การศึกษาระบาดวิทยาของไวรัสไข้หวัดใหญ่และการตรวจหาไวรัสในระบบทางเดินหายใจของผู้ป่วยด้วยวิธีเมตาจีโนมิกส์

นางสาวสลิลภร ปรัชญางค์ปรีชา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
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EPIDEMIOLOGY OF INFLUENZA VIRUS AND METAGENOMIC ANALYSIS OF VIRUSES IN HUMAN RESPIRATORY TRACT SPECIMENS

Miss Slinporn Prachayangprecha

A Dissertation Submitted in Partial Fulfillment of the Requirements

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By	Miss Slinporn Prachayangprecha
Field of Study	Biomedical Sciences
Thesis Advisor	Professor Yong Poovorawan, M.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Graduate School

(Associate Professor Sunait Chutintaranond, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Chintana Chirathaworn, Ph.D.)

.....Thesis Advisor

(Professor Yong Poovorawan, M.D.)

.....Examiner

(Assistant Professor Sunchai Payungporn, Ph.D.)

.....Examiner

(Professor Rujipat Samransamraujkit, M.D.)

.....External Examiner

(Associate Professor Teeraporn Chinchai, Ph.D.)

สลิลภร ปรัชญางค์ปรีชา : การศึกษาระบาดวิทยาของไวรัสไข้หวัดใหญ่และการตรวจหาไวรัสในระบบทางเดินหายใจของผู้ป่วยด้วยวิธีเมตาจีโนมิกส์ (EPIDEMIOLOGY OF INFLUENZA VIRUS AND METAGENOMIC ANALYSIS OF VIRUSES IN HUMAN RESPIRATORY TRACT SPECIMENS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ. ยง ภู่วรวรรณ, 122 หน้า.

โรคติดเชื้อในระบบทางเดินหายใจนั้นเป็นปัญหาสุขภาพที่พบได้ในเด็กและผู้ใหญ่ทั่วโลก โดยโรคนี้จะทำให้เกิดอาการในระบบทางเดินหายใจทั้งส่วนบนและส่วนล่าง การศึกษาพบว่าไวรัสเป็นสาเหตุสำคัญของการติดเชื้อในระบบทางเดินหายใจ และหนึ่งในสาเหตุหลักของโรคนี้คือไวรัสไข้หวัดใหญ่ งานศึกษาวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความชุกของภูมิคุ้มกันต่อเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในช่วงระยะเวลาปี 2009-2010 ซึ่งเป็นช่วงเวลาหลังการระบาดครั้งใหญ่ในกลุ่มประชากรทั่วไปและบุคลากรทางการแพทย์โดยใช้เทคนิค Hemagglutination (HI) ในเขตอำเภอชุมแพ จังหวัดขอนแก่น โดยเก็บตัวอย่างจากบุคลากรทางการแพทย์จำนวน 255 ตัวอย่าง และประชากรทั่วไปจำนวน 307 ตัวอย่าง จากตัวอย่างที่เก็บได้จากประชากรทั่วไปในอำเภอชุมแพ จังหวัดขอนแก่น ช่วงเดือนธันวาคมในปี 2009 พบว่าตัวอย่างทั้งหมด 36% มีความชุกของแอนติบอดีต่อไวรัสไข้หวัดใหญ่ A(H1N1)pdm09 (positive HI titers ≥ 40) ในขณะที่ตัวอย่างจากปี 2008 ซึ่งเป็นปีก่อนการระบาดมีเพียงจำนวน 2 จาก 100 ตัวอย่าง ที่มีแอนติบอดีต่อไวรัสไข้หวัดใหญ่ A(H1N1)pdm09 ในตัวอย่างบุคลากรทางการแพทย์ 255 ตัวอย่างพบว่ามีความชุกของแอนติบอดี 48% ซึ่งสูงกว่าประชากรทั่วไปเนื่องจากในกลุ่มบุคลากรทางการแพทย์ที่มีความเสี่ยงสูงในการประกอบอาชีพมากกว่า นอกจากนี้ยังมีการเก็บตัวอย่างจากบุคลากรทางการแพทย์หลังจากการระบาดของไวรัสไข้หวัดใหญ่ A(H1N1)pdm09 แต่ระลอกในเดือนมิถุนายนและธันวาคม 2010 ซึ่งมีตัวอย่างจำนวน 397 และ 366 ตัวอย่างตามลำดับ HI titers หลังการระบาดในระลอกสองและสามพบ positive HI titers 22% และ 26% ตามลำดับ ส่วนในกลุ่มตัวอย่างที่ได้รับวัคซีนไข้หวัดใหญ่ในช่วงเดือนกุมภาพันธ์และมีนาคม 2010 พบว่ามีค่า GMT ของ HI titers สูงกว่าอย่างมีนัยยะสำคัญเมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้ฉีดวัคซีน ส่วนการศึกษาระบาดวิทยาของเชื้อไวรัสไข้หวัดใหญ่ในกลุ่มผู้ป่วยที่ได้รับการวินิจฉัยด้วยอาการในระบบทางเดินหายใจพร้อมกับไข้ (influenza-like-illness) จำนวนทั้งหมด 17,416 ตัวอย่าง โดยตัวอย่างทั้งหมดนั้นจะได้รับการตรวจหาไวรัสไข้หวัดใหญ่ด้วยวิธี real-time RT-PCR ผลการศึกษาพบว่าผู้ป่วยมีการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอมากกว่าชนิดบี แม้ว่าจำนวนผู้ป่วยที่ตรวจพบไวรัสไข้หวัดใหญ่มีแนวโน้มว่าจะสูงในช่วงฤดูฝน แต่การวิเคราะห์พบว่าไม่มีความสัมพันธ์อย่างมีนัยยะสำคัญทางสถิติกับปัจจัยทางอากาศเช่น อุณหภูมิ ความชื้นสัมพัทธ์ หรือ ปริมาณน้ำฝน ลักษณะของการระบาดของไข้หวัดใหญ่นั้นสามารถพบได้ตลอดทั้งปีแต่จะมีช่วงที่มีการระบาดหนักในช่วงหน้าฝนซึ่งจะพบผู้ป่วยจำนวนมากในช่วงเวลานี้ของทุกปี ยกเว้นปีหลังการระบาดครั้งแรกของเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ที่พบการระบาดในช่วงเวลาที่แตกต่างจากปีอื่นๆคือช่วงต้นปี 2010 ซึ่งอาจเป็นเพราะประชากรไทยยังไม่มีภูมิคุ้มกันต่อเชื้อไวรัสใหม่ชนิดนี้ เมื่อวิเคราะห์ดูช่วงอายุของผู้ป่วยในช่วงปีที่มีการระบาดของไวรัสไข้หวัดใหญ่ A(H1N1)pdm09 พบว่ามีค่าเฉลี่ยของอายุเท่ากับ 19 เช่นเดียวกับการศึกษาอื่นว่าพบการติดเชื้อ pH1N1 ส่วนใหญ่ในผู้ป่วยอายุน้อยซึ่งตรงข้ามกับไข้หวัดใหญ่ตามฤดูกาลสายพันธุ์อื่นการศึกษาส่วนนี้ทำให้สามารถเห็นลักษณะช่วงเวลาการระบาดของไวรัสไข้หวัดใหญ่อย่างชัดเจน จากข้อมูลนี้สามารถแนะนำได้ว่าช่วงเวลาเหมาะสมที่สุดในการฉีดวัคซีนคือเดือนเมษายนถึงเดือนพฤษภาคมเพื่อที่จะลดความรุนแรงของการระบาดของเชื้อไวรัส นอกจากไวรัสไข้หวัดใหญ่แล้วยังมีไวรัสอีกหลายชนิดอื่นเป็นสาเหตุของโรคในระบบทางเดินหายใจ ดังนั้นจึงเป็นที่น่าสนใจว่าในตัวอย่างที่ตรวจไม่พบไวรัสชนิดหลักที่ทำให้เกิดอาการโรคในระบบทางเดินหายใจจะสามารถพบไวรัสชนิดใหม่โดยการใช้เทคนิคเมตาจีโนมิกส์หรือไม่ เนื่องจากในปัจจุบันเทคนิคนี้เป็นอีกทางเลือกหนึ่งที่สามารถนำมาใช้ในด้านการตรวจวินิจฉัย ด้วยเทคนิคนี้สามารถค้นหาเชื้อไวรัสชนิดใหม่โดยไม่ต้องอาศัยไพรเมอร์ที่จำเพาะ แต่ความไวของเทคนิคนี้ยังเป็นที่กังขาว่าสามารถเทียบเคียงกับความไวและความจำเพาะของ PCR ได้หรือไม่ ดังนั้นเปรียบเทียบประสิทธิภาพของเทคนิคเมตาจีโนมิกส์กับ real-time RT-PCR ในการตรวจหาเชื้อไวรัสในกลุ่มผู้ป่วยเด็กจำนวน 81 คนที่มีอาการป่วยด้วยโรคในระบบทางเดินหายใจ ผลการวิเคราะห์พบว่าเทคนิค NGS มีความไวในการตรวจจับเชื้อไรโนไวรัสและฮิวแมนเมตานิวโมไวรัสเทียบเท่ากับเทคนิค real-time RT-PCR แต่พบว่ามีความไวในการตรวจเชื้อเอนเทอโรไวรัสและโบคาไวรัสน้อยกว่า ปัจจุบันนั้นเทคโนโลยี NGS มีการพัฒนาให้มีการหาลำดับเบสด้วยความรวดเร็วถูกต้องแม่นยำมากขึ้น พร้อมกับต้นทุนที่ต่ำลงนั้นจึงทำให้เทคนิคนี้เป็นที่น่าสนใจสำหรับการนำมาประยุกต์ใช้ในการตรวจวินิจฉัยโรคในอนาคต

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5287830520 : MAJOR BIOMEDICAL SCIENCES
KEYWORDS: INFLUENZA / SERODIAGNOSIS / SURVEILLANCE / METAGENOMIC / HIGH-THROUGHPUT NUCLEOTIDE SEQUENCING / REAL TIME RT-PCR / THAILAND

SLINPORN PRACHAYANGPRECHA: EPIDEMIOLOGY OF INFLUENZA VIRUS AND METAGENOMIC ANALYSIS OF VIRUSES IN HUMAN RESPIRATORY TRACT SPECIMENS. ADVISOR: PROF. YONG POOVORAWAN, M.D., 122 pp.

Acute respiratory infections are the cause of immense clinical problem worldwide, affect both adults and children every seasonal cycle. These respiratory tract symptoms involved both upper and lower respiratory tract. Many studies found that viruses are generally associated with the infection, and Influenza viruses are the one that find to be one of the most common viruses that cause respiratory illness. Overall, this thesis aimed to detect antibodies against influenza A(H1N1)pdm09, between July 2009 and November 2010, after its emergence in 2009. Seroprevalence among general population and medical personnel was established in three cross-sectional studies at the end of each wave of the pandemic in Khon Kaen province, Thailand, by performing HI assay. Sample of 255 medical personnel and 307 members of the general population were collected after the first peak of outbreak during second week of December 2009. The results of the HI test demonstrated that HI titers among Healthcare personnel (48%) were higher than general population (36%) due to the higher occupational risk, while only two of the 100 stored sera from 2008 contained antibodies (HI titers $\geq$40) against pandemic influenza. Furthermore, serum specimens from medical personnel were collected after the end of each wave, for another two consecutive waves. After the second wave in June 2010, 397 serum samples were collected, and 366 serum samples were collected after the third wave of the pandemic in December 2010. The positive HI titers after the second and third waves were 22.4%, and 25.7%, respectively. Also, the medical personnel (n=146) who had received the influenza A(H1N1)pdm09 monovalent vaccine between February and March 2010 showed significant higher GMT when compare to unvaccinated groups. This showed that annual influenza vaccination can induce an immune response against the virus and is the most effective way to prevent against influenza infection. These findings show that seasonal influenza strain in Chumphae and the predominant influenza strain from each wave was influenza A(H1N1)pdm09. These results also represent the severity of the attack rate in each wave. To investigate seasonal pattern, distribution of influenza cases in patients (n=17,416) diagnosed with influenza-like-illness (ILI) during June 2009- July 2014. All samples were subjected to real-time RT-PCR, as laboratory confirmation, for detection of influenza viruses. Results during the period showed that influenza virus type A predominated over type B. The seasonal pattern of influenza activity in Thailand tended to peak during rainy season, although no significant correlation with environmental factor such as average temperature, average relative humidity, and rainfall. We found that influenza activity in Thailand usually presents as an annual cycle, except during the 2009 pandemic and its aftermath, when the circulation of influenza A(H1N1)pdm09 caused the normal pattern to break. Most likely this was the result of the Thai population's lack of immunity toward the new pandemic strain. At the emergence of influenza A(H1N1)pdm09 in 2009, the mean age of infected patients was 19 years, which correlates with other studies that have reported that influenza A(H1N1)pdm09, in contrast to seasonal influenza, specifically targets younger age groups. This observation of influenza's annual incidence pattern provides a better understanding of its occurrence, suggesting that vaccination campaigns should be started before the influenza season begins in order to reduce transmission. Recently, metagenomic sequencing is one of the method that being explore as a tool in clinical diagnosis because its potential for identifying known or new viruses without specific primers. These advantages include speed and the ability to generate large volumes of sequencing data, but sensitivity of deep sequencing is still in question when compare to specificity and sensitivity of PCR primer and probe. The last part involves comparing efficiency of next generation sequencing approach and real time diagnostic RT-PCR of respiratory viruses in a cohort of Children (n=81) with respiratory disease in Thailand. Results revealed that next generation sequencing approach was at least as sensitive as diagnostic real time RT-PCR for rhinovirus and human metapneumovirus and maybe less sensitive than real time RT-PCRs for entero- and bocavirus detection. Moreover, an advantage of using a next-generation sequencing approach to detect viruses in clinical specimens is that it can also be used to obtain information regarding the virus species and/or type of virus. Considering its declining cost and development for increasing sequencing depth, this approach, combined with bioinformatics analysis, can be an alternative method for virus identification in clinical and public health setting in future.

Field of Study: Biomedical Sciences Student's Signature .....

Academic Year: 2014 Advisor's Signature .....

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LIST OF ABBERVIATIONS

ARTI = Acute respiratory tract complications

EV = Enterovirus

HRV = Human rhinovirus

hRSV = Human respiratory syncytial virus

hAdV = Human adenovirus

hMPV = Human metapneumovirus

hPIV = Human parainfluenza virus

hCoV = Human coronavirus

pH1N1 = Pandemic influenza virus H1N1

ILI = Influenza-like illness

PCR = Polymerase chain reaction

RT-PCR = Reverse transcription polymerase chain reaction

rRT-PCR = Real-time reverse transcription polymerase chain reaction

C_T = Threshold cycle

DNA = Deoxyribonucleic acid

RNA = Ribonucleic acid

vRNPs = Viral ribonucleic acid nucleoproteins

PB2 = Polymerase basic protein 2

PB1 = Polymerase basic protein 1

PA = Polymerase acidic protein

HA = Hemagglutinin

NA = Neuraminidase

NP = Nucleocapsid protein

M = Matrix protein

NS = Non-structural protein

bp = Base pair

kb = Kilobase

nt = Nucleotide

ELISA = Enzyme-linked immunosorbent assay

MN = Microneutralization

HI = Hemagglutination inhibition

RDE = Receptor destroying enzyme

GMT = Geometric mean titer

BLAST = Basic local alignment search tool

m = Month

yr = Year

CDC = Centers for Disease Control

MOPH = Thai Ministry of Public Health

WHO = World Health Organization

CHAPTER I

Introduction

Acute respiratory infections are the cause of immense clinical problem worldwide, affect both adults and children, and involving upper and/or lower respiratory tract which are the cause of immense economic burden around the world (1, 2). Viruses are generally the main cause for respiratory tract infections (3-5). Respiratory infections can be caused by various respiratory viruses, most of them are rhinovirus (HRV), influenza virus, human coronavirus (hCoV), human adenovirus (hAdV), human metapneumovirus (hMPV), human respiratory syncitial virus (hRSV) or human parainfluenza virus (hPIV). Although most of virus infection only affect upper respiratory tract and are not serious in healthy individual, but viral infection in lower respiratory tract can cause pneumonia (6), even in patient without chronic lung conditions, resulted in hospitalization, especially in patient with chronic respiratory conditions. This thesis will focus mainly on influenza viruses which are the major causes of respiratory tract diseases in the human population, cause up to 500,000 deaths per year worldwide (7). Influenza is highly contagious diseases which cause annual epidemic, despite effective vaccine and antiviral medicine, and periodic pandemics that have tremendous impact to morbidity and mortality as experienced throughout human history. Therefore, surveillance of epidemiology of seasonal influenza viruses in the population will help understand the pattern of influenza transmission and infection, assist in designing suitable methods of intervention prior to the next influenza pandemic. Additionally, this study has provided an insight into sensitivity aspects of deep-sequencing approach for virus diagnosis in clinical and public health settings by comparing with standard real time diagnostic (RT)-PCR assays, also with evidence for the detection of viruses that may associated with patients' condition.

Part 1: Serological analysis of human pandemic influenza (H1N1) in Thailand during 2009-2010

Research Questions

1. How many individuals have the protective antibody in Chumphae population after the human pandemic influenza (H1N1) outbreak in 2009?

2. Is there any significant difference in seroprevalence against Human Pandemic Influenza (H1N1) among age group?

3. Are there any different in HI (hemagglunitation inhibition) antibody response among health care personnel that divided into three groups? (high, intermediate and low risk; based on their areas of work)

Objectives

1. To determining the prevalence of Seroprotective HI titers against pandemic influenza (H1N1) 2009 among population in Chumphae, Khon Kaen province.

2. To examine distributions of antibodies against the influenza virus in different age groups.

3. To investigate rate of seropositivity in health care personnel which were at a higher risk for infection than the general population.

Part 2: Epidemiology of seasonal influenza virus in Thailand

Research Questions

1. What are the incidences of influenza infection in ILI (influenza-like-illness) patients?
2. Do meteorological factors affect influenza seasonal activities in Thailand?
3. Is there any different in influenza seasonality pattern in Bangkok, Thailand in comparison with countries with similar climate?

Objectives

1. To establish epidemiological profiles of influenza viruses in Thailand.
2. To investigate the seasonal pattern of influenza activity in Thailand.
3. To determine whether meteorological factors are associated with influenza virus incidence.

Part 3: Application of next-generation sequencing in diagnostic detection of respiratory viruses

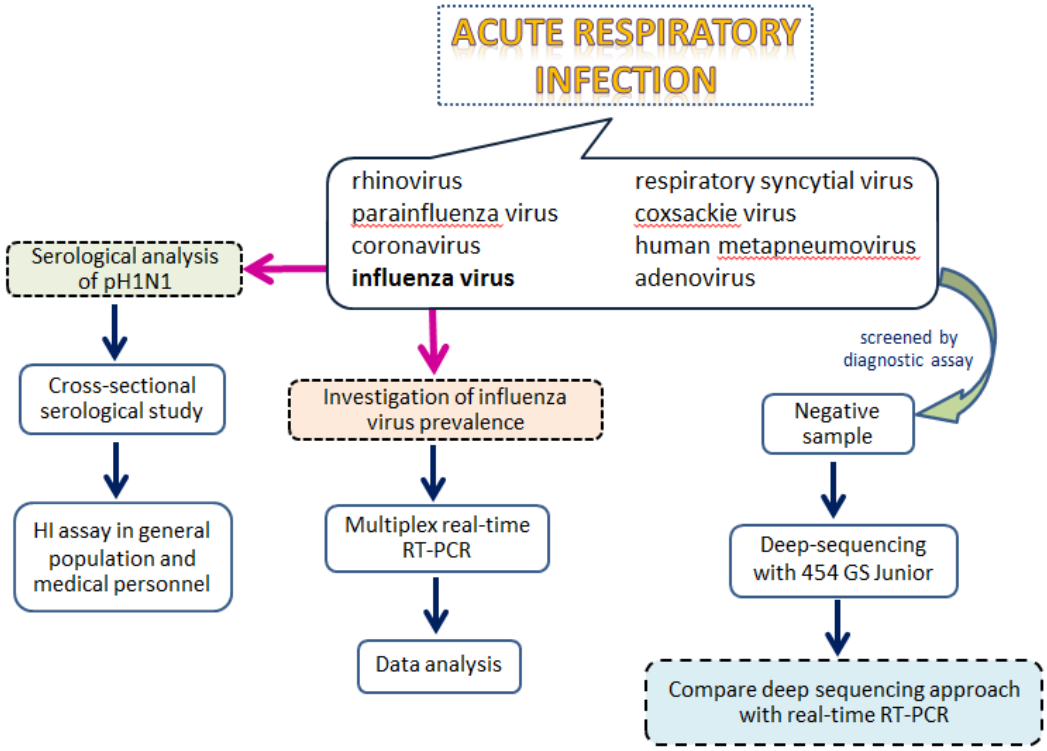
Research Questions

1. Will deep-sequencing approaches comparable with the gold standard real-time reverse transcriptase PCR (real-time RT-PCR) assays?
2. Are there any new viruses that may associate with patients' with respiratory condition after diagnostic screening is negative?

Objectives

1. To obtain insight into sensitivity aspects of deep-sequencing approach when compare with standard real time diagnostic PCR assays, in a cohort of clinical nasopharyngeal aspirates of children with respiratory disease.

Conceptual framework



CHAPTER II

Review of related literatures

Influenza virus

There are three genuses in this virus family, influenza A, B and C, based on their antigenic differences in the nucleocapsid (NP) and matrix protein (M), but types that cause significant disease in humans are A and B. Influenza A is the most common subtype responsible for the serious epidemics and pandemics in human history. Influenza B viruses, which infect only humans (8) and seals (9), cause infections about 20 % of the yearly cases, but does not cause pandemics. The virus is easily spread from person to person by droplets and fomite causing mild to severe illness. Seasonality of the influenza incidence can be commonly found in north and south hemispheres, and usually occurs all year round on equator.

1. Clinical

Symptoms caused by influenza virus often referred as influenza-like–illness (ILI), unless diagnostic test confirms as other respiratory viruses because a wide range of respiratory viruses can also cause a similar symptoms (2, 10, 11), although the typically dominant virus that cause ILI are influenza viruses [10]. Case definition can be defined as an abrupt onset of fever (>38°C) and/or malaise/ headache/ muscle pain with cough and/or sore throat (12). Although most of people recover within few weeks, but in older people and risk group such as young, elderly and chronically ill patients may take much longer as they are more at risk at complications (13). The infection is usually an infection of upper respiratory tract. Sometimes virus can replicate in lower respiratory tract, causing a viral pneumonia that can lead to death (14, 15). However, pneumonia can cause by secondary infection with bacteria after the virus infection weaken the host immune system; *Streptococcus pneumonia* and *Staphylococcus aureus*, both are the most frequent and life threatening (16-18).

2. The viruses

Influenza A virus belongs to the family *Orthomyxoviridae* which is a lipid enveloped virus with a size of approximately 80-100 nm in diameter (Figure1), and are pleomorphic in shape. The envelope is covered with a spike glycoproteins, HA (hemagglutinin) and NA (neuraminidase), which can be seen clearly under the electron microscope (EM). The hemagglutinin is the most antigenic surface protein of virus, and has been a main target for the body's immune system's responses. Function of the HA is to allow the virus to enter the cell by attach to the cell receptor which is sialic acid. About 80 percent of proteins are HA and the remainders are another antigen, NA. The NA has an important role to release the new virus from the cells by the cleavage of sialic acid residues from proteins and glycolipids.

