ระบาดวิทยาและการวิเคราะห์ลักษณะทางพันธุกรรม
ของ Human Respiratory Syncytial Virus (RSV) และ Human Enterovirus D68 (EV-D68) ใน
ผู้ป่วยเด็กไทยที่มีการติดเชื้อระบบทางเดินหายใจส่วนล่าง

นางสาวไอลดา ทองปาน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PREVALENCE AND MOLACULAR GENETIC ANALYSIS OF HUMAN RESPIRATORY SYNCYTIAL VIRUS AND ENTEROVIRUS 68 AMONG CHILDREN WITH ACUTE LOWER RESPIRATORY TRACT INFECTION IN THAILAND

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for the Degree of Doctor of Philosophy Program in Biomedical Sciences
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Thesis Title	PREVALENCE AND MOLACULAR GENETIC ANALYSIS OF HUMAN RESPIRATORY SYNCYTIAL VIRUS AND ENTEROVIRUS 68 AMONG CHILDREN WITH ACUTE LOWER RESPIRATORY TRACT INFECTION IN THAILAND
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ไอลดา ทองปาน : ระบาดวิทยาและการวิเคราะห์ลักษณะทางพันธุกรรมของ Human Respiratory Syncytial Virus (RSV) และ Human Enterovirus D68 (EV-D68) ในผู้ป่วยเด็กไทยที่มีการติดเชื้อระบบทางเดินหายใจส่วนล่าง (PREVALENCE AND MOLACULAR GENETIC ANALYSIS OF HUMAN RESPIRATORY SYNCYTIAL VIRUS AND ENTEROVIRUS 68 AMONG CHILDREN WITH ACUTE LOWER RESPIRATORY TRACT INFECTION IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ. ยง ภู่วรวรรณ, หน้า.

เชื้อไวรัส Respiratory Syncytial Virus หรือ RSV ทำให้เกิดการติดเชื้อในระบบทางเดินหายใจส่วนล่างที่รุนแรงในเด็กทารกและเด็กเล็กทั่วโลก การอธิบายเชิงระบาดวิทยา วิวัฒนาการ และการแพร่กระจายของเชื้อ RSV อาศัยข้อมูลทางพันธุกรรมของไวรัสโดยละเอียดเพื่อนำไปสู่กลยุทธ์ใหม่ในการควบคุมการระบาด และการพัฒนาวัคซีน การศึกษาระบาดวิทยาของเชื้อ RSV ในประเทศไทยในช่วง 6 ปีติดต่อกัน (มกราคม 2555 ถึงธันวาคม 2560) เริ่มต้นจากการคัดเลือกตัวอย่าง 3306 ตัวอย่าง จากผู้ป่วยเด็กอายุต่ำกว่า 5 ปีที่มีอาการติดเชื้อทางเดินหายใจเฉียบพลัน ระหว่างเดือนมกราคม 2555 ถึงธันวาคม 2558 ด้วยเทคนิค semi-nested RT-PCR ในเวลาต่อมาได้ทำการพัฒนาวิธีการตรวจหาเชื้อ RSV รวมทั้งตรวจหาเชื้อ human metapneumovirus หรือ hMPV ในผู้ป่วยทุกกลุ่มอายุ ซึ่งเป็นผู้ป่วยในกรุงเทพมหานคร ในช่วงเดือนมกราคม 2559 ถึงธันวาคม 2560 ด้วยเทคนิค multiplex real-time RT-PCR ผลการวิจัยพบว่าในปี พ.ศ. 2555-2558 ตัวอย่างที่ให้ผลบวกต่อเชื้อ RSV คิดเป็น 8.4% (277/ 3,306) และผลการวิเคราะห์ลำดับนิวคลีโอไทด์ของยีน G protein ด้วย phylogenetic tree พบว่าจีโนไทป์ที่ระบาดในช่วงนั้น คือ NA1, ON1 และ BA (BA9, BA10 และ BA-C) และในปี พ.ศ. 2559-2560 ตัวอย่างทั้งหมด 8842 ตัวอย่าง พบตัวอย่างที่ให้ผลบวกต่อเชื้อ RSV คิดเป็น 11.4% (1011/8842) และเชื้อ hMPV คิดเป็น 3.6% (318/8842) ของผู้ป่วยทุกกลุ่มอายุ ซึ่งการติดเชื้อ RSV และ hMPV ส่วนใหญ่พบได้ในเด็กเล็ก ๆ แต่ก็สามารถพบได้ในกลุ่มผู้ใหญ่ สำหรับจีโนไทป์ที่พบระบาดสำหรับเชื้อ RSV คือ ON1 และ BA ในขณะที่จีโนไทป์ที่พบระบาดสำหรับเชื้อ hMPV คือ A2, B1 และ B2 ผลงานวิจัยยังได้อธิบายถึงวิวัฒนาการของเชื้อ RSV ในระดับจีโนม โดยสร้างจีโนมที่สมบูรณ์หรือเกือบสมบูรณ์ของจีโนไทป์ ON1 จำนวน 10 สายพันธุ์ ที่ระบาดในประเทศไทย ในช่วง 7 ปี (พ.ศ. 2554-2560) ด้วยเทคนิค RT-PCR ผลจากการเปรียบเทียบสายพันธุ์ท้องถิ่นกับสายพันธุ์ทั่วโลกแสดงให้เห็นว่าสายพันธุ์ ON1 ที่พบในประเทศไทยกระจายร่วมกับสายพันธุ์อื่นๆที่พบอยู่ทั่วโลก แสดงถึงข้อจำกัดในรูปแบบวิวัฒนาการแบบท้องถิ่น และจากการวิเคราะห์อัตราการเปลี่ยนแปลงของลำดับนิวคลีโอไทด์แต่ละ open reading frames (ORFs) พบว่า G protein และ NS2 protein มีค่าอัตราการเปลี่ยนแปลงของลำดับนิวคลีโอไทด์ที่สูง ซึ่งจากการศึกษาจีโนมนี้ ยังพบว่าอัตราวิวัฒนาการของเชื้อ RSV ให้ค่าแม่นยำมากขึ้น เมื่อเทียบกับการวิเคราะห์ในแต่ละ ORFs แสดงให้เห็นถึงความสำคัญของความผันแปรของจีโนม RSV และรูปแบบการเคลื่อนไหวทั่วโลก ข้อมูลที่นำเสนอนี้ได้ขยายความเข้าใจเรื่องระบาดวิทยาของเชื้อ RSV ในประเทศไทย นอกจากนี้ลำดับจีโนม ON1 ที่รายงานในที่นี้ยังขยายฐานความรู้ในการศึกษาเพิ่มเติมเกี่ยวกับการแพร่กระจายของเชื้อไวรัสในระดับชุมชน ซึ่งการศึกษาเหล่านี้จะสามารถนำไปสู่กลยุทธ์ใหม่ในการควบคุมการระบาดของไวรัส และการพัฒนาวัคซีนในอนาคต

Enterovirus D68 (EV-D68) มีความสัมพันธ์กับการติดเชื้อทางเดินหายใจส่วนล่างที่รุนแรง และความผิดปกติของระบบประสาท รวมทั้งความผิดปกติของเส้นประสาทไขสันหลังอักเสบและความผิดปกติของเส้นประสาทในซ่องท้อง เพื่อตรวจสอบว่าอุบัติการณ์ที่เพิ่มขึ้นของ EV-D68 เกิดขึ้นในเอเชียตะวันออกเฉียงใต้หรือไม่ จึงได้เก็บตัวอย่าง respiratory specimen ที่เหลือจากการตรวจทางคลินิกของผู้ป่วยที่มีอาการเกี่ยวกับระบบทางเดินหายใจ ซึ่งมีอายุต่ำกว่าหรือเท่ากับ 5 ปี ตั้งแต่ช่วงเดือนมกราคม พ.ศ. 2555 ถึงเดือนธันวาคม พ.ศ. 2557 ด้วยเทคนิค RT-PCR และตรวจสอบลำดับนิวคลีโอไทด์บริเวณ 5'UTR / VP2 นอกจากนี้เรายังได้รวบรวมข้อมูลระบาดวิทยาของเชื้อไวรัสชนิดนี้ตั้งแต่ปี พ. ศ. 2552 ซึ่งได้รับการวินิจฉัยครั้งแรกในประเทศไทย จากการตรวจสอบเชื้อ EV-D68 จำนวน 837 ราย พบว่ามีผู้ป่วย 5 ราย (0.6%) ที่ติดเชื้อ EV-D68 ผู้ป่วยทุกรายมีภาวะปอดอักเสบจากเชื้อไวรัส และต้องเข้ารับการรักษาในโรงพยาบาล การวิเคราะห์วิวัฒนาการของลำดับนิวคลีโอไทด์ของส่วน VP4 / VP2 พบว่าสายพันธุ์ EV-D68 ที่ระบาดในประเทศไทย ระหว่างปีพ. ศ. 2555 ถึง พ.ศ. 2557 มีความสัมพันธ์ใกล้เคียงกับสายพันธุ์ที่รายงานในประเทศญี่ปุ่น สหราชอาณาจักรจีน และประเทศฝรั่งเศส ซึ่งการเฝ้าระวังการติดเชื้อในระบบทางเดินหายใจอย่างรุนแรงที่เกี่ยวข้องกับ EV-D68 และการวินิจฉัยอย่างรวดเร็วต่อเชื้อชนิดนี้ เป็นสิ่งสำคัญในการสนับสนุนและอำนวยความสะดวกในการป้องกันและควบคุมโรค

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5687831520 : MAJOR BIOMEDICAL SCIENCES
KEYWORDS: EPIDEMIOLOGY / RESPIRATORY SYNCYTIAL VIRUS / HUMAN METAPNEUMOVIRUS / AGE GROUP ANALYSIS / VIRAL GENOTYPE / ENTEROVIRUS D68 / EVOLUTION

I-LADA THONGPAN: PREVALENCE AND MOLACULAR GENETIC ANALYSIS OF HUMAN RESPIRATORY SYNCYTIAL VIRUS AND ENTEROVIRUS 68 AMONG CHILDREN WITH ACUTE LOWER RESPIRATORY TRACT INFECTION IN THAILAND. ADVISOR: PROF. YONG POOVORAWAN, M.D., pp.

Respiratory syncytial virus (RSV) causes acute lower respiratory tract infection in infants and young children worldwide. A clear description of local RSV molecular epidemiology, evolution, and transmission requires detailed sequence data and can inform new strategies for virus control and vaccine development. To investigate the RSV burden in Thailand over six consecutive years (January 2012 to December 2017). There were two different methods to detect RSV in the present study. First, we screened 3306 samples obtained from children ≤5 years old with acute respiratory tract infection using semi-nested reverse-transcription polymerase chain reaction (RT-PCR) during January 2012 to December 2015. Second, we screened 8842 samples using real-time RT-PCR to determine the burden of RSV, including hMPV infections in patients in all age groups, who presented with influenza-like illnesses in Bangkok, Thailand, during January 2016 to December 2017. In 2012-2015, 8.4% (277/3,306) of the specimens tested positive for RSV, and then genotyped RSV by sequencing the G glycoprotein gene and performed phylogenetic analysis to determine the RSV antigenic subgroup. The result revealed that NA1, ON1 and BA (BA9, BA10 and BA-C) were the circulating RSV genotypes. Of 8842 specimens to testing between 2016 and 2017, RSV was detected in 1011 (11.4%) specimens and hMPV was detected in 318 (3.6%) specimens. The most commonly RSV and hMPV infections were observed in young children, but both virus infections can sporadically occur in adult groups. For RSV and hMPV strains, ON1 and BA were the circulating RSV genotypes, while A2, B1, and B2 were the circulating hMPV genotypes. The current work described RSV genome evolution and transmission, we have generated 10 complete or nearly complete genomes of ON1 in Thailand, over a 7-year period using a RT-PCR. Comparison of local versus global strains demonstrated that most ON1 variants observed locally in Thailand were also seen in other parts of the world. The nucleotide substitution rates for the individual open reading frames (ORFs) were highest in the regions encoding the attachment (G) glycoprotein and the NS2 protein. The analysis of RSV full genomes, compared to subgenomic regions, provided more precise estimates of the RSV sequence changes and revealed important patterns of RSV genomic variation and global movement. The data presented expand our understanding of the epidemiology of RSV infection in Thailand. Moreover, the new RSV genomic sequences reported here expand our knowledge base for the further study on transmission of virus at the local community level. The outcome of these studies can support new strategies for RSV control and vaccine use and development.

Enterovirus D68 (EV-D68) is associated with severe lower respiratory tract infection and neurological abnormalities including acute myelitis and cranial nerve dysfunction. To determine whether an increased incidence of EV-D68 occurs in Southeast Asia, we retrospectively tested specimens collected from Thai pediatric patients who were less than 5 years of age and presented with acute respiratory tract infections between 2012 and 2014. RT-PCR and nucleotide sequencing of the 5'-UTR/VP2 region were used to identify EV-D68. We also examined the epidemiological pattern of EV-D68 since 2009, when it was first identified in Thailand, and compiled records of clinical manifestations in children with confirmed EV-D68 infection. From 837 samples, 5 samples (0.6%) tested positive for EV-D68. All patients presented with viral pneumonia and required hospitalization. Phylogenetic analysis of the VP4/VP2 regions revealed that EV-D68 strains circulating in Thailand between 2012 and 2014 were closely related to strains reported in Japan, United Kingdom, China, and France. Continued surveillance of probable EV-D68-associated severe respiratory tract infection and the development of a rapid diagnostic test for EV-D68 are essential in supporting awareness and facilitating disease prevention and control.

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

Academic Year: 2017 Advisor's Signature .....

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LIST OF ABBREVIATIONS

RTIs	=	Respiratory tract infections
LRTI	=	Lower respiratory tract illness
RSV	=	Respiratory Syncytial Virus
INF-A	=	Influenza A
INF-B	=	Influenza B
PIVs	=	Parainfluenza viruses
HRVs	=	Human rhinoviruses
HAdVs	=	Human adenoviruses
hMPV	=	Human metapneumovirus
RT-PCR	=	Reverse transcription polymerase chain reaction
RNA	=	Ribonucleic acid
EV-D68	=	Enterovirus D68
BRSV	=	Bovine respiratory syncytial virus
ORSV	=	Ovine respiratory syncytial virus
AMPV	=	Avian metapneumovirus
PIFV	=	Parainfluenzavirus
UV	=	Ultraviolet
ORFs	=	Open reading frames
mRNAs	=	Messenger RNAs
GAGs	=	Glycosaminoglycans
ICAM-1	=	Intracellular adhesion molecule
DFA	=	Direct immunofluorescence assay
EIA	=	Enzyme-linked immunoassay
HVR2	=	Second hypervariable region
VTM	=	Viral transport media

ILI	=	Influenza-like illness
BLAST	=	Basic Local Alignment Search Tool
SLAC	=	Single-likelihood ancestor counting
FEL	=	Fixed-effects likelihood
IFEL	=	Internal fixed-effects likelihood
REL	=	Random effects likelihood
FUBAR	=	Fast, Unconstrained Bayesian AppRoximation
mg	=	Milligram
µL	=	Microliter
cDNAs	=	Complementary DNA
GAPDH	=	Glyceraldehyde-3-Phosphate Dehydrogenase
ML	=	Maximum Likelihood
SD	=	Standard deviation
CV	=	Coefficient of variation
CDS	=	Coding sequence
tMRCA	=	Time to most recent common ancestor
BEAST	=	Bayesian Evolutionary Analysis Sampling Trees
NP	=	Nasopharyngeal
GTR	=	General-time reversible
MCMC	=	Markov chain Monte Carlo
HPD	=	Highest posterior density
BSP	=	Bayesian skyline plots
EPS	=	Effective population size

CHAPTER I
GENERAL INTRODUCTION

1.1 Background and rationale

Worldwide, viral respiratory tract infections are significant causes of morbidity and mortality, particularly in infants and children (1). Viral respiratory tract infections, including infection with influenza viruses, cause a public health problem in people of all ages (2). A variety of viruses, including respiratory syncytial virus (RSV), influenza A and B (INF-A, INF-B), parainfluenza viruses (PIVs), human rhinoviruses (HRVs), and human adenoviruses (HAdVs) have already been recognized as the most common cause of acute respiratory tract infections (RTIs) in hospitalized patients. Moreover, the newly-recognized viruses such as human metapneumovirus (hMPV), severe acute respiratory syndrome coronavirus, human coronavirus NL63 and HKU1, parainfluenza 4, and human bocavirus have been associated with respiratory infection. In the epidemiological study, the predominant virus associated with acute RTIs among infants and young children is RSV, which decreases significantly with age (3, 4).

