycolipids, is mediated by the viral surface glycoprotein HA (Wang, Tian, Chen, & Ma, 2007). HA forms a homotrimer with each monomer composed of an HA1 and HA2 subunit. HA1 is the receptor-binding subunit of HA and represents the major antigenic sites that undergo constant antigenic variations due to frequent amino acid substitutions and insertion/deletions (Nerome et al., 1998). In contrast, the hydrophobic N-terminus of HA2 is the fusion peptide, which is the most conserved and play role for inducing fusion of viral envelope and endosomal host membrane (Ni, Kondrashkina, & Wang, 2013). Genetic reassortment is a crucial process of evolution for segmented RNA viruses, including influenza B virus, which effectively generates new recombinant genome most fit for viral adaptation (Chen & Holmes, 2008; S. E. Lindstrom et al., 1999). Previous studies

revealed that the rates of antigenic drift and evolution of influenza type B viruses are lower than in influenza type A (Krystal et al., 1983). Influenza A viruses are able to undergo antigenic shift by genetic reassortment between different subtypes (Cox & Bender, 1995), while influenza B viruses resort to various mechanism of deletion, insertion, and substitution within different co-circulating strains (Nerome et al., 1998). This antigenic drift allows influenza B virus to escape host immunity and continue to adapt/evolve without antigenic shift (S. E. Lindstrom et al., 1999; McCullers, Wang, He, & Webster, 1999; Nerome et al., 1998), thus explaining the limited virus diversity and pandemic potential (Barr et al., 2010; McCullers et al., 1999).

Although the Ministry of Public Health in Thailand encouraged individuals ≥65 years, those with underlying medical conditions (asthma, heart diseases, diabetes, etc.), and pregnant women to receive yearly influenza vaccination, universal vaccination is not implemented and vaccination coverage is relatively low. Data on individuals seeking vaccination from private healthcare facilities are lacking. As a result, the rising prevalence and increasingly severe cases of influenza B virus infection have been reported (Byarugaba et al., 2013; Gutierrez-Pizarraya et al., 2012; Yang et al., 2012). A study of prevalence and epidemiological data among influenza B virus in Thailand was previously described (Chittaganpitch et al., 2012), but molecular characterization and genetic evolutionary profiles of influenza B virus in Thailand remained unclear. In this study, we determined the prevalence of influenza B virus infection in Thailand from individuals with influenza-like illness during January 2010 to February 2014. We also characterized the gene segments of influenza B virus on the basis of genetic clustering, phylogenetic topology and pairwise amino acid variations.

Materials and Methods

Study population and sample collection

From January 2010 to February 2014, a total of 14,418 upper respiratory specimens from patients with influenza-like illness were collected from Bangkok, Khon Kaen and Surat Thani provinces in Thailand. The inclusion criteria were fever (> 38°C) combined with respiratory symptoms such as cough, sore throat and runny nose. The specimens were collected in the viral transport medium and sent to the Center of Excellence in Clinical Virology, Faculty Medicine, Chulalongkorn University for testing of respiratory viruses. All samples were stored at -70°C.

Ethical consideration

This research was performed on respiratory specimens stored as anonymous. All patient identifiers were removed to protect patient confidentiality and no personal information appeared in any part of the document in this study. The Institutional Review Board of the Faculty of Medicine at Chulalongkorn University approved the research protocol (IRB number 337/57). The IRB waived the need for consent because the samples were anonymous.

Influenza B screening by one-step multiplex real-time RT-PCR

Viral RNA was extracted from samples by using a commercially available Viral Nucleic Acid Extraction Kit (RBC Bioscience Co, Taiwan) following the manufacturer's instruction. Influenza virus detection was performed with one-step multiplex real-time RT-PCR assays based on TaqMan probes as previously described (Chieochansin. T, 2009; Hoffmann, Stech, Guan, Webster, & Perez, 2001; Suwannakarn et al., 2008). In addition, the GAPDH gene served as an internal control, while the matrix (M) gene of influenza A and B was amplified to characterize the types of influenza virus. In brief, the 15 μl reaction volume contained 7.5 μl of 2X reaction buffer (included dNTPs), 1.825 μl of RNAse-free H_2O, 0.3 μl of Superscript III enzyme mix (Taq DNA polymerase and reverse transcriptase; Invitrogen), 0.375 μl of 10 nmol/L each reverse and forward primer, and 2 μl of template RNA. Amplification was performed on Rotor-Gene3000 (Corbett Research, New South

Wales, Australia) with a single reverse transcription step of 50°C for 45 min, "hot start PCR" at 95°C for 2 minutes, followed by 50 amplification cycles of denaturation for 30 seconds, primer annealing at 55°C for 10 seconds, extension at 60°C for 10 seconds, and a final extension step at 72°C for 20 seconds.

Conventional PCR and sequencing

One sample positive for influenza B virus was randomly chosen each month for whole genome analysis. Viral cDNA was synthesized using the M-MLV reverse-transcription system (Promega, Madison, WI) and 1 mM universal primers as described (Hoffmann et al., 2001). The whole genome sequences were amplified by primer sets for influenza B virus (Table S1). Briefly, the reaction volume contained 10 ml of 2.5 X Eppendorf mastermix (5Prime, Hamburg, Germany), 0.25 mM MgCl2, 0.5 mM forward and reverse primers, 2 μl of cDNA template and RNAse-free H_2O to the final volume of 25 μl. Amplification was carried out in a thermal cycler (Eppendorf, Germany) under the following conditions: initial denaturation at 94°C for 3 minutes, 40 cycles of 30 seconds denaturation at 94°C, 30 seconds of primer annealing at 50°C (for PB2, PB1, and PA genes) and 55°C (for HA, NP, NA, MP, and NS genes), 90 seconds of extension at 72°C, and further extension for 7 minutes at 72°C. PCR products were separated on a 2% agarose gel with a 100-bp DNA ladder and visualized on a UV trans-illuminator. PCR products were gel-purified using the HiYield Gel DNA Fragment Extraction kit (RBC Bioscience Co, Taiwan). DNA sequencing was performed by First BASE Laboratories Sdn Bhd (Selangor, Malaysia).

Phylogenetic analysis

SeqMan II program of the DNAStar software (v6.0) was used for nucleotide sequence assembly. Genome sequences were aligned using ClustalW implemented in the BioEdit program (v7.2.0). MEGA program (v6.06) was used for the phylogenetic tree construction by applying the neighbor-joining method with Kimura's two-parameter distance model and 1,000 bootstrap replicates. Sequences representative from different areas of the world available in GenBank and GISAID databases and those of Southern hemisphere vaccine strains recommended by WHO for the

influenza seasons from 2006 to 2014 (Table S2) were included in phylogenetic analysis. The latter virus sequences were also used as references to compare the amino acid substitutions with influenza B Thailand strains. The relative amino acid frequency in the analysis of the genome signatures for each gene was done using WebLogo (Crooks, Hon, Chandonia, & Brenner, 2004).

Meteorological data

Thailand is located in a tropical area between latitudes 5° 37′N to 20°27′N and longitudes 97° 22′E to 105° 37′E. Its climate is characterized by the rainy season (mid-May to mid-October) due to the southwest monsoon, winter season (mid-October to mid-February) due to the northeasterly wind, and hot dry summer or pre-monsoon season (mid-February to mid-May) (Manisan, 1995).

Statistical analysis

Statistical data analysis was carried out using the Statistical Package for Social Sciences version 19.0 (SPSS Inc., Chicago, USA). The Chi-square test was used to analyze demographic patient factors. All data were considered statistically significant at a p-value less than 0.05.

Accession numbers

The whole genome sequences of the influenza B isolates in Thailand during 2012 to 2014 are available in GenBank (Accession numbers KM100190-KM100333). The complete gene sequences of all strains in 2010 to 2011 have been previously deposited in GenBank (Accession numbers JX512971-JX513210).

Results

Demographic profile and seasonality

Among 14,418 patients with influenza-like illness, a total of 3050 patient samples tested positive for influenza viruses. We found 1387 samples positive for influenza A H1N1 pdm09 (45.5%), 1192 for influenza A H3N2 (39.1%), and 471 for influenza B (15.4%). Seasonality of each virus varied from year to year (Figure 6A). From 2010, influenza A H1N1 pdm09 was the prevalent subtype among influenza-positive samples. In the second half of 2011 and most of 2013, influenza A H3N2 virus predominated. Influenza B, however, was detected yearly between 2010 and 2014. Overall, influenza B infection accounted for approximately 3.2% of all patient samples. Approximately 47.8% with influenza B were male and 52.2% were female (1:1.09 ratio), which was not a statistically significant difference (p = 0.725) (Table 8). For individuals in which age information was available, we stratified them into five different age groups. Those between 5–19 years represented 22.7% of all patients, but they constituted the majority (41.2%) of all influenza B infection (p < 0.0001). Specimens tested positive for influenza B virus were nasal (73.5%), nasopharyngeal (18.3%), and throat (8.3%) swabs. The majority of influenza B cases were collected in Bangkok (89.4%). Most influenza B occurred in 2012, while fewest cases were found in 2013 (Figure 6B). From 2010 to 2012, influenza B generally peaked between July and September and coincided with the local rainy season. The annual incidence of influenza B compared to all influenza-positive cases was 2.6% in 2010 (141/5326), 2.2% in 2011 (56/2545), 8.3% in 2012 (222/2676), 0.37% in 2013 (11/3000), and 4.71% in the first two months of 2014 (41/871).

Table 8 Demographic characteristics of patients (N = 14,418)

Parameter	Variable	No. specimens (%)	No. patients (%)	% positive rate	*P* value [a]
Gender	Male	7002 (48.6)	225 (47.8)	3.22	0.725
	Female	7416 (51.4)	246 (52.2)	3.32	
Age (Years)	< 5	4029 (27.9)	46 (9.7)	1.15	<0.0001
	5 - 19	3280 (22.7)	194 (41.2)	5.92	
	20 - 44	2713 (18.8)	99 (21.0)	3.65	
	45 - 64	993 (6.8)	39 (8.3)	3.93	
	> 65	429 (2.9)	7 (1.5)	1.64	
	N/A	2974	86		
Type of specimens	Nasopharyngeal swab	2275 (15.8)	86 (18.3)	3.78	<0.0001
	Nasopharyngeal aspirate	5 (0.1)	0	0	
	Nasal swab	7475 (51.8)	346 (73.5)	4.63	
	Throat swab	4663 (32.3)	39 (8.3)	0.84	
Provinces	Bangkok	8916 (61.8)	421 (89.4)	4.73	<0.0001
	Khon Kaen	4652 (32.3)	39 (8.3)	0.84	
	Surat Thani	850 (5.9)	11 (2.3)	1.3	

[a] At least one of the expected values is smaller than 0.05, the *P* value is calculated by the Chi square test; N/A Information not available.

Table 8. Demographic characteristics of patients
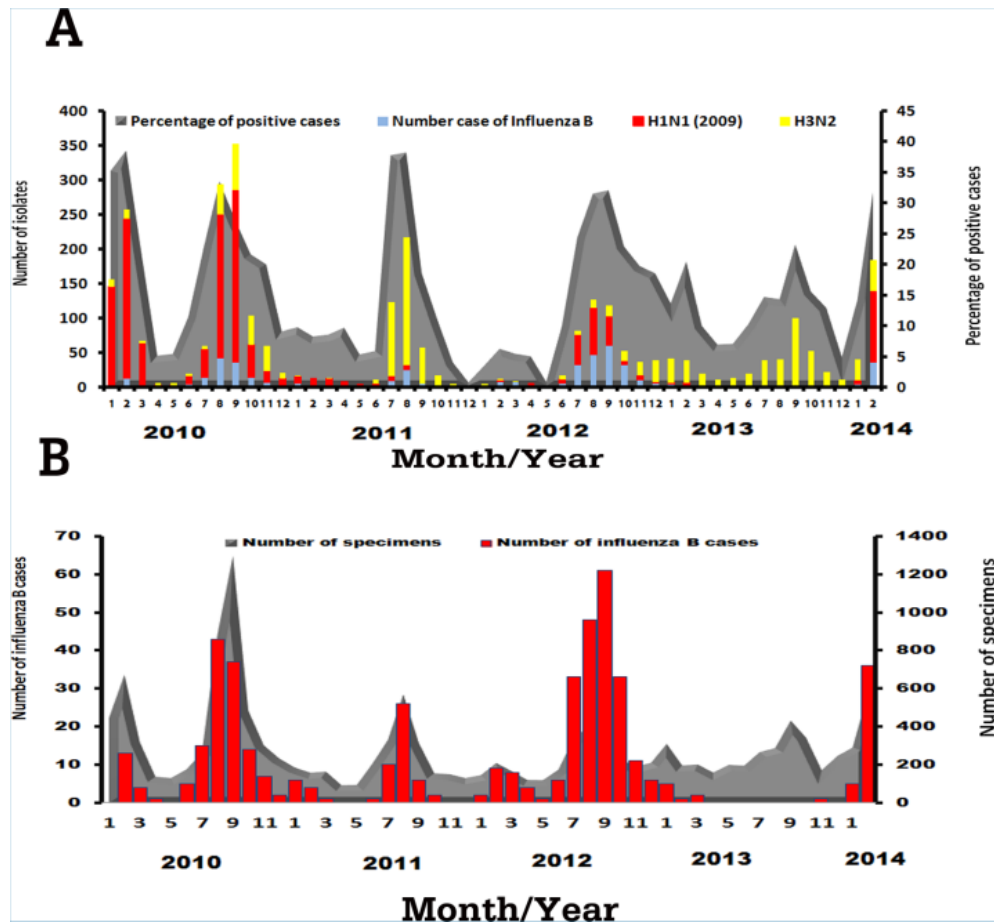
Figure 6. *Incidence of influenza A and B viruses identified from clinical samples between 2010 and 2014. (A) The distribution of the influenza isolates for each month, including influenza B (light blue color), influenza A(H1N1)pdm09 (red color), and influenza A(H3N2) (yellow color) shown as bars (left scale). From the total number of specimens collected every month, the percent of influenza-positive cases are shown as grey color area under the curve (right scale). (B) Bar graph illustrating only the total number of influenza B infection monthly (left scale) relative to the number of total specimens collected each month are shown in gray (right scale).*

Sequence and phylogenetic analysis

To identify the lineage of the influenza B virus circulating in Thailand during these years, 51 samples which tested positive for influenza B virus by real-time RT-PCR were randomly selected (at least one isolate per month when available) and the entire haemagglutinin (HA) gene was sequenced. Evaluation of the HA nucleotide sequences revealed that all influenza B isolates from 2010 belonged to the B/Victoria lineage (Figure 7). Interestingly, co-circulation of influenza B/Victoria lineage (68.2%) and B/Yamagata lineage (31.8%) was observed during 2011–2012. From 2013 to February 2014, however, all isolates belonged to the B/Yamagata lineage. Although the 51 random isolates represented only one/tenth of all influenza B cases during this period, these data suggested a lineage shift from B/Victoria to B/Yamagata during the past 4 years in Thailand.
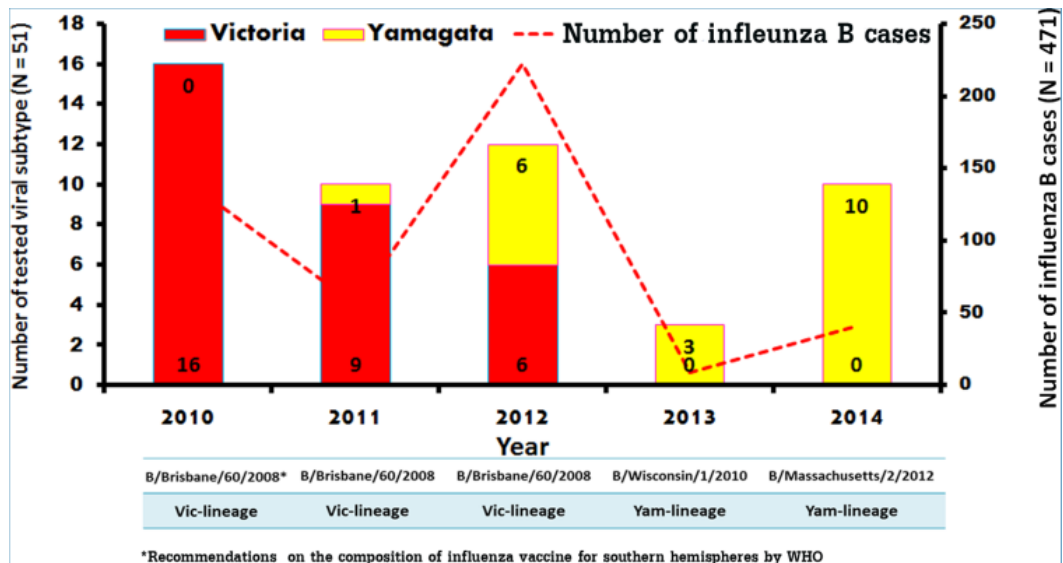
Figure 7. *Analysis of the numbers and influenza B strains of randomly sampled sequences from January 2010 to February 2014. Number of B/Victoria and B/Yamagata lineage strains found are displayed in red and yellow bars, respectively (left scale). Total number of influenza B positive samples for each year is indicated by dot-line (right scale).The strains B/Brisbane/60/2008, B/Wisconsin/1/2010, and B/Massachusetts/2/2012 included in the Southern hemisphere vaccines for the given year are indicated by the asterisk. Vic denotes Victoria and Yam denotes Yamagata.*

To fully characterize these circulating influenza B strains, the entire viral genomes of the 51 isolates were sequenced. For comparison, we also included 2 additional full-length influenza B sequences from samples previously isolated in 2006 and 2008. The designations, collection dates, and relevant details are shown in Table 9. Individual gene sequences from these 53 isolates were then compared to other strains previously isolated between 2010 and 2011 (GenBank accession numbers JX512971-JX513210), 2012 to 2014 (GenBank accession numbers KM100190-KM10033), and several established vaccine and reference strains.

Table 9. *Influenza B virus clinical isolates sequenced in this study*

Name of isolate	Collection date	Location	Age (yr)	Sex	Clade	Genes sequenced
B/Thailand/CU-243/2006	2006	-	-	-	Vic-3	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-364/2008	2008	-	-	-	Vic-3	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H1400/2010	1-Feb-10	Bangkok	6	Male	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B2201/2010	24-Feb-10	Bangkok	8	Male	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B2271/2010	5-Mar-10	Bangkok	5	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B2320/2010	20-Mar-10	Bangkok	33	Male	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H1896/2010	29-Apr-10	Surat Thani	-	Female	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B2372/2010	4-Jun-10	Bangkok	17	Female	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B2390/2010	17-Jun-10	Bangkok	7	Female	Vic-5	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B2432/2010	9-Jul-10	Bangkok	16	Female	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B2504/2010	9-Jul-10	Bangkok	6	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H2132/2010	5-Aug-10	Surat Thani	-	Female	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B2660/2010	14-Aug-10	Bangkok	-	Female	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B3153/2010	1-Sep-10	Bangkok	14	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H2584/2010	30-Sep-10	Bangkok	12	Female	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-C1262/2010	5-Oct-10	Khon Kaen	10	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H2738/2010	17-Nov-10	Bangkok	25	Male	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-C1451/2010	8-Dec-10	Khon Kaen	14	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B4504/2011	1-Jan-11	Bangkok	7	Female	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B4585/2011	4-Feb-11	Bangkok	33	Female	Vic-5	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H2933/2011	12-Feb-11	Bangkok	3	Male	Yam-3	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-C1768/2011	22-Mar-11	Khon Kaen	-	Female	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H3002/2011	9-Jul-11	Bangkok	-	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B5522/2011	27-Aug-11	Bangkok	7	Female	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H3052/2011	29-Aug-11	Bangkok	1	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B5671/2011	15-Sep-11	Bangkok	34	Female	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B5734/2011	23-Sep-11	Bangkok	34	Female	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B5910/2011	4-Nov-11	Bangkok	50	Male	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B6078/2012	19-Feb-12	Bangkok	-	Male	Yam-2	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B6096/2012	13-Mar-12	Bangkok	8	Male	Yam-3	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B6148/2012	4-Apr-12	Bangkok	33	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B6240/2012	23-May-12	Bangkok	30	Male	Vic-1B	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B6257/2012	14-Jun-12	Bangkok	33	Female	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H3313/2012	16-Jul-12	Bangkok	9	Female	Yam-3	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H3349/2012	20-Aug-12	Bangkok	2	Female	Yam-2	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B6975/2012	20-Sep-12	Bangkok	8	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H3456/2012	24-Oct-12	Bangkok	7	Female	Yam-2	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B7215/2012	19-Nov-12	Bangkok	5	Male	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-H3496/2012	14-Dec-12	Bangkok	11	Male	Yam-3	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B7337/2012	18-Dec-12	Bangkok	55	Female	Vic-1A	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B8332/2013	11-Feb-13	Bangkok	9	Female	Yam-2	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-B8813/2013	19-Dec-13	Bangkok	1	Male	Yam-2	PB2, PB1, PA, HA, NP, NA, MP, NS
B/Thailand/CU-A585/2013	25-Dec-13	Khon Kaen	56	Female	Yam-2	HA, NA, MP

HA nucleotide sequence variations in clinical isolates

Phylogenetic analysis of the HA nucleotide sequences identified 6 genetic clades of influenza B/Victoria lineage and 3 genetic clades of the B/Yamagata lineage (Figure 8). The majority of the clinical isolates grouped into B/Victoria clades 1A and 1B, especially isolates from 2010 to 2012. These clades shared the nucleotide sequences coding for the amino acid substitutions N165K, N75K, and S172P on the HA. Specifically, the B/Victoria clade 1A strains encoded an additional I146V substitution, while clade 1B strains encoded an additional L58P substitution. The B/Victoria clade 1A comprises the B/Brisbane/60/2008, a 2010–2012 Southern hemisphere vaccine strain. Moreover, 2 of the 51 isolates grouped into B/Victoria clade 5, which is characterized by the T37I substitution. Interestingly, the 2 isolates from 2006 and 2008 formed B/Victoria clade 3, as was B/Malaysia/2506/2004 included in the 2006 Southern hemisphere influenza vaccine (>99.3% nucleotide and amino acid similarities).

The remaining influenza B isolates clustered with B/Yamagata clades 2 and 3. Sixteen of the 51 isolates (99.31% nucleotides and 99.77% amino acid identity) clustered in clade 2, which is characterized by the P108A substitution. A notable strain in this clade included the B/Massachusetts/2/2012, which was chosen for the 2014 Southern hemisphere vaccine. Along with 4 isolates, clade 3 included the B/Wisconsin/01/2010 vaccine strain for 2013. Members in clade 3 possessed S150I, N165Y, and G229D substitutions and shared >99.2% nucleotide and amino acid homology.
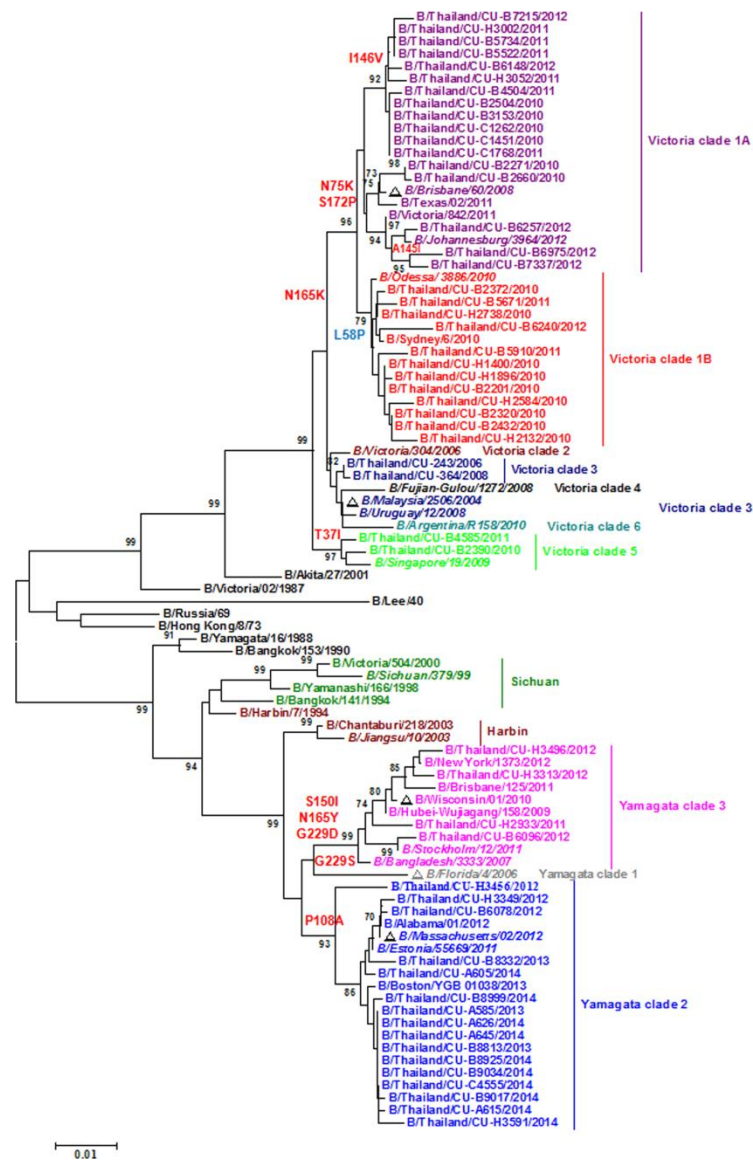
Figure 8. *Phylogenetic analysis of the HA nucleotide sequences from influenza B strains isolated in Thailand from 2006–2014. The 53 HA sequences of influenza B (names beginning with B/Thailand/CU) were compared with those from the vaccine strains of southern hemisphere which are recommended by WHO (noted with triangles) and reference strains of the clades previously reported by WHO Influenza Centre London (italic font). The phylogenetic tree was generated by the neighbor-joining method with 1,000 bootstrap replicates. Branch values >70 are indicated. The scale bar represents approximately 1% nucleotide change between close relatives.*

HA protein sequence variations in clinical isolates

As sequence variations on the HA protein can influence both receptor-binding and antigenic epitopes, we examined amino acid changes in these clinical isolates. Structural studies of the B/Hong Kong/8/73 HA protein, which represent an earlier strain before influenza B diverged into B/Victoria and B/Yamagata, had identified 4 major epitopes on HA1, the subunit of HA important for antigenic variation. They are the 120-loop, the 150-loop, the 160-loop, the 190-helix, and their respective surrounding regions (Wang et al., 2007). The 33 HA sequences of B/Victoria lineage and 20 HA sequences of B/Yamagata lineage in this study showed diverse amino acid substitutions on these epitopes (Table 10). For example, the HA1 residues 179–181 near the 120-loop encode amino acids TKG (179TKG181) in strains isolated between 1972–1982 (Crooks et al., 2004), but all isolates of the B/Victoria lineage in this study were 179TEG181. In addition, 4 of the B/Yamagata isolates also have 179TEG181, while 16 isolates have a novel 179AEG181. In the 150-loop, all B/Victoria strains have 148NGN150 similar to the early influenza B strains. However, 4 and 16 B/Yamagata isolates had 148SKI150 and 148SKS150, respectively. Within the 160-loop, isolates of both lineages had amino acid insertions in this region when compared to B/Hong Kong/8/73.

Comparison of the 33 isolates of B/Victoria lineage and 20 isolates of B/Yamagata lineage with the vaccine strains B/Brisbane/60/2008 (Victoria lineage) and B/Florida/4/2006 (Yamagata lineage) (Dreyfus et al., 2012) showed identity at residues F95, W158, H191 and Y202 (Figure S1). These four amino acids form the base of the receptor-binding site on the HA protein and are highly conserved among all known sequences of influenza B virus HA (Wang et al., 2007). However, all of our B/Yamagata isolates had I136R substitution, another receptor binding residue previously observed in 98% of B/Yamagata strains (Ni et al., 2013), while all isolates of B/Victoria lineage have I136K substitution. Finally, another important attribute of the HA protein is its glycosylation sequence. Our B/Yamagata isolates possessed a novel potential N-linked glycosylation at residue 197, which was not present in any of the vaccine or reference strains chosen for comparison.

Table 10. *Amino acid substitutions found in the HA protein of influenza B virus clinical isolates in this study* [a].

Residues at site [b]	B/HK/73	Victoria (# of sequences)	Yamagata (# of sequences)
HA1 subunit			
120-Loop			
48	Q	E (33)	R (4), K (16)
56	N	K (31), R (2)	D (20)
71	K	K (33)	M (20)
75	T	K (29), N (4)	T (19), I (1)
116	N	H (32), N (1)	K (2), N (18)
122	R	H (33)	Q (20)
125	T	I (33)	I (20)
129	T	N (32), S (1)	K (20)
179-181	TKG	TEG (33)	TEG (4), AEG (16)
150-Loop			
148-150	NGN	NGN (33)	SKI (4), SKS (16)
160-Loop			
Insertion at 162 - 163	-	NDK (30), NDN (3)	NDY (4), DNN (16)
190-Helix			
195	E	E (33)	K (20)
199	V	A (32), E (1)	K (20)
206	K	K (33)	N (20)
230	N	N (33)	D (20)
232	A	T (33)	T (9), R (11)
235	E	G (33)	G (20)
Receptor binding site			
136	I	K (33)	R (20)
HA2 subunit			
132	D	E (33)	D (20)
158	N	D (33)	N (20)

[a] The defined residue positions on the antigenic epitope according to Ni et al., 2013 [24].

[b] The residues are numbered according to that of B/HK/73 HA [23].

NA sequence variations in clinical isolates

Frequent reassortment of gene segments often complicates lineage assignment, therefore we analyzed the lineage in all subsequent gene segments using previously proposed group designation (Chen & Holmes, 2008). The phylogenetic tree of NA nucleotide sequences classified them into two distinct groups (groups II and III). Group II consisted of the viruses from HA Victoria and HA Yamagata lineages. All fifty-three isolates clustered in this group (Figure 9). In contrast, group III consisted of viruses with the HA sequences belonging to Victoria lineage. The NA sequences of the B/Victoria clade 1 strains, which included the B/Brisbane/60/2008 vaccine, were characterized by I204V and N220K substitutions, whereas B/Victoria clade 3 strains contained K285E change. The remaining clinical isolates clustered within B/Yamagata clades 2 and 3. Clade 2, which included B/Florida/4/2006, was characterized by T106I and S295R substitutions, while clade 3, which included B/Wisconsin/1/2010, was distinguished by Q42R, T125K, and K186R substitutions. Therefore, comparative analysis of the NA sequences obtained from the clinical isolates differed from the HA phylogenetic tree.

There are several important conserved residues in the NA active site of influenza B virus (Colman, Varghese, & Laver, 1983). The NA protein of influenza B clinical isolates have eight catalytic residues (R116, D149, R150, R223, E275, R292, R374, and Y409) and 11 framework residues (E117, R154, W177, S178, D197, I221, E226, H273, E276, N293, E428) (Figure S1). None of the 53 NA protein sequences we analyzed displayed substitutions in the active site and their surrounding residues. In addition to the 4 recognized potential N-linked glycosylation sites on the NA protein at positions 56, 64, 144, and 284, 5 of our isolates have a new glycosylation site at residue 463.
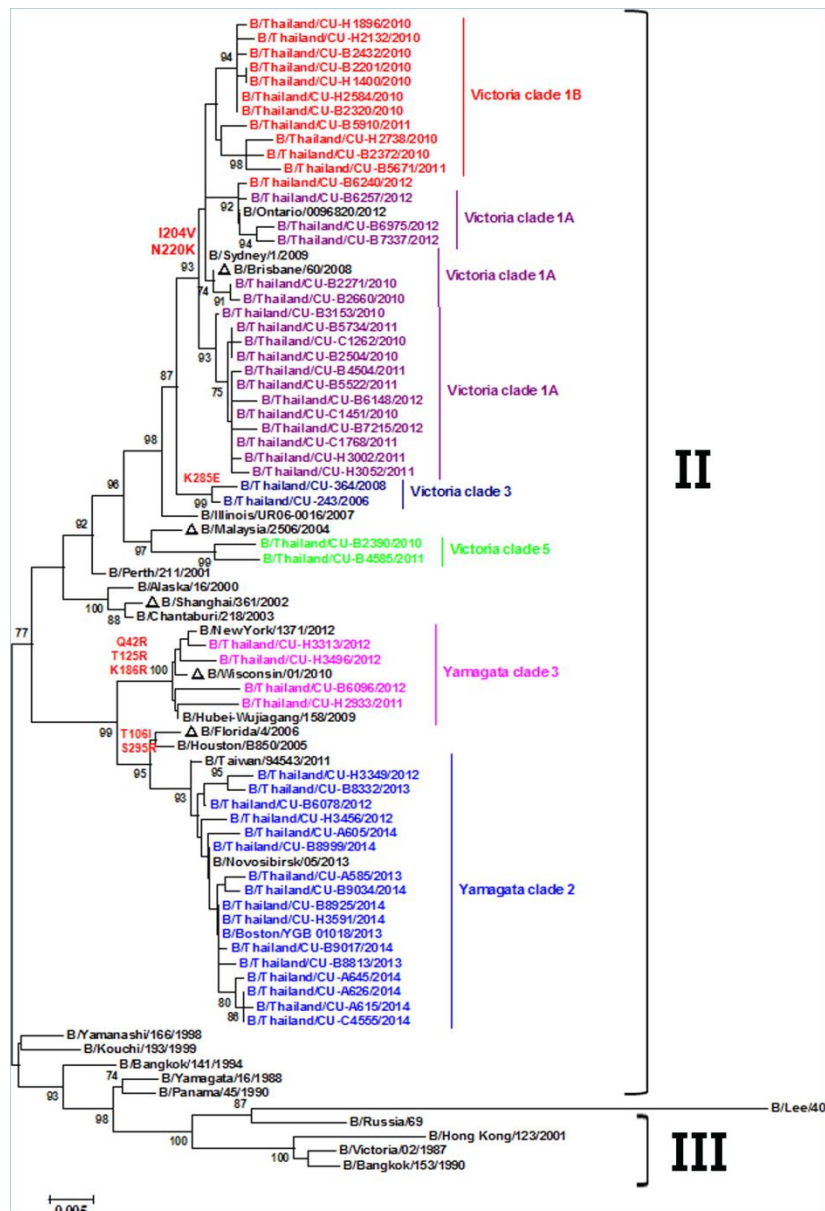
Figure 9. *Phylogeny analysis of the NA nucleotide sequences from influenza B strains isolated in Thailand from 2010–2014. Trees were constructed using neighbor-joining method in MEGA (V.6.06). Bootstrap values (1,000 replicates) >70 are indicated on the branches. Analysis was based on nucleotide 1,402 base pairs. The scale bar represents approximately 0.5% nucleotide change between close relatives. The sequences isolated in this study are denoted by /Thailand/CU. The vaccine strains are preceded by open triangles.*

Other influenza B gene segments

For the three influenza B polymerase genes (PB1, PB2, and PA), we were able to obtain full sequences from 46 strains. The polymerase genes are classified into three distinct groups (I, II, and III). Based on PB1 and PB2, all Yamagata and Victoria strains belonged to groups II and III, respectively. Phylogenetic trees of the PB2 and PB1 gene sequences appeared very similar (Figures S2 and S3, respectively). The PA and the NP genes of either Victoria or Yamagata strains, however, all clustered in group II (Figures S4 and S5). For the M gene, we were able to obtain sequences from 49 strains. Its phylogenetic tree also showed that all isolates clustered into group II (Figure S6). Since both Victoria and Yamagata isolates clustered into the same group II based on the phylogenetic analysis of the PA, NP, and M genes, it appeared likely that even after gene reassortment by some clinical isolates these genes remained associated. Furthermore, analysis of the 46 NS gene sequences in this study indicated that while the reference strains B/Victoria/02/1987 and B/Yamagata/16/1988 formed group III, all of the clinical isolates clustered together to form a separate group IV (Figure S7). A summary of the genetic analysis for each gene segment is shown in Table 11. The six internal genes of influenza B virus displayed three distinct evolutionary profiles: (i) PB1 and PB2 genes; (ii) PA, NP and M genes; and (iii) NS gene. This observation suggests that the PA, NP, M, and NS genes evolved and reassorted independently of the HA gene.

Table 11. Summary of the whole genome analysis and phylogenetic patterns of influenza B virus isolated in Thailand between 2010 and 2014

No. of strain (Year of isolation)	HA	NA	PB1	PB2	PA	NP	M	NS
2 (2006 - 2008)	Vic-3	II	III	III	II	II	II	IV
2 (2010 - 2011)	Vic-5	II	III	III	II	II	II	IV
17 (2010 - 2012)	Vic-1A	II	III	III	II	II	II	IV
12 (2010 - 2012)	Vic-1B	II	III	III	II	II	II	IV
4 (2011 - 2012)	Yam-3	II	II	II	II	II	II	IV
9 (2012 - 2014)	Yam-2	II	II	II	II	II	II	IV

Vic, strains clustered with B/Victoria/2/87; Yam, strains grouped in B/Yamagata/16/88.
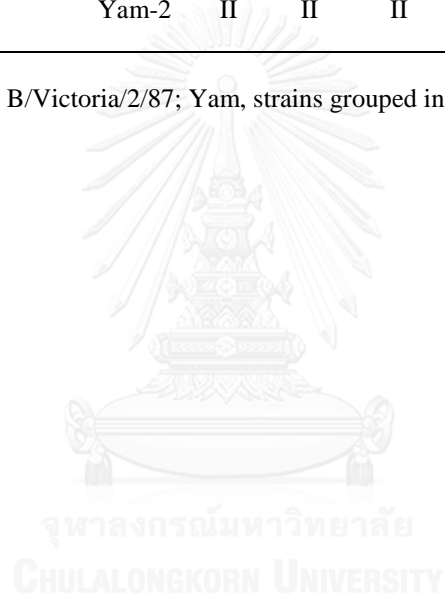
Overall profile of amino acid variations observed among the clinical isolates

A number of previous studies have described and characterized point mutations in the genome of influenza B viruses (Crooks et al., 2004; Dreyfus et al., 2012; Rota et al., 1990). To yield a better understanding of the total amino acid mutations found in all viral genes among our isolates, all 378 sequenced reads obtained from all genes were analyzed. The amino acid substitutions in the PB1, PB2, PA, HA, NP, NA, NB, M1, BM1, NS1, and NS2 are summarized (Figure 10). PB1, PB2, PA, NB, and BM1 displayed intermediate variation throughout their protein sequences, while the HA protein accumulated the most diversity of amino acid changes. In contrast, the NA protein appeared the most constant among the influenza B proteins.

Discussions

In this study, we surveyed the incidence of influenza B virus from 14,418 respiratory tract samples obtained between 2010 and 2014. We found an annual rate of influenza B infection in Thailand averaged 3.27% during this period similar to the rate of 3.68% found in the U.S. (WHO, 2014) and 3.0% in Singapore (Virk et al., 2014). The majority of influenza B virus infection affected children and adolescent between 5–19 years, which was reflective of the epidemiological data in the Japan (Kimura et al., 2011) and Finland (Heikkinen, Ikonen, & Ziegler, 2014). Although influenza infection can occur year-round, environmental factors can influence host susceptibility and increase viral spread (Dowell, 2001; Hemmes, Winkler, & Kool, 1960; Lowen, Mubareka, Steel, & Palese, 2007). The current study suggested that the relative humidity and rainfall was positively correlated with a higher prevalence of influenza B cases [data not shown] as was seen in an earlier study in Thailand (Chittaganpitch et al., 2012) and Hong Kong (Chan et al., 2009). Rain and cold weather have also been associated with seasonal influenza transmission due to close-contact and contribute to an increased risk of person-to-person transmission (Alonso et al., 2007). The seasonal patterns observed in this study further support a proposed influenza vaccination schedule of March and April in Thailand (Chittaganpitch et al., 2012).
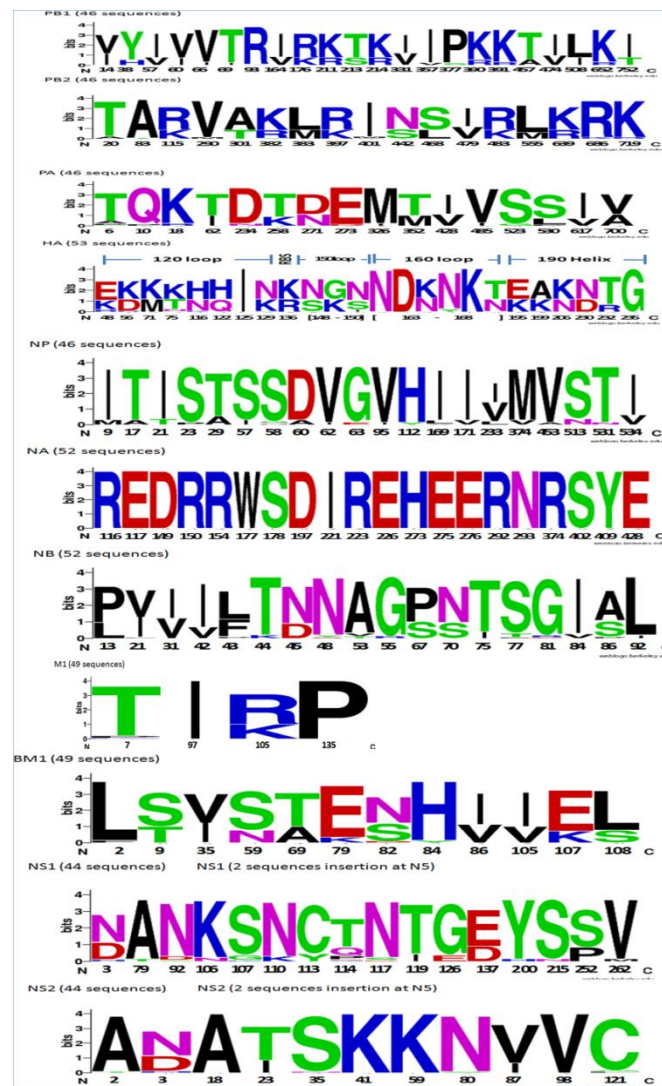
Figure 10. Amino acid residues of influenza B virus proteins isolated in Thailand during 2010–2014. (A) PB1, (B) PB2, (C) PA, (D) HA, (E) NP, (F) NA, (G) NB, (H) M1, (I) BM1, (J) NS1 and (K) NS2. The graphics were generated using WebLogo3. The relative frequency of the corresponding amino acid at a given position is proportional to the residue height. Residue positions are indicated on the x-axis. Amino acids are colored according to their chemical properties: polar amino acids (G,S,T,Y,C) are green, basic (K,R,H) blue, acidic (D,E) red, amide polar (Q, N) purple, and hydrophobic (A,V,L,I,P,W,F,M) amino acids are black.

Our whole genome sequences contribute to the growing collection of the most recent circulating strains of influenza B in Southeast Asia. Although randomly sampling one clinical isolate per month may be too few, the available data revealed changing patterns of prevalence for influenza A and B viruses in Thailand. We observed that the types or subtypes of influenza A (H1N1 pdm09, or H3N2) and B viruses have fluctuated over the past four seasons. When an influenza A virus season is severe and prolonged, influenza B diversity and severity were generally reduced (Chen & Holmes, 2008). This might have been the case in 2013 when we observed an extremely low rate of influenza B (0.37%) despite the high A/H3N2 infection in the same period. In addition, A/H1N1 pdm09 virus emerged worldwide since 2009 and this infection peaked in Thailand dramatically in 2010 until mid-2011. This might have contributed to the low level of influenza B cases in 2011.

The molecular characterization of eight gene segments of influenza B virus was previously classified (Hiromoto et al., 2000; McCullers, Saito, & Iverson, 2004; Nerome et al., 1998), but the tendency for influenza to reassort complicates viral classification. Therefore, genes other than HA were classified into groups. Five internal genes and NA could be broadly classified into three groups (I, II, and III), while the NS gene could be characterized into four groups (I–IV). Phylogenetic division of HA (6 Victoria and 3 Yamagata clades) reveals that the circulating influenza B strains during this time were Victoria clades 1A, 1B, 3, 5 and Yamagata clades 2 and 3. All of the NA sequences belonged to group II, the same group as B/Yamagata/88. This suggests that reassortment of the HA and NA genes may have occurred consistent with a previous report (McCullers et al., 2004). Meanwhile, the phylogeny of PB1 and PB2 genes clustered into group II and III, which correspond with the HA of B/Yamagata/88 and B/Victoria/87 lineages, respectively. The evolution of the PB1 and PB2 genes were similar to the HA gene (McCullers et al., 2004). In contrast, all of the PA, NP, and M genes, regardless of the lineage, were in group II. This suggests reassortment of these genes in influenza B virus (S. E. Lindstrom et al., 1999). The PA, NP, and M genes had similar evolutionary patterns suggesting possible functional association among the proteins (McCullers et al., 1999). The NS gene sequences belonging to both HA Victoria and Yamagata lineages

were classified into group IV, and phylogeny of the NS tree was absolutely divergent compared to the NP and M genes. Therefore, the NS gene exhibited the pattern of genetic reassortment which was distinct from those of the NP or M genes.

The N-linked glycosylation plays a major role in stabilizing the HA structure, to protect the HA protein from being hydrolyzed by the enzyme and to evade antibody recognition. The diversity in glycosylation on the HA1 epitope is known to result in an antigenic change (Wang, Cheng, Lu, Tian, & Ma, 2008). In this study, the N-linked glycosylation at residue N123 on HA1 no longer existed in both the B/Yamagata and B/Victoria lineage strains due to T125I substitution. This residue is located on the strong antigenic determinant 120-loop and the alteration may help the virus escape neutralizing antibody from the host (Ni et al., 2013). Meanwhile, the B/Victoria lineage strains have a glycosylation site at HA1 233 shared by none of the B/Yamagata lineage strains.

The four major antigenic sites of influenza B HA are on the 120-loop, the 150-loop, the 160-loop and the 190-helix and their surrounding regions (Wang et al., 2008). Analysis of the HA sequences showed frequent amino acid substitution on these four major epitope residues. The 120-loop epitope dictates the antigenicity of HA1 and as a result most of the mutations were found here. Fewer substitutions were observed on the 150-loop and the 160-loop among the isolates. The numbers of amino acid mutation contributing to the HA structure were sufficiently large enough to escape antigen recognition of neutralizing antibodies, but still small enough to maintain the structural integrity of the protein, especially the receptor-binding site to ensure potential binding of host cell receptors (Ni et al., 2013).