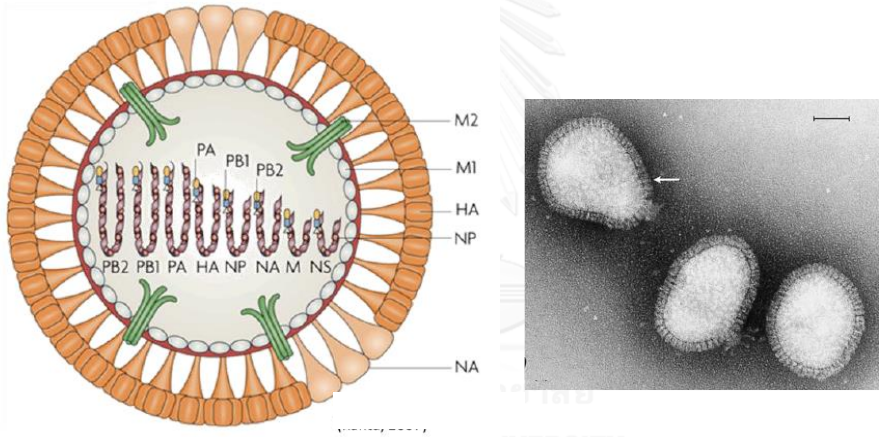


Figure 1. *Influenza A virus particle. (a) A schematic of the influenza virus (19). (b) Electron micrographs of negatively stained influenza virus. The surface HA and NA antigens are arrowed. Scale bar = 50 nm.(20)*

2.1 Genome

Influenza A genome composed of eight segments negative single-stranded RNA that encode 11 viral genes as shown in Table 1 (13, 21), 13 kb in size. Genes encoded for viral vRNPs (viral RNA nucleoproteins) are polymerase basic protein 2 (PB2), polymerase basic protein 1(PB1; alternate reading frame PB1-F2), and polymerase acidic protein (PA). These three complexes play role as viral RNA dependent RNA polymerase initiates the RNA synthesis to replicate the viral genome. The segments which encode for structural proteins are hemagglutinin (HA), neuraminidase (NA) and nucleocapsid protein (NP). The M segment encodes both matrix protein (M1) and the ion channel protein (M2) which creates a low pH environment in virion in order to release ribonucleoprotein into the cell's cytoplasm. The RNP is then transported to the nucleus where viral RNA replication is initiate. The NS segment encodes the non-structural (NS) protein NS1 and spliced product NS2, which acts as a nuclear export protein (NEP). The influenza viruses non-structural protein NS1 has been identified as a common factor which the virus uses to antagonize host immune system responses by interfering with IFN production (22). The NS2 role is known as the nuclear export protein (NEP). The role is performed by transporting newly synthesized viral ribonucleoproteins (RNPs) from the nucleus (23).

Table 1. *Characteristics and functions of genes of influenza.*

Segment	Length(bp)	Gene Product	Size (a.a.)	Function
1	2350	PB2	759	Cap binding, endonuclease
2	2350	PB1	757	RNA polymerase
3	2250	PA	716	RNA polymerase subunit, proteolysis
4	1780	HA	560	Attachment to sialic acid, membrane fusion
5	1575	NP	498	Encapsidates RNAs, nuclear import of RNA
6	1420	NA	450	Sialidase activity, release of virus
7	1050	M1	252	Major structural protein, virus assembly
		M2	96	Proton channel, viral uncoating
8	900	NS1	230	Down-regulate anti-viral response
		NS2	121	Nuclear export of RNPs

2.2 Replication

After attachment of HA glycoprotein and sialic acid residues on the cell membrane, virions are taken into cell by receptor-mediated endocytosis (24) and then transported through endosomes. In order to release viral genome into host cell cytoplasm, an increasingly of acidic pH by virus M2 ion channel, induces a change in the HA conformation (25). This rearrangement consequently disassembled virion into the cytoplasm. Then RNP:RNA complex will enter the cell nucleus through the nuclear pore as an intact for transcription and replicated by polymerase complex. The new virion will be released by budding from the

infected cell. This process involved the catalytic function of the NA for cleaving from viral and cellular surface glycoprotein, and preventing aggregation of progeny virions.

2.3 Genetic variation

To classify into subtypes, the surface HA and NA protein combinations are used for viral characterization. There are 18 different HA subtypes (H1-18) and 11 different NA subtypes (N1-11) which make many possible HA and NA subtypes (26, 27). Until now, only seven subtypes are reported to infect humans (H1N1, H2N2, H3N2, H5N1, H7N7, H7N9 and H9N2). Only H1, H2 and H3 are found transmit easily between humans. Others are found to be infecting animals, including birds, pigs, horses, seals, cats, dogs and recently discovered H17-H18 in bats (Figure 2) (28). Although, human and swine can be infected with same subtypes (like human influenza virus subtype H1N1 and swine influenza virus subtype H1N1) but the viruses itself are antigenically very *different* between the different hosts as the receptor binding efficiency of HA is specific to host sialic acid receptor (29-31). Many studies suggested that migrating aquatic fowls are the major source of influenza viruses reservoir although not all of these subtypes are virulent in their natural host. Most of pandemic human influenza virus strains resulting from genetic reassortment with viral genes from water fowl gene pool (32-37). There is an assumption that the human pandemic strains have arisen from the close association between human and animal, increasing the chances of exchange of viruses between human and other domestic animals, such as pigs and birds (38, 39). Many studies suggested that reassortment and adaptation between avian and mammal influenza are likely to occur in an intermediate host like the pig (34, 40). Because pig's epithelial cells contain both avian-like and human-like receptors so they are susceptible for both avian and human viruses (41, 42). The processes of reassortment are likely occurred from sharing habitat between wild and domestic animals and/or live animal trading market. A1997 outbreak of H5N1 avian influenza was the first avian viruses that successfully cross specie to infect in human. After that, there are reports of H7N7, H7N9, H9N2, H10N7 and H10N8 that caused illness in humans (43, 44). These cases are rare, and most of the cases usually involved direct

contact with infected birds and apparently failed to spread efficiently in human population (45-49).

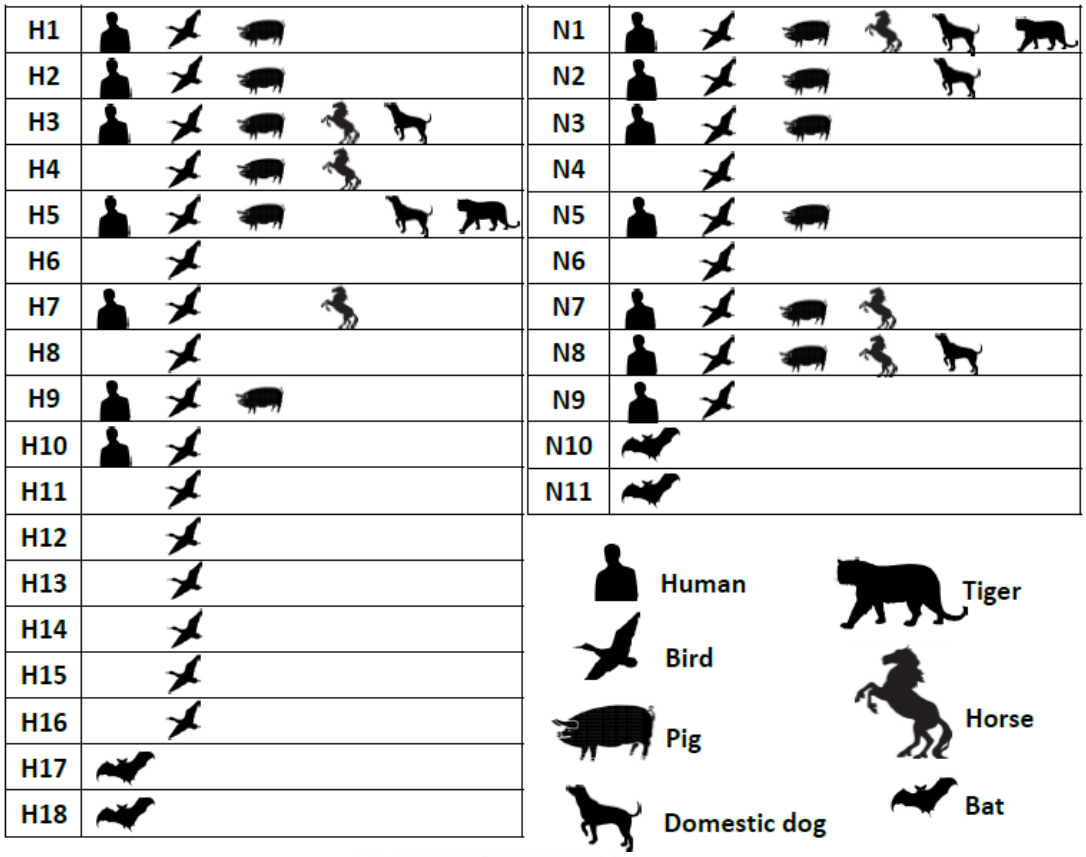


Figure 2. *Examples of HA an NA subtypes identified in influenza A virus infection.*

The Influenza virus can easily undergo genetic variation because it has several segmented genomes which allow reassortment of gene segments between two or more different subtypes, with genetic composition that differ from general circulation strains (34).

Genetic drift is a gradual process of small change due to the high mutation rate of RNA-dependent RNA, allowing influenza to accumulate the replication error and change slightly so pre-existing antibodies would no longer recognize new strains. Many studies showed evidence that HA protein is subjected to most intense positive selection pressure because the virus gradually accumulate the mutation on this gene, to evade from host's immune response (50-52). If the exchange pieces are HA and NA, it will produce a major

change in the influenza virus and give rise to a new subtype which can lead to an epidemic outbreak or pandemics (53), this process is called genetic shift. However, after the outbreak for some period of time, pandemic influenza virus will usually become a seasonal influenza virus.

Pandemic influenza

Throughout history, there were unpredicted 4 pandemics of influenza A resulting in high number of illnesses and deaths. Such pandemics occurred in 20th century: 1918, 1957 and 1968; and in the 21st Century: 2009–2010. The lethal pandemic in 1918 (H1N1 Spanish flu) killed about 50 million people worldwide. After that, there were the less devastating pandemics in 1957 (H2N2 Asian flu) and 1968 (H3N2 Hong Kong flu) (54). Theses 3 outbreaks associated with severe disease progression even in young healthy adults (55).These strains of viruses show that the genetic shift and successful adaptation of animal influenza viruses to humans could result in global influenza pandemics. In early March 2009, there was an emergence of a new strain of virus from North America, which rapidly became responsible for the spread of respiratory illnesses around the world because most of the world's population have not had pre-existing immunity (56, 57). Although pandemic (H1N1) 2009 was relatively mild, the virus caused highest attack rate and increasing the risk of severe illness mainly in children and young adults (<65 years old), contrast to finding in seasonal influenza (58). Research has revealed that this strain of influenza resulted from a triple reassortant between human, avian, and swine influenza viruses (59). After that, the human pandemic influenza virus 2009 (H1N1) was a dominant circulating strain in each influenza season in most parts of the world. On 10 September 2010, the World Health Organization (WHO) has announced that pH1N1 has entered the post-pandemic period as the number of cases began to decrease and started to behaved like seasonal influenza (60). Currently, epidemic from seasonal influenza virus strains continue to cause local outbreaks of influenza A viruses are subtype H1N1, H3N2 and influenza B viruses.

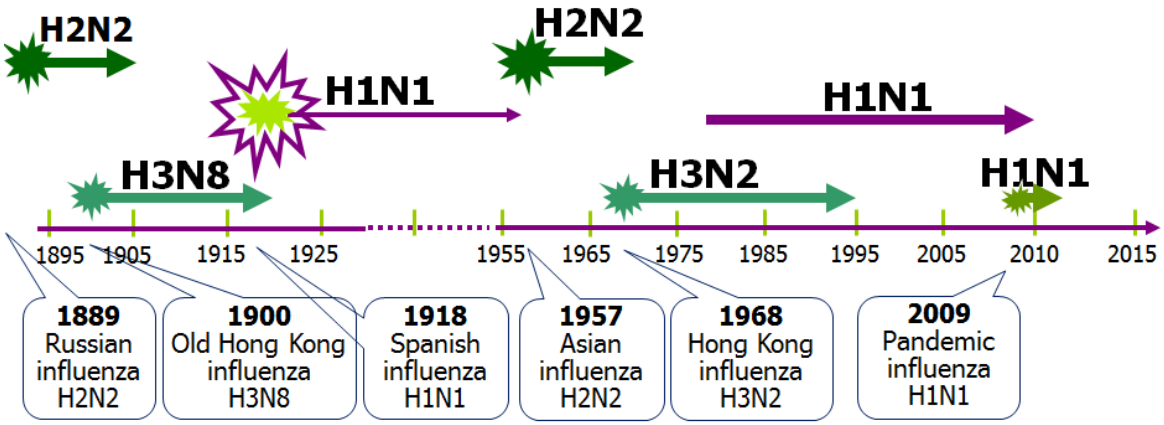


Figure 3. Timeline of human influenza pandemics.

Laboratory diagnostic for influenza virus

For predicting health trends and managing influenza virus infection, World health organization (WHO) recommended that the monitoring of influenza virus should be conducted as a routine surveillance for rapid identification of important changes in influenza virus. Serological surveys can show an evidence for past infection and can be used as a method for virus detection, by presence of specific antibody and four-fold or greater rising titer of antibody.

To determine the circulating strains, specific laboratory testing that will exclude other respiratory pathogens will be needed. The detection can target against viral antigen, nucleic acid or antibody (61-63). Rapid diagnosis can reduce hospitalization and chance to develop complications by treating them with antiviral medicine. Nowadays, nucleic acid amplification methods have been used for the detection of respiratory viruses (62, 64). These assays show greater sensitivity than other conventional methods such as virus culture and antigen detection assays. The most widely used method for molecular screening is real-time reverse transcriptase PCR (real-time RT-PCR), as a standard golden method, by developing an in-house assay or from commercial kit. This highly specific and sensitive method capable of detect various types of influenza virus in single test by using set of primers and fluorescence probes, can be used to identify genetic materials of influenza virus in terms of qualification and quantification. Nevertheless, reagents are expensive and required specialized equipment.

Various assays can be utilized for antibody detection, such as Enzyme-linked immonosorbant assay (ELISA), HI (hemagglutination-inhibition) test and MN (microneutralization) test. The microneutralization test is a gold standard technique for measuring influenza specific neutralizing antibodies in sera. However, this technique required expertise technical skill and more labor intensive. Another serological method, HI test, is the fundamental tool to determine serological evidence in sera samples and commonly used to predict and evaluation of vaccine efficacy. The HI test presents more simple process, less expensive which appropriate for large scale analysis. This assay is based on binding ability of HA protein to receptors on the erythrocyte surface lead to agglutination. The presence of neutralizing antibodies in sera against HA will inhibit hemagglutination proportionally to the concentration of antibodies (65, 66).

Viral metagenomics

In recent years, next-generation sequencing (NGS) has been widely used for virus detection and virus discovery when patients or animals got disease with unknown Etiological agents, whole genome construction from a poorly characterized virus, including human genetic mutation (67-74), as these technologies can be performed in large scale analysis and also gradually become more cost effective with improving sequence chemistry. These technological advance systems can be utilized to explore a comprehensive sequencing of DNA sequences within a given sample, with or without prior information about sequences contrast to traditional sequencing approach. These techniques are capable of expand our knowledge in cause of human disease, whether it cause by virus or other organism. Although this technology is very useful in many research areas and possibility to use in routinely clinical practice, there are concerns about how to handle with data management and result analysis with the bioinformatics pipeline which the need to improve the algorithm is essential. Many platforms are available as bench top sequencers; such as MiSeq (Illumina), 454 GS Junior (Roche), SOLiD (Applied biosystems) and Ion Torrent's Personal Genome Machine (Life Technologies).The performance of various NGS technologies has been reviewed elsewhere (75, 76).

The aim of this thesis is to observe seasonal influenza epidemiological profile in Thailand during June 2009 to July 2014, together with the cross-sectional serological survey of influenza A(H1N1)pdm09. This epidemiological data will assist in understanding which factors contribute to virus emergence in tropical countries, and thus in developing strategies for outbreak prevention. Moreover, sensitivity of deep sequencing approach was compared with routinely used real time RT-PCR assays. For last part of this thesis, as mentioned earlier, viruses are the most cause of acute respiratory tract infection, so it will be interesting to investigate if the negative samples that routinely screened by diagnostic assay will find new virus or not. Moreover, deep sequencing techniques has been used to detect or characterize causative agents in diseases other than traditional diagnostic assays, as this approach will provide more speed and requires little to no prior knowledge of the organism. However, sensitivity of deep sequencing is still in question when compare to specific PCR primer and probe. To obtain an insight, sensitivity of next generation sequencing approach will be compared with routinely used real time RT-PCR assays. This thesis was divided into 3 parts as follows;

CHAPTER III

SEROLOGICAL ANALYSIS OF HUMAN PANDEMIC INFLUENZA (H1N1) IN THAILAND

(Part1.1)

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Slinporn Prachayangprecha[1], Jarika Makkoch[1], Sunchai Payungporn[2], Thaweesak Chieochansin[1], Chanpim Vuthitanachot[3], Viboonsuk Vuthitanachot[3], Apiradee Theamboonlers[1], Yong Poovorawan[1]

[1]Center of Excellence in Clinical Virology, Department of Pediatrics and [2]Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, and [3]Chumphae Hospital, Chumphae District, Khon Kaen Province, Thailand

CHAPTER III

Part 1.1: Serological analysis of human pandemic influenza (H1N1) in Thailand.

Summary

The study was aimed at determining the prevalence of pandemic influenza (H1N1) 2009 among patients with respiratory tract diseases during July-December 2009 using real-time reverse transcription polymerase chain reaction. Haemagglutination inhibition (HI) assay was performed to detect antibody titres against pandemic influenza in 255 medical personnel, 307 members of the general population during the second week of December 2009 in Khon Kaen province, Thailand, and in 100 stored sera collected from people of different age-groups during 2008. The results showed that the pandemic (H1N1) 2009 had occurred during July-December 2009. The results of the HI test after the wave of this outbreak showed that 123 (48%) of the 255 sera collected from the medical personnel, 109 (36%) of the 307 sera obtained from the general population, and only two of the 100 stored sera from 2008 contained antibodies (HI titres ≥40) against pandemic influenza. Antibody against the pandemic (H1N1) 2009 was found in at least one-third of the population. In conclusion, the prevalence of virus and serological data obtained from the study can be used as the serological background level of the Thai population after the July-December pandemic. Finally, the serological data might be useful for outbreak-prevention and control strategies and for the management of vaccination for the pandemic (H1N1) 2009 in Thailand.

Introduction

The pandemic influenza (H1N1) 2009 has emerged from North America since mid-March 2009 and rapidly became responsible for the spread of respiratory illness around the world. Since then, it has spread to over 208 countries, and the total number of laboratory-confirmed cases of the pandemic (H1N1) 2009 has exceeded 414,000, with at least 18,114 deaths worldwide (28 May 2010) (77). Research has revealed that the strain of virus is a triple reassortant among human, avian and swine influenza viruses (59). Further evidence has also supported that the haemagglutinin protein of this pandemic strain was derived from swine (78). An H1N1 virus was first identified from samples obtained from humans in 1918 when there was an outbreak of pandemic influenza A virus (H1N1) which spread rapidly across the world and caused millions of deaths worldwide. This worldwide outbreak occurred in several waves with increasing virulence (79). Transmission patterns of influenza are different according to weather conditions, such as temperature and humidity (80). As an example, in the Northern hemisphere, infections mostly occur from November through March whereas infections occur in the Southern hemisphere from July to December, and infections in the tropics tend to be spread throughout the year (81-83). In Thailand, like other tropical countries, the rate of infection due to influenza is normally sporadic throughout the year. It also tends to follow a biphasic seasonal pattern with an increase in the infection rate from June to August and from January to March (84).

The first confirmed cases of pandemic (H1N1) 2009 in Thailand were declared by the Bureau of Emerging Infectious diseases, Department of Disease Control, Ministry of Public Health, on 12 May 2009(85). The pandemic spread through overcrowded public areas, such as Pattaya and schools in Bangkok, and the virus was probably transmitted from person to person by droplet transmission (86). The frequency of pandemic (H1N1) 2009 infection was generally higher among children and young adults compared to seasonal influenza (87). Antigenic and genetic characterization of the pandemic (H1N1) 2009 virus has shown differences between pandemic (H1N1) 2009 and seasonal H1N1 influenza virus. Hence, the vaccine against human seasonal influenza virus (H1N1) is unlikely to provide cross-reactive immune response against the novel pandemic (H1N1) 2009 virus (88). Hemagglutinin (HA)

and neuraminidase (NA) are important glycoproteins on the surface of influenza viruses, and these act as surface antigens that can induce an immune response. Hence, administration of antibodies against these surface antigens can protect the host from infection; however, if the existing antibodies are directed against a different antigenic type or subtype of the influenza virus, they might not protect the host from the antigenic variant of the virus. The pH1N1 spread worldwide because previously developed antibodies to the antigenic site of this particular virus were not present in most of the people (89, 90).

To determine the host's immune response, various laboratory methods can be applied, such as haemagglutination inhibition (HI) test, enzyme-linked immunosorbent assay (ELISA), and microneutralization (MN) test. The HI test is a widely-used technique for measuring specific antibody against influenza as it presents a relatively simple and inexpensive method which has been extensively used in epidemiological studies of influenza virus infection

In this study, we have investigated pandemic H1N1 influenza by performing real-time reverse transcription polymerase chain reaction (rRT-PCR) for the detection of virus in throat or nasopharyngeal swab specimens obtained from randomly-selected patients with respiratory tract diseases. Using the HI test, we have serologically screened medical personnel and the general population in Chumphae district, Khon Kaen province, Thailand, for specific antibodies against the pandemic (H1N1) 2009 virus. Thus, the evaluation of antibody response to the pandemic (H1N1) 2009 among the population might be useful for the management of vaccine. Data will help prioritize certain groups within the population and could also assist in the recommendation for vaccine and management of vaccine strategies.

Material and methods

Sources of specimens

Chumphae in KhonKaen province was selected as the site for epidemiological studies and surveillance on the pandemic (H1N1) 2009. Chumphae district, with a population of approximately 650,000, was selected for representing a suburban area of Thailand. The district is located in the northeast of Thailand, approximately 449 km from Bangkok (Figure 4) Specimens and sera collected from the participants were sent to the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, within 48 hours. The sera were kept at -20 °C until tested.

Figure 4. *Map of Thailand showing location of Chumphae district.*

Nasopharyngeal/throat swab specimens

We collected nasopharyngeal or throat swab samples from 20 patients (15 outpatients and 5 inpatients) with symptoms of acute respiratory tract infection, such as fever, sore throat, cough, rhi-norrhoea, nasal congestion. Samples were collected each week during

July 2009–December 2009. The specimens were collected in 2 mL of viral transport medium containing antibiotics (penicillin G (2 × 106 U/L) and streptomycin 200 mg/L) kept on ice and sent to the Center of Excellence in Clinical Virology within 48 hours for the diagnosis of influenza infection. All the samples were screened for the pandemic (H1N1) 2009, human seasonal influenza A (H1N1 and H3N2), and influenza B virus by rRT-PCR (91).

Population for testing of HI antibody

People diagnosed with immunodeficiency, bleeding disorder, and any chronic respiratory diseases, such as bronchial asthma, bronchiectasis, or not willing to participate in the study, were excluded. The population to be studied was divided into the following two groups:

General population

Three hundred and seven cross-sectional serum specimens were collected from healthy volunteers, devoid of respiratory symptoms, residing in Chumphae district by invitation for screening of antibody against the pandemic (H1N1) 2009. They comprised 185 males and 122 females, with a median age of 30 (range 5–92) years. These serum samples were included in the study protocol to represent the baseline for HI seropositivity in the general population and then used for comparison with healthcare personnel.

Healthcare personnel

Two hundred and fifty-five sera were collected by notification from healthcare workers at the Chumphae Hospital, Khon Kaen province, for screening the pandemic (H1N1) 2009. Their median age was 32 (range 19–56) years. Sera were divided into three groups based on their areas of work.

High-risk group: Specimens were collected from healthcare personnel who had close contact with patients with respiratory tract disease or collected specimens from patients with clinical respiratory tract infection during the outbreak of the pandemic influenza (H1N1) 2009 or had contact with a person with laboratory-confirmed pandemic (H1N1) 2009.

Intermediate risk group: Specimens were collected from healthcare personnel who worked in a high-risk area but were not directly exposed to patients with respiratory tract disease or collected specimens from patients with clinical respiratory tract infection

Low-risk group: Specimens were collected from healthcare personnel who work in the hospital but were directly exposed to patients, such as office workers, electricians, secretaries, and accountants.

Control baseline group

One hundred serum specimens were collected from individuals aged 11–86 years from Chum Saeng district of Nakhon Sawan province (Fig. 4) in March 2008 before the pandemic (H1N1) 2009. Stored serum samples were selected from anonymous specimens with only the age range known. These specimens were collected for a study project on antibody to avian influenza (H5N1).