1.2 Studies of the thesis

This thesis focused mainly on the molecular epidemiology of RSV strains circulating in Thailand during the study period (2012-2017) (Figure 1). RSV is best known for its clinical significance cause of infant bronchiolitis, but it can infect all age-groups, leading upper and lower respiratory tract to produce a subclinical illness to severe pneumonia and death (5). Classification of RSV can be subtyped into RSV-A and RSV-B on the basis of the antigenic properties of its protein surface, and both subtypes were detected in hospitalized infants and elderly individuals (6, 7). This study provided the

prevalence of RSV in Thailand. The outcome of an epidemiological study may play a crucial role to help prevention of RSV infections associated with acute RTIs in Thai children. Moreover, this study also provided a high sensitivity and specificity approach for the diagnosis of RSV infection as well as hMPV infection, which is simple and rapid for the large surveillance scale of a viral infection. The multiplex (one-step) *TaqMan* real-time RT-PCR assay was developed to identify RSV and hMPV infections. Since we investigated the genetic diversity of RSV in our country, we concluded that ON1 rapidly replaced the NA1 genotype to become the most prevalent RSVA genotype circulating in Thailand. Then, we characterized the molecular evolution of the genomic RNA of ON1 genotype to understand the novel RSV-A evolutionary dynamics. ON1 genotype is characterized by a 72-nucleotide duplication in the second hypervariable region (HVR2) on the part of the G gene first described by Eshaghi et al, in Ontario, Canada in 2010 (8) and has spread so quickly across parts of worldwide including Thailand. The knowledge about circulated genotypes in local place may help to develop the vaccine.

During the preparation of acute RTIs samples, the re-emergence of Enterovirus D68 (EV-D68) shows the largest outbreak which occurred across the United States and Canada in 2014, which affected more than 1,100 people. Shortly after the North American outbreak, there were reports of EV-D68 emergence among other countries including Norway, Denmark, and the Netherlands. The most common clinical manifestation of EV-D68 is the upper and lower respiratory tract infection, and the majority of the affected population are children. In this study, we also investigated the epidemiological profile, clinical significance and virological characteristics of EV-D68 in Thailand.

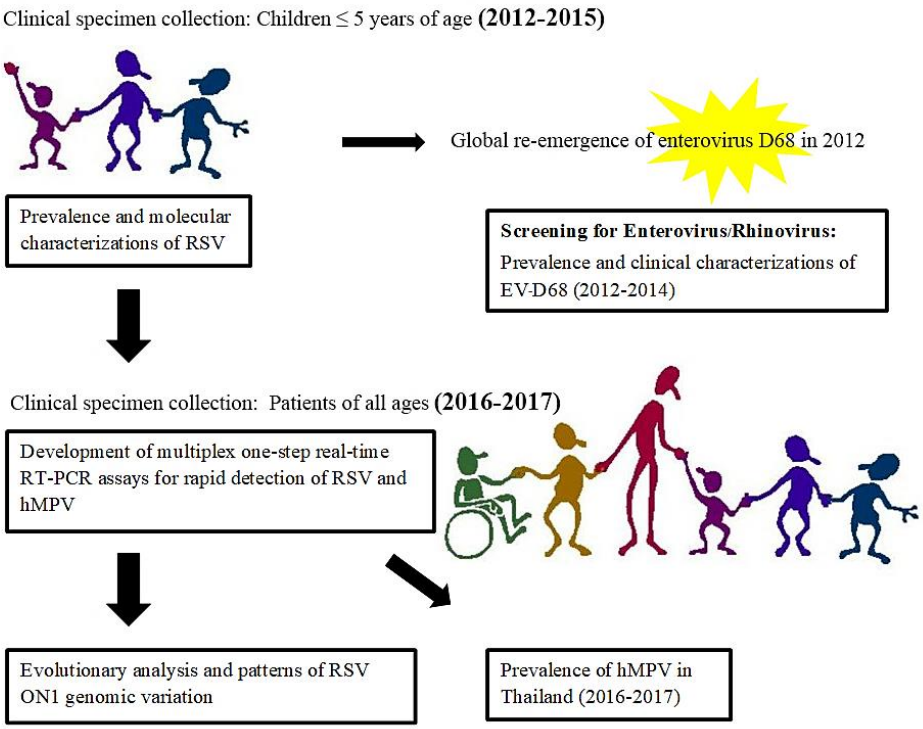

Figure 1 Flow chart of research methodology presented in this thesis.

1.3 Objectives

Therefore the objectives of this study were to:

(i) Evaluate the prevalence and molecular characterization of RSV during the study period.

(ii) Develop a rapid, sensitive and specific assay based on the multiplex (One-step) *TaqMan* real-time RT-PCR assay for use in RSV and hMPV detection.

(iii) Investigate the evolutionary patterns of ON1 genotype derived from Whole-Genome Sequencing.

(iv) Evaluate the prevalence and phylogenetic characterization of EV-D68 in pediatric patients with acute RTIs in Thailand

CHAPTER II
LITERATURE REVIEW

2.1 History

Human respiratory syncytial virus (RSV) was first isolated from a chimpanzee in 1955, it was called chimpanzee coryza (9). Thereafter, the same virus was recovered from infants with respiratory illness, and serologic studies indicated that RSV infection in infants and children was common (10). RSV is now recognized as the most important in patients with lower respiratory tract illness (LRTI) worldwide, outranking all other microbial cause of pneumonia and bronchiolitis in infants. However, RSV can also infect and cause disease in individuals of all ages and severe disease in the elderly (11). Worldwide, acute respiratory infection is the leading cause of mortality due to infectious disease and RSV remains one of the pathogens considered most important for vaccine development. In addition, RSV is hampered vaccine development by its poor growth *in vivo* and its physical instability.

2.2 Infectious Agent

2.2.1 Classification of RSV

RSV is an envelope with negative single stranded RNA viruses. It is a member of the genus *Orthomyxovirus*, subfamily *Pneumovirinae,* family *Paramyxoviridae*, and in the order *Mononegavirales*. Subfamily *Pneumovirinae* contains human metapneumovirus (hMPV) and avian metapneumovirus (AMPV). The other subfamily of family *Paramyxoviridae* is parainfluenzavirus (PIFV) (12).

2.2.2 Virion

RSV consists of a nucleocapsid surrounded by a lipid envelope derived from portions of the host cell membranes during budding (Figure 2). Virions appear to be irregular spherical particles that are of 100 to 350 nm in diameter and long filamentous forms with diameters ranging from 60 to 200 nm and reaching lengths of up to 10 μm when visualized by electron microscopy (Figure 3).

The RSV genome is composed of a single-stranded negative-sense RNA encoding 11 viral proteins (Figure 4). There are non-structural (NS2, NS1), nucleocapsid (N), phosphoprotein (P), matrix (M1, M2), small hydrophobic (SH), surface attachment glycoprotein (G), surface fusion glycoprotein (F), second matrix (M2), and RNA-dependent RNA polymerase (L) in the order 3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5'. The virion matrix is composed of M proteins with the N, P, and L proteins associating with genomic RNA to form the nucleocapsid. The lipid envelope that surrounds the nucleocapsid is derived from portions of the host cell membranes by budding and contains three major genes that encode surface transmembrane glycoproteins: G, F, and SH.

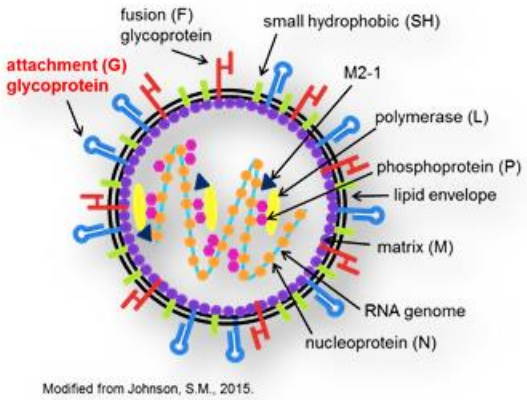

Figure 2 Idealized diagram of the respiratory syncytial particle. The G, SH and F proteins are present in homo-oligomers that constitute the glycoprotein spikes. The M protein underlines the lipid bilayer. The proteins of nucleocapid are not depicted individually (13, 14).

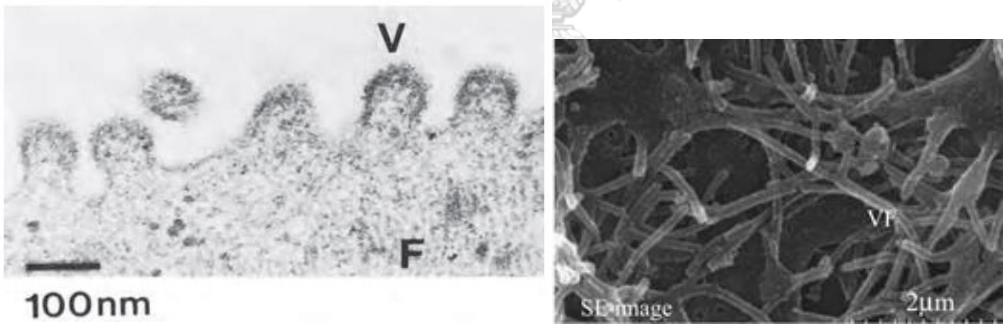

Figure 3 The electron micrographs of respiratory syncytial virus-infected cells. **(A)** Negatively stained electron micrograph of budding RSV virions, **V** indicates a budding virion and **F** indicates filamentous cytoplasmic structures that likely are nucleocapsids. **(B)** Field emission scanning electron micrographs of the RSV-infected cells, illustrating viral filamentous structures that are thought to form at sites of virus budding and may yield filamentous particles.

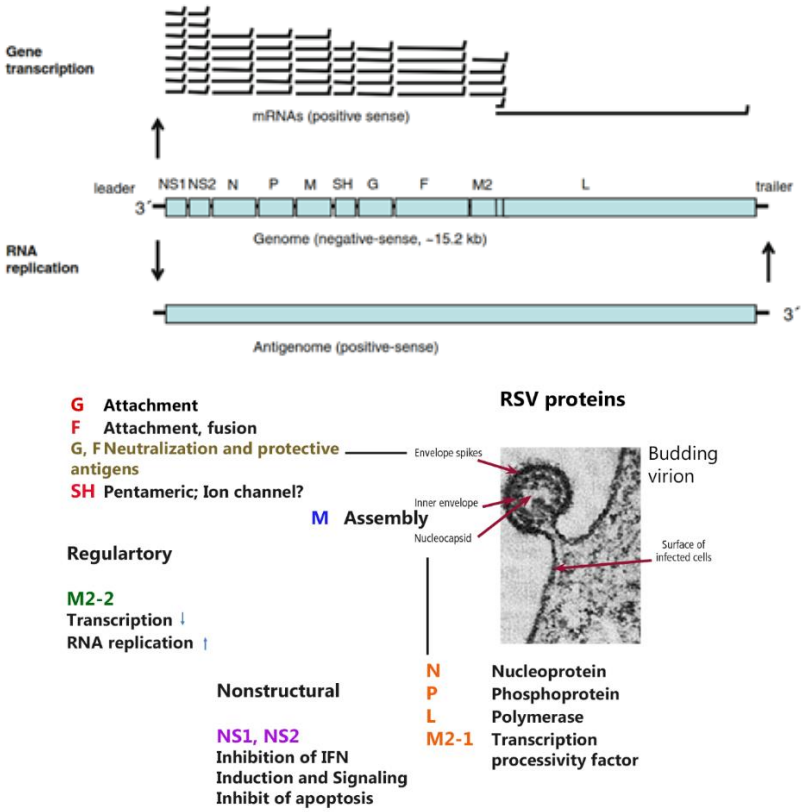

Figure 4 The proteins of RSV and the viral genomic RNA. The upper shows the 3' to 5' negative-sense RNA genome, encoding 11 viral proteins. The overlapping open reading frames (ORFs) of the M2 messenger RNAs (mRNAs) are indicated over the M2 genes (M2-1 and M2-2). The lower shows the location of the proteins in the virus particle and major functions are indicated when known.

2.2.3 Replicative cycle

For efficient attachment to cells, RSV involves binding to cellular glycosaminoglycans (GAGs), especially heparin sulfate and chondroitin sulfate B. The G and F proteins each can bind to GAGs (15). A number of additional potential receptor molecules for RSV have been tentatively identified, including intracellular adhesion molecule (ICAM-1), RhoA, the CX3CR1 fractalkine receptor (16), and annexin II. More recently, efficient RSV infection *in vitro* and in mouse model was shown to depend on binding to cellular protein nucleolin (17), which also have been identified as a co-receptor for human parainfluenza virus 3 (HPIV-3). Unexpectedly, nucleolin is mediated by binding

to the F protein rather than by binding to the G protein. This suggests that efficient attachment and infection by RSV depends on two different binding events mediated by G and F.

RSV can enter the host cell by fusion of the viral envelope with the cell plasma membrane with clathrin-mediated endocytosis (18). Genome transcription and replication occur in the cytoplasm (Figure 5). RSV mRNAs and proteins could be detected intracellularly at 4 to 6 hours after infection and reach a peak accumulation by 15 to 20 hours. The progeny virus begins to leave the cell by 10 to 12 hours post-infection, reaches its peak within 24 hours, and continues until the cells deteriorate by 30 to 48 hours. Transcription and RNA replication occur concurrently but the role of M2-2 is determined the balance between RNA synthesis and transcription (19).

RSV assembly and budding occur at the plasma membrane (20). The budding processes in the circumscribed regions on the cell surface (21). The minimal viral protein (the F, M, N, and P proteins) are required for the production of virus-like particles capable of delivering the viral genome to target cells and these proteins expression induced the initial stages of filament formation (22).