The NA active site contains 19 highly conserved residues common to all influenza A and B viruses (Colman et al., 1983). A total of 53 NA protein sequences have eight catalytic residues (R116, D149, R150, R223, E275, R292, R374, and Y409) that directly contact the sialic acid and 11 framework residues (E117, R154, W177, S178, D197, I221, E226, H273, E276, N293, E428) that support the enzymatic binding pocket. Reports indicated that E117V/A, D197N/E/Y, I221T, H273Y, R292K, and R374K mutations of influenza B NA could contribute to reduced

susceptibility of oseltamivir and zanamivir (Burnham et al., 2014; Oakley et al., 2010; Yen et al., 2006). The NA protein sequences obtained from all of the influenza B isolates did not have any changes in the active site or their surrounding residues, therefore these strains likely remained susceptible to neuraminidase inhibitor.

The existing influenza B vaccine has the limitation in that it does not cross-protect between the two distinct influenza B lineages and as a result the vaccine efficacy decreased when the included vaccine strain did not match the circulating epidemic strain (Beran et al., 2009). The influenza vaccine recommended by WHO and used in Thailand from 2010 to 2012 comprised of only the B/Victoria/87 lineage (B/Brisbane/60/2008), and although the sequence closely matched that of our clinical isolates, it did not protect infection by the B/Yamagata strains. This may explain why we observed an increased incidence of B/Yamagata (8.3%) in 2012. Thus, a quadri-valent influenza vaccine that consists of H1N1, H3N2 and two lineages of influenza B viruses should be recommended to better provide protection from influenza B infection. In summary, the phylogenetic tree of all gene segments and the mutations identified at various positions on epitopes will provide a better understanding of influenza B evolution and lead to a better vaccine development strategy.

CHAPTER III

EVIDENCE FOR INFLUENZA B VIRUS LINEAGE SHIFTS AND REASSORTANTS CIRCULATING IN THAILAND IN 2014-2016

(Part 3.3)

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CHAPTER III

Part 3.3: Evidence for influenza B virus lineage shifts and reassortants circulating in Thailand in 2014–2016

Summary

Towards the surveillance of seasonal influenza viruses between August 2015 and June 2016, respiratory samples (n = 3390) were collected from Thai patients with influenza-like illness. One-hundred fifty-seven (4.6%) samples tested positive for influenza B virus by real-time reverse-transcription polymerase chain reaction (RT-PCR). While the influenza B virus Yamagata lineage strains were more prevalent than the Victoria lineage strains in 2015 (77.5% vs. 22.5%), the Victoria lineage strains appeared to dominate the first half of 2016 (62.3%). To better assess possible lineage shift in this transition period, 73 influenza B virus strains circulating between March 2014 and May 2016 were randomly selected for hemagglutinin (HA) and neuraminidase (NA) gene sequencing. Phylogenetic analysis of the HA gene showed clustering in Yamagata clade 3 (61.6%), Victoria clade 1 (20.6%), and Yamagata clade 2 (17.8%). Analyses of both the HA and NA segments together, however, demonstrated that 5 influenza B strains (6.8%) were of mixed lineages. Our findings suggest that the circulating strains of the Victoria and Yamagata lineages underwent another lineage shift in 2016. The identification of mutations and reassortment of influenza B virus underscores the importance of careful surveillance and the selection of optimal vaccine strains.

Influenza virus infection from annual epidemics and occasional pandemics contributes significantly to the morbidity and mortality worldwide (Krammer & Palese, 2015). Current vaccine formulation includes both influenza A and B virus subtypes/strains. While influenza A virus typing depends on the combination of the hemagglutinin (HA) and the neurominidase (NA) genes, influenza B virus is genetically and antigenically divided into two lineages, Victoria and Yamagata, based on the phylogenetic analysis of the HA gene (Hay, Gregory, Douglas, & Lin, 2001). Both lineages of the influenza B virus co-circulate throughout the world including Thailand (Ambrose & Levin, 2012; Horthongkham et al., 2016). New influenza B virus strains constantly evolve because the segmented viral genome has allowed the genetic reassortment between viruses of different lineages during co-infection (McCullers et al., 2004; McCullers et al., 1999). Our previous study initially observed a transitional co-circulation period of influenza B virus in Thailand between 2010 and 2014 in which the Yamagata lineage strains began to replace the once-prevalent Victoria lineage strains (Tewawong, Suwannakarn, et al., 2015). Here, we examined the most recent prevalence of influenza B virus lineages and assessed reassortment patterns between the HA and the NA genes of the circulating influenza B virus strains.

This study was approved by the Institute Review Board of the Faculty of Medicine at Chulalongkorn University (IRB No. 581/58). A total of 3390 respiratory samples from patients with influenza-like illness were sent to King Chulalongkorn Memorial Hospital in Bangkok between August 2015 and June 2016 for testing. Virus detection directs from these clinical samples was performed using real-time RT-PCR as previously described (Suwannakarn et al., 2008). Influenza B virus-positive samples were further subjected to real-time PCR with melting curve analysis to identify potential co-infection and to determine strain lineage (Tewawong et al., 2016).

In this study, 661 samples (19.5%) tested positive for influenza A and B viruses. Among the 504 (76.2%) influenza A virus-positive samples, 325 (64.5%) were H3N2 and 179 (35.5%) were H1N1 pdm2009. More importantly, 157 samples (23.8%) tested positive for influenza B virus. For the second half of 2015, influenza B

virus of the Yamagata lineage (n = 62, 77.5%) was more frequently detected than the Victoria lineage (n = 18, 22.5%) (Figure 11). In 2016, however, Victoria lineage strains (n = 48, 62.3%) surpassed the Yamagata lineage strains (n = 29, 37.7%).

We next compiled the prevalence of influenza B virus in Thailand within the last 5 years (from February 2010 to July 2015) for which lineage data were available (Tewawong et al., 2016). The Victoria lineage (n = 311, 42.1%) and the Yamagata lineage (n = 428, 57.9%) strains co-circulated during this period (Figure 11). From February 2010 to July 2012, there were more Victoria than Yamagata lineage strains. Very few Victoria strains were detected between February 2013 and the end of 2014. The relative absence of the Victoria strains gradually changed and in 2016 comprised most of the influenza B virus detected. Therefore, analysis of the data over the past 6 years showed that lineage shifts between Victoria and Yamagata strains had occurred twice, most recently in 2016. Such lineage shift to predominantly Victoria in 2016 was also observed in Europe (Control, 2016) and elsewhere around the world (Organization, 2016).
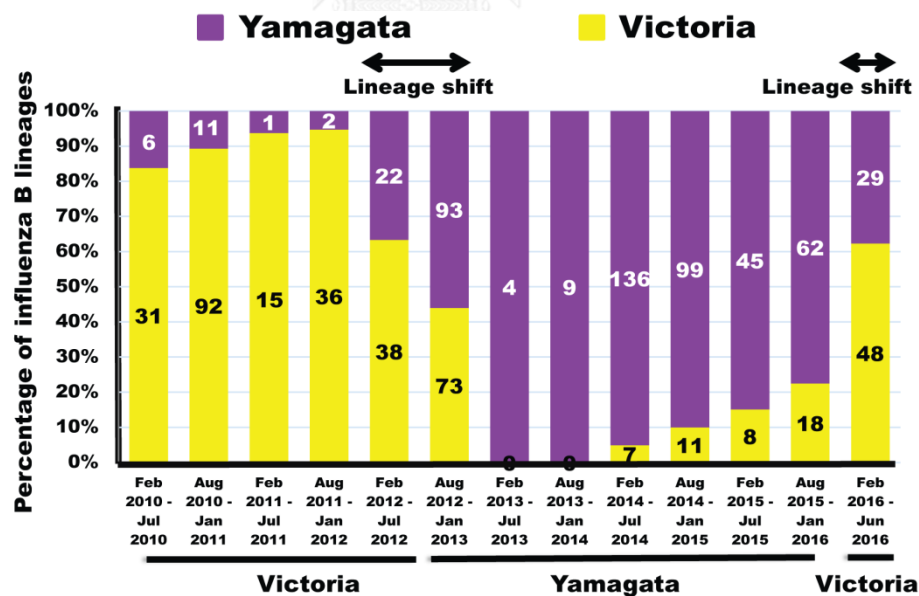
Figure 11. The distribution of influenza B virus by lineage identified from clinical samples between 2010 and 2016 (n = 23.329). The numbers of Yamagata (purple) or Victoria (yellow) lineage strains are indicated in the bar graphs.

To characterize the influenza B virus strains circulating in Thailand in more details, we examined the sequences of the entire HA and NA genes. Seventy-three influenza B virus strains collected between March 2014 and May 2016 (17 of which were from August 2015 and after) were randomly chosen (Table S3). Their HA and the NA genes were amplified by RT-PCR as previously described (Tewawong, Suwannakarn, et al., 2015), sequenced (GenBank accession numbers are indicated in Supplementary Table S3), and compared to the HA and NA sequences of 32 reference and vaccine strains available from the Influenza Virus Sequence Database in NCBI (http://www.ncbi.nlm.nih.gov) and the Global Initiative on Sharing All Influenza Data (GISAID) database (http://platform.gisaid.org/). Phylogenetic analysis and genetic distance calculation were performed using MEGA software version 6.06 (Tamura et al., 2013). Trees were reconstructed using the best-fitted Kimura's two-parameter nucleotide substitution model as determined by the Bayesian Information Criterion. Bootstraping for tree topologies was generated using the neighbor-joining approach with 1000 replicates to determine the statistical significance of branching.

Phylogenetic analysis of the HA gene showed that the circulating viruses belonged to Yamagata clade 3 (61.6%) and clade 2 (17.8%), and Victoria clade 1 (20.6%) (Figure 12). Interestingly, the analysis of the NA phylogenetic tree revealed five influenza B virus strains which appeared to be reassortants (Figure 13). Three Yamagata lineage reassortants (B/Thailand/CU-B10127/2014, B/Thailand/CU-B11705/2015, and B/Thailand/CU-B12998/2015) belonged to clade 3 (same as the B/Phuket/3073/2013 strain, which was included in the 2015 Southern Hemisphere influenza vaccine), but they possessed the NA gene of the Victoria lineage. Both the mean HA nucleotide and amino acid homologies between these reassortants and the vaccine strain were ≥ 99.3% (Table 12). The mean NA nucleotide and amino acid homologies of the reassortants compared to the B/Brisbane/60/2008 strain of Victoria clade 1 were ≥ 96.8% and ≥ 96.1%, respectively.

Meanwhile, 2 Victoria lineage reassortant strains (B/Thailand/CU-B14987/2016 and B/Thailand/CU-B15115/2016) belonged to clade 1 together with the 2016 Southern Hemisphere influenza vaccine strain B/Brisbane/60/2008, but possessed the NA gene of the Yamagata lineage. The mean HA nucleotide and amino

acid homologies between these reassortant strains and the vaccine strain were ≥ 98.4% and 99.5%, respectively. The mean NA nucleotide and amino acid homologies between the reassortants and the vaccine strain B/Massachusetts/02/2012 (Yamagata clade 2) were ≥ 97.3% and 97.2%, respectively. Although additional reassortment of genes encoding the nucleoprotein, the matrix protein, and polymerase may be possible (Dudas, Bedford, Lycett, & Rambaut, 2015; Hiromoto et al., 2000), the reassortment of the surface glycoprotein HA and NA genes enabled a significant increase in genetic diversity in addition to natural mutations.
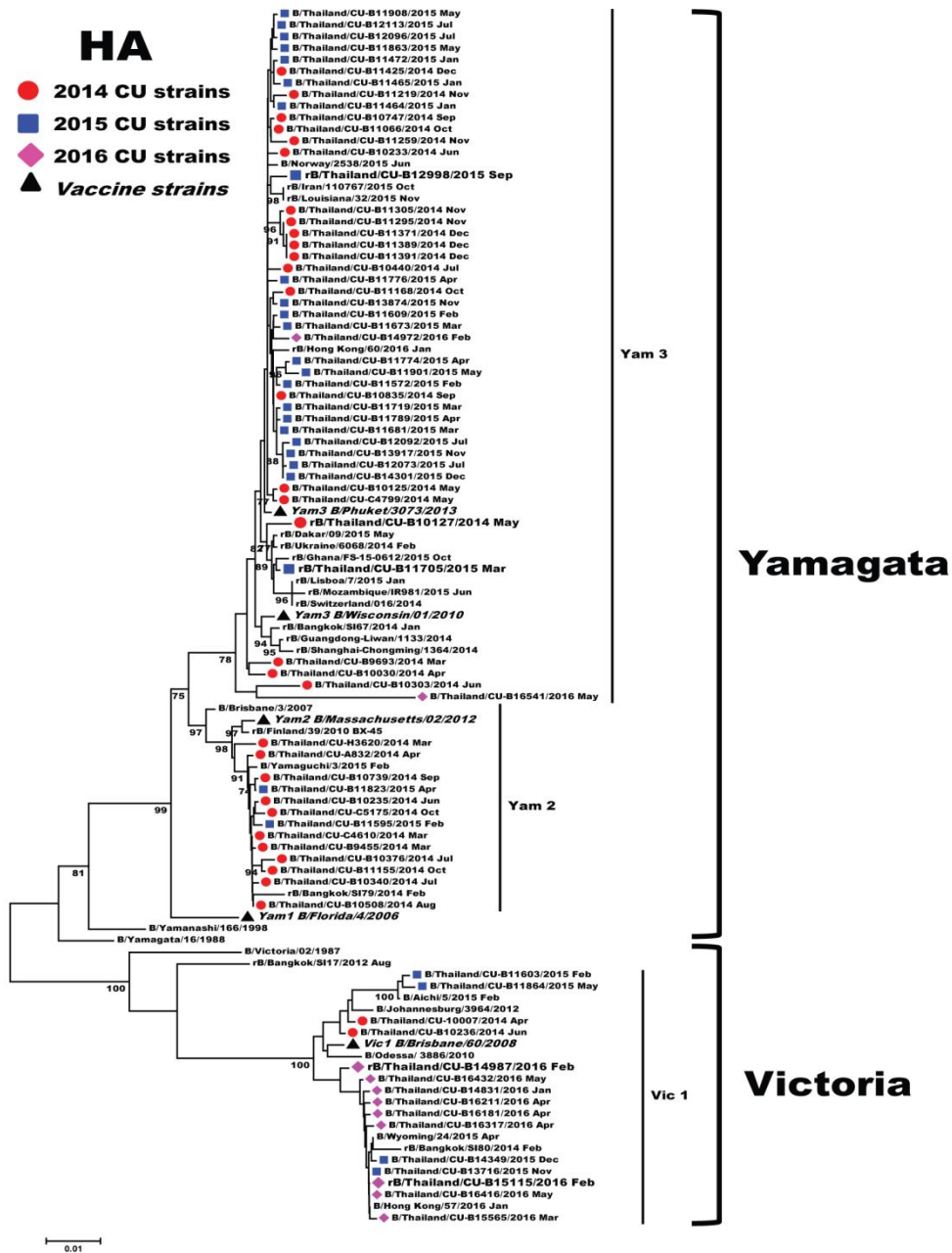
Figure 12. *Phylogenetic analysis of the influenza B virus HA gene. The neighbor-joining tree was constructed from nucleotide alignments of the HA gene using MEGA software. The genetic distances were calculated according to the Kimura two-parameter best-fit model. Branch values > 70 are indicated at the nodes. The scale bar represents approximately 1% nucleotide difference between close relatives.*
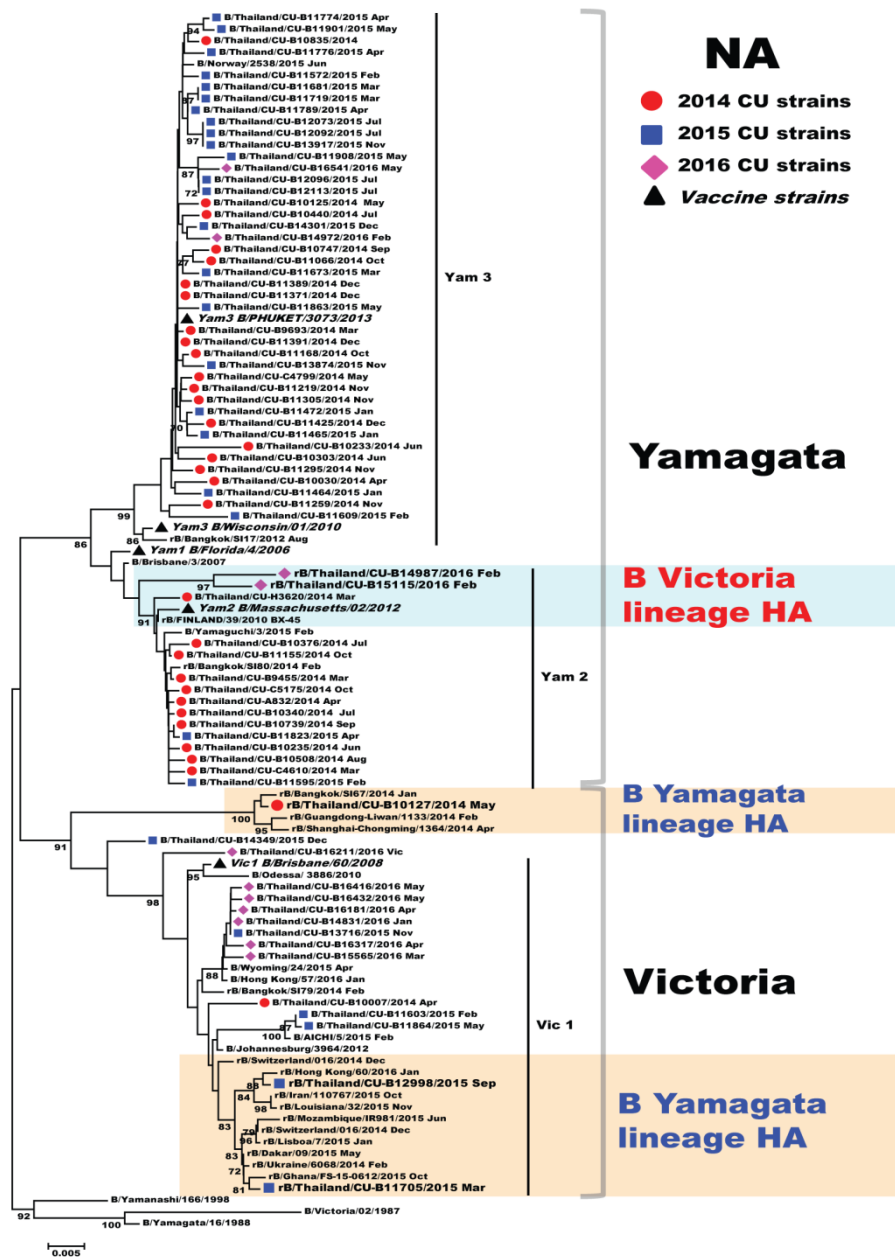
Figure 13. *Phylogenetic analysis of the influenza B virus NA gene. Phylogeny tree was reconstructed from nucleotide alignments of the NA gene using MEGA software. The genetic distances were calculated according to the Kimura two-parameter best-fit model. Branch values > 70 are indicated at the nodes. The scale bar represents approximately 0.5% nucleotide difference between close relatives.*

Previous structural studies of the HA protein of the B/Hong Kong/8/73 strain have identified four major epitope domains comprising the 120-loop, 150-loop, 160-loop, and 190-helix, which collectively form part of the receptor binding site (Wang

et al., 2007). Our study showed that the HA sequence of 45 Yamagata clade 3 strains, 15 Victoria clade 1 strains, and 13 Yamagata clade 2 strains possessed numerous amino acid substitutions in these domains (Table S4) in agreement with previous reports (Horthongkham et al., 2016; Oong et al., 2015; Tewawong, Suwannakarn, et al., 2015). Interestingly, we found a novel mutation in the 120-loop at position N129D in all 9 Victoria lineage strains from 2016, which contributed to antigenic drift (Ni et al., 2013; Wang et al., 2008). Additionally, one reassortant strain (B/Thailand/CU-B10127/2014) in this study showed nucleotide sequence change in the NA gene corresponding to amino acid D342S mutation, which has been shown by others to be associated with a 28-fold increase in the half maximal inhibitory concentration (IC_{50}) of the antiviral drug peramivir (Hurt et al., 2016). Taken together, these evidences suggest important changes in the circulating influenza B viruses.

The HA and NA genes encode surface glycoproteins important for influenza virus infectivity, virulence, and immune evasion. Diversification of influenza B virus through reassortment might contribute to changes in the antigenic properties and produce viruses with optimized match of the HA and NA functional activities (Mitnaul et al., 2000). In the current study, low prevalence of reassortants (6.8%) in the population of influenza B virus strains implies that either the HA and NA protein combination in the reassortants was incompatible with virus viability or that co-infection by both lineages was not widespread. It is increasingly recognized that host immunity to the NA protein is necessary in order to elicit protective antibody response and enhance protection against influenza challenge (Lambert & Fauci, 2010). Antibodies generated against the NA protein aggregate the virus on the host cell surface, inhibit viral release, and are often directed against conserved NA epitopes (Marcelin, Sandbulte, & Webby, 2012). Higher NA inhibition strongly correlates with protection from infection and reduction in disease severity (Memoli et al., 2016; Monto et al., 2015).

Table 12 *Sequence homology of HA and NA genes of five influenza B reassortant strains compared with nucleotide and amino acid sequences of vaccine strains*

strain	lineage		HA Homology (%)						NA Homology (%)					
	HA	NA	BR/60/2008		MA/02/2012		PH/3073/2013		BR/60/2008		MA/02/2012		PH/3073/2013	
			NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA
B/Thailand/CU-B10127/2014	Yam 3	Vic 1	88.9	93.3	97.1	98.1	**99.3**	**99.8**	**96.8**	**96.1**	94.1	93.6	94.3	94.2
B/Thailand/CU-B11705/2015	Yam 3	Vic 1	88.9	93.3	97.1	98.1	**99.4**	**99.8**	**98.9**	**98.1**	94.3	94.2	94.3	93.8
B/Thailand/CU-B12998/2015	Yam 3	Vic 1	88.4	92.6	96.9	97.8	**99.5**	**99.3**	**98.7**	**98.3**	94.1	94.4	94.2	94.0
B/Thailand/CU-B14987/2016	Vic 1	Yam 2	**98.4**	**99.5**	89.3	93.1	88.6	93.1	96.6	96.1	**97.3**	**97.2**	96.2	95.7
B/Thailand/CU-B15115/2016	Vic 1	Yam 2	**98.9**	**99.5**	88.8	93.1	88.1	93.1	96.4	96.4	**98.1**	**97.2**	96.3	95.1

Table 12. *Sequence homology of HA and NA genes of five influenza B reassortant strains compared with nucleotide and amino acid sequences of vaccine strains*

Influenza B virus reassortant strains isolated in the U.S. and China were initially described in 1999 (S. E. Lindstrom et al., 1999; McCullers et al., 1999). Subsequently, reassortants in Southeast Asia emerged between 2000 and 2002 (Barr et al., 2003). Recently, the reassortment of the NA gene in four strains of influenza B virus was reported in Thailand, the majority of which appeared in 2014 (Horthongkham et al., 2016). Our surveillance data revealed that influenza B viruses of Victoria clade 1 gradually re-emerged in Thailand and have increasingly replaced the circulating Yamagata clades 2 and 3 strains within the past few years. It is possible that the prevalence of influenza B virus lineage circulating in any given year may be driven by the strain included in the traditional trivalent vaccine. Although co-circulation of the Victoria and Yamagata lineage strains will continue to allow opportunities for reassortment, widespread use of the tetravalent influenza vaccine with both influenza B virus lineages may negate previous selective pressure responsible for driving the periodic lineage shifts. Due to continued emergence of the influenza B virus reassortants, vigilant surveillance of the circulating strains will remain important in the epidemiology and management of influenza virus.

CHAPTER IV

ASSESSING ANTIGENIC DRIFT OF SEASONAL INFLUENZA A(H3N2) AND A(H1N1)PDM09 VIRUSES

(Part 4.1)

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CHAPTER IV

Part 4.1 Assessing antigenic drift of seasonal influenza A(H3N2) and A(H1N1)pdm09 viruses

Summary

Under selective pressure from the host immune system, antigenic epitopes of influenza virus hemagglutinin (HA) have continually evolved to escape antibody recognition, termed antigenic drift. We analyzed the genomes of influenza A(H3N2) and A(H1N1)pdm09 virus strains circulating in Thailand between 2010 and 2014 and assessed how well the yearly vaccine strains recommended for the southern hemisphere matched them. We amplified and sequenced the HA gene of 120 A(H3N2) and 81 A(H1N1)pdm09 influenza virus samples obtained from respiratory specimens and calculated the perfect-match vaccine efficacy using the $P_{epitope}$ model, which quantitated the antigenic drift in the dominant epitope of HA. Phylogenetic analysis of the A(H3N2) HA1 genes classified most strains into genetic clades 1, 3A, 3B, and 3C. The A(H3N2) strains from the 2013 and 2014 seasons showed very low to moderate vaccine efficacy and demonstrated antigenic drift from epitopes C and A to epitope B. Meanwhile, most A(H1N1)pdm09 strains from the 2012–2014 seasons belonged to genetic clades 6A, 6B, and 6C and displayed the dominant epitope mutations at epitopes B and E. Finally, the vaccine efficacy for A(H1N1)pdm09 (79.6–93.4%) was generally higher than that of A(H3N2). These findings further confirmed the accelerating antigenic drift of the circulating influenza A(H3N2) in recent years.

Introduction

Influenza A virus is a major cause of acute respiratory disease in humans and is responsible for ~250,000–500,000 deaths annually worldwide (Thompson et al., 2003). Pandemic influenza A virus infection resulted in significant morbidity and mortality in 1918 (H1N1), 1957 (H2N2), 1968 (H3N2), and 2009 (H1N1) (Pariani et al., 2013). Subtypes of influenza A viruses are defined by the surface proteins hemagglutinin (HA) and neuraminidase, two major viral targets for the host immune system (Nicholson, Wood, & Zambon, 2003). The HA protein of the influenza virus is cleaved by the protease enzyme in the host cells into two subunits: HA1 and HA2. The HA1 subunit plays a major role in binding to host receptor or neutralizing antibodies and represents major antigenic sites (defined as epitopes A, B, C, D, and E). In contrast, the HA2 subunit induces fusion of the viral envelope and endosomal host membrane (Krystal et al., 1982). The accumulation of amino acid mutations on the antigenic sites of HA1 reduces antibody recognition and drives antigenic drift (Bush, Bender, Subbarao, Cox, & Fitch, 1999; J. C. De Jong, Rimmelzwaan, Fouchier, & Osterhaus, 2000; Fantoni et al., 2014; J. W. Huang & Yang, 2011).

The yearly updated trivalent influenza vaccine consists of inactivated virus with HA from types A(H1N1)pdm09 and A(H3N2), and one type B that best match the predicted circulating strains. Due to the high mutation rate of the influenza virus, however, typical vaccine efficacies are only around 50–60%, while complete protection against influenza-like illness is rarely achieved. An epidemiological study conducted in Thailand demonstrated an influenza vaccine efficacy among vaccinated children of 55–64% (Kittikraisak et al., 2015). Therefore, epidemiological studies examining how antigenic drifts affect vaccine efficacy are required for an optimal vaccine design each flu season.

Towards assessing the influenza antigenic drift, differences between the vaccine strain and the circulating strain are quantified by the number of amino acid changes in the dominant epitope, a region on the HA1 protein recognized by the neutralizing antibodies. This $P_{epitope}$ model has been used to estimate the antigenic distance for influenza virus and was shown to correlate with the vaccine efficacy to a

greater degree than phylogenetic analyses or antisera hemagglutination inhibition assay (Gupta, Earl, & Deem, 2006). To better understand the molecular evolution of influenza and assess how genetic drift affected vaccine efficacy, we examined the antigenic epitopes of HA1 of influenza A(H3N2) and A(H1N1)pdm09 circulating in Thailand from 2010 to 2014.

Materials and Methods

Specimen collection and preparation

Respiratory samples were obtained from Thai patients with influenza-like symptoms by the Center of Excellence in Clinical Virology at Chulalongkorn University. The inclusion criteria were fever (> 38°C) combined with respiratory symptoms such as cough, sore throat and runny nose. A total of 18,018 samples, collected from January 2010 to December 2014 in Thailand, were screened for influenza A and B virus by one-step multiplex real-time polymerase chain reaction (RT-PCR) and subtypes H1 and H3 were identified by specific primers as previously described (Hoffmann et al., 2001; Suwannakarn et al., 2008). Part of the surveillance data has been previously reported (Prachayangprecha et al., 2013; Tewawong, Suwannakarn, et al., 2015). Samples tested positive for seasonal influenza A (H1 and H3) were then randomly selected for HA gene analysis. All samples were stored anonymously and acquired with permission from the Director of King Chulalongkorn Memorial Hospital. The study protocol was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (IRB No. 337/57) and the need for consent was waived because the samples were anonymous. This study was conducted according to the principles expressed in the Declaration of Helsinki and the IRB waived the need for consent because the samples were de-identified and anonymous.

Nucleic acid extraction, PCR, and sequencing

Viral RNA was extracted using a commercial viral nucleic acid extraction kit (RBC Bioscience, New Taipei City, Taiwan) according to the manufacturer's instructions. Viral RNA was transcribed into cDNA using the ImProm-II reverse transcription system (Promega, Madison, WI) and 1 µM universal 12 primers (Hoffmann et al., 2001). The HA sequences were amplified by PCR with the primer sets for human influenza A(H3N2) and A(H1N1)pdm09 virus (Table S5). Briefly, a total reaction volume of 25 µl contained 10 µl of 2.5x mastermix (5Prime, Hamburg, Germany), 0.25 mM MgCl2, 0.5 µM each of forward and reverse primers, 2 µl of cDNA template and RNase-free H2O. The PCR parameters were 94°C for 3 minutes, followed by a total of 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds, and 72°C for 7 minutes. PCR products were visualized on a 2% agarose gel and purified using the HiYield gel DNA fragment extraction kit (RBC Bioscience, New Taipei City, Taiwan). DNA sequencing was performed by First BASE Laboratories Sdn Bhd (Selangor, Malaysia).

Nucleotide sequence accession numbers

The HA sequences of influenza A(H3N2) and A(H1N1)pdm09 obtained in the 2010 season in Thailand had previously been deposited in GenBank (S2 Table), while the HA sequences of the influenza A(H3N2) (in 2011–2014) and A(H1N1)pdm09 (in 2011–2014) isolates were submitted to GenBank under accession numbers KP335865 to KP335981 and KP941680 to KP941741, respectively. Moreover, the HA sequences of reference and southern hemisphere vaccine strains from influenza A(H3N2) and A(H1N1)pdm09 viruses included in phylogenetic analysis were obtained from the GenBank and GISAID databases. Their accession numbers were also included in the Table S6.

Phylogenetic analysis and antigenic characterization

The HA sequences were edited and assembled using SeqManPro (DNASTAR, Madison, WI). The ClustalX v.2.1 was used for the alignment of protein and nucleotide sequences (Larkin et al., 2007). The Akaike information criterion and maximum likelihood value indicated that the HKY+G model was the best fit model (Hasegawa, Kishino, & Yano, 1985). A phylogenetic tree of the HA1 coding nucleotide sequences was generated by Molecular Evolutionary Genetic Analysis (MEGA) version 6.06 (Tamura et al., 2013) using a maximum likelihood tree by the HKY+G model with 1,000 bootstrap replicates; only bootstrap values over 50 were shown. The amino acid residues in the five epitopes (A—E) of A(H3N2) (A/Aichi/2/1968) and A(H1N1)pdm09 (A/California/04/2009) viruses were previously identified (Deem & Pan, 2009; Macken, Lu, Goodman, & Boykin, 2001). The relative amino acid frequency in the epitope of HA1 was performed using WebLogo (Crooks et al., 2004).

Measurement of selection pressure

The selective pressure on encoding HA1 A(H3N2) and A(H1N1)pdm09 was examined by calculating the ratio of synonymous and non-synonymous substitutions (dN/dS, defined as ω) across lineage on a codon-by-codon basis. The individual site-specific selection pressure and ω were estimated using the single likelihood ancestor counting (SLAC) and fixed effects likelihood (FEL) methods contained in the HYPHY package (Delport, Poon, Frost, & Kosakovsky Pond, 2010). All analyses utilized the Datamonkey online tool (http://www.datamonkey.org). The value of ω was estimated based on the neighbor-joining trees under the HKY85 substitution model. The significance level for a positively selected site by either SLAC/FEL or both methods was accepted at 0.1.

Prediction of glycosylation sites

The NetNGlyc 1.0 server was used to predict potential N-linked glycosylation sites (amino acids Asn-X-Ser/Thr, whereby X is any amino acid except Asp or Pro) (Gupta R, 2004). A threshold value of >0.5 for the average potential score suggests glycosylation.

Estimation of vaccine efficacy using the P_{epitope} model

We estimated the vaccine efficacy of the influenza A(H3N2) and A(H1N1)pdm09 seasonal influenza viruses using the P_{epitope} method (Falchi et al., 2011; Fantoni et al., 2014; Pan, Subieta, & Deem, 2011; Shao et al., 2014). Since the vaccine efficacy is linearly correlated with the antigenic distance between the vaccine strain and the dominant circulating strains, the antigenic distance, defined as P_{epitope}, is calculated by the fraction of amino acid substitutions in the dominant HA epitope (Gupta et al., 2006). The association between vaccine efficacy and P_{epitope} is given by E = −2.47 × P_{epitope} + 0.47 for influenza A(H3N2) virus and by E = −1.19 × P_{epitope} + 0.53 for influenza A(H1N1)pdm09 virus (Pan et al., 2011). The influenza A(H3N2) vaccine efficacy with $P_{\text{epitope}} = 0$ is 47% as a perfect match between vaccine and virus (Gupta et al., 2006). For the influenza A(H1N1)pdm09 virus, the vaccine efficacy is 53% when $P_{\text{epitope}} = 0$ (Pan et al., 2011).

Results

Phylogenetic analysis of A(H3N2)

Among the 18,018 respiratory samples of unknown etiology, 673 samples (3.7%) tested positive for influenza B and 3,034 samples (16.8%) tested positive for influenza A. The latter comprised 1,394 (46%) A(H3N2) and 1,640 (54%) A(H1N1)pdm09. From these, random selection resulted in the analysis of the HA gene from 120 A(H3N2) and 81 A(H1N1)pdm09 samples.

Comparison of the HA gene was performed on the A(H3N2) strains circulating during the 2010 (N = 3), 2011 (N = 24), 2012 (N = 16), 2013 (N = 41), and 2014 (N = 36) seasons and sequences from the southern hemisphere vaccine and

reference strains. Phylogenetic analysis of the HA1 sequence showed that A(H3N2) strains from the 2010 season belonged to genetic clade 1 and shared amino acid substitutions at P162S, I260M, and R261Q (Figure 14). These 2010 strains clustered with A/Perth/16/2009, the reference vaccine strain for 2010, 2011, and 2012 (99.2% nucleotide and 98.9% amino acid identities). Meanwhile, the strains from the 2011 and 2012 seasons belonged to genetic clade 3 (3A, 3B, 3C.1, and 3C.2) and shared amino acid substitutions at N145S and V223I. Most strains (57.5%) belonged to sub-clade 3C.1 as defined by Q33R and N278K when compared to A/Victoria/361/2011, a vaccine strain for 2013 (Table S7). The A(H3N2) strains from the 2013 and 2014 seasons grouped into clades 3C.2 and 3C.3. Most sub-clade 3C.2 strains (N = 66, 85.7%) possessed N145S and V186G compared to the A/Victoria/361/2011, the reference vaccine strain for 2013. In contrast, sub-clade 3C.3 was characterized by T128A, A138S, R142G, and F159S compared to A/Victoria/361/2011 (vaccine strain for 2013) and A/Texas/50/2012 (vaccine strain for 2014).
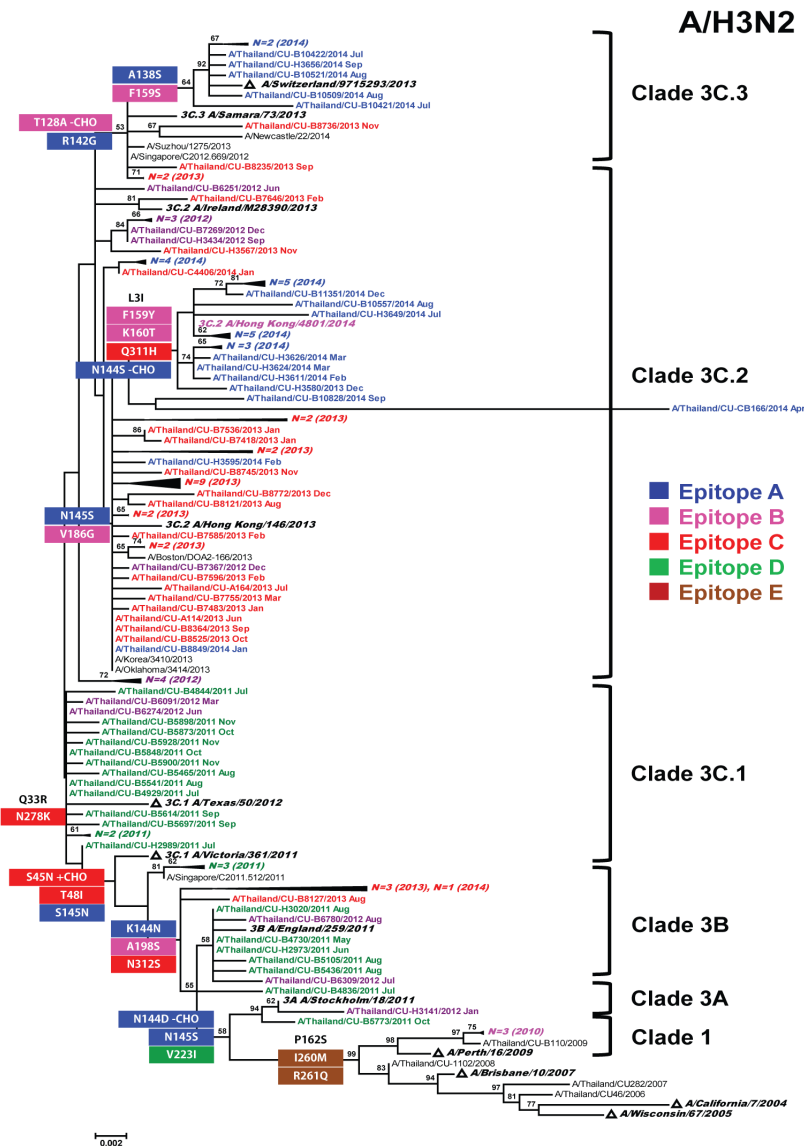
Figure 14. *Phylogenetic analysis of HA1 nucleotide sequences of influenza A(H3N2). Sequences from 120 strains isolated in Thailand during 2010–2014 (designated A/Thailand/CU) were compared to the reference strains of known clades reported by WHO Influenza Center London (bolded) and the southern hemisphere vaccine strains recommended by WHO (denoted with triangles). The phylogenetic tree was generated by the maximum likelihood method using HKY+G model with 1,000 bootstrap replicates implemented in MEGA (version 6.06). Branch values >50 are indicated at the nodes. The signature amino acid changes to each clade are indicated in different colors by epitopes (A through E). CHO denotes site-specific glycosylation. Scale bar represents approximately 0.2% nucleotide difference between close relatives.*

The overall HA1 nucleotide identities among the A(H3N2) strains compared to the given vaccine strains over the period examined were >97%, while the amino acid identities were >96% (Table 13). The nucleotide and amino acid similarities between A(H3N2) strains from the 2011 and 2012 seasons and A/Perth/16/2009 were >97.6 and >96.3%, respectively. Meanwhile, the nucleotide and amino acid similarities between the 2013 strains and A/Victoria/361/2011 were 98.7% and 97.7%, respectively. The A(H3N2) strains in 2014 were closely related to A/Texas/50/2012 (98.2% nucleotide and 96.9% amino acid identities)

Phylogenetic analysis of A(H1N1)pdm09

To assess the evolution of the A(H1N1)pdm09 during the same period, circulating strains in 2010 (N = 18), 2011 (N = 7), 2012 (N = 5), 2013 (N = 7), and 2014 (N = 44) seasons were also compared to the vaccine and reference sequences (Figure 15). There were distinct phylogenetic groups of A(H1N1)pdm09 strains between 2010 to 2014. Among the A(H1N1)pdm09 strains, 69% belonged to clade 6 viruses, while 31% grouped into clades 1, 4, 5, and 7. The HA1 sequence of A(H1N1)pdm09 viruses isolated in the 2013–2014 season clustered in genetic clades 6B and 6C. Although both sub-clades were related to the A/California/07/2009 vaccine strain (recommended every year since 2010) and shared > 98.2% nucleotide and > 97.4% amino acid sequence homology, they were slightly different from A/California/07/2009 in that they shared D97N and S185T substitutions (Table S8). Moreover, sub-clade 6B possessed additional K163Q, K283E, and A256T substitutions, while clade 6C possessed V234I, M257V, and K283E substitutions. No changes were observed in the A(H1N1)pdm09 at residues Y98, T133, W150, H180, and Q223, which are conserved and important in the HA receptor binding pocket of the influenza virus (Stevens et al., 2006).

Table 13. *Comparison of influenza A(H3N2) nucleotide and amino acid similarities between the vaccine and the circulating Thai strains.*

Year	Clade	No. of strain	Vaccine strain	% identity of HA1	
				Nucleotide	**Amino acid**
2010	1	3	A/Perth/16/2009 (clade 1)	99.2	98.9
2011	3A	1	A/Perth/16/2009	97.9	96.8
(*N* = 24)	3B	6			
	3C.1	17			
2012	3A	1	A/Perth/16/2009	97.6	96.3
(*N* = 16)	3B	2			
	3C.1	6			
	3C.2	7			
2013	3C.2	37	A/Victoria/361/2011 (clade 3C.1)	98.7	97.7
(*N* = 41)	3C.3	4			
2014	3C.2	29	A/Texas/50/2012 (clade 3C.1)	98.2	96.9
(*N* = 36)	3C.3	7			

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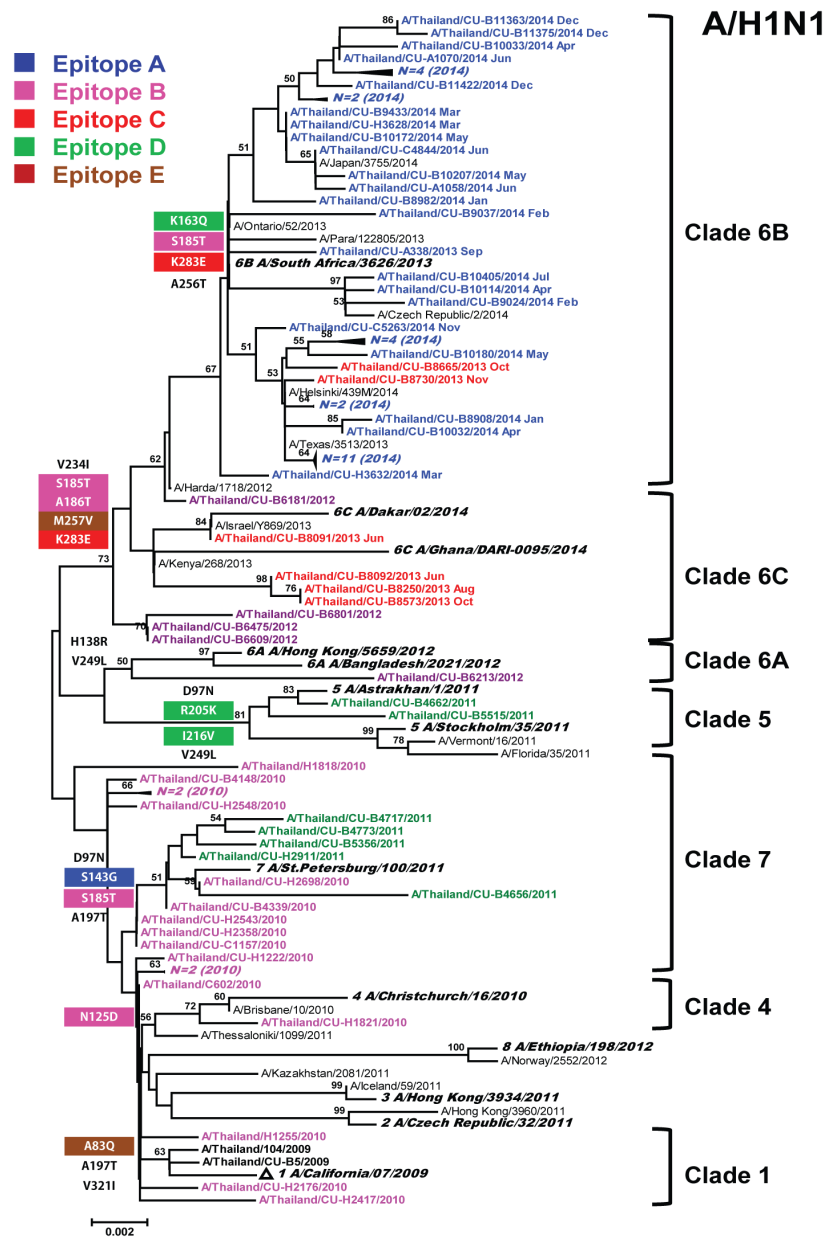
Figure 15. *Phylogenetic analysis of the HA1 nucleotide sequences of influenza A(H1N1)pdm09. Sequences from 81 strains isolated in Thailand between 2010 and 2014 (designated A/Thailand/CU) were compared to the reference strains of known clades reported by WHO Influenza Center London (bolded) and the southern hemisphere vaccine strains recommended by WHO (denoted with triangles). Phylogenic tree was generated using maximum likelihood method by HKY+G model. Bootstrap values of 1,000 replicates >50 are indicated at the nodes. Also at the nodes are the signature amino acid changes in different colors according to epitopes. Scale bar represents approximately 0.2% nucleotide difference between close relatives.*

Antigenic characterization

The receptor binding site (RBS) on the HA comprised several highly conserved amino acid residues (Y98, T133, W150, H180, and Q223; numbered according to HA1). Residues at the terminal sialic acid receptor binding sites (RBSs) of all A(H3N2) strains were I226 and S228, while all A(H1N1)pdm09 strains possessed D204 (numbered according to HA0). Additionally, differences in the residues on the A(H3N2) and A(H1N1)pdm09 HA protein were located on the antigenic sites, which comprised epitopes A to E (Tables S7 and S8). We summarized the relative frequencies of the residues found on the dominant epitope domain on the HA1 of A(H3N2) and A(H1N1)pdm09 (Figure 16). Overall, the A(H3N2) strains displayed more diversity from the accumulated epitope mutations than the A(H1N1)pdm09 strains.
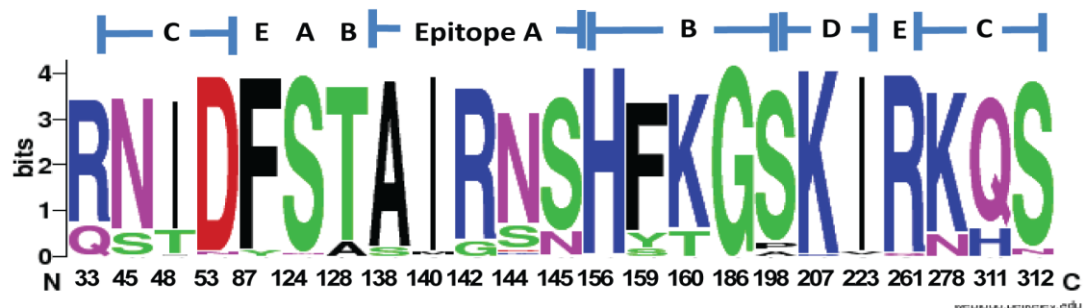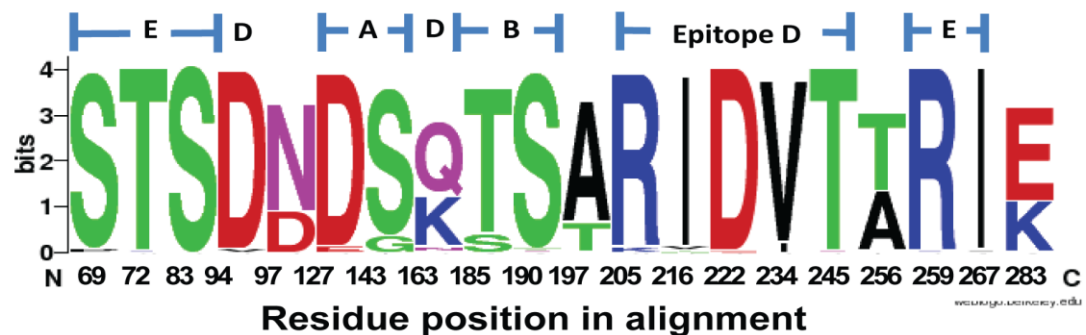
Figure 16. *Frequency of amino acid residues found on epitopes A through E in the HA1 protein of influenza virus identified in Thailand during 2010–2014.Residue positions along the x-axis for (A) influenza A(H3N2) are based on the A/Perth/16/2009 strain and (B) influenza A(H1N1)pdm09 on the A/California/07/2009. Relative frequency of the amino acid residue at a given position is proportional to the residue height. Residues were colored according to their chemical properties. Polar residues (G, S, T, Y, and C) are green; basic polar residues (K, R, and H) are blue; acidic polar residues (D and E) are red; amide polar residues (Q and N) are purple; and hydrophobic residues (A, V, L, I, P, W, F, and M) are black. Graphics were generated using WebLogo3.*

Selection pressure on A(H3N2) and A(H1N1)pdm09

Assuming that the influenza HA protein is subjected to selection pressure in order to evade the host cell recognition, the rate of change was assessed by the ω values in which ω < 1 meant that negative or purifying selection was present, ω = 1 when selection pressure was neutral, and ω >1 when there was positive selection (Kryazhimskiy & Plotkin, 2008). Analysis showed that the overall ω values of the coding HA1 regions of A(H3N2) and A(H1N1)pdm09 were 0.34 and 0.31, respectively. Since the majority of residues in the HA1 domain showed ω < 1, this suggested that the amino acids in the HA epitope domain were under purifying selection. Although overall positive selection was not present, specific sites of positive selection were found using SLAC and FEL methods (Table 14). Among the 329 codons in the HA1 domain of the A(H3N2) circulating strains, SLAC showed that only codon 33 (Q/R) was a positively selected site (ω = 3.78, P = 0.04). FEL identified codons 33 (Q/R), 144 (K/N/D/S), and 198 (A/S) as positively selected sites (ω = 8.02 × 1014, P = 0.02; infinity, P = 0.09; and 34.5 × 1014, P = 0.07; respectively). For A(H1N1)pdm09 strains, only one codon 197 (A/T) was a positively selected site (ω = 8.21 × 1014, P = 0.08) as shown by FEL.