Laboratory methods

Detection of influenza viruses by real-time RT-PCR

RNA was extracted from 200 µL of each nasopharyngeal swab using the Viral Nucleic Acid Extraction kit (RBC Bioscience Co, Taiwan) according to the protocol of the manufacturer. The extracted RNA was used as a template for the detection of infection due to influenza virus. Primers, specific TaqMan probes, and thermal profiles for the detection were as previously described (87, 91, 92). rRT-PCR was performed using the SuperScript III Platinum One-Step RT-PCR system (Invitrogen, Foster city, CA) in a Rotor-Gene 3000 (Corbett Research, New South Wales, Australia).

Propagation of influenza virus

The pandemic (H1N1) 2009 virus used in the study was A/Thailand/CU-H88/09 (accession numbers: HM446345 and HM446344). The influenza virus was propagated by inoculation of virus stock into the allantoic cavity of 10-day old embryonated chicken eggs. The inoculated eggs were placed in an egg incubator at 37 °C for 48 hours. The allantoic fluid was harvested and subjected to centrifugation at 3,000 rpm for 10 minutes. The supernatant was tested for viral titers by haemagglutination assay (HA). Virus-propagation procedures were carried out in a biosafety level 2+ (BSL2+) laboratory.

Haemagglutination inhibition assay

To abolish non-specific HIs in serum samples, sera were treated with RDE (receptor destroying enzyme) produced by *Vibrio cholera* Ogawa type 558 (Denka Seiken, Co. Ltd., Tokyo, Japan) following the specifications of the manufacturer. Briefly, serum and RDE were mixed in a ratio of 1:3 and incubated at 37 °C for 18 hours. Subsequently, the mixture was incubated at 56°C for 30 minutes to inactivate the RDE and complement system, followed by 10-fold dilution with phosphate-buffered saline (PBS). Two-fold serial dilutions of RDE-treated sera (25 μL) were incubated with eight HA units of pandemic influenza virus (25 μL) per well in a V-shaped 96-well plate (Greiner Bio-One GmbH, Kremsmuenster, Austria) for 30 minutes, followed by addition of 50 μL of 0.5% turkey red blood cells and incubation at room temperature for 30 minutes (65). HI titers of ≥1/40 were considered positive antibody response.

Statistical analysis

Data were analyzed using the SPSS software for windows (version 17). Chi-square test was used for comparing the different groups.

Ethics

The Ethics Committee of the Faculty of Medicine, Chulalongkorn University, approved the protocol of the immunological study. Permission for pandemic influenza H1N1 surveillance was granted by the director of Chumphae Hospital, KhonKaen, to facilitate outbreak control and establish preventive measures. All participants were informed about the objectives of the study, and their written consents were obtained before collection of specimens.

Results

Detection of influenza viruses by real-time RT-PCR

Five hundred and fourteen specimens were collected from children (233 patients) and adults (281 patients), aged 25 days-86 years. The number of samples and percentage positive for the pandemic (H1N1) 2009 are depicted in Figure 5. From July, the numbers of positive samples increased and reached the highest peak by the end of August 2009 and

started to decline from September to December 2009. In contrast, the seasonal influenza strains (H3N2 and influenza B) circulated in a low level throughout the year.

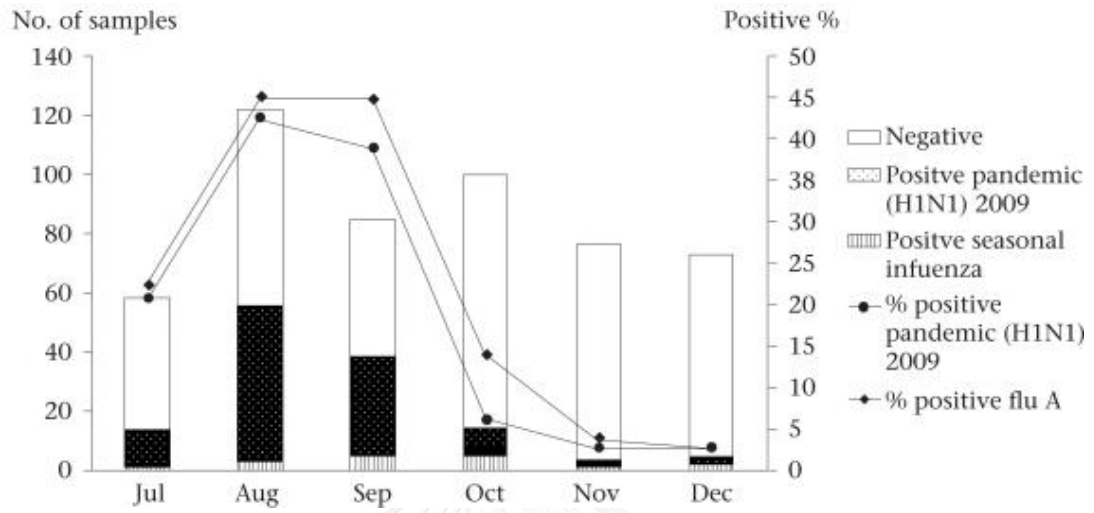

Figure 5. *Positive percentage of nasopharyngeal or throat swab samples for pandemic (H1N1) 2009 and human seasonal influenza viruses from Chumphae Hospital, Khon Kaen province, Thailand, between July and December 2009.*

HI antibodies against pandemic (H1N1) 2009 in healthcare personnel

Based on the HI test performed on 255 healthcare personnel at the Chumphae Hospital, 123 samples (48%) contained specific antibodies (HI titres ≥40) against the pandemic (H1N1) 2009. A comparison between groups of medical personnel showed that the percentage of samples displaying specific antibodies against the pandemic (H1N1) 2009 was 58% (70/120) in the high-risk group, 35% (29/82) in the intermediate risk group, and 45% (24/53) in the low-risk group. Data are summarized in the table 2. A comparison between antibody-positive risk groups showed a significant ($p<0.05$) difference between the high-risk group and the intermediate risk group (Table 2)

Table 2. *Hemagglutination inhibition (HI) antibodies titers against human pandemic influenza (H1N1) among health care personnel in Chumphae hospital, Khon Kaen province, Thailand.*

HI titers	High Risk N= 120 (47.1%)	Intermediate Risk N= 82 (32.5%)	Low Risk N= 53 (21.0%)
20 to ≤ 10, %	41.7	64.6	54.7
(No.)	(50)	(53)	(29)
40, %	31.7	18.3	22.6
(No.)	(38)	(15)	(12)
80, %	19.2	12.2	17.0
(No.)	(23)	(10)	(9)
160, %	6.7	-	5.7
(No.)	(8)		(3)
320, %	0.8	3.6	-
(No.)	(1)	(3)	
640, %	-	1.2	-
(No.)		(1)	
%positive (≥40)	58.4	35.3	45.3
(No.)	(70)	(29)	(24)

Antibody response between high-risk group and intermediate risk group was significant ($p<0.05$); HI titres of ≥40 were used as positive; HI=Haemagglutination inhibition

HI antibodies against pandemic (H1N1) 2009 in the general population

The results of the HI test performed on 307 members of the general population, aged 5–92 years (age: 5–10 years: n=18; 11–20 years: n=24; 21–30 years: n=11, 31–40 years: n=23; 41–50 years: n=41, 51–60 years: n=31, 61–70 years: n=30 and over 70 years: n=26) in Chumphae district showed that 109 samples (36%) displayed specific antibodies (HI titres ≥40) against the pandemic (H1N1) 2009. Details of the positive HI antibody response (HI titres >40) among different age-groups are shown in Figure 6.

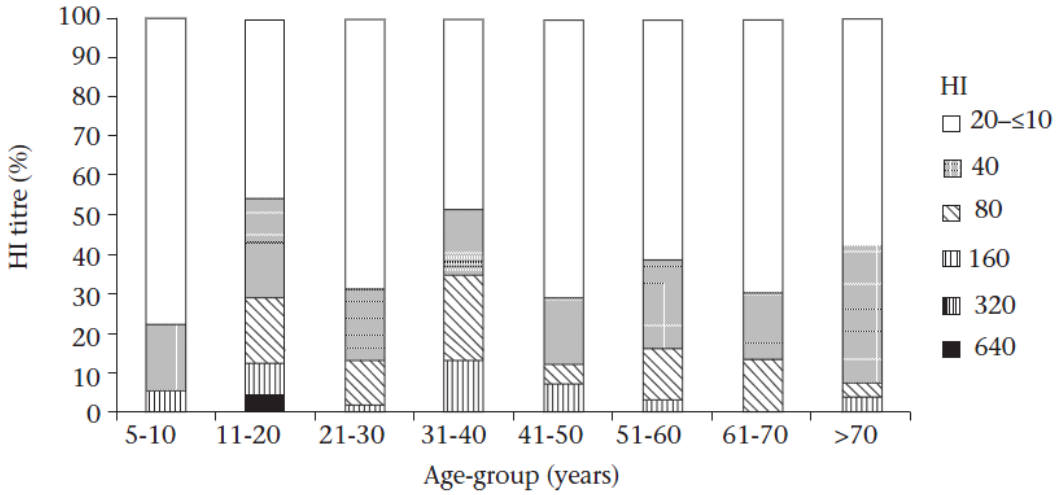

Figure 6. *Percentage of HI antibody titers against pandemic (H1N1) 2009 in relation to age among the general population in Chumphae district, KhonKaen province, Thailand, 2009.*

HI antibodies against pandemic (H1N1) 2009 in 2008 control group

The results of the HI test showed that two (2%) of 100 anonymous control sera collected during 2008 had HI titres of ≥40 against the pandemic (H1N1) 2009. The positive specimens were derived from two individuals aged over 50 years.

Discussion

In this study, we have performed the HI assay for determining the antibody levels against the pandemic (H1N1) 2009 to better understand the infection rate and estimated immunity in the population. High numbers of patients diagnosed with the pandemic (H1N1) 2009 were observed during July–September 2009 and then decreased towards the end of the year (Fig. 5). The serum samples collected for HI test in December showed high seropositivity against the human pandemic (H1N1) 2009, implying that approximately one-third of the Chumphae population developed antibodies against the pandemic (H1N1) 2009 at that point in time.

According to a previous study, the HI test in paired serum samples of patients with influenza-like illness (ILI) diagnosed with the pandemic (H1N1) 2009 showed a significant four-fold increase in antibody titers upon comparison between acute and convalescent sera (93). To determine cross-reactivity between seasonal influenza and pandemic (H1N1) 2009, the HI test was performed on acute and convalescent sera of patients with ILI and healthy young adults collected before the outbreak. The results showed that there were no cross-reactivity between antibody against the seasonal and pandemic (H1N1) 2009.

The HI test performed on sera of the medical personnel demonstrated a high percentage (48%) of samples with a positive HI titre against the pandemic (H1N1) 2009. In all probability, these individuals were at a higher risk for infection than the general population due to exposure to clinical specimens of respiratory tract disease and close contact with infected patients.

A comparison between the individual groups of the medical personnel showed that the high-risk group exhibited the highest percentage (58%) of antibody titres whereas the intermediate risk group and the low-risk group presented a lower percentage of antibody titres at 35% and 45% respectively. In the intermediate risk group, the positive HI percentages were lower than those found in the low-risk group. The lower HI titres in the intermediate group might be due to the level of exposure and personal awareness, including knowledge of appropriate protection, such as gloves, respiratory mask, and protective clothing while the low-risk group limited their protection to washing their hands. However, the antibody response

in the intermediate risk group did not show a significant difference from that in the low-risk group.

The HI titers among the general population showed that 36% of the Chumphae residents already had developed immune response against the pandemic (H1N1) 2009 virus. Yet, other studies have shown a lower rate of seropositivity (11% and 13% in studies of China and Singapore respectively) (94, 95), which might have been due to the differences in geographic location since various factors, such as overcrowding, climate, and personal hygiene, can affect the transmission efficiency of virus. Hence, the number of people infected with the pandemic (H1N1) 2009 may be different in some areas studied.

The elevated seroprevalence of the pandemic (H1N1) 2009 in the 11-20-year age-group (54%) may be explained by schools serving as gathering places for children and young adults in class-rooms and group activity which create ideal conditions for the transmission of the pandemic (H1N1) 2009 virus, such as lack of or improper handwashing and good personal hygiene (96). The 31-40-year age-group exhibited 52% seroprevalence. These individuals tend to work in air-conditioned offices, use public transport, and attend meetings and other social functions, all of which favour transmission of the virus (97). The elderly have possibly acquired partial immunity since around 1977 when the H1N1 strain with a similar epitope was re-introduced into humans (56). In this study, 42% of the elderly aged over 70 years displayed antibodies against the pandemic (H1N1) 2009, which may have been elicited by previous exposure to a virus containing a similar epitope. Thus, the elderly had acquired some level of immunity against the pandemic influenza A (H1N1) strain. This could explain the lower rate of infection among the elderly people when compared with seasonal influenza and, likewise, why the new pandemic strain tended to affect the younger age-group (87).

In this study, a comparison of the stored serum samples collected in 2008 showed that the HI titer was positive in only two specimens derived from individuals in the age range of 51-60 years and over 70 years. A previous study conducted in the United States established that one-third of people born before 1950 had antibody against the pandemic (H1N1) 2009 (56). However, our baseline data from 2008 revealed a very low prevalence of antibody when compared with the results from sera collected in 2009. This finding was similar

to the findings from Japan and China obtained from sera collected before the outbreak (78, 94).

The results of the HI test showed that the population in Chumphae area had developed immunity against the pandemic (H1N1) 2009. This level of immunity among the population can probably lower the infection rate during the next wave due to an effect called 'herd immunity' (herd immunity=1-1/R0) (98). Herd immunity can protect non-immunized individuals from infection. The estimated basic reproduction number (R_0) of the pandemic (H1N1) 2009 was 1.4-2.1 (99-101). Once the herd immunity reaches 30-50% of the population, it will be sufficient to lower and control the infection rate of the pandemic (H1N1) 2009. As with previous patterns of pandemic, the virus can still cause sporadic infection in non-immunized individuals, although there is no severe outbreak and, at some point, the virus will become a common strain that continually circulates in the human population.

The HI assay was conducted in this study for determining the antibody levels against human pandemic influenza instead of the microneutralization assay. This would provide suitable data on neutralizing antibodies in sera but requires sophisticated laboratories for cell culture. The HI test represents a simpler screening assay and is a less-expensive process, providing acceptable serological data which would be feasible and attractive for large-scale analysis (102).

Conclusions

Based on the percentage of the pandemic (H1N1) 2009 cases diagnosed and the antibody levels against the pandemic (H1N1) 2009 virus measured, this study has shown that the Chumphae population had gained some level of immunity to the pandemic (H1N1) 2009 during the outbreak between July and December. Thus, the next pandemic (H1N1) 2009 wave may not impact Thailand as severely as suspected, and the disease will become seasonal influenza in the near future. This serological study has provided useful serological data that could help prioritize population groups for vaccination against the pandemic (H1N1) 2009 virus. Moreover, ongoing serological analysis would be essential for the

recommendation of vaccine and for the development of strategies to prevent future epidemics or pandemics.

CHAPTER III

EPIDEMIOLOGICAL AND SEROLOGICAL SURVEILLANCE OF HUMAN PANDEMIC INFLUENZA A VIRUS INFECTIONS DURING 2009–2010 IN THAILAND

(Part 1.2)

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Slinporn Prachayangprecha, Jarika Makkoch, Chanpim Vuthitanachot[1], Viboonsuk Vuthitanachot[1], Sunchai Payungporn[2], Thaweesak Chieochansin, Apiradee Theamboonlers, Yong Poovorawan

Center of Excellence in Clinical Virology, Department of Pediatrics and [2]Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok; and [1]Chumphae Hospital, Khon Kaen, Thailand

CHAPTER III

Part 1.2: Epidemiological and Serological Surveillance of Human Pandemic Influenza A Virus Infections during 2009–2010 in Thailand

Summary

Since April 2009, the outbreak of human pandemic influenza A (H1N1) virus (pH1N1) infection has spread from North America to other parts of the world, and currently, pH1N1 is the predominant circulating strain of influenza viruses. Our objectives were to perform a serological survey of medical personnel at the Chumphae Hospital in Thailand and to investigate the prevalence of pH1N1 in randomly selected patients diagnosed with respiratory tract disease. Prevalence of pH1N1 in the patients was determined by performing real-time reverse transcription-polymerase chain reaction. The study was carried out between July 2009 and November 2010. Seroprevalence of hemaglutination inhibition (HI) titers among medical personnel was established in three cross-sectional studies at the end of each wave of the pandemic by performing HI assay to detect antibodies against pH1N1. Infection by the pH1N1 peaked between July and October 2009; the second wave was from January to March 2010 and the third wave from June to November 2010. The HI titers after the first, second, and third waves were 48.2%, 22.4%, and 25.7%, respectively. After the second and third waves, 52.1% and 45.3% of the medical personnel who had received pH1N1 vaccination had HI titers ≥40. These findings show that seasonal influenza strain in Chumphae and the predominant influenza strain from each wave was pH1N1. HI assay results also represent the severity of the attack rate in each wave.

Introduction

Since April 2009, the outbreak of human pandemic influenza A (H1N1) 2009 virus (pH1N1) infection has spread from North America to other parts of the world, and this H1N1 strain has replaced the currently circulating seasonal influenza virus. On September 10, 2010, the World Health Organization announced the beginning of the post-pandemic period of pH1N1 infection (60).

In Thailand, the first confirmed case of pH1N1 infection was reported on May 12, 2009 by the Bureau of Emerging Infectious Diseases, Department of Disease Control, Ministry of Public Health (49). In the outbreak, pH1N1 cases were reported predominantly from schools and crowded communities; the high infection rate among children and young adults may be the result of group activities in schools (87).

Symptoms of pH1N1 infection are usually mild and similar to those of common cold, which presents with fever, headache, sore throat, and nasal congestion. However, patients with pH1N1 infection having underlying complications or chronic diseases are at an increased risk of hospitalization. The new strain of H1N1 has resulted from triple reassortment of human, avian, and swine influenza viruses, and thus, is antigenically distinct from the currently circulating strains of seasonal influenza H1N1 virus (4,5).

Hemagglutinin (HA) and neuraminidase (NA) are important glycoproteins on the surface of influenza viruses, and these act as surface antigens that can induce an immune response. Hence, administration of antibodies against these surface antigens can protect the host from infection; however, if the existing antibodies are directed against a different antigenic type or subtype of the influenza virus, they might not protect the host from the antigenic variant of the virus. The pH1N1 spread worldwide because previously developed antibodies to the antigenic site of this particular virus were not present in most of the people (6,7).

Protective antibody titers against an infection would develop either in response to annual vaccination or because of natural infection with the virus. Vaccination against circulating strains, which usually change every year because of antigenic drift in the virus, would optimize protection against the viral infection.

In this study, we conducted a cross-sectional serological survey of medical personnel at the Chumphae Hospital, Khon Kaen province, Thailand, by performing the hemagglutination inhibition (HI) assay. These patients represented the high-risk group. We also investigated the prevalence of pH1N1 infection by performing real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) on nasal or throat swab samples obtained from randomly selected patients diagnosed with respiratory tract disease.

Materials and Methods

Specimen collection

Chumphae district of Khon Kaen province in northeast Thailand was chosen as the site for epidemiological studies and surveillance on pH1N1 as it represents a suburban area of Thailand. The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Permission for pH1N1 surveillance was granted by the director of the Chumphae Hospital. The participants were informed about the objective of this study, and their written consents were obtained. All the specimens and sera were sent to the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University within 48 h of collection and were stored at -20°C until analysis.

Nasopharyngeal/throat swab sample collection

Nasopharyngeal or throat swab samples were collected on a weekly basis between July 2009 and November 2010. Samples were collected from approximately 20 patients (15 outpatients and 5 inpatients), who presented with symptoms of acute respiratory tract infection such as fever, sore throat, cough, rhinorrhea, and nasal congestion. The samples were then transported to the Center of Excellence in Clinical Virology in a viral transport medium (placed on ice) containing antibiotics (penicillin G, 2 × 106 U/L and streptomycin, 200 mg/L) within 48 h of collection. The samples were screened for pH1N1, human seasonal influenza A (H1N1 and H3N2) viruses, and influenza B virus by performing real-time RT-PCR analysis of the samples.

Study populations for HI antibody testing

Medical personnel: Cross-sectional serum samples were obtained from medical personnel of the Chumphae Hospital, including cleaners, electricians, and people who volunteered after every wave of the pandemic influenza. After the first wave in the second week of December 2009, 255 samples were obtained. After the second wave in June 2010, 397 serum samples were collected, and 366 serum samples were collected after the third wave of the pandemic in December 2010. The participants were volunteers. In addition to the blood samples collected, pH1N1 vaccination history, health status, and informed consents were also obtained from the participants.

Laboratory methods

Detection of influenza viruses by real-time RT-PCR

RNA was extracted from 200 mL of the nasopharyngeal swab using the Viral Nucleic Acid Extraction Kit (HiYield™; RBC Bioscience Corp., Taipei, Taiwan) according to the manufacturer's instructions. The influenza virus was detected using the extracted RNA as a template. Primers, specific TaqMan probes (BioDesign Co., Thailand), and thermal profiles for the reaction were the same used in previous studies (3,8,9). Real-time RT-PCR was performed using the SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, Foster City, Calif., USA) in Rotor-Gene 3000 (Corbett Research, New South Wales, Australia).

Influenza virus propagation

The virus used in this study was A/Thailand/CU-H88/09 (accession numbers, HM446345 and HM446344). The virus was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs placed in an egg incubator at 36.59 °C for 48 h. Allantoic fluid was then harvested, and the virus titers were determined by the HI assay.

HI assay

The serum samples were treated with receptor destroying enzyme (RDE) obtained from the bacterium Vibrio cholerae Ogawa type 558 (Denka Seiken, Tokyo, Japan) according to the manufacturer's instructions. The sera were diluted tenfold with phosphate buffered saline (PBS). RDE-treated sera were twofold serially diluted in a V-shaped 96-well plate

(Greiner Bio-One GmbH, Kremsmuenster, Austria) and incubated with 25 mL of 8 HA units of virus for 30min; this was followed by the addition of 50 mL of 0.5% turkey erythrocytes to the sera and incubation at room temperature for 30 min (10). Samples with HI titers greater than or equal to 1:40 were considered seropositive. Statistical analysis: We calculated the geometric mean titer (GMT) for the titer values obtained from the HI test for medical personnel who enrolled in HI test only; HI titers below 40 were excluded from the calculation. Analysis was performed using SPSS software, version 17 for Windows. *P*-value <0.05 was considered statistically significant.

Results

Detection of influenza viruses by real-time RT-PCR

In total 1,355 patients with age ranging from 25 days to 86 years were screened for influenza virus infections. The number of samples and percentage positive for the pandemic (H1N1) 2009 are depicted in Figure 7. During the study, there were three waves of influenza activity dominated by pH1N1. The activity of pH1N1 peaked for the first time between July and October 2009 (103), for the second time between January and March 2010, and for the third time between June and September 2010. By October 2010, influenza activity had started to decrease.

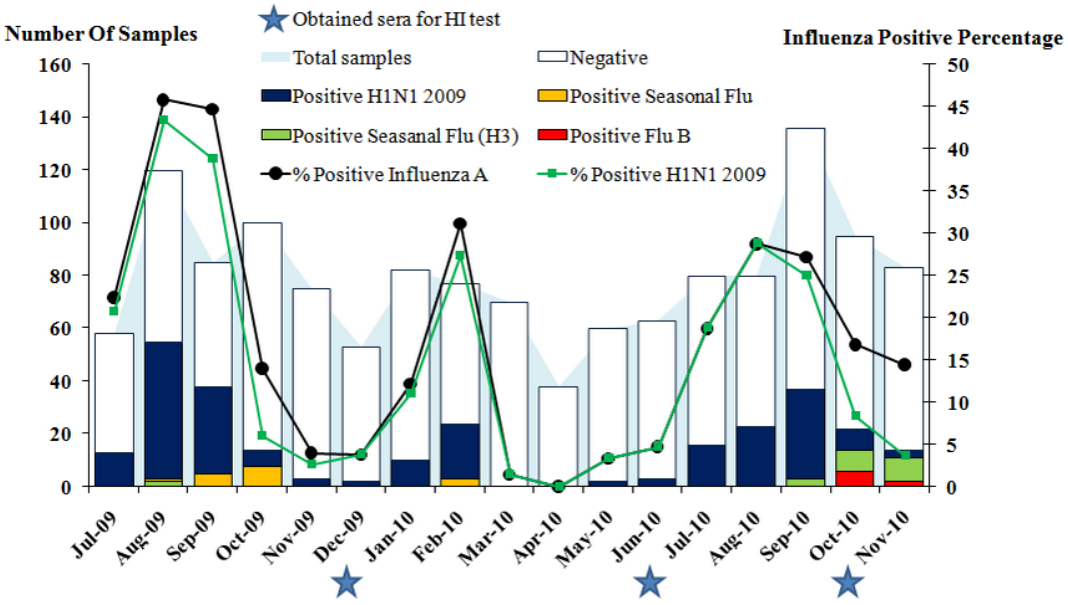

Figure 7. *Positive percentage of nasopharyngeal or throat swab samples for pandemic (H1N1) 2009 and human seasonal influenza viruses from Chumphae Hospital, KhonKaen province, Thailand, July 2009 – November 2010.*

HI assay

Table 3 shows the characteristics of the medical personnel observed after every wave of the pandemic influenza. The percentage of antibody-positive medical personnel is shown in Table 4.