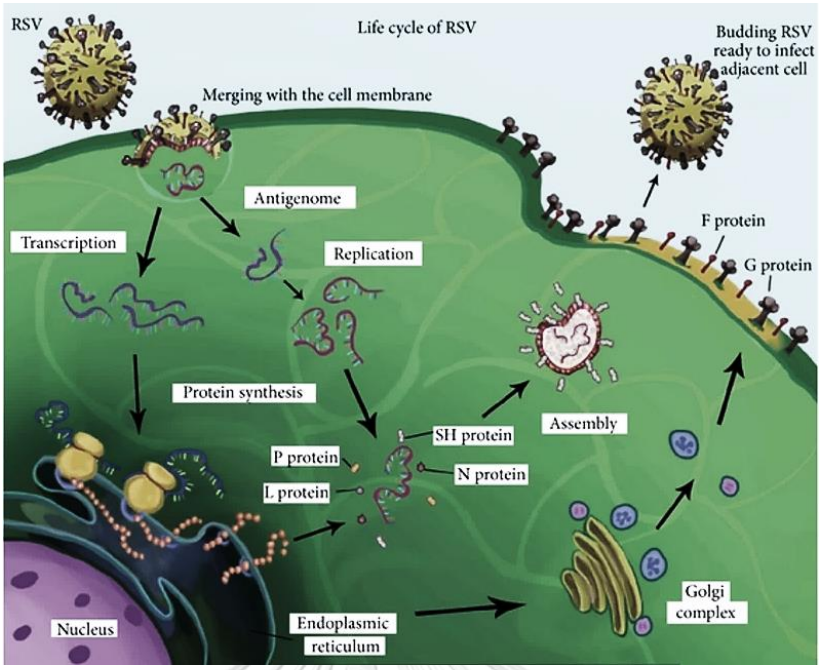

Figure 5 A schematic representation of RSV life cycle (23).

2.2.4 Antigenic subgroups and diversity

The RSV strains can be divided into two distinct serological subgroups A and B (RSV-A and RSV-B). These groups of an RSV can be further identified by genetic classification of the G protein. The differences of nucleotide sequences of HVR2 of the RSV G gene is the most variable viral protein and has been used to classify genotypes of both the RSV subgroups. RSV-A consists of eleven genotypes, GA1-GA7, SAA1, NA1-NA2 and the 72-nucleotide duplication genotype, ON1 (24-26). RSV-B can be divided into 23 RSV-B genotypes, GB1-GB4, SAB1-SAB4, URU1-URU2, BAI-BAXII, and THB (8, 24, 27-34). The RSV infection may be characterized by multiple genotypes and new variants that may replace older ones in subsequent epidemics. These patterns of circulating strains showed that the different areas have been various predominant patterns are related to epidemic community (35, 36). Currently, a new group B strain with a 60-nucleotide duplication within the G gene was first observed in Buenos Aires, Argentina, in 1999 (named BA genotype) and spread around the world in 3–4 years. The prevalent of

BA strains have replaced RSV-B (37). Another notable G gene change, named ON1 is characterized by a 72-nucleotide duplication in the HVR2 on part of the G gene first described by *Eshaghi et al*, in Ontario, Canada in 2010 (8). This genotype has spread so quickly across parts of worldwide (Figure 6) (38).

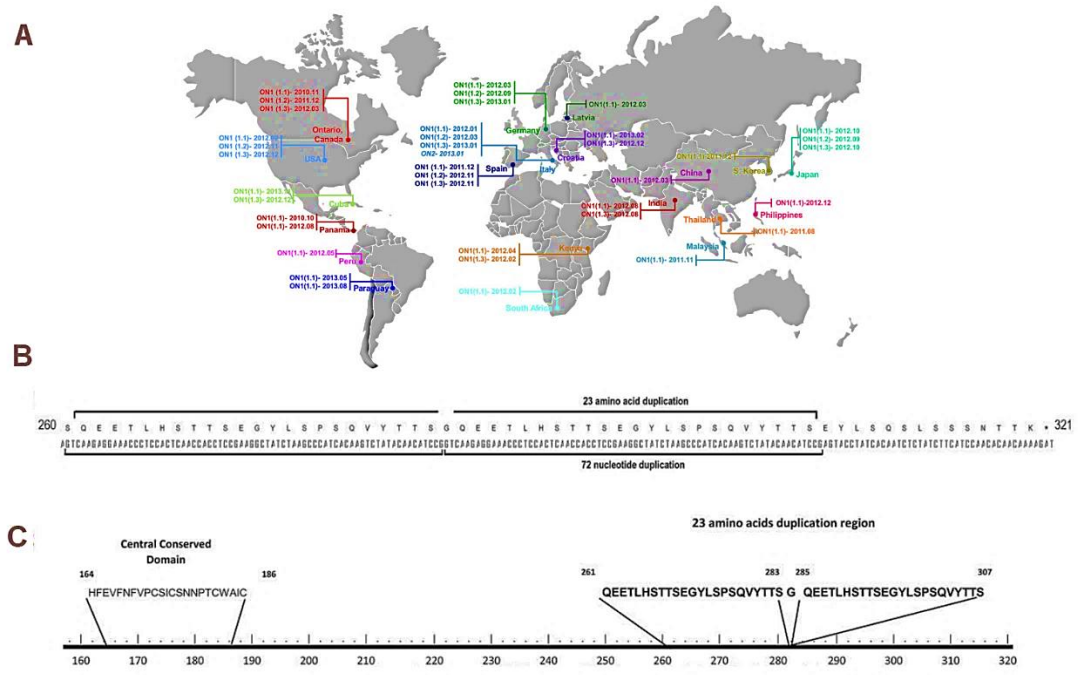

Figure 6 Rapid spread of the novel respiratory syncytial virus A ON1 genotype. **A)** RSV-A ON1 RSV strains do spread rapidly and widely **B)** The 72-nucleotide duplication is indicated by 2 horizontal solid lines below the sequences. **C)** The central conserved domain and second variable region are identified with graphical representation of G protein. Duplicated AA sequences are highlighted in boldface.

2.2.5 Epidemiology

1) Infection of infants and young children

Globally, RSV estimates an annual incidence of approximately 34 million episodes of RSV-associated acute LRT in children younger than 5 years (39). RSV infection was detected in 43% of those hospitalized with diagnosis of bronchiolitis and in 25% of those with pneumonia. RSV infection is more common in infancy than is infection with other respiratory viruses. Prospective studies have demonstrated that the infection rate to be 50-69% during the first year of life are RSV-infected, and virtually all are infected by age of 2 years. Reinfection by RSV is most common, particularly during the first few years of life (40, 41) more so than with other respiratory viruses. One study found that LRT could during either the first or second infection early in life, whereas for subsequent infections there was a considerable reduction in disease severity reflecting increasing protective immunity (40).

2) Infection of Adults

RSV reinfected adults at a rate of approximately 5% to 10% per year (11). Reinfection is more frequent in adults with increased exposure to the virus, such as during a typical RSV season, health care workers, and family members of sick children. RSV is deemed to be second to influenza as a cause of illness in elderly and high-risk adults. However, severe disease and even death due to RSV can rarely occur in adults (42).

RSV is an important cause of morbidity and mortality in the elderly (>65 years of age). It is estimated to cause on average 78% of RSV-related respiratory and circulatory deaths occurred in persons aged 65 years or older (43).

3) Epidemics

In temperate climates, RSV occurs annually in epidemics of 4 to 5 months duration in the winter and early spring, although continuous activity also has been reported (44). Worldwide, the RSV season varies from location to location (45). RSV activity is greatest during periods of moderate humidity and either cool (2°C to 6°C) or warm (24°C to 30°C) temperature (46, 47). In addition, RSV is highly infectious and easily spread by contact, especially in settings of close contact such as kissing, shaking hands, and sharing cups or utensils with others. Not surprisingly, RSV is introduced into families primarily by a young school-aged child, after which infection spreads to siblings and adults with high frequency. In one prospective study, approximately 40% of all family members older than 1 year were infected after the introduction of RSV (48).

An evaluation of the RSV strains circulating in distinct geographic regions during the same years found that five to seven distinct lineages or genotypes representing both antigenic subgroups circulated during the same season (49). In a same year, the pattern of genotypes frequently is different in different locations. The pattern of local strains gradually changes in successive years. There typically are one or two dominant local genotypes that are replaced in dominance in successive years. In addition, a predominance of one or two genotypes can be shifts occurring in 1- or 2-year cycles (50). This presumably reflect an advantage of the heterologous strain in evading previously induced immunity (51), but reinfection by the same subgroup also frequently occurs.

2.2.8 Virus evolution

The simplest way to examine the role of mutation in virus evolution is to measure the rate of its occurrence. RSV isolates collected over 44 years were analyzed using a variety of techniques, including nucleotide sequencing, bioinformatics analysis,

phylogenetic reconstruction and evolutionary modelling to provides insights into the epidemiology and evolution of these phylogenetically closely related viruses. RSV clades in order to further understanding of multiple co-circulating RSV lineages.

Most epidemiological and evolutionary studies conducted worldwide for RSV have concentrated in part of the G protein, which possessed one of the most changes observed among a total of 11 proteins encoded in the 15.2 kb RSV genome (24, 26). In previous studies revealed that the evolutionary rate of RSV has been calulated by partial G gene sequence data, with rates of $1.83x10^{-3}$ and $1.95x10^{-3}$ substitutions/site/year determined for subtypes A and B respectively. The G protein is under evolutionary pressure by interactions with adaptive immune responses (52).

The evolutionary and transmission dynamics was characterized to investigate using the HVR2 for ON1 genotype revealed that ON1 evolutionary rate of 4.12×10^{-3} is similar to the BA genotype of 4.7×10^{-3} (37, 53). The other analyses of evolutionary rate of ON1 in many countries revealed evidence of high mutation rates, include Italy (4.04×10^{-3}), Germany (5.5×10^{-3}), Japan (6.6×10^{-3}) and Ontario (3.12×10^{-3}) (53). These evolutionary differences of ON1 at a local level could indicate the effects of population genetic structure and different host immune response. However, the comparative study using whole genomic to investigate in the RSV-A ON1 evolution would be worth conducting. Agoti et al, (54) showed that RSV genome with identical G regions, nucleotide differences were found occurring outside the G region. Of 7 sets of them showed at least 1 up to 9 nucleotide differences across the full genome. Thus, this work is the increase in resolution provided by WGS for monitoring virus transmission over short times (Figure 7). Improved understanding of transmission chains can help with infection control.

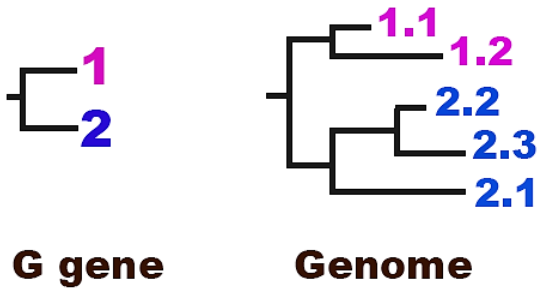

Figure 7 Comparison of genomes of viruses with identical G protein. The genome phylogeny is to increase the resolution for tracking virus transmission chains, although the clusters determined with the G region is similar to the patterns observed with the full-genome sequences.

2.2.6 Clinical Features

Bronchiolitis and pneumonia are the primary manifestations of RSV. Cough may appear simultaneously but occurs more often after an interval of 1 to 3 days. At that time, there also may be sneezing and a low-grade fever. Soon after the cough has developed, the child have wheezing; if the disease is mild, the symptoms may not progress beyond this stage (55). There are many cases of tachypnea, diffuse inspiratory crackles, and expiratory wheezes. In most instances, uneventful recovery occurs after an illness of 7 to 12 days. Among infants and children who require hospitalization with RSV, clinical manifestations from bronchiolitis or asthma exacerbation to severe pneumonia (56). Cough is present in most, fever 52% to 86%, and wheezing in about half of cases (57). In more severe cases, the coughing and wheezing progress, and the child becomes dyspneic. Hyperexpansion of the chest is evident, and there may be intercostal and subcostal retractions. In advanced disease, the child tired and hypoxia becomes more extreme, and then listlessness and respiratory failure occur (57).

2.2.7 Diagnosis

The gold standard is isolation of the virus in cell culture from nasal swabs, nasal washes, or nasopharyngeal aspirates (58); however, the lability of RSV, the expense of the technique, and the increasing availability of assays that rely on nucleic acid detection have made cell culture an increasingly infrequent choice, even for diagnostic virology laboratories in academic centers (59).

More rapid diagnosis can be made by the detection of viral antigen in nasal swabs or washes. In the direct immunofluorescence assay (DFA) reacted with virus-specific fluorescence-labeled antibodies, and visualized by microscopy, but the method is time-consuming and requires expertise and a fluorescence microscope. Alternatively, antigen in secretions can be detected by antigen-capture enzyme-linked immunoassay (EIA), in which the sample is incubated with immobilized virus-specific antibodies to capture viral antigen, which is then detected with a second, enzyme-linked antibody (60). Both DFA and EIA for RSV are commercially available. Serologic methods are used infrequently and provide retrospective information, since antibody rises are detected by analysis of sera collected 2 to 4 weeks post-infection (60).

RT-PCR of viral RNA for detection of RSV is a useful method of diagnosis for both pediatric and adult populations, because the sensitivity of these assays allows detection of even small quantalities of virus (61). Although, the procedure is somewhat time-consuming (3 to 6 hours), requires technical expertise, specialized equipment and the elimination of false-positive results due to cross contamination, but they are more sensitive than virus culture or antigen assay. Real-time reverse transcriptase PCR (RT-PCR) quantification technique for clinical studies would be a rapid and sensitive tool for detection of RSV compared to conventional techniques and enables improvements to the

quantitation of viral load. Therefore, a rapid and sensitive tool for large-scale samplings has been effectively for yearly outbreaks of RSV, a very important option for patient management, in particular for the prevention of specific nosocomial infections.

2.2.9 Treatment and prevention

1) Symptomatic interventions and supportive care

Inpatient treatment of RSV infection requires considerable supportive care: mechanical removal of secretions, proper positioning of the infant, administration of humidified oxygen, intravenous fluids, and in the most severe cases, respiratory assistance with medical ventilation.

2) Antiviral interventions

Ribavirin, a nucleotide analog, is a broad spectrum antiviral compound the mode of action of which remains unclear, but which exhibits potent activity against RSV in the cell culture and experimental animals. Currently, the America Academy of Pediatrics recommends that ribavirin not be used routinely for the management of bronchiolitis, although it could be considered for use in children at risk with severe disease. RSV disease in young infants can be reduced by prophylaxis with RSV-neutralizing antibodies. Therefore, these antibodies also have been evaluated for therapy of established infection. The most recent studies have utilized the RSV F Mab palivizumab and the more potent motavizumab administered intravenously. Small molecule antiviral drugs specific for RSV have been developed that target the F, G, N, or L proteins. Four different small molecule inhibitors are undergoing clinical trials for RSV (www.clinicaltrials.gov, accessed June, 2011).

3) Prevention

Infection by RSV can be reduced by hand washing, limiting exposure to infected individuals, and avoiding self-inoculation of nasal and conjunctival mucosa. Nosocomial spread of RSV has been shown to be reduced by the use of gloves and gowns by caregivers, strict observance of hand washing, active surveillance of RSV infection, limiting visitors during the RSV season, and cohorting of infected patients and caregivers.

2.3 Relationship between respiratory syncytial virus and human metapneumovirus

The newly identified hMPV is a recently discovered respiratory pathogen of the family *Paramyxoviridae*, as is RSV (62), although retrospective serological studies have revealed the existence of hMPV antibodies among the human population has been circulating since at least the 1950s. (63). The hMPV is now recognized as a frequent cause of acute respiratory tract infections in the pediatric populations (64). Children under 5-years-of-age, elderly adults, and immunocompromised patients are at increased risk for severe hMPV infection, and the virus has also been reported to be an infrequent cause of respiratory tract infection in healthy children (62). Around 12% of all RTIs in children are caused by hMPV, is deemed to be second to RSV (64-67). hMPV RNA (13 kDa) encodes nine proteins that generally correspond to those of RSV, but they do not encode the NS1 and NS2 and the gene order also is different from that of RSV (Figure 8).