Table 14. Positively selected sites on HA1 of influenza A virus among the Thai strains between 2010 and 2014.

Subtype	SLAC				FEL			
	position	dN/dS[a]	Normalized dN/dS	*P*-value[b]	position	dN/dS[a]	Normalized dN/dS	*P*-value[b]
H3N2	33	3.78	11.14	0.04	33	8.02x10^{14}	16.3	0.02
					144	Infinity	11	0.09
					198	34.5x10^{14}	11.5	0.07
H1N1 pdm 09	N/D	-	-	-	197	8.21x10^{14}	46.83	0.08

SLAC, single likelihood ancestor counting; FEL, fixed effects likelihood; N/D, not detected.

[a] dN/dS or ω is the ratio of synonymous to non-synonymous substitutions.

[b] *P*-value from the SLAC and FEL results for positive selection level.

Prediction of glycosylation sites

Ten potential glycosylation sites on the HA1 of A(H3N2) clades 1 and 3A strains were identified at amino acid positions 8, 22, 38, 63, 122, 126, 133, 165, 244, and 285. The K144N substitution occurred in clades 3B and 3C.1 strains, resulting in an increase in the number of glycosylation sites. The S45N substitution appeared in clades 3C.1 and 3C.2 strains, contributing to an increase in the number of glycosylation sites. Loss of a glycosylation site occurred in clade 3C.3 strains due to T128A mutation. The majority of A(H1N1)pdm09 strains possessed 6 potential glycosylation sites in the HA1 domain at amino acid positions 10, 11, 23, 87, 276, and 287, which were present in all but clade 6A. The mutation at N11S among clade 6A strains resulted in a loss of potential glycosylation site at this position. We did not find the D222G mutation commonly associated with increased virulence.

Estimation of vaccine efficacy for A(H3N2) and A(H1N1)pdm09

To assess the effect of the accumulated mutations on the HA1 domain on the vaccine efficacy in a given year, the P_{epitope} method was used to evaluate how closely the vaccine strain resembled the circulating strain (Table 15). Theoretically, when P_{epitope} in the dominant epitope is higher than 0.19, the vaccine efficacy becomes negative [10]. For 2010, the P_{epitope} between A(H3N2) strains and the A/Perth/16/2009 vaccine strain was 0.045 (epitope E; mutation 261), which suggested a worst-case vaccine efficacy against these strains of 75.96% (E = 35.7% of 47%, P_{epitope} = 0). For the 2011 and 2012 seasons, HA1 sequences showed antigenic drifts mainly on epitopes A and C (Figure S8). The P_{epitope} of 0.148 (dominant epitope = C substitutions 45, 48, 278, and 312) suggested a worst-case vaccine efficacy against these strains of 22.1% (E = 10.4% of 47%, P_{epitope} = 0) of that of a perfect match with the A/Perth/16/2009 vaccine strain. Consequently, vaccine efficacy declined by more than half. For 2013, P_{epitope} of 0.095 from 30 strains (dominant epitope = B mutations 156 and 186) suggested a worst-case vaccine efficacy against these strains of 49.9% (E = 23.4% of 47%, P_{epitope} = 0) of that of a perfect match with the A/Victoria/361/2011 vaccine strain. For 2014, the HA1 sequences mostly had a

dominant mutation in epitope B (128, 159, 186, and 198) and the P_{epitope} of 0.191 with respect to A/Texas/50/2012 vaccine strain, suggesting that the latter poorly matched the multiple circulating strains present that year. The 2015 vaccine strain A/Switzerland/9715293/2013, which belongs to clade 3C.3, was also examined for its ability to match the A(H3N2) strains of the same clade circulating in 2014. The resulting P_{epitope} value of 0.052 gave an estimated worst-case vaccine efficacy against strains of 72.3%. However, clade 3C.2 strains of A(H3N2) in 2014 and A/Switzerland/9715293/2013 showed a value of > 0.19, indicating a negative vaccine efficacy against these strains.

In contrast, comparison between the A(H1N1)pdm09 strains circulating between 2010–2014 and A/California/07/2009 vaccine strain yielded the P_{epitope} of 0.045. This was attributed to an amino acid substitution at 185 on the dominant epitope B (Figure S9) and suggested a worst-case vaccine efficacy against these strains of 89.7% (E = 47.6% of 53%, $P_{\text{epitope}} = 0$) of that of a perfect match with the vaccine strain (Table 16). In all, 12.3% of the strains obtained between 2010–2014 (10/81) possessed dominant mutation in epitope E at position 83, which gave an estimated worst-case vaccine efficacy against the virus of 93.4% (E = 49.5% of 53%, $P_{\text{epitope}} = 0$) of a perfect match with the vaccine strain. In summary, HA1 sequences of A(H1N1)pdm09 from recent years showed antigenic changes mainly on epitope B and one or two amino acid mutations on other epitopes. Taken together, these results suggested that the past and current vaccine provided optimal protection against A(H1N1)pdm09 strains that circulated in Thailand.

Table 15. *Efficacy among the vaccine strains and number of mutations found on the dominant epitope of influenza A(H3N2) circulating in Thailand.*

Year	Vaccine strain	No. of strain	Dominant epitope	No. of mutation	$p_{epitope}$	Efficacy	Vaccine efficacy (100%)
2010	A/Perth/16/2009	2	E	1	0.0455	0.3577	75.96
($N = 3$)		1	A	1	0.0526	0.3400	72.34
2011	A/Perth/16/2009	6	A	2	0.1053	0.2100	44.68
($N = 24$)		14	C	4	0.1481	0.1041	22.14
		4	A	3	0.1579	0.0800	17.02
2012	A/Perth/16/2009	3	A	2	0.1053	0.2100	44.68
($N = 16$)		11	C	4	0.1481	0.1041	22.14
		2	A	3	0.1579	0.0800	17.02
2013	A/Victoria/361/2011	30	B	2	0.0952	0.2348	49.95
($N = 41$)		2	A	2	0.1053	0.2100	44.68
		3	C	3	0.1111	0.1956	41.61
		4	B	3	0.1429	0.1171	24.92
		1	A	3	0.1579	0.0800	17.02
		1	B	4	0.1905	−0.0005	−0.10
2014	A/Texas/50/2012	1	B	2	0.0952	0.2348	49.95
($N = 36$)		8	B	3	0.1429	0.1171	24.92
		17	B	4	0.1905	−0.0005	−0.10
		9	B	5	0.2381	−0.1181	−25.13
		1	A	7	0.3684	−0.4400	−93.62
	A/Switzerland/9715293/2013	6	A	1	0.0526	0.3400	72.34
		1	B	2	0.0952	0.2348	49.95
		8	A	3	0.1579	0.0800	17.02
		1	B	4	0.1905	−0.0005	−0.10
		14	A	4	0.2105	−0.0500	−10.64
		4	B	5	0.2381	−0.1181	−25.13
		1	A	5	0.2632	−0.1800	−38.30
		1	A	11	0.5263	−0.8300	−176.60

Table 16. *Vaccine efficacy and number of mutations in dominant epitope of influenza A(H1N1)pdm09 circulating in Thailand compared with A/California/07/2009 vaccine strain.*

Year	No. of strain	Dominant epitope	No. of mutations	$p_{epitope}$	Efficacy	Vaccine efficacy (53%)	Vaccine efficacy (100%)
2010 (N=18)	11	B	1	0.0455	0.4759	47.59	89.79
	1	C	2	0.0606	0.4579	45.79	86.39
	6	E	1	0.0294	0.4950	49.50	93.40
2011 (N = 7)	2	A	2	0.0833	0.4308	43.08	81.29
	3	B	1	0.0455	0.4759	47.59	89.79
	1	B	2	0.0909	0.4218	42.18	79.59
	1	E	1	0.0294	0.4950	49.50	93.40
2012	5	B	1	0.0455	0.4759	47.59	89.79
2013 (N = 7)	3	B	1	0.0455	0.4759	47.59	89.79
	1	C	2	0.0606	0.4579	45.79	86.39
	3	E	2	0.0588	0.4600	46.00	86.79
2014	44	B	1	0.0455	0.4759	47.59	89.79

Discussion

Since its emergence in 1968, influenza A(H3N2) strain has been the predominant circulating influenza subtype between 2011 and 2014 (Kilbourne, 2006). In contrast, the A(H1N1) subtype was first documented in Thailand in May 2009 and continued to circulate until 2010. Although the (H1N1)pdm09-like A/California/7/2009 has remained the recommended vaccine strain for the past several years, we identified changes on the HA1 of A(H1N1)pdm09 Thai strains belonging to epitopes B (S185T) and E (P83S). Additional observed changes at residues 97 (D97N) and 197 (A197T) previously implicated in the adaptive mutation or immune escape continued to persist (71% and 18%, respectively) (Melidou, Gioula, Exindari, Chatzidimitriou, & Malisiovas, 2015). In this study, the vaccine efficacy for A(H1N1)pdm09 strains of 79.6–93.4% was higher than that for the A(H3N2). This is concordant with previous studies, which estimated the vaccine efficacy using serologically based methods and suggested a moderate to high vaccine effectiveness for influenza A(H1N1)pdm09 during the 2010–2014 seasons (Rondy et al., 2015; Widgren et al., 2013).

We found that influenza clusters on the phylogenetic tree is mostly chronological. The influenza A(H3N2) strains found in Thailand belonging to at least two phylogenetic sub-clades (1 and 3), which co-circulated between 2011 and 2014. During the 2010 season, only strains in clade 1 were identified. In 2011–2012, A(H3N2) sub-clade 3C strains appeared. Accumulated amino acid variations on epitope C in the HA1 domain allowed it to drift away from the A/Perth/16/2009-like strain (the vaccine strain of 2010, 2011, and 2012 seasons). As a result, the vaccine strain was changed to the A/Victoria/361/2011-like strain for 2013 season. However, all A(H3N2) strains circulated in 2013 and 2014 seasons belonged to the new emerging sub-clades 3C.2 (A/Hong Kong/146/2013-like strain) and 3C.3 (A/Samara/73/2013-like strain), which were different from the sub-clade 3C.1 A/Victoria/361/2011 and A/Texas/50/2012 strains used for the vaccines in those years. This was consistent with the P_{epitope} >0.19 obtained from the analysis, which revealed high antigenic drift and subsequently resulted in negative vaccine efficacy.

Genetic evolution of influenza virus appears gradual, but antigenic changes were found to occur more abruptly (Smith et al., 2004). For example, one single amino acid substitution in the case of N145K on the HA1 of A(H3N2) can characterize the difference between clades. It is also known that the antigenic variation of H3 occurs more frequently than H1. The average amino acid substitution rate of the HA protein is 3.6 per year for A(H3N2) and 2.45 per year for A(H1N1) (Klein, Serohijos, Choi, Shakhnovich, & Pekosz, 2014; Smith et al., 2004). One reason may be that more individuals are susceptible to the relatively novel A(H1N1)pdm09 strain, and therefore the weaker immune pressure has resulted in the slow rate of the viral evolution.

New influenza variants are thought to drift considerably from the parental strain when they displayed four or more amino acid mutations on at least two epitope domains on the HA1 protein (Wilson & Cox, 1990). Alternatively, antigenic drift variants can result from a change in the antigenic site in combination with a mutation in the RBS, which interacts with the sialic acid on the cell surface (Shih, Hsiao, Ho, & Li, 2007). We found that the HA1 sequences from the A(H3N2) strains during the 2011–2012 season possessed seven amino acid changes on four epitopes including epitope C (S45N, T48I, N278K, and N312S), epitope B (A198S), epitope D (V223I), and Q33R. In the 2013–2014 seasons, antigenic drift also occurred due to at least four amino acid mutations on epitope B combined with additional mutations on epitope A (R142G), epitope B (T128A), and epitope C (N278K). It is noteworthy that T128A had previously been observed in Fujian strain, which was associated with high mortality rate in children (Bragstad, Nielsen, & Fomsgaard, 2008).

Further analysis of the HA1 from A(H3N2) strains revealed three positively selected codons (33, 144, and 198), suggesting that these sites were immune-escaped mutants. The N144D substitution was not unique to the strains found in Thailand as it was observed among isolates in Europe and Africa during 2010–2011 (Eshaghi et al., 2014). The resulting glycosylation at position 144 was previously implicated in the antigenic change in A/Fujian/411/02-like strains from the 2002–2003 seasons (Nakajima, Nobusawa, Nagy, & Nakajima, 2005). Compared to A/Perth/16/2009, two amino acid mutations involving A128T and N45S could effectively alter the

glycosylation pattern, providing evolutionary advantage to the virus including more effective masking of viral epitopes, stabilization of polymeric HA structures, regulation of the receptor binding domain, and balancing the binding activity of HA with the release activity of neuraminidase (Sun, Wang, Zhao, Chen, & Li, 2012). This was evident when it was observed that the loss of the glycosylation site at 128 of HA1 was associated with loss of antibody recognition (Suzuki, 2011).

The vaccine efficacy between A/Perth/16/2009 vaccine strain and A(H3N2) strains circulated in Thailand in 2010 of 75.96% is consistent with a moderate vaccine efficacy reported for the trivalent inactivated influenza vaccines in 2010–2011 in Thailand (Dawood et al., 2014) and a moderate protection against subtype-specific A/H3 reported in the U.S. (Eick-Cost et al., 2012). During the 2011–2012 seasons, the dominant epitope change from A to C relative to the A/Perth/16/2009 subsequently resulted in a decline in the percentage of perfect-match vaccine efficacy (44.6% and 22.1%, respectively). Furthermore, the antigenic sites of the circulating A(H3N2) strains in 2013 and 2014 drifted from epitope C to B compared to the vaccine strains. In 2013, the reference vaccine strain had to be changed from A/Perth/16/2009 to A/Victoria/361/2011, which appeared to moderately improve the perfect-match vaccine efficacy (49.9%). This was consistent with the results from an epidemiological cohort study showing vaccine efficacy in the 2011–2012 (55%) and 2012–2013 (64%) seasons among Thai children (Kittikraisak et al., 2015). Meanwhile, the vaccine strain chosen in 2014 (A/Texas/50/2012-like) did not improve the perfect-match vaccine efficacy, which was fairly low (approximately 24.9%). Moreover, the $P_{epitope}$ values for the majority of the HA1 sequences in 2014 (75%) was > 0.19 [10] and, therefore, the vaccine efficacy became negative. This has occurred in the past whereby the outbreak of the Sydney/5/79 strain yielded the value of $P_{epitope}$ of 0.238; hence, the vaccine efficacy was −17% compared with the 1997–1998 northern hemisphere influenza vaccine (Bridges et al., 2000). Taken together, the emergence of multiple circulating strains in 2014 contributed to the reduced vaccine efficacy in Thailand that year and was reflected in the weekly morbidity and mortality report from the U. S. Centers for Disease Control and Prevention, which

suggested that the 2014–15 influenza vaccine strain A/Texas/50/2012 was essentially ineffective against the circulating A(H3N2) strains (Flannery et al., 2015).

There are several limitations in this study. Since there is no general consensus on the epitope regions for A(H1N1)pdm09, we estimated the antigenic drift and vaccine efficacy based on the mutation of the dominant epitope by mapping epitopes A-E from H3 onto the pandemic A/California/04/2009 strain. Our results therefore require validation using alternative models with differently defined epitope regions (Caton, Brownlee, Yewdell, & Gerhard, 1982; J. W. Huang, Lin, & Yang, 2012). The greater number of A(H3N2) strains analyzed in this study may have contributed to more mutations observed on the epitope domains of the A(H3N2) than the A(H1N1). Finally, the assessment of vaccine efficacy relied on the comparison of the circulating influenza strains to the vaccine strains chosen annually, therefore this measurement is not absolute as antigenic diversity have not always been predictive of the vaccine effectiveness. In conclusion, continued influenza surveillance, molecular evolution analysis, and antigenic distance measurement of the dominant influenza A strains in circulation will help refine the interpretation of vaccine efficacy and improve the yearly influenza vaccine.

CHAPTER IV

GENETIC AND ANTIGENIC CHARACTERIZATION OF HEMAGGLUTININ OF INFLUENZA A/H3N2 VIRUS FROM THE 2015 SEASON IN THAILAND

(Part 4.2)

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CHAPTER IV

Part 4.2 Genetic and antigenic characterization of hemagglutinin of influenza A/H3N2 virus from the 2015 season in Thailand

Summary

Antigenic changes in the HA1 domain of the influenza A/H3N2 hemagglutinin (HA) present a challenge in the design of the annual influenza vaccine. We examined the genetic variability in the nucleotide and amino acid of encoding HA1 sequences of the influenza A/H3N2 virus during the 2015 influenza season in Thailand. Toward this, the HA genes of 45 influenza A/H3N2 strains were amplified and sequenced. Although a clade 3C.3a strain (A/Switzerland/9715293/2013) was chosen for the 2015 vaccine, phylogenetic analysis demonstrated that strains belonging to clade 3C.2a (96 %) instead of clade 3C.3a (4 %) were circulating that year. Sequence analysis showed that seven codons were under positive selection, five of which were located inside the antigenic epitopes. The percentages of the perfect match vaccine efficacy (VE) estimated by the $P_{epitope}$ model against circulating strains suggested antigenic drift of the dominant epitopes A and B, which contributed to reduced VE of the 2015 vaccine. However, the 2016 vaccine strain (A/Hong Kong/4801/2014) was closely related and well matched against the circulating strain (mean of VE = 79.3 %). These findings provide data on the antigenic drift of the influenza A/H3N2 virus circulating in Thailand and further support continual monitoring and surveillance of the antigenic changes on HA1.

Introduction

Since influenza A/H3N2 emergence in 1968, its rapid spread has affected multiple countries (Kilbourne, 2006). The influenza virus has continually evolved by accumulated point mutations, gene reassortment, RNA genome recombination, and the production of defective interfering particles (Webster et al., 1992). Influenza hemagglutinin (HA) protein is cleaved by the host protease into two subunits: HA1 and HA2. The HA1 region is recognized by the host antibodies, which drives the accumulation of amino acid mutations due to positive selection pressure and results in antigenic drift (Illingworth, Fischer, & Mustonen, 2014). The HA1 domain of influenza A/H3N2 virus forms a globular head that comprises five epitopes: A, B, C, D, and E (Krystal et al., 1982). These epitope regions have been well characterized and are significantly involved in the host immune recognition. Amino acid residues on the epitopes correlated with the seasonal influenza severity as a result of the antigenic drift of the HA1 sequence, which can be assessed by phylogenetic analysis. Quantitating the antigenic distance (referred to as the P_{epitope}) between the vaccine and the circulating strain provides a direct correlation to the efficacy of the influenza vaccine (Air, Els, Brown, Laver, & Webster, 1985; E. T. Munoz & Deem, 2005). Mapping the antigenicity of the HA1 of influenza A/H3N2 virus has shown that antigenically distinct clusters remain dominant for approximately 3 years (Smith et al., 2004). The ongoing antigenic drift of the influenza virus significantly contributes to the emergence of the new strains and the reduced effectiveness of the vaccine (Boni, 2008).

Previously, laboratory surveillance of influenza virus to assess the antigenic drift in the HA1 domain of influenza virus was performed using the P_{epitope} method (Tewawong, Prachayangprecha, et al., 2015). The influenza A/H3N2 strains from 2013–2014 seasons showed very low to moderate vaccine efficacy and revealed antigenic drift from epitopes C and A to B. In this study, we examined the genetic variability and quantify the antigenic drift of HA1 gene of influenza A/H3N2 strains circulating in Thailand in the 2015 season.

Methods

A total of 4332 respiratory samples from patients with influenza-like illness (ILI) between January and December 2015 were collected from Bangkok for routine testing and Khon Kaen Province (an influenza virus sentinel surveillance site). ILI was defined by high fever (≥38 °C) and respiratory tract symptoms (cough, nasal congestion, sore throat, and sneezing). The samples were collected in viral transport media and sent to The Center of Excellence in Clinical Virology at King Chulalongkorn Memorial Hospital for testing of respiratory viruses. All samples were stored anonymously and acquired with permission from the Director of King Chulalongkorn Memorial Hospital. The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 581/58). Viral RNA was extracted and tested for influenza A and B using one-step multiplex real-time PCR (Suwannakarn et al., 2008). A total of 45 influenza A/H3N2 strains were randomly selected for the analysis of the HA gene segment as described previously (Tewawong, Prachayangprecha, et al., 2015). Sequences were submitted to the GenBank database under the accession numbers KU558934–KU558978. The 33 HA sequences of other Thai strains were obtained from GenBank and GISAID databases for comparison. The phylogenetic tree was generated from the neighbor-joining method using maximum likelihood model with bootstrap analysis of 1000 using MEGA version 6.06 software package (Tamura et al., 2013). The vaccine efficacy is given by $E = -2.47 \times P_{\text{epitope}} + 0.47$ for influenza A/H3N2 virus based on antigenic distance parameter P_{epitope} (Gupta et al., 2006). The NetNGlyc 1.0 server was used to predict potential N-linked glycosylation sites (Gupta R, 2004). The selective pressure on the HA1 was examined using single likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), internal branch fixed-effects likelihood (IFEL), and mixed effects model of evolution (MEME) methods contained in the HYPHY package (Delport et al., 2010). The substitution rates of the HA1 sequences were estimated using Markov chain Monte Carlo (MCMC) method in Bayesian Evolutionary Analysis Sampling Trees (BEAST) software 1.7.4 (Drummond & Rambaut, 2007).

Results and discussion

From 4332 samples, 543 (12.5 %) were typed as influenza A and 154 (3.6 %) were influenza B (Figure 17a). Among the influenza A-positive cases, 484 (69.4 %) were identified as A/H3N2 and 59 (8.5 %) were H1N1 pdm2009. The influenza A/H3N2 virus was the most common strain found during the 2015 season and the peak incidence was highest in the rainy season (June to August).

Phylogenetic analysis of the HA1 showed that most influenza A/H3N2 strains identified (96 %) belonged to genetic clade 3C.2a, while three strains isolated early in 2015 clustered in clade 3C.3a (Figure 17c). Surveillance reports in the U.S. and Europe also showed similar findings (Control, 2015; Lednicky et al., 2016). When compared to the 2015 southern hemisphere vaccine strain A/Switzerland/9715293/2013 (clade 3C.3a), the average nucleotide and amino acid sequence similarity on the HA1 were 98.1 and 96.2 %, respectively. However, the majority of the Thai strains were more similar to the A/Hong Kong/4801/2014 (clade 3C.2a), which was the 2016 vaccine strain for southern hemisphere (99.5 % nucleotide and 99.3 % amino acid sequence similarity).

The amino acid residue differences located on the HA1 antigenic sites A through E from the Thai strains in 2015 season are summarized in the Table S10. Significant differences exist on the dominant antigenic sites A and B between the Thai A/H3N2 strains and A/Switzerland/9715293/2013 vaccine strain. These changes included T128A, A138S, I140R, R142G, S144N, Y159S, T160K, and G186V. Another variation outside these epitopes also included H311Q. Key residues of the receptor-binding domain and NeuAc(α2-6)-Gal linkage specific for the H3 subtype (98Y, 136H, 153W, 183H, and 195Y; 226I and 228S) were highly conserved similar to a previous study (S. Lindstrom et al., 1996). The N144S and K160T residue changes inside epitopes A and B resulted in the loss and gain of potential glycosylation site, respectively (Control, 2015; Lednicky et al., 2016). Thus, the antigenic drift of the circulating strains in the 2014–2015 influenza seasons in the northern hemisphere has been reported to decrease the vaccine effectiveness. We estimated the substitution rate of 3.681×10^{-3} $(2.727-4.751 \times 10^{-3})$ per site per year

for the HA1 domain. The overall dN/dS ratio of selective pressure using SLAC method was 0.339, which did not provide evidence for positive selection. The SLAC method also identified only one codon at 261, while the FEL method found four positively selected sites (codons 3, 198, 261, and 312). The alternative MEME method detected four sites under positive selection pressure (3, 4, 261, and 312). To test the selection pressure on internal branches of the HA1 tree, the IFEL method identified five positively selected sites (codons 3, 4, 159, 197, and 261) (Table S9). In our study, the positively selected site codons are located inside epitope B (159, 197, and 198), epitope C (312), epitope E (261), and outside major antigenic sites (codons 3 and 4) of the HA1 domain.

The $P_{epitope}$ model has been used to estimate the influenza A vaccine efficacy and the selection of the suitable strains for annual vaccine candidate. For 2015, the $P_{epitope}$ between A/H3N2 Thai strains and the A/Switzerland/9715293/2013 vaccine strain showed a mean value of 0.2194, which is >0.19 and indicates a negative vaccine efficacy against these strains (Table 17). In contrast, the $P_{epitope}$ of the current strains was 0.0394 (0.000–0.210), which estimated a vaccine efficacy of 79.3 % (−10.64–100 %) of a perfect match with the A/Hong Kong/4801/2014. These results suggest that A/Hong Kong/4801/2014 is a significantly more effective vaccine strain than the A/Switzerland/9715293/2013 (p = 0.0005, paired t test, 2-tailed) (Figure 17b).

Table 17. *Amino acid residue differences on the dominant epitopes of influenza A/H3N2 and the antigenic distance using the $P_{epitope}$ model*

Vaccine strain	No. of strains	Dominant Epitope	No. of mutations	Residue differences					$P_{epitope}$
A/Switzerland/9715293/2013	1	A	1	140					0.0526
(2015 vaccine strain)	2	A	2	124	140				0.1053
	42	A	4	138	140	142	144		0.2105
	4	A	5	138	140	142	144	168	0.2632
	2	A	5	135	138	140	142	144	0.2632
	24	B	5	128	159	160	186	197	0.2381
	2	B	5	128	158	159	160	186	0.2381
	1	B	5	128	159	160	186	198	0.2381
								Mean	**0.2194**
A/Hong Kong/4801/2014	30	None	0						0.0000
(2016 vaccine strain)	1	A	1	135					0.0526
	1	A	1	168					0.0526
	21	A	1	142					0.0526
	1	A	2	135	142				0.1053
	3	A	2	142	168				0.1053
	1	A	3	138	142	144			0.1579
	2	A	4	124	138	142	144		0.2105
	2	B	1	158					0.0476
	2	B	1	160					0.0476
	2	B	1	197					0.0476
	1	B	1	198					0.0476
	1	B	2	160	197				0.0952
	1	C	1	51					0.0370
	1	C	1	304					0.0370
	1	C	1	276					0.0370
	1	D	1	208					0.0244
	1	D	1	171					0.0244
	4	E	1	261					0.0455
	1	E	3	2	57	81			0.0909
								Mean	**0.0394**
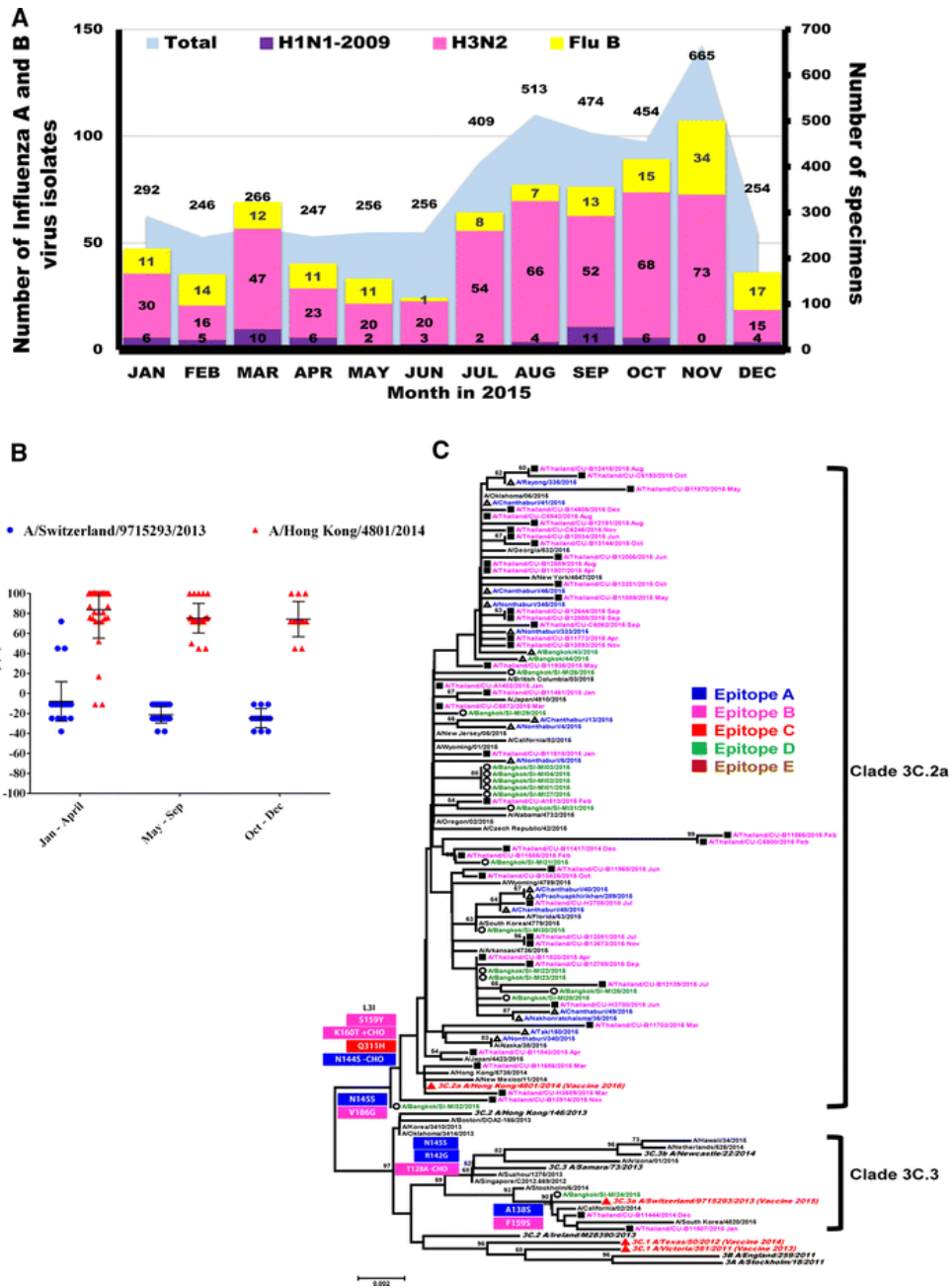
Figure 17. *(A). The total number of ILI per month (indicated above the area shaded blue) positive for H1N1 pdm09 (purple), H3N2 (pink), and influenza B (yellow) is indicated as bar graphs. (B).The vaccine efficacy afforded by A/Switzerland/9715293/2013 (circles) and A/Hong Kong/4801/2014 (triangles) against the 2015 Thai strains using the P$_{epitope}$ model. The bars of the vaccine efficacy (mean and standard error of the mean) were determined from the data points. (C). Phylogenetic tree of the HA1 nucleotide sequences of influenza A/H3N2. Sequences from 45 strains isolated in this study (filled squares) and 33 others isolated in*

Thailand in 2015 from GenBank (open circles) and GISAID (open triangles) databases were compared to the WHO-recommended southern hemisphere vaccine strains (filled triangles) and the reference strains of known clades reported by the WHO Influenza Center London (bolded). The signature amino acid changes to each clade are indicated in different colors by epitopes (A through E). CHO denotes site-specific glycosylation. Scale bar represents approximately 0.2 % nucleotide difference between close relatives (Color figure online).

Conclusion

This study found that the majority of influenza A/H3N2 virus belonging to clade 3C.2a was circulating in Thailand in the 2015 season. The high degree of antigenic drift in the HA1 domain primarily linked to the dominant epitopes A and B suggests that the vaccine strain A/Switzerland/9715293/2013 (clade 3C.3a) offered suboptimal protection from influenza A in 2015. The World Health Organization subsequently recommended the A/Hong Kong/4801/2014 (clade 3C.2a) as the vaccine strain in 2016 for the southern hemisphere. These findings highlight the importance of influenza surveillance and the characterization of genetic and antigenic variations in assisting the timely selection of the influenza strains to include in the annual vaccine.

CHAPTER V

EVOLUTION OF THE NEURAMINIDASE GENE OF

SEASONAL INFLUENZA A AND B VIRUSES

IN THAILAND BETWEEN 2010 AND 2015

(Part 5.1)

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CHAPTER V

Part 5.1: Evolution of the neuraminidase gene of seasonal influenza A and B viruses in Thailand between 2010 and 2015

Summary

The neuraminidase inhibitors (NAIs) oseltamivir and zanamivir are commonly used for the treatment and control of influenza A and B virus infection. However, the emergence of new influenza virus strains with reduced susceptibility to NAIs may appear with the use of these antivirals or even naturally. We therefore screened the neuraminidase (NA) sequences of seasonal influenza virus A(H1N1), A(H1N1)pdm09, A(H3N2), and influenza B virus strains identified in Thailand for the presence of substitutions previously reported to reduce susceptibility to NAIs. We initially examined oseltamivir resistance (characterized by the H275Y mutation in the NA gene) in 485 A(H1N1)pdm09 strains circulating in Thailand and found that 0.82% (4/485) had this substitution. To further evaluate the evolution of the NA gene, we also randomly selected 98 A(H1N1)pdm09, 158 A(H3N2), and 69 influenza B virus strains for NA gene amplification and sequencing, which revealed various amino acid mutations in the active site of the NA protein previously shown to be associated with reduced susceptibility to NAIs. Phylogenetic analysis of the influenza virus strains from this study and elsewhere around the world, together with the estimations of nucleotide substitution rates and selection pressure, and the predictions of B-cell epitopes and N-linked glycosylation sites all provided evidence for the ongoing evolution of NA. The overall rates of NA evolution for influenza A viruses were higher than for influenza B virus at the nucleotide level, although influenza B virus possessed more genealogical diversity than that of influenza A viruses. The continual surveillance of the antigenic changes associated with the NA protein will not only contribute to the influenza virus database but may also provide a better understanding of selection pressure exerted by antiviral use.

Introduction

The World Health Organization (WHO) have highlighted that the influenza virus causes approximately 3 to 5 million cases of influenza every year, which contribute to 250,000 to 500,000 deaths and 200,000 hospitalizations annually (Thompson et al., 2003). Since 1977, the seasonal influenza A(H1N1), A(H1N1)pdm09, A(H3N2), and the influenza B virus have co-circulated globally (Fiore et al., 2010; Su et al., 2015). Antivirals against influenza virus are effective for the prevention of these viral infections and have been shown to reduce the duration of infection, the severity of illness, and mortality (Fiore et al., 2011; Hayden et al., 1999; Jain et al., 2009).

Hemagglutinin (HA) and neuraminidase (NA) are the two major surface glycoproteins of the influenza virus. HA is a homo-trimeric type I integral membrane protein that plays a role in the attachment of the virion to the host receptors and is targeted by the host immune response (W. Li et al., 2011; Sauter et al., 1989). NA is a tetrameric type II integral membrane protein with sialidase activity responsible for releasing the newly produced viral particles (W. Li et al., 2011; Palese, Tobita, Ueda, & Compans, 1974).

Current treatment for influenza virus infection is limited to a single class of antivirals, namely neuraminidase inhibitors (NAIs) (Kim et al., 1997; Schild et al., 1974). Although the structure of the catalytic and antigenic sites of NA protein of the influenza virus was identified in 1983 (Colman et al., 1983), the continual evolution of the NA gene has resulted from nucleotide substitutions, insertions, and deletions (Webster et al., 1992). The relatively low fidelity of the influenza virus RNA polymerase contributes to the high rate of replication errors, which occur at approximately 1 in 10^4 bases per replication cycle (Steinhauer & Holland, 1987). Thus, each round of replication leads to a population with more variants (Webster et al., 1992). The resulting changes in the NA protein can modify the virus so that it can escape the host's immune system or be resistant to antiviral drugs and persist in the human population (Abed, Baz, & Boivin, 2006; Schild et al., 1974).

Currently, clinically approved NAIs include oseltamivir, zanamivir, peramivir, and laninamivir (M. D. de Jong et al., 2014; T. C. Li, Chan, & Lee, 2015). However, reports of emerging resistance to NAIs among some circulating strains of influenza virus have appeared (AVWG., 2016; Baz et al., 2009; Hatakeyama et al., 2007; Hurt et al., 2011; Leang et al., 2014; Meijer et al., 2009; M. Richard et al., 2011). Therefore, careful surveillance of the genetic variability of the NA gene may provide important insight into the evolution of the influenza virus. In this study, we examined for the presence of NA substitutions associated with reduced susceptibility to NAIs among influenza A and B viruses identified in Thailand. We further identified the B-cell epitopes and the potential N-linked glycosylation sites of the NA proteins, and to determine the evolutionary dynamics of the NA genes of strains of seasonal A(H1N1), A(H1N1)pdm09, A(H3N2), and influenza B viruses circulated in Thailand. The findings will aid in the understanding of the evolution of the viruses and provide surveillance data on NAI-resistant influenza virus strains. The antiviral susceptibility monitoring is important to provide the information about pandemic preparedness strategies for appropriate outbreak treatment and control.

Materials and methods

Ethical consideration

Respiratory samples were collected from patients with influenza-like illness (ILI) and analyzed at the Center of Excellence in Clinical Virology at King Chulalongkorn Memorial Hospital as part of the routine influenza surveillance program. The study protocol was approved by the Institutional Review Board (IRB) of the Faculty of Medicine at Chulalongkorn University (IRB No. 581/58). The study was conducted in accordance with the Declaration of Helsinki, and the IRB waived the need for consent because the samples were de-identified and anonymous. All the samples were acquired with permission from the Director of King Chulalongkorn Memorial Hospital.

Clinical samples

The matrix (M) and HA genes PCR positive respiratory samples (nasal and nasopharyngeal swabs and aspirates, throat swabs, and bronchoalveolar lavage) for influenza A(H1N1)pdm09, A(H3N2), and B viruses between 2010 and 2015 in Thailand were used from the current study (Tewawong et al., 2016). From 707 samples found to be positive for A(H1N1)pdm09 between November 2010 and December 2015, a total of 485 were randomly chosen for analysis of oseltamivir resistance (H275Y mutation in the NA gene) using real-time reverse transcription polymerase chain reaction (RT-PCR) for the NA gene and direct sequencing. In addition, influenza virus-positive respiratory samples [A(H1N1)pdm09 (N = 98), A(H3N2) (N = 158), and influenza B virus (N = 69)] collected by the Center of Excellence in Clinical Virology during the 2012 to 2015 influenza seasons were randomly selected for NA gene amplification and sequencing from original materials without prior virus isolation.

NA amplification and sequence analysis

The NA segment of strains of A(H3N2), A(H1N1)pdm09, and influenza B virus were amplified using conventional PCR assays according to previously reported protocols and primer sets (Chutinimitkul et al., 2008; Makkoch et al., 2012;

Tewawong, Suwannakarn, et al., 2015). Briefly, viral RNA was extracted from the respiratory samples using a commercial viral nucleic acid extraction kit (GeneAll Biotechnology, Seoul, Korea). Thereafter, cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega, Madison, WI) and 1 µM of either universal primers (Uni12 primer 5'-AGCAAAAGCAGG-3') for influenza A virus or (FluB primer 5'-AGCAGAAGCA-3') for influenza B virus. The PCR master mix contained 5 µl PRIME MasterMix (5Prime, Hamburg, Germany), 0.25 mM of $MgCl_2$, 0.5 µM each of forward and reverse primers, 2 µl of cDNA template, and nuclease-free water to a final volume of 25 µl. Amplification was performed in a thermal cycler under the following conditions: initial denaturation at 94°C for 3 minutes, 40 cycles of 30 seconds of denaturation at 94°C, 30 seconds of primer annealing at 55°C, 90 seconds of extension at 72°C, and final extension for 7 minutes at 72°C. The PCR products were visualized on 2% agarose gel stained with ethidium bromide. The expected PCR products were purified using an Expin Combo GP kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's protocol. The NA sequencing was performed by First BASE Laboratories (Selangor, Malaysia).

The NA sequences of A(H3N2) (KP336040 to KP336156 and KX151186 to KX151226), A(H1N1)pdm09 (KX151227 to KX151324), and influenza B virus (KX151325 to KX151393) in the present study were deposited in GenBank (Table S11). The accession numbers associated with the 753 NA sequences of A(H3N2) (1998-2015), A(H1N1)pdm09 (2009-2015), seasonal A(H1N1) (2004-2009) and influenza B virus (1990-2015) circulated in Thailand, of which 118 were NA sequences of seasonal A(H1N1) reference strains obtained from the databases of GenBank and the Global Initiative on Sharing All Influenza Data (GISAID) (Table S11). We analyzed for the presence of NA substitutions associated with either NAI-resistant genotype in different NA subtypes (E119V/I/A/G, H274Y, R292K, and N294S: N2 numbering) or reduced susceptibility genotype to NAIs (Q136K, D151E/V/D, D198N/G/E/Y, I222V/T/K/R/M, S246N, E276D, and R371K: N2 numbering) among influenza A and B viruses.

Detection of oseltamivir resistance in A(H1N1)pdm09 strains

A real-time RT-PCR assay for detection of oseltamivir-resistant A(H1N1)pdm09 (H275Y) strains has been published previously (Payungporn et al., 2011). Briefly, 10 μl reaction mixture comprised 3 μl of RNA, 0.75 μM of each primer, 0.25 μM of each probe, 2.5 mM of $MgSO_4$, 5 μl of 2X reaction buffer (Invitrogen, Carlsbad, CA), 0.2 μl of SuperScript III RT/ Platinum Taq High Fidelity Enzyme Mix (Invitrogen, Carlsbad, CA), and water. Amplification and data analysis were performed using the LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) under the following condition: reverse transcription at 50°C for 45 minutes, initial denaturation at 95°C for 10 minutes, 50 cycles of 15 seconds of denaturation at 95°C, and 40 seconds of annealing and extension at 60°C. The threshold cycle (C_t) values obtained from both FAM and JOE channels were used to calculate the relative quantities by employing the delta-delta C_t ($\Delta\Delta C_t$) method.