Table 3. Demographic data of medical personnel.

	After		
	first wave	second wave	third wave
Total no.	255	397	366
Age			
15-30	115	68	29
31-40	76	66	33
>40	64	263	304
Sex			
Male: no. (%)	59 (23.1)	90 (22.7)	76 (20.8)
Female: no. (%)	196 (76.9)	307 (77.3)	290 (79.2)
pH1N1 monovalent vaccination			
Yes: no. (%)	-	71 (17.9)	75 (20.5)
No: no. (%)	-	326 (82.1)	291 (79.5)

Table 4. Hemagglutination inhibition (HI) titers to pH1N1 among medical personnel in Chumphae hospital, KhonKaen province, Thailand, 2009-2010.

Health care personnel	Seroprotective HI titer No. (%)	GMT (95% CI)
After the first wave	123 (48.2)	62.8 (42.9-76.3)
(n = 255)		
Age (no.)		
15-30 (115)	69 (60)	70.9 (49.1-92.8)
31-40 (76)	28 (36.8)	60.9 (39.2-82.7)
>40 (64)	26 (40.6)	46.9 (40.3-53.5)
After the second wave	89 (22.4)	117.2 (69.42-164.92)
(n = 397)		
Age (no.)		
15-30 (68)	24 (35.3)	89.8 (35.6-143.9)
31-40 (66)	8 (12.1)	61.7 (47.3-76.0)
>40 (263)	57 (21.7)	143.4 (74.8-212.0)
- Monovalent pH1N1 vaccination	37 (52.1)	166.1 (68.1-264.1)
(Three months after vaccination)		
(n = 71; mean age = 51.1 years)		
- Unvaccinated group	52 (16.0)	91.4 (55.4-127.4)
(n=326; mean age = 46.8 years)		
After the third wave	94 (25.7)	104.3 (69.1-139.6)
(n = 366)		
Age (no.)		
15-30 (29)	11 (37.9)	102.9 (42.0-163.9)
31-40 (33)	7 (21.2)	72.5 (32.6-112.3)
>40 (304)	76 (25.0)	108.1 (65.7-150.5)
- Monovalent pH1N1 vaccination	34 (45.3)	94.8 (65.6-124.0)
(nine months after vaccination)		
(n = 75; mean age = 52.6 years)		
- Unvaccinated group	74 (25.4)	97.4 (53.9-140.9)
(n=291; mean age = 51.7 years)		

HI antibodies against pH1N1 after the first wave

The HI assay was performed on samples collected from 255 medical personnel; 123 samples (48.2%) showed positive HI titers (HI titers ≥40) against pH1N1. Mean age was 34.0 years (range, 19–56 years). GMT after the first wave was 62.8 (95%CI, 49.2–76.3). Results of comparison between the risk groups have been reported elsewhere (103).

HI antibodies against pH1N1 after the second wave

Of the 397 study participants, 89 (22.4%) showed positive HI titers. Their mean age was 47.6 years (range, 14–87 years). GMT of medical personnel after the second wave was 117.2 (95% CI, 69.4–164.9). Of the 83 samples obtained from the participants who had been tested after the first wave for the presence of antibody against pH1N1, 26 (31.3%) showed HI titers against pH1N1 (GMT=56.6). However, the results of the assay conducted in June 2010 for HI titers against pH1N1 showed that only 6 participants of this cohort had antibody titers against pH1N1 (GMT=89.8).

HI antibodies against pH1N1 after the third wave

In total 94 of the 366 medical personnel showed positive HI titers (25.7%). The GMT for the medical personnel (mean age, 54.5 years; range, 17–86 years) calculated after the third wave was 104.3 (95% CI, 69.1–139.6).

Monovalent pH1N1 vaccination (calculation of GMT 3 months after vaccination)

Seventy-one participants had been vaccinated with pH1N1 monovalent vaccine (Sanofi Pasteur, Lyon, France) between February and March, 2010. Mean age was 51.1 years (range, 21–75 years), and 37 (52.1%) of the 71 participants had a positive titer in the HI test. GMT of the vaccinated participants was 166.1 (95% CI, 68.1–264.1). In the unvaccinated participants (n = 326), 52 had positive protective HI titers (≥40), with a GMT of 91.4 (95% CI, 55.4–127.4). GMT of the vaccinated cohort was significantly higher than that of the unvaccinated group ($P < 0.05$).

Discussion

In this study, we investigated the prevalence of influenza virus from July 2009 to November 2010. The pH1N1strain of influenza virus has become the predominant influenza strain circulating in the Chumphae district and has taken the place of the circulating seasonal influenza strains (influenza A/H1, A/H3, and B) since July 2009. The pH1N1 has shown a tendency to continue its activity and become the standard circulating seasonal strain in the future. In Thailand, influenza activity occurs in a biphasic pattern with sporadic infection throughout the year (84) as seen in other temperate countries of the northern hemisphere. Influenza is more prevalent in the rainy season than in the winter, as has been observed in other tropical countries (81).

Serological tests were performed for determining the antibody levels against the pandemic (H1N1) 2009 to better understand the infection rate and estimated immunity in the general population and on medical personnel who represented the high-risk group susceptible to infectious disease. Furthermore, if the medical personnel acquire an infection, it may be transmitted to patients, such as those with underlying chronic medical conditions, pregnant women, and immunosuppressed and immunocompromised patients, who are at a high risk of acquiring infections.

The baseline serum samples collected from individuals in Chum Saeng district, Nakorn Sawan Province in 2008 had shown very low positive HI titers (2%) against pH1N1 (103). Furthermore, tests showed no cross-reactivity between the seasonal influenza virus and the H1N1 virus in the HI assay, even though pH1N1 are antigenically distinct from the current seasonal strains and vaccination against the seasonal strain cannot provide protection against pH1N1 (93). In the previous study, HI assay performed after the first wave in the general population that had a lower risk of infection than the medical personnel did, showed that 36% of the study participants exhibited HI titers against pH1N1 with high seroprevalence in the age group of 11–20 years (103).

According to a previous study (103), 48.2% (123/255) of the study participants developed HI antibody titers after the first wave, while 22.41% (89/397) of the participants developed the same against pH1N1 after the second wave that occurred 6 months later. After

the third wave, 25.7% of the participants showed HI antibody titers. The HI titers after the first wave were high, and this can be explained by the fact that pH1N1 had spread globally in 2009. Although serum samples were collected 4 months after the first wave had peaked, at that point in time, the majority of the population had not developed antibodies against the virus. After the second and third waves, the HI titers were lower than that observed after the first wave, possibly because a proportion of the population had been infected during the first wave and had developed immunity against pH1N1.

Mean age of the participants in the first wave was lower (34.0 years) than that of the participants in the second and third waves (47.6 years and 54.5 years, respectively).The number of participants in the age group of 15–20 years in the first wave was higher than that in the second and third waves, and the number of participants in the age group above 40 years was lower in the first wave than in the second and third waves. This might also explain the higher HI titers after the first wave, as the pH1N1 infection rates were higher among the young adults as shown in Table 4. Seroprotective HI titers observed in the age group of 15–30 years were higher than those observed in other age groups during all the three waves.

This phenomenon can also be explained by the persistence of antibodies against HA, whose titers can decrease to two- and tenfold relative to the peak titers observed after the first 6 months (90). Hence, antibodies produced during the first wave did not persist long enough to be detectable during the second wave, and consequently, the overall rate of seropositive samples was low. On the other hand, the percentage of HI titers correlated with the pH1N1 peak during the second and third waves because HI titers observed at the peak of the second and third waves were lower than those in the first wave, indicating low infection rate. This may be another reason why HI titers observed after the first wave was higher than those observed after the two subsequent waves.

GMT calculated after the first wave (62.8) was lower than that calculated after the second (117.2) and third wave (104.3), indicating that although the number of persons who developed immune response during the second wave was low, GMT of the HI titers was higher after the second wave than after the first wave. This finding may be attributed to a

booster effect in the individuals, since serum samples were collected after the peak of the second and third waves.

In the group that had the second blood sample drawn from the same participants (83/397), 26 were seropositive as per the previous HI assay; however, only 6(21.4%) participants displayed HI titers after the second wave. This phenomenon also showed that the levels of the protective antibody had declined over time. The incubation period of the influenza virus during an infection is rather short (2–5 days); however, the body's immune response, which includes the release of antibodies, is initiated a few weeks after the onset of infection, and lasts for short durations.

To optimize protection against influenza infections, protective antibodies against circulating influenza strains are required. Hence, annual influenza vaccination is required to assure that a rapid and effective immune response can be initiated in the hosts.

Of all the participants who had received 1 dose of pH1N1 monovalent vaccine after the second wave, 52.1% showed seroprotective antibody levels (HI titers of at least 40). This showed that annual influenza vaccination can induce an immune response against the virus. However, in the third wave, only 45.3% of the participants showed seroprotective titers, since the influenza vaccination period was from February–March 2010.This showed a decline in the seroprotective levels of the antibodies after 6 months; this finding was in contrast to that of a US trial (104) in which more than 93% of the adults below the age of 65 years showed seroprotective HI levels after 1 dose of the monovalent pH1N1 vaccine.

HI assay is used to screen patients for seroprotective antibody levels and to estimate the infection rate in the population by measuring the antibodies against HA, which is the major target for most of the neutralizing antibodies. HI assaying is widely used for influenza epidemiological surveillance and vaccine studies because it specifically detects HA. In contrast, microneutralization assays are more sensitive, but expert laboratory staff are required to ascertain health and safety (105). In conclusion, to determine the seroprevalence in a defined population, a serological surveillance study was conducted to estimate changes in the seroprotective antibody titers due to the viral infection occurring in pandemic waves. Continuous epidemiological surveillance of pH1N1 prevalence in the population will help

understand the pattern of transmission of influenza and the pH1N1 infection and will assist in designing suitable methods of intervention before the next pH1N1 wave.

CHAPTER IV

EPIDEMIOLOGY OF SEASONAL INFLUENZA IN BANGKOK BETWEEN 2009 AND 2012

(Part 2.1)

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Slinporn Prachayangprecha, Jarika Makkoch, Kamol Suwannakarn, Preeyaporn Vichaiwattana,

Sumeth Korkong, Apiradee Theamboonlers, Yong Poovorawan

Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Thailand

CHAPTER IV

Part 2.1: Epidemiology of seasonal influenza in Bangkok between 2009 and 2012

Summary

This study investigated influenza activity in Bangkok, Thailand between June 2009 and June 2014. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed to detect influenza viruses among patients with influenza-like illnesses. Of the 6417 patients tested, influenza virus infection was detected in 42% (n = 2697) of the specimens. Influenza A pH1N1 viruses comprised the predominant strain between 2009 and 2010, and seasonal influenza (H3) had a high prevalence in 2011. Laboratory data showed a prevalence and seasonal pattern of influenza viruses. In 2009, influenza activity peaked in July, the rainy season. In 2010, influenza activity happened in two phases, with the initial one at the beginning of the year and another peak between June and August 2010, which again corresponded to the rainy period. Influenza activity was low for several consecutive weeks at the beginning of 2011, and high H3N2 activity was recorded during the rainy season between July and September 2011. However, from the beginning of 2012 through July 2012, pH1N1, influenza H3N2, and influenza B viruses continuously circulated at a very low level. In conclusion, the seasonal pattern of influenza activity in Thailand tended to peak during rainy season between July and September.

Introduction

Influenza viruses are major causes of respiratory tract disease in humans, presenting a significant burden to global public health. In addition to seasonal influenza, occasional outbreaks of pandemic influenza have occurred on a global scale. These outbreaks have invariably been caused by a virus to which the majority of people have not yet had the opportunity to develop sufficient immunity. During early April 2009, while the pH1N1 virus was spreading across the world, there were increasing numbers of pH1N1 infection cases during many influenza seasons in various regions of the world. By August 2010, the World Health Organization (WHO) declared the end of the pandemic phase (106), as there was no immediate concern about the situation. Since then, the pH1N1 virus has been considered with the same level of concern as seasonal influenza and still continues to circulate among human populations.

In Thailand, the first confirmed case of the pH1N1 virus occurred in May 2009 and was reported by the Bureau of Emerging Infectious Diseases, Department of Disease Control, Ministry of Public Health. Thailand is a tropical country in the northern hemisphere with a climate that regularly alternates between wet and dry seasons. The rainy season normally lasts from May to October. The dry season usually lasts from November to April, and the cooler season lasts from November to February. Seasonal influenza prevalence in tropical climates is different from that in temperate climates. However, there are diverse occurrences of influenza among countries in the same climate zone, with the majority of cases occurring during the respective rainy seasons (107-109). In temperate areas, on the other hand, influenza activity usually peaks during the winter months (110).

Globally circulating strains of influenza viruses vary and can include Influenza A, H3N2 viruses, or Influenza B viruses that are usually identified every year, with the dominant strains of influenza changing from one year to the next influenza viruses are continuously evolving by a process called antigenic drift and antigenic shift; they are mainly subject to antigenic drift, which is caused by an accumulation of changes in the viral antigenic determinants. This is a likely result of host selection pressure, as the two viral genes most prone to mutation are hemagglutinin (HA) and neuraminidase (NA), which code for the

external glycoproteins located on the surface of influenza viruses. These gradual changes in its antigenic sites enable the virus to escape the host's immune response. Hence, predominant strains or subtypes of influenza can circulate for some period of time and will decrease their infection rate as the population acquires protective antibodies sufficiently specific to prevent infection by those subtypes or strains, until the virus begins to undergo antigenic change, a process called herd immunity. Thus, as antibody levels may decline and the viruses evolve, the most efficient protection from influenza is an annual vaccination.

The purpose of this study was to observe seasonal influenza activity along with influenza occurrence and weather conditions based on data collected between June 2009 and July 2012. This epidemiological data will assist in understanding which factors contribute to virus emergence in tropical countries, and thus in developing strategies for outbreak prevention.

Materials and Methods

Geographic location

Bangkok, the capital of Thailand, is located in the central part of the country; with a population of 8 million, it is Thailand's most densely populated city. Overall, the climate is tropical monsoon from May to October and dry between November and April, with the lowest and highest temperatures ranging between 18 and 39 degrees Celsius; the average temperature throughout the year is around 29 degrees Celsius with an average relative humidity of 74%.

Specimen collection

In total, 6417 nasopharyngeal or throat swabs were collected from patients with ILI who had attended hospitals located in Bangkok, Thailand between June 2009 and July 2012. All specimens were obtained during the patients' routine examinations for respiratory tract viruses for management and treatment. The inclusion criteria for influenza-like illnesses were based on symptoms such as fever, sore throat, cough, nasal congestion, and myalgia. During transport to the Center of Excellence in Clinical Virology, all samples were stored in a transport medium (penicillin G (2 x 106 U/L), streptomycin 200 mg/L) at 4°C, and then transferred to -

20°C within 24 hours. The samples were tested for influenza A (pH1N1, seasonal H1N1, seasonal H3N2), and influenza B viruses by real-time reverse transcription polymerase chain reaction (RT-PCR).

This study examined patients' samples once routine service had been finalized. Patient identification, including personal information and hospitalization or admission number, was deleted from the data records to protect patient confidentiality. The research protocol was approved by the Ethics Committee of the Faculty of Medicine Chulalongkorn University (IRB No. 350/54). Permission to use those data was granted by the director of Chulalongkorn Hospital, and all information was used for research purposes only. The Institutional Review Board waived the need for consent because the data used was kept anonymous.

Meteorological Data

Data on temperature and average rainfall were provided by the Thai Meteorological Department. These data were collected daily in the Bangkok metropolitan area for the entire duration of this study. Average temperature, average relative humidity, and rainfall were measured in degrees Celsius and millimeters, respectively.

Laboratory methods

Detection of influenza viruses by real-time RT-PCR assay

RNA was extracted using the Viral Nucleic Acid Extraction Kit (RBC Bioscience Co, New Taipei City, Taiwan) according to the manufacturer's instructions. To create amplification of the target genes, primers were designed by retrieve nucleotides of various influenza strains from the GenBank database. After the alignment of the nucleotide, the most conserve region was selected as the primer to amplify cDNA during the assay. Degenerate primers were designed for nucleotide data sets with a low conservative region to ensure sequence coverage. The first reaction included primers and probes designed to specifically detect influenza A or B viruses; the second reaction was designed to detect pH1N1 and seasonal H1, H3, H5 subtypes. TaqMan probes, primers, and thermal profiles for the reaction were as described previously (87, 91, 92). RT-PCR was performed using the SuperScript III Platinum One-Step RT-PCR system (Invitrogen, Foster City, USA) in a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia). With this method, only the type and subtype of influenza

viruses could be identified. Samples were defined as positive cases upon confirmation of any influenza virus by real-time RT-PCR.

Data analysis

The epidemiological surveillance study was conducted based on recorded information from routine service. All data were depicted as a weekly percentage of laboratory-confirmed influenza cases by influenza type and distribution of influenza positive cases by age group. Statistical Pearson correlation analysis was performed by using SPSS software for Windows version 17.

Results

Of the 6417 samples collected between June 2009 and July 2012, 3102 were from males and 3315 were from females; the age of the patients ranged from one day to 98 years, with a mean age of 23. Due to insufficient quantity of cellular material, the housekeeping gene could not be amplified in 136 specimens. Six thousand and fifty specimens, comprised of 3035 males and 3246 females, were included in the analysis. Of those, 2969 (42%) were found positive for influenza viruses. Samples positive for influenza A virus were subtyped into seasonal H1N1, seasonal H3N2, pandemic (H1N1) 2009 viruses, and influenza B virus. None of the specimens were positive for H5N1.

Prevalence distribution

The pH1N1 virus was the most common influenza strain found in positive samples. During the study period, 40% of ILI cases had influenza A virus infection. Seasonal H1N1 subtype was identified in 0.2%, pH1N1 in 30%, and H3N2 in 10%. Three per cent of tested samples were positive for the influenza B virus. Figure 7 shows the total number of patients with acute respiratory tract symptoms per week, as well as percentages of pH1N1 and influenza A (H3). In June 2009 (week 24), influenza activity – most notably pH1N1 – started to increase until August 2009 (week 35). Of the 1621 specimens tested during this wave of influenza activity, 813 (54%) were positive for pH1N1.

Moreover, the small proportions of co-circulating strains were influenza A (H3) and, to a lesser extent, influenza A (H1). After August 2009 (week 35), influenza activity declined for

several months with a small number of positive cases of pH1N1 and influenza A (H1) remaining.

The low activity of influenza viruses continued until early 2010, when influenza activity started to increase with the same predominant strain, pH1N1, as during the previous year. In the course of this wave, which lasted until early March (week 10), 373 of 849 (44%) specimens were positive for pH1N1. Low levels of influenza B viruses as co-circulating strains were also detected in this wave, which lasted approximately three months. From the end of March 2010 (week 12) to the end of May (week 21), influenza activity remained low, with minor pH1N1 activity. By August 2010 (week 31), high levels of pH1N1 were detected. During this wave, influenza B viruses and seasonal influenza (H3) co-circulated until October 2010 (week 40), when the overall activity started to decrease. Out of the 983 specimens tested during this time, 371 (38%) cases were positive for pH1N1, 67 (7%) for influenza B viruses, and 75 (8%) for seasonal influenza (H3). From the end of October 2010 (week 42) to July 2011 (week 26), pH1N1 prevalence remained very low. From July to mid-September 2011 (weeks 26-37), 328 of 859 (38%) collected specimens harbored influenza A virus subtype H3N2, with 43 (5%) samples positive for influenza B, and 14 (2%) for pH1N1. From mid-September 2011 through June 2012, the frequency of influenza virus infections continued to decrease, with little influenza activity. Of a total of 171 specimens collected at the beginning of 2012, 35 (20%) specimens were positive for influenza B, 26 (15%) were positive for pH1N1, and 15 (9%) for influenza A (H3N2). From late June to the end of July, the numbers of pH1N1-positive cases increased. There were a total of 51 influenza-positive cases from the 159 specimens tested. The predominant influenza viruses during this month were the pH1N1 and influenza B viruses, which account for 23 (14%) and 20 (13%) positive cases, respectively; 8 (5%) specimens were positive for the influenza A virus subtype H3N2.

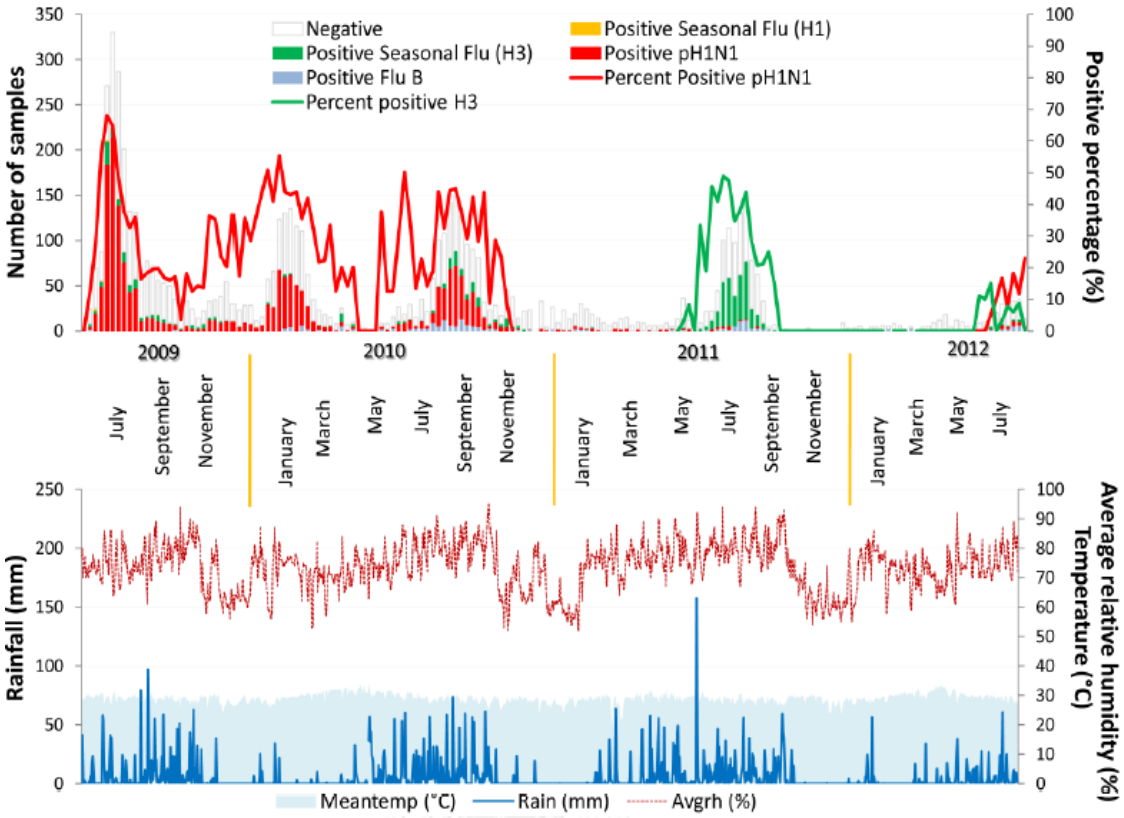

Figure 8. Weekly percentage of laboratory confirmed influenza cases by type from influenza-like illness patient visits to hospital in Bangkok area, and daily average temperature, average rainfall in Bangkok from June 2009 to July 2012.