Similar to RSV strains, hMPV strains can be classified into two genetically and antigenically distinct groups (A and B), with each group divided into genetic subgroups 1 and 2 (64, 65). In comparison to RSV, the clinical features with hMPV in children are is quite similar to RSV. Both upper and lower respiratory tract infections in children have been associated with hMPV, such as fever, cough, tachypnea, wheezing and hypoxia (68). Although RSV and hMPV share similar clinical spectrum, hMPV induces a different

expression of proinflammatory and immune mediators compared to RSV (69-71), suggesting that the contribution of host immunity to pathogenesis of lung disease are viral specific.

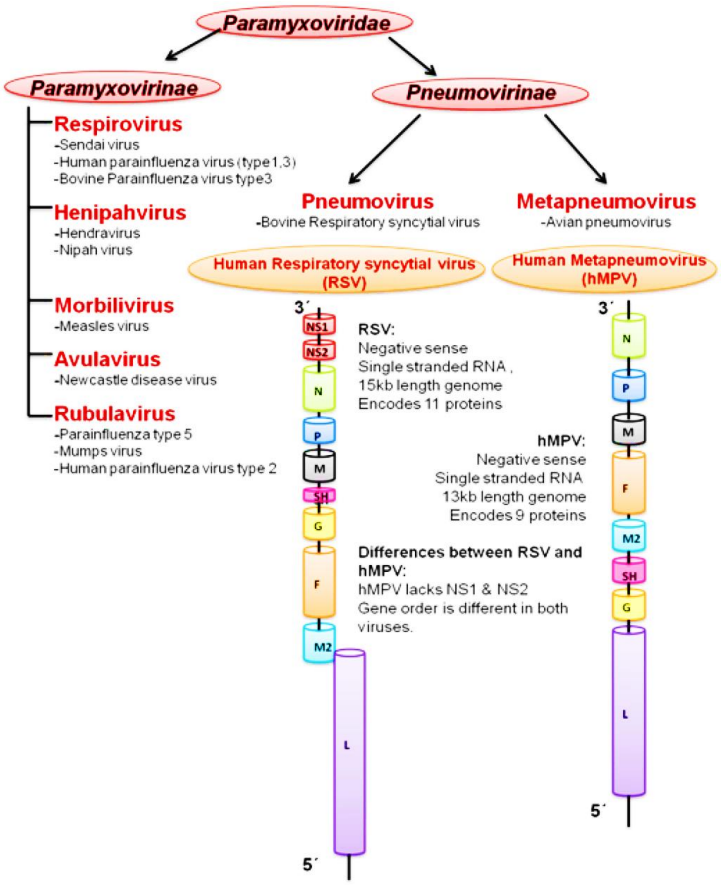

Figure 8 Representative members of *Paramyxoviridae* family and the genomic organization of RSV and hMPV.

2.4 Global re-emergence of enterovirus D68

Enterovirus D68 (EV-D68) belongs to the family *Picornaviridae* and genus *Enterovirus* in the species *Enterovirus D* (Figure 9). It was first isolated in the United States in 1962 from respiratory specimens obtained from four pediatric patients with pneumonia and bronchiolitis (72). Since then, EV-D68 infections have been reported in small numbers and accounted for only 26 reports among enteroviruses detected in the United States

during 36 years surveillance period from 1970-2005 (73). Between 2008 and 2010, clusters of EV-D68 were reported in the Philippines, Japan, the Netherlands, and the United States (CDC, 2011). In the recent years, EV-D68 infections have emerged in Italy (2010-2012), France (2009-2010), China (2009-2012), New Zealand (2010), Great Britain (2009-2010), Kenya (2008-2011), Japan (2005-2010), the Netherlands (2010), and Thailand (2009-2011) (74-82). The large outbreak in the United States and Canada in 2014 likely are affected much more than the 1,100 laboratory-confirmed cases (83). After that, reports of EV-D68 occurred in Europe including Norway, Denmark and the Netherlands (84-86).

The spectrum of diseases caused by EV-D68 ranged from mild to severe respiratory tract infection, and fatalities have been reported (87). EV-D68 is unique among enteroviruses because it shares some biological features with rhinovirus. While most enteroviruses tolerate low pH and grow well at 37°C, EV-D68 is acid-labile and thrives at 33 °C (88). Clinically, EV-D68 infection affects the upper and lower respiratory tract and mainly children (83). The EV-D68 infection has also been associated with severe acute myelitis (84) and cranial nerve dysfunction (89, 90). To date, there is neither an effective treatment nor a prophylactic vaccine specific for EV-D68. There are no specific treatments for EV-D68 and no antiviral targets available. Therefore, rapid and accurate diagnosis is important.

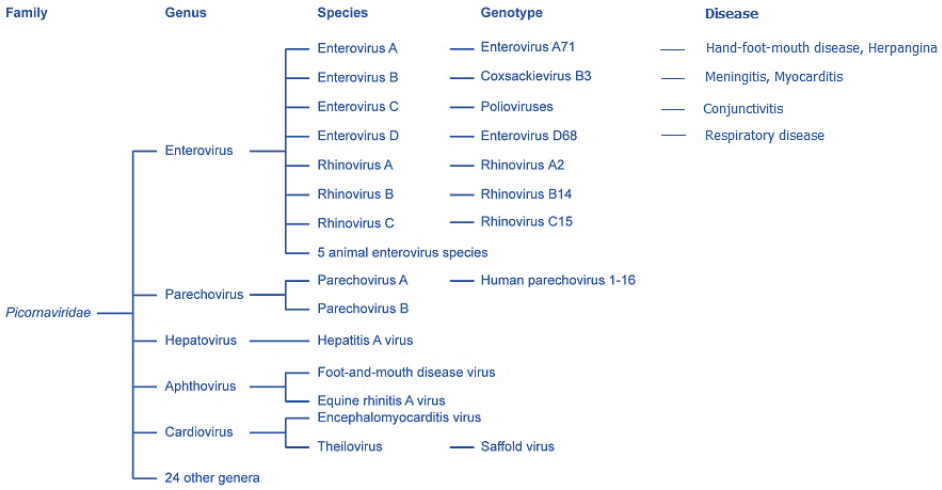

Figure 9 Members of the *Picornaviridae* family as classified based on Maximum Likelihood tree of capsid proteins by Knowles NJ (91).

In recent years, the circulation of EV-D68 has risen worldwide as exemplified by the outbreak in the United States. Our study examined the prevalence of EV-D68 in Thailand in children less than five years of age as they appeared to be most vulnerable to infection (83). The EV-D68 prevalence of 1.4% found in Thailand between 2006 and 2011 (92). It is lower than the rates reported in Europe in recent years: 7.7% in Germany, 2% in the Netherlands, 17% in Norway, and 11% in Denmark (85, 86, 93, 94). Our data suggest that the prevalence of EV-D68 in Thailand varies annually between 0-4.3% (mean = 1.5%) and slightly above the 0.45% found in China (77) and 0.87% in Japan (80). Continued monitoring for severe respiratory tract infection, especially in children may help control EV-D68 and other emerging enteroviruses.

2.5 Significance of the study

A detailed knowledge of the epidemiology means by which evolution rate can be modulated may lead to the development of new therapeutic approaches for the treatment of the common respiratory viruses associated with acute RTIs. Continued surveillance of probable virus-associated severe respiratory tract infection and the development of rapid diagnostic test are essential in assisting awareness and facilitating disease prevention and control.

CHAPTER III
METHODS AND RESULTS

Part 1: Prevalence and molecular characterization of human respiratory syncytial virus during 2012 to 2015 in Thailand

(Published in Peer J. 2017;5: e3970)

RSV is a major cause of severe respiratory infections in infants and young children worldwide with mortality rates exceeding 70%. A remarkable feature of RSV is its ability to cause recurring infections throughout a person's life (95). Although RSV infections in healthy adults are limited to the upper respiratory tract (96), is the most frequent cause of bronchiolitis and pneumonia in infants and children (97). However, no safe and effective vaccine yet exists.

RSV is a member of the genus *Pneumovirus,* subfamily *Pneumovirinae*, family *Paramyxoviridae*, and order *Mononegavirales*. It has a single-stranded negative-sense RNA genome of approximately 15.2 kb. The lipid envelope that surrounds the nucleocapsid contains three virally encoded surface transmembrane glycoproteins: G, F, and SH (13, 14). Proteins G and F are important for attachment and fusion with the target cell, respectively, and they are involved in the host immune response to RSV (14). The RSV strains can be divided into two distinct serological groups: group A (RSV-A) and group B (RSV-B), based on reactions between the glycoproteins and monoclonal antibodies (98). Both RSV groups can co-circulate during epidemics, although RSV-A identified more frequently (99).

The RSV groups are further divided into genotypes by genetic classification of the second hypervariable region (HVR2) of the G gene, located at the protein's C-terminus. RSV-A consists of eleven genotypes: GA1 to GA7 (24, 25), SAA1 (26), NA1 to NA4 (27,

100), and ON1 (8). RSV-B can be divided into 22 genotypes: GB1 to GB4 (24), SAB1 to SAB4 (26, 28), URU1 and URU2 (29), BA1 to BA10 (30), BA-C (100), and THB (34). The genotypic characterization of RSV infections has indicated that there are rather complex patterns of circulation, as multiple genotypes from both groups have been found to co-circulate in single epidemic seasons, and new genotypes may replace previously predominant genotypes in consecutive epidemic seasons. The data on the circulating strains indicate that different regions have experienced different predominant patterns, which are related to the epidemic community (35, 36).

An RSV-B strain with a 60-nucleotide duplication within the G gene HVR2 was first observed in 1999 in Buenos Aires, Argentina, and named genotype BA (101). Subsequently, it spread around the world within 3–4 years. The BA strains have completely replaced the other RSV-B genotypes (37). Another notable RSV-A strain, namely ON1, is characterized by a 72-nucleotide duplication in the G gene HVR2, and it was first identified in 2010 by Eshaghi et al, in Ontario, Canada (8). Genotype ON1 spread rapidly across many parts of the world: Japan, South Korea, Germany, South Africa, Malaysia, Italy, India, Kenya, Latvia, Spain, the Philippines, Peru, Panama, Croatia, and the USA (32, 38, 100, 102-111). In Thailand, a molecular and demographic study of RSV infections in 2007 during the most active season for respiratory tract infections (June to December) (112) showed that genotype GA2 of RSV-A and genotype BA4 of RSV-B were circulating. Also, our previous study (34) (from June 2010 to December 2011) on the epidemiology of RSV infections revealed that NA1 strains were originally the predominant genotype and ON1 strains were first detected in 2011. Almost all of the RSV-B strains were genotype BA9.

In this study, we aimed to investigate the diversity of RSV strains in Thailand (following on from our previous report in 2010–2011 (34)) over 4 consecutive years from

2012–2015. This involved monitoring the seasonal epidemic progression of subgroup and genotype patterns.

Materials and Methods

Ethical considerations

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB 609/59). The need for consent was waived by the IRB since this study involved the use of anonymous residual specimens.

Population study

The sample of nasopharyngeal aspirates was collected from the inpatients and outpatients who presenting with influenza-like illness (ILI) or diagnosed as suspected cases of RSV infection (younger than 5 years of age) at the King Chulalongkorn Memorial Hospital and the Hospitals located in Bangkok (Bangpakok 9 International Hospital). We also included the samples from Chum Phae Hospital (Khon Kaen, Thailand) from January 2012 to 2015. These clinical samples were subjected for routine respiratory virus diagnosis and then keep at the Center of Excellent in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The patients presenting an ILI defined as the presence of high body temperature (more than 38ºC) and respiratory tract symptoms such as a runny nose, cough, sore throat and difficulty breathing. All patient samples were transferred to 2 mL viral transport media (VTM) modified according to the World Health Organization (WHO) recommendation and stored at -70ºC until RNA extraction.

Sample size estimation

The RSV prevalence values have been reported by many countries, it is as follows: China, 7.6%; Korea, 17.4%; Vietnam, 23.8%; Thailand 16.3%. This calculator uses the following formula for the sample size n:

$$n = \frac{Z\alpha^2 P(1-P)}{e^2}$$

n = required a sample size

$Z\alpha$ = confidence level at 95% (standard Z value of 1.96 (two-tail))

P = incidence proportion RSV infection estimated from previous study (average value of 16.3%)

e = acceptable margin of error at 5% (standard value of 0.05)

Therefore, a total of 210 samples were required for the RSV screening to achieve the statistically supported results with 5% acceptable error.

RNA extraction and reverse transcription

RNA was extracted using the Viral Nucleic Acid Extraction Kit (RBC Bio-science) and cDNA synthesized using an ImProm-II Reverse Transcription System (Promega) according to the manufacturer's instructions. Briefly, RNA and random hexamer primers were incubated at 70 ˚C for 5 min, followed by extension for 2 h at 42 ˚C and inactivation at 70 ˚C for 15.

PCR amplification and DNA sequencing

For RSV screening, semi-nested PCR using a primer set covering the G/F region was performed as previously published (34). The first-round amplification is the cycling conditions with initial denaturation at 94°C for 3 minutes. Then 40 cycles of PCR in each cycle of denaturation for 30 seconds at 94°C is used, then annealing for 30 seconds at 55°C and elongation for 1 minute at 72°C and ended with the final extension cycle at 72°C for 10 minutes. For second-round PCR, the cycling conditions use the same profile in the first-round amplification, followed by 30 cycles. Expected products (about 840 bp for RSV-

A and 720 bp for RSV-B) were visualized by ethidium bromide staining following electrophoresis on a 2% agarose gel. Positive samples were selected for sequencing. The purified PCR products were subjected to direct sequencing of the G gene by First BASE Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia).

Phylogenetic tree construction and analysis of partial nucleotide sequences of the G gene

Nucleotide sequences were visualized using Chromas Lite (v2.01), assembled using SeqMan (DNASTAR), analyzed using the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), aligned using BioEdit (v7.0.9.0) and ClustalW. For genotype assignment, 211 strains of RSV-A (n =117) and RSV-B (n = 94) in which the partial nucleotide sequences for the G gene were successfully obtained were subjected to phylogenetic analysis. RSV-A strain A2 (genotype GA1, GenBank accession number M74568) and RSV-B strain B1 (genotype GB1, GenBank accession number AF013254) served as reference strains. Phylogenetic trees were constructed using the neighbor-joining method implemented in MEGA (v6.0) with 1,000 bootstrap replicates. Differences between (inter-) and within (intra-) genotypes were evaluated by pairwise nucleotide distance (p-distance) calculations. All RSV sequences obtained from this study were deposited in the GenBank database under the accession numbers KY327937–KY328054 (RSV-A) and KY328055–KY328148 (RSV-B)

Results

RSV prevalence

In this study, RSV was identified in 8.4% (277/3,306) of all samples tested, of which 57.4% (159/277) were RSV-A and 42.6% (118/277) were RSV-B (Table 1). Cyclical pattern was reflected by the predominance of RSV-A in 2012, RSV-B in 2013 and 2014, and the subsequent return of RSV-A predominance in 2015. Co-infection with RSV-A and RSV-B was extremely rare (0.4%, 1/277). RSV infection appeared to peak in the rainy months of July to November with the highest incidence (32%, 24/75) in October 2014 (Figure 10).