Phylogenetic analysis

The nucleotide sequences of the coding regions of NA genes of seasonal A(H1N1) (1–1410), A(H1N1)pdm09 (1–1407), A(H3N2) (1–1407) and influenza B virus (8–1408) viruses were aligned using ClustalX version 2.1 (Larkin et al., 2007). The Bayesian Information Criterion (BIC) and the maximum-likelihood value indicated the best-fit model for the seasonal A(H1N1) (T92+G+I), A(H1N1)pdm09 (T92+G), A(H3N2) (GTR+G), and influenza B virus (T92+G) datasets (Nei, 2000). The phylogenetic trees of the NA nucleotide sequences were constructed using Molecular Evolutionary Genetics Analysis (MEGA) version 6.06 (Tamura et al., 2013) employing maximum-likelihood tree with 1,000 bootstrap replicates. Bootstrap values >70% were shown.

Estimation of nucleotide substitution rates

The best nucleotide substitution model was estimated using jModelTest software version 2.1.3 (Darriba, Taboada, Doallo, & Posada, 2012) and a GTR model with Gamma-distributed rate variation among sites was selected. The overall rates of

evolutionary change (substitutions/site/year) and the relative genetic diversity of the NA genes were determined using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in Bayesian Evolutionary Analysis Sampling Trees (BEAST) software version 1.8.2 (Drummond, Suchard, Xie, & Rambaut, 2012).

Sequence dataset comprised seasonal A(H1N1) (N=144), A(H1N1)pdm09 (N=306), A(H3N2) (N=373), and influenza B virus (N=255) from 989 viruses circulating in Thailand and 89 closely related reference strains from around the world with known sampling dates. For each analysis, a strict clock and uncorrelated log-normal relaxed clock model were both used under a GTR+G or GTR+G+I substitution model. The Bayesian skylines (BSP) and Gaussian Markov Random Field (GMRF) were used as coalescent prior. Two independent Bayesian MCMC analyses were run for 100 million states, sampling every 1,000 states. The convergence and effective sample sizes (ESSs) were assessed using Tracer software version 1.6, and ESSs values of ≥ 200 were accepted. The maximum clade credibility (MCC) tree was generated using TreeAnnotator software version 1.8.2 with 10% burn-in, and Figtree software version 1.4.2 was used to visualize the annotated trees. The uncertainty in each parameter estimate was reported using 95% highest posterior density (HPD) intervals.

Estimation of selection pressure

In order to identify the selection pressure associated with the NA gene, the ratio of nonsynonymous substitutions (dN) and synonymous substitutions (dS) was estimated (dN/dS, defined as ω) using the single-likelihood ancestor counting (SLAC) method. Codon by codon basis of positively selected sites was further identified in HYPHY software (Delport et al., 2010) using the SLAC, the fixed effects likelihood (FEL), and the mixed effects model of evolution (MEME) methods. All the analyses were performed using the Datamonkey online tool. For each method, each codon with a P-value of < 0.1 was determined to be a positively selected site.

Prediction of potential N-linked glycosylation sites

The N-linked glycosylation sites were predicted using the NetNGlyc 1.0 Server (Gupta R, 2004). For each of the NA proteins, the consensus sequences associated with N-linked glycosylation sites in proteins (i.e., amino acids Asn-X-Ser/Thr, where X is any amino acid except for Asp or Pro) were examined using the artificial neural networks on the server. A threshold value of >0.5 for the mean potential score was treated as indicative of glycosylation.

Prediction of B-cell epitopes

In order to examine how the amino acid mutations found in the NA proteins of the 989 seasonal influenza A and B viruses circulated in Thailand may affect the antigenic properties of the NA protein, we predicted B-cell epitopes in the NA proteins using the BepiPred 1.0 Server. This server predicts the location of linear B-cell epitopes using a combination of a hidden Markov model and the propensity scale method (Larsen, Lund, & Nielsen, 2006). The alignment of the NA protein sequences was submitted to the server and residues were annotated as being a part of linear B-cell epitopes when the score was above a particular threshold. The score threshold of 0.35 indicated the presence of an epitope, which corresponds to a sensitivity of 0.49 and a specificity of 0.75.

Results

Genotypic analysis of the neuraminidase inhibitors resistance among influenza A and B viruses

In the years after the influenza A virus pandemic of 2010, 485 samples tested positive for A(H1N1)pdm09 of which 4 (0.82%) were oseltamivir-resistant (H275Y) strains (Figure S10 and Table S12). We next screened NA sequences for the presence of substitutions previously reported to reduce susceptibility to NAIs by the WHO expert working group on surveillance of influenza antiviral susceptibility (AVWG) 2014 (19-22; 24). Numbering is based on an alignment of NAs from the following reference strains: A/Brisbane/59/2007 (H1N1), A/California/7/2009(H1N1) pdm09, A/Perth/16/2009 (H3N2), and B/Yamanashi/166/1998. The NA sequences of seasonal A(H1N1) viruses circulated from 2004 to 2009 in Thailand showed that the resistance rate to oseltamivir was 17% (20/118) (Table 18). Between 2009 and 2015, 6.4% of viruses circulating in Thailand of A(H1N1)pdm09 were H275Y strains, whereas, 1.4% had an S247N mutation. The A(H3N2) viruses circulating in Thailand had amino acid substitutions at positions D151N/G (1.7%) and I222T/V (1.2%), which are in the active site of the NA protein and may affect enzyme activity. The NB sequences of influenza B virus circulated in Thailand between 1990 and 2015 had an amino acid mutation at position D197N (0.4%), and an amino acid substitution at position A395E (1.7%) was also found. In addition, we found that the NA protein in influenza B was mutated at positions A245S (0.4%), K360R (0.9%), and A395V/T/D/S (12.5%).

Table 18. *The frequency of NA amino acid substitutions associated with reduced inhibition by NAIs among influenza A and B viruses circulated in Thailand in 1998-2015.*

Type/Subtype (year of circulation)	Amino acid substitution [a]	No. mutant viruses/no. total (%) [b]
A(H1N1) (2004-2009)	H275Y	20/118 (16.95)
A(H1N1)pdm09 (2009-2015)	S247N	4/296 (1.35)
	H275Y	19/296 (6.42)
Influenza B virus (1990-2015)	D197N	1/232 (0.43)
	A395E	4/232 (1.72)

[a] Numbering is based on an alignment of NAs from the following reference strains: A/Brisbane/59/2007 (H1N1), A/California/7/2009 (H1N1)pdm09, A/Perth/16/2009 (H3N2), and B/Yamanashi/166/1998.

[b] The total number of NA sequences of influenza A and B viruses identified in Thailand and deposited on the NCBI and GISAID.

Phylogenetic analysis

To examine the genetic variability of the NA genes of influenza A and B viruses circulating in Thailand, we conducted a phylogenetic analysis of NA of 118 seasonal A(H1N1), 296 A(H1N1)pdm09, 343 A(H3N2), and 232 influenza B viruses. The designation of the clades for NA phylogenetic trees of influenza viruses in the present study is based on WHO influenza center London (McCauley J, 2016). In addition, we screened for the presence of NA molecular markers associated with reduced NAIs susceptibility.

The seasonal A(H1N1) viruses circulating in Thailand from 2004 to 2006 were oseltamivir-susceptible and belonged to clades 1, 2A, and 2C (Figure 18). However, the strains from 2007 to 2009 were mostly oseltamivir-resistant (54.05%, 20/37) and belonged to clade 2B. Most of these oseltamivir-resistant viruses (95%, 19/20) from 2007 to 2009 had a D354G mutation in the coding region when compared with the reference strain (A/Brisbane/59/2007).

For A(H1N1)pdm09, strains with mutation S247N circulated in 2010 belonged to clade 2 (Figure 19). They possessed amino acid mutations at positions N248D, I389V, and V394I compared with the reference (strain A/California/07/2009) (Table 19). In contrast, the H275Y oseltamivir-resistant strains that were circulated in other years were clustered separately: clade 1 (2009 season), clades 2, 5, and 7 (2010 season), clade 6A (2012 season), and clade 6B (2014 season). Most of the H275Y oseltamivir-resistant strains had the additional NA mutations at positions V106I, V241I, N248D, and N369K.

For A(H3N2), the strains demonstrated a typical ladder-like gradual evolution, with the replacement of old strains by newer ones (Figure 20). The strains with a I222T/V mutation were grouped into clade 3C.2; these two strains maintained the NA gene signature amino acid substitutions L81P, D93G, S367N, K369T, N402D, and I464L. The strains with a D151N/G mutation were clustered separately in clades 3C.2 and 3C.3. Most of these strains (83.3%) had amino acid mutations at positions L81P, D93G, S367N, K369T, N402D, and I464L.

For NA sequences of influenza B virus, the A395E NAI-resistant strains belonged to Victoria lineage (clade 1) (Figure 21). Only one D197N NAI-resistant isolate was classified in Yamagata lineage (clade 2). Most of the strains with A395V/T/D substitutions were found in the Victoria lineages. The additional amino acid substitutions found in the NA proteins of suspected NAI-resistant of influenza B viruses from Thailand are summarized (Table 19).
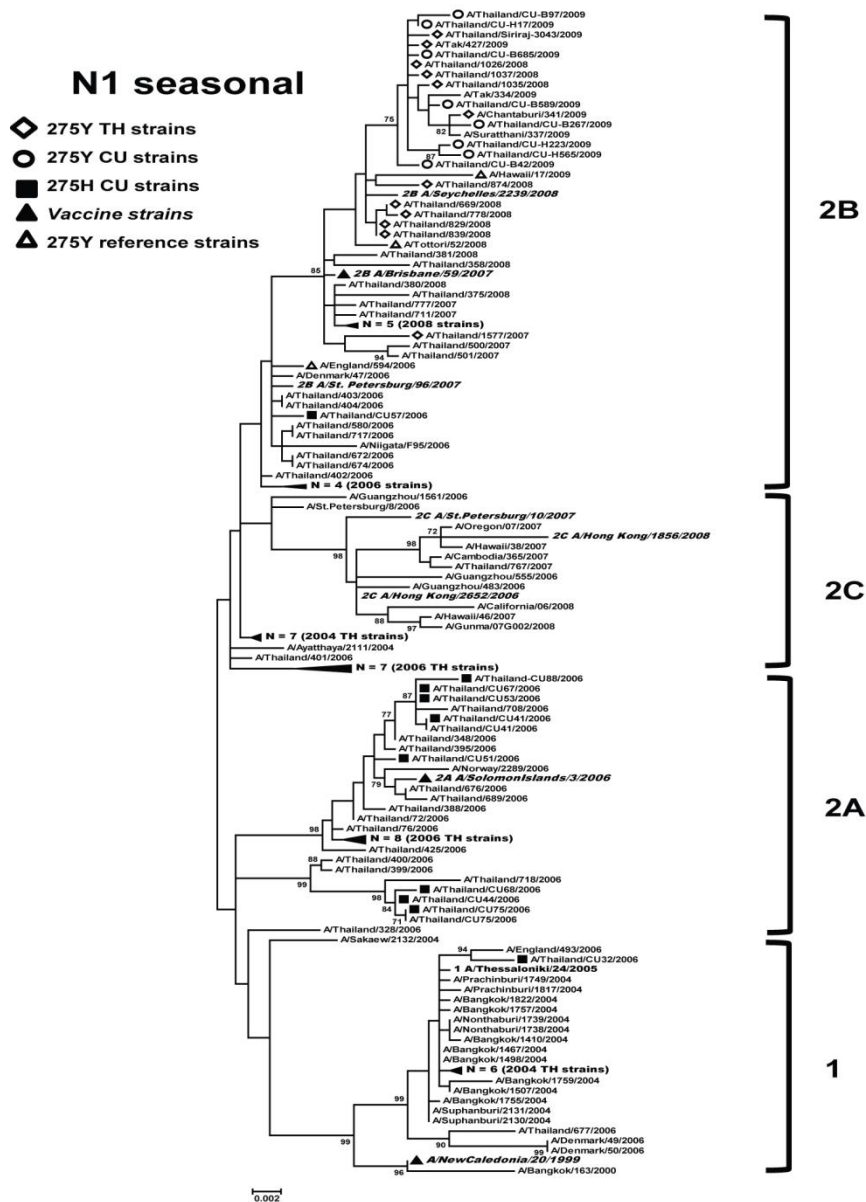
Figure 18. Maximum-likelihood phylogenetic tree of the NA gene of seasonal A(H1N1) influenza viruses circulated in Thailand. NA sequences from 118 A(H1N1) strains circulated in Thailand between 2000 and 2009 were compared to the reference strains of known clades reported by WHO Influenza Center London (bolded) and the southern hemisphere vaccine strains recommended by WHO (denoted as ▲). Bootstrap values >70% are shown at the branch nodes. Scale bar represents approximately 0.2% nucleotide difference between close relatives.
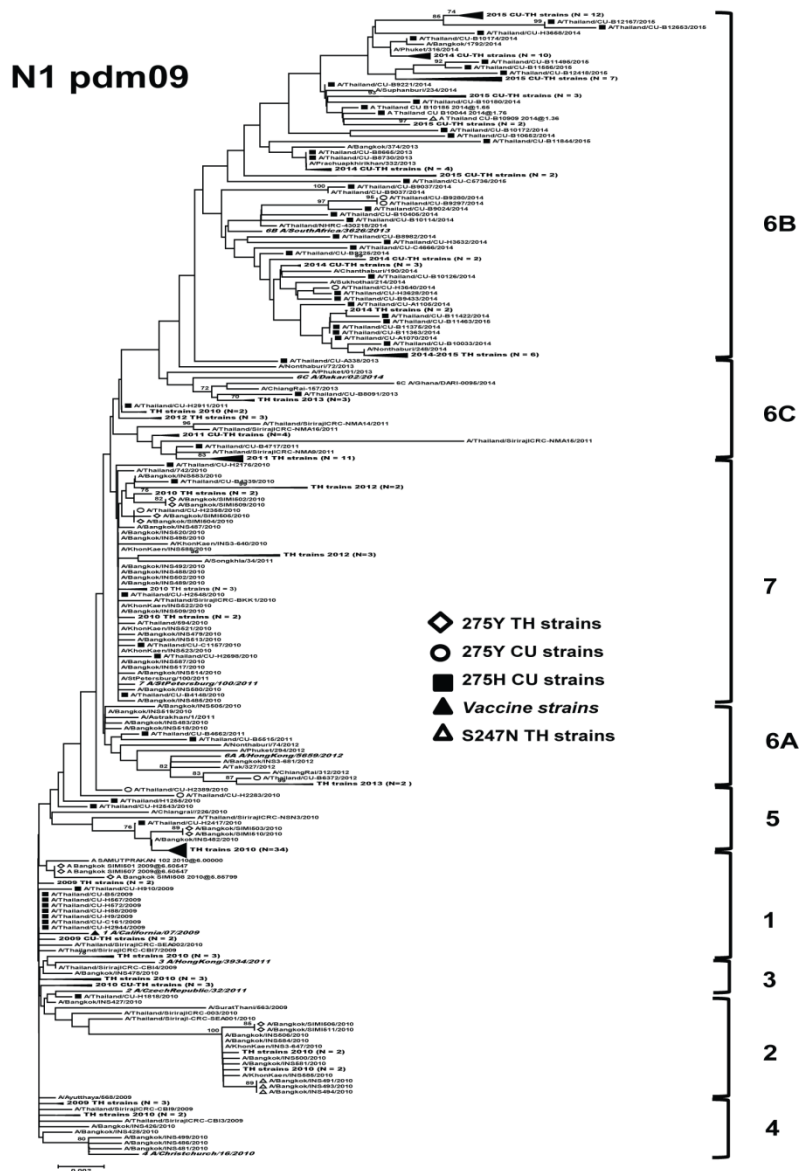
Figure 19. Maximum-likelihood phylogenetic tree of the NA gene of A(H1N1)pdm09 influenza viruses circulated in Thailand. NA sequences from 296 A(H1N1)pdm09 strains circulated in Thailand between 2009 and 2015 were compared to the reference strains of known clades reported by WHO Influenza Center London (bolded) and the southern hemisphere vaccine strains recommended by WHO (denoted as ▲). Bootstrap values >70% are shown at the branch nodes. Scale bar represents approximately 0.2% nucleotide difference between close relatives.
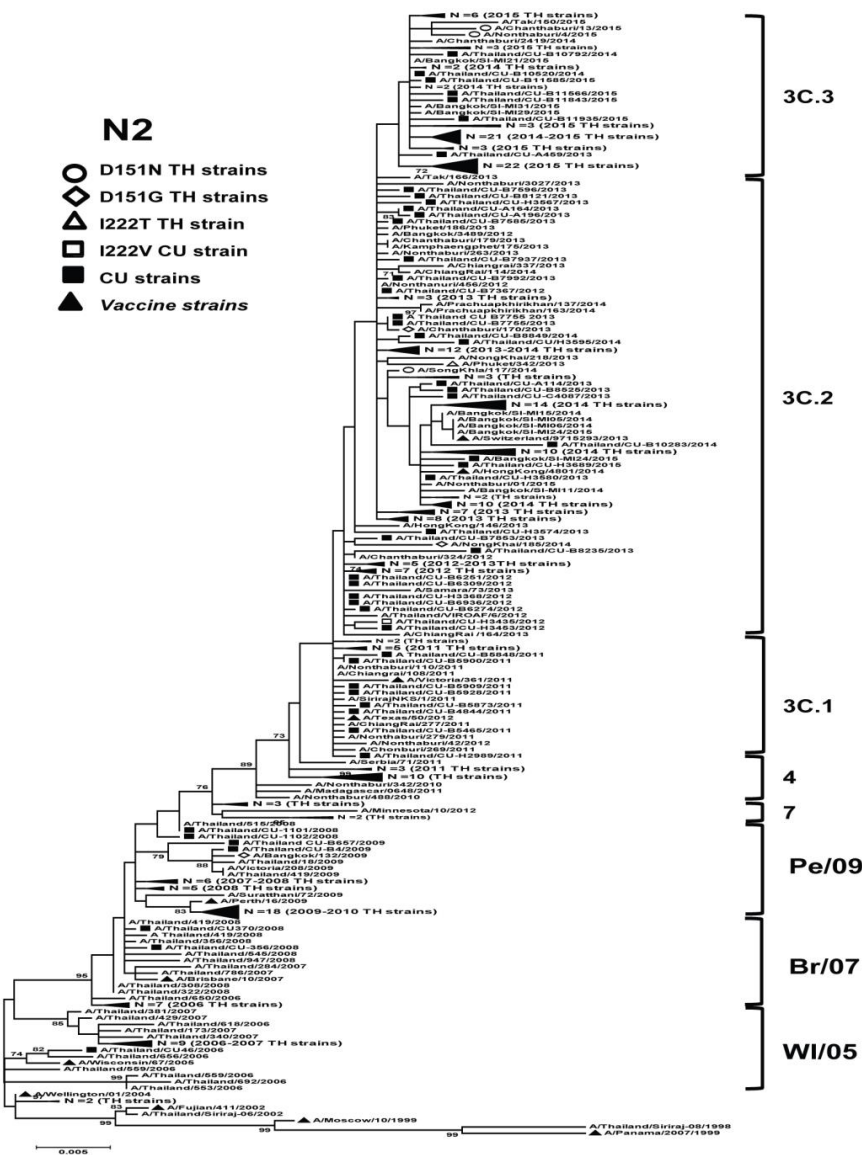
Figure 20. Maximum-likelihood phylogenetic tree of the NA gene of A(H3N2) influenza viruses circulated in Thailand. NA sequences from 343 A(H3N2) strains circulated in Thailand between 2002 and 2015 were compared to the southern hemisphere vaccine strains recommended by WHO (denoted as ▲). Bootstrap values >70% are shown at the branch nodes. Scale bar represents approximately 0.2% nucleotide difference between close relatives. Pe/09 denotes A/Perth/16/2009, Br/07 denotes A/Brisbane/10/2007, and WI/05 denotes A/Wisconsin/67/2005.
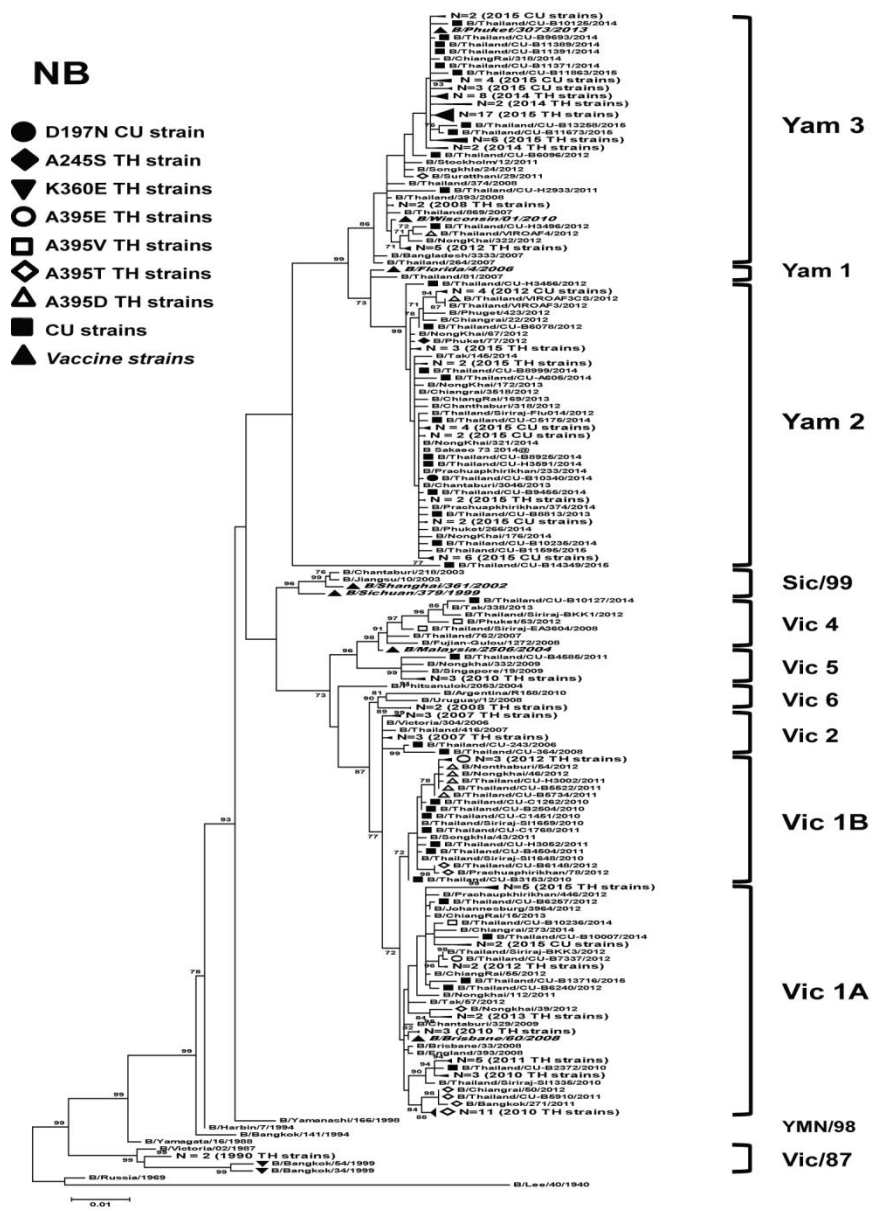
Figure 21. Maximum-likelihood phylogenetic tree of the NA gene of influenza B viruses circulated in Thailand. NA sequences from 232 influenza B strains circulated in Thailand between 1990 and 2015 were compared to the southern hemisphere vaccine strains recommended by WHO (denoted as ▲). Bootstrap values >70% are shown at the branch nodes. Scale bar represents approximately 0.2% nucleotide difference between close relatives. Yam denotes Yamagata, Sic/99 denotes B/Sichuan/379/1999, Vic denotes Victoria, and YMN/98 denotes B/Yamanashi/166/1998.

Table 19. *NA amino acid substitutions associated with reduced inhibition by NAIs among influenza A and B viruses circulated in Thailand.*

Position	Reference strain [a]	Observed residues (number of strains)	Major[b] (%)
A(H1N1) seasonal (H275Y: 20 sequences)			
221-222	KQ	KQ (16), KR (2), EQ (2)	80
354	D	G (19), D (1)	95
A(H1N1)pdm09 (S247N: 4 sequences)			
248	N	D (4)	100
389	I	V (3), I (1)	75
394	V	V (3), I (1)	75
A(H1N1)pdm09 (H275Y: 19 sequences)			
43-44	QN	QS (9), QN (8), KN (2)	47.4
106	V	I (16), V (3)	84.2
241	V	I (12), V (7)	63.2
248	N	D (19)	100
369	N	K (13), N (6)	68.4
Influenza B virus (D197N and A395E: 5 sequences)			
41-42	SP	SP (4), SQ (1)	80
49-50	TM	TM (4), IM (1)	80
125	N	N (4), T (1)	80
198	N	N (4), S (1)	80
204	V	V (4), I (1)	80
219-220	NK	NK (4), KN (1)	80
320	D	D (4), E(1)	80
358	E	E (4), A (1)	80
378	G	E (4), G (1)	80
389	A	A (4), T (1)	80
395-396	AF	EF (4), AL (1)	80
404	K	K (4), E (1)	80
463	D	N (4), D (1)	80

[a] Numbering is based on an alignment of NAs from the following reference strains: A/Brisbane/59/2007 (H1N1), A/California/7/2009 (H1N1)pdm09, A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008.

[b] Denotes the proportion of the most commonly observed residues for a given position.

Evolutionary rate and ancestral time analysis

The NA phylogenic trees of influenza A(H1N1)pdm09 and A(H3N2) viruses showed according to the timescale analyses (Figure S11). Based on the phylogenetic trees, posterior probabilities, marginal likelihoods, Bayes factor and convergence in terms of ESS values, the best-fit models for the NA gene of influenza A(H1N1)pdm09 and A(H3N2) data sets were the strict clock models with constant population size. Based on these models, the rates of nucleotide substitution for the NA genes of A(H1N1)pdm09 and A(H3N2) were estimated to be 3.49×10^{-3} substitutions/site/year (95% highest posterior density, HPD: $3.12 \times 10^{-3} – 3.88 \times 10^{-3}$) and 3.18×10^{-3} substitutions/site/year (95% HPD: $2.64 \times 10^{-3} – 3.72 \times 10^{-3}$), respectively. Estimating time to the most recent common ancestor (TMRCA) for (H3N2) gene and for the A(H1N1)pdm09 gene were 49.38 years (95% HPD: 48.84 – 49.93) and 9.52 years (95% HPD: 8.70 – 10.45), respectively.

In contrast, the best-fit models for the NA genes of influenza seasonal A(H1N1) and influenza B virus data sets were relaxed uncorrelated log-normal molecular clock models with constant population size (Table 20). Based on the timescale analyses, NA phylogenies of seasonal A(H1N1) and influenza B virus are shown (Fig S11). The rates of nucleotide substitutions of NA genes for the seasonal A(H1N1) and influenza B virus genes were estimated to be 6.26×10^{-3} substitutions/site/year (95% HPD: $3.79 \times 10^{-3} – 9.15 \times 10^{-3}$) and 1.61×10^{-3} substitutions/site/year (95% HPD: $1.14 \times 10^{-3} – 1.70 \times 10^{-3}$), respectively. The mean of the root age for the seasonal A(H1N1) gene and for the influenza B virus gene were estimated to be 14.55 years (95% HPD: 13.85 – 15.62) and 108.13 years (95% HPD: 89.31 – 133.14), respectively.

Table 20. *Estimating time to the most recent common ancestor (TMRCA) and nucleotide substitution rate of the NA genes among influenza A and B viruses circulating in Thailand and globally*

Types/subtypes	Clock Model		TMRCA of root height		Nucleotide substitution rate $\times 10^{-3}$ (subs/site/year)	
			Mean	95%HPD Interval	Mean	95%HPD Interval
A(H1N1)seasonal	Uncorrelated relaxed clock	lognormal	14.55	13.85 - 15.62	6.26	3.79 - 9.15
A(H1N1)pdm09	Strict clock		9.52	8.70 - 10.45	3.18	2.64 - 3.72
A(H3N2)	Strict clock		49.38	48.84 - 49.93	3.49	3.12 - 3.88
B	Uncorrelated relaxed clock	lognormal	108.13	89.31 - 133.14	1.61	1.14 - 1.70

Selection pressure analysis

We investigated the selection pressure in NA gene of influenza A and B viruses for understanding its evolutionary dynamics. The positive selection in NA genes indicates to the viral adaptation to the new human host, potential evasion of the host immune system, and balancing functionality between HA and NA. The overall global ω values of the A(H1N1)pdm09 (0.31) and influenza B virus (0.30) genes were higher than those of the seasonal A(H1N1) (0.26) and A(H3N2) (0.24) genes (Table 21). Although overall positive selection was not present, specific sites of positive selection were found (Table 21). A statistically significant result in each method was defined (Table S13). The results of the MEME analyses indicated that the number of positively selected sites for the A(H3N2) and influenza B virus genes were higher than for both the A(H1N1)pdm09 and seasonal A(H1N1) genes.

Table 21. *The positively selected sites from selection pressure analysis of the NA gene of Thai seasonal influenza isolates*

Types/Subtypes	Global ω by SLAC	No. of positively selected sites (codon position)		
		SLAC	FEL	MEME
A(H1N1)seasonal	0.26	3 (77, 222, 452)	5 (77, 222, 249, 344, 452)	7 (7, 77, 222, 249, 266, 344, 452)
A(H1N1)pdm09	0.31	1 (463)	7 (13, 34, 48, 188, 270, 451, 463)	6 (13, 34, 48, 188, 366, 463)
A(H3N2)	0.24	4 (93, 215, 401, 468)	9 (4, 43, 93, 141, 150, 215, 401, 464, 468)	12 (4, 43, 93, 141, 181, 215, 267, 271, 401, 402, 464, 468)
B	0.30	3 (73, 395, 404)	9 (68, 73, 106, 220, 248, 358, 395, 404, 465)	13 (15, 27, 41, 51, 73, 106, 107, 219, 220, 358, 395, 404, 465)

Global ω denotes the ratio of nonsynonymous substitutions (dN) to synonymous substitutions (dS).

SLAC, single-likelihood ancestor counting; FEL, fixed effects likelihood; MEME, mixed effects model of evolution

Potential N-linked glycosylation sites

We predicted the potential N-linked glycosylation sites of the NA proteins of the 118 seasonal A(H1N1), 296 A(H1N1)pdm09, 343 A(H3N2), and 232 influenza B virus strains circulated in Thailand (Table 22). The NA proteins from seasonal A(H1N1) viruses had nine conserved N-linked glycosylation sites when compared with the reference strain (A/New Caledonia/20/1999), while those from A(H1N1)pdm09 viruses revealed eight glycosylation sites when compared with the reference strain (A/California/07/2009). Interestingly, for the viruses in sub-clades 6B and 6C, an N-linked glycosylation site was observed at position 42 of the A(H1N1)pdm09 protein and at position 386 in all the strains except for those in sub-clades 6A and 6B.

Regarding the NA proteins of A(H3N2) viruses, seven N-linked glycosylation sites were conserved, except for the strains in clade A/Brisbane/10/2007, which had lost an N-linked glycosylation site at position 329. The viruses from Thailand in clade A/Wisconsin/67/2005 were found to have an N-linked glycosylation site at position

93. Surprisingly, the clade 3A, 3B, 3C.2, 3C.3, 4 and 6 of the A(H3N2) viruses circulating in Thailand that started emerging early in the 2011 season have an N-linked glycosylation site at position 367 but not at position 402 (3B, 3C and 4). For the NA proteins of influenza B viruses, four N-linked glycosylation sites were found, and the viruses in Yamagata lineage clade 3 also had an additional N-linked glycosylation site at position 463.

Table 22. *Potential N-linked glycosylation sites of NA proteins in seasonal influenza strains circulating in Thailand.*

NA position	NA sequences	N-Gly Score[a]	Jury agreement[b]	N-Gly results[c]	Clade
A(H1N1) seasonal (N = 118)					
44	NHTG	0.671	(8/9)	+	All
58	NSTW	0.599	(8/9)	+	All
63	NHTY	0.560	(8/9)	+	All
70	NNTN	0.688	(8/9)	+	All
88	NSSL	0.767	(9/9)	+++	All
146	NGTV	0.687	(9/9)	++	All
235	NGSC	0.736	(9/9)	++	All
434	NTTI	0.610	(7/9)	+	All
455	NWSW	0.272	(9/9)	---	All
A(H1N1)pdm09 (N = 296)					
42	NQSQ	0.629	(8/9)	+	Only clade 6B, 6C
50	NQSV	0.554	(6/9)	+	All
58	NNTW	0.569	(6/9)	+	All
63	NQTY	0.681	(9/9)	++	All
68	NISN	0.737	(9/9)	++	All
88	NSSL	0.738	(9/9)	++	All
146	NGTI	0.660	(9/9)	++	All
235	NGSC	0.728	(9/9)	++	All
386	NFSI	0.294	(9/9)	---	All except clade 6A and 6B
A(H3N2) (N = 343)					
61	NITE	0.756	(9/9)	+++	All
70	NTTI	0.545	(6/9)	+	All
86	NWSK	0.604	(8/9)	+	All
93	NITG	0.685	(9/9)	++	Only clade WI/05
146	NDTV	0.642	(9/9)	++	All
200	NATA	0.361	(9/9)	--	All
234	NGTC	0.755	(9/9)	+++	All
329	NDSS	0.488	(5/9)	-	All except Br/07
367	NETS	0.540	(5/9)	+	3A, 3B, 3C.2, 3C.3, 4 and 6
402	NRSG	0.407	(6/9)	-	All except 3B, 3C and 4
Influenza B virus (N = 232)					
56	NASN	0.592	(7/9)	+	All
64	NRSA	0.685	(8/9)	+	All
144	NGTR	0.816	(9/9)	+++	All
284	NKTI	0.704	(9/9)	++	All
463	NMTL	0.425	(6/9)	-	Only clade Yam-3

[a] The potential score is the averaged output of nine neural networks

[b] The Jury agreement indicate how many of the nine networks support the prediction

[c] The N-Glyc result indicates (+) potential N-glycosylated sites > 0.5 threshold, (++) potential N-glycosylated sites > 0.5 threshold and Jury agreement (9/9), (+++) potential N-glycosylated sites > 0.75 threshold and Jury agreement (9/9), (-) non-glycosylated sites < 0.5 threshold, (- -) non-glycosylated sites < 0.5 threshold and Jury agreement (9/9), and (- - -) non-glycosylated sites < 0.32 threshold.

Prediction of B-cell epitopes

For the seasonal A(H1N1), 24 residues were associated with the B-cell epitope profile. Significant antigenic variation between the clade 2C viruses from Thailand and the reference strain (A/New Caledonia/20/1999) was found at residues 99–101. Seasonal A(H1N1) strains in clade 2B did not have epitopes at residues 284–285, while strains in clade 2C did not have epitopes at residues 450–452 (Figure S12).

Meanwhile, 26 residues in the A(H1N1)pdm09 strains were found to be associated with the B-cell epitope profile in A/California/07/2009. Most of the viruses circulating in Thailand did not have epitopes at residues 102–104 and 110, except for the strains in sub-clades 6B and 6C. The strains in sub-clade 6B had epitopes at residues 224–228, whereas the strains in sub-clade 6A showed a loss of antigenicity at residues 385–388.

The A(H3N2) of the strain in clade A/Perth/16/2009 had B-cell epitopes at residues 301–303, 305, 307–308, and 310. The viruses from Thailand in clades 3B, 3C.1, 3C.2, 3C.3, and 4 had epitopes at residues 76–84.

The influenza B virus NA protein of Vic87 had 23 predicted B-cell epitope residues that are recognized by human antibodies. The epitopes of the NA protein of strains in Yamagata lineage clade 3 and Victoria lineage clade 5 differed from those of Vic87, as they had epitopes at residues 69–77 and 401–403, respectively. None of Yamagata strains identified in this study possessed epitopes at residues 48–53.

Evolutionary dynamics of NA segments of seasonal influenza A and B viruses

The phylogenetic trees of NA showed that each year and for each types/subtypes of influenza virus, strains from multiple clades were co-circulating (Figure 22). For A(H3N2), A(H1N1)pdm09, and seasonal A(H1N1) genes, the emergence of new clades/sub-clades occurred over short periods of time (with mean durations of 1.39, 1.97, and 2.35 years, respectively). In contrast, the NB genes of influenza B viruses persisted unchanged over 3 and 8 seasons for the Victoria and Yamagata lineages, respectively (with mean durations of 3.38 and 8.68 years).

Population dynamics of the NA genes of seasonal A(H1N1), A(H3N2), and influenza B viruses (Figures 22A, 22C, and 22D) demonstrated the constant sizes of the populations during the period from 2002 to 2015. In contrast, the NA gene of the A(H1N1)pdm09 viral population indicated alternating of periods of exponential growth and oscillating patterns of relative genetic diversity after the early pandemic spread of the A(H1N1)pdm09 strain (Figure 22B). This pattern indicated increases in genetic diversity followed by bottlenecks between the seasons. Interestingly, there was a marked decrease in the relative genetic diversity at the end of 2012 until the mid-2014 season, with periods during which the numbers of A(H1N1)pdm09 infections decreased (Figure S10), while the number of A(H3N2) infections increased. Overall, the relative genetic diversity of the NA gene of A(H1N1)pdm09 during the 2009–2011 seasons and of influenza B virus was higher than that of seasonal A(H1N1), post-pandemic A(H1N1)pdm09, and A(H3N2).
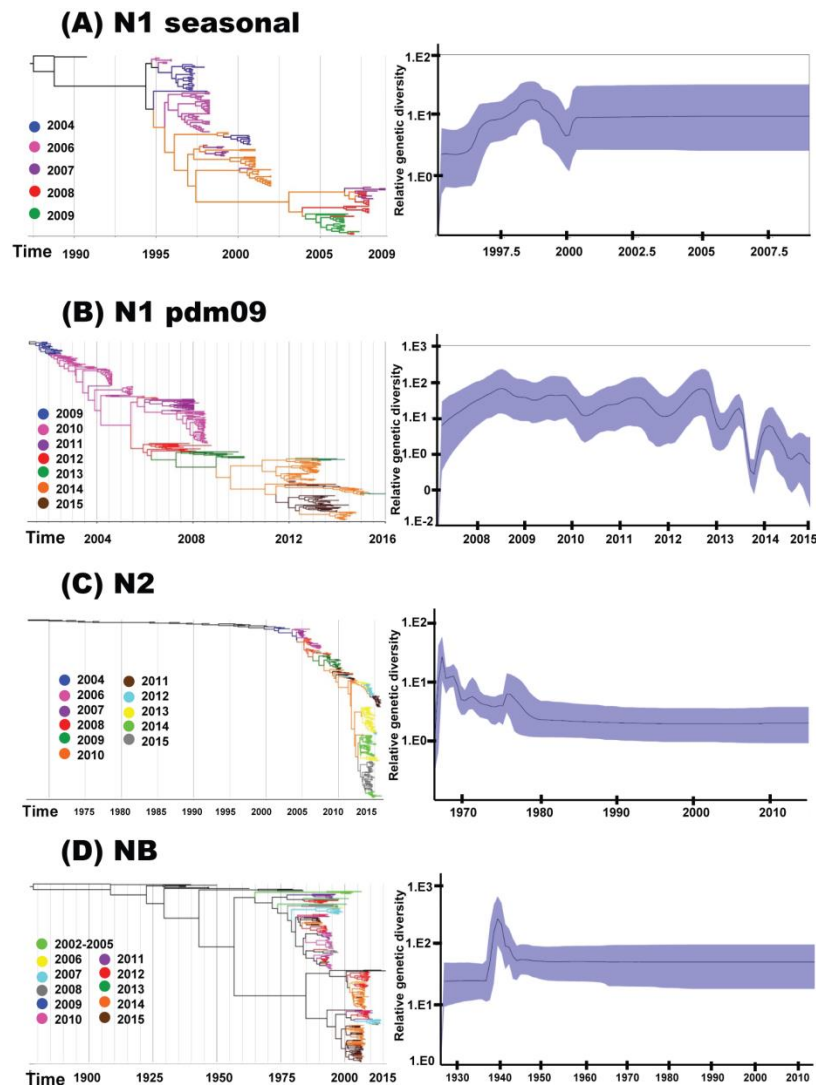
Figure 22. Temporal phylogeny and population dynamic diversity of seasonal influenza viruses circulating in Thailand. *Evolution of the NA genes of (A) seasonal A(H1N1) (2004-2009), (B) A(H1N1)pdm09 (2009-2015), (C) A(H3N2) (2004-2015), and (D) influenza B virus (2002-2015). Phylogenetic trees were constructed using uncorrelated lognormal relaxed or strict clock model with branches colored by year of virus isolation and the relative genetic diversity of NA segments using the Gaussian Markov Random Field (GMRF) or Bayesian skyline (BS) model. Solid black lines in GMRF or BS plot denoted mean relative genetic diversity, while blue shade represents the 95% HPD intervals.*

Discussion

Strains of A(H1N1)pdm09 have co-circulated with A(H3N2) and influenza B viruses to a varying extent during most influenza seasons. Published surveillance data have demonstrated that the prevalence of oseltamivir resistance characterized by the mutation at residue H275Y in NA gene of A(H1N1)pdm09 viruses is still low (<3%) (W. Huang et al., 2015; T. C. Li et al., 2015; Okomo-Adhiambo et al., 2015). In Thailand, during the second and third waves of the A(H1N1)pdm09 pandemic from January 2010 to October 2010, the prevalence of oseltamivir-resistant strains was 0.44% (Chutinimitkul et al., 2008). The present study showed that the prevalence of oseltamivir-resistant strains during the post-pandemic period was slightly higher than that during the pandemic (0.82%; 4/485). Based on the $\Delta\Delta C_t$ method calculation using real-time RT-PCR, a mix of the wild-type susceptible strain (275H) and resistant strain (275Y) was detected in all the H275Y-positive samples and the relative quantities of RNA of the latter virus were higher than those of the former virus. This finding indicates that the resistant strains have potentially advantageous mutations that allow them to become dominant when the antiviral is used (Ghedin et al., 2012). Furthermore, the majority of individuals infected with the H275Y variants were in "at risk" groups (i.e. individuals with hematologic cancers, allergic rhinitis, and the elderly) who are more likely to have the strain due to their immunocompromised status (Hurt et al., 2012).

Hurt and colleagues found that a novel influenza A(H1N1)pdm09 variant with mildly reduced oseltamivir and zanamivir sensitivity has been detected in more than 10% of community specimens in Singapore and more than 30% of samples from northern Australia (Hurt et al., 2011). In Thailand, the prevalence of this S247N mutation in A(H1N1)pdm09 was lower (1.35%) than in Australia and Singapore, which might be due to sampling size bias. In the NA gene of the A(H1N1)pdm09 circulated in Thailand, the H275Y strains had permissive mutations (define as mutations that allowed the virus to tolerate subsequent occurrences of H275Y) at residues V241I (63%) and N369K (68%). It has been suggested that these variants might be able to replicate and transmitted more efficiently (Butler et al., 2014).

The earliest reports of the detection of oseltamivir-resistant seasonal A(H1N1) were in November 2007 from France, Norway, and the United Kingdom (Meijer et al., 2009). In the present study, the proportion of H275Y strains during the period from 2007 to 2009 was 54.05% (20/37); which is consistent with findings from other studies (Dharan et al., 2009; Hurt, 2014; Meijer et al., 2009). The H275Y mutation can confer cross-resistance to peramivir, but susceptibility to zanamivir and laninamivir is not significantly affected (T. C. Li et al., 2015). As noted in a previous study, the H275Y seasonal A(H1N1) strains with reduced viral fitness had permissive substitutions at V234M (100%), R222Q (90%), K329E (100%), and D344N (100%), and compensatory mutation D354G (95%), which maintained the functionality of the NA protein (Duan et al., 2014).

The surveillance data on seasonal influenza reveal that the incidence of oseltamivir and zanamivir resistance was low in A(H3N2) strains (1–3%) and rare (<1%) in influenza B virus strains (Takashita et al., 2015; Whitley et al., 2013). Drug resistance characterized by E119V or R292K mutation (according to N2 numbering) was not observed in the present study. However, the catalytic site mutation (D151N/G) and framework mutation (I222T/V) of the NA protein were found. The NA protein of influenza B virus in Thailand between 1990 and 2015 showed D197N mutation, which is associated with reduced susceptibility to oseltamivir and zanamivir (Hatakeyama et al., 2007). In influenza B virus, the A395E substitution located outside the active site of NA is associated with reduced susceptibility to oseltamivir and peramivir (Leang et al., 2014). Interestingly, we found a high frequency of NA mutations A395V/T/D/S in the influenza B viruses circulating in Thailand. Mutations at the active site of the NA protein in strains of both A(H3N2) and influenza B virus may affect the half maximal inhibitory concentration (IC_{50}) of the NAIs. In present study, the antiviral susceptibility of the viruses with D151N/G, I222T/V, and A395E/V/T/D/S NA substitutions cannot be concluded by genotypic methods. Therefore, the phenotypic assay to confirm the NAI resistance profile of these NA substitutions should be needed.

The continual genetic drift of influenza virus resulting from cumulative mutations may introduce novel N-linked glycosylation sites and alter antigenic

epitopes of progeny viruses. The loss or gain of glycosylation may also change the NA protein's substrate specificity profiles and correct molecule folding, which affects the NA protein's enzymatic activity (Saito & Kawano, 1997). Moreover, glycosylation of the NA protein may change the antigenic properties of the virus and glycosylation may affect its pathogenicity (S. Li, Schulman, Itamura, & Palese, 1993). Therefore, potential N-linked glycosylation sites in the NA proteins of seasonal influenza A and B circulated in Thailand were assessed.