Age stratification

Between June 2009 and July 2012, influenza virus activity was divided into four waves: June to August 2009, January 2010 to March 2010, August to October 2010, and July to September 2011. To determine the case distribution per age group during these periods, the percentages of influenza-positive cases related to age group were compared to the pandemic (H1N1) 2009 virus targeted school-age children and adolescents (between five and 24 years of age) in 2009 during the first wave of pH1N1 (mean age = 19 years). In 2010, the prevalence of the pandemic (H1N1) 2009 virus was still predominant in the school-age group, but the older age group was increasingly affected when compared with the first period. But in 2011, the proportion of positive cases among those of working age (25-49 years) and the elderly groups (over 50 years) increased, as the predominant strain had shifted from

pandemic (H1N1) 2009 to seasonal influenza H3. In 2012, most positive cases were among children and adolescents (5-24 years) and the working age group (25-49 years).

Weather conditions and prevalence

The weather in Bangkok and its surrounding areas was reviewed for the study period between June 2009 and July 2012 and was compared with influenza activity in this area as shown in Figure 7. The overall percentages of influenza-positive cases among acute respiratory tract infection patients are depicted in Figure 8 as percent positive in each age group from each year.

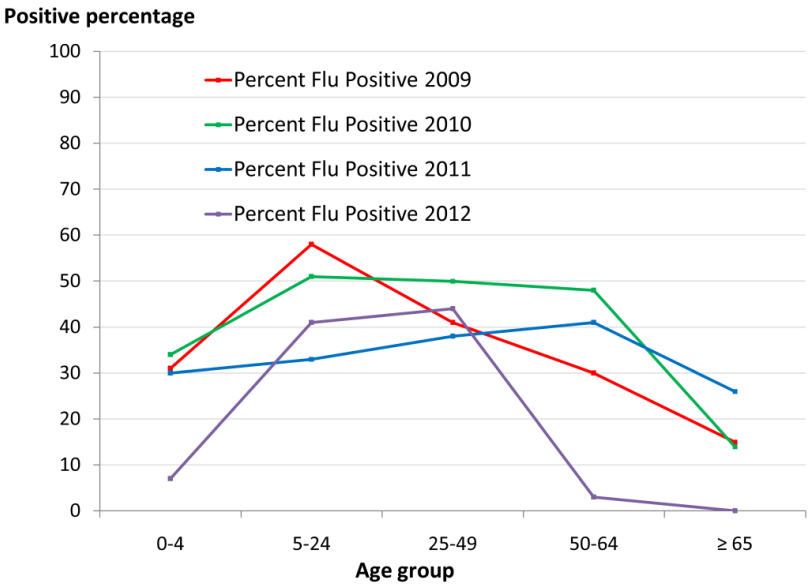

Figure 9*. Percent positive of influenza by age group from June 2009 to July 2012.*

In the first wave of infection, between June and August 2009, the average temperature in Bangkok, the capital of Thailand was 29.2°C (range 26.7 to 31.4°C), and rainfall averaged at 8.1 millimeters per day (range 0 to 96.6 millimeters per day). This period constituted the rainy season. During this period, the pandemic (H1N1) 2009 virus was the most prevalent. The seasonal H3N2 virus was also detected throughout this season. Between January 2010 and March 2010, the pandemic (H1N1) 2009 virus was still predominant. The average temperature in this period was 29.1°C (range 25.7 to 31.8°C). The average rainfall in this period was lower than during the previous period, 1.30 millimeters per day (range 0 to 34 millimeters per day). In the second period of infection, the prevalence of seasonal H3N2 and

B virus decreased in comparison with the rainy season, with seasonal H1N1 absent; however, there was a high frequency of pandemic (H1N1) 2009.

During the following wave, from August to October 2010, the average temperature and rainfall was 29.4°C (range 24.9 to 30.4°C) and 10.97 millimeters per day (range 0.0 to 73.5 millimeters per day), respectively. The average rainfall during this season was higher than in the previous year. During this period, the pandemic (H1N1) 2009 virus was still predominant. During the wave in 2011 from July to September, the average temperature was 28.4°C (range 26.1 to 31.1°C), with average rainfall of 9.11 millimeters per day (range 0 to 56.1 millimeters per day). During this period, seasonal influenza H3 was predominant.

Between January and July 2012, the average temperature and rainfall was 29.62°C (range 21.8 to 40°C) and 1.5 millimeters per day (range 0 to 56.4 millimeters per day), respectively. The correlation analysis between average temperature, average rainfall, and average relative humidity did not show any significant correlation with the number of influenza-positive cases from 2009 through 2012 ($r = -0.091$, $r = 0.189$ and $r = 0.238$; $p > 0.05$, respectively).

Discussion

This study indicated that most patients who attended a hospital during the influenza season were diagnosed with influenza viruses that caused acute respiratory tract infections. This study also indicated that influenza viruses are important causes of respiratory tract disease, in addition to other known viruses associated with respiratory tract infection such as rhinovirus, adenovirus, respiratory syncytial virus, and parainfluenza virus (111, 112). Also, influenza A virus strains circulated during this study, with pH1N1 and seasonal influenza H3 predominant in 2009 through 2010 and 2011, respectively.

From the end of 2011 through July 2012, the number of patients admitted to a hospital with acute respiratory tract diseases decreased due to lower overall public awareness of pH1N1, because the symptoms of influenza were relatively mild in patients without any underlying disease. The number of positive cases may therefore be underestimated.

In 2009, the prevalence of pH1N1 was much higher than at the peak of the influenza season in 2010. This may be due to a large proportion of the population not yet having developed antibodies against pH1N1, which at that time constituted a new pandemic strain. In this study, cases of pH1N1 were detected in June 2009. The first confirmed case in Thailand occurred in May 2009 and was reported by the Bureau of Emerging Infectious Diseases, Department of Disease Control, Ministry of Public Health. Upon its emergence, a large number of positive cases were confirmed in this period. The seasonal H1N1 virus decreased in prevalence and, since the emergence of the pandemic (H1N1) 2009 virus, has been detected rarely.

At the emergence of pandemic (H1N1) in 2009, the mean age of infected patients was 19 years, which correlates with other studies that have reported that pH1N1, in contrast to seasonal influenza, specifically targets younger age groups (113, 114). This may be explained by the fact that older adults have developed some level of protective antibody against pH1N1 due to previous infection with the related H1N1 virus (56).

The influenza positive rate of the 5-24 year age group decreased in the following year because this group had acquired some level of seroprotective antibody after pH1N1 infection, while the infection became more prevalent in older age groups (115). Thus, in 2010, the mean age of patients was 19 years, which increased to 29 years in 2011. It was observed that once the pandemic (H1N1) 2009 virus had become the seasonal flu, people's attentiveness to good personal hygiene dropped in comparison with the first infection period.

In 2011, the seasonal influenza (H3) was the major circulating strain, affecting the 50-64 year age group, which correlates with numerous studies that have reported that most seasonal influenza viruses tend to attack older people due to their weaker immune systems and causes more severe complications (116). However, from the beginning of 2012 through June, the overall influenza activity in Bangkok declined, with few patients attending the hospital. From late June to July, influenza activity started to increase. This may be because influenza activity is usually most pronounced during the rainy season, despite the apparent lack of any significant correlation between influenza positive cases and rainfall or any other two environmental factors in this study.

Influenza activity in Bangkok and its surroundings was different from other regions in Thailand. The predominant strain in Nakhon Si Thammarat province (south of Thailand) was seasonal influenza (H3), with lower levels of the pH1N1 and influenza B virus co-circulating during the 2010 season (117), while the pH1N1 virus predominated during the same season in Khon Kaen province (northeast of Thailand) with low-level co-circulation of seasonal influenza (H3) and influenza B viruses (118). In Bangkok, pH1N1 was a predominant strain throughout the year with co-circulation of seasonal influenza (H3) and influenza B viruses. However, during the 2011 season, seasonal influenza (H3) became the predominant strain.

Despite minor differences in influenza activity patterns at the same time of year, which might have been influenced by weather variations, the peak influenza activity trend was similar (84, 119). In south Thailand, the climate has only two seasons, rainy and dry (120), with more prolonged periods of rain than in other regions. In the northeast, on the other hand, the climate is usually semi-humid and dry with generally little rainfall compared to other regions. In the neighboring countries and other countries in tropical climate zones, influenza activity has been reported to peak in the rainy season (121-124). Also, in this study, the peak of influenza activity was usually observed during and immediately after the rainy season, which might have had a direct or indirect effect on influenza seasonality. However, other environmental factors such as temperature, humidity, El Niño, and the annual variability of climate have been reported to affect influenza prevalence (125, 126).

Continuing surveillance of influenza circulation patterns could constitute part of the epidemiological data required for preventive measures such as vaccination campaigns for high-risk groups among the Thai population. Although epidemiological studies will assist in improving strategies aimed at future prevention of epidemics or pandemics, the best prevention strategies are raising awareness about the importance of personal hygiene.

MOPH introduced a national influenza surveillance system in cooperation with the US CDC (127). The following year, in cooperation with the WHO, Thailand implemented its first National Strategic Plan for Avian Influenza Control and Pandemic Influenza Preparedness (128). Thanks to these initiatives, Thailand has brought its domestic influenza surveillance and vaccination programs up to the global standard.

During the 2009 influenza pandemic, the Center of Excellence in Clinical Virology of the Faculty of Medicine of Chulalongkorn University began to collect influenza surveillance data from four Thai provinces to better understand patterns of seasonality and typology of influenza in Thailand. We analyze the data in this study and compare it against national surveillance data from the US and Australia to assess the similarities and differences between influenza activity in different climates. We believe that this information is important for Thailand's current influenza strategy and the future of public health policymaking in Thailand.

CHAPTER IV

INFLUENZA ACTIVITY IN THAILAND AND OCCURRENCE IN DIFFERENT CLIMATES

(Part 2.2)

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Slinporn Prachayangprecha [1], Preeyaporn Vichaiwattana [1], Sumeth Korkong [1], Joshua A Felber [2], Yong Poovorawan [1]

Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University[1]

Josef Korbel School of International Studies, University of Denver[2]

CHAPTER IV

Part 2.2: Influenza activity in Thailand and occurrence in different climates

Summary

This study observed influenza activity between June 2009 and July 2014 in Thailand, a country in the Northern hemisphere with a tropical climate, and compared the results to activity in the United States (US) and Australia, which represent temperate climates in the Northern and Southern hemispheres, respectively. From Thailand, a total of 17,416 specimens were collected from patients exhibiting influenza-like illnesses and subjected to real-time PCR (RT-PCR) for the detection of influenza viruses. For comparison, laboratory confirmations of influenza originating from the US and Australia were obtained from the US CDC's FluView surveillance reports and the Australian Government's Department of Health and Ageing websites. We found that, generally, the influenza season in Thailand starts with the rainy season. This observation of influenza's annual incidence pattern provides a better understanding of its occurrence, suggesting that vaccination campaigns should be started before the influenza season begins in order to reduce transmission.

Introduction

In the last decade, the Kingdom of Thailand has rapidly evolved in terms of influenza control and pandemic influenza preparedness. Since 2004, these have been the explicit goals for a series of government-led initiatives aimed at reducing the burden of influenza in Thailand, both for the advancement of public health and the maintenance of national security. Thus, the Thai Ministry of Public Health (MOPH), in collaboration with the World Health Organization (WHO) and the United States Centers for Disease Control (CDC), has established itself at the forefront of global influenza surveillance and research. Now, after ten years of national surveillance by two, independent studies, it is useful to review and analyze the data for the benefit of future public health policymaking in Thailand. In 2004, the impetus for a national initiative on influenza control and pandemic preparedness was found when a new strain of highly pathogenic avian influenza (Influenza A H5N1) swept through Thailand, causing an estimated THB 25.24 billion in economic damages within its first three months (129). Between 2004 and 2006, Thailand recorded 3 more rounds of H5N1 outbreaks in humans before government interventions finally contained the problem by 2007 (130). Nevertheless, avian and swine influenza viruses remain endemic among Thai fauna, where they circulate and may possibly recombine to form novel strains. Influenza A(H1N1)pdm09, for example, was a novel, reassortant strain estimated to have caused between 151,700 and 575,400 human deaths in its first twelve months, with over half occurring in Africa and Southeast Asia (131). Based on these experiences and others, the government of Thailand is convinced that influenza represents a major threat to both public health and national security. Thus, since 2004, influenza has been the focus of public health interventions in Thailand. Vaccination has proven to be the most effective strategy for preventing influenza transmission and reducing morbidity (132). The WHO makes annual recommendations on the viral strains that should be vaccinated against for both the Northern and Southern hemispheres (133). Their recommendations are based on global influenza surveillance data, including genetic characterizations of circulating viruses, while the vaccines are based on the virion's surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (134). In 2004, the Thai MOPH introduced a national influenza

surveillance system in cooperation with the US CDC (127). The following year, in cooperation with the WHO, Thailand implemented its first National Strategic Plan for Avian Influenza Control and Pandemic Influenza Preparedness (128). Thanks to these initiatives, Thailand has brought its domestic influenza surveillance and vaccination programs up to the global standard.

During the 2009 global influenza pandemic, the Center of Excellence in Clinical Virology of the Faculty of Medicine of Chulalongkorn University began to collect influenza surveillance data from four Thai provinces to better understand patterns of seasonality and typology of influenza in Thailand. We analyze the data in this study and compare it against national surveillance data from the US and Australia to assess the similarities and differences between influenza activity in different climates. We believe that this information is important for Thailand's current influenza strategy and the future of public health policymaking in Thailand.

Materials and Methods

Geographic Location

Thailand is a tropical country located between latitudes 5° 37′N and 20°27′N and between longitudes 97° 22′E and 105° 37′E. Annual temperatures range between 0.8 and 44.5 degrees Celsius, with an average temperature of 27 degrees and an average relative humidity of 76% (135). The United States and Australia, on the other hand, are temperate countries located well within the Northern and Southern hemispheres, respectively.

Specimen collection

Between June 2009 and July 2014, 17,416 nasopharyngeal swab samples were taken during hospitalizations and outpatient services at geographically disparate hospitals in Thailand. Locations included Bangkok (n= 10,361), Chumphae district, Khon Kaen province (n=5,736), Surin province (n=119) and Thungsong district, Nakorn Sri Thammarat province (n=1,200) (Figure 9). We did not analyze influenza activity in each hospital or province separately, as the goal of our surveillance was to determine activity based on the entire sample. All specimens were collected individually from medically diagnosed cases of influenza-like illness (ILI), which was determined through the presence of high fever (>38°C)

and respiratory tract symptoms. Participants were selected routinely based on these criteria alone and not differentiated by demographic factors such as age, sex, ethnicity, etc. Once collected, each sample was maintained at 4°C within a standard viral transport medium containing the antibiotics penicillin (G 2 x 106 U/L) and streptomycin (200 mg/L) and sent for laboratory analysis within 48 hours to the Center of Excellence in Clinical Virology in Bangkok. Laboratory work involved subjecting each sample to RNA extraction and influenza virus detection through real-time RT-PCR (91, 92). These procedures were conducted at the King Chulalongkorn Memorial Hospital by researchers from the Faculty of Medicine of Chulalongkorn University. Data for this study was obtained from previous reports (103, 117) and routine surveillance data acquired by the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University. This research was carried out according to university's ethical guidelines and was exempted by the Institutional Review Board of the Faculty of Medicine of Chulalongkorn University.

Source of data for Influenza in the temperate zone

Cross-country comparison data were obtained from two sources chosen based on their reliability, quality, coverage and accessibility. To compare our influenza data to the northern hemisphere, we used the annual influenza reports from the United States provided by the US Centers for Disease Control and Prevention (136). To compare our influenza data to the southern hemisphere, we used the annual influenza reports from Australia provided by the Australian Department of Health and Ageing (137). We believe that these sources are ideal for comparison purposes because both collected data routinely from a large, nationally representative sample and published the data freely online. We further believe that the comparison is useful as both countries are geographically and demographically large in their respective hemispheres. As a medium-sized country located near the equator, Thailand should frequently examine similarities and differences between itself and both hemispheres.

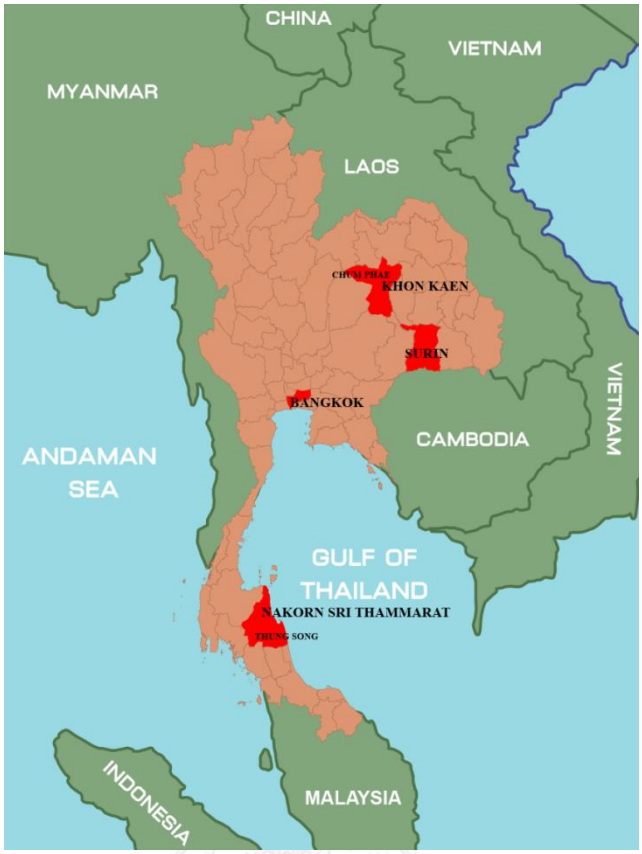

Figure 10. Map of Thailand. Location of specimen collection; Chum Phae, Khon Kaen provinces, Surin province, Bangkok and Thung Song, Nakorn Sri Thammarat province.

Results

Influenza activity in Thailand

A total of 17,416 nasopharyngeal swab samples were collected from June 2009 to July 2014: 3,435 in 2009, 4,343 in 2010, 2,262 in 2011, 2,025 in 2012, 2,995 in 2013 and 2,356 in 2014. Through RT-PCR testing, 4,553 (26.1%) of the samples were found to be positive for influenza viruses. Of the positives, 3,723 (81.8%) tested positive for influenza A viruses while 830 (18.2%) tested positive for influenza B viruses. Of influenza A subtypes, A(H1N1)pdm09 contributed 2,458 (66.0%) of all influenza A positives while A(H3N2) accounted for 1,222 (32.8%). We also observed that seasonal, pre-pandemic A(H1N1) disappeared from our survey after 2011, though it was responsible for a small number of influenza A positives in 2009 and 2010.

In 2009, 1,318 of 3,435 (38.4%) samples were found to be influenza positive, with influenza A(H1N1)pdm09 accounting for 74.4% of all positive samples. Nevertheless, despite 2009 being the pandemic year, influenza B still accounted for 12.7% of positives. In 2010, 1,444 of 4,343 samples (33.2%) were tested positive for influenza viruses, with influenza A(H1N1)pdm09 responsible for 67% of all positives and influenza B, 21.1%. In 2011, 528 of 2,262 samples (23.3%) were confirmed for influenza and 66.5% of those were positive for influenza A(H3N2). Influenza B was responsible for 12.9%. In 2012, 346 of 2,025 samples (17.1%) were influenza positive, of which 45.7% were influenza B viruses and 54.3%, influenza A. In 2013, 410 of 2,995 samples (13.7%) were influenza positive, mostly for A(H3N2), which contributed 92.7% of all positives. In 2014, 507 of 2,356 samples (21.5%) tested positive, with influenza A(H1N1)pdm09 accounting for 57.8% of positives, while influenza B contributed 24.1%.

Comparative influenza activity between tropical and temperate countries

Figure 10 illustrates the number of specimens that were positive for influenza viruses from 2009 to 2014 in the United States, Australia and Thailand. The total number of patients per week with acute respiratory tract symptoms is also shown to compare against the cases of laboratory confirmed influenza viruses by type and subtype.

During the 2009 pandemic year, influenza surveillance in the US reported different periods of peak activity compared to Thailand and Australia. While the US experienced new infections almost continuously, Thailand and Australia showed only sporadic new cases just three to four months after the beginning of the outbreak. Furthermore, although the total number of new infections in the US was considered low in 2010, confirmed cases increased until the end of the year and continued into 2011. This is in contrast to Thailand, where influenza circulation produced two waves of infection, with the first wave lasting for a few months at the beginning of 2010 and the second occurring from August to September. Similarly, Australia recorded high levels of influenza activity from August to October.

During 2011, overall influenza activity in Thailand and Australia mirrored the previous year except that the curve began a few weeks earlier for Australia. In 2012, influenza activity in Thailand and Australia mirrored 2011, while in the US it started at the beginning of the

year and lasted until June. In 2013, the US had a similar experience to 2012, but showed an increasing trend since early December, while Australia reported that the influenza season arrived later than in previous years. In Thailand, influenza activity remained at a low level. Between January and July of 2014, influenza activity in Thailand was similar to the US, beginning early in the year, and Australia's influenza season started earlier than in 2013.

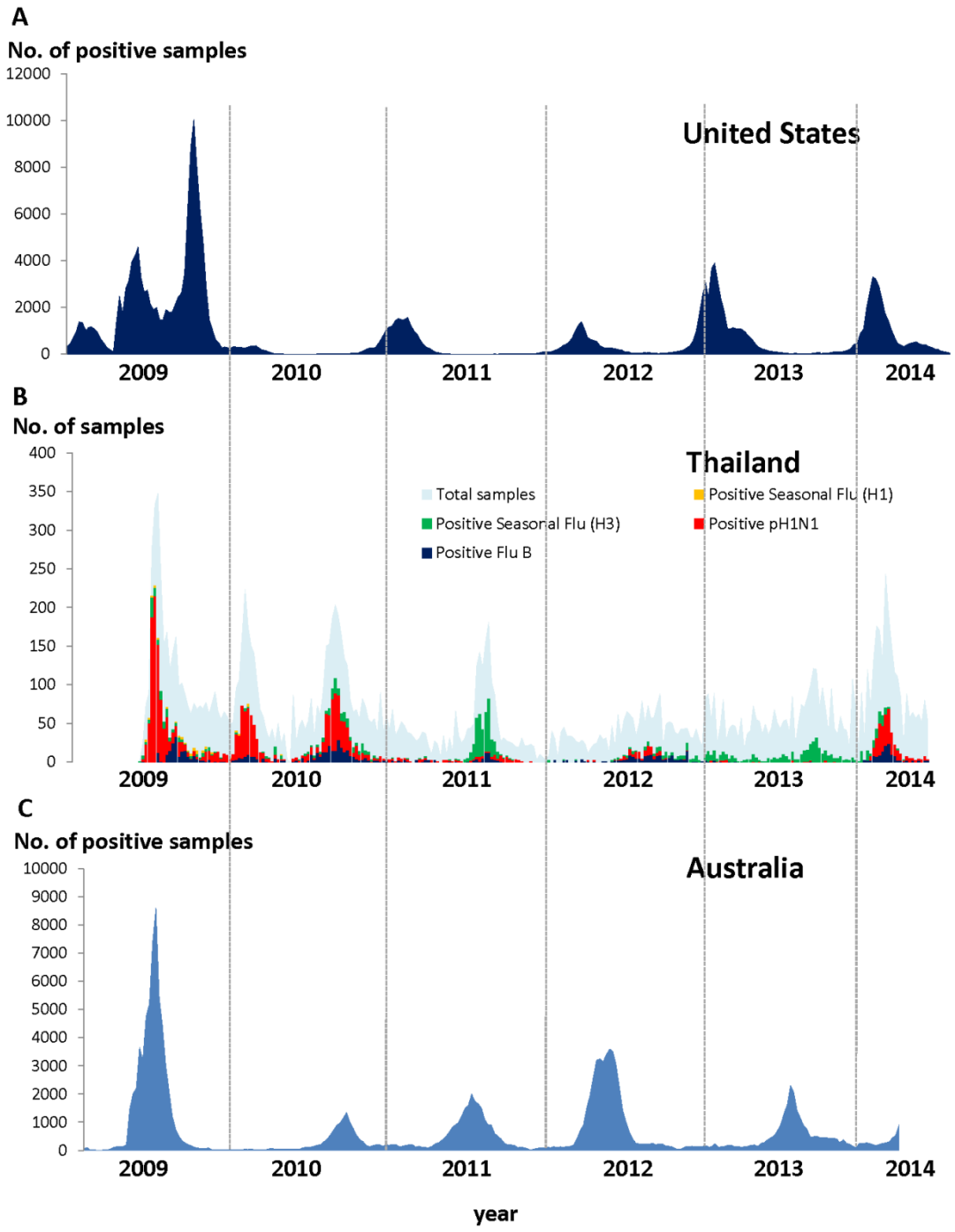

Figure 11. Weekly influenza activity during 2009 -2014 influenza season. A: United States, 2009- July 2014; B: Thailand, July 2014 - July 2014; C: Australia, 2009 – July 2014.