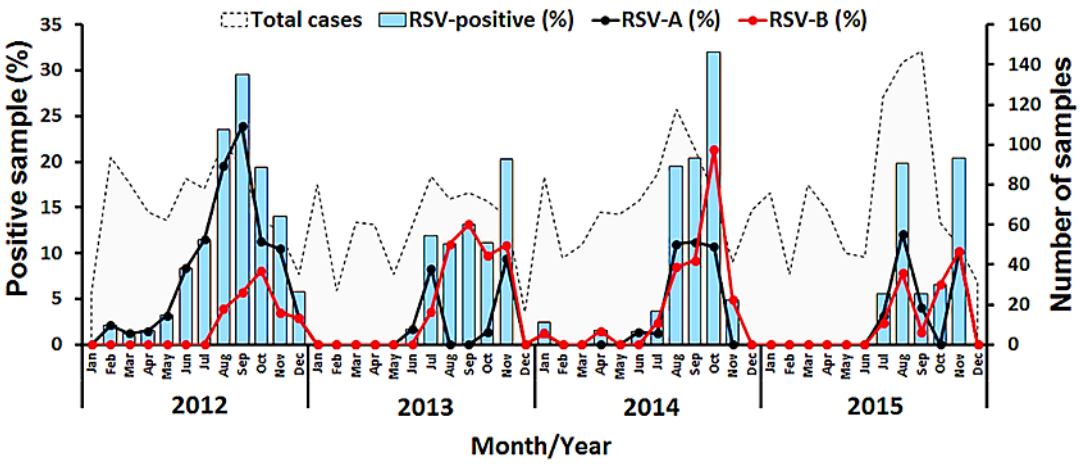

Figure 10 Seasonal distribution of RSV detected between 2012 and 2015 in this study.
The number of samples examined by month is shown as gray peaks (right scale). RSV positive rates (bar graphs) for RSV-A (black line) and RSV-B (red line) are also shown (left scale).

The distribution of the RSV subgroups during each epidemic season is shown in Table 1. We observed an alternating pattern involving RSV-A and RSV-B. In 2012, the positive rate of RSV-A (81.9%, 77/94) was higher than that of RSV-B (18.1, 17/94). In 2013, RSV-B-positive cases (70%, 35/50) were more frequent than RSV-A (30%, 15/50). Subsequently, RSV-B infections decreased slightly for all of 2014 (53.9%, 41/76).

Beginning in 2015, RSV-A (56.9%, 33/58) became the predominant subgroup. As can be seen in Figure 10, the epidemic curves of RSV subgroups in Thailand revealed a circulation pattern that involved subgroup A being predominant (2012), then B (2013), followed by B (2014), and finally A again (2015).

Table 1 Number of RSV samples identified between 2012 and 2015 from among 3,306 patients with acute respiratory tract infections.

Year	No. of samples	RSV-positive (%)	Groups	
			RSV-A (%)	RSV-B (%)
2012	834	94 (11.3)	77 (81.9)	17 (18.1)
2013	707	50 (7.1)	15 (30)	35 (70)
2014	864	86 (8.8)	35 (40.7)	41 (59.3)
2015	901	57 (6.3)	32 (56.1)	25 (43.1)
Total	3,306	277 (8.4)	159 (57.4)	118 (42.4)

Genotyping and phylogenetic analysis

RSV-positive strains from subgroups A and B were selected for sequencing in the phylogenetic analysis (based on sequences of the G gene HVR2). Unfortunately, we were unable to genotype 24% (67/278) of the strains due to insufficient quantities of RNA. The phylogenetic analysis of the HVR2 nucleotide sequences revealed (using bootstrap values of >70%) that 34.8% (48/118) of the RSV-A strains belonged to genotype NA1 (Figure 11). This genotype included the major epidemic strains that caused RSV-A infections in 2012, but it was rarely found after 2013, and undetected between 2014 and 2015. Importantly, the analysis of the newly emerged ON1 strains showed (using bootstrap values of >50%) that 59.3% (70/118) were grouped with the reference strains.

The ON1 strain became the dominant genotype in subgroup A in 2013, and it then completely replaced the other RSV-A genotypes from 2014.

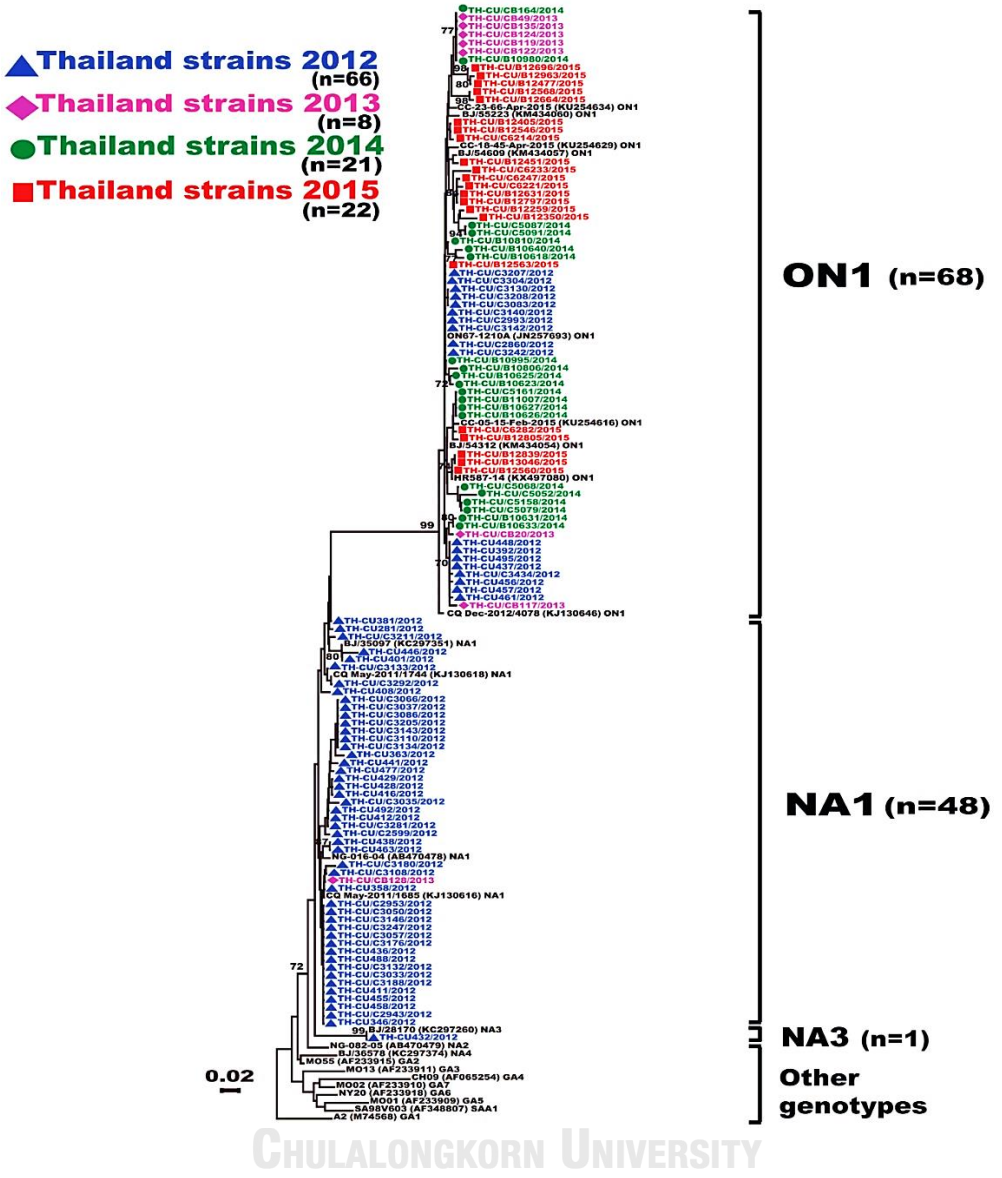

Figure 11 Phylogenetic tree of RSV-A based on the partial nucleotide sequences of the second hypervariable region (HVR2) of the G gene. The phylogenetic tree was constructed using the neighbor-joining algorithm in MEGA 6.0 software. Only bootstrap values >70% are displayed at the branch nodes. Years of virus isolation are color-coded for 2012 (blue triangle), 2013 (pink diamond), 2014 (green circle), and 2015 (red square). Other genotypes consist of GA1 to GA7, SAA1, NA2 and NA4. The scale bar represents the number of nucleotide substitutions per site between close relatives. The number of strains are in parentheses.

The phylogenetic tree of RSV-B strains is shown in Figure.12; 86/93 (92.5%) of these strains were shown (using bootstrap values of >70%) to belong to the genotype BA9, 4/93 (4.3%) to genotype BA10, and 3/93 (3.2%) to genotype BA–C (Figure 12). BA9 strains became the dominant genotype in RSV-B epidemics at the end of the study period. In addition, three strains were clustered (using bootstrap values of >70%) in genotype BA-C, which was the genotype of RSV-B viruses in 2009 in Beijing. Moreover, BA10 and BA-C strains were sporadically discovered in 2012, and they reoccurred in 2015. Almost all of the RSV-positive cases were single-strain infections, and only one RSV-positive case (TH-CU448) involved a co-infection with two subgroups, genotypes ON1 and BA9.

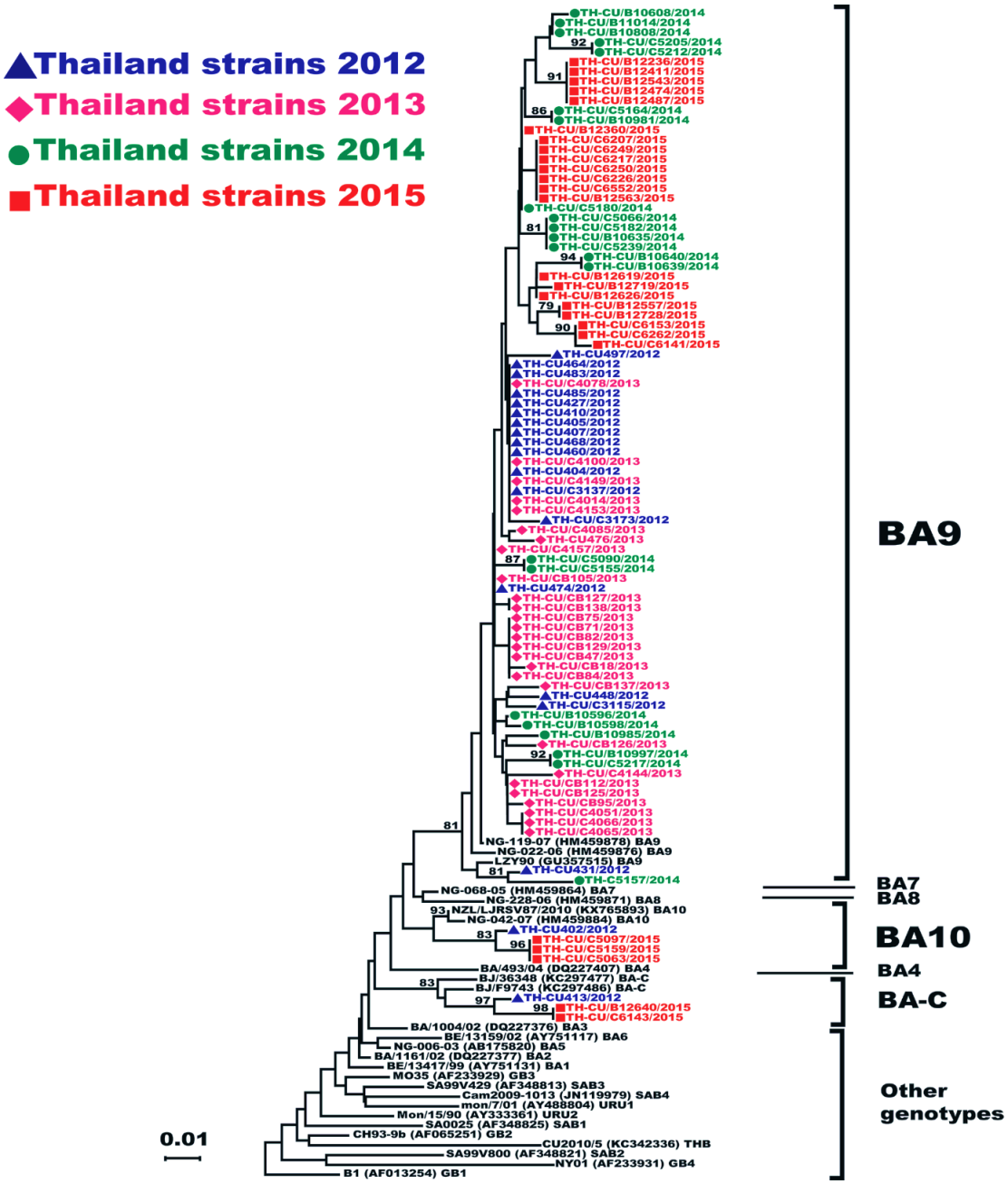

Figure 12 Phylogenetic tree of the RSV-B nucleotide sequences based on the second hypervariable region (HVR2) of the G gene. The phylogenetic tree was constructed using the neighbor-joining algorithm in MEGA 6.0 software. Bootstrap values >70% are displayed at the branch nodes. Years of virus isolation are color-coded for 2012 (blue triangle), 2013 (pink diamond), 2014 (green circle), and 2015 (red square). Other genotypes consist of GB1 to GB4, SAB1 to SAB4, URU1, URU2, THB, BA1 to BA3, BA5 and BA6. The scale bar represents the number of nucleotide substitutions per site between close relatives. The number of strains are in parentheses.

The p-distances indicated that there was a high degree of diversity among the NA1 strains. They ranged from 0.004 to 0.06, with a mean p-distance of 0.03. The p-distances for the ON1 strains were between 0.003 and 0.06, with a mean p-distance of 0.03. Thus, the maximum p-distance of the NA1 strains was the same as that of the ON1 strains.

In addition to assessing intra-genotype diversity, p-distances were calculated between all the RSV-A strains detected. NA3 had mean p-distances of 0.062 and 0.083 when compared to NA1 and ON1, respectively. ON1 and NA1 had much a lower mean p-distance (0.050), which highlights the fact that ON1 emerged from NA1. A range of p-distances (0.000–0.067) was observed for BA9. The p-distance between the two BA10 strains was 0.010. The p-distance between the BA-C strains was 0.030. BA-C had mean p-distances of 0.090 and 0.080 when compared to BA9 and BA10, respectively. In contrast, BA9 and BA10 had much a lower mean p-distance (0.062).

Mutational analysis

The comparison with prototype A2 strain indicated that the predicted G proteins of all the NA1 strains comprised 297 amino acids and that of all the ON1 strains had a 24-amino acid duplication in the HVR2, giving a G protein length of 321 amino acids (Figure 13). In the multiple sequence alignment analysis, multiple site-specific mutations among the NA1 and ON1 strains were identified, including S222P/T, P226L, E233K/M/R, I244R, L258H/Y, M262E/K, F265L/I, S269T/A, N273Y/H, P274L/I, S280Y/H, P310L, P313S/Y, S314P/L, P316S, P317S, P320T/S/A, and R321K/Q (Figure 13). Differences between the ON1 and NA1 strains were identified at three substitution sites: E232G/R, T253K, and P314L.

Regarding the N-glycosylation sites within the G gene HVR2, the Thai RSV-A strains had four common N-glycosylated sites (N237, N251, N273, and N318; Figure 13,

red shading). However, almost all (41/48, 85.4%) of the NA1 strains had lost the N-glycosylation site at position 237 due to an N237D/Y substitution. The majority of NA1 strains (41/48, 85.4%) had gained a new N-glycosylation site at position 273 because of Y273N and P274L/I substitutions. It is noteworthy that only three common N-glycosylation sites occurred in the NA1 strains, due to incompatibility between the sites N237 and N273. In ON1 strains, the two most conserved N-glycosylation sites were located at the sites N237 and N318, which were present in 100% and 94.3% (66/70) of the ON1 strains, respectively. They had lost both N-glycosylation sites at N251 and N273 as a result of N253K and N273Y/H substitutions.