We observed that the overall rate of nucleotide substitution was higher for the NA gene of the seasonal A(H1N1) viruses than for the NA genes of the A(H1N1)pdm09, A(H3N2), and influenza B viruses similar to previous studies. For example, A(H3N2) viruses collected between 1968 and 2011 worldwide (N = 286) had a mean nucleotide substitution rate of 3.27×10^{-3} substitutions/site/year (Westgeest et al., 2014). In North America, studies have reported rates of (3.11–12.50) x 10^{-3} (Nelson et al., 2006) and 5.41 x 10^{-3} (Rambaut et al., 2008). For influenza B viruses, the nucleotide substitution rate of the NA gene in the present study is comparable to those reported in Australia and New Zealand (mean nucleotide substitution rate of 2.04×10^{-3} substitutions/site/year for the Victoria lineage and 2.25×10^{-3} substitutions/site/year for the Yamagata lineage) (Vijaykrishna et al., 2015) and Malaysia (2.5-3.4 x 10^{-3}) (Oong et al., 2015). In contrast, the mean nucleotide substitution rate of the NA gene of A(H1N1)pdm09 in the present study was slightly lower than a previous study of 5.21×10^{-3} substitutions/site/year (Su et al., 2015) and 5.27 x 10^{-3} (Makkoch et al., 2012), which may be attributed to the study sample size and sequence diversity of the strains.

The mean of the global ω values was similar for all the NA genes of the strains of seasonal influenza virus in Thailand. Since the majority of the NA protein residues of influenza A and B viruses showed ω < 1, it suggests that the amino acids in this protein are under purifying selection. These findings are consistent with previous studies, which reported the overall ω values for the NA genes of 0.30 for seasonal A(H1N1), 0.32 for A(H1N1)pdm09, 0.29 for A(H3N2), and 0.20–0.31 for influenza B virus (Su et al., 2015; Suzuki, 2006; Vijaykrishna et al., 2015; Westgeest et al., 2014). The selection pressure of NA of A(H3N2) in German was 0.21, while in Taiwan it

was 0.37 (Lin et al., 2011; Mostafa et al., 2016). Moreover, the findings showed that NA gene had highest a number of amino acid residue under the positive selection. The large ω values observed for the NA of A(H1N1)pdm09 may indicate adaptation of the virus to its new human host or due to the observed imbalance in HA and NA functionality in some viruses (R. Xu et al., 2012), while the large ω values for the NA gene of influenza B may be due to broad NA-related immunity (Wan et al., 2013). Moreover, the positively selected sites in the NA segment of influenza viruses in Thailand indicate the potential to evade the host immune response and NAIs (Suzuki, 2006). The positively selected sites in the NA proteins [seasonal A(H1N1): 77, 249, 344, and 452; A(H1N1)pdm09: 48, 270, 366, 451, and 463; A(H3N2): 43, 93, 141, 150, 181, 267, 271, 401, 402, and 464; and influenza B virus: 51, 68, 73, 106, 107, 248, 395, 404, and 465] may represent the antigenic sites (Colman et al., 1983).

These defined epitopes were associated with the findings on B-cell epitope profiles predicted using the BepiPred 1.0 Server. In addition, the codon at residues 93 and 402 of the A(H3N2) protein (which were associated with the loss of an N-linked glycosylation site) were under positive selection pressure, reflecting the potential for the virus to evade the host immune system. The positively selected codon at residue 141 in A(H3N2) is linked to a calcium ion binding site, which plays an important role in stabilizing the conformation of the NA protein (Takahashi, Suzuki, Hidari, Miyamoto, & Suzuki, 2003). The mutation at codon 395 in influenza B virus conferred an increase in the IC_{50} of oseltamivir and peramivir and was under positive selection pressure. In addition, we found V106I and N248D mutations in the NA protein, which are located at the subunit interfaces and the primary calcium ion binding site that is associated with the stability of A(H1N1)pdm09 virus at a low pH (Takakuwa et al., 2013).

The MCC tree from the Bayesian timescale phylogenetic analysis of the NA from seasonal influenza viruses circulating in Thailand is consistent with previous studies and highlights how circulating strains are continually drifting (Su et al., 2015; Vijaykrishna et al., 2015; Westgeest et al., 2014) whereby strains which are unable to evade the host immune system are eliminated (Nelson & Holmes, 2007). The evolutionary patterns of the NA gene indicated a more rapid genetic drift for influenza

A than influenza B virus due to higher nucleotide substitution rate, although there was more genetic diversity in influenza B than in influenza A virus. Therefore, there is an inverse relationship between the evolutionary rate and genealogical diversity, which is similar to the evolutionary pattern of the HA gene of seasonal influenza viruses across the globe (Bedford et al., 2015).

In conclusion, this study found evidence for the ongoing evolution of the NA protein, including amino acid mutations in the active site that were associated with reduced susceptibility to NAIs and evolutionary parameters including the nucleotide substitution rate, the selection pressure, and the genetic diversity. Continual monitoring of the antigenic changes and evolutionary dynamics of the NA segments of the influenza virus will assist public health and clinical recommendations for antiviral use.

CHAPTER V

NUERAMINIDASE INHIBITORS SUSCEPTIBILITY AND NEURAMINIDASE ENZYME KINETICS OF HUMAN INFLUENZA A AND B VIRUSES CIRCULATING IN THAILAND IN 2010-2015

(Part 5.2)

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CHAPTER V

Part 5.2: Neuraminidase Inhibitor Susceptibility and Neuraminidase Enzyme Kinetics of Human Influenza A and B Viruses Circulating in Thailand in 2010-2015

Summary

The amino acid substitutions located either within or in close proximity to neuraminidase (NA) active site may affect overall virus fitness. We identified NA substitutions in influenza A(H3N2) and B viruses circulating in Thailand between 2010 and 2015 previously reported to be associated with reduced inhibition by NAIs. To study the effect of these substitutions on the NA enzymatic properties and virus characteristic, we generated recombinant influenza viruses containing either wild type (WT) NA or NA with a single I222V, S331G, and S331R [influenza A(H3N2)] or S342S, A395T, A395V, and A395S NA substitution (influenza B). Recombinant influenza A and B viruses (7 + 1) were generated in genetic background of A/Puerto Rico/8/1934 (A/RP/8, H1N1) or B/Yamanashi/166/1998 (B/YAM) viruses, respectively. In contrast to previous reports, all of recombinant influenza A(H3N2) and B viruses carrying NA substitutions were susceptible to NAIs. The K_{m} and $V_{\max}$ of A/PR8-S331G and A/PR8-S331R viruses were higher and those of B/Yam-D342S virus were lower as compared to those of WT virus. The yields of A/PR8-S331R and A/PR8-S331G viruses in MDCK cells were significantly lower than those of A/PR8-WT virus at 6 and 12 hpi. Although replication efficiency of recombinant influenza B viruses in MDCK cells was low at 6 and 12 hpi, it reaches levels comparable to those of WT at 48 hpi. Influenza B/YAM-D342S and B/YAM-A395V viruses reverted to WT NA after three passages. Our results suggest that NA substitutions that found outside active sites of NA may affect the replication capacity, depending on NA enzyme activity and stability.

Introduction

Influenza A and B viruses are important human respiratory pathogens causing annual epidemics worldwide. Anti-influenza prophylaxis and treatment with antiviral drugs are used to prevent and control influenza virus infections, particularly when vaccines are ineffective or unavailable (Fiore et al., 2011). The report from the Centers for Diseases Control and Prevention (CDC) indicated the high level of resistances (>99%) to adamantanes among circulating seasonal influenza A(H3N2) and A(H1N1)pdm09 viruses (CDC, 2016). Therefore, existing options for prophylaxis and treatment of influenza virus infection are limited to a single class of antiviral drug, neuraminidase (NA) inhibitors (NAIs). The NAIs, oral oseltamivir, inhaled zanamivir, and intravenous peramivir are approved by the United States Food and Drug Administration and are recommended for the prophylaxis and treatment of patients infected influenza A and B viruses (CDC, 2016).

The NA is a tetrameric type II integral membrane protein with sialidase activity that is responsible for releasing of the newly produced viral particles from the virus-infected cells (W. Li et al., 2011; Palese et al., 1974). Based on NA sequence similarity, the NA genes of influenza A and B viruses are classified into Group I and Group II, respectively. The NA of influenza A is further classified into two Subgroups (Subgroup I and Subgroup II). The Subgroup 1 contains N2, N3, N6, N7 and N9 and Subgroup 2 consists of N1, N4, N5, and N8 NA subtypes (J. Xu et al., 2012). The highly conserved among influenza A and B viruses framework (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, and E425) (in N2 numbering) and catalytic (R118, D151, R152, R224, E276, R292, R371, and Y406) residues are forming NA active site (Burmeister, Ruigrok, & Cusack, 1992; Colman, Hoyne, & Lawrence, 1993; Colman et al., 1983). The NA substitutions located either within or in close proximity to NA active site can cause reduced or highly reduced inhibition of influenza viruses by NAIs , affect NA enzymatic properties, replication efficiency, transmissibility, and overall virus fitness in vitro and in vivo (Burnham, Baranovich, & Govorkova, 2013; Govorkova, 2013; Nguyen, Fry, & Gubareva, 2012; Yen et al., 2006). The NA substitutions are NA subtype specific and drug-specific and they have a frequency at varying levels in any residues. Although the novel NA substitutions

were reported at novel residues, the effect of these substitutions on NAI susceptibility at a different genetic background was usually undetermined. The NA substitutions at residue 222 (T/V/M/L) confer reduced susceptibility to NAIs and were found in seasonal A(H1N1), pandemic A(H1N1)pdm09, and highly pathogenic A(H5N1) influenza viruses. The I222T/V NA substitutions were reported to cause reduced susceptibility of highly pathogenic influenza A(H5N1) to NAIs with the IC50 of oseltamivir ranging from 40 to 75 nM (McKimm-Breschkin et al., 2013). The antiviral surveillance studies identified novel NA substitutions (S331R for influenza A(H3N2) viruses; D342S and A395E for influenza B viruses) associated with reduced inhibition by oseltamivir and peramivir (higher IC_{50} values) (Hurt et al., 2016; Leang et al., 2013; Leang et al., 2014; WHO, 2012). These changes are located outside the NA active site (Colman et al., 1983; Leang et al., 2014). Recent study demonstrated that influenza A(H1N1)pdm09, containing the I427T and Q313R double NA substitutions, which are not part of the NA active site, possessed decreased NAIs susceptibility, altered NA properties and viral fitness (Tu et al., 2017).

Previously, we reported NA amino acid substitutions at 222 and 331 residues of influenza A(H3N2) viruses, and at 342 and 395 residues of influenza B viruses circulating in Thailand (Tewawong N, 2017). However, the effect of these NA substitutions on susceptibility to NAIs, enzymatic properties, and viral fitness was not determined. Here, we applied reverse genetics and generated the recombinant influenza A and B viruses carrying either wild type (WT) NA or NA with a single I222V, S331G, and S331R [influenza A(H3N2)] or S342S, A395T, A395V, and A395S substitution which we subsequently used to study the NA properties (activity, enzyme kinetic, thermostability), susceptibility to NAIs, and virus replication kinetic and genetic stability of NA substitutions during passages in MDCK cells.

Methods

Clinical samples, influenza virus and cells

The novel NA substitutions of influenza A(H3N2) and B PCR-positive respiratory samples from Thailand were previously identified (Table 23) (Tewawong N, 2017). Substitution-encoding NA gene sequences from these samples were amplified. These clinical samples were from the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University in Thailand as part of the influenza surveillance program. The study protocol was approved by the Institutional Review Board (IRB) of the Faculty of Medicine at Chulalongkorn University (IRB No. 581/58).

Influenza B/Brisbane/60/2008 (B/BR/60/08) virus was obtained from the St. Jude Children's Research Hospital repository. Madin-Darby canine kidney cells (ATCC, Manassas, VA) were maintained in Minimum Essential Medium Eagle supplemented with 5% of fetal bovine serum and antibiotics and antimycotics mixture. Human embryonic kidney 293T cells (ATCC, Manassas, VA) were maintained in Opti-MEM reduced serum medium glutaMAX supplement without antibiotics.

Cloning of NA in pHW2000 and pAD3000 plasmids

Influenza viral RNA was extracted using Qiagen RNeasy kit (Qiagen, CA). The full length NA genes of influenza A(H3N2) and influenza B viruses were amplified using one-step RT-PCR as previously described (Hoffmann et al., 2002; Hoffmann et al., 2001). After separation and purification (QIAquick Gel Extraction Kit, Qiagen, CA), gel-purified product of NA gene of influenza A(H3N2) and B viruses were digested with *Bsa*I and *BsmB*I restriction enzymes (New England Biolabs), purified and ligated (T4 DNA ligase, NE Biolabs) into the *BsmB*I digested pHW2000 and pAD3000 plasmids, respectively. Plasmids were transformed into One Shot Chemically Competent Escherichia coli (Invitrogen, Carlsbad, CA) and positive clones were inoculated into terrific broth with ampicillin. The positive clones were purified using the Hispeed Plasmid Maxi Kits (Qiagen, CA) and determined the DNA

concentration of plasmid by both UV spectrophotometry at 260 nm and analysis on agarose gel. The constructed plasmids were sequenced to ensure their identity to the field strains.

Table 23. The novel NA substitutions of influenza A(H3N2) and B PCR-positive samples from Thailand used in the present study

Influenza virus	Subtype /lineage	Substitution in NA [a]	Recombinant influenza virus [b]	Accession number [c]
A/Thailand/CU-B11870/2015	H3N2	Wild type	A/PR8-WT	KX151204
A/Thailand/CU-B7418/2013	H3N2	I222V	A/PR8-I222V	KP336079
A/Thailand/CU-B11518/2015	H3N2	S331G	A/PR8-S331G	KX151190
A/Thailand/CU-B12139/2015	H3N2	S331R	A/PR8-S331R	KX151213
B/Thailand/CU-B11776/2015	B/Yam	D342S	B/YAM-D342S	KX151370
B/Thailand/CU-B5910/2011	B/Vic	A395T	B/YAM-A395T	JX513088
B/Thailand/CU-H3002/2011	B/Vic	A395D	B/YAM-A395D	JX513200
B/Thailand/CU-B10236/2014	B/Vic	A395V	B/YAM-A395V	KX151337

[a] Numbering is based on an alignment of NAs from the following reference strains: A/Perth/16/2009 (H3N2), and B/Yamanashi/166/1998 (AVWG., 2016).

[b] Designations of recombinant influenza viruses used in the study.

[c] GenBank database accession numbers for NA.

Generation of recombinant influenza A and B viruses

Recombinant influenza viruses (7 + 1) carrying NA from A(H3N2) or B viruses were generated in a homogenous genetic background of A/Puerto Rico/8/1934 (H1N1) (A/PR8) and B/Yamanashi/166/1998 (B/YAM) influenza viruses, respectively. The eight plasmids were transfected into 293T cells as previously described (Hoffmann et al., 2002; Hoffmann, Neumann, Kawaoka, Hobom, & Webster, 2000). The NA gene segment was amplified from the extracted viral RNA of rescued virus using one-step RT-PCR. The NA sequences of recombinant viruses were performed by Hartwell Center Bioinformatics and Biotechnology at St. Jude Children's Research Hospital by using BigDye Terminator (version 3) chemistry and

synthetic oligonucleotides. Samples were analyzed on Applied Biosystems 3700 DNA analyzers. The NA sequences were analyzed by SeqMan program in the DNAStar software (http://www.dnastar.com/). Recombinant viruses were grown in the allantoic cavity of 10-day-old embryonated chicken eggs (Marshall Durbin, Bermingham, AL) for 72 h at 35 °C (influenza A viruses) or 33 °C (influenza B viruses). The virus-containing allantoic fluid was stored at -80°C until use. The 50% tissue culture infectious dose (TCID$_{50}$) of recombinant influenza viruses was determined in MDCK cells incubated for 72 h at 35 °C (influenza A viruses) or 33 °C (influenza B viruses). The TCID$_{50}$ values were calculated by Reed-Muench (Reed LJ, 1938). The HA titers were determined by using packed 0.5% chicken red blood cells.

Susceptibility to NAIs

The NAIs (oseltamivir carboxylate, zanamivir, and peramivir) were dissolved in sterile distilled water, and aliquots were stored at -20°C until use. A fluorescence-based NA inhibition assay was used to determine the 50% inhibitory concentration (IC$_{50}$) values as previously described (Gubareva, Webster, & Hayden, 2002). The IC$_{50}$ of viruses were determined by the curve fitting model in GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Phenotypic NAI susceptibility of influenza A and B viruses was evaluated based on the fold-change in IC$_{50}$ values compared to that in susceptible virus from the same NA subtype/lineage as previously described by the WHO Influenza Antiviral Working group (WHO, 2012): "normal inhibition" ($\leq$ 10-fold change for A and $\leq$ 5-fold change for B viruses); "reduced inhibition" (10–100 fold change for A and 5-50 fold change for B viruses); "highly reduced inhibition" ($\geq$ 100-fold change for A and $\geq$ 50-fold change for B viruses).

NA enzyme activity and thermostability

The NA activity of recombinant viruses was measured by modified fluorometric assay using fluorogenic substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma-Aldrich, St Louis, MO) at a final concentration of 100 μM (Gubareva et al., 2002; Potier, Mameli, Belisle, Dallaire, & Melancon, 1979). Fluorescence was measured on a Synergy 2 multimode microplate reader (BioTek Instruments, Winooski, VT) using excitation and emission wavelength

of 360 and 460 nm, respectively. The NA activity was normalized against the HA titer and revealed as the quantity of 4-methylumbelliferone (4-MU, μg) formed per 1 HA unit of virus. The thermostability of the NA was determined by incubating viruses at 33, 37, 45, and 55°C for 15 and 30 min using gradient thermocycler. The NA activity was determined and expressed as a percentage of the NA activity of the virus at 4°C. The NA activity of each virus calculated based on the mean of triplicate assays.

NA enzyme kinetic assay

NA enzyme kinetics (V_{max} and K_m) of recombinant influenza A and B viruses carrying a single NA substitution were determined as previously described (Marathe, Leveque, Klumpp, Webster, & Govorkova, 2013). The optimum dilution of recombinant virus was determined to assess NA kinetic properties. We measured the NA enzyme kinetics at pH 6.5 with enzyme buffer and final concentration of MUNANA substrate ranging from 0.98 to 1000 μM. The reaction was examined at 37°C in a total volume of 150 μl, and fluorescent signal was detected every 60 sec for 60 min using wavelength of 360 and 460 nm. The MUNANA substrate affinity (V_{max}) and velocity of catalysis (K_m) values were calculated by fitting to the data appropriate Michaelis-Menten equations by using non-linear regression in GraphPad Prism 5 software.

Replication kinetic

The multi-step growth curves were determined for each recombinant influenza virus in MDCK cells. Confluent cells monolayers were infected with recombinant virus at the multiplicity of infection (MOI) of 1 and 0.001 $TCID_{50}$/cell. The supernatant were collected at 6, 12, and 24 h (for MOI of 1 $TCID_{50}$/cell) and 24, 48, and 72 h (for MOI of 0.001 $TCID_{50}$/cell) after virus inoculation (hpi) and stored at -70°C until use. Virus titers were determined by $TCID_{50}$ assay in MDCK cells.

Statistical analysis

The results of the NA activity, IC_{50} values, enzyme kinetic parameters (K_m and V_{max}), and virus titers were compared by a two-tailed student's t-test. The results were considered significantly different if the P value was < 0.05.

Results

NAI susceptibility profiles

We determined the NAI susceptibility profiles of recombinant 7 + 1 influenza A and B viruses carrying a single NA substitution using NA inhibition assay (Table 24). When compared to the IC_{50} value of A/PR8-WT virus, the fold changes of IC_{50} values of recombinant influenza A viruses were <10 fold. These results demonstrated that A/PR8 viruses carrying NA from A(H3N2) virus with a single I222V, S331R, and S331G NA substitutions were susceptible to NAIs. Regarding the recombinant B/YAM viruses the fold changes of IC_{50} values were <5 when compared to that of B/BR/60/08 virus. These results revealed that B/YAM viruses carrying NA from currently circulating B virus with a single D342S, A395T, A395V, and A395D NA substitution were susceptible to NAIs.

Table 24. *NAIs susceptibility profile of recombinant influenza A and B viruses carrying a single NA substitution*

Recombinant influenza virus	Oseltamivir		Zanamivir		Peramivir	
	mean IC_{50} ± SD, nM [a]	Fold change [b]	mean IC_{50} ± SD, nM	Fold change	mean IC_{50} ± SD, nM	Fold change
A/PR8-WT	0.3 ± 0.0	1.0	0.4 ± 0.1	1.0	0.1 ± 0.02	1.0
A/PR8-I222V	0.3 ± 0.1	1.0	0.5 ± 0.1	1.3	0.1 ± 0.01	1.0
A/PR8-S331G	0.5 ± 0.5	1.6	0.4 ± 0.1	1.0	0.3 ± 0.3	3.0
A/PR8-S331R	0.1 ± 0.1	0.3	0.4 ± 0.1	1.0	0.2 ± 0.06	2.0
B/BR/60/08 [c]	16.1 ± 7.8	1.0	3.0 ± 0.6	1.0	0.5 ± 0.33	1.0
B/YAM- D342S	8.3 ± 4.2	0.5	0.9 ± 0.3	0.3	0.4 ± 0.12	0.8
B/YAM-A395T	17.2 ± 4.5	1.1	3.4 ± 0.5	1.1	0.6 ± 0.08	1.2
B/YAM-A395D	12.3 ± 2.7	0.8	2.5 ± 0.5	0.8	0.5 ± 0.36	1.0
B/YAM-A395V	18.3 ± 7.2	1.1	1.2 ± 0.3	0.4	0.8 ± 0.12	1.6

[a] IC50 value is the concentration of NAI that inhibit viral NA activity by 50% and the values were obtained from three independent experiments.

[b] Fold changes compared to that of WT (NAI susceptible) influenza viruses.

[c] This reference virus was used as WT influenza B virus

NA enzyme activity

We investigated the effect of NA amino acid substitutions on the NA activity of recombinant 7 + 1 influenza A/PR8 and B/YAM viruses using a modified fluorescence-based assay. The NA enzyme salidase activity was normalized against the viral titer and expressed as the quantity of 4-MU (μg) per 1 log $TCID_{50}$/mL (Table 25). The A/PR8-S331G and A/PR8-S331R NA substitutions decreased significantly NA activity compared with the A/PR8-WT NA activity ($P < 0.001$ and $P < 0.01$, respectively), whereas the I222V NA substitution slightly increased the NA activity (Table 25). By contrast, the B/YAM-D342S and B/YAM-A395T NA substitutions had significantly higher NA activity than did the B/BR/60/08 ($P < 0.01$). The B/YAM-A395D NA substitution decreased NA activity, whereas the B/YAM-A395V NA substitution had increased NA activity compared with the B/BR/60/08 (Table 25). Taken together, residue changes on the NA protein differentially affected the enzyme activity as measured by our assay.

NA enzyme kinetic

To investigate the effect of NA amino acid substitutions on NA enzymatic kinetic, we measured the K_m and V_{max} from recombinant influenza A and B viruses using the fluorogenic MUNANA substrate (Table 25). The affinity against the MUNANA substrate (K_m) of A/PR8 virus carrying S331G and S331R NA substitutions was significantly increased ($P < 0.01$). The D342S and A395T NA substitutions of B/YAM viruses led to the Km that was decreased ($P < 0.01$ and $P < 0.05$, respectively). The S331G substitution in the NA of A/PR8 induced 1.8 fold increase of the V_{max} ($P < 0.001$), while A/PR8 virus carrying S331R substitution was slightly increased the V_{max} ($P < 0.01$) (Table 25). In contrast, the B/YAM virus carrying D342S and A395T NA substitutions were found to reduce the V_{max} ($P < 0.05$). The higher V_{max} of the S331G and S331R viruses may facilitate virus release and spread but the higher K_m has a low affinity for its substrate. This result revealed that these substitutions contribute to reduce the NA enzyme activity.

Table 25. *NA activity and NA enzyme kinetics of recombinant influenza A and B viruses*

Recombinant influenza virus	Virus yield		Enzymatic property			
	($\log_{10}$ $TCID_{50}$/mL)	HA titer	NA activity [a]	K_m (µM) [b]	V_{max} (µM/min) [c]	V_{max} ratio vs WT
A/PR8-WT	8.87 ± 0.48	4096	41.6 ± 0.7	55.3 ± 3.3	0.08 ± 0.00	1.0
A/PR8-I222V	9.31 ± 0.13	4096	45.8 ± 0.2	59.2 ± 0.8	0.09 ± 0.01	1.1
A/PR8-S331G	8.31 ± 0.38	512	4.3 ± 0.6 [***]	71.7 ± 1.1 [**]	0.14 ± 0.00 [***]	1.8
A/PR8-S331R	9.37 ± 0.14	4096	31.9 ± 0.9 [**]	80.5 ± 5.2 [**]	0.11 ± 0.01 [**]	1.4
B/BR/60/08	5.62 ± 0.14	256	30.2 ± 3.0	20.3 ± 1.1	0.09 ± 0.01	1.0
B/YAM- D342S	7.50 ± 0.00	1024	50.3 ± 0.9 [**]	13.3 ± 0.1 [**]	0.07 ± 0.00 [*]	0.8
B/YAM-A395T	7.50 ± 0.00	1024	58.2 ± 0.5 [**]	17.8 ± 0.4 [*]	0.08 ± 0.00 [*]	0.9
B/YAM-A395D	7.50 ± 0.00	512	24.6 ± 0.9	31.5 ± 6.9	0.11 ± 0.01	1.2
B/YAM-A395V	7.50 ± 0.00	512	42.1 ± 5.3	18.1 ± 0.1	0.10 ± 0.00	1.1

a Values are the mean $\pm$ SD from three independent experiments and expressed as the quantity of 4-MU (µg) per 1 log TCID50/mL.

b, c Values indicate means of Km and Vmax from three independent experiments $\pm$ SD.

*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared to WT virus, student's t-test.

NA thermostability

The NA amino acid substitutions can contribute to NA protein stability (Burnham et al., 2013). Therefore, we determined the thermostability of mutant NAs by measuring the NA activity of the recombinant viruses after 15 and 30 min of incubation at different temperatures. For recombinant influenza A viruses, the NA activity of A/PR8-I222V virus was stable at 33°C to 55°C, whereas the A/PR8-S331G and A/PR8-S331R viruses showed lower activity at 55°C (Figure 23A, B). By contrast, the NA activity of B/YAM carrying 395 (T/V/D) changes decreased when compared to that of B/BR/60/08. Virus carrying D342S NA substitution possessed high NA activity that was thermostable (Figure 23C, D).
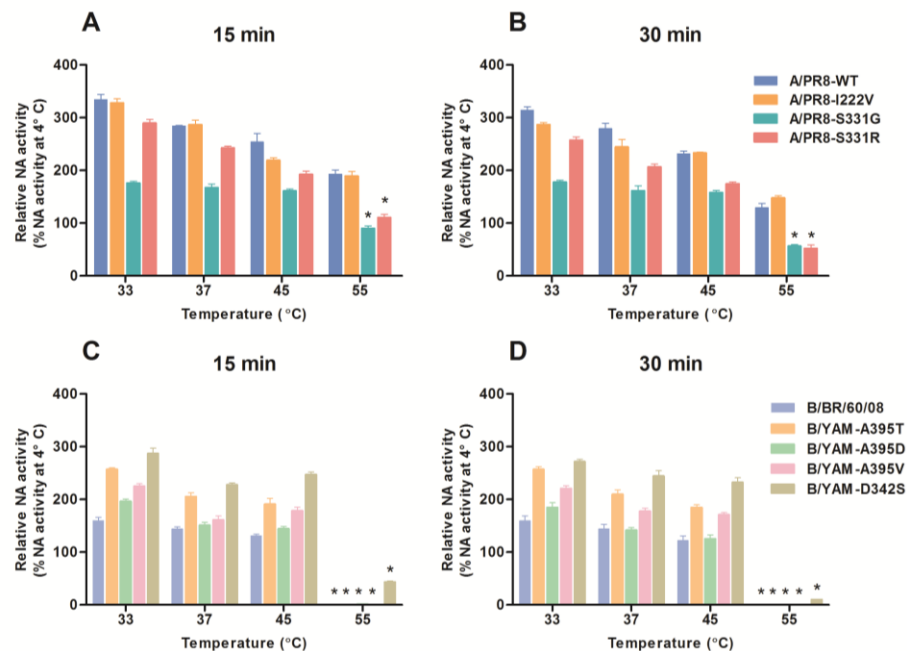
*Figure 23. The NA thermostability of the recombinant influenza A and B viruses carrying a single NA substitution. Influenza A/PR8 (A, B) and B/YAM (C, D) viruses were incubated for 15 and 30 min at indicated temperatures. Residual NA enzyme activity was assayed and is presented as a percentage of the NA activity at 4°C. The bars represent the mean of the relative NA activity ± SD for different temperatures from three experiments, * P < 0.05.*

Replication kinetic

To investigate the virus fitness *in vitro*, we measured the replication capacity of recombinant influenza A and B viruses with NA amino acid substitutions in MDCK cells. At 6 and 12 hpi, the growth of A/PR8-I222V virus was similar to that of A/PR8-WT but the A/PR8-S331G and A/PR8-S331R viruses had significantly lower viral yields than A/PR8-WT virus ($P < 0.05$) (Figure 24A). At 48 hpi, the yields of all recombinant influenza A viruses were comparable (range, 6.8 - 7.5 $\log_{10}TCID_{50}$/mL) (Figure 24B). At 6 and 12 hpi, recombinant influenza B viruses had significantly lower viral yield than B/BR/60/08 virus ($P < 0.05$) (Figure 24C). At 48 hpi, all of influenza B viruses replicated to significantly higher virus titers in MDCK cells than that for B/BR/60/08 virus ($P < 0.05$) (Figure 24D). At 72 hpi, the viral titers

of recombinant B viruses were 4.2 and 5.2 $\log_{10}$ $TCID_{50}$/mL. These results demonstrated that S331G, S331R (A/PR8 background), and S342S, A395T, A395V, and A395S (B/YAM background) NA substitutions reduced the NA enzyme activity and thermostability and impaired the replication kinetic in MDCK cells at12 hpi.
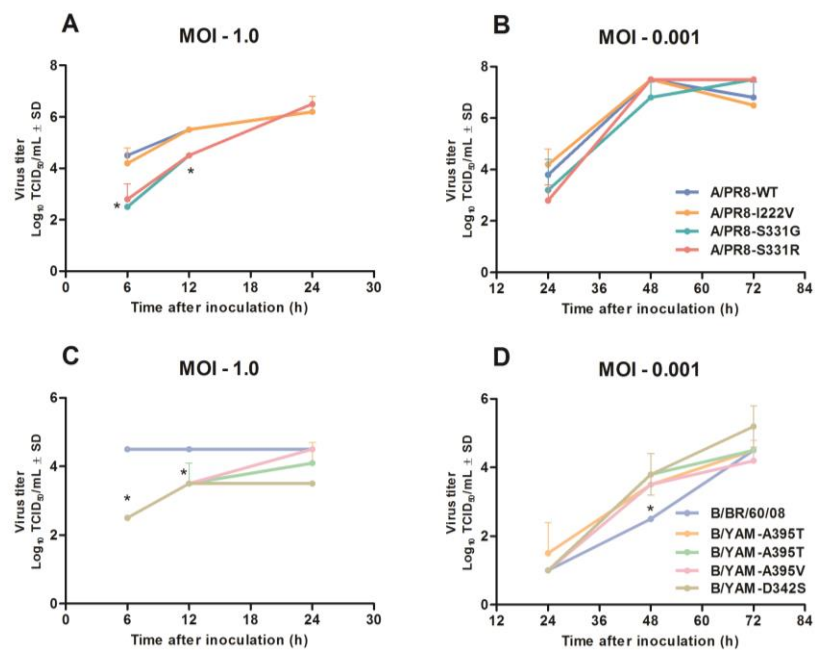
Figure 24. *Replication kinetics of recombinant influenza A and B viruses carrying a single NA substitution in MDCK cells. MDCK cells were infected with recombinant viruses at MOI of 1 (A, C) or 0.001 (B, D). Virus titer was determined by $TCID_{50}$ assay in MDCK cells after incubation for 72 h at 35°C (influenza A viruses) or 33°C (influenza B viruses). Each data point represents mean virus titers ($log_{10}TCID_{50}$/mL) ± SD in triplicate experiments. * P < 0.05.*

NA genetic stability

To determine whether NA substitutions introduced into A/PR8 and B/YAM genetic background can be stably maintained *in vitro*, the NA genes were amplified from viruses before and after three passages in the MDCK cells and NA sequences were determined. The I222V, S331G and S331R NA substitutions were genetically stable in N2 proteins in the background of A/PR8 virus. A diverse pattern was observed for influenza B viruses. Thus, A395T and A395D NA substitutions were genetically stable and maintained unchanged during three passages, although D342S and A395V NA substitutions were unstable and reverted to WT NA after three passages (Table 26). These findings indicated that genetically unstable D342S and A395V NA substitutions of influenza B viruses more likely cannot emerge at a high frequency.

Table 26. Genetic stability of NA substitutions among recombinant influenza A and B viruses after subsequent three passages in MDCK cells

Recombinant influenza virus	Passaging in MDCK cells	
	0 passage	**3rd passage**
A/PR8-WT	ND [a]	ND
A/PR8-I222V	V	V
A/PR8-S331G	G	G
A/PR8-S331R	R	R
B/BR/60/08	ND	ND
B/YAM-D342S	S	D
B/YAM-A395T	T	T
B/YAM-A395D	D	D
B/YAM-A395V	V	A

[a] Not done

Discussion

In this study, we investigated the effect of the novel NA substitutions on NAIs susceptibility, NA enzymatic properties, and replication capacity *in vitro*. We generated recombinant influenza A and B viruses (7 + 1) containing NA glycoprotein from the viruses currently circulating in Thailand in the homogeneous genetic background of A/PR8 and B/YAM viruses, respectively. The conserved framework I222 residue in NA of influenza virus provides support to the catalytic residues (Burmeister et al., 1992). In our study, the A/PR8-I222V virus was susceptible to NAIs in phenotypic NA inhibition assay and possessed slightly higher NA enzymatic activity. It was previously reported that influenza A(H3N2) virus with a single I222V NA amino acid substitution was susceptible to oseltamivir and possessed slightly higher NA enzymatic activity as compared to WT virus (Baz, Abed, McDonald, & Boivin, 2006). Our data revealed that recombinant A/PR8-I222V virus has minor effect on the NA enzymatic properties and the virus replication efficiency in MDCK cells. However, I222V NA substitution has a compensatory effect on the influenza A(H3N2) virus carrying E119V, and existence of these two substitutions resulted in partially improved viral fitness in cells culture and high level of oseltamivir resistance (Simon et al., 2011).

Regarding the S331G and S331R, D342S and A395T/D/V NA substitutions, these are found outside the NA active site, and showed normal inhibition by NAIs. The S331G and S331R NA substitutions impacted the kinetic properties of the NA enzyme, affecting the affinity to bind to MUNANA substrate (K_m). The velocity (V_{max}) of the A/PR8-S331G and A/PR8-S331R viruses nearly doubled compared to that of A/PR8-WT virus, demonstrating the compensatory effect seen in the lower replication capacity of viruses carrying S331G and S331R substitutions than those with WT and I222V NAs. The significant decrease of NA activity and increase K_m value demonstrated that S331G and S331R NA substitutions is deleterious the NA functions. The NA activity of A/PR8-S331G, A/PR8-S331R, and B/YAM-A395D viruses has been decreased, while they showed lower viral titer than those of the WT at 6 and 12 hpi in MDCK cells. The A/PR8-S331G and A/PR8-S331R viruses were characterized by a loss of NA activity at high temperatures (55°C), while PR8-WT

and A/PR8-I222V viruses did not. This data indicated that viral replication capacity *in vitro* is dependent on NA activity (Matrosovich, Matrosovich, Carr, Roberts, & Klenk, 2003) and thermostability of viruses (Burnham et al., 2013). The B/YAM-D342S, B/YAM-A395T, and B/YAM-A395V viruses showed an increase NA activity while K_m and V_{max} values also decreased. This data suggested the inverse relationship between NA activity and K_m and V_{max} values and these substitutions are advantageous the NA functions. Moreover, they revealed significant higher viral yield at 48 hpi in MDCK cells than WT virus. The recombinant influenza A viruses carrying NA substitutions were more stable at high temperature (55°C) than B viruses. The A395V and D342S NA substitutions in B/YAM background were unstable, and after three passages *in* vitro reverted to WT NA. This data indicated that D342 or A395 amino acids are more suitable for the overall viral fitness than S342 or V395.

There are few limitations in this study. Since we cannot generate recombinant B/YAM virus with WT NA protein using reverse genetics, we used B/BR/60/2008 virus that is genetically similar to viruses circulating in Thailand and compared the NA enzymatic properties and viral replication efficiency *in vitro*. Our results therefore require validation using B/YAM-WT NA virus. The NA of influenza A(H3N2) positive specimens with I222V, S331G, and S331R NA substitutions were generated in A/PR8 (H1N1) backbone, the replication efficiency in MDCK cells maybe different that seen in natural isolates because of the effect of HA-NA balance (Little et al., 2015).

In conclusion, the recombinant influenza A and B viruses containing different NA substitutions found outside the NA active site showed normal inhibition by NAIs, but demonstrated differences in the NA enzymatic properties and virus replication capacity *in vitro*. Continuous influenza antiviral surveillance is important for the identification of the molecular markers of antiviral resistance and characterization of the NA enzyme functions and viral fitness.

Supporting Information

Table S1. *Primer sets used for conventional PCR amplification of the whole genome of influenza B isolates.*

Primer Name	Nucleotide Sequences (5'-3')	PCR product (bp)
PB2-F5'	AGC AGA AGC GGA GCC TTT TCA AG	
PB2-R1082	CCG TCC CAT ATT CCA ATC TTC TG	1082
PB2-F902	CAG CCA TAG ACG GAG GTG ATG T	
PB2-R1907	TGA TAA GGC TCC CCA TTG CTC CTT	1005
PB2-F1716	GTT CCA ATG GGA TGC ATT TGA AG	
PB2-R3	ATG ACC AGT AGA AAC ACG AGC A	584
PB1-F5'	AGC AGA AGC GGA GCC TTT AAG ATG	
PB1-R1024	CCG GTG CTA TAC TAC AAA AAT CCC	1024
PB1-F896	GCA TGA CAG TAA CAG GAG ACA AT	
PB1-R1946	CCA TGT GCT GGG GTT ATA TCT GC	1050
PB1-F1667	ACA CCT ACA AAT GCC ACA GGG GAG	
PB1-R3'	TGA CCA GTA GAA ACA CGA GCC TTT	639
PA-F5'	AGC AGA AGC GGT GCG TTT GAT TTG	
PA-R1114	ATG TTA ATC CAT CCC CTG TGG CCC	1114
PA-F1010	GGA AGC TTT GGA GAG ACT GTG TAA A	
PA-R1836	GGG GCT ATT TAC TCT GTC TCC C	826
PA-F1622	TTG GCT CCC TAT TTG TGA GTG GG	
PA-R3'	AGT AGA AAC ACG TGC ATT TTT GAT TC	578
HA-F5'	AGC AGA AGC AGA GCA TTT TCT AA	
HA-R1142	GCA ATC ATT CCT TCC CAT CCT CC	1142
HA-F997	ATA GGA AAT GCC CAA TAT GGG GT	
HA-R3'	CGT TTC TTT GTA ATG ATG ACA AG	703

Table S1. Table (Continue)

Primer Name	Nucleotide Sequences (5'-3')	PCR product (bp)
NP-F5'	AGC AGA AGT ACA GCA TTT TCT TG	
NP-R916	TCT TCA ATG TCT GCA ATC CCT GG	916
NP-F786	GGC AGA CAG AGG GCT ATT GAG AG	
NP-R3'	AGT AGA AAC AAC AGC ATT TTT TA	779
NA-F5'	AGC AGA AGC AGA GCA TCT TCT CAA A	
NA-R1165	TTA GAC ATC GTT CGA GAG TAC CA	1165
NA-F1014	ACT TAT TTG GAC ACC CCC AGA CC	
NA-R3'	AGT AGT AAC AAG AGC ATT TTT CAG A	386
M-F5'	ACC AGA AGC ACG CAC TTT CTT AA	
M-R3'	TGA CCA GTA GAA ACA ACG CAC	1000
NS-F5'	AGC AGA AGC AGA GGA TTT GTT TAG	
NS-R3'	ATG ACC AGT AGT AAC AAG AGG ATT	900

Table S2. *Accession numbers of influenza B sequences in GenBank and GISAID used to construct phylogenetic trees of 8 genes.*

Gene	Accession No.
PB1	CY018763 CY018771 CY019537 CY040455 CY115189 CY153888 CY171829 CY176319 CY156664 CY115157 CY115349 CY155704 CY115253 CY119944 CY033882 EF626642 EU305611 AJ781186 X266881 DQ792895
PB2	CY018764 CY018772 CY019538 CY115198 CY115390 CY171854 CY175504 CY176128 CY175856 CY040456 CY033883 CY115158 CY115190 DQ792894 EF626643 FJ461688 EU305612 JX266889 AJ781207
PA	CY018762 CY018770 CY019536 CY172132 CY171964 CY176358 CY153551 CY153887 CY155887 CY115404 CY040454 CY033881 CY115156 CY115188 DQ792896 EF626641 JX266897 KJ532166
HA	AFH58304 AFH58348 AJ784040 AJ784056 AY504602 AGX18589 CY019675 CY019611 CY022221 DQ792897 CY018757 CY018765 CY019531 CY019619 CY153730 CY154530 CY022221 CY018677 CY156938 CY018453 CY115383 CY171959 CY173809 CY040449 CY033876 CY115151 CY115183 CY033844 CY149981 CY040441 M10298 KC891793 KC813773 KC892118 EPI406272 EPI171429 EPI366580 EPI193056 EPI301334 EPI340834 EPI271913 EPI406983 EU305614 EF626636 EPI 211559
NP	CY018760 CY018768 CY019534 CY175092 CY171786 CY172050 CY153693 CY155901 CY115282 CY175852 CY040452 CY033879 CY115154 CY115186 DQ792898 EF626639 KJ532157 JX266940
NA	CY018759 CY018767 CY019533 CY018471 CY018639 CY018343 CY115385 CY030777 CY018431 CY018351 CY171849 CY176291 CY150172 CY040451 CY033878 CY115153 CY115185 CY019677 CY019613 CY022223 DQ792899 EF626638 EF541477 JX266945 KF234468 KC478983
M	CY018758 CY018766 CY019532 CY153275 CY153443 CY033941 CY153315 CY172096 CY176322 CY155899 CY115176 CY040450 CY033877 CY115152 CY115184 CY019676 CY019612 CY022222 DQ792900 EF626637 JX266954 JX266956 KC813859 AB120273
NS	CY018761 CY018769 CY019535 CY171963 CY153886 CY033872 CY173797 CY153782 CY040453 CY033880 CY115155 CY115187 DQ792901 EF626640 KC891879 KC891927 KC892030 KJ532186

Table S3. *Details of patients and influenza B virus analyzed during March 2014 to May 2016 (N = 73)*

Name of strain	Gender	Age (Yr)	Collection date	Isolation source	Accession number		Clade	
					HA	NA	HA	NA
B/Thailand/CU-H3620/2014	Female	6	3-Mar-2014	Nasopharyngeal swab	KT725657	KX151325	Yam 2	Yam 2
B/Thailand/CU-C4610/2014	Male	10	6-Mar-2014	throat swab	KT725655	KX151326	Yam 2	Yam 2
B/Thailand/CU-B9455/2014	Male	6	11-Mar-2014	Nasal swab	KT725654	KX151327	Yam 2	Yam 2
B/Thailand/CU-B9693/2014	Male	47	15-Mar-2014	Nasal swab	KT725656	KX151328	Yam 3	Yam 3
B/Thailand/CU-B10007/2014	Male	27	3-Apr-2014	Nasal swab	KT725661	KX151329	Vic 1	Vic 1
B/Thailand/CU-B10030/2014	Female	53	18-Apr-2014	Nasal swab	KT725662	KX151330	Yam 3	Yam 3
B/Thailand/CU-A832/2014	Male	29	23-Apr-2014	throat swab	KT725663	KX151331	Yam 2	Yam 2
B/Thailand/CU-B10125/2014	Female	34	6-May-2014	Nasal swab	KT725666	KX151332	Yam 3	Yam 3
B/Thailand/CU-B10127/2014	Female	43	6-May-2014	Nasal swab	KT725665	KX151333	Yam 3	Vic1
B/Thailand/CU-C4799/2014	Male	11	28-May-2014	throat swab	KT725667	KX151334	Yam 3	Yam 3
B/Thailand/CU-B10233/2014	Male	73	14-Jun-2014	Nasal swab	KT725668	KX151335	Yam 3	Yam 3
B/Thailand/CU-B10235/2014	Female	41	16-Jun-2014	Nasal swab	KT725669	KX151336	Yam 2	Yam 2
B/Thailand/CU-B10236/2014	Male	38	16-Jun-2014	Nasal swab	KT725670	KX151337	Vic 1	Vic 1
B/Thailand/CU-B10303/2014	Male	56	28-Jun-2014	Nasal swab	KT725671	KX151338	Yam 3	Yam 3
B/Thailand/CU-B10340/2014	Male	9	1-Jul-2014	Nasal swab	KT725672	KX151339	Yam 2	Yam 2
B/Thailand/CU-B10376/2014	Female	36	7-Jul-2014	Nasal swab	KT725673	KX151340	Yam 2	Yam 2
B/Thailand/CU-B10440/2014	Female	9	30-Jul-2014	Nasal swab	KT725674	KX151341	Yam 3	Yam 3
B/Thailand/CU-B10508/2014	Male	8	4-Aug-2014	Nasal swab	KT725675	KX151342	Yam 2	Yam 2
B/Thailand/CU-B10739/2014	Female	53	1-Sep-2014	Nasal swab	KT725678	KX151343	Yam 2	Yam 2
B/Thailand/CU-B10747/2014	Male	36	1-Sep-2014	Nasal swab	KT725679	KX151344	Yam 3	Yam 3
B/Thailand/CU-B10835/2014	Male	73	11-Sep-2014	Nasal swab	KT725681	KX151345	Yam 3	Yam 3
B/Thailand/CU-B11066/2014	Male	56	15-Oct-2014	Nasal swab	KT725684	KX151346	Yam 3	Yam 3
B/Thailand/CU-B11155/2014	Female	47	21-Oct-2014	Nasal swab	KT725685	KX151347	Yam 2	Yam 2
B/Thailand/CU-B11168/2014	Female	61	25-Oct-2014	Nasal swab	KT725686	KX151348	Yam 3	Yam 3
B/Thailand/CU-C5175/2014	Male	5	30-Oct-2014	throat swab	KT725687	KX151349	Yam 2	Yam 2
B/Thailand/CU-B11219/2014	Female	7	5-Nov-2014	Nasal swab	KT725688	KX151350	Yam 3	Yam 3
B/Thailand/CU-B11259/2014	Male	19	14-Nov-2014	Nasal swab	KT725689	KX151351	Yam 3	Yam 3
B/Thailand/CU-B11295/2014	Female	4	20-Nov-2014	Nasal swab	KT725690	KX151352	Yam 3	Yam 3
B/Thailand/CU-B11305/2014	Female	4	21-Nov-2014	Nasal swab	KT725691	KX151353	Yam 3	Yam 3
B/Thailand/CU-B11371/2014	Male	18	4-Dec-2014	Nasal swab	KT725695	KX151354	Yam 3	Yam 3
B/Thailand/CU-B11389/2014	Male	43	8-Dec-2014	Nasal swab	KT725696	KX151355	Yam 3	Yam 3
B/Thailand/CU-B11391/2014	Male	69	9-Dec-2014	Nasal swab	KT725697	KX151356	Yam 3	Yam 3
B/Thailand/CU-B11425/2014	Female	54	19-Dec-2014	Nasal swab	KT725698	KX151357	Yam 3	Yam 3
B/Thailand/CU-B11464/2015	Female	56	5-Jan-2015	Nasal swab	KT725701	KX151358	Yam 3	Yam 3
B/Thailand/CU-B11465/2015	Male	42	5-Jan-2015	Nasal swab	KT725702	KX151359	Yam 3	Yam 3
B/Thailand/CU-B11472/2015	Female	55	6-Jan-2015	Nasal swab	KT725703	KX151360	Yam 3	Yam 3
B/Thailand/CU-B11572/2015	Female	55	4-Feb-2015	Nasal swab	KT725705	KX151361	Yam 3	Yam 3
B/Thailand/CU-B11595/2015	Male	44	10-Feb-2015	Nasal swab	KT725706	KX151362	Yam 2	Yam 2
B/Thailand/CU-B11603/2015	Female	33	13-Feb-2015	Nasal swab	KT725707	KX151363	Vic 1	Vic1