Discussion

We analyzed differing patterns of influenza activity in the United States, Australia and Thailand. We chose to compare Thailand against the US and Australia because those countries represent temperate Northern and Southern hemisphere climates, respectively, while Thailand is a tropical Northern hemisphere country that is relatively close to the equator. In temperate regions, influenza activity has been associated with low temperatures and humidity (109), which explains why temperate countries typically experience influenza seasons during winter. In tropical climates, however, factors such as high temperatures, high humidity and high levels of precipitation are often linked to increased influenza activity (112, 138-140). In fact, year-round influenza activity is more common in countries with tropical influenza seasonality (83). Nevertheless, whether influenza activity will be particularly high or low in each season is still hard to predict due to the influence of many variables, including host factors, environmental factors and the virulence and/or pathogenicity of each strain (109, 141).

Our study has shown that Thailand's annual influenza activity during our period of observation is more similar to Australia than the US, despite Thailand being located in the Northern hemisphere. We found that influenza activity in Thailand usually presents as an annual cycle, except during the 2009 pandemic and its aftermath, when the circulation of influenza A(H1N1)pdm09 caused the normal pattern to break. Most likely this was the result of the Thai population's lack of immunity toward the new pandemic strain. Although in normal years there is a pattern of influenza seasonality in Thailand, except in 2012 and 2013, which we cannot clearly see the distinct cyclic peak like previous years because there were lower total ILI cases during 2012-2013, low influenza activity can still be detected outside of influenza seasons. For countries with smaller populations, such as Thailand and Australia, detecting influenza activity year-round may be more difficult than in larger countries like the US. Therefore, an increase in the number of testing specimens would likely improve influenza detection outside of influenza seasons (142).

Interestingly, our data shows that the percentage of influenza positives obtained from our sampling of medically diagnosed cases of influenza-like illness has been

decreasing over time. Except in 2014, for which we only have 7 months of data, the percentage of influenza positives decreased each year since 2009. This could also be an indication that other pathogens are becoming more important over time in causing influenza-like illnesses.

Thailand has three seasons: summer from February to May, the rainy season from June to September and the cool season from October to January. Influenza activity in Thailand tends to increase during the rainy season, which is typical for countries located in tropical latitudes (121, 122). Thus, in order to effectively reduce annual influenza transmission, seasonal influenza vaccination campaigns should be started before late summer, prior to the influenza season. Nevertheless, it is still beneficial for people to be vaccinated during the influenza season. Another method to effectively reduce overall influenza transmission is to immunize children and young adults who experience the highest risk of contracting the virus due to personal hygiene and group activities involving close contact (92, 143, 144). Because most schools in Thailand open in May, vaccinations usually start from April to May and may reduce influenza-related morbidity and mortality. Vaccination is also recommended for sub-populations that are especially at risk, such as children under 5 years of age, adults aged 65 years and over, pregnant women, healthcare workers and people with underlying health conditions such as chronic respiratory disease, chronic heart disease and obesity (145). Annually, the WHO makes recommendations on influenza vaccine compositions for both the Northern and Southern hemispheres, with the chosen strains that are the good match with circulating strains. Vaccine production according to WHO guidelines for the southern hemisphere is usually conducted in time to be distributed just as the influenza season is starting in Thailand. However, it is important to remember that, even if there is some difference in the season's timing or if the vaccine supply does not meet peak demand, the best vaccine should always be the available one, regardless of which hemisphere it is recommended for, as it can still be beneficial for influenza prevention.

In conclusion, Thailand is tropical country which experiences annual influenza activity that tends to peak during the rainy season, which is typical for many other tropical

countries. With Thailand's first domestic vaccine production facility to be completed in the near future, we believe that it is valuable to report on current surveillance data and influenza trends so that the operations of this factory can be planned for maximum cost-effectiveness. Aside from closely monitoring influenza activity, seasonal influenza vaccination with southern hemisphere strains is recommended for certain high-risk groups before the influenza season begins as part of a strategic vaccination program.

CHAPTER V

EXPLORING THE POTENTIAL OF NEXT-GENERATION SEQUENCING IN DETECTION OF RESPIRATORY VIRUSES

(Part 3)

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Slinporn Prachayangprecha[1], Claudia M. E. Schapendonk[2], Marion P. Koopmans[2,3], Albert D. M. E. Osterhaus[2,4], Anita C. Schürch[2], Suzan D. Pas[2], Annemiek A. van der Eijk[2], Yong Poovorawan[1], Bart L. Haagmans[2], Saskia L. Smits[2,4]

[1]Center of Excellence in Clinical Virology, Department of Pediatrics, Chulalongkorn University and Hospital, Bangkok, Thailand; [2]Department of Viroscience, Erasmus Medical Center, Rotterdam, the Netherlands; [3]Virology Division, Centre for Infectious Diseases Research, Diagnostics and Screening, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; [4]Viroclinics Biosciences, Rotterdam, the Netherlands

CHAPTER V

Part 3: Exploring the potential of next-generation sequencing in detection of respiratory viruses

Summary

Efficient detection of human respiratory viral pathogens is crucial in the management of patients with acute respiratory tract infection. Sequence-independent amplification of nucleic acids combined with next-generation sequencing technology and bioinformatics analyses is a promising strategy for identifying pathogens in clinical and public health settings. It allows the characterization of hundreds of different known pathogens simultaneously and of novel pathogens that elude conventional testing. However, major hurdles for its routine use exist, including cost, turnaround time, and especially sensitivity of the assay, as the detection limit is dependent on viral load, host genetic material, and sequencing depth. To obtain insights into these aspects, we analyzed nasopharyngeal aspirates from a cohort of 81 Thai children with respiratory disease for the presence of respiratory viruses using a sequence-independent next-generation sequencing approach and routinely used diagnostic real-time reverse transcriptase PCR (real-time RT-PCR) assays. With respect to the detection of rhinovirus and human metapneumovirus, the next generation sequencing approach was at least as sensitive as diagnostic real-time RT-PCR in this small cohort, whereas for bocavirus and enterovirus, next-generation sequencing was less sensitive than real-time RT-PCR. The advantage of the sequencing approach over real-time RT-PCR was the immediate availability of virus-typing information. Considering the development of platforms capable of generating more output data at declining costs, next-generation sequencing remains of interest for future virus diagnosis in clinical and public health settings and certainly as an additional tool when screening results from real-time RT-PCR are negative.

Introduction

Laboratories nowadays largely perform viral species-specific assays for virus diagnosis in clinical samples to increase sensitivity of detection and reduce the time needed for diagnosis. However, an etiological agent cannot be identified in many cases despite the use of a wide range of sensitive diagnostic assays (5, 146-149), possibly because of limited detection of divergent viruses due to high specificity of the assays. New perspectives for research and diagnostic applications of virus detection have opened up with recent advances in sequence-independent amplification techniques combined with next-generation deep sequencing platforms. These technologies are well known for their enormous output of sequence data at a relatively modest cost.

Sequence-independent deep-sequencing approaches have been applied to various fields in virology, among which virus discovery, whole viral genome reconstruction, and minority variant analyses (150-154). Sequence-independent amplification of nucleic acids combined with next-generation sequencing technology and bioinformatics analyses is a promising strategy for rapid identification of pathogens in clinical and public health settings. It would allow the characterization of hundreds of different known pathogens simultaneously and also detect novel pathogens that elude conventional testing. The general idea is that it is unlikely, however, that genomics-based tools will be used in a clinical diagnostic setting shortly (155). Its major hurdles are cost-effectiveness; high-throughput formats for clinical settings; turnaround time; the requirement for investments in bioinformatics tools, databases, and data management; training of personnel; and the reporting and interpretation of guidelines upon the identification of viruses of which the clinical relevance is not clear (156). In addition, issues regarding patient privacy need to be resolved before transition of genomics-based tools from a research setting to the clinic. Yet, the costs for deep-sequencing are still declining and thorough comparisons of sequence-independent deep sequencing approaches to current diagnostic assays are scarce.

Here, we describe the comparison of a sequence-independent next-generation sequencing approach to diagnostic real-time reverse transcriptase PCR (real-time RT-

PCR) assays in a cohort of Thai children with respiratory disease. The data indicate that a sequence-independent next-generation sequencing approach is a relatively efficient tool for the simultaneous detection of multiple respiratory viruses, albeit slower than routine diagnostic real-time RT-PCR assays.

Materials & Methods

Sample collection.

The nasopharyngeal aspirates included in this study were collected for diagnostic testing from children with respiratory illness (n=261) from 2010 to 2013 and kept at the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (see Table S1 in the supplemental material). Out of 261 patients screened by standard diagnostic assays in Thailand for influenza A and B viruses (92) and (nested) in-house PCR assays for human respiratory syncytial virus (RSV; (157)), rhinovirus (HRV; (158)), enterovirus (HEV; (158, 159)), adenovirus (hAdV; (160)), human metapneumovirus (hMPV), human parainfluenza virus (hPIV; (161)), and human coronavirus (hCoV), 89 had no diagnosis, and samples from 81 of these patients were available for additional studies. As the (nested) in house RT-PCR assays in Thailand are not based on real-time PCR technology, it was assumed that this initial diagnostic screening may not have been optimal. The age distribution of the enrolled patients was between 8 days and 14 years. Patients were categorized into 1 of 4 groups: infant (<2 years), preschool age (2 to 5 years), primary school age (6 to 11 years), and secondary school age (12 to 15 years). Of the 81 patients, 66.7% were infants (n = 54), 25.9% were in preschool (n = 21), 3.7% were in primary school (n = 3), and 3.7% were in secondary school (n = 3) (see Table S1). The clinical severity of each disease case was defined as mild (a pediatric patient with acute respiratory tract infection [ARTI] complications without abnormal breath sounds and who did not require intubation) (9.9%; n = 8), moderate (patients with ARTI symptoms and abnormal breath sounds who required intubation) (60.5%; n = 49), or severe (patients with ARTI complications with abnormal breath sounds and who required intubation) (28.4%; n = 23) (see Table S1).

Ethics statement

In compliance with relevant laws and institutional guidelines, ethical approval was obtained from the institutional review board of the Faculty of Medicine, Chulalongkorn University (IRB192/2557). The study was conducted on the stored clinical specimens as anonymous. Patient identifiers including personal information (name, address) and hospitalization numbers were removed from these samples to protect patient confidentiality and neither appeared in any part of the documents in this study. Permission for specimen utilization was granted by the Director of King Chulalongkorn Memorial Hospital, Thailand.

Sequence-independent next generation sequencing

Depletion of host nucleic acids, isolation of viral nucleic acids, sequence-independent amplification and next-generation sequencing with a 454GS Junior (Roche) was carried out as previously described (162-164) on 81 available nasopharyngeal aspirates. Briefly, nasopharyngeal aspirates were centrifuged and filtrated through a 0.45 μm-pore filter, after which samples were treated with Omnicleave endonuclease (Epicentre, Illumina). RNA and DNA were extracted using the Nucleospin RNA XS kit (Macherey-Nagel) and the High Pure viral nucleic acids kit (Roche). After first- and second-strand syntheses, random PCR amplification was performed and PCR products were purified using the MinElute PCR purification kit (Qiagen) (163, 164). Subsequently, 12 samples were pooled for each library preparation and unique sequence tags were added to the PCR products of each sample using the GS FLX Titanium rapid library MID adaptor kit, and a library of DNA fragments was prepared using a GS FLX Titanium library preparation kit (454 Life Sciences, Roche). The libraries of DNA fragments were sequenced on a454 GS Junior instrument (454 Life Science)

Assembly

Exhaustive iterative assembly of sequences is part of a virus discovery pipeline written in the python 2.7 programming language, which includes trimming of reads and initial assembly with Newbler (454GS Assembler version 2.7; Roche), with standard parameters. Trimmed reads and initial contigs were subjected to assembly by CAP3 (version date, 21 December 2007) with standard parameters. The resulting singletons and contigs were iteratively assembled by CAP3 until no new contigs were formed. Subsequently, the trimmed reads were mapped back to the identified taxonomic units with Newbler (454 GSMapper version 2.7; Roche) using a minimum length of 75 nucleotides and otherwise standard parameters. The resulting contigs and singletons were filtered with DustMasker, which is part of the NCBI BLAST 2.2.25 suite of tools for sequences that contain >60% low-complexity sequences.

Metagenome analysis

After filtering the low-complexity sequences, the remaining taxonomic units were subjected to a BLASTN search against a database that contained only nucleotide sequences from birds (Aves, taxonomic identification [taxID] 8782), carnivores (Carnivora, taxID 33554), primates (Primates, taxID 9443), rodents (Rodentia, taxID 9989), and ruminants (Ruminantia, taxID 9845) with an E value cutoff value of 0.001 for the subtraction of potential host sequences. Sequences without hits in the host BLAST were then subjected to a BLASTN search against the entire nucleotide database with an E value cutoff value of 0.001. Due to limited capacity, all the sequences without hits were then subjected to a BLASTX search against sequences present in the GenBank nr database. BLAST hits were categorized by assigning taxonomic categories. The sensitivity of a deep-sequencing approach for detecting viruses is dependent on sequencing depth. In this study, the average number of reads analyzed per sample was ~10,000; inherently, the detection limit lies at ~0.01% of viral reads in the metagenome, which results in 1 viral read in the metagenomic data set per sample. The whole sequence-independent next-generation sequencing approach, including analysis, takes up to 5 days.

Diagnostic real time PCR assays

Total nucleic acid was extracted from an aliquot (200 µl) of the 81 nasopharyngeal aspirates using the MagNA Pure LC total nucleic acid isolation kit and the MagNA Pure LC isolation station (Roche) and eluted in 50 µl of elution buffer, according to the manufacturer's instructions. Subsequently, the 81 samples were screened for the presence of human rhinovirus, enterovirus, and human metapneumovirus by a real-time RT-PCR with primers and probes used in the routine molecular viral diagnostics setting of Erasmus Medical Center essentially as described previously (165), except hMPV-probe-2 was not used. For bocaviruses, 4 µl extracted nucleic acid was amplified by a real-time PCR, as described previously (166).

Phylogenetic analysis

Alignments and phylogenetic trees were prepared with MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) and Molecular Evolutionary Genetics Analysis version 6 (MEGA6) (167) with corresponding sequences of representative members of the respective virus families (see Table S3 in the supplemental material). Neighbor-joining phylogenetic trees were created with 1,000 bootstrap replicates using p-distance (HRV and HBoV) and maximum-likelihood composite models (EV and hMPV) as described previously (168-170).

Results

Eighty-one nasopharyngeal aspirates from Thai children with acute respiratory tract complications (ARTI) who visited two hospitals located in Thailand between 2010-2013 (Supplementary Table S1) were analyzed by random amplification combined with deep sequencing (162-164). The taxonomic content of the different samples varied substantially (Figure 12A) and showed no strong correlations with patient age or disease severity (data not shown). Although not significantly different, the moderate and severe disease cases seemed to have higher mean viral contents in the metagenome than did mild disease cases, in contrast to the bacterial contents, which were similar in mild, moderate, and severe disease cases (Figure 12B-C). The identified mammalian viral sequences belonged to the families Anelloviridae (~57% of patients), Picornaviridae

(~51%), Herpesviridae (~12%), Orthomyxoviridae (~5%), Paramyxoviridae (~20%), Parvoviridae (~21%), Adenoviridae (~9%), Papillomaviridae (~4%), and Retroviridae (~1%) (Table 5; Supplementary Table S2). Single infections and multiple infections occurred in 28% versus 60% of children (48% and 25% when only clinically well-established infectious respiratory pathogens able to induce disease on their own were calculated).

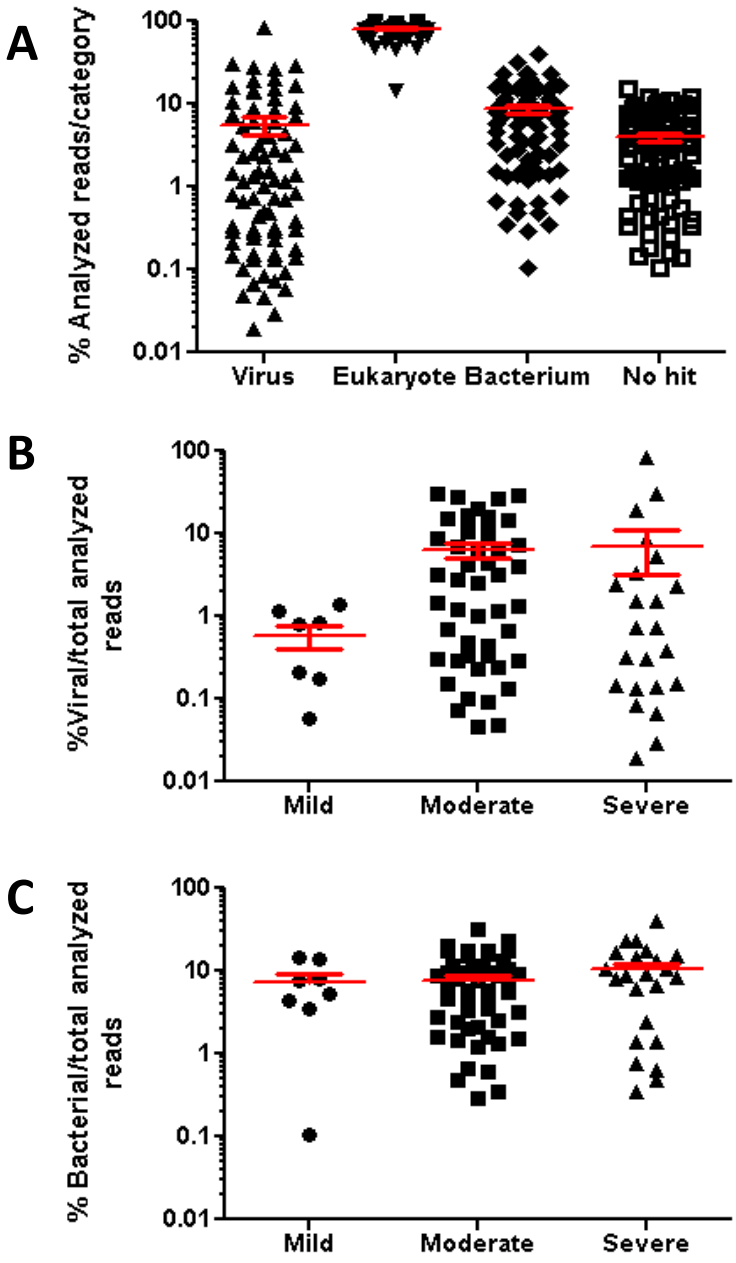

Figure 12. Overview of the metagenomic content. (A) Relative abundance of the main broad taxonomic categories in metagenomic sequences obtained from nasopharyngeal aspirates of 81 Thai children. The percentage of viral (B) and bacterial (C) reads of the total number of analyzed reads was displayed against disease category (explained in Materials and Methods).

Table 5. *Frequency of mammalian viruses found in patients.*

Mammalian virus	Number of patients	Mild (n=8)	Moderate (n=49)	Severe (n=23)
Anellovirus	46	3 (37.5%)	29 (59.2%)	14 (60.1%)
Rhinovirus	28	3 (37.5%)	21 (42.9%)	4 (17.4%)
Bocavirus	17	1 (12.5%)	12 (24.5%)	4 (17.4%)
Enterovirus	13	0	13 (26.5%)	0
Respiratory syncytial virus	11	1 (12.5%)	9 (18.4%)	1 (4.3%)
Herpesvirus	10	0	7 (14.3%)	3 (13%)
Adenovirus	7	0	3 (6.1%)	4 (17.4%)
Influenza virus	4	2 (25%)	1 (2%)	1 (4.3%)
Metapneumovirus	4	0	2 (4.1%)	2 (8.6%)
Papillomavirus	3	0	2 (4.1%)	1 (4.3%)
Parainfluenza virus	1	0	1 (2%)	0
Human endogenous retrovirus	1	0	1 (2%)	0
Parechovirus	1	1 (12.5%)	0	0

To obtain insight into the sensitivity of the deep-sequencing approach compared with that of the real-time RT-PCR assays routinely used for virus detection in clinical settings, we performed diagnostic real-time RT-PCRs for rhinovirus, enterovirus, hMPV, and bocavirus on the entire sample set. These viruses were chosen based on genome composition exemplifying both RNA (negative and positive stranded) and DNA viruses with genome sizes ranging from ~5.5 to 13 kb. A total of 23, 15, 3, and 21 patients were identified as positive for rhinovirus, enterovirus, hMPV, and bocavirus, respectively (Table6; Supplementary Table S2). In general, a strong correlation was observed between the threshold cycle (C_T) and percentage of viral reads identified by next-generation sequencing (Supplementary Table S2; Figure 13A-D). These data suggest that the deep-sequencing approach was at least as sensitive as real-time RT-PCRs for rhinovirus and

human metapneumovirus detection. However, the sequencing approach may be less sensitive than real-time RT-PCRs for enterovirus and bocavirus detection.

The sensitivity of a deep-sequencing approach for detecting viruses is dependent on sequencing depth (155). In this study, the average amount of reads that could be analyzed per sample was ~10,000 (range 452-34,116 reads per sample); inherently the detection limit was ~0.01% (range 0.22-0.003%) viral reads in the metagenome (Figure 13A-D). Upon exclusion of all samples with less than 10,000 analyzed deep-sequencing reads (Figure 13E), the overall correlation between C_T value and percentage of viral reads identified by deep-sequencing is very strong. A subset of samples was detected by only one of the two assays (Figure 13A-E). The next generation sequencing approach was negative in a small amount of samples (n=8) with C_T values > 30 (range 30.4-34.7) (Table 6; Figure 13E; Supplementary Table S2). Conversely, in practically all cases in which real time RT-PCR did not detect viral RNA/DNA and next generation sequencing was positive (n=10), a low number of virus-positive reads (mean 4.2, range 1-14) was obtained in the next generation sequencing approach (Table 6;Supplementary Table S2; Figure 13A-E). We checked whether any of the detected viral reads by deep sequencing were identical between the 81 analyzed samples. This was the case for one bocavirus read of 159 bp which was identical in samples from patients CU58 and CU66. These patients contained, however, 34 and 1367 bocavirus reads, respectively, and this single identical read does not change the interpretation of our data. In addition to exclusion of all samples with <10,000 analyzed next generation sequencing reads, samples that contained one or only a set of identical HRV, EV, hMPV, or HBoV reads were assumed to be negative by the deep-sequencing approach (Figure 13F). The overall strong correlation between C_T value and percentage of viral reads identified by sequencing remained.