The comparison with prototype B1 strain indicated that the predicted G proteins of most of the Thai RSV-B strains comprised 312 amino acids, though some strains had a G protein length of 319 amino acids. The Thai BA strains had multiple site-specific mutations, including L237P, S247P, L257S/T/P, and E258K. Four site-specific substitutions were confined to BA9 strains, comprising P219L, T229I, I281T, and H287Y. Specific substitutions including T222P, T223L, T238R, T239I, T256A, and Q282R were only found in BA-C (Figure 14).

Two potential N-glycosylation sites, N296 and N310, were conserved in all the BA9 and BA10 strains except for one strain of BA9 (TH_CU/B10985/2014) (Figure 14). The BA-C lost both N-glycosylation sites as a result of the S297P and T312N/Y substitutions.

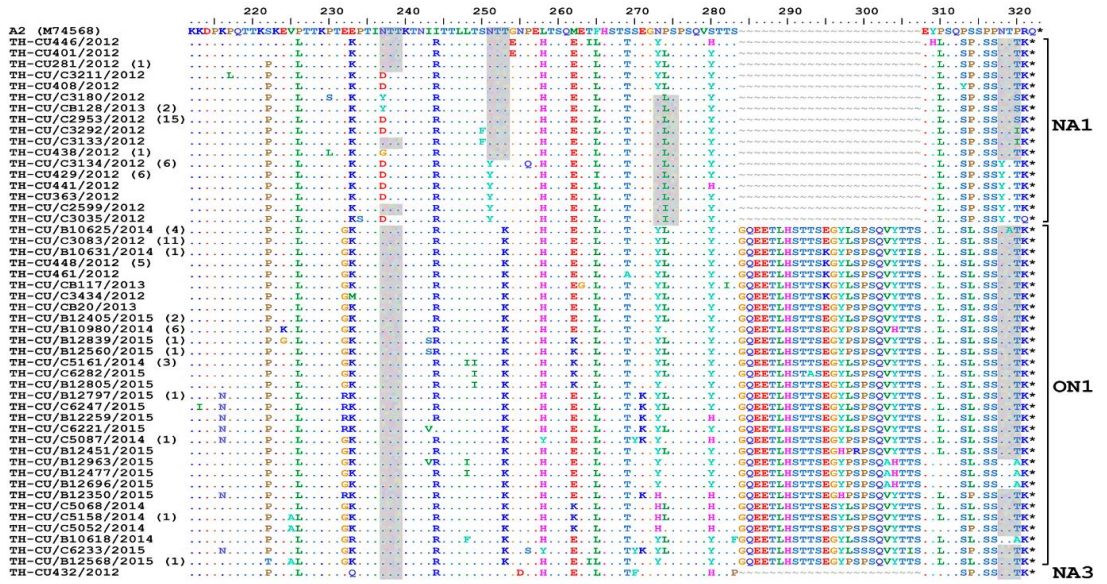

Figure 13 Alignment of the deduced amino acid residues encoding the second hypervariable region (HVR2) of the RSV-A G protein. RSV-A strains identified in this study compared to the reference A2 strain. The strain genotypes (NA1, ON1, and NA3) are indicated on the right (brackets). The number of additional strains with identical sequence as shown is indicated in parentheses to the right of the strain name. The presence of potential N-linked glycosylation sites are shaded. Asterisks indicate the positions of stop codons. Dots indicate identical residues. Tildes indicate missing residues.

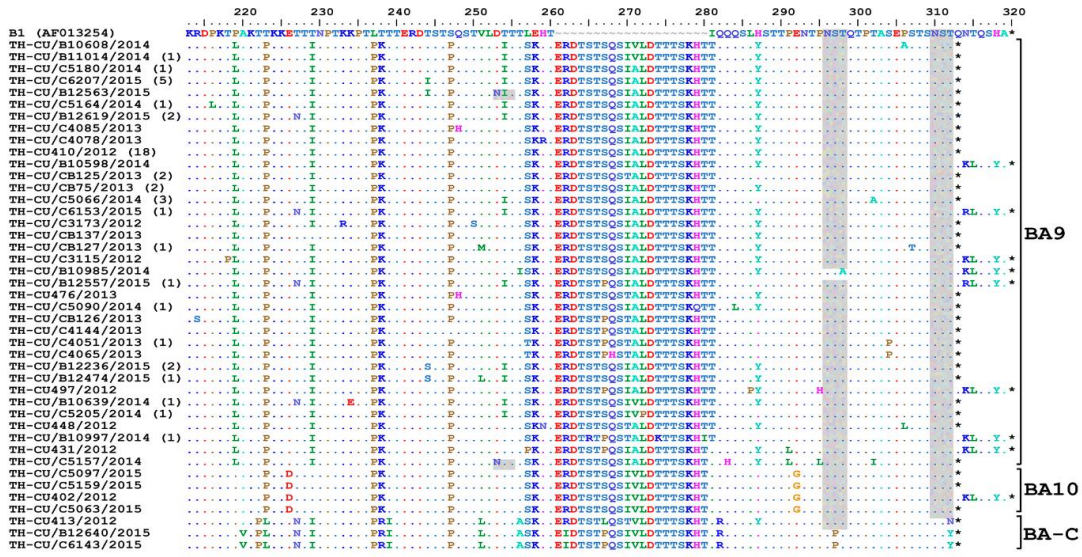

Figure 14 Alignment of the deduced amino acid residues encoding the second hypervariable region (HVR2) of the RSV-B G protein. RSV-B strains identified in this study compared to the reference B1 strain. The strain genotypes (BA9, BA10, and BA-C) are indicated on the right (brackets). The number of additional strains with identical sequence as shown is indicated in parentheses to the right of the strain name. The presence of potential N-linked glycosylation sites are shaded. Asterisks indicate the positions of stop codons. Dots indicate identical residues. Tilde indicate missing residues.

Selection pressure analysis

The positive selection analyses that used the SLAC model indicated that the overall dN/dS ratios were 1.13, 1.11, and 0.65 for genotypes NA1, ON1, and BA9, respectively (Table 2). Overall, site-specific identification revealed four sites (233, 274, 298, and 314) in the Thai ON1 strains. Among these four sites, the strongly site-specific P274L and L298P substitutions were identified in at least three selection models. One site (237) in the Thai NA1 strains and one site (244) in the Thai BA9 strains were identified as being under positive selection pressure, but these sites were identified only by single selection models.

Table 2 Selection pressure analysis by single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), internal fixed-effects likelihood (IFEL), and random effects likelihood (REL) methods.

		SLAC		FEL		IFEL		REL	
Genotype (Mean dN/dS)	Codon	dN/dS	p-value	dN/dS	p-value	dN/dS	p-value	dN/dS	Bayesian factor
NA1 (1.13)	237	-	-	-	-	-	-	6.665	97643.700
	273	-	-	-	-	66.265	0.084	-	-
	296	-	-	-	-	168.21	0.078	-	-
ON1 (1.11)	233	-	-	17.361	0.064			1.778	50.153
	274*	10.685	0.050	27.016	0.075	36.324	0.067	-	-
	298*	21.605	0.002	55.119	0.012	58.489	0.023	14.822	14894.500
	314	8.800	0.080	-	-			-	-
BA9 (0.65)	244	-	-	-	-	67.675	0.040	-	-

* Indicate strong site-specific substitutions with at least three selection models

Part 2: Development of the multiplex (one-step) *TaqMan* real-time reverse transcription-PCR assay for rapid detection of human respiratory syncytial virus and human metapneumovirus during 2016 to 2017 in Thailand

(Manuscript in preparation)

Sensitive nucleic acid detection based on method takes advantages to a better understanding of the epidemiology and natural history of the virus. The real-time RT-PCR assay was successfully implemented for diagnosis of RSV and hMPV infections in a routine diagnostic laboratory. It was easy to detect the sample, rapidly diagnostic. Moreover, molecular methods offer highly sensitivity in diagnosis is essential for not only timely therapeutic intervention but also the identification more accurate detected these viruses in populations, especially in older adults, and the avoidance of unnecessary antibiotic treatment.

Epidemiological studies have shown that the predominant viral cause of acute RTIs among infants and young children is RSV and that the incidence of RSV infection decreases significantly with age (3, 4). RSV is known for its tendency to cause bronchiolitis in infants, but it can infect all age groups, causing both upper and lower respiratory tract infection and a spectrum of illness ranging from subclinical infection to pneumonia, which may result in mortality in certain patient groups (5). RSV can be subtyped into RSV-A and RSV-B according to the antigenic properties of its proteins, and both subtypes are found in hospitalized infants and elderly individuals (6, 7).

The newly identified hMPV is a recently discovered respiratory pathogen of the family *Paramyxoviridae*, as is RSV (62). The hMPV is now recognized as a frequent cause of acute respiratory tract infections in the pediatric populations (113, 114). Children under 5-years-of-age, elderly adults, and immunocompromised patients are at increased risk for severe hMPV infection, and the virus has also been reported to be an infrequent cause of respiratory tract infection in healthy children (66, 115, 116). Similar to RSV strains, hMPV

strains can be classified into two genetically and antigenically distinct groups (A and B), with each group divided into genetic subgroups 1 and 2 (117). Both antigenic group A and group B have been reported to co-circulate in urban areas during epidemic periods with varied patterns of predominance, but epidemiological data about the prevalence of hMPV infection Thailand remains limited (118, 119).

The present study aimed to undertake a retrospective review of the viral causes of respiratory tract infection from a large patient population of all age groups who attended hospital in Bangkok, Thailand, with an influenza-like illness during the two-year period from January 2016 to December 2017. The viruses investigated included RSV, hMPV, and influenza viruses, including the presence of viral co-detection and viral genotypes. The secondary aim of the study was to evaluate the age distribution of viral infections and to determine whether strains of RSV and hMPV circulating in adults in this urban population were similar to those circulating in children.

Materials and Methods

Ethical Considerations

This study was conducted on specimens collected during routine clinical examinations that were stored in a confidential and anonymized way. Patient identifiers including personal information (including name and address) and hospital number were removed from these samples to protect patient confidentiality, and no personal patient details appeared in any part of the data used in this study. The local Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University approved the research protocol (IRB number 609/59), the IRB waived the need for consent because the samples were de-identified and this was a retrospective study.

Specimens collection

From January 2016 to December 2017, nasopharyngeal airway (NPA) swabs were obtained from all patients who visited Bangpakok 9 International Hospital, Bangkok, Thailand due to an influenza-like infection. A case of influenza-like infection was defined according to the US Centers for Disease Control and Prevention guidelines for influenza surveillance, as a progression of fever (>38°C) and either symptom of a cough, sore throat, or pharyngitis. All patient samples were collected in viral transport media with the addition of antibiotics (2 x 10^6 U/L of penicillin G and 200 mg/L of streptomycin) and transported within 24 hours to the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, for routine respiratory virus diagnostic testing. All respiratory specimens were divided into aliquots and stored at -70°C until further analysis was required.

The design of primers and probes

Nucleotide sequences were directed to a region located on the M gene of RSV and the F gene of hMPV (Table 3). The M gene sequence of RSV and F gene sequence of hMPV were retrieved from NCBI (National Center for Biotechnology Information) and then aligned using the multiple sequence alignment CLUSTAL W via BioEdit software version 7.0.9. The result from the alignment should reveal many conserved regions. Both of primers and probes were generated using the primer design software version 9.1 (Ruslan Kalendar, Institute of Biotechnology, University of Helsinki, Finland) and the Primer Express Software version 5.0 (Applied Biosystems). RSV-probe was labeled with the 5' reporter dye 6-carboxy-fluorescein (FAM) and the 3' quencher dye Black Hole Quencher-1 (BHQ-1). hMPV-probe was labeled with the 5' reporter dye 6-carboxy-fluorescein (HEX) and the 3' quencher dye Black Hole Quencher-1 (BHQ-1). The primers and probes used in this study are shown in Table 3.

Table 3 Primers and probes used in multiplex assays for the detection of viral pathogens.

Virus	Primer/Probe	Sequence 5'- 3'	Target region	Position	Strand
RSV (A/B)	RSV_F3251	GGCAAATATGGAAACATACGTGAA	M gene	3251-3274	Sense
	RSV_R3334	TCTTTTTCTAGGACATTGTAYTGAACAG	M gene	3334-3361	Antisense
	RSV_P3303	FAM-CTGTGTATGTGGAGCCTTCGTGAAGCT-BHQ1	M gene	3303-3329	Sense
hMPV (A/B)	hMPV_F3604	CAARTGYGACATTGCTGAYCTRAA	F gene	3604-3628	Sense
	hMPV_R3683	ACTGCCGCACAACATTTAGRAA	F gene	3683-3662	Antisense
	HMPV_P3630	JOE-TGGCYGTYAGCTTCAGTCARTTC -BHQ1	F gene	3630-3643	Sense

***In vitro* RNA synthesis**

RNA transcripts of the RSV and hMPV were used as standards to determine assay sensitivity. RSV (strain TH-CU178), hMPV (strain TH-B19819) cDNAs were processed using RNA extraction and cDNA synthesis as described above. The entire M gene and F

gene were amplified using F and R primers (Table 3). Thermal cycling conditions were 50°C for 30 minutes for reverse transcription, initial denaturation at 94°C for 10 minutes, 45 cycles of three steps 30 seconds at 94 °C, 30 seconds at 55°C, 1 minutes at 72 °C and final extension at 72 °C for 10 minutes. PCR products were cloned into pGEM-T Easy Vector System (Promega, CA, USA). The resulting plasmid constructs were confirmed by PCR and sequence analysis by First BASE Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia).

In vitro-synthesized RNA was made using a T7 riboprobe kit (Promega, Leiden, The Netherlands) as per manufacturer's instructions. *In vitro*-synthesized RNA was quantified using a Nanophotometer (IMPLEN, Germany), and the RNA copy number was calculated. The *in vitro*-transcribed RNA was then serially diluted tenfold and used to determine the analytical sensitivity of the real-time RT-PCR.

Virus detection using real-time RT-PCR

RNA was extracted from 200 µL of specimens using the commercial Viral Nucleic Acid Extraction Kit (RBC Bioscience, Taipei, Taiwan), according to the manufacturer's instructions. The detection of RSV and hMPV were performed using an in-house multiplex *TaqMan* (one-step) real-time RT-PCR assay. To obtain the fluorescence signals threshold cycles (Ct), the final concentration of primers and probes were optimized. For the viruses tested, RSV and hMPV, specimens with cycle threshold values ≤38 were considered positive. The positive control was the cDNA from the recombinant T7 promoter. Primers and the probe were targeted to a region located on the M gene of RSV, and the F gene of hMPV (Table 1). The RSV-probe was labeled with the 5' reporter dye 6-carboxy-fluorescein (FAM) and the 3' quencher dye Black Hole Quencher-1 (BHQ-1). The hMPV-

probe was labeled with the 5' reporter dye 6-carboxy-fluorescein (HEX) and the 3' quencher dye Black Hole Quencher-1 (BHQ-1).