B/Thailand/CU-B11609/2015	Male	52	18-Feb-2015	Nasal swab	KT725708	KX151364	Yam 3	Yam 3
B/Thailand/CU-B11673/2015	Male	42	3-Mar-2015	Nasal swab	KT725711	KX151365	Yam 3	Yam 3
B/Thailand/CU-B11681/2015	Male	52	5-Mar-2015	Nasal swab	KT725712	KX151366	Yam 3	Yam 3
B/Thailand/CU-B11705/2015	Female	28	14-Mar-2015	Nasal swab	KT725713	KX151367	Yam 3	Vic1
B/Thailand/CU-B11719/2015	Female	40	18-Mar-2015	Nasal swab	KT725714	KX151368	Yam 3	Yam 3
B/Thailand/CU-B11774/2015	Female	92	1-Apr-2015	Nasal swab	KT725716	KX151369	Yam 3	Yam 3
B/Thailand/CU-B11776/2015	Female	30	2-Apr-2015	Nasal swab	KT725717	KX151370	Yam 3	Yam 3
B/Thailand/CU-B11789/2015	Female	37	4-Apr-2015	Nasal swab	KT725718	KX151371	Yam 3	Yam 3
B/Thailand/CU-B11823/2015	Male	31	17-Apr-2015	Nasal swab	KT725720	KX151372	Yam 2	Yam 2
B/Thailand/CU-B11863/2015	Male	66	4-May-2015	Nasal swab	KT725721	KX151373	Yam 3	Yam 3
B/Thailand/CU-B11864/2015	Female	37	4-May-2015	Nasal swab	KT725722	KX151374	Vic 1	Vic1
B/Thailand/CU-B11901/2015	Female	47	19-May-2015	Nasal swab	KT725723	KX151375	Yam 3	Yam 3
B/Thailand/CU-B11908/2015	Male	16	20-May-2015	Nasal swab	KT725724	KX151376	Yam 3	Yam 3
B/Thailand/CU-B12073/2015	Female	27	2-Jul-2015	Nasal swab	KX602166	KX151377	Yam 3	Yam 3
B/Thailand/CU-B12092/2015	Male	40	8-Jul-2015	Nasal swab	KX602167	KX151378	Yam 3	Yam 3
B/Thailand/CU-B12096/2015	Male	15	9-Jul-2015	Nasal swab	KX602168	KX151379	Yam 3	Yam 3
B/Thailand/CU-B12113/2015	Male	15	13-Jul-2015	Nasal swab	KX518699	KX151380	Yam 3	Yam 3
B/Thailand/CU-B12998/2015	Male	27	30-Sep-2015	Nasal swab	KX518700	KX151383	Yam 3	Vic1
B/Thailand/CU-B13716/2015	Female	38	11-Nov-2015	Nasal swab	KX602168	KX151386	Vic 1	Vic 1
B/Thailand/CU-B13874/2015	Female	79	18-Nov-2015	Nasal swab	KX518701	KX151387	Yam 3	Yam 3
B/Thailand/CU-B13917/2015	Female	36	23-Nov-2015	Nasal swab	KX602170	KX151388	Yam 3	Yam 3
B/Thailand/CU-B14301/2015	Female	49	2-Dec-2015	Nasal swab	KX518702	KX151391	Yam 3	Yam 3
B/Thailand/CU-B14349/2015	Male	12	9-Dec-2015	Nasal swab	KX602171	KX151393	Vic 1	Vic 1
B/Thailand/CU-B14831/2016	Male	33	21-Jan-2016	Nasal swab	KX518703	KX518707	Vic 1	Vic 1
B/Thailand/CU-B14972/2016	Male	37	5-Feb-2016	Nasal swab	KX602172	KX602179	Yam 3	Yam 3
B/Thailand/CU-B14987/2016	Female	6	8-Feb-2016	Nasal swab	KX518704	KX518708	Vic 1	Yam 2
B/Thailand/CU-B15115/2016	Male	52	10-Feb-2016	Nasal swab	KX518705	KX518709	Vic 1	Yam 2
B/Thailand/CU-B15565/2016	Female	33	11-Mar-2016	Nasal swab	KX602173	KX602180	Vic 1	Vic1
B/Thailand/CU-B16181/2016	Female	20	12-Apr-2016	Nasal swab	KX602174	KX602181	Vic 1	Vic 1
B/Thailand/CU-B16211/2016	Female	18	23-Apr-2016	Nasal swab	KX602175	KX602182	Vic 1	Vic 1
B/Thailand/CU-B16317/2016	Female	34	27-Apr-2016	Nasal swab	KX518706	KX518710	Vic 1	Vic 1
B/Thailand/CU-B16416/2016	Male	35	10-May-2016	Nasal swab	KX602176	KX602183	Vic 1	Vic 1
B/Thailand/CU-B16432/2016	Female	34	14-May-2016	Nasal swab	KX602177	KX602184	Vic 1	Vic 1
B/Thailand/CU-B16541/2016	Male	11	20-May-2016	Nasal swab	KX602178	KX602185	Yam 3	Yam 3

Table S4. *Amino acid mutations found in the epitope domain of HA1 protein of influenza B virus strains in this study.*

Residues at site	B/HK/73	Victoria 1 (N = 15)	Yamagata 2 (N = 13)	Yamagata 3 (N = 45)
120 Loop				
48	Q	E (100%)	K (100%)	R (97.7%), K (2.3%)
56	N	K (100%)	D (100%)	D (100%)
71	K	K (100%)	M (100%)	M (100%)
75	T	K (100%)	T (100%)	T (100%)
116	N	H (100%)	N (100%)	K (97.7%), R (2.3%)
122	R	H (100%)	Q (100%)	Q (100%)
125	T	I (100%)	I (100%)	I (100%)
129	T	N (26.7%), D (73.3%)	K (100%)	K (97.7%), N (2.3%)
179-181	TKG	TEG (93.3%), TEE (6.7%)	AEG (100%)	TEG (100%)
150 Loop				
148-150	NGN	NGN (100%)	SKS (100%)	SKI (97.7%), NGN (2.3%)
160 Loop				
Insertion at 162 - 163	-	NDKN (100%)	DNN (100%)	DNY (97.7%), DNN (2.3%)
190 Helix				
195	E	E (100%)	K (100%)	K (100%)
199	V	A (100%)	K (100%)	K (100%)
206	K	K (86.7%), N (13.3%)	N (100%)	N (100%)
230	N	N (100%)	D (100%)	D (100%)
232	A	T (100%)	R (92.3%), T (7.7%)	T (100%)
235	E	G (100%)	G (100%)	G (100%)
Receptor binding site				
136	I	K (100%)	R (100%)	R (97.7%), K (2.3%)

The defined residue positions on the epitope domains of HA1 protein according to Ni et al., 2013

Ni F, Kondrashkina E, Wang Q (2013) Structural basis for the divergent evolution of influenza B virus hemagglutinin. Virology 446: 112–122. doi: 10.1016/j.virol.2013.07.035

Table S5. *Primers used for conventional PCR amplification of the HA gene of influenza A(H3N2) and A(H1N1)pdm09 strains circulating in Thailand.*

Primer Name	Nucleotide Sequences (5'→3')	PCR product (bp)
HA_F5'	AGCAAAAGCAGGGGAAAATAAAAGCA	797
H3_R797	TCCCGGATTTACTATTGTCCA	
H3_F598	TTGACAAATTGTACATTTGGGG	566
H3_R1164	GCTTTTGAGATCTGCTGCTTG	
H3_F1013	CACTCTGAAATTGGCAACAGG	688
HA_R3'	AGTAGAAACAAGGGTGTTTTTAACTAC	
HA_F1	AGCAAAAGCAGGGGAAAATAAAAGCA	694
H1_R694	TCTTGATGACCCCACAAAAACATA	
H1_F510	AGCTTCTACARAAATTTAATATGGCT	616
H1_R1126	CATCCATCTACCATCCCTGTCCA	
H1_F923	AACACCCAAGGGTGCTATAAACA	625
H1_R1548	ATTTTGGGTAGTCATAAGTCCCATT	
H1_F1398	GATTCAAATGTGAAGAACTTATATGA	382
H1-R1780	AGTAGAAACAAAGGGTGTTTTTTCTCATGT	

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Table S6. *Accession numbers in GenBank and GISAID of HA influenza A(H3N2) and A(H1N1)pdm09 gene sequences used for phylogenetic analysis.*

Name	Accession No.			
A/H3N2-TH strains (2010)	CY074950	CY074958	CY074966	
A/H1N1-TH strains (2010)	CY080307	CY080299	CY089429	CY089437
	CY081156	CY074982	CY080323	CY080339
	CY088801	CY088809	CY088816	CY088823
	CY088830	CY088838	CY089447	CY089455
	CY089463	CY080315	CY080331	
A/H3N2 reference and vaccine strains	KC892248	KC535440	KC882860	KC892582
	GQ293081	EU021268	EU021276	EU625364
	EPI318272	EPI346607	EPI353906	EPI467994
	EPI426061	EPI539576	EPI460558	EPI540526
	EPI539874	EPI319276	EPI326115	EPI232453
	EPI367105	EPI160218	EPI165489	GQ902809
A/H1N1 reference and vaccine strains	EPI319447	EPI326206	EPI280344	EPI319590
	EPI382424	EPI466626	EPI539470	EPI541029
	EPI316435	EPI319527	EPI450810	EPI466545
	EPI331059	EPI239666	EPI253705	EPI279895
	FJ969540	GQ205436	GQ166661	

Table S7. *Amino acid changes observed in the antigenic sites (epitopes A through E) of the HA protein of 120 influenza A(H3N2) strains.*

Amino acid position	33	45	48	53	87	124	128	138	140	142	144	145	156	159	160	186	198	207	223	261	278	311	312
Antigenic sites	C	C	C	E	A	B	A	A	A	A	A	B	B	B	B	B	B	D	D	E	C	C	C
A/Perth/16/2009	Q	S	T	D	F	S	T	A	I	R	K	N	H	F	K	G	A	K	V	R	N	Q	N
A/Victoria/361/2011	.	N	I	.	.	.	.	.	.	.	N	.	Q	.	.	V	S	.	I	.	.	.	S
A/Texas/50/2012	R	N	I	.	.	.	N	.	.	.	N	.	.	.	.	V	P	.	I	.	K	.	S
A/Thailand/CU-H1285/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	.
A/Thailand/CU-H1443/2010	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	.
A/Thailand/CU-H1817/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	.
A/Thailand/CU-B4730/2011	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-H2973/2011	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-B4844/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H2989/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B4836/2011	.	.	.	N	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-B4929/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5021/2011	.	N	I	.	.	.	.	.	.	M	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-B5105/2011	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-H3020/2011	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-B5436/2011	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5465/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5541/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5593/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5614/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5697/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5735/2011	.	N	M	.	.	.	.	.	.	M	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-B5773/2011	.	.	.	.	.	.	.	.	.	.	D	S	.	.	.	.	.	.	I	.	.	.	.
A/Thailand/CU-B5848/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5873/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5898/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	G
A/Thailand/CU-B5900/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5909/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B5928/2011	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-C2417/2011	.	N	I	.	.	.	.	.	.	M	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-H3141/2012	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.
A/Thailand/CU-B6091/2012	R	N	I	.	.	.	.	.	.	.	N	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B6251/2012	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B6274/2012	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B6309/2012	.	.	.	N	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-B6780/2012	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-H3368/2012	R	N	I	.	.	.	.	.	T	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B6936/2012	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3434/2012	R	N	I	.	Y	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3435/2012	R	N	I	.	.	.	.	.	.	G	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3453/2012	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7189/2012	R	N	I	.	Y	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3490/2012	R	N	I	.	Y	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7235/2012	R	N	I	.	Y	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7269/2012	R	N	I	.	Y	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7367/2012	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7418/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7483/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7536/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7585/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7596/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7646/2013	.	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	S	.	I	.	K	.	S

Strain																								
A/Thailand/CU-B7755/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7765/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7853/2013	R	N	I	.	.	.	A	.	.	G	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7885/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7937/2013	R	N	I	.	.	.	A	.	.	G	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A7/2013	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A24/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A114/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B7992/2013	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	L	.	.	.	S
A/Thailand/CU-A134/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A153/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A164/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	R	I	.	K	.	S
A/Thailand/CU-A166/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A182/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A196/2013	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	R	I	.	K	.	S
A/Thailand/CU-B8121/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8127/2013	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	.	.	S
A/Thailand/CU-B8222/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A305/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-C4087/2013	R	N	I	.	.	.	.	.	.	.	D	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8235/2013	R	N	I	.	.	G	A	.	.	G	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8236/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8364/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8518/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8525/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A411/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A459/2013	R	N	I	N	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3567/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3574/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8736/2013	R	N	I	.	.	.	A	.	.	G	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8745/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8772/2013	.	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-C4364/2013	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3580/2013	R	N	I	.	.	.	.	.	.	.	S	S	.	.	.	T	.	S	.	I	.	K	H	S
A/Thailand/CU-H3584/2013	R	N	I	G	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-A598/2014	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B8849/2014	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-C4406/2014	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-C4492/2014	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3595/2014	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-C4507/2014	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-C4546/2014	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3611/2014	R	D	I	.	.	.	.	.	.	.	S	S	.	.	.	T	.	S	.	I	.	K	H	S
A/Thailand/CU-H3624/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	.	.	T	.	S	.	I	.	K	H	S
A/Thailand/CU-H3626/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	.	.	T	.	S	.	I	.	K	H	S
A/Thailand/CU-CB166/2014	R	N	I	N	.	.	.	.	.	.	T	I	.	.	.	.	.	S	.	I	.	K	H	S
A/Thailand/CU-C4655/2014	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B10282/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	.	.	T	.	S	.	I	.	K	H	S
A/Thailand/CU-B10283/2014	R	N	I	.	.	.	A	S	.	G	N	S	.	S	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-H3649/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	.	Y	T	.	S	.	I	.	K	H	S
A/Thailand/CU-B10345/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	.	.	T	.	S	.	I	.	K	H	S
A/Thailand/CU-B10421/2014	.	.	.	.	.	.	A	S	.	G	N	S	Q	S	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B10422/2014	R	N	I	.	.	.	A	S	.	G	N	S	.	S	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B10509/2014	R	N	I	.	.	.	A	S	.	G	N	S	.	S	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B10520/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	.	Y	T	.	S	.	I	.	K	H	S
A/Thailand/CU-B10521/2014	R	N	I	.	.	.	A	S	.	G	N	S	.	S	.	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B10557/2014	.	N	I	.	.	.	.	.	.	.	S	S	.	.	Y	T	.	S	.	I	.	K	H	S
A/Thailand/CU-H3656/2014	R	N	I	.	.	.	A	S	.	G	N	S	.	S	.	.	.	S	.	I	.	K	.	S

A/Thailand/CU-B10755/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	Y	T	.	P	.	I	.	K	H	S
A/Thailand/CU-B10792/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	Y	T	.	S	.	I	.	K	H	S
A/Thailand/CU-B10828/2014	R	N	I	.	.	.	.	.	.	.	N	S	.	.	.	.	P	.	I	.	K	H	S
A/Thailand/CU-B10952/2014	R	N	I	.	.	.	A	S	.	G	N	S	.	S	.	.	S	.	I	.	K	.	S
A/Thailand/CU-B10975/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	Y	T	.	P	.	I	.	K	H	S
A/Thailand/CU-B11055/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	Y	T	.	S	.	I	.	K	H	S
A/Thailand/CU-B11065/2014	R	N	M	.	.	.	.	.	.	.	S	S	.	Y	T	.	P	.	I	.	K	H	S
A/Thailand/CU-B11201/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	Y	T	.	P	.	I	.	K	H	S
A/Thailand/CU-B11202/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	Y	T	.	S	.	I	.	K	H	S
A/Thailand/CU-B11284/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	Y	T	.	P	.	I	.	K	H	S
A/Thailand/CU-H3680/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	Y	T	.	S	.	I	.	K	H	S
A/Thailand/CU-B11351/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	Y	T	.	S	.	I	.	K	H	S
A/Thailand/CU-B11367/2014	R	N	I	.	.	.	.	.	.	.	S	S	.	.	T	.	S	.	I	.	K	H	S

Table S8. *Amino acid changes observed in the antigenic sites (epitopes A through E) of the HA protein of 81 influenza A(H1N1)pdm09 strains.*

Amino acid position	69	72	83	94	97	127	143	163	185	190	197	205	216	222	234	245	256	259	267	283
Antigenic sites	E	E	E	D		A	A	D	B	B		D	D	D		D		E	E	
A/California/07/2009	S	T	P	D	D	D	S	K	S	S	A	R	I	D	V	T	A	R	I	K
A/Thailand/C602/2010	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B2357/2010	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B4148/2010	.	.	S	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B4339/2010	.	.	S	.	.	.	G	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-C1157/2010	.	.	S	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H1222/2010	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H1786/2010	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H1821/2010	.	.	S	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H2176/2010	.	.	S	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
A/Thailand/CU-H2283/2010	.	.	S	V	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H2358/2010	.	.	S	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H2389/2010	.	.	S	V	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H2417/2010	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H2543/2010	.	.	S	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H2548/2010	.	.	S	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-H2698/2010	.	.	S	.	.	.	G	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/H1255/2010	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Thailand/H1818/2010	.	.	S	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	E
A/Thailand/CU-H2911/2011	.	.	S	.	.	.	G	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B4656/2011	.	.	S	.	.	E	G	.	T	.	T	.	.	.	.	.	.	.	V	.
A/Thailand/CU-B4662/2011	.	.	S	N	.	.	.	.	.	.	.	K	V	.	.	.	.	.	.	.
A/Thailand/CU-B4717/2011	.	.	S	.	.	.	G	R	.	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B4773/2011	.	.	S	.	.	.	G	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B5356/2011	.	.	S	.	.	E	G	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B5515/2011	.	.	S	N	.	E	G	.	T	.	T	K	V	.	.	.	.	.	.	.
A/Thailand/CU-B6181/2012	.	.	S	.	N	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B6213/2012	.	.	S	.	N	.	.	.	T	.	.	K	.	.	.	.	.	.	.	.
A/Thailand/CU-B6475/2012	.	.	S	.	N	.	.	.	T	.	.	.	.	.	.	.	.	.	.	E
A/Thailand/CU-B6609/2012	.	.	S	.	N	.	.	.	T	.	.	.	.	.	.	.	.	.	.	E
A/Thailand/CU-B6801/2012	.	.	S	.	N	.	.	.	T	.	.	.	.	.	I	N	.	.	.	E
A/Thailand/CU-B8091/2013	.	.	S	.	N	.	.	.	T	.	.	.	.	.	I	.	.	.	.	E
A/Thailand/CU-B8092/2013	.	.	S	.	N	.	.	N	T	.	.	.	.	.	I	.	.	.	.	E
A/Thailand/CU-B8250/2013	P	.	S	.	N	.	.	N	T	.	.	.	.	.	I	.	.	.	.	E
A/Thailand/CU-A338/2013	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B8573/2013	P	.	S	.	N	.	.	N	T	.	.	.	.	.	I	.	.	.	.	E
A/Thailand/CU-B8665/2013	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	.
A/Thailand/CU-B8730/2013	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B8906/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E

Strain																			
A/Thailand/CU-B8908/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B8981/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B8982/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B9024/2014	.	K	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B9037/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B9221/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B9225/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B9433/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-H3628/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-H3632/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	.
A/Thailand/CU-B10114/2014	.	.	S	.	N	.	G	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10032/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10033/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10044/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10126/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10172/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10174/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10180/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10207/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-A1058/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	E	.	T	.	.	E
A/Thailand/CU-A1070/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-C4844/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10185/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-A1105/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10405/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10578/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10658/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B10888/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	.	.	.	E
A/Thailand/CU-B10909/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	.
A/Thailand/CU-H3658/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-C5062/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	K	.	E
A/Thailand/CU-A1205/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B11070/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-C5149/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-C5169/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-A1282/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B11233/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B11291/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-C5263/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	I	.	.	E
A/Thailand/CU-A1344/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B11363/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B11375/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E
A/Thailand/CU-B11422/2014	.	.	S	.	N	.	.	Q	T	.	.	.	.	.	.	.	T	.	.	E

Table S9. *Positively selected sites on the hemagglutinin-1 (HA1) domain of influenza A/H3N2 among strains isolated in Thailand, 2015.*

Codon	SLAC dN-dS	SLAC *p*-value	FEL dN-dS	FEL *p*-value	MEME ω$^+$	MEME *p*-value
3	7.781	0.107	28.763	**0.026**	>100	**0.039**
4	0.671	0.667	9.624	0.608	>100	**0.021**
198	6.106	0.165	22.25	**0.086**	>100	0.11
261	9.685	**0.056**	27.557	**0.041**	>100	**0.058**
312	7.179	0.177	23.253	**0.049**	>100	**0.067**

dN/dS or ω is the ratio of synonymous to non-synonymous substitutions.

P-value from the SLAC, FEL, and MEME results (for positive selective pressure).

Statistically significant values are reported in bold.

Table S10. *Amino acid residue differences in the antigenic sites (epitopes A through E) of the HA protein from influenza A/H3N2 strains isolated in Thailand in 2015.*

Amino acid position	3	4	124	128	135	138	140	142	144	159	160	168	172	186	197	198	208	242	261	262	276	304	311	312
Antigenic sites	A	P	S	B	A	A	A	A	A	B	B	A	D	B	B	B	D	D	E	E	C	C	C	C
3C.3a A/Switzerland/9715293/2013	**L**	P	S	A	A	S	R	G	N	S	K	M	E	V	Q	S	R	I	R	S	K	A	Q	S
3C.2a A/Hong Kong/4801/2014	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	**H**	.
A/Thailand/CU-B11417/2014 Dec	.	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11444/2014 Dec	.	.	N	.	.	A	I	.	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B11461/2015 Jan	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11507/2015 Jan	I	.	N	.	.	A	I	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
A/Thailand/CU-B11518/2015 Jan	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-A1402/2015 Jan	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11566/2015 Feb	V	Q	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11585/2015 Feb	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-C5500/2015 Feb	V	Q	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-A1513/2015 Feb	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-H3689/2015 Mar	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	K	.	.	.	.	.	.	.	H	.
A/Thailand/CU-C5572/2015 Mar	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11686/2015 Mar	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	Q	.	.	.	H	.
A/Thailand/CU-B11703/2015 Mar	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	R	P	.	.	.	.	.	.	H	.
A/Thailand/CU-B11773/2015 Apr	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11807/2015 Apr	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11820/2015 Apr	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11843/2015 Apr	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	V	.	.	.	.	H	.
A/Thailand/CU-B11870/2015 May	I	.	.	T	.	A	I	K	S	Y	T	.	G	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11889/2015 May	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	G
A/Thailand/CU-B11935/2015 May	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B11968/2015 Jun	.	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-H3700/2015 Jun	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12006/2015 Jun	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12034/2015 Jun	N	.	N	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12081/2015 Jul	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.

Table S10. *Amino acid residue differences in the antigenic sites of the HA protein from influenza A/H3N2 strains isolated in Thailand in 2015*

Table S10. (*Continued*)

Amino acid position	3	4	124	128	135	138	140	142	144	159	160	168	172	186	197	198	208	242	261	262	276	304	311	312
Antigenic sites			A	B	A	A	A	A	A	B	B	A	D	B	B	B	D	D	E	E	C	A	C	C
3C.3a A/Switzerland/9715293/2013	**L**	**P**	**S**	**A**	**T**	**S**	**R**	**G**	**N**	**S**	**K**	**M**	**E**	**V**	**Q**	**S**	**R**	**I**	**R**	**S**	**K**	**A**	**Q**	**S**
3C.2a A/Hong Kong/4801/2014	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12139/2015 Jul	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-H3708/2015 Jul	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	L	.	.	.	H	.
A/Thailand/CU-C5942/2015 Aug	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12191/2015 Aug	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12415/2015 Aug	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12589/2015 Aug	I	.	.	T	.	A	I	K	S	Y	T	V	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12644/2015 Sep	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-C6062/2015 Sep	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12788/2015 Sep	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B12888/2015 Sep	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B13144/2015 Oct	N	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B13201/2015 Oct	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B13425/2015 Oct	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-C6193/2015 Oct	I	.	.	T	.	A	I	K	S	Y	T	V	G	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B13673/2015 Nov	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Thailand/CU-C6246/2015 Nov	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	Q	.	.	.	H	.
A/Thailand/CU-B13893/2015 Nov	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Thailand/CU-B13914/2015 Nov	.	.	.	T	.	A	I	R	S	Y	T	I	.	G	R	.	.	.	.	N	.	.	H	.
A/Thailand/CU-B14505/2015 Dec	I	.	.	T	K	A	I	R	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI01/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI02/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI03/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI04/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	G	.	.	.	.	.	H	.
A/Bangkok/SI-MI21/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI22/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI23/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI24/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table S10. (*Continued*)

Amino acid position	3	4	124	128	135	138	140	142	144	159	160	168	172	186	197	198	208	242	261	262	276	304	311	312
Antigenic sites			A	B	A	A	A	A	A	B	B	A	D	B	B	B	D	D	E	E	C	C	C	C
3C.3a A/Switzerland/9715293/2013	L	P	S	A	A	S	R	G	N	S	K	M	E	V	Q	S	R	I	R	S	K	A	Q	S
3C.2a A/Hong Kong/4801/2014	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI25/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI26/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	T	H	.
A/Bangkok/SI-MI27/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI28/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI29/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI30/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	L	.	.	.	H	.
A/Bangkok/SI-MI31/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Bangkok/SI-MI32/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Tak/150/2015	I	.	.	T	K	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Chanthaburi/13/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Nonthaburi/5/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	R	.	H	.
A/Nonthaburi/4/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Chanthaburi/49/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Chanthaburi/48/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	R	.	.	.	L	.	.	.	H	.
A/Bangkok/44/2015	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Chanthaburi/41/2015	I	.	.	T	.	A	I	K	S	Y	X	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Bangkok/43/2015	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	N
A/Chanthaburi/40/2015	I	.	.	T	.	A	I	R	S	Y	X	.	.	G	.	.	.	.	L	.	.	.	H	.
A/Chanthaburi/46/2015	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Nakhonratchaisima/35/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Prachuapkhirikhan/289/2015	I	.	.	T	.	A	I	R	S	Y	X	.	.	G	.	.	.	.	L	.	.	.	H	.
A/Nonthaburi/345/2015	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.
A/Nonthaburi/340/2015	I	.	.	T	.	A	I	R	S	Y	T	.	.	G	.	.	.	.	.	.	.	.	H	.
A/Rayong/335/2015	I	.	.	T	.	A	I	K	S	Y	T	V	.	G	R	.	.	.	.	.	.	.	H	.
A/Nonthaburi/333/2015	I	.	.	T	.	A	I	K	S	Y	T	.	.	G	R	.	.	.	.	.	.	.	H	.

Table S11. *The accession numbers of neuraminidase sequences were used in present study Influenza A(H3N2)*

Virus name	Accession number
A/California/7/2004	EPI367106
A/Wisconsin/67/2005	EPI160220
A/Brisbane/10/2007	EPI165491
A/Perth/16/2009	EPI182942
A/Victoria/361/2011	EPI353905
A/Switzerland/9715293/2013	EPI540525
A/Moscow/10/99	EPI103336
A/Panama/2007/1999	DQ487337
A/Beijing/353/1989	DQ508835
A/Beijing/32/1992	CY033608
A/England/42/1972	CY113087
A/Fujian/411/2002	CY088485
A/Hong Kong/1/1968	CY112251
A/Sichuan/2/1987	CY112398
A/Sydney/5/1997	CY112887
A/Texas/1/1977	CY113263
A/Victoria/3/1975	CY113183
A/Wuhan/359/1995	CY112823
A/Wellington/01/2004	CY012106
A/Israel/1/11/2011	EPI319251
A/Norway/1330/2010	EPI302230
A/Stockholm/18/2011	EPI318273
A/England/259/2011	EPI346608
A/Texas/50/2012	KC892237
A/Hong Kong/146/2013	EPI426062
A/Ireland/M28390/2013	EPI467995
A/Hong Kong/4801/2014	EPI539577
A/Samara/73/2013	EPI460559
A/Newcastle/22/2014	EPI541460
A/Madagascar/0648/2011	EPI319277
A/Serbia/71/2011	EPI326116
A/Alabama/05/2010	KC535442
A/Iowa/19/2010	KC882859
A/Minnesota/10/2012	KC892316
A/Victoria/208/2009	EPI232452
A/Bangkok/1/1979 1979	CY121002
A/Bangkok/SI-MI01/2015	KP877373
A/Bangkok/SI-MI02/2015	KP877374

A/Bangkok/SI-MI03/2015	KP877375
A/Bangkok/SI-MI04/2015	KP877376
A/Bangkok/SI-MI05/2014	KP877377
A/Bangkok/SI-MI06/2014	KP877378
A/Bangkok/SI-MI07/2014	KP877379
A/Bangkok/SI-MI08/2014	KP877380
A/Bangkok/SI-MI09/2014	KP877381
A/Bangkok/SI-MI10/2014	KP877382
A/Bangkok/SI-MI11/2014	KP877383
A/Bangkok/SI-MI12/2014	KP877384
A/Bangkok/SI-MI13/2014	KP877385
A/Bangkok/SI-MI14/2014	KP877386
A/Bangkok/SI-MI15/2014	KP877387
A/Bangkok/SI-MI16/2014	KP877388
A/Bangkok/SI-MI17/2014	KP877389
A/Bangkok/SI-MI18/2014	KP877390
A/Bangkok/SI-MI19/2014	KP877391
A/Bangkok/SI-MI20/2014	KP877392
A/Bangkok/SI-MI21/2015	KP877393
A/Bangkok/SI-MI22/2015	KP877394
A/Bangkok/SI-MI23/2015	KP877395
A/Bangkok/SI-MI24/2015	KP877396
A/Bangkok/SI-MI25/2015	KP877397
A/Bangkok/SI-MI26/2015	KP877398
A/Bangkok/SI-MI27/2015	KP877399
A/Bangkok/SI-MI28/2015	KP877400
A/Bangkok/SI-MI29/2015	KP877401
A/Bangkok/SI-MI30/2015	KP877402
A/Bangkok/SI-MI31/2015	KP877403
A/Bangkok/SI-MI32/2015	KP877404
A/Thailand/173/2007	AB501496
A/Thailand/308/2008	AB501490
A/Thailand/315/2008	AB501497
A/Thailand/322/2008	AB501493
A/Thailand/340/2007	AB501499
A/Thailand/380/2007	AB501503
A/Thailand/381/2007	AB501504
A/Thailand/429/2007	AB501506
A/Thailand/447/2008	AB501491
A/Thailand/461/2007	AB501507
A/Thailand/515/2008	AB501487
A/Thailand/536/2008	AB501498

A/Thailand/545/2008	AB501488
A/Thailand/764/2008	AB501508
A/Thailand/CU-1101/2008	EU625366
A/Thailand/CU-1102/2008	EU625367
A/Thailand/CU-1103/2008	EU625368
A/Thailand/CU-A114/2013	KP336092
A/Thailand/CU-A134/2013	KP336094
A/Thailand/CU-A153/2013	KP336095
A/Thailand/CU-A164/2013	KP336097
A/Thailand/CU-A166/2013	KP336096
A/Thailand/CU-A182/2013	KP336099
A/Thailand/CU-A196/2013	KP336098
A/Thailand/CU-A24/2013	KP336091
A/Thailand/CU-A305/2013	KP336103
A/Thailand/CU-A411/2013	KP336112
A/Thailand/CU-A459/2013	KP336114
A/Thailand/CU-A598/2014	KP336121
A/Thailand/CU-A7/2013	KP336090
A/Thailand/CU-B10282/2014	KP336134
A/Thailand/CU-B10283/2014	KP336133
A/Thailand/CU-B10345/2014	KP336136
A/Thailand/CU-B10421/2014	KP336137
A/Thailand/CU-B10422/2014	KP336138
A/Thailand/CU-B10509/2014	KP336139
A/Thailand/CU-B10520/2014	KP336141
A/Thailand/CU-B10521/2014	KP336140
A/Thailand/CU-B10557/2014	KP336142
A/Thailand/CU-B106/2009	GQ983550
A/Thailand/CU-B10755/2014	KP336144
A/Thailand/CU-B10792/2014	KP336145
A/Thailand/CU-B10828/2014	KP336146
A/Thailand/CU-B10952/2014	KP336147
A/Thailand/CU-B10975/2014	KP336148
A/Thailand/CU-B110/2009	GQ902811
A/Thailand/CU-B11055/2014	KP336149
A/Thailand/CU-B11065/2014	KP336150
A/Thailand/CU-B11201/2014	KP336151
A/Thailand/CU-B11202/2014	KP336152
A/Thailand/CU-B11284/2014	KP336154
A/Thailand/CU-B11351/2014	KP336155
A/Thailand/CU-B11367/2014	KP336156
A/Thailand/CU-B1672/2009	GU271976

A/Thailand/CU-B1697/2009	GU271984
A/Thailand/CU-B4/2009	GQ902795
A/Thailand/CU-B4730/2011	KP336040
A/Thailand/CU-B4836/2011	KP336044
A/Thailand/CU-B4844/2011	KP336042
A/Thailand/CU-B4929/2011	KP336045
A/Thailand/CU-B5021/2011	KP336046
A/Thailand/CU-B5105/2011	KP336047
A/Thailand/CU-B5436/2011	KP336049
A/Thailand/CU-B5465/2011	KP336050
A/Thailand/CU-B5541/2011	KP336051
A/Thailand/CU-B5593/2011	KP336053
A/Thailand/CU-B5614/2011	KP336052
A/Thailand/CU-B5697/2011	KP336054
A/Thailand/CU-B5735/2011	KP336055
A/Thailand/CU-B5773/2011	KP336056
A/Thailand/CU-B5848/2011	KP336057
A/Thailand/CU-B5873/2011	KP336058
A/Thailand/CU-B590/2009	GQ902819
A/Thailand/CU-B5900/2011	KP336059
A/Thailand/CU-B5909/2011	KP336060
A/Thailand/CU-B5928/2011	KP336061
A/Thailand/CU-B6091/2012	KP336064
A/Thailand/CU-B6251/2012	KP336065
A/Thailand/CU-B6274/2012	KP336066
A/Thailand/CU-B6309/2012	KP336067
A/Thailand/CU-B657/2009	GQ902827
A/Thailand/CU-B6780/2012	KP336068
A/Thailand/CU-B6936/2012	KP336070
A/Thailand/CU-B7189/2012	KP336074
A/Thailand/CU-B7235/2012	KP336076
A/Thailand/CU-B7269/2012	KP336077
A/Thailand/CU-B7367/2012	KP336078
A/Thailand/CU-B7418/2013	KP336079
A/Thailand/CU-B7483/2013	KP336080
A/Thailand/CU-B7536/2013	KP336081
A/Thailand/CU-B7585/2013	KP336082
A/Thailand/CU-B7596/2013	KP336083
A/Thailand/CU-B7646/2013	KP336084
A/Thailand/CU-B7755/2013	KP336085
A/Thailand/CU-B7765/2013	KP336086
A/Thailand/CU-B7853/2013	KP336087

A/Thailand/CU-B7885/2013	KP336088
A/Thailand/CU-B7937/2013	KP336089
A/Thailand/CU-B7992/2013	KP336093
A/Thailand/CU-B8121/2013	KP336100
A/Thailand/CU-B8127/2013	KP336101
A/Thailand/CU-B8134/2013	KP336102
A/Thailand/CU-B8222/2013	KP336104
A/Thailand/CU-B8235/2013	KP336106
A/Thailand/CU-B8236/2013	KP336107
A/Thailand/CU-B8364/2013	KP336108
A/Thailand/CU-B8518/2013	KP336109
A/Thailand/CU-B8525/2013	KP336110
A/Thailand/CU-B8736/2013	KP336115
A/Thailand/CU-B8745/2013	KP336116
A/Thailand/CU-B8772/2013	KP336118
A/Thailand/CU-B8849/2014	KP336122
A/Thailand/CU-C2417/2011	KP336062
A/Thailand/CU-C4087/2013	KP336105
A/Thailand/CU-C4364/2013	KP336120
A/Thailand/CU-C4406/2014	KP336123
A/Thailand/CU-C4492/2014	KP336124
A/Thailand/CU-C4507/2014	KP336126
A/Thailand/CU-C4546/2014	KP336127
A/Thailand/CU-C4655/2014	KP336132
A/Thailand/CU-CB166/2014	KP336131
A/Thailand/CU-H1071/2009	CY074944
A/Thailand/CU-H1285/2010	CY074952
A/Thailand/CU-H1443/2010	CY074960
A/Thailand/CU-H16/2009	GU271992
A/Thailand/CU-H1817/2010	CY074968
A/Thailand/CU-H2973/2011	KP336041
A/Thailand/CU-H2989/2011	KP336043
A/Thailand/CU-H3020/2011	KP336048
A/Thailand/CU-H3141/2012	KP336063
A/Thailand/CU-H3368/2012	KP336069
A/Thailand/CU-H3434/2012	KP336071
A/Thailand/CU-H3435/2012	KP336072
A/Thailand/CU-H3453/2012	KP336073
A/Thailand/CU-H3490/2012	KP336075
A/Thailand/CU-H3567/2013	KP336111
A/Thailand/CU-H3574/2013	KP336113
A/Thailand/CU-H3580/2013	KP336117

A/Thailand/CU-H3584/2013	KP336119
A/Thailand/CU-H3595/2014	KP336125
A/Thailand/CU-H3611/2014	KP336128
A/Thailand/CU-H3624/2014	KP336129
A/Thailand/CU-H3626/2014	KP336130
A/Thailand/CU-H3649/2014	KP336135
A/Thailand/CU-H3656/2014	KP336143
A/Thailand/CU-H3680/2014	KP336153
A/Thailand/CU-124/2006	EU021285
A/Thailand/CU-228/2006	EU021275
A/Thailand/CU-231/2006	EU021283
A/Thailand/CU-259/2006	EU021279
A/Thailand/CU-260/2006	EU021281
A/Thailand/CU-272/2007	EU021271
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Virus name	Accession number
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A/Cambodia/365/2007	EPI157505
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A/England/594/2006	FJ445066
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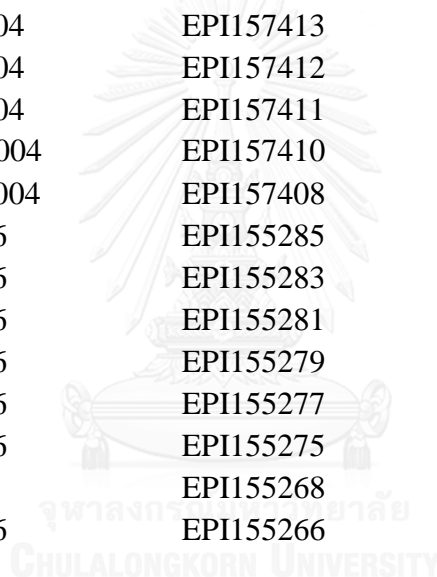
Influenza A(H1N1)pdm09

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A/PRACHUAP KHIRI KHAN/332/2013	EPI 529418
A/Song Khla/139/2014	EPI524286
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A/Surat Thani/16/2013	EPI 439227
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A/Chiang Rai/312/2012	EPI 406031
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A/Thailand/594/2010	EPI 276899
A/CHIANG RAI/226/2010	EPI271972
A/SAMUTPRAKAN/102/2010	EPI 231481
A/Surat Thani/563/2009	EPI231343
A/Ayutthaya/568/2009	EPI 221043
A/Thailand/2944/2009	EPI179068

Influenza B

Virus name	Accession number
B/Victoria/02/1987	CY018759
B/Yamagata/16/1988	CY018767
B/Yamanashi/166/1998	CY019533
B/Lee/40 1940	DQ792899
B/Russia/1969	EF626638
B/Shanghai/361/2002	EF541477
B/Malaysia/2506/2004	CY040451
B/Florida/4/2006 2006	CY033878
B/Brisbane/60/2008	CY115153
B/Wisconsin/01/2010	CY115185
B/Jiangsu/10/2003	CY033846
B/Johannesburg/3964/2012	EPI406273
B/Brisbane/33/2008	CY149983
B/England/393/2008	EPI211560
B/Victoria/304/2006	AGX18593
B/Uruguay/12/2008	EPI172515
B/FujianGulou/1272/2008	EPI366582
B/Singapore/19/2009	EPI193057
B/Argentina/R158/2010	EPI301333
B/Harbin/7/1994	CY040443
B/Sichuan/379/99	AJ784087
B/Bangladesh/3333/2007	AFH58308
B/Stockholm/12/2011	EPI340833
B/Bangkok/141/1994	CY019677
B/Bangkok/153/1990	CY019613
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B/Bangkok/54/1999	AY139055
B/Chantaburi/218/2003	CY022223
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Table S12. *Clinical data and sequencing results of each patient with oseltamivir resistant strains (275Y) of influenza A H1N1 pdm09*

Age	Sex	AA mutation	Ct GAPDH	Ct JOE	Ct FAM	Ratio	Genetic mutant
19	Male	H275Y	29	26	28	4	TAC
32	Female	H275Y	27	9	19	1024	TAC
64	Female	H275Y	29	13	24	2048	TAC
4	Male	H275Y	25	10	17	128	TAC

Table S13

A. *Positively selected sites on the neuraminidase of influenza A/H1N1 pdm09 among strains isolated in Thailand, 2009-2015.*

Codon	SLAC dN-dS	SLAC p-value	FEL dN-dS	FEL p-value	MEME ω^+	MEME p-value
13	6.329	0.133	3.959	**0.039**	>100	**0.056**
34	6.201	0.148	4.239	**0.057**	>100	**0.078**
48	6.344	0.132	4.27	**0.074**	>100	**0.098**
188	4.888	0.23	3.542	**0.059**	>100	**0.079**
270	5.86	0.2	3.981	**0.093**	>100	0.118
366	2.727	0.479	1.531	0.62	>100	**0.009**
451	4.691	0.271	2.776	**0.098**	>100	0.124
463	8.239	**0.033**	5.886	**0.013**	>100	**0.000**

B. *Positively selected sites on the neuraminidase of seasonal influenza A/H1N1 among strains isolated in Thailand, 2009-2015.*

Codon	SLAC dN-dS	SLAC *p*-value	FEL dN-dS	FEL *p*-value	MEME ω⁺	MEME *p*-value
7	-7.864	0.998	-27.072	0.084	>100	**0.012**
77	3.657	**0.088**	11.702	**0.079**	>100	**0.036**
222	8.798	**0.006**	33.293	**0.006**	>100	**0.01**
249	2.451	0.199	7.726	**0.074**	>100	**0.097**
266	1.93	0.274	9.57	0.121	>100	**0.045**
344	2.767	0.193	9.633	**0.055**	>100	**0.075**
452	5.606	**0.033**	19.616	**0.018**	>100	**0.024**

C. *Positively selected sites on the neuraminidase of influenza A/H3N2 among strains isolated in Thailand*

Codon	SLAC dN-dS	SLAC *p*-value	FEL dN-dS	FEL *p*-value	MEME ω⁺	MEME *p*-value
4	-5.582	0.988	-3.225	**0.047**	>100	**0**
43	3.88	0.179	2.196	**0.082**	41.261	**0.02**
93	4.092	**0.078**	1.601	**0.038**	>100	**0.055**
141	3.263	0.166	1.649	**0.038**	>100	**0.054**
150	2.369	0.214	1.157	**0.096**	>100	0.12
181	-0.529	0.87	-0.337	0.672	>100	**0**
215	3.586	**0.094**	1.72	**0.039**	>100	**0.056**
267	1.097	0.492	0.221	0.87	66.257	**0.023**
271	2.72	0.223	1.264	0.106	>100	**0.009**
401	3.602	**0.091**	1.53	**0.049**	>100	**0.068**
402	2.175	0.302	1.059	0.137	>100	**0.088**
464	2.972	0.143	1.562	**0.032**	>100	**0.001**
468	3.623	**0.088**	1.837	**0.032**	>100	**0.046**

D. *Positively selected sites on the neuraminidase of influenza B among strains isolated in Thailand*

Codon	SLAC dN-dS	SLAC *p*-value	FEL dN-dS	FEL *p*-value	IFEL dN-dS	IFEL *p*-value	MEME ω[+]	MEME *p*-value
15	-	-	-	-	0.313	0.024	>100	0.005
27	-	-	-	-	0.321	0.018	>100	0.04
41	-	-	-	-	0.151	0.073	>100	0.007
51	-	-	-	-	0.244	0.067	>100	0.077
68	-	-	0.162	0.096	0.228	0.078	-	-
73	0.447	0.036	0.483	0.058	-	-	14.964	0.033
106	-	-	0.365	0.055	0.387	0.090	5.167	0.075
107	-	-	-	-	0.280	0.064	>100	0.085
219	-	-	-	-	0.144	0.066	>100	0.011
220	-	-	0.113	0.051	0.216	0.027	>100	0.092
248	-	-	0.153	0.056	0.220	0.047	-	-
358	-	-	0.363	0.04	0.446	0.035	>100	0.052
395	0.633	0.002	0.615	0.002	0.646	0.008	>100	0.004
404	0.284	0.092	0.255	0.085	0.690	0.009	>100	0.035
465	-	-	0.237	0.093	-	-	>100	0.099

dN/dS or ω is the ratio of synonymous to non-synonymous substitutions.