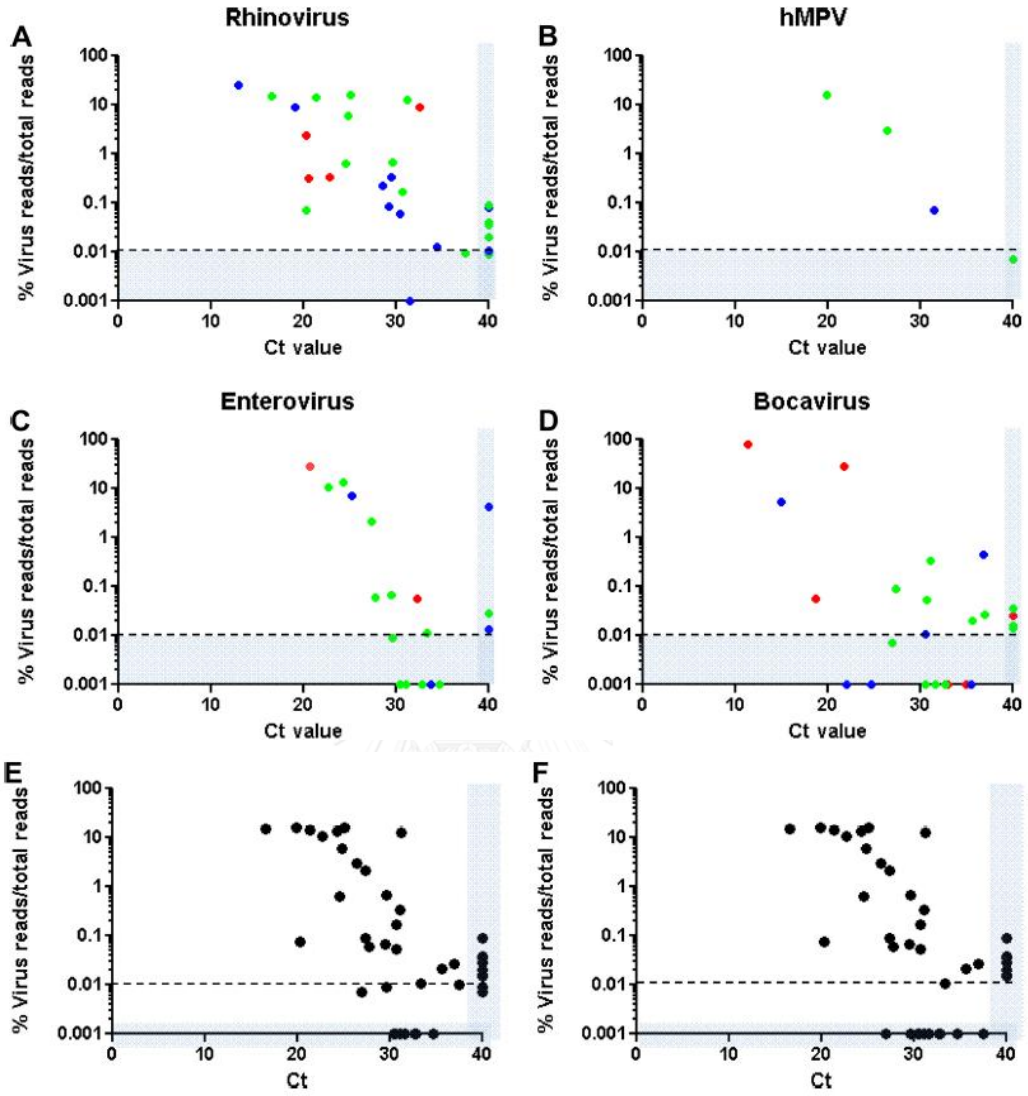

***Figure 13**. Correlation between deep-sequencing and real-time RT-PCR assays. The percentages of viral reads of the total number of analyzed reads are displayed against the real-time RT-PCR C_T values for rhinovirus (A), human metapneumovirus (B), enterovirus (C), and bocavirus (D). The theoretical next-generation sequencing detection limit (based on an average of 10,000 analyzed reads per sample) of 0.01% is indicated by dashed lines. Red, blue, and green dots indicate samples for which <5,000, ≥5,000 and <10,000, or ≥10,000 reads, respectively, were analyzed. Samples depicted on the x axis were negative by next-generation sequencing and positive by real-time RT-PCR. (E) A combination of panels A to D depicting the percentages of viral reads of the total number of analyzed reads displayed against the real-time RT-PCR C_T values for all*

samples and viruses analyzed with >10,000 analyzed deep sequence reads. (F) A combination of panels A to D depicting the percentages of viral reads of the total number of analyzed reads displayed against the real-time RT-PCR C_T values for all samples with >10,000 analyzed deep sequence reads and with samples that contained one or only a set of identical HRV, EV, hMPV, or HBoV reads assumed to be negative by the deep-sequencing approach.

Table 6. Summary of deep-sequencing and real-time PCR data.

Patient no.	Total no. of analyzed reads	HRV[a]		EV[b]		hMPV[c]		HBoV[d]	
		%[e]	C_T[f]	%	C_T	%	C_T	%	C_T
CU4	6,301	0.079[g]							
CU12	10,220	0.039[g]							
CU14	7,063								21.94[h]
CU20	11,213							0.054	30.67
CU36	17,533	0.165	30.65		34.71[h]				
CU37	4,067								32.93[h]
CU38	5,477		31.45[h]		33.73[h]				
CU40	7,314	1.477	29.62						
CU41	16,268	13.751	25.04	0.0061	27.70				
CU42	13,690	10.270	31.21					0.088	27.40
CU43	11,208			0.018	29.64				32.64[h]
CU44	1,287								34.90[h]
CU46	7,384								24.67[h]
CU51	9,779							0.460	36.81
CU52	2,554							82.341	11.36
CU56	11,035	0.009[g]						0.027	36.99
CU57	13,928	5.916	24.79					0.007	26.99
CU58	10,007							0.340	31.12
CU62	941	0.319	20.50						
CU66	4,798							28.491	21.74
CU70	10,482			10.99	22.66				
CU71	8,605		31.47[h]						
CU72	7,565	0.225	28.50	0.013[g]					35.51[h]
CU106	10,086	13.960	21.31						
CU113	9,451	0.011[g]							

Patient no.	Total no. of analyzed reads	HRV[a]		EV[b]		hMPV[c]		HBoV[d]	
		%[e]	C_T[f]	%	C_T	%	C_T	%	C_T
CU115	12,716			2.139	27.35			0.016[g]	
CU121	15,163	0.092[g]			30.38[h]				32.65[h]
CU127	25,231	0.020[g]		0.067	29.44	3.028	26.44		
CU132	34,116							0.015[g]	
CU134	11,504	0.635	24.59	13.543	24.33				
CU136	12,848								30.57[g]
CU149	38,800							0.026[h]	
CU151	5,060			7.016	25.18				
CU153	5,382			4.236[g]					
CU157	3,490							0.057	18.7
CU168	9,536							0.01	30.55
CU171	4,545	0.33	22.74	29.131	20.63				
CU173	452	8.85	32.47						
CU177	3,466	2.424	20.25	0.058	32.3				
CU183	5,939	0.084	29.25						
CB1	6,944	8.77	19.09						
CB5	8,335	0.06	30.39			0.072	31.47		
CB6	10,311	0.01	37.4						
CB7	7,828	0.013	34.34						
CB9	7,474							5.312	14.99
CB10	13,903					0.007[g]		0.036[g]	
CB11	14,479	0.007[g]			32.80[h]			0.021	35.62
CB14	15,705	15.39	16.48						

[a] HRV, human rhinovirus.

[b] EV, human enterovirus.

[c] hMPV, human metapneumovirus.

[d] HBoV, human bocavirus.

[e] Percentage of virus reads out of the total number of analyzed reads.

[f] C_T, real-time PCR cycle threshold value.

[g] Samples positive only by deep sequencing.

[h] Samples positive only by real-time PCR.

An advantage of using a deep-sequencing approach to detect viruses in clinical specimens is that it could also be used to obtain information regarding virus species and/or type of virus that was identified, in contrast to the real time PCR assays used in this study. Indeed, we obtained virus type information (Supplementary Table S2). Near full-length rhino-, entero-, human metapneumo-, and bocavirus genomes were obtained from 11 different patients in which >10% of the analyzed reads (mean C_T value 20.7; range 11.4 to 31.2) were of the described viruses (Table 6; Supplementary Table S2). The genetic relationships of these viruses with representative viral genomes of the respective viral families were assessed (Figure 13A-D).Rhinoviruses from patients CB14, CB19, and CU106 showed highest nucleotide identity (91.9%, 90.6%, and 83.6%) with HRV-A types 23, 61, and 88, respectively. The rhinoviruses obtained from patients CU41 and CU42 showed highest nucleotide identity (~97.6 and 67%) with HRV-C types4 and 9, respectively in the phylogenetic tree (Figure 14A). Rhinovirus CU42 may constitute a new rhinovirus C type (type 55) as it displays highest nucleotide identity (~74%) to HRV-51, HRV-26and HRV-36 (171). Three near-complete enterovirus genomes were obtained from patients CU70, CU134, and CU171, which showed highest nucleotide identity to enterovirus 68 strains from Japan and New Zealand (Figure 14B). The human metapneumovirus genome from patient CB22 was most closely related (~98% nucleotide identity) to a genetic group A hMPV from China (Figure 14C). The two bocavirus strains from patients CU52 and CU66 were most closely related (~99%nucleotide identity) to HBoV-1 strains previously identified in Thailand (Figure 14D). Thus virus type information was available in the next-generation sequencing data.

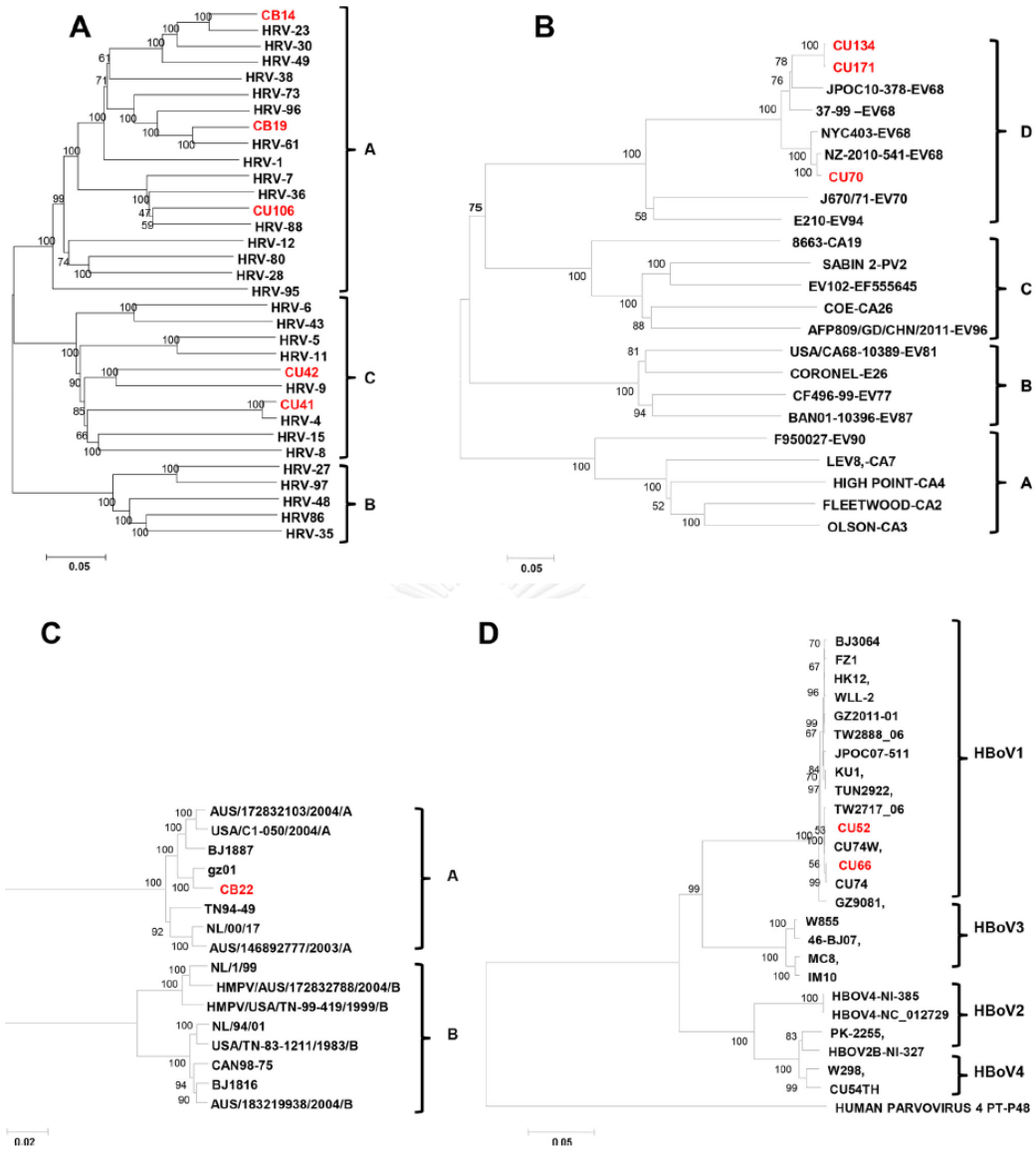

Figure 14. *Phylogenetic analysis of obtained genome sequences. (A) A phylogenetic tree of the near-complete rhinovirus genomes from patients CU41, CU42, CU106, CB14, and CB19 and representative human rhinoviruses (corresponding to nucleotides [nt] 132 to 6677 of HRV-23) was generated using MEGA6 with the neighbor-joining method with p-distance parameter and 1,000 bootstrap replicates. Bootstrap values are shown. (B) A phylogenetic tree of the near-complete enterovirus genomes from patients CU70, CU134, and CU171 and representative human enteroviruses (corresponding to nt 126 to 7143 of EV-NZ-2010-541) was generated using MEGA6 with the neighbor-joining method with the maximum-likelihood composite parameter and 1,000 bootstrap replicates. Bootstrap values are shown. (C) A phylogenetic tree of the near-complete*

hMPV genome from patient CB22 and representative human metapneumoviruses (corresponding to nt 32 to 13080 of hMPV-gz01) was generated using MEGA6 with the neighbor-joining method with p-distance parameter and 1,000 bootstrap replicates. Bootstrap values are shown. (D) A phylogenetic tree of the near-complete bocavirus genomes from patients CU52 and CU66 and representative human bocaviruses (corresponding to nt 61 to 5201 of HBoV-CU74) was generated using MEGA6 with the neighbor-joining method with the maximum-likelihood composite parameter and 1,000 bootstrap replicates. Bootstrap values are shown. GenBank accession numbers we used are shown in Supplementary Table S3.

Discussion

In principle, random amplification combined with next-generation sequencing would be advantageous in clinical viral diagnostics, as there is no need to design specific primers to amplify target sequences. It avoids the design and validation of several tens or hundreds of specific primers targeting multiple viral pathogens and does not require continuous adaptation of the primer sequences with the description of new variants and species. Instead it allows the characterization of hundreds of different known pathogens simultaneously without a priori knowledge of the virus present and provides in-depth virus species/type information.

A recent review on the application of next-generation sequencing technology in clinical diagnostics describes several major drawbacks, among which the fact that random amplification results in amplification of all nucleic acids, including host nucleic acids, suggesting that an analytical sensitivity similar to that of diagnostic PCRs cannot be expected even with increased depth of sequencing and specific pathogen enrichment steps applied (155). Our data indicate that a strong correlation existed between C_T value from diagnostic PCRs and percentage of viral reads identified by deep-sequencing for rhino-, entero-, boca-, and human metapneumoviruses, which is in line with previous observations (172). Using a next-generation sequencing platform that generated on average 10,000 reads per sample, our deep-sequencing approach was at least as sensitive (if not more) as real time diagnostic PCRs for rhinovirus and human metapneumovirus detection. However, the deep-sequencing approach seemed less sensitive than real time PCRs for entero- and bocavirus detection. The detection of RNA viruses with the deep-sequencing approach was generally better than for DNA viruses, which could be due to genome size differences and/or the nucleic acid isolation procedure as the removal of host DNA was more substantial in the RNA-isolation than in the DNA isolation procedure. As shown by Wylie and coworkers (173), increasing the sequencing depth using other next-generation sequencing platforms with increased sequence output may increase the sensitivity of the next-generation sequencing approach even further. Thus, achieving an analytical sensitivity comparable to that of

diagnostic real-time RT-PCRs using next-generation sequencing platforms may actually be possible. A common belief is that the necessary genetic information required for accurate virus typing is unpredictable when partial sequences are acquired due to insufficient genome coverage (155). This, however, depends on a number of variables, including the divergence of viruses within the virus families under study and which part of the genome is sequenced. In our study, typing information was readily available from next-generation sequencing data from 11 different patients in whom >10% of the analyzed reads (mean C_T value, 20.7; range, 11.4 to 31.2) were of the described viruses. In line with previous observations, virus type information was obtained even when only a few sequence reads were available (173). For example, most enterovirus-positive samples contained viral reads that were typed as enterovirus D type 68. Until recently, reports of respiratory infections due to HEV 68 were rare, but over the past 3 years, outbreaks in Japan, the Philippines, and the Netherlands as well as epidemic clusters in the United Kingdom have implicated HEV 68 as an emerging respiratory pathogen (174-178). Our data confirm previous observations regarding enterovirus 68 infections in Thailand (159). In addition, we could type most of the rhinovirus-positive samples as being A, B, or C and obtained near-full length genome sequences which could be typed as A23, A61, and A88, C4 and a new type which we designated C55 (171). All hMPV strains were typed as A, the human bocaviruses were type I and for example RSV and human herpesvirus infections could be typed as well.

The next-generation sequencing approach identified a number of viruses that are usually not screened for in respiratory infections using routine diagnostic assays, including Herpesviridae. It is of note that herpesviruses were identified in 12% of the samples analyzed here (mean, 0.24% of analyzed reads; range, 0.007% to 2%) and that they always occurred in combination with at least one other viral infection. This may be due to the detection of latent viruses, but it is also possible that respiratory disease caused by other viruses results in the reactivation of latent herpesviruses. However, it cannot be ruled out that human herpesviruses are involved in respiratory disease. Cytomegalovirus (CMV) infections have been described to cause pneumonia sporadically, and in this

study, CMV was detected at relatively high levels using next generation sequencing (~2% of the analyzed reads) in an 8-month-old patient (CU188) and seemingly dominated the simultaneous RSV infection.

Another prominent observation was the detection of anelloviruses in respiratory samples from over half of the children with respiratory illness. Anellovirus infections are commonly acquired during early childhood during which the virus establishes a chronic productive infection with long-lasting detectable viremia (179). They are endemic worldwide and can be detected in blood and various tissues in the body, including cerebrospinal and bronchoalveolar lavage fluid (179). Many investigations have been carried out to unravel their epidemiological, clinical and pathogenic properties, but at present their causative role in disease is not considered likely. Previously, we have shown that the presence of human anelloviruses in vitreous fluid of patients with seasonal hyperacute panuveitis (SHAPU), a potentially blinding ocular disease occurring in Nepal that principally affects young children, was significantly higher compared to non-SHAPU patients. The data suggested that anelloviruses observed in vitreous fluid samples of uveitis patients, but not patients with retinal detachment, most likely originated from the systemic anellovirus pool upon inflammation-induced disruption of the blood-ocular barrier (180). Likewise, inflammation in the respiratory tract system, caused by an infection for example, may cause increased permeability of the capillary barrier, resulting in edema and influx of inflammatory cells and the concomitant increase of anelloviruses in the respiratory tract.

One of the major questions is how to interpret next-generation sequencing data in terms of what is clinically relevant for the patient. The comparison of the next-generation sequencing data to real-time RT-PCR C_T values showed that the interpretation may not need to be very different than the interpretation of C_T values, as there is a very strong correlation between the percentage of viral reads detected and the C_T value obtained from the same sample. However, as the sensitivity of the next-generation sequencing assay compared to that of the real-time RT-PCR assays is not the same for each RT-PCR assay, the next-generation sequencing assay may need to be validated with each real-

time RT-PCR assay. In addition, our data show that multiple viral infections occurred substantially more often than did single-virus infections (60% versus 28%, respectively). Even when taking only well-established respiratory pathogens into account, 25% of the cases were multiple infections. In most cases, one of the detected viruses seemed to dominate. Although major hurdles for the routine use of next generation sequencing approaches do exist, including cost, labor intensity, and especially turnaround time, the sensitivity of a next generation sequencing assay may actually reach that of current diagnostic routine real-time RT-PCR assays, and it potentially provides more information regarding virus species/type, thus remaining of interest for virus diagnosis in clinical and public health settings. Although it might not be used for routine diagnostics at present, a sequence-independent next-generation sequencing assay may be applied to samples that remain negative with routine diagnostics and, as such, may function as an additional diagnostic tool with the added value of surveillance for (re)emerging viruses.

Supplemental material

Table S1. *Patient information*

Patient	Gender	Age	Collection date	Diagnosis	Respiratory distress	Comorbidities	Category	Symptom
CU4	F	1 yr	2010/07/23	Infectious mononucleosis	No	Allergic rhinitis	Infant	Mild
CU8	M	2 yr	2010/07/28	Pneumonia	Yes	NA	Pre-school	Moderate
CU9	M	5 yr	2010/07/28	Pneumonia	Yes	Epilepsy	Primary school	Severe
CU12	M	4 m	2010/08/02	Pneumonia	Yes	NA	Infant	Moderate
CU14	M	10 m	2010/08/04	Pneumonia	Yes	Dandy Walker syndrome	Infant	Severe
CU15	M	1 m	2010/08/04	Pneumonia	Yes	Prematurity	Infant	Severe
CU19	M	2 m	2010/08/11	Pneumonia	Yes	Prematurity	Infant	Severe
CU20	M	11 m	2010/08/11	Pneumonia	Yes	X-linked CGD	Infant	Moderate
CU22	F	1 yr	2010/08/11	Pneumonia	Yes	Prematurity	Infant	Severe
CU24	M	1 m	2010/08/11	Pneumonia	Yes	Cyanotic heart disease	Infant	Severe
CU25	M	2 m	2010/08/16	Pneumonia	Yes	Prematurity	Infant	Moderate
CU26	M	5 yr	2010/08/23	Pneumonia	Yes	Epilepsy	Pre-school	Severe
CU32	F	2 m	2010/10/22	Pneumonia	Yes	Endophthalmitis	Infant	Severe
CU33	F	3 m	2010/10/22	Pneumonia	Yes	Prematurity	Infant	Severe
CU35	F	27 days	2010/11/09	Pneumonia	Yes	NA	Infant	Mild
CU36	M	1 yr	2010/11/12	Pneumonia	Yes	Down syndrome	Infant	Moderate
CU37	M	1 yr	2010/12/15	Pneumonia	Yes	Congenital hydrocephalus	Infant	Severe
CU38	M	1 yr	2010/12/15	Pneumonia	Yes	Congenital hydrocephalus	Infant	Severe
CU40	M	3 yr	2011/01/04	Pneumonia	Yes	NA	Pre-school	Moderate
CU41	M	1 yr	2011/01/04	Pneumonia	No	Reactive airway disease	Infant	Moderate
CU42	F	2 yr	2011/01/11	Pneumonia	Yes	NA	Pre-school	Moderate
CU43	M	1 yr	2011/01/12	Pneumonia	Yes	Epilepsy	Infant	Moderate

Patient	Gender	Age	Collection date	Diagnosis	Respiratory distress	Comorbidities	Category	Symptom
CU44	M	2 yr	2011/01/31	Pneumonia	Yes	Chronic lung disease	Pre-school	Moderate
CU45	M	2 yr	2011/04/11	Pneumonia	Yes	Acute respiratory distress syndrome	Pre-school	Severe
CU46	M	1 yr	2011/04/20	Pneumonia	Yes	NA	Infant	Moderate
CU48	F	5 m	2011/04/26	NA	NA	NA	Infant	NA
CU49	M	5 m	2011/06/16	Pneumonia	Yes	Acyanotic heart disease	Infant	Severe
CU50	M	2 yr	2011/06/17	Pneumonia	Yes	Cyanotic heart disease	Pre-school	Moderate
CU51	F	2 m	2011/06/17	Pneumonia	Yes	Laryngomalacia, vallecular cyst	Infant	Severe
CU52	F	13 yr	2011/06/27	Pneumonia	Yes	Pulmonary TB	Secondary school	Severe
CU55	M	1 yr	2011/07/11	Pneumonia	Yes	Dandy Walker syndrome	Infant	Severe
CU56	M	3 yr	2011/07/19	Pneumonia	Yes	NA	Pre-school	Moderate
CU57	F	6 m	2011/07/19	Pneumonia	Yes	Acyanotic heart disease	Infant	Moderate
CU58	F	1 yr	2011/07/15	Pneumonia	Yes	Multiple congenital anomalies	Infant	Moderate
CU62	F	1 yr	2011/07/15	URI	No	Cyanotic heart disease	Infant	Mild
CU66	F	9 m	2011/07/21	Pneumonia	Yes	Prematurity	Infant	Moderate
CU68	F	2 yr	2011/07/22	Pneumonia	Yes	Epilepsy	Pre-school	Moderate
CU70	F	1 yr	2011/07/25	Pneumonia	Yes	NA	Infant	Moderate
CU71	F	1 yr	2011/07/25	Pneumonia	No	Bechwidth widerman syndrome	Infant	Moderate
CU72	M	2 yr	2011/07/27	Pneumonia	Yes	Bronchiolitis obliterans	Pre-school	Moderate
CU100	M	1 m	2011/08/15	Pneumonia	Yes	NA	Infant	Moderate
CU103	M	11 yr	2011/08/16	URI	No	Dilated cardiomyopathy	Primary school	Mild