The PCR conditions included 2 µL RNA added to the reaction mixture included SensiFAST Probe No-ROX One-Step Kit (Bioline USA Inc., Taunton, MA). There was 10 µmol of each of the primers and 10 µmol of the probes, with the profile: 1 cycle for 20 min at 42°C to activate reverse transcription, and followed by initial denaturation for 3 minutes at 95°C, followed by 50 cycles for 10 seconds at 95°C and 20 seconds at 60°C. This assay has a limit of detection of 100 copies per reaction for both viruses. There were no cross-reactions between the two viruses and other respiratory viruses, including influenza A virus, influenza B virus, adenovirus, enterovirus, rhinovirus, and coronavirus.

Reverse transcription and PCR amplification

Both RSV and hMPV RNAs, which were positive real-time RT-PCR detection, were selected to identify their groups and genotypes. Subsequently, those RNAs were synthesized to cDNA using the ImProm-II Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, RNA and random hexamer primers were incubated at 70°C for 5 minutes, followed by extension for 2 hours at 42°C and inactivation at 70°C for 15 minutes.

RSV RT-PCR

For RSV strains, the amplification of the partial G (inclusive of the second hypervariable region [HVR2]) and F genes was performed using semi-nested RT-PCR, as previously described (34). Amplification was performed in a DNA thermal cycler under the following conditions: initial denaturation at 94°C for 3 minutes; 40 cycles of denaturation at 94°C for 20 seconds; annealing at 55°C for 20 seconds; elongation at 72°C for 90 seconds; and a final extension at 72°C for 10 minutes. Identical amplification

parameters were carried out in the second-round PCR for 30 cycles. The PCR amplicons (approximately 840 bp for RSV-A and 720 bp for RSV-B) were visualized by 2% agarose gel electrophoresis and purified using the GeneAll® Expin™ gel extraction kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The purified PCR products were randomly sequenced by First BASE Laboratories Sdn Bhd (Seri Kembangan, Selangor Darul Ehsan, Malaysia).

hMPV RT-PCR

For hMPV strains, the cDNA was templated to PCR analysis to detect the partial F-gene of hMPV. The amplification of PCR was performed using nested PCR, as previously described (120). The PCR conditions involved initial denaturation at 95°C for 3 minutes; 35 cycles at 95°C for 1 minute: at 55°C for 1 minute; at 72°C for 1 minute; and a final extension at 72°C for 3 minutes. When the first PCR was negative, the identical amplification parameters were carried out in a nested PCR. The amplified DNA fragments were visualized by 2% agarose gel electrophoresis and purified using a GeneAll® Expin™ gel extraction kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The purified PCR products were sequenced by First BASE Laboratories Sdn Bhd (Seri Kembangan, Selangor Darul Ehsan, Malaysia). Some of the hMPV strains could not be typed due to a low viral load.

Sequence comparison and a phylogenetic tree for RSV and hMPV genotypes

Sequence data for each clinical strain were formatted and assembled using the SeqMan program of DNASTAR Software (version 5.0). RSV and hMPV were genotyped to study the distribution and diversity of RSV and hMPV genotypes in this study compared to other RSV and hMPV reference strains, respectively (S1 and S2 Tables). Nucleotide sequences of G protein of RSV and the F gene of hMPV were multiply aligned by using

ClustalW implemented in BioEdit (version 7.0.9). Phylogenetic trees were constructed using the Maximum Likelihood (ML) method implemented in the MEGA program (version 6.0). The reliability of the ML tree method based on the Tamura–Nei model was estimated using 1,000 bootstrap pseudo-replications.

Nucleotide sequence accession numbers

The GenBank accession numbers of the nucleotide sequences obtained in the present study are MH447703 - MH447725 for RSV-A, MH447726 - MH447818 for RSV-B and MH447819 - MH447950 for hMPV.

Data analysis

The association between virus prevalence and the patient age at infection was evaluated with one-way analysis of variance (ANOVA) using SPSS software (version 22.0). A p-value of <0.05 was considered statistically significant.

Statistical analysis

Reproducibility was computed using the standard deviation (SD), the coefficient of variation (CV), and Ct-values for eight 10-fold dilutions of control plasmids. Sensitivity, specificity, and positive and negative predictive values of the two assays were conducted at the two-tailed 5% significance level. Data analyses were performed using the Statistical Package for the Social Sciences version 22.0 (SPSS Inc., Chicago, IL).

Results

Interpretation of multiplex real-time RT-PCR detection

The fluorescent signals obtained from each detection channels revealed a clear signal without any unanticipated noises as shown in figure. The presence of RSV and hMPV was detected by using *TagMan* probes labeled with the FAM and HEX fluorescent dyes, respectively (Figure 15). Samples yielding a positive FAM signal have been interpreted as RSV and samples yielding a positive HEX signal as hMPV.

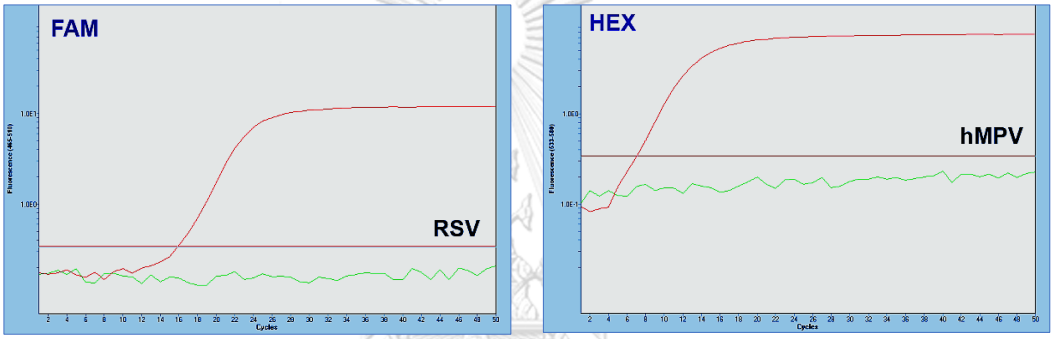

Figure 15 Interpretation of RSV and hMPV detection by multiplex real-time RT-PCR assay.

Specificity and qualitative assessment of assays

The specificity of the multiplex real-time RT-PCR assay was estimated for testing the cross-reactivity of the probes and primers against other RNA viruses including influenza A virus (H3N2 and H1N1), influenza B virus, human enteroviruses (CA4, CA5, CA8, CA9, CA10, CB1, CB2, CB4, CB5, D68), Adenovirus, human rhinovirus (A, C), Coronavirus (OC43, HKU1, NL63, 229E) and unknown specimens, which were negative for RSV and hMPV. The results show yielded only a FAM fluorescent signal of partial M gene indicating RSV, while a HEX florescent signal presented only hMPV specimen (Table 4). No cross-reactivity was observed with other respiratory viruses. These findings indicate high specificity of the primers and probes used in the multiplex real-time RT-PCR assay.

Table 4 Different respiratory viruses tested by real-time RT-PCR

Virus	Isolate code	Fluorescent signal	
		FAM-RSV (Ct value)	HEX-HMPV (Ct value)
Enterovirus CA4	A2496	–	–
Enterovirus CA5	A2195	–	–
Enterovirus CA8	A2487	–	–
Enterovirus CA9	A1927	–	–
Enterovirus CA10	A2337	–	–
Enterovirus CB1	A1907	–	–
Enterovirus CB2	A1721	–	–
Enterovirus CB4	A1532	–	–
Enterovirus CB5	A2272	–	–
Influenza A virus (H3N2)	B25287	–	–
Influenza A virus (H3N2)	B25464	–	–
Influenza A virus (H3N2)	B25502	–	–
Influenza A virus (H3N2)	B25698	–	–
Influenza A virus (H3N2)	B26185	–	–
Influenza A virus (H1N1)	B21633	–	–
Influenza A virus (H1N1)	B21428	–	–
Influenza A virus (H1N1)	B25276	–	–
Influenza A virus (H1N1)	B25494	–	–
Influenza A virus (H1N1)	B24083	–	–
Influenza B virus	B25308	–	–
Influenza B virus	B25670	–	–
Influenza B virus	B26096	–	–
Influenza B virus	B26338	–	–
Influenza B virus	B26467	–	–
Human metapneumovirus A	B24501	–	24
Human metapneumovirus A	B24522	–	24
Human metapneumovirus A	B17484	–	24
Human metapneumovirus A	B17519	–	24
Human metapneumovirus A	B19630	–	28

Human metapneumovirus B	B24460	–	28
Human metapneumovirus B	B24587	–	26
Human metapneumovirus B	B24515	–	25
Human metapneumovirus B	B19820	–	25
Human metapneumovirus B	B20201	–	32
RSVA	B26518	24	–
RSVA	B26269	20	–
RSVA	B26261	19	–
RSVA	B25740	23	–
RSVA	B25597	18	–
RSVB	B26249	21	–
RSVB	B26158	24	–
RSVB	B26155	21	–
RSVB	B26074	21	–
RSVB	B26067	23	–
Adenovirus	B3601	–	–
Adenovirus	B3616	–	–
Enterovirus D68	TU73	–	–
Enterovirus D68	TU48	–	–
Enterovirus D68	CB108	–	–
Rhinovirus A	TU39	–	–
Rhinovirus C	TU53	–	–
Coronavirus OC43	C4317	–	–
Coronavirus HKU1	B8244	–	–
Coronavirus NL63	B7479	–	–
Coronavirus 229E	B8003	–	–

Sensitivities and detection limits of assays

To assess the detection limit of the assay, the results were performed using *in vitro*-transcribed RNA from template of both lineage A and B viruses. The standard curve was plotted using tenfold serial dilutions of the *in vitro*-synthesized RNA in duplicate from 10^8 copies per reaction to 10 copies per reaction to check the PCR efficiency of the assay

(Figure 16). The slope of the standard curve was found to be -3.3724 for RSV and to be -4.0431 for hMPV. For the analytical sensitivity of the assay, RSV was detected at least 100 copies of *in vitro*-synthesized RNA per reaction and hMPV was detected at least 10 copies of *in vitro*-synthesized RNA per reaction (Figure 17).

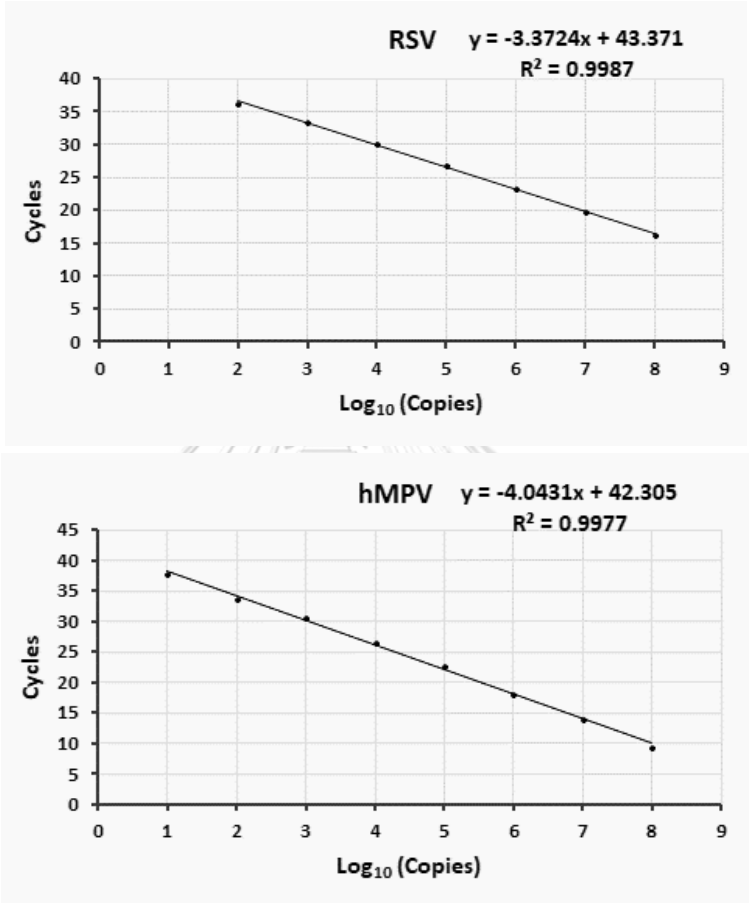

Figure 16 Standard curves of multiplex (one-step) real-time RT-PCR assays. The multiplex (one-step) real-time *TagMan* RT-PCR assays were tested using synthesized in vitro target viral RNA transcripts ranging from 10 to 10^8 copies/reaction.

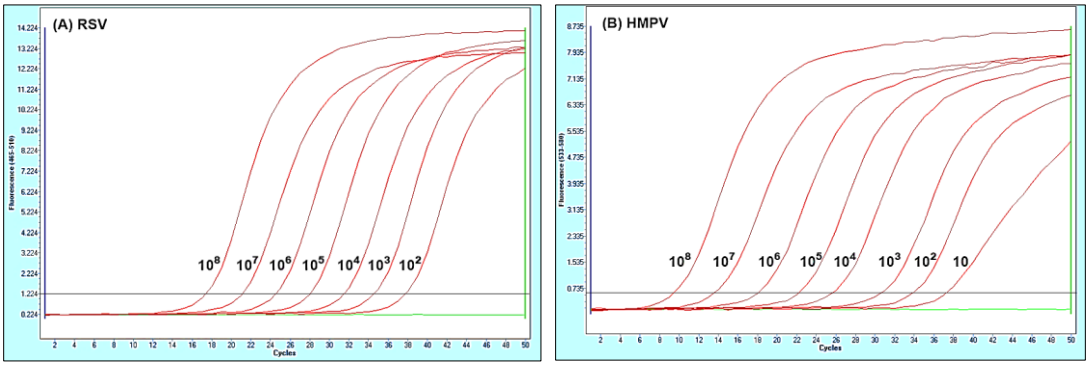

Figure 17 Sensitivity of the multiplex real-time RT-PCR assay for RSV (A) and hMPV (B) obtained from the amplification of 10-fold serially diluted the transcribed RNA standards.

Assay precision

The serial dilution viral RNA standards (from 10^6-10^3 copies/reaction) were tested in triplicate within a single run to determine intra-assay variation of both monoplex and multiplex (one-step) real-time RT-PCR assays (Table 5). The mean coefficients of variations (CVs) of intra-assay variability of the monoplex real-time RT-PCR range from 1.66-3.43% for RSV and 1.21-2.06% for hMPV. The mean CVs of intra-assay variability of the multiplex real-time RT-PCR range from 0.47-5.46% for RSV and 0.59-0.63% for hMPV.

To determine inter-assay variation of both monoplex and multiplex real-time RT-PCR assays, each standard dilution tested in three independent runs were performed (Table 5). The mean CVs of inter-assay variability of the monoplex real-time RT-PCR were 0.52-2.15% for RSV and 0.52-1.67% for hMPV. The mean CVs of inter-assay variability of the multiplex real-time RT-PCR were 1.60-4.25% for RSV and 0.35-1.70% for hMPV.

Table 5 Intra- and inter-assay reproducibility of the monoplex and multiplex real-time RT-PCR.