P-value from the SLAC, FEL, and MEME results (for positive selective pressure).

Statistically significant values are reported in bold.

Figure S1. Haemagglutinin and neuraminidase protein sequence alignments for influenza B virus isolates

HA protein alignment for B/Victoria lineage strains in Thailand, 2010 – 2014

Reference strains, Vaccine strains, Thailand strains; <u>Potential N-linked glycosylation sites</u>; <mark>Receptor binding site</mark>

```
                                        110       120       130       140       150       160       170       180       190       200
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Malaysia/2506/2004               RTKIRQLPNLLRGYEHIRLSTHNVINAENAPGGSYKIGTSGSCPNVTNGNGFFATMAWAVPKNDNNKTATNSLTIEVPYICTEGEDQITVWGFHSDNEAQ
B/Thailand/CU-243/2006             ....................................................................................................T.
B/Thailand/CU-364/2008             ....................................................................................................T.
B/Singapore/19/2009                ....................................................................................................T.
B/Thailand/CU-B2390/2010           ......................................................................A.............................T.
B/Thailand/CU-B4585/2011           ...........................................................K......P..................................T.
B/Brisbane/60/2008                 ..............................I............................K......P..............................D.T.
B/Johannesburg/3964/2012           ...........................................................K......P..................................T.
B/Thailand/CU-B2271/2010           ..............................I............................K......P..................................T.
B/Thailand/CU-B2660/2010           ..............................I............................K......P..................................T.
B/Thailand/CU-B2504/2010           ...........................................................K......P..................................T.
B/Thailand/CU-B3153/2010           ...........................................................K......P..................................T.
B/Thailand/CU-B4504/2011           ...........................................................K......P..................................T.
B/Thailand/CU-B5522/2011           ....................................................E......K......P..................................T.
B/Thailand/CU-C1262/2010           ...........................................................K......P..................................T.
B/Thailand/CU-C1451/2010           ...........................................................K......P..................................T.
B/Thailand/CU-C1768/2011           ...........................................................K......P..................................T.
B/Thailand/CU-H3002/2011           ....................................................E......K......P................I.................T.
B/Thailand/CU-H3052/2011           .................K..................................E......K......P..................................T.
B/Thailand/CU-B6148/2012           ....................................................E......K......P..................................T.
B/Thailand/CU-B6257/2012           ...........................................................K......P..................................T.
B/Thailand/CU-B6975/2012           ....................................................T......K......P..................................T.
B/Thailand/CU-B7215/2012           ....................................................E......K......P..................................T.
B/Thailand/CU-B7337/2012           ....................................................T......K......P..................................T.
B/Thailand/CU-B5734/2011           ....................................................E......K......P..................................T.
B/Odessa/3886/2010                 ...........................................................K......P..................................T.
B/Thailand/CU-B2201/2010           ...........................................................K......P..................................T.
B/Thailand/CU-B2320/2010           ...........................................................K......P..................................T.
B/Thailand/CU-B2372/2010           ...........................................................K......P..................................T.
B/Thailand/CU-B2432/2010           ...........................................................K......P..................................T.
B/Thailand/CU-B5671/2011           ...........................................................K......P..................................T.
B/Thailand/CU-H2738/2010           ...........................................................K......P..................................T.
B/Thailand/CU-B5910/2011           ...........................................................K......P..................................T.
B/Thailand/CU-B6240/2012           ..............N..........S.................................K....DP....................................T.
B/Thailand/CU-H1400/2010           ...........................................................K......P..................................T.
B/Thailand/CU-H1896/2010           ...........................................................K......P..................................T.
B/Thailand/CU-H2132/2010           ...........................................................K......P..................................T.
B/Thailand/CU-H2584/2010           ....................................................E......K......P..................................T.
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                                        210       220       230       240       250       260       270       280       290       300
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Malaysia/2506/2004               MAKLYGDSKPQKFTSSANGVTTHYVSQIGGFPNQTEDGGLPQSGRIVVDYMVQKSGKTGTITYQRGILLPQKVWCASGRSKVIKGSLPLIGEADCLHEKY
B/Thailand/CU-243/2006             ....................................................................................................
B/Thailand/CU-364/2008             ....................................................................................................
B/Singapore/19/2009                ....................................................................................................
B/Thailand/CU-B2390/2010           ....................................................................................................
B/Thailand/CU-B4585/2011           .....................................................................N..............................
B/Brisbane/60/2008                 ....................................................................................................
B/Johannesburg/3964/2012           ....................................................................................................
B/Thailand/CU-B2271/2010           ....................................................................................................
B/Thailand/CU-B2660/2010           ..............................................Y.....................................................
B/Thailand/CU-B2504/2010           ....................................................................................................
B/Thailand/CU-B3153/2010           ....................................................................................................
B/Thailand/CU-B4504/2011           ....................................................................................................
B/Thailand/CU-B5522/2011           ....................................................................................................
B/Thailand/CU-C1262/2010           ....................................................................................................
B/Thailand/CU-C1451/2010           ....................................................................................................
B/Thailand/CU-C1768/2011           ....................................................................................................
B/Thailand/CU-H3002/2011           ....................................................................................................
B/Thailand/CU-H3052/2011           ....................................................................................................
B/Thailand/CU-B6148/2012           ......................................................E.............................................
B/Thailand/CU-B6257/2012           ....................................................................................................
B/Thailand/CU-B6975/2012           ....................................................................................................
B/Thailand/CU-B7215/2012           ....................................................................................................
B/Thailand/CU-B7337/2012           ....................................................................................................
B/Thailand/CU-B5734/2011           ....................................................................................................
B/Odessa/3886/2010                 ....................................................................................................
B/Thailand/CU-B2201/2010           ....................................................................................................
B/Thailand/CU-B2320/2010           ....................................................................................................
B/Thailand/CU-B2372/2010           ..............................................M.....................................................
B/Thailand/CU-B2432/2010           ....................................................................................................
B/Thailand/CU-B5671/2011           .E..................................................................................................
B/Thailand/CU-H2738/2010           ....................................................................................................
B/Thailand/CU-B5910/2011           ....................................................................................................
B/Thailand/CU-B6240/2012           ....................................................................................................
B/Thailand/CU-H1400/2010           ....................................................................................................
B/Thailand/CU-H1896/2010           ....................................................................................................
B/Thailand/CU-H2132/2010           ..............................................S.....................................................
B/Thailand/CU-H2584/2010           ....................................................................................................
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        310       320       330       340      HA2        360       370       380       390       400
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Malaysia/2506/2004      GGLNKSKPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLN
B/Thailand/CU-243/2006    ....................................................................................................
B/Thailand/CU-364/2008    ....................................................................................................
B/Singapore/19/2009       ....................................................................................................
B/Thailand/CU-B2390/2010  ....................................................................................................
B/Thailand/CU-B4585/2011  ....................................................................................................
B/Brisbane/60/2008        ....................................................................................................
B/Johannesburg/3964/2012  ....................................................................................................
B/Thailand/CU-B2271/2010  ....................................................................................................
B/Thailand/CU-B2660/2010  ....................................................................................................
B/Thailand/CU-B2504/2010  ....................................................................................................
B/Thailand/CU-B3153/2010  ....................................................................................................
B/Thailand/CU-B4504/2011  ....................................................................................................
B/Thailand/CU-B5522/2011  ....................................................................................................
B/Thailand/CU-C1262/2010  ....................................................................................................
B/Thailand/CU-C1451/2010  ....................................................................................................
B/Thailand/CU-C1768/2011  ....................................................................................................
B/Thailand/CU-H3002/2011  ....................................................................................................
B/Thailand/CU-H3052/2011  ....................................................................................................
B/Thailand/CU-B6148/2012  ....................................................................................................
B/Thailand/CU-B6257/2012  ....................................................................................................
B/Thailand/CU-B6975/2012  ....................................................................................................
B/Thailand/CU-B7215/2012  ....................................................................................................
B/Thailand/CU-B7337/2012  ....................................................................................................
B/Thailand/CU-B5734/2011  ....................................................................................................
B/Odessa/3886/2010        ....................................................................................................
B/Thailand/CU-B2201/2010  ....................................................................................................
B/Thailand/CU-B2320/2010  ....................................................................................................
B/Thailand/CU-B2372/2010  ....................................................................................................
B/Thailand/CU-B2432/2010  ....................................................................................................
B/Thailand/CU-B5671/2011  ....................................................................................................
B/Thailand/CU-H2738/2010  ....................................................................................................
B/Thailand/CU-B5910/2011  ....................................................................................................
B/Thailand/CU-B6240/2012  ....................................................................................................
B/Thailand/CU-H1400/2010  ....................................................................................................
B/Thailand/CU-H1896/2010  ....................................................................................................
B/Thailand/CU-H2132/2010  ....................................................................................................
B/Thailand/CU-H2584/2010  ....................................................................................................
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        410       420       430       440       450       460       470       480       490       500
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Malaysia/2506/2004      SLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVEIGNGCFETKHKCNQTCLDRIA
B/Thailand/CU-243/2006    ....................................................................................................
B/Thailand/CU-364/2008    ....................................................................................................
B/Singapore/19/2009       ....................................................................................................
B/Thailand/CU-B2390/2010  ....................................................................................................
B/Thailand/CU-B4585/2011  ....................................................................................................
B/Brisbane/60/2008        ....................................................................................................
B/Johannesburg/3964/2012  ....................................................................................................
B/Thailand/CU-B2271/2010  ....................................................................................................
B/Thailand/CU-B2660/2010  ....................................................................................................
B/Thailand/CU-B2504/2010  ....................................................................................................
B/Thailand/CU-B3153/2010  ....................................................................................................
B/Thailand/CU-B4504/2011  ....................................................................................................
B/Thailand/CU-B5522/2011  ....................................................................................................
B/Thailand/CU-C1262/2010  ....................................................................................................
B/Thailand/CU-C1451/2010  ...........................................................................I........................
B/Thailand/CU-C1768/2011  ....................................................................................................
B/Thailand/CU-H3002/2011  ....................................................................................................
B/Thailand/CU-H3052/2011  ....................................................................................................
B/Thailand/CU-B6148/2012  ....................................................................................................
B/Thailand/CU-B6257/2012  ....................................................................................................
B/Thailand/CU-B6975/2012  ....................................................................................................
B/Thailand/CU-B7215/2012  ....................................................................................................
B/Thailand/CU-B7337/2012  ....................................................................................................
B/Thailand/CU-B5734/2011  ....................................................................................................
B/Odessa/3886/2010        ....................................................................................................
B/Thailand/CU-B2201/2010  ....................................................................................................
B/Thailand/CU-B2320/2010  ....................................................................................................
B/Thailand/CU-B2372/2010  ....................................................................................................
B/Thailand/CU-B2432/2010  ....................................................................................................
B/Thailand/CU-B5671/2011  ....................................................................................................
B/Thailand/CU-H2738/2010  ....................................................................................................
B/Thailand/CU-B5910/2011  ....................................................................................................
B/Thailand/CU-B6240/2012  ....................................................................................................
B/Thailand/CU-H1400/2010  ....................................................................................................
B/Thailand/CU-H1896/2010  ....................................................................................................
B/Thailand/CU-H2132/2010  ....................................................................................................
B/Thailand/CU-H2584/2010  ....................................................................................................
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                510       520       530       540       550       560       570
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
B/Malaysia/2506/2004       AGTFDAGEFSLPTFDSLNITAASLNDDGLDNHTILLYYSTAASSLAVTLMIAIFVVYMVSRDNVSCSICL*
B/Thailand/CU-243/2006     ...................................................................*
B/Thailand/CU-364/2008     ...................................................................*
B/Singapore/19/2009        ...................................................................*
B/Thailand/CU-B2390/2010   ...................................................................*
B/Thailand/CU-B4585/2011   ...................................................................*
B/Brisbane/60/2008         ...................................................................*
B/Johannesburg/3964/2012   ...................................................................*
B/Thailand/CU-B2271/2010   ...................................................................*
B/Thailand/CU-B2660/2010   ...................................................................*
B/Thailand/CU-B2504/2010   ...............................................................I...*
B/Thailand/CU-B3153/2010   ...............................................................I...*
B/Thailand/CU-B4504/2011   ...............................................................I...*
B/Thailand/CU-B5522/2011   ...............................................................I...*
B/Thailand/CU-C1262/2010   ...............................................................I...*
B/Thailand/CU-C1451/2010   ...............................................................I...*
B/Thailand/CU-C1768/2011   ...............................................................I...*
B/Thailand/CU-H3002/2011   ...............................................................I...*
B/Thailand/CU-H3052/2011   ...............................................................I...*
B/Thailand/CU-B6148/2012   ...............................................................I...*
B/Thailand/CU-B6257/2012   ...................................................................*
B/Thailand/CU-B6975/2012   ...................................................................*
B/Thailand/CU-B7215/2012   ...............................................................I...*
B/Thailand/CU-B7337/2012   ...................................................................*
B/Thailand/CU-B5734/2011   ...............................................................I...*
B/Odessa/3886/2010         ...................................................................*
B/Thailand/CU-B2201/2010   ...................................................................*
B/Thailand/CU-B2320/2010   ...................................................................*
B/Thailand/CU-B2372/2010   ...................................................................*
B/Thailand/CU-B2432/2010   ...................................................................*
B/Thailand/CU-B5671/2011   ...................................................................*
B/Thailand/CU-H2738/2010   ...................................................................*
B/Thailand/CU-B5910/2011   ...................................................................*
B/Thailand/CU-B6240/2012   .....................P.............................................*
B/Thailand/CU-H1400/2010   ...................................................................*
B/Thailand/CU-H1896/2010   ...................................................................*
B/Thailand/CU-H2132/2010   ...................................................................*
B/Thailand/CU-H2584/2010   ...................................................................*
```