Patient	Gender	Age	Collection date	Diagnosis	Respiratory distress	Comorbidities	Category	Symptom
CU105	M	9 yr	2011/08/17	Pneumonia	No	Epilepsy	Primary school	Moderate
CU106	M	2 yr	2011/08/17	Pneumonia	Yes	NA	Pre-school	Moderate
CU113	M	7 m	2011/08/22	Pneumonia	Yes	Severe laryngomalacia	Infant	Severe
CU115	F	4 yr	2011/08/22	Pneumonia	Yes	Asthma	Pre-school	Moderate
CU121	F	3 yr	2011/08/25	URI	No	NA	Pre-school	Mild
CU127	F	2 yr	2011/08/29	Pneumonia	No	NA	Pre-school	Moderate
CU132	F	8 d	2011/08/30	Pneumonia	Yes	Maternal PROM	Infant	Moderate
CU134	M	2 yr	2011/08/31	Pneumonia	Yes	Swallowing dysfunction	Pre-school	Moderate
CU135	M	14 yr	2011/08/31	URI	No	Herpes encephalitis	Secondary school	Mild
CU136	F	6 m	2011/09/02	URI	No	Biliary atresia	Infant	Mild
CU138	F	1 yr	2011/09/05	Pneumonia	Yes	Cyanotic heart disease	Infant	Moderate
CU143	F	2 yr	2011/09/06	Pneumonia	No	Crouzon's syndrome	Pre-school	Moderate
CU149	M	10 m	2011/09/08	Pneumonia	Yes	TE fistula	Infant	Moderate
CU150	F	1 yr	2011/09/08	Pneumonia	Yes	Cyanotic heart disease	Infant	Moderate
CU151	M	4 yr	2011/09/12	URI	Yes	Reactive airway disease	Pre-school	Moderate
CU153	F	5 yr	2011/09/12	Pneumonia	Yes	Spastic CP	Pre-school	Moderate
CU157	M	7 m	2011/09/14	Pneumonia	Yes	G6PD	Infant	Moderate
CU168	F	6 m	2011/09/22	URI	No	Biliary atresia	Infant	Mild
CU169	F	1 m	2011/09/22	Pneumonia	Yes	NA	Infant	Severe
CU171	M	6 m	2011/09/23	Pneumonia	Yes	Tracheal stenosis	Infant	Moderate
CU173	M	8 yr	2011/09/26	Pneumonia	Yes	Asthma	Primary school	Moderate
CU177	F	2 yr	2011/09/26	Pneumonia	Yes	Cerebral palsy	Pre-school	Moderate
CU183	F	4 m	2011/10/03	Pneumonia	Yes	NA	Infant	Moderate
CU188	F	8 m	2011/10/04	URI	Yes	Heart failure	Infant	Moderate

Patient	Gender	Age	Collection date	Diagnosis	Respiratory distress	Comorbidities	Category	Symptom
CB1	M	7 m 22d	2013/06/24	Pneumonia	Yes	NA	Infant	Moderate
CB5	M	6 m 5d	2013/06/24	Pneumonia	Yes	NA	Infant	Severe
CB6	M	1 m 23d	2013/06/24	Pneumonia	Yes	NA	Infant	Severe
CB7	M	1 m 17d	2013/06/24	Pneumonia	Yes	NA	Infant	Moderate
CB9	M	10 m 13d	2013/06/24	Pneumonia	Yes	NA	Infant	Severe
CB10	F	4 m 29d	2013/06/24	Pneumonia	Yes	NA	Infant	Moderate
CB11	F	1m 17d	2013/06/24	Pneumonia	Yes	NA	Infant	Severe
CB14	M	12 yr 3m	2013/06/24	Pneumonia	Yes	NA	Secondary school	Moderate
CB15	M	2 yr 1m	2013/07/06	Pneumonia	Yes	Chronic lung disease	Pre-school	Moderate
CB17	F	3m	2013/07/06	Pneumonia	Yes	Chronic lung disease	Infant	Moderate
CB19	M	1 yr 11m	2013/07/06	Pneumonia	Yes	NA	Infant	Moderate
CB21	M	1 yr 5m	2013/07/19	Pneumonia	Yes	NA	Infant	Moderate
CB22	F	1 yr 3m	2013/07/19	Pneumonia	Yes	Hydroce-phalus	Infant	Severe
CB23	F	11 m	2013/07/19	Pneumonia	Yes	NA	Infant	Moderate
CB24	M	9 m	2013/07/19	Pneumonia	Yes	NA	Infant	Moderate

Table S2. Summary of deep-sequencing data and real-time-PCR data

Patient	Mammalian virus (no. of reads)	Total no. analyzed reads	Total no. viral reads	%Viral/total reads	HRV reads	EV reads	hMPV reads	HBoV reads	HRV Ct	EV Ct	hMPV Ct	HBoV Ct	Symptoms
CU4	HRV-C (5)	6301	13	0.21	5	-	-	-	-	-	-	-	Mild
CU8	Anellovirus (1323)	6294	1758	27.93	-	-	-	-	-	-	-	-	Moderate
CU9	-	7852	182	2.32	-	-	-	-	-	-	-	-	Severe
CU12	HRV-C (4), Anellovirus (22)	10220	122	1.19	4	-	-	-	-	-	-	-	Moderate
CU14	Anellovirus (151)	7063	174	2.46	-	-	-	-	-	-	-	21.94	Severe
CU15	RSV-A (242)	6340	212	3.34	-	-	-	-	-	-	-	-	Severe
CU19	hADV-C (1)	6816	2076	30.46	-	-	-	-	-	-	-	-	Severe
CU20	HBoV-1 (6), Anellovirus (35)	11213	73	0.65	-	-	-	6	-	-	-	30.67	Moderate
CU22	HPV-107 (1), Anellovirus (7)	9214	766	8.31	-	-	-	-	-	-	-	-	Severe
CU24	-	8855	6	0.07	-	-	-	-	-	-	-	-	Severe
CU25	RSV (1)	667	1	0.15	-	-	-	-	-	-	-	-	Moderate
CU26	hADV-C (1), Anellovirus (1)	9415	29	0.31	-	-	-	-	-	-	-	-	Severe
CU32	-	3744	5	0.13	-	-	-	-	-	-	-	-	Severe
CU33	Anellovirus (1)	13193	19	0.14	-	-	-	-	-	-	-	-	Severe
CU35	-	5275	62	1.18	-	-	-	-	-	-	-	-	Mild
CU36	HHV-6B (20), HRV-B (29), Anellovirus (69), HPV-5b (2)	17533	200	1.14	29	-	-	-	30.65	34.71	-	-	Moderate
CU37	Anellovirus (45), Flu A-H1N1 (1)	4067	62	1.52	-	-	-	-	-	-	-	32.93	Severe
CU38	Anellovirus (8), HHV-6B (25)	5477	40	0.73	-	-	-	-	31.45	33.73	-	-	Severe
CU40	HRV-C,B (108)	7314	109	1.49	108	-	-	-	29.62	-	-	-	Moderate
CU41	HRV-C (2237), EV-B (10), Anellovirus (1008)	16268	3273	20.12	2237	10	-	-	25.04	27.70	-	-	Moderate
CU42	HRV-C (1406), HBoV-1 (12), Anellovirus (29)	13690	1448	10.58	1406	-	-	12	31.21	-	-	27.40	Moderate
CU43	Anellovirus (15), EV-C (2)	11208	33	0.29	-	2	-	-	-	29.64	-	32.64	Moderate
CU44	-	1287	0	0.00	-	-	-	-	-	-	-	34.90	Moderate
CU45	Anellovirus (102), hADV-C (1)	8885	134	1.51	-	-	-	-	-	-	-	-	Severe
CU46	Anellovirus (89)	7384	99	1.34	-	-	-	-	-	-	-	24.67	Moderate
CU48	hADV-C (1)	8475	24	0.28	-	-	-	-	-	-	-	-	NA
CU49	hADV-C (4), HHV-5 (3), Anellovirus (1)	9584	37	0.39	-	-	-	-	-	-	-	-	Severe
CU50	hPIV-3 (3), Anellovirus (1022)	14528	1040	7.16	-	-	-	-	-	-	-	-	Moderate
CU51	hADV-C (1), HBoV-1 (45)	9779	73	0.75	-	-	-	45	-	-	-	36.8	Severe
CU52	HBoV-1 (2103), Anellovirus (34)	2554	2167	84.85	-	-	-	2103	-	-	-	11.36	Severe
CU55	Anellovirus (1)	6959	2	0.03	-	-	-	-	-	-	-	-	Severe
CU56	HBoV-1 (3), hADV-C (1), HRV-C (1), Anellovirus (1)	11035	32	0.29	1	-	-	3	-	-	-	36.99	Moderate
CU57	HRV-A (824), HHV-5 (1), Anellovirus (14), HBoV-1 (1)	13928	847	6.08	824	-	-	1	24.79	-	-	26.99	Moderate
CU58	HBoV-1 (34)	10007	34	0.34	-	-	-	34	-	-	-	31.1	Moderate
CU62	HRV-A (3), Anellovirus (5)	941	8	0.85	3	-	-	-	20.50	-	-	-	Mild
CU66	HBoV-1 (1367)	4798	1373	28.62	-	-	-	1367	-	-	-	21.74	Moderate
CU68	-	7353	454	6.17	-	-	-	-	-	-	-	-	Moderate
CU70	EV-D (1152), Anellovirus (54)	10482	1209	11.53	-	1152	-	-	-	22.66	-	-	Moderate

Patient	Mammalian virus (no. of reads)	Total no. analyzed reads	Total no. viral reads	%Viral/total reads	HRV reads	EV reads	hMPV reads	HBoV reads	HRV Ct	EV Ct	hMPV Ct	HBoV Ct	Symptoms
CU71	Anellovirus (270)	8605	345	4.01	-	-	-	-	31.47	-	-	-	Moderate
CU72	HRV-A (1), HPV-110 (1), EV-D (17), Anellovirus (12)	7565	34	0.45	17	1	-	-	28.50	-	-	35.51	Moderate
CU100	Human endogenous retrovirus (1), Anellovirus (2)	7667	7	0.09	-	-	-	-	-	-	-	-	Moderate
CU103	Anellovirus (6)	10432	6	0.06	-	-	-	-	-	-	-	-	Mild
CU105	Anellovirus (1)	14808	7	0.05	-	-	-	-	-	-	-	-	Moderate
CU106	HHV-5 (6), HRV-A (1408)	10086	1422	14.10	1408	-	-	-	21.31	-	-	-	Moderate
CU113	HRV-A (1), Anellovirus (2)	9451	8	0.08	1	-	-	-	-	-	-	-	Severe
CU115	EV-D (276), HBoV-1 (2)	12716	361	2.84	-	272	-	2	-	27.35	-	-	Moderate
CU121	HRV-A (14), Flu A-H3N2 (88), Flu B (7), Human parechovirus (2)	15163	124	0.82	14	-	-	-	-	30.38	-	32.65	Mild
CU127	EV-A (17), HRV-A (5), hMPV-A (764), Anellovirus (10)	25231	818	3.24	5	17	764	-	-	29.44	26.44	-	Moderate
CU132	HBoV-1 (5)	34116	46	0.13	-	-	-	5	-	-	-	-	Moderate
CU134	HRV-B (73), EV-D (1558)	11504	1695	14.73	73	1558	-	-	24.59	24.33	-	-	Moderate
CU135	-	1712	0	0.00	-	-	-	-	-	-	-	-	Mild
CU136	-	12848	23	0.18	-	-	-	-	-	-	-	30.57	Mild
CU138	Anellovirus (11)	5388	38	0.71	-	-	-	-	-	-	-	-	Moderate
CU143	Flu-A H1N1 (1), Anellovirus (1)	6041	14	0.23	-	-	-	-	-	-	-	-	Moderate
CU149	RSV-A (20), HBoV-1 (1), Anellovirus (4)	3880	39	1.01	-	-	-	1	-	-	-	-	Moderate
CU150	Anellovirus (12)	6316	19	0.30	-	-	-	-	-	-	-	-	Moderate
CU151	EV-D (355)	5060	357	7.06	-	355	-	-	-	25.18	-	-	Moderate
CU153	EV-D (228), RSV-A(1)	5382	238	4.42	-	228	-	-	-	-	-	-	Moderate
CU157	RSV-A(591), HBoV-1 (2)	3490	593	16.99	-	-	-	2	-	-	-	18.70	Moderate
CU168	Flu-A H3N2 (23), RSV-A (2), HBoV-1 (1), Anellovirus (3)	9536	135	1.42	-	-	-	1	-	-	-	30.55	Mild
CU169	-	8529	27	0.32	-	-	-	-	-	-	-	-	Severe
CU171	HRV-C (15), EV-D (1324), Anellovirus (44)	4545	1388	30.54	15	1324	-	-	22.74	20.63	-	-	Moderate
CU173	HRV-A (40), Anellovirus (3)	452	69	15.27	40	-	-	-	32.47	-	-	-	Moderate
CU177	HRV-A (84), EV-D (2)	3466	89	2.57	84	2	-	-	20.25	32.30	-	-	Moderate
CU183	RSV-A (528), HRV-C (5), HHV-5 (1)	5939	534	8.99	5	-	-	-	29.25	-	-	-	Moderate
CU188	HHV-5 (149), RSV-A (7)	7289	303	4.16	-	-	-	-	-	-	-	-	Moderate
CB1	HRV-A (609), Anellovirus (1)	6944	615	8.86	609	-	-	-	19.1	-	-	-	Moderate
CB5	hMPV-A (6), HRV-A (5), Anellovirus (2)	8335	13	0.16	5	-	6	-	30.4	-	31.47	-	Severe
CB6	HRV-A (1), Anellovirus (1)	10311	2	0.02	1	-	-	-	37.4	-	-	-	Severe
CB7	HRV-A (1)	7828	8	0.10	1	-	-	-	34.3	-	-	-	Moderate
CB9	HBoV-1 (397)	7474	400	5.35	-	-	-	397	-	-	-	14.99	Severe
CB10	HBoV-1 (5), RSV-B (1), hMPV-A (1)	13903	10	0.07	-	-	1	5	-	-	-	-	Moderate
CB11	HBoV-1 (3), HHV-4 (2), HRV-A (1)	14479	20	0.14	1	-	-	3	-	32.80	-	35.62	Severe
CB14	HRV-A (2417), Anellovirus (1)	15705	2484	15.82	2417	-	-	-	16.48	-	-	-	Moderate
CB15	RSV-B (212)	6586	212	3.22	-	-	-	-	-	-	-	-	Moderate
CB17	RSV-B (4), HBoV (2), EV-C (3), Anellovirus (11)	26657	65	0.24	-	3	-	-	-	33.37	-	-	Moderate
CB19	HRV-A (2062), Anellovirus (9)	7948	2076	26.12	2062	-	-	-	12.94	-	-	-	Moderate
CB21	HRV-A (24)	7227	24	0.33	24	-	-	-	29.5	-	-	-	Moderate
CB22	hMPV-A (2070), Anellovirus (253)	12880	2435	18.91	-	-	2070	-	-	31.03	19.87	31.62	Severe
CB23	HRV-A (10), HHV-5 (1), Anellovirus (49)	13596	66	0.49	10	-	-	-	20.24	-	-	-	Moderate
CB24	EV-D (3), HHV (1), Anellovirus (1)	10698	5	0.05	-	3	-	-	-	-	-	-	Moderate

Table S3. Genbank accession numbers

Virus	Accession no.
Human rhinovirus A23	DQ473497
Human rhinovirus A30	DQ473512
Human rhinovirus A49	DQ473496
Human rhinovirus A38	DQ473495
Human rhinovirus A73	DQ473492
Human rhinovirus A96	FJ445171
Human rhinovirus A61	FJ445144
Human rhinovirus A1	FJ445111
Human rhinovirus A7	DQ473503
Human rhinovirus A36	DQ473505
Human rhinovirus A88	DQ473504
Human rhinovirus A12	EF173415
Human rhinovirus A28	DQ473508
Human rhinovirus A80	FJ445156
Human rhinovirus A95	FJ445170
Human rhinovirus C5	EF582386
Human rhinovirus C9	GQ223228
Human rhinovirus C4	EF582385
Human rhinovirus C15	GU219984
Human rhinovirus C8	GQ223227
Human rhinovirus C11	EU840952
Human rhinovirus C6	EF582387
Human rhinovirus C43	JX074056
Human rhinovirus C51	JF317015
Human rhinovirus B27	EF173421
Human rhinovirus B97	FJ445172
Human rhinovirus B48	DQ473488

Virus	Accession no.
Human rhinovirus B35	DQ473487
Human rhinovirus B86	FJ445164
HMPV/AUS/172832103/2004/A	KC403978
HMPV/USA/C1-050/2004/A	KC562243
BJ1887	DQ843659
HMPVgz01	GQ153651
TN94-49	JN184400
NL/00/17 type A2	FJ168779
HMPV/AUS/146892777/2003/A	KC403981
NL/1/99	AY525843
HMPV/AUS/172832788/2004/B	KF530164
HMPV/USA/TN-99-419/1999/B	KC562234
NL/94/01 subtype B2	FJ168778
HMPV/USA/TN-83-1211/1983/B	KC562244
CAN98-75	AY297748
BJ1816	DQ843658
HMPV/AUS/183219938/2004/B	KF530178
Human enterovirus 68, strain: JPOC10-378	AB601883
Human enterovirus 68 isolate 37-99 from France	EF107098
Human enterovirus 68 strain NYC403 from USA	JX101846
Human enterovirus 68 isolate NZ-2010-541	X070222
Human enterovirus 70 strain J670/71	DQ201177
Human enterovirus 94 isolate E210	DQ916376
Human coxsackievirus A19 strain 8663	AF499641
Poliovirus type 2 genome (strain Sabin 2 (P712, Ch, 2ab))	X00595
Human enterovirus 102	EF555645
Human coxsackievirus A21 (strain Coe) genomic RNA	D00538
Human enterovirus C96 isolate AFP809/GD/CHN/2011	KF495604

Virus	Accession no.
Human enterovirus 81 strain USA/CA68-10389	AY843299
Human echovirus 26 strain Coronel	AY302550
Human enterovirus 77 gene for polyprotein	AJ493062
Human enterovirus 87 strain BAN01-10396	AY843305
Human enterovirus 90 isolate F950027	AY773285
Human coxsackievirus A7 strain LEV8	JQ041367
Human coxsackievirus A4 strain High Point	AY421762
Human coxsackievirus A2 strain Fleetwood	AY421760
Human coxsackievirus A3 strain Olson	AY421761
Human Bocavirus BJ3064	DQ988933
Human bocavirus strain FZ1	GQ455988
Human bocavirus isolate HK12	EF450728
Human bocavirus WLL-2	EF441262
Human bocavirus strain GZ2011-01	JN128954
Human bocavirus isolate TW2888_06,	EU984237
Human bocavirus DNA, complete genome, strain: JPOC07-511	AB481080
Human bocavirus isolate KU1	FJ695472
Human bocavirus strain TUN2922	JF327787
Human bocavirus isolate CU74W,	EU262979
Human bocavirus isolate CU74	EF203922
Human bocavirus isolate GZ9081	EF203922
Human bocavirus isolate GZ9081	JN794566
Human bocavirus 3 strain W855	FJ948861
Human bocavirus 3 isolate 46-BJ07	HM132056
Human bocavirus 3 strain MC8	GQ867666
Human bocavirus 3 strain IM10	GQ867667
Human bocavirus 4 strain HBoV4-NI-385	FJ973561

Virus	Accession no.
Human bocavirus 4	NC_012729
Human bocavirus 2 isolate PK-2255	FJ170279
Human bocavirus 2 strain HBoV2B-NI-327	FJ973559
Human bocavirus 2 strain W298	FJ948860
Human bocavirus 2 strain CU54TH	GU048663
Human parvovirus 4 isolate PT-P48	HQ113143

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APPENDIX A

PRIMER SEQUENCES

Primers and probes for influenza virus detection by real-time RT-PCR

Target Gene	Primer/Probe	Sequence (5'→3')
GAPDH	GAPDH-F	GTGAAGGTCGGAGTSAACGG
	GAPDH-P	HEX-CGCCTGGTCA**C**CAGGGCTGC-BHQ1
	GAPDH-R	TCAATGAAGGGGTCATTGATGG
M Flu A	FluA-M-F	CATGGARTGGCTAAAGACAAGACC
	FluA-M-P	FAM-ACGC**T**CACCG**T**GCCC**A**GT-BHQ1
	FluA-M-R	AGGGCATTYTGGAYAAAGCGTCTA
M Flu B	FluB-M-F	AAGCATCACATTCACACAGGGC
	FluB-M-P	Cy5-ATGGTCTCAGCTATGAACACAGCAA-BBQ
	FluB-M-R	CCTTCYCCATTCTTTTGACTTGC
H1-2009	H1-09-F	AACACCCAAGGGTGCTATAAACA
	H1-09-P	ROX-TACATCCGATCACAATTGGAAAATGTCC-BBQ
	H1-09-R	CATCCATCTACCATCCCTGTCCA
H1 seasonal	H1_F	ACTACTGGACTCTGCTKGAA
	H1_P	FAM-TTGAGGCAAATGGAAATCTAATAGC-BHQ1
	H1_R	AAGCCTCTACTCAGTGCGAA
H3 seasonal	H3_F	GCTACTGAGCTGGTTCAGARTTCCT
	H3_P	HEX-AGAT**G**C**T**C**T**A**T**T**G**G**G**AGACC-BHQ1
	H3_R	AGGGTAACAGTTGCTGTRGGC
H5 avian	H5-F	TGGAAAGTGTAARAAACGGAACGT
	H5-P	Cy5-A**C**TC**C**A**C**TT**A**T**T**T**C**CT**C**T**C**T-BHQ3
	H5-R	TGCTAGGGAACTCGCMACTG

********** Under line and bold = LNA residue **********

Primers and probes used in the routine molecular viral diagnostics setting of Erasmus Medical Center (Hoek et al. 2012)

Viral Pathogen	Primer/Probe	Sequence (5'→3')
Rhinovirus	rhino fwd 45	CGAAGAGTCTACTGTGCTCACCTT
	rhino fwd 5	GAAGATCCTATTGCGCTTAGCTGT
	rhino fwd Ag	GGTGTGAAGAGCCCCGTGTG
	rhino fwd Bg	GGTGTGAAGACTCGCATGTGC
	rhino rev	GTAGTCGGTCCCATCCC
	rhinoprobe 4	TCCTCCGGCCCCTGAATGYGG-BHQ-1
Enterovirus	entero-fwd-D	GACATGGTGYGAAGAGTCTATTGA
	entero-probe-TM	CGGAACCGACTACTTTGGGTGTCCGTGTTTC
	entero-rev-TM	GATTGTCACCATAAGCAGCCA
hMPV	hmpv-fwd-TM	CATATAAGCATGCTATATTAAAAGAGTCTC
	hmpv-probe-2	FAM-TGTAATGATGAGGGTGTCACTGCGGTTG-BHQ-1
	hmpv-probe-TM	FAM-TGCAATGATGAGGGTGTCACTGCGGTTG-BHQ-1
	hmpv-rev-TM	CCTATTTCTGCAGCATATTTGTAATCAG

VITA

The author of this thesis was born on 30 April 1987 in Bangkok, Thailand. She received her Bachelor degree of Science Major in Biochemistry in 2008 from Faculty of Science, Chulalongkorn University. In 2009, she enrolled in the Royal Golden Jubilee Scholar (TRF) in graduate program for degree of Doctor of Philosophy Program in Biomedical Science, Chulalongkorn University under supervision of Prof. Dr. Yong Poovorawan. Her research focuses on the epidemiology of influenza virus. She also did metagenomic analysis of viruses from patients with respiratory illness. This work was carried out at the Erasmus MC department of Viroscience (Rotterdam, the Netherlands) under supervision of Dr. Bart L. Haagmans and Dr. Saskia L. Smits.

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