HA protein alignment for B/Yamagata lineage strains in Thailand, 2010 – 2014

Reference strains, Vaccine strains, Thailand strains; Potential N-linked glycosylation sites; Receptor binding site

```
                HA1     10        20        30        40        50        60        70        80        90        100
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006          DRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSYFANLKGTRTRGKLCPDCLNCTDLDVALGRPMCVGTTPSAKASILHEVKPVTSGCFPIMHD
B/Wisconsin/1/2010        .............................................................................................R......
B/Bangladesh/3333/2007    .............................................................................................R......
B/Stockholm/12/2011       ........................A....................................................................R......
B/Thailand/CU-H2933/2011  ...........................................................................................IR......
B/Thailand/CU-B3313/2012  .............................................................................................R......
B/Thailand/CU-H3496/2012  .............................................................................................R......
B/Thailand/CU-B6096/2012  ........................A....................................................................R......
B/Massachusetts/2/2012    .............................................K...............................................R......
B/Estonia/55669/2011      .............................................K...............................................R......
B/Thailand/CU-H3349/2012  .............................................K...............................................R......
B/Thailand/CU-H3456/2012  .............................................K.......................I.......................R......
B/Thailand/CU-B6078/2011  .............................................K...............................................R......
B/Thailand/CU-A585/2013   .............................................K...............................................R......
B/Thailand/CU-B8813/2013  .............................................K...............................................R......
B/Thailand/CU-B8332/2013  .............................................K...............................................R......
B/Thailand/CU-A605/2014   .............................................K...............................................R......
B/Thailand/CU-A615/2014   ...........M.................................K...............................................R......
B/Thailand/CU-A626/2014   .............................................K...............................................R......
B/Thailand/CU-A645/2014   .............................................K...............................................R......
B/Thailand/CU-B8925/2014  .............................................K...............................................R......
B/Thailand/CU-B8999/2014  .............................................K...............................................R......
B/Thailand/CU-B9017/2014  .............................................K...............................................R......
B/Thailand/CU-B9034/2014  .............................................K...............................................R......
B/Thailand/CU-C4555/2014  .............................................K...............................................R......
B/Thailand/CU-H3591/2014  .............................................K...............................................R......
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              110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006      RTKIRQLPNLLRGYENIRLSTQNVIDAEKAPGGPYRLGTSGSCPNATSKSGFFATMAWAVPKDNNKNATNPLTVEVPYICTEGEDQITVWGFHSDDKTQM
B/Wisconsin/1/2010    ...........................................I.......Y...................................
B/Bangladesh/3333/2007 ...........................................I.......Y...................................
B/Stockholm/12/2011   ...........................................I.......Y.....Q.............................
B/Thailand/CU-H2933/2011 ........................................I.......Y...................................N...
B/Thailand/CU-B3313/2012 ..............K.........................I.......Y...................................N...
B/Thailand/CU-H3496/2012 ..............K.........................I.......Y...................................N...
B/Thailand/CU-B6096/2012 ........................................I.......Y.....Q.............................N...
B/Massachusetts/2/2012 ......A...............................................................A..............
B/Estonia/55669/2011  ......A...............................................................A..............
B/Thailand/CU-H3349/2012 ......A...............................................................A...........N...
B/Thailand/CU-H3456/2012 ......A...............A...............................................A...........N...
B/Thailand/CU-B6078/2011 ......A...............................................................A...........N...
B/Thailand/CU-A585/2013 ......A...............................................................A...........N...
B/Thailand/CU-B8813/2013 ......A...............................................................A...........N...
B/Thailand/CU-B8332/2013 ......A.....................................A.........................A...........N...
B/Thailand/CU-A605/2014 ......A...............................................................A...........N...
B/Thailand/CU-A615/2014 ......A...............................................................A...........N...
B/Thailand/CU-A626/2014 ......A...............................................................A...........N...
B/Thailand/CU-A645/2014 ......A...............................................................A...........N...
B/Thailand/CU-B8925/2014 ......A...............................................................A...........N...
B/Thailand/CU-B8999/2014 ......A...............................................................A...........N...
B/Thailand/CU-B9017/2014 ......A...............................................................A...........N...
B/Thailand/CU-B9034/2014 ......A...............................................................A...........N...
B/Thailand/CU-C4555/2014 ......A...............................................................A...........N...
B/Thailand/CU-H3591/2014 ......A...............................................................A...........N...


              210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006      KNLYGDSNPQKFTSSANGVTTHYVSQIGSFPDQTEDGGLPQSGRIVVDYMMQKPGKTGTIVYQRGVLLPQKVWCASGRSKVIKGSLPLIGEADCLHEKYG
B/Wisconsin/1/2010    .S..................D...............................................................
B/Bangladesh/3333/2007 ....................D...............................................................
B/Stockholm/12/2011   ....................D..................V............................................
B/Thailand/CU-H2933/2011 ....................D............................................................
B/Thailand/CU-B3313/2012 .S..................D...........................R.................................E..
B/Thailand/CU-H3496/2012 .S..................D.............................................................E..
B/Thailand/CU-B6096/2012 ....................D..................V............................................
B/Massachusetts/2/2012 ....................G...............................................................
B/Estonia/55669/2011  ....................G...............................................................
B/Thailand/CU-H3349/2012 ....................G............................................................
B/Thailand/CU-H3456/2012 .............I......G...........................R................................
B/Thailand/CU-B6078/2011 ....................G............................................................
B/Thailand/CU-A585/2013 ....................G...R........................................................
B/Thailand/CU-B8813/2013 ....................G...R........................................................
B/Thailand/CU-B8332/2013 ....................G............................................................
B/Thailand/CU-A605/2014 ....................G............................................................
B/Thailand/CU-A615/2014 ....................G...R........................................................
B/Thailand/CU-A626/2014 ....................G...R........................................................
B/Thailand/CU-A645/2014 ....................G...R........................................................
B/Thailand/CU-B8925/2014 ....................G...R........................................................
B/Thailand/CU-B8999/2014 ....................G...R........................................................
B/Thailand/CU-B9017/2014 ....................G...R.............................E..........................
B/Thailand/CU-B9034/2014 ....................G...R........................................................
B/Thailand/CU-C4555/2014 ....................G...R........................................................
B/Thailand/CU-H3591/2014 .....E..............G...R........................................................


              310       320       330       340       HA2       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006      GLNKSKPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNS
B/Wisconsin/1/2010    ...................................................................................
B/Bangladesh/3333/2007 ..................................................................................
B/Stockholm/12/2011   ...................................................................................
B/Thailand/CU-H2933/2011 ................................................................................
B/Thailand/CU-B3313/2012 ..........K.....................................................................
B/Thailand/CU-H3496/2012 ..........K.....................................................................
B/Thailand/CU-B6096/2012 ................................................................................
B/Massachusetts/2/2012 ................................................................................
B/Estonia/55669/2011  .................................................................................
B/Thailand/CU-H3349/2012 ................................................................................
B/Thailand/CU-H3456/2012 ................................................................................
B/Thailand/CU-B6078/2011 ................................................................................
B/Thailand/CU-A585/2013 ................................................................................
B/Thailand/CU-B8813/2013 ................................................................................
B/Thailand/CU-B8332/2013 .....................E..........................................................
B/Thailand/CU-A605/2014 ................................................................................
B/Thailand/CU-A615/2014 ................................................................................
B/Thailand/CU-A626/2014 ................................................................................
B/Thailand/CU-A645/2014 ................................................................................
B/Thailand/CU-B8925/2014 ................................................................................
B/Thailand/CU-B8999/2014 ................................................................................
B/Thailand/CU-B9017/2014 ................................................................................
B/Thailand/CU-B9034/2014 ................................................................................
B/Thailand/CU-C4555/2014 ................................................................................
B/Thailand/CU-H3591/2014 ................................................................................
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                                         410       420       430       440       450       460       470       480       490       500
                                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006                    LSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVEIGNGCFETKHKCNQTCLDRIAA
B/Wisconsin/1/2010                  ....................................................................................D...................
B/Bangladesh/3333/2007              ....................................................................................D...................
B/Stockholm/12/2011                 ....................................................................................D...................
B/Thailand/CU-H2933/2011            ....................................................................................D...................
B/Thailand/CU-B3313/2012            ....................................................................................D...................
B/Thailand/CU-H3496/2012            ....................................................................................D...................
B/Thailand/CU-B6096/2012            ....................................................................................D...................
B/Massachusetts/2/2012              ....................................................................................D...................
B/Estonia/55669/2011                ....................................................................................D...................
B/Thailand/CU-H3349/2012            ....................................................................................D...................
B/Thailand/CU-H3456/2012            ....................................................................................D...................
B/Thailand/CU-B6078/2011            ....................................................................................D...................
B/Thailand/CU-A585/2013             ....................................................................................D...................
B/Thailand/CU-B8813/2013            ....................................................................................D...................
B/Thailand/CU-B8332/2013            ....................................................................................D...................
B/Thailand/CU-A605/2014             ....................................................................................D...................
B/Thailand/CU-A615/2014             ....................................................................................D...................
B/Thailand/CU-A626/2014             ....................................................................................D...................
B/Thailand/CU-A645/2014             ....................................................................................D...................
B/Thailand/CU-B8925/2014            ....................................................................................D...................
B/Thailand/CU-B8999/2014            ....................................................................................D...................
B/Thailand/CU-B9017/2014            ....................................................................................D...................
B/Thailand/CU-B9034/2014            ....................................................................................D...................
B/Thailand/CU-C4555/2014            ....................................................................................D...................
B/Thailand/CU-H3591/2014            ....................................................................................D...................


                                         510       520       530       540       550       560       570
                                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006                    GTFNAGEFSLPTFDSLNITAASLNDDGLDNHTILLYYSTAASSLAVTLMLAIFIVYMVSRDNVSCSICL*
B/Wisconsin/1/2010                  ...................................................................*
B/Bangladesh/3333/2007              ...................................................................*
B/Stockholm/12/2011                 ...................................................................*
B/Thailand/CU-H2933/2011            ...................................................................*
B/Thailand/CU-B3313/2012            ...................................................................*
B/Thailand/CU-H3496/2012            ...................................................................*
B/Thailand/CU-B6096/2012            ...................................................................*
B/Massachusetts/2/2012              ...................................................................*
B/Estonia/55669/2011                ...................................................................*
B/Thailand/CU-H3349/2012            ...................................................................*
B/Thailand/CU-H3456/2012            ...........................................................K.......*
B/Thailand/CU-B6078/2011            ...................................................................*
B/Thailand/CU-A585/2013             ...................................................................*
B/Thailand/CU-B8813/2013            ...................................................................*
B/Thailand/CU-B8332/2013            ...................................................................*
B/Thailand/CU-A605/2014             ...................................................................*
B/Thailand/CU-A615/2014             ...................................................................*
B/Thailand/CU-A626/2014             ...................................................................*
B/Thailand/CU-A645/2014             ...................................................................*
B/Thailand/CU-B8925/2014            ...................................................................*
B/Thailand/CU-B8999/2014            ...................................................................*
B/Thailand/CU-B9017/2014            ...................................................................*
B/Thailand/CU-B9034/2014            ...................................................................*
B/Thailand/CU-C4555/2014            ...................................................................*
B/Thailand/CU-H3591/2014            ...................................................................*
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NA protein alignment for influenza B virus isolated in Thailand, 2010 – 2014

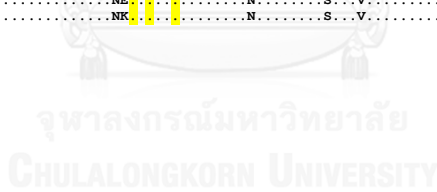**Reference strains, Vaccine strains, Thailand strains;** <u>Potential N-linked glycosylation sites</u>; `Catalytic site and framework residues`

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                             10        20        30        40        50        60        70        80        90       100
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006        MLPSTIQTLTLFLTSGGVLLSLYVSASLSYLLYSDILLKFSQTEITAPIMPLDCANASNVQAVNRSAAKGVTLLLPEPEWTYPRLSCPGSTFQKALLISP
B/Perth/211/2001        ..........................................P.....T.................T...................................
B/Thailand/CU-B6078/2012 ...............................................................H....................................
B/Thailand/CU-H3349/2012 ..................M............................................H...........K........................
B/Thailand/CU-H3456/2012 ...................................................................................................
B/Thailand/CU-A585/2013 ...................................................................................................
B/Thailand/CU-B8813/2013 ...................................................................................................
B/Thailand/CU-B8332/2013 ....................A.........................................H...R.................................
B/Thailand/CU-A605/2014 ..................M...................I.............................................................
B/Thailand/CU-A615/2014 .......................................K...........................................................
B/Thailand/CU-A626/2014 .......................................K...........................................................
B/Thailand/CU-A645/2014 ..............................................................H....................................
B/Thailand/CU-B8925/2014 ...................................................................................................
B/Thailand/CU-B8999/2014 ...................................................................................................
B/Thailand/CU-B9017/2014 ...................................................................................................
B/Thailand/CU-B9034/2014 ...................................................................................................
B/Thailand/CU-C4555/2014 .......................................K...........................................................
B/Thailand/CU-H3591/2014 ...................................................................................................
B/Wisconsin/1/2010      ......................................R...................T.........................................
B/Thailand/CU-H2933/2011 ......................................R..V................T...F.....................................
B/Thailand/CU-B6096/2012 ......................................R...................T...IP....................................
B/Thailand/CU-H3313/2012 ......................................R...................T.........................................
B/Thailand/CU-H3496/2012 ....................M.....R...............................T.........................................
B/Malaysia/2506/2004    ........................................PS.....T.................T...................................
B/Thailand/CU-243/2006  ..........................................P.....T.................T...................................
B/Thailand/CU-364/2008  ..........................................P.....T.................T...................................
B/Brisbane/60/2008      ..........................................P.....T.................T...................................
B/Thailand/CU-B2271/2010 ..................................G.......P.....T.................T..............................I....
B/Thailand/CU-B2660/2010 ..................................G.......P.....T.........L..T..................................I....
B/Thailand/CU-B3153/2010 ..........................................P.....T.................T...................................
B/Thailand/CU-C1262/2010 ..........................................P.....T.................T...................................
B/Thailand/CU-C1451/2010 ..........................................P.....T.................T...................................
B/Thailand/CU-B2504/2010 ..........................................P.....T.................T...................................
B/Thailand/CU-B4505/2011 ..........................................P.....T.................T...................................
B/Thailand/CU-B5522/2011 ..........................................P.....T.................T...................................
B/Thailand/CU-B5734/2011 ..........................................P.....T.................T...................................
B/Thailand/CU-C1768/2011 ..........................................P.....T.................T...................................
B/Thailand/CU-H3002/2011 ..........................................P.....T.................T...................................
B/Thailand/CU-H3052/2011 ...................L......................P.....T.................T...................................
B/Thailand/CU-B6148/2012 ..........................................P.....T.................T...................................
B/Thailand/CU-B7215/2012 ..........................................P.....T.................T...................................
B/Thailand/CU-B6257/2012 ..........................................P.....T.................T...F.............................
B/Thailand/CU-B6975/2012 ..........................................P..M..T.................T...................................
B/Thailand/CU-B7337/2012 ..........................................P.....T.................T...S.............................
B/Thailand/CU-B2201/2010 ..........................................P.....T.................T.........................Q.........
B/Thailand/CU-B2320/2010 ..........................................P.....T.................T...................................
B/Thailand/CU-B2372/2010 ...................L......................P.....T.S...............T...F.............................
B/Thailand/CU-B2390/2010 .......M.....L......................PP.....T.................T...................................
B/Thailand/CU-B2432/2010 ..........................................P.....TI................T...................................
B/Thailand/CU-H1400/2010 ..........................................P.....T.................T.........................Q.........
B/Thailand/CU-H1896/2010 ..........................................P.....T.................T...................................
B/Thailand/CU-H2132/2010 ..........................................P.....T.................T...................................
B/Thailand/CU-H2584/2010 ..........................................P.....T.T...............T...................................
B/Thailand/CU-H2738/2010 ...................L......................P.....T.S...............T...F.............................
B/Thailand/CU-B4585/2011 .......M.....L...............L......PP.....T.................T...................................
B/Thailand/CU-B5671/2011 .................L..L.....................P.....T.S...............T...F.............................
B/Thailand/CU-B5910/2011 ..........................................P.....T.................T...F.............................
B/Thailand/CU-B6240/2012 ..........................................P.....T.................T...................................
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                    110       120       130        140       150       160       170       180       190       200
                    ....|....|....|....|....|....|    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006    HRFGETKGNSAPLIIREPFIACGPTECKHFALTHYAAQPGGYYNGTREDRNKLRHLISVKLGKIPTVENSIFHMAAWSGSACHDGKEWTYIGVDGPDSNA
B/Perth/211/2001    .............................K.........................G.........................................EN..
B/Thailand/CU-B6078/2012  .....I...................................................................................N..
B/Thailand/CU-H3349/2012  .....I...................................................................................
B/Thailand/CU-H3456/2012  .....I...................R...............................................................
B/Thailand/CU-A585/2013   .....I...................................................................................
B/Thailand/CU-B8813/2013  .....I...................................................................................
B/Thailand/CU-H8332/2013  .....I...................................................................................
B/Thailand/CU-A605/2014   .....I...................................................................................
B/Thailand/CU-A615/2014   .....I...................................................................................
B/Thailand/CU-A626/2014   .....I...................................................................................
B/Thailand/CU-A645/2014   .....I...................................................................................
B/Thailand/CU-B8925/2014  .....I...................................................................................
B/Thailand/CU-B8999/2014  .....I...................................................................................
B/Thailand/CU-B9017/2014  .....I...................................................................................
B/Thailand/CU-B9034/2014  .....I...................................................................................
B/Thailand/CU-C4555/2014  .....I...................................................................................
B/Thailand/CU-H3591/2014  .....I...................................................................................
B/Wisconsin/1/2010        .........................K......................................................R............
B/Thailand/CU-H2933/2011  .......................V...K....................................................R............
B/Thailand/CU-B6096/2012  .........................K......................................................R............
B/Thailand/CU-H3313/2012  .........................K......................................................R............
B/Thailand/CU-H3496/2012  .........................K......................................................R............
B/Malaysia/2506/2004      .........................K.............................G.................................N..
B/Thailand/CU-243/2006    .........................S.............................G.................................N..
B/Thailand/CU-364/2008    ....A....................S.............................G.................................N..
B/Brisbane/60/2008        .........................N.............................G.................................N..
B/Thailand/CU-B2271/2010  .........................N.............................G.................................N..
B/Thailand/CU-B2660/2010  .........................N.............................G.................................N..
B/Thailand/CU-B3153/2010  .........................N.............................G.................................N..
B/Thailand/CU-C1262/2010  .........................N.............................G...........................I.....N..
B/Thailand/CU-C1451/2010  .........................N.............................G.................................N..
B/Thailand/CU-B2504/2010  .........................N.............................G.................................N..
B/Thailand/CU-B4505/2011  .........................N.............................G.................................N..
B/Thailand/CU-B5522/2011  .........................N.............................G.................................N..
B/Thailand/CU-B5734/2011  .........................N.............................G.................................N..
B/Thailand/CU-C1768/2011  .........................N.............................G.................................N..
B/Thailand/CU-H3002/2011  .........................N.............................G.................................N..
B/Thailand/CU-H3052/2011  .........................N.............................G.................................N..
B/Thailand/CU-B6148/2012  .........................N.............................G.................................N..
B/Thailand/CU-B7215/2012  .........................N.............................G.................................N..
B/Thailand/CU-B6257/2012  .........................N.............................G.................................N..
B/Thailand/CU-B6975/2012  .........................N.............................G.................................N..
B/Thailand/CU-B7337/2012  .........................N.............................G.................................N..
B/Thailand/CU-B2201/2010  .........................N.............................G.................................N..
B/Thailand/CU-B2320/2010  .........................N.............................G.................................N..
B/Thailand/CU-B2372/2010  .........................N.............................G.................................ND.
B/Thailand/CU-B2390/2010  .....A...................K.............................G.................................N..
B/Thailand/CU-B2432/2010  .........................N.............................G.................................N..
B/Thailand/CU-H1400/2010  .........................N.............................G.................................N..
B/Thailand/CU-H1896/2010  .........................N.............................G.................................N..
B/Thailand/CU-H2132/2010  .........................N.................R..........G.................................N..
B/Thailand/CU-H2584/2010  .........................N.............................G.................................N..
B/Thailand/CU-H2738/2010  .........................N.............................G...................L.............ND.
B/Thailand/CU-B4585/2011  .........................K.............................G.................................N..
B/Thailand/CU-B5671/2011  .........................N.............................G.................................ND.
B/Thailand/CU-B5910/2011  .........................N.............................G.................................N..
B/Thailand/CU-B6240/2012  .........................N.............................G.................................N..
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                 210       220       230        240       250       260       270       280       290       300
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006      LLKIKYGEAYTDTYHSYAKNILRTQESACNCIGGDCYLMITDGPASGISECRFLKIREGRIIKEIFPTGRVKHTEECTCGFASNKTIECACRDNSYTAKR
B/Perth/211/2001      .................N.........N........S...........................................................
B/Thailand/CU-B6078/2012  ...................................V.............................................R.....
B/Thailand/CU-H3349/2012  ...................................V.............................................R.....
B/Thailand/CU-H3456/2012  ...................................V.............................................R.....
B/Thailand/CU-A585/2013   ...................................V.............................................H.....
B/Thailand/CU-B8813/2013  ...................................V.............................................R.....
B/Thailand/CU-H8332/2013  ...................................V.............................................R.....
B/Thailand/CU-A605/2014   ..........................R........V.............................................R.....
B/Thailand/CU-A615/2014   ...................................V.............................................R.....
B/Thailand/CU-A626/2014   ...................................V.............................................R.....
B/Thailand/CU-A645/2014   ...................................V.............................................R.....
B/Thailand/CU-B8925/2014  ...................................V.............................................R.....
B/Thailand/CU-B8999/2014  ...................................V.............................................R.....
B/Thailand/CU-B9017/2014  ...................................V.............................................R.....
B/Thailand/CU-B9034/2014  ...................................V.............................................H.....
B/Thailand/CU-C4555/2014  ...................................V.............................................R.....
B/Thailand/CU-H3591/2014  ...................................V.............................................R.....
B/Wisconsin/1/2010        ..................................................................................
B/Thailand/CU-H2933/2011  ..................................................................................
B/Thailand/CU-B6096/2012  ..................................................................................
B/Thailand/CU-H3313/2012  ..................................................................................
B/Thailand/CU-H3496/2012  ..................................................................................
B/Malaysia/2506/2004      .................N.........N.......S...V...................I.......................
B/Thailand/CU-243/2006    .................NK........N.......S...V.................................E.........
B/Thailand/CU-364/2008    .................NK........N.......S...V.................................E.........
B/Brisbane/60/2008        ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B2271/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B2660/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B3153/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-C1262/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-C1451/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B2504/2010  ...V.............NK........N........V...........................................
B/Thailand/CU-B4505/2011  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B5522/2011  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B5734/2011  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-C1768/2011  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-H3002/2011  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-H3052/2011  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B6148/2012  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B7215/2012  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B6257/2012  ...V.............NK........N.......S...V.........................................R.....
B/Thailand/CU-B6975/2012  ...V.............NK........N.......S...V.........................................R.....
B/Thailand/CU-B7337/2012  ...V.............NK........N.......S...V.........................................R.....
B/Thailand/CU-B2201/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B2320/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B2372/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B2390/2010  .................N.........N.......S...V...........................................
B/Thailand/CU-B2432/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-H1400/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-H1896/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-H2132/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-H2584/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-H2738/2010  ...V.............NK........N.......S...V...........................................
B/Thailand/CU-B4585/2011  .........F.......N.........N.......S...V...........................................
B/Thailand/CU-B5671/2011  ...V.............NK........E.N.....S...V...................V.......................
B/Thailand/CU-B5910/2011  ...V.............NE........N.......S...V...........................................
B/Thailand/CU-B6240/2012  .................NK........N.......S...V.........................................R.....
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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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            310       320       330        340       350       360       370       380       390       400
                                           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B/Florida/4/2006                           PFVKLNVETDTAEIRLMCTETYLDTPRPNDGSITGPCESDGDKGSGGIKGGFVHQRMASKIGRWYSRTMSKTKRMGMGLYVKYDGDPWTDSEALALSGVM
B/Perth/211/2001                           ..........................D.........N.......................................................D........
B/Thailand/CU-B6078/2012                   ...................................................................................................
B/Thailand/CU-H3349/2012                   ..............................................................................................G........
B/Thailand/CU-H3456/2012                   .........................................................T.........................................
B/Thailand/CU-A585/2013                    ...........K.................................................F.....................................
B/Thailand/CU-B8813/2013                   ...................................................................................................
B/Thailand/CU-H8332/2013                   ...................................................................................................
B/Thailand/CU-A605/2014                    ...................................................................................................
B/Thailand/CU-A615/2014                    .....................................N.............................................................
B/Thailand/CU-A626/2014                    ...................................................................................................
B/Thailand/CU-A645/2014                    ...................................................................................................
B/Thailand/CU-B8925/2014                   ...................................................................................................
B/Thailand/CU-B8999/2014                   ...................................................................................................
B/Thailand/CU-B9017/2014                   ...................................................................................................
B/Thailand/CU-B9034/2014                   ...................................................................................................
B/Thailand/CU-C4555/2014                   ...................................................................................................
B/Thailand/CU-H3591/2014                   ...................................................................................................
B/Wisconsin/1/2010                         ...................................N...............................................................
B/Thailand/CU-H2933/2011                   ...................................N...............................................................
B/Thailand/CU-B6096/2012                   ......................K............N..E............................................................
B/Thailand/CU-H3313/2012                   ...................................N...............................................................
B/Thailand/CU-H3496/2012                   ...................................N...............................................................
B/Malaysia/2506/2004                       ........................D..........N.......................................A..D...F....
B/Thailand/CU-243/2006                     ...................D...D...........N..................E....................A..D...F....
B/Thailand/CU-364/2008                     ...................D...D...........N.................................E.....A..D...F....
B/Brisbane/60/2008                         ...................D...............N..................E.............E.....A..D...F....
B/Thailand/CU-B2271/2010                   ...................D...............N..................E.............E.....A..D...F....
B/Thailand/CU-B2660/2010                   ...................D...............N..................E.............E.....A..D...F....
B/Thailand/CU-B3153/2010                   ...................D...............N..................E.............E.....A..D...F....
B/Thailand/CU-C1262/2010                   ...................D...............N..................E.............E.....A..D...F....
B/Thailand/CU-C1451/2010                   ...................D...............N..................E.............E.....A..D...F....
B/Thailand/CU-B2504/2010                   ...................D...............N..................E.............E.....A..D...F....
B/Thailand/CU-B4505/2011                   ...................D...............N..................E.............E.....A..D...F....
B/Thailand/CU-B5522/2011                   ...................D...............N..................E.............E...E.A..D..DF....
B/Thailand/CU-B5734/2011                   ...................D...............N..................E.............E...E.A..D..DF....
B/Thailand/CU-C1768/2011                   ...................D...............N..................E.............E.....A..D..DF....
B/Thailand/CU-H3002/2011                   ...................D...............N..................E.............E...E.A..D..DF....
B/Thailand/CU-H3052/2011                   ...................D...............N..................E.............E.....A..D...F....
B/Thailand/CU-B6148/2012                   ...................D...............N.E................E.............E.....A..D..TF....
B/Thailand/CU-B7215/2012                   ...................D...............N..................E.............E...E.A..D..EF....
B/Thailand/CU-B6257/2012                   ...................D...............N..................K.............E.....A..D...F....
B/Thailand/CU-B6975/2012                   ...................D...............N..................K.............E.....A..D...F..A.
B/Thailand/CU-B7337/2012                   ...................D...........N...N..................K.............E.....A..D...F....
B/Thailand/CU-B2201/2010                   ...................D.......D.......N..................E.............E.....A..D..TF....
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B/Thailand/CU-B2372/2010                   ..I................D.......D.......N..................E.............E.....A..D...F....
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B/Thailand/CU-H2132/2010                   ...................D.......D.......N..................E.............E.....A..D..TF....
B/Thailand/CU-H2584/2010                   ...................D.......D.......N..................E.............E.....AE.D..TF....
B/Thailand/CU-H2738/2010                   ...................D.......D.......N..................E.............E.....A..D...F....
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B/Perth/211/2001       .......................................E.........................*
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B/Thailand/CU-B8999/2014 ..................................................................*
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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY
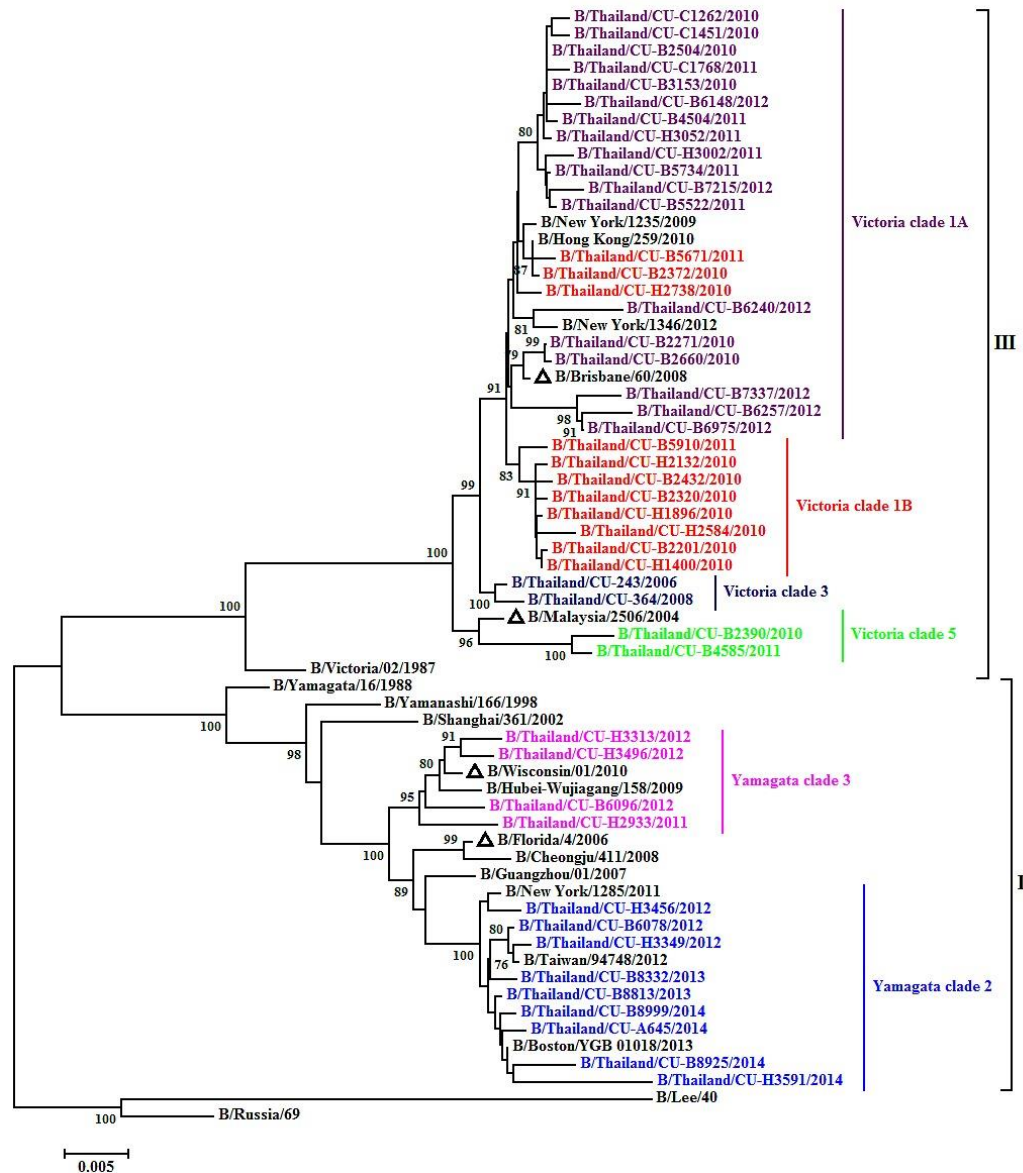
Figure S2. *Phylogenetic trees of PB2 gene of influenza B viruses of 44 samples isolated in Thailand, 2010–2014. Trees were constructed using Neighbor Joining analysis in MEGA (V.6.06). Bootstrap values (1000 replicates) > 70 are indicated on the branch. The scale bar shows the mutation rate between each two sequences. The sequences studied in this study are represented by name of taxa "Thailand". All vaccine strains are marked as open triangles.*
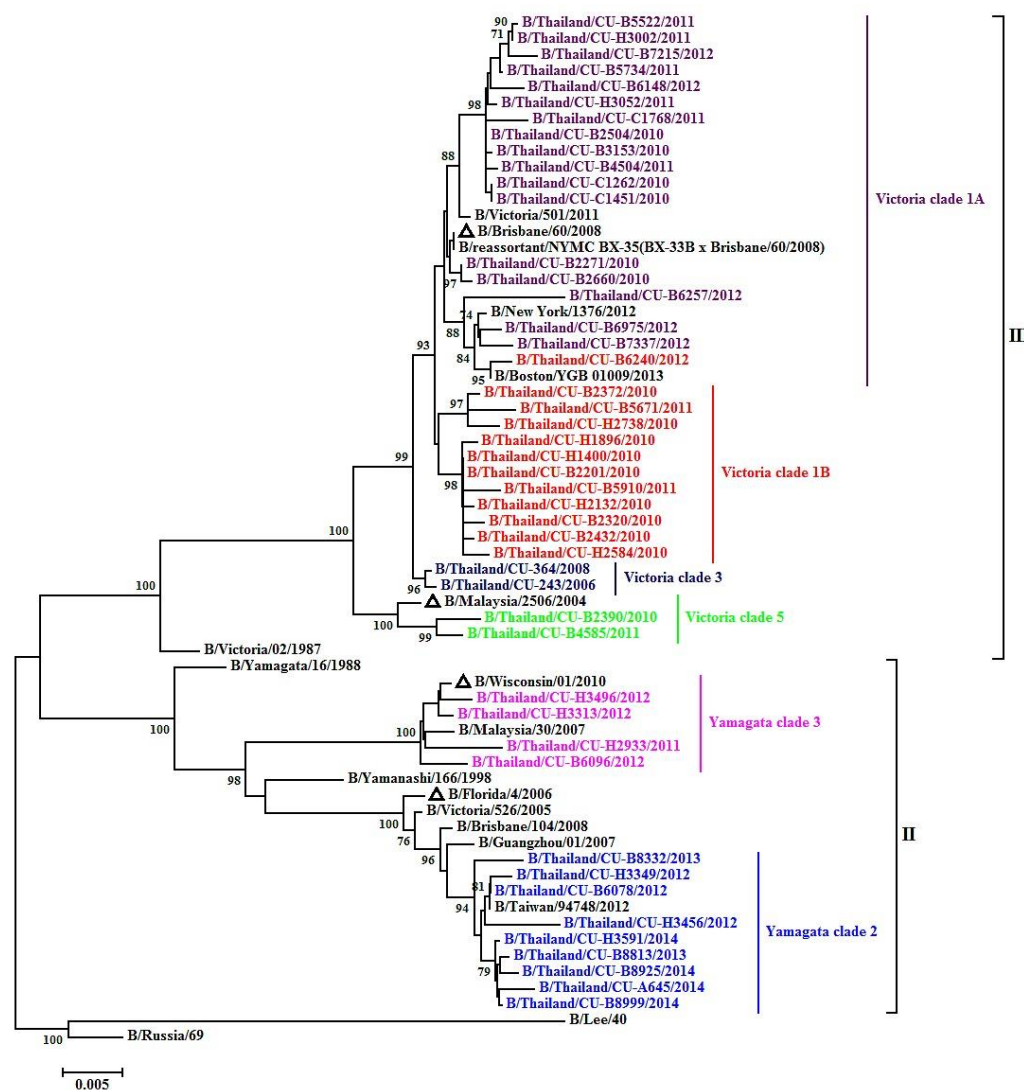
Figure S3. *Phylogenetic trees of PB1 gene of influenza B viruses of 44 samples isolated in Thailand, 2010–2014. Trees were constructed using Neighbor Joining analysis in MEGA (V.6.06). Bootstrap values (1000 replicates) > 70 are indicated on the branch. The scale bar shows the mutation rate between each two sequences. The sequences studied in this study are represented by name of taxa "Thailand". All vaccine strains are marked as open triangles.*
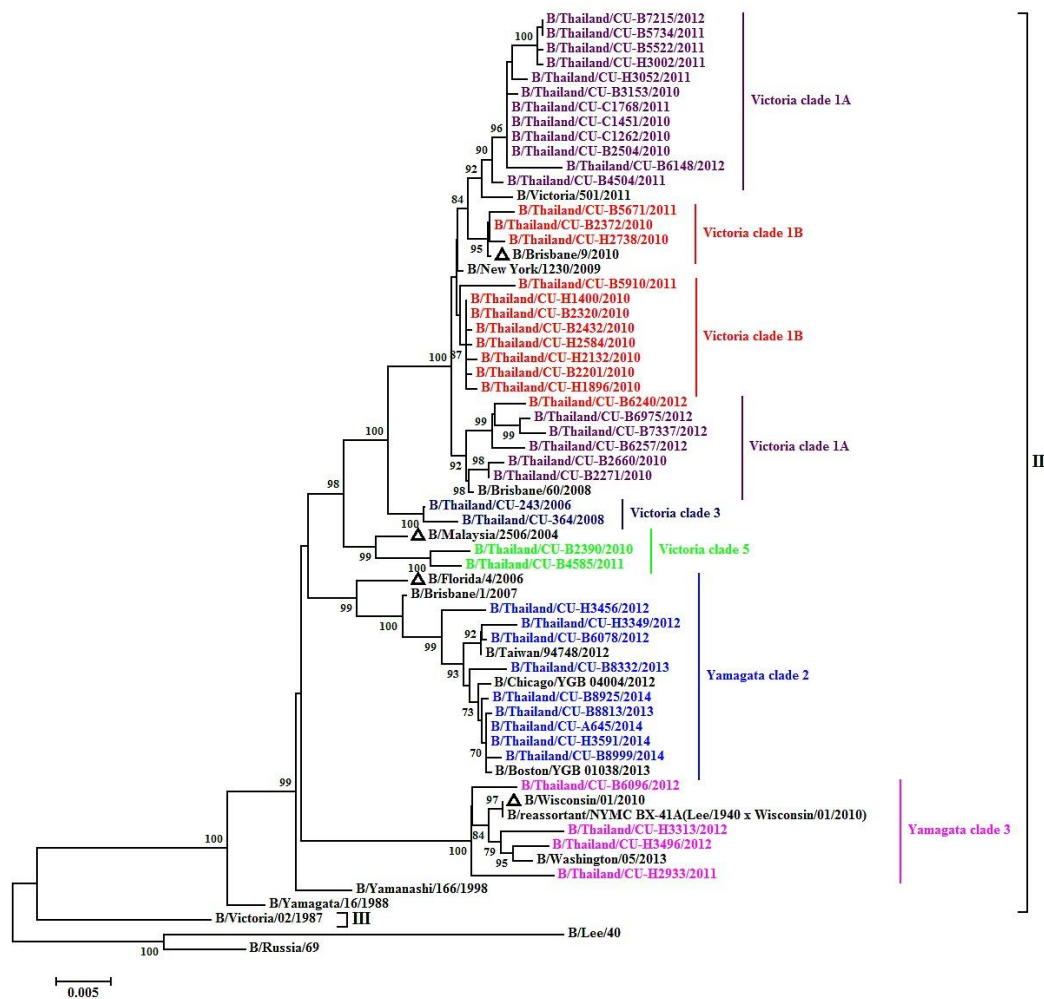
Figure S4. *Phylogenetic trees of PA gene of influenza B viruses of 44 samples isolated in Thailand, 2010–2014. Trees were constructed using Neighbor Joining analysis in MEGA (V.6.06). Bootstrap values (1000 replicates) > 70 are indicated on the branch. The scale bar shows the mutation rate between each two sequences. The sequences studied in this study are represented by name of taxa "Thailand". All vaccine strains are marked as open triangles.*
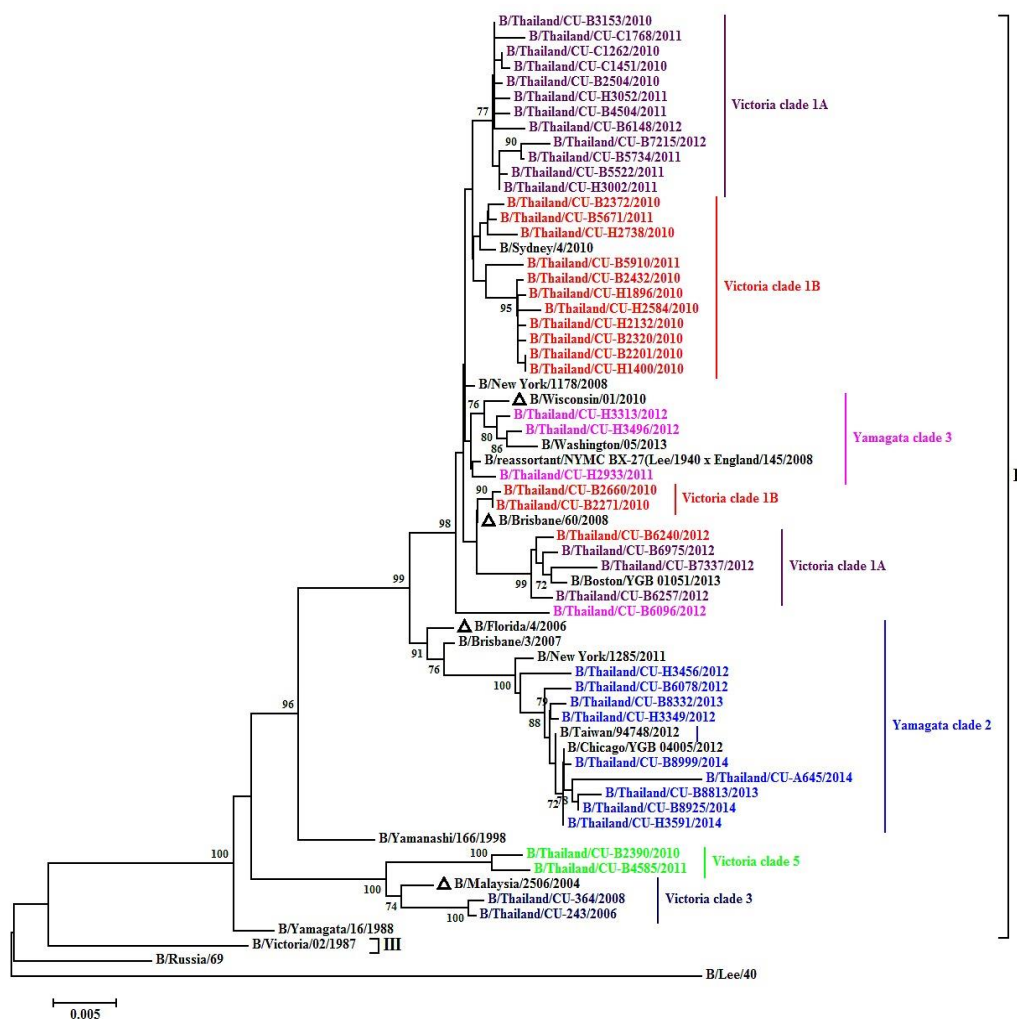
Figure S5. *Phylogenetic trees of NP gene of influenza B viruses of 44 samples isolated in Thailand, 2010–2014. Trees were constructed using Neighbor Joining analysis in MEGA (V.6.06). Bootstrap values (1000 replicates) > 70 are indicated on the branch. The scale bar shows the mutation rate between each two sequences. The sequences studied in this study are represented by name of taxa "Thailand". All vaccine strains are marked as open triangles.*
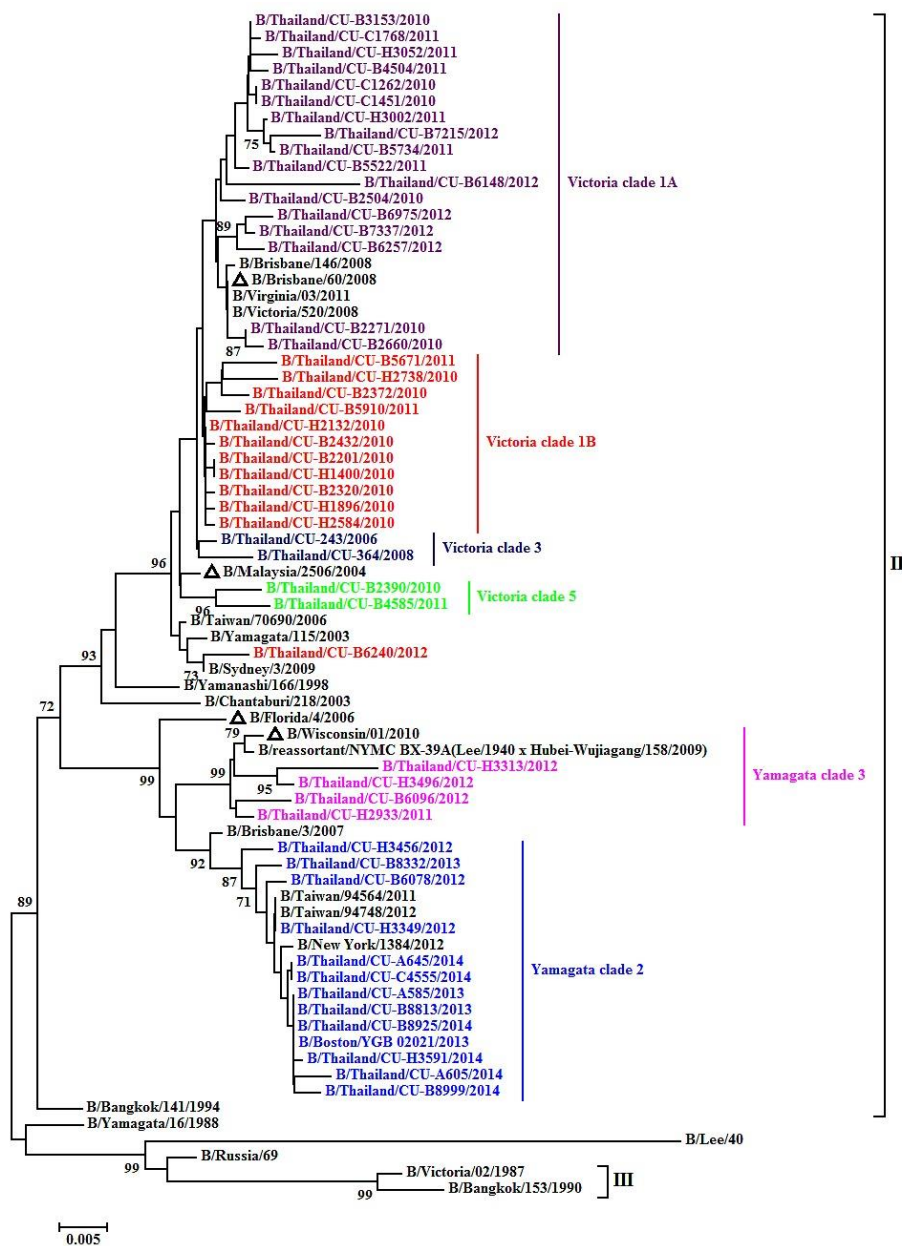
Figure S6. *Phylogenetic trees of M gene of influenza B viruses of 47 samples isolated in Thailand, 2010–2014. Trees were constructed using Neighbor Joining analysis in MEGA (V.6.06). Bootstrap values (1000 replicates) > 70 are indicated on the branch. The scale bar shows the mutation rate between each two sequences. The sequences studied in this study are represented by name of taxa "Thailand". All vaccine strains are marked as open triangles.*
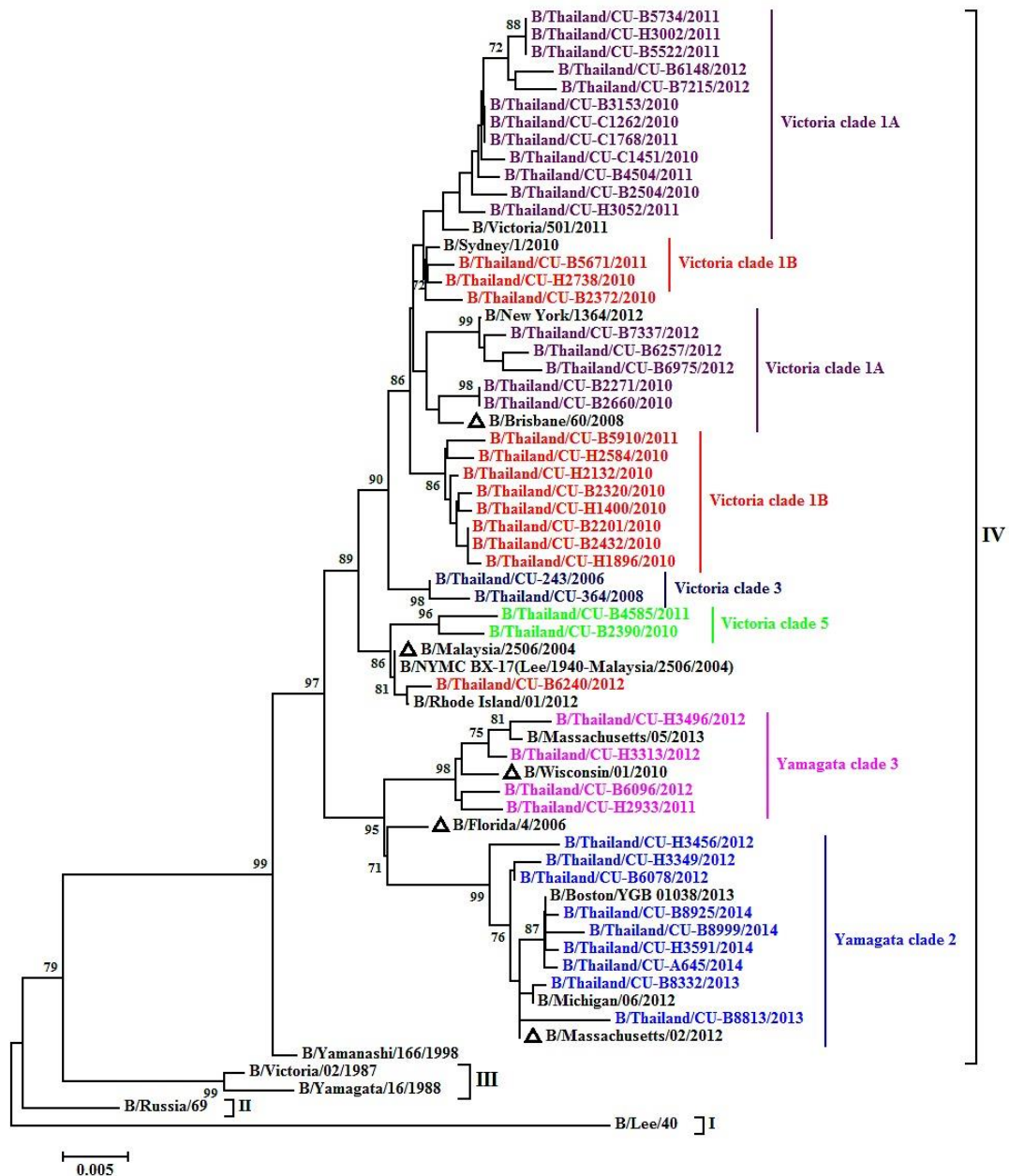
Figure S7. Phylogenetic trees of NS gene of influenza B viruses of 44 samples isolated from Thailand, 2010–2014. Trees were constructed using Neighbor Joining analysis in MEGA (V.6.06). Bootstrap values (1000 replicates) > 70 are indicated on the branch. The scale bar shows the mutation rate between each two sequences. The sequences studied in this study are represented by name of taxa "Thailand". All vaccine strains are marked as open triangles.

Year	Vaccine strain	No of strain	Dominant Epitope	Differing Residues
2010	A/Perth/16/2009	2	E	261
(N=3)		1	A	124
2011	A/Perth/16/2009	6	A	144 145
(N=24)		1	A	124 144 145
		3	A	140 144 145
		14	C	45 48 278 312
2012	A/Perth/16/2009	3	A	144 145
(N=16)		1	A	124 144 145
		1	A	140 144 145
		11	C	45 48 278 312
2013	A/Victoria/361/2011	1	A	143 145
(N=41)		1	A	144 145
		1	A	124 142 145
		30	B	156 186
		1	B	156 160 186
		1	B	156 186 187
		2	B	128 156 186
		1	B	128 156 157 186
		2	C	45 48 278
		1	C	278 297 304
2014	A/Texas/50/2012	1	A	126 133 144 145 146 150 152
(N=36)		1	B	128 186
		8	B	128 186 198
		5	B	128 159 160 186
		12	B	128 159 186 198
		1	B	128 156 159 186 198
		8	B	128 159 160 186 198
2014	A/Switzerland/9715293/2013	6	A	140
(N=36)		1	B	156 186
		8	A	138 140 142
		14	A	138 140 142 144
		1	B	128 159 186 198
		1	A	135 138 140 142 144
		4	B	128 159 160 186 198
		1	A	10 126 133 138 140 142 144 145 146 150 152

Figure S8. *Positions of mutations in the dominant epitope of HA1 influenza A(H3N2) compared with vaccine strains.*
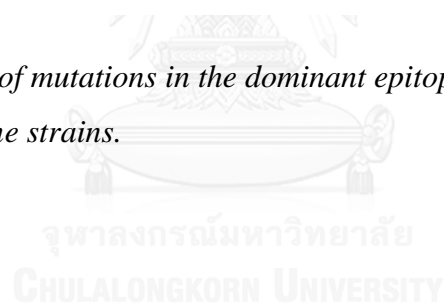
Year	No. of strain	Dominant Epitope	No. of mutation	Differing Residues	
2010	9	B	1	185	
(N=18)	2	B	1	190	
	1	C	2	38	277
	6	E	1	83	
2011	2	A	2	127	143
(N=7)	3	B	1	185	
	1	B	2	183	185
	1	E	1	83	
2012	5	B	1	185	
2013	3	B	1	185	
(N=7)	1	C	2	283	302
	2	E	2	83	69
	1	E	2	83	263
2014	44	B	1	185	

Figure S9. *Positions of mutations in the dominant epitope of HA1 influenza A(H1N1)pdm09 compared with A/California/07/2009 vaccine strain.*
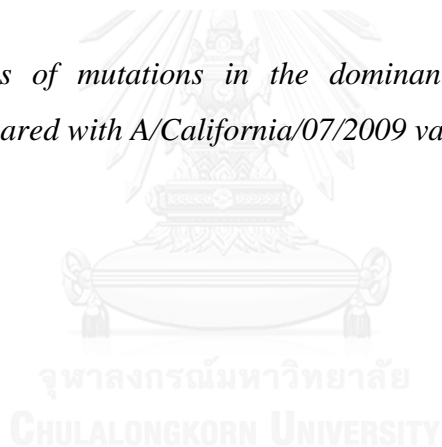
Figure S10. Incidence of influenza A and B viruses identified from clinical samples between 2010 and 2015 (N= 19,859). A total of 3,995 (20.12%) influenza viruses were isolated in Thailand. 1581 (39.57%), 1,673 (41.88%), and 741 (18.55%) were subtyped as influenza A (H3N2), A (H1N1)pdm09, and influenza B viruses, respectively. Post-pandemic of A/H1N1 pdm09, 4 of the 485 samples (0.82%) were positive for the oseltamivir resistant strains.
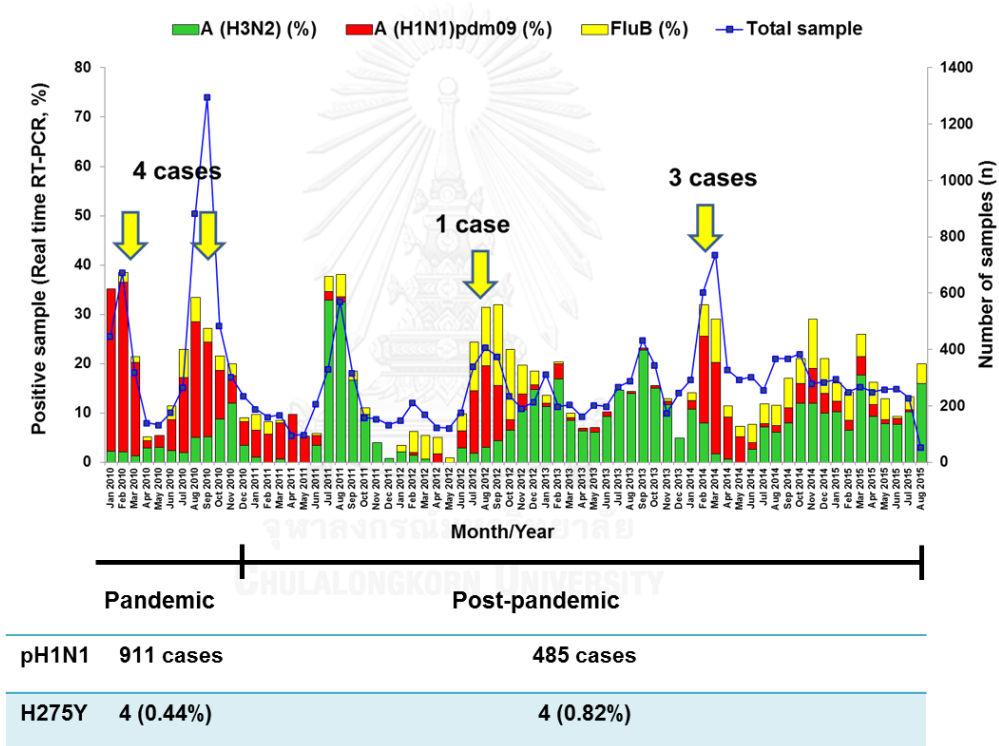
Figure S11. *A maximum clade credibility tree from Bayesian timescale phylogenetic analysis of NA genes (dataset N1 seasonal n = 144 sequences, length = 1410 nt.; N1 pdm09 n = 306 sequences, length = 1407 nt.; N2 n = 373 sequences, length = 1407 nt.; NB n = 255 sequences, length = 1398 nt.). The posterior probabilities and node ages of the key nodes are depicted above the respective nodes. The major recent clusters are marked by vertical lines.*

S2A) N1 seasonal

S2B) N1 pdm09

S2C) N2
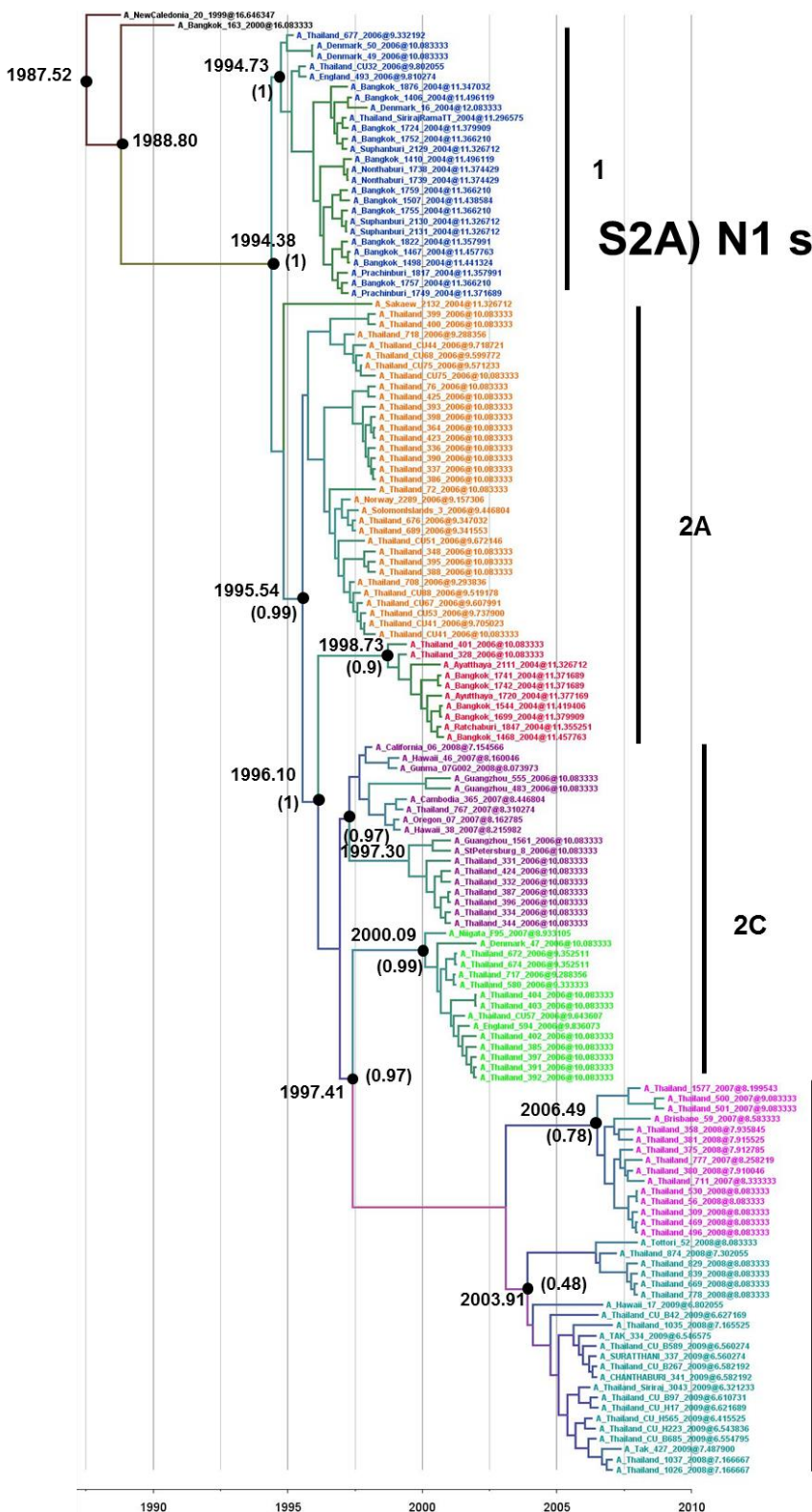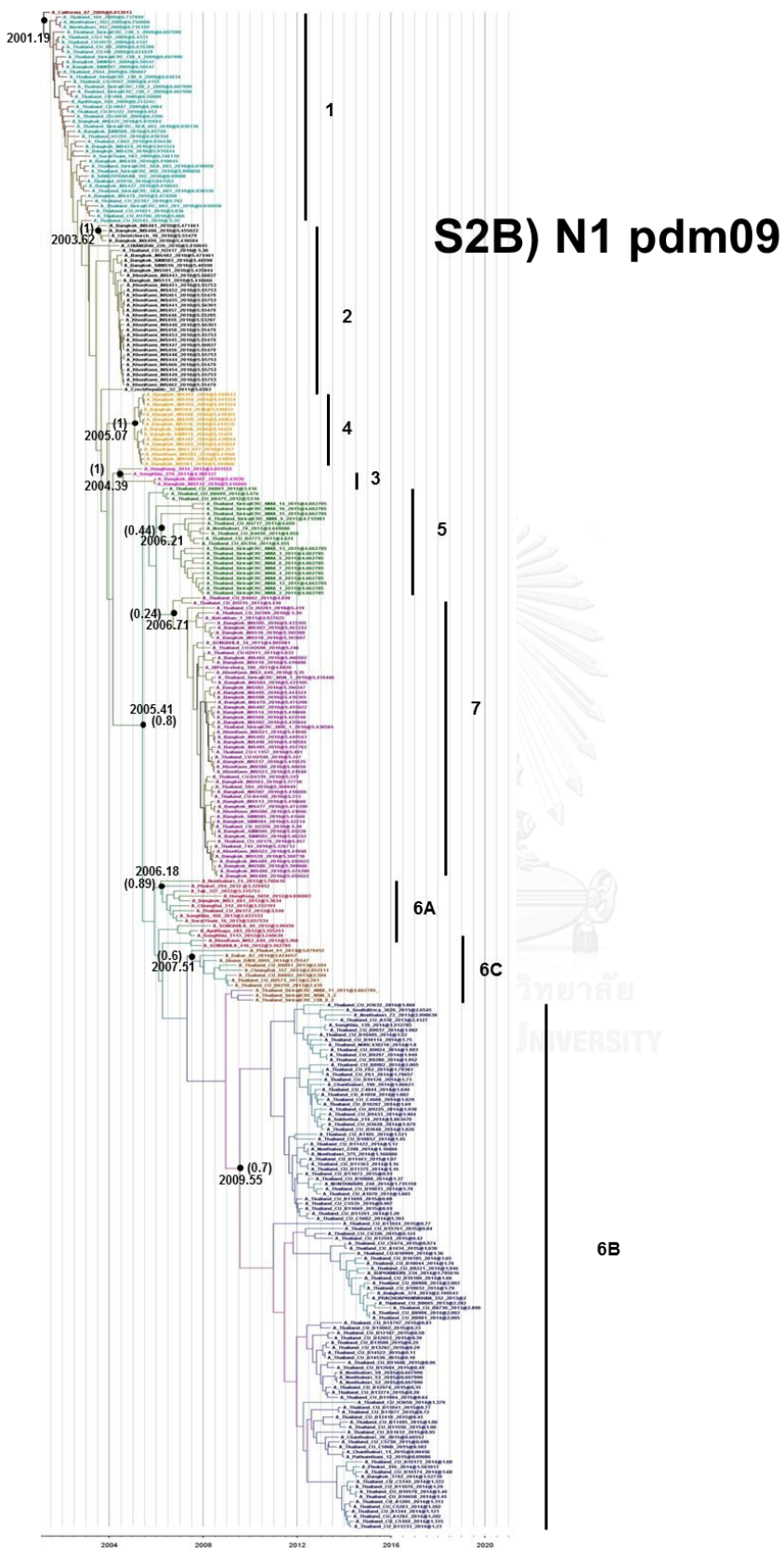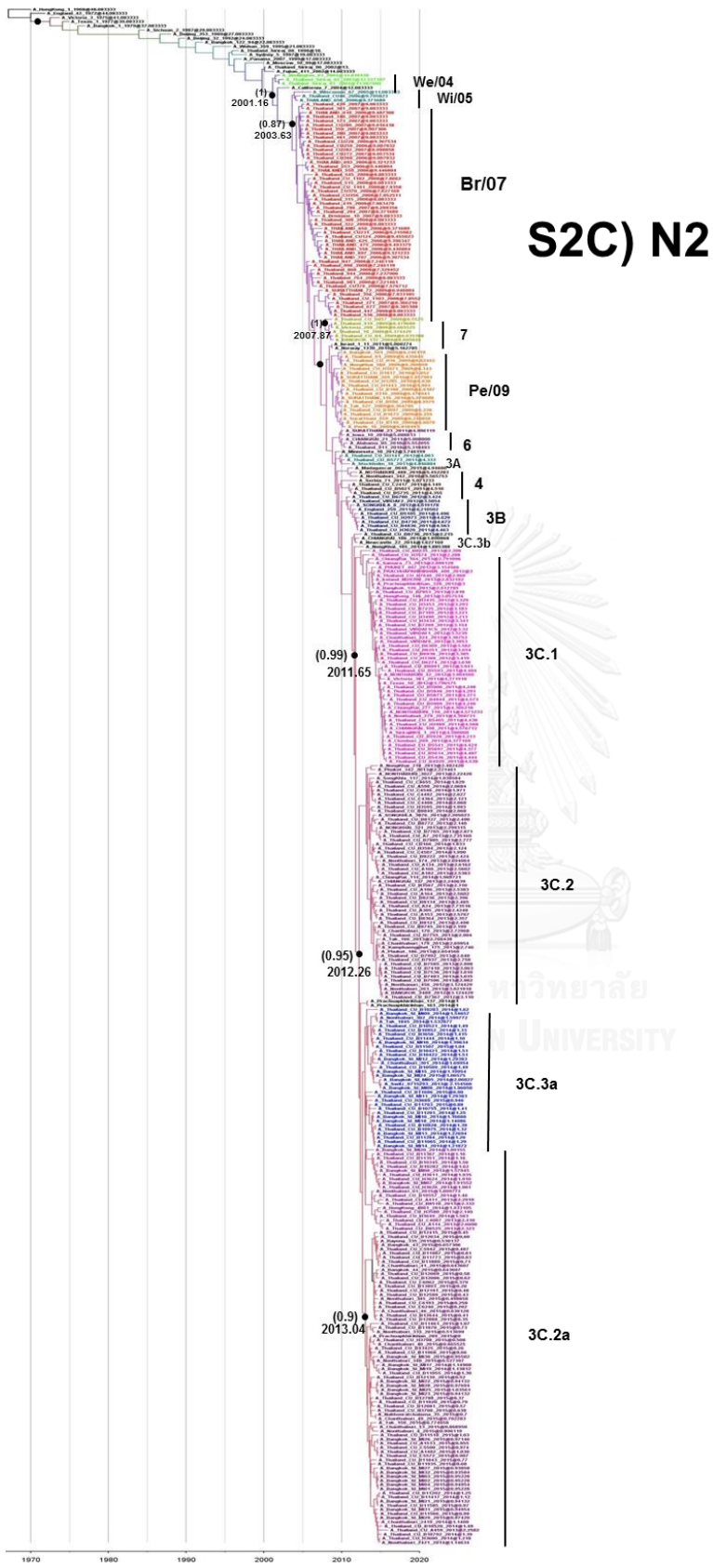
S2D) NB
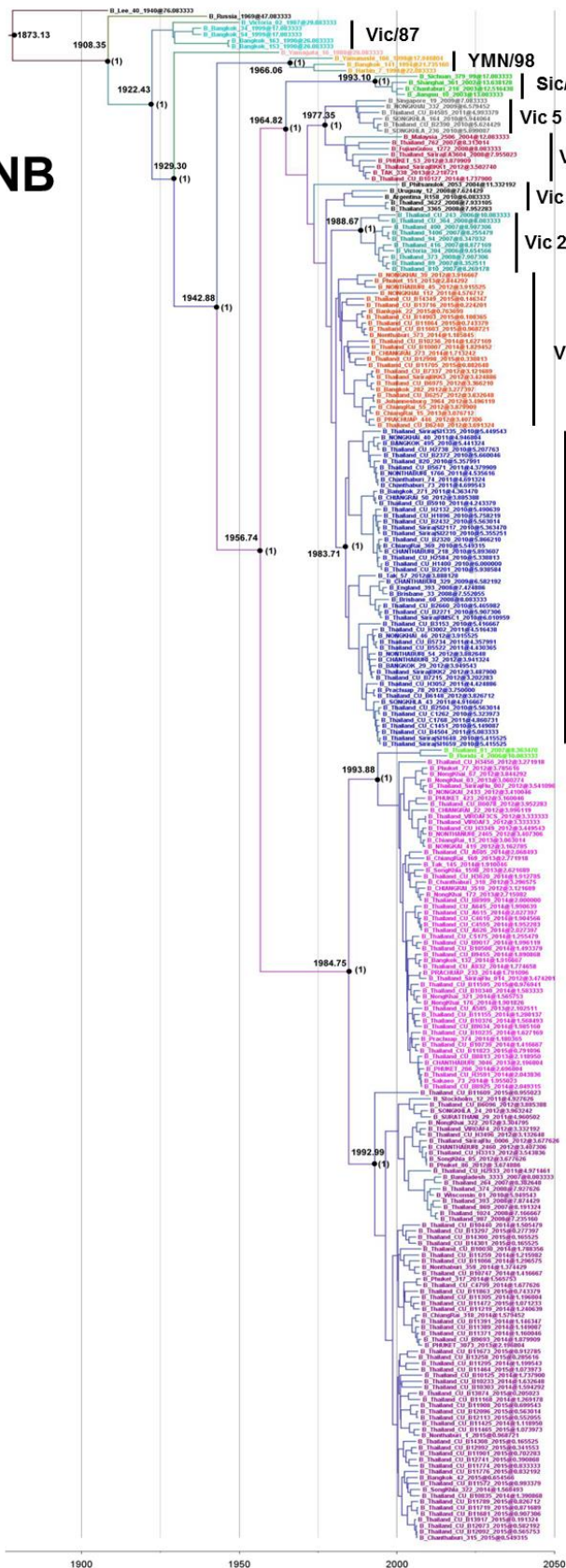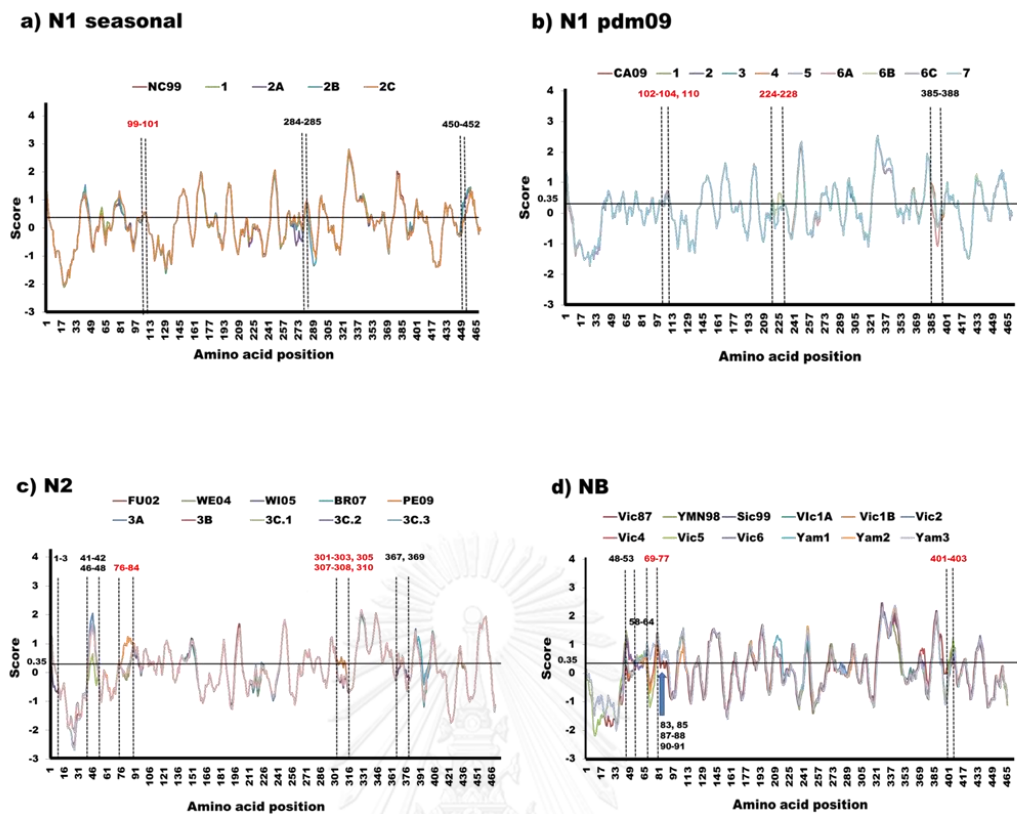
Figure S12. *Predicted B-cell epitopes of NA proteins of influenza A/H1N1 seasonal (a), A/H1N1 pdm09 (b), A/H3N2 (c), and influenza B (d) viruses. The BepiPred score above the 0.35 threshold is shown by horizontal line. Epitope difference between t5he vaccine (A/New Caledonia/20/1999 for N1 seasonal, A/California/07/2009 for N1 pdm09, A/Fujian/411/2002 for N2, and B/Victoria/02/1987 for NB) and Thai strains are indicated by dotted vertical lines. The substituted amino acid contribute to loss B-cell epitope are denoted a black letter, while that gain of the antigen property are denoted red letter.*

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VITA

The author of this thesis was born on December 3, 1981 in Bangkok, Thailand. She received bachelor degree of medical technology major in 2003 from Faculty of Allied Health Sciences, Thammasat University. In 2004, she received the German Academic Exchange Service (DAAD) and the Southeast Asian Ministers of Education Organization Tropical Medicine (SEAMEO-TROPMED) scholarships for a Master's thesis research at Mahidol University, which sought to identify mimotope using phage-displayed random peptide libraries against monoclonal antibodies specific to house dust mite under supervision of Assoc. Prof. Pongrama Ramasoota. After graduation, she joined a biotech company that managed and provided stem cell banking services. Later, she accepted a faculty position in the Department of Microbiology in the Faculty of Medical Technology, Rangsit University. Her responsibilities included teaching microbiology to undergraduate students in both classroom and laboratory, and conducted research in the antibiotic resistance and toxin production in pathogenic bacteria. In 2013, she enrolled in the 100th anniversary Chulalongkorn University Fund for doctoral scholarship in graduated program for degree of doctor of philosophy program in Faculty of Medicine, Chulalongkorn University under supervision of Prof. Yong Poovorawan. Her Ph.D. work examines the evolutionary patterns and epidemiological dynamics of influenza A and B viruses, the rapid identification of influenza B lineage, and the study of potential vaccine efficacy by assessing the antigenic epitope on the HA1 domain of circulating seasonal influenza A. She also did the reverse genetics technique to produce the neuraminidase mutant influenza A and B viruses for testing the neuraminidase inhibitors susceptibility and enzyme kinetic. This work was performed at department of infectious disease, St. Jude children's research hospital (Memphis, TN) under supervision of Dr. Richard J. Webby and Dr. Elena A. Govorkova. The sponsorship of this work was Overseas Research Experience Scholarship for Graduate Students, Chulalongkorn University.

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