Virus Standard	Concentration (copies/ul)	Intra-assay						Inter-assay					
		Monoplex rRT-PCR			Multiplex rRT-PCR			Monoplex rRT-PCR			Multiplex rRT-PCR		
		Mean Ct	SD	CV%	Mean Ct	SD	CV%	Mean Ct	SD	CV%	Mean Ct	SD	CV%
RSV	1,000,000	23.94	0.82	3.43	23.27	0.11	0.47	23.76	0.51	2.15	22.44	0.72	3.21
	100,000	28.27	0.47	1.66	25.66	1.40	5.46	28.40	0.48	1.69	26.75	1.02	3.81
	10,000	31.59	0.77	2.44	28.87	1.00	3.46	31.93	0.37	1.16	30.33	1.29	4.25
	1,000	34.92	0.69	1.98	33.26	0.62	1.86	35.25	0.36	1.02	33.74	0.54	1.60
hMPV	1,000,000	15.64	0.19	1.21	15.97	0.10	0.63	15.60	0.26	1.67	15.92	0.27	1.70
	100,000	19.44	0.33	1.70	19.90	0.13	0.65	19.33	0.17	0.88	20.12	0.24	1.19
	10,000	23.29	0.48	2.06	24.58	0.16	0.65	23.43	0.17	0.73	24.65	0.16	0.65
	1,000	27.22	0.41	1.51	28.76	0.17	0.59	27.07	0.14	0.52	28.71	0.10	0.35

Patient Characteristics

We retrospectively tested 8842 stored respiratory samples obtained from patients with ILI presenting between January 2016 and December 2017. All patients were divided into seven groups to assess the distribution of respiratory virus infection with regard to the specific patient's age, as follows: 1916 (21.7%) infant (≤2 yrs, mean age 1.2±0.6), 1541 (17.4%) pre-school (3 to 5 yrs, mean age 3.8±0.8), 1253 (14.2%) primary-school (6 to 12 yrs, mean age 8.4±1.9), 371 (4.2%) secondary school children (13 to 18 yrs, mean age 15.2±3.2), 1148 (13.0%) early adult (19 to 30 yrs, 25.4±3.2), 2164 (24.5%) middle adult (31 to 60 yrs, 41.4±8.1) and 446 (5.0%) elderly adult (>60 yrs, 72.0±9.1). The population study was more female than a male with a ratio of 1:0.9.

RSV and hMPV prevalence

Out of the 8,842 specimens tested, 15.0% (1,329/8,842) were positive for one or more viruses. Results are summarized in Table 6. The most commonly identified virus was RSV (11.4%, 1011/8842), which found in 14.1% in 2016 and 9.0% in 2017. A low frequency

has been detected by hMPV in 3.6% (318/8842) that contained 2.7% in 2016, 4.4% in 2017 (Table 6).

Table 6 RSV and hMPV identified between 2016 and 2017 from patients with respiratory tract infections.

Years of study	No. of samples	RSV-positive (%)	hMPV-positive (%)
Jan-Dec 2016	4178	590 (14.1)	114 (2.7)
Jan-Dec 2017	4664	421 (9.0)	204 (4.4)
Total	8842	1011 (11.4)	318 (3.6)

Age distribution of RSV and hMPV infections

The age association of the viral incidence is shown in Figure 18. In infants between 0–2 years old, RSV was the most common pathogen (21.2%) (Table 7 and Figure 18). The incidence of RSV infection significantly decreased with increasing the age of the patients ($p<0.0001$) dropping to 7.6% in children older than 5-years-of-age. Similar to RSV infection, the main hMPV-infected cases were children, particularly children aged between 0–5 years, who accounted for between 5.5–5.7% for hMPV-identified patients. Both RSV and hMPV infections were also presented in adults, with a lower incidence rate than in children. Regarding multiple infections, children in secondary school group (mean age, 15.2 ± 1.7yrs.) had the highest rate of multiple infections when compared to the other age groups ($p=0.0313$).

Table 7 The frequency of hospitalizations associated with a respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) in 2016-2017 among 8,842 specimens with influenza-like illness (ILI) presenting with all age groups.

Characteristics		No. of tested specimens (N=8842)	RSV (%) (N=1011)	hMPV (%) (N=318)	Multiple infections (%) (N=157) [a]
Age	≤2 years	1916 (21.7)	406 (21.2)	105 (5.5)	29 (1.5)
	3-5 years	1541 (17.4)	238 (15.4)	88 (5.7)	29 (1.9)
	6-12 years	1253 (14.2)	100 (8.0)	25 (2.0)	25 (2.0)
	13-18 years	371 (4.2)	19 (5.1)	10 (2.7)	11 (3.0)
	19-30 years	1148 (13.0)	66 (5.7)	20 (1.7)	20 (1.7)
	31-60 years	2164 (24.5)	144 (6.7)	53 (2.4)	34 (1.6)
	>60 years	446 (5.0)	38 (8.5)	17 (3.8)	9 (2.0)
p-value			**0.0262**	0.5695	**0.0313**
Sex	Male	4288 (48.5)	514 (50.8)	160 (50.3)	80 (51)
	Female	4554 (51.5)	497 (49.2)	158 (49.7)	77 (49)

Numbers in bold represent significant difference among groups (p value < 0.05). [a] The previously published influenza virus infections (January 2016 and June 2017 in Thailand) (121) was extended additional studies on the multiple infections in this study.

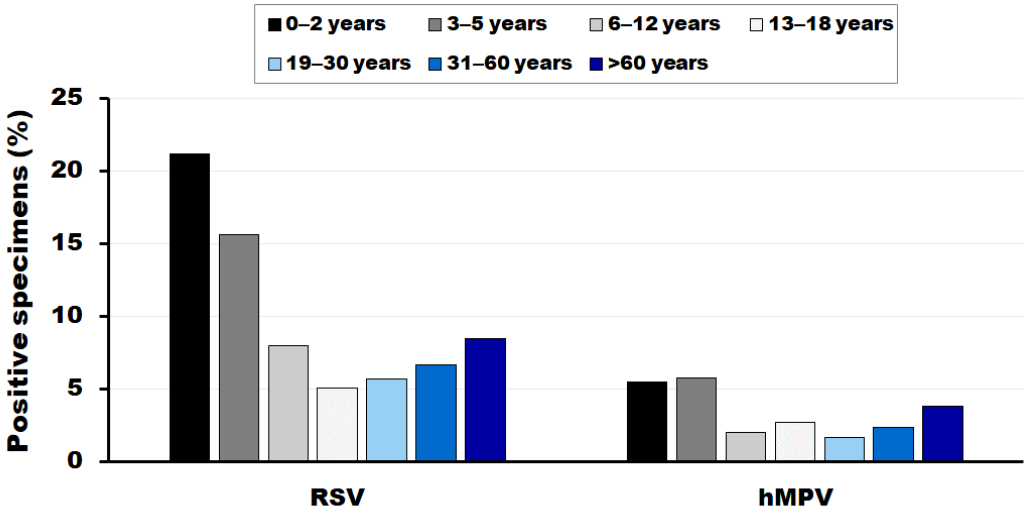

Figure 18 Distribution of virus infection by patient age for RSV and hMPV; infant (0-2 yrs), pre-school (3-5 yrs), primary school (6-12 yrs), secondary school (13-18 yrs), early adult (19-30 yrs), middle adult (31-60 yrs) and elderly adult (<60 yrs) during a 2-year period (2016-2017).

Seasonal prevalence of RSV and hMPV infections

The seasonal distribution of detected viruses during the two-year study period is shown in Figure 19 (Figure 19). The total number of cases of influenza-like infection peaked from June to December during both years, 2016 and 2017, with September having the highest activity. RSV infection appeared to peak in the rainy months between July and November with the highest incidence being 37% (206/555) in August 2016 and the highest incidence being 17.3% (136/784) in September 2017 (Figure 19). The peak number of hMPV infections overlapped with but occurred later than the peak for RSV infections (Figure 19). Also, RSV and hMPV infections showed sporadic peak activity throughout the year.

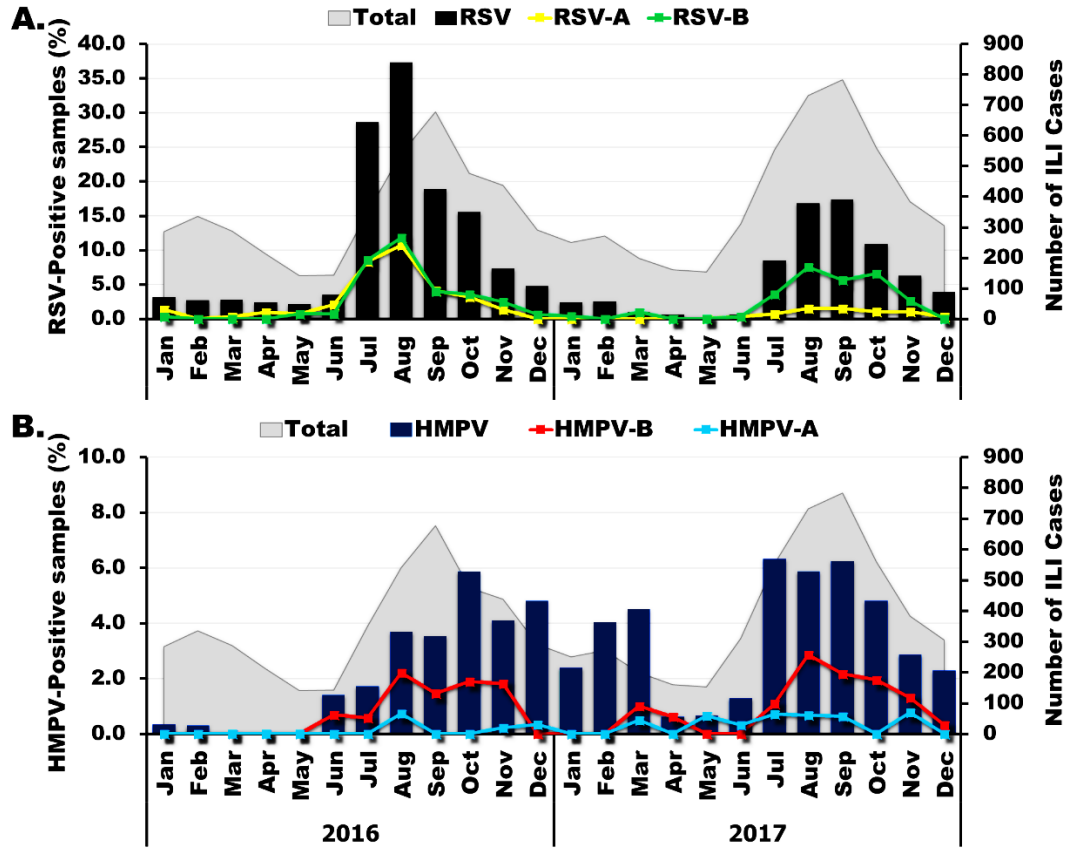

Figure 19 Seasonal distribution of those viruses detected between 2016 and 2017 in this study. The number of samples examined by month is shown as a gray area (right scale). (A) RSV positive rates (bar graphs) for RSV-A (yellow line) and RSV-B (green line) are shown (left scale). (B) hMPV positive rates (bar graphs) for hMPV-A (blue line) and hMPV-B (red line) are shown (left scale).

Subgroup distribution patterns

In this study, RSV was identified in 11.4% of all samples tested, of which 36.3% (177/488) were RSV-A, and 66.4% (324/488) were RSV-B. The cyclical pattern of RSV was the co-dominance with RSV-A and RSV-B in 2016, and the subsequent of RSV-B predominance was in 2017 (Figure 19). Co-infection with RSV-A and RSV-B was sporadically case (1.3%, 13/1011). The predominant group of hMPV detected in all ages during two years was hMPV-B with 80.3% (106/132) and low circulation rate of hMPV-A with 19.7% (26/132) (Figure 19).

Genotyping and phylogenetic analysis

Analysis of the partial G gene sequence showed that 100% (23/23) of RSV-A were genotype ON1 (Figure 20). This genotype was predominant during a 2-year study period. For RSV-B, all were BA9 (100%, 93/93) (Figure 20). We found a high degree of inter-groups diversity between ON1 and BA9 with 2.170-2.441, whereas the closely genetic variation showed in intra-genotype with p-distances ranged from 0-0.073 and 0-0.071 within the ON1 and BA9 genotypes, respectively.

Phylogenetic analysis of 132 hMPV strains in Thailand confirmed two main genetic lineages, A and B. Interestingly, all the strains were clustered as A2, B1, and B2, and subgroup A1 was not identified (Figure 21). The strains from subgroups B1 cocirculated with 74% (98/132). Only 6% (8/132) from subgroup B2 and 20% (26/132) from subgroup A2 were detected during a 2-year study period. The hMPV-detected strains were the closely genetic variation that showed intra-genotype (0.001-0.019) and inter-genotype (0.087-0.116) genetic diversity (measured by p-distance) among hMPV-A and hMPV-B strains in this study.

Additionally, the phylogenetic analysis also revealed that the samples were all taken from adults with RSV infections or hMPV infections genetically similar to their infected children (Figure 20 and Figure 21).

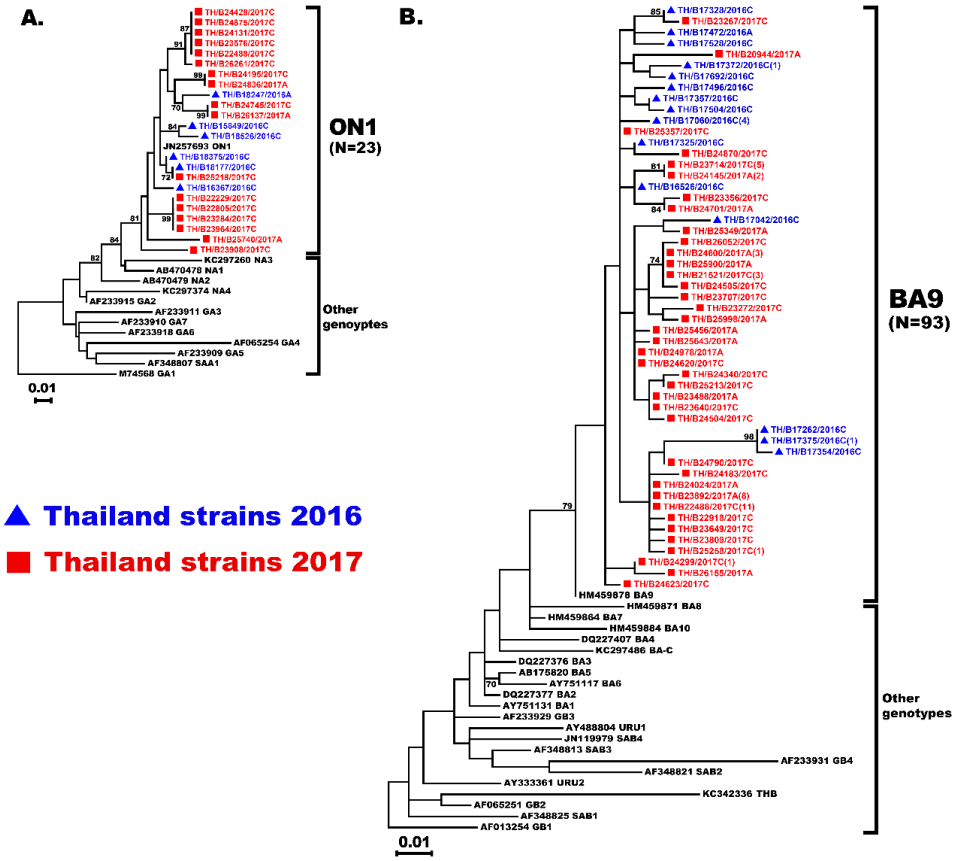

Figure 20 Phylogenetic tree of the RSV nucleotide sequences based on the second hypervariable region (HVR2) of the G gene. Phylogenetic trees were constructed by ML method implemented in the MEGA program (v6). The reliability of the ML tree method based on the Tamura-Nei model was estimated using 1000 bootstrap pseudo-replications. Bootstrap values >70% are displayed at the branch nodes. Years of virus isolation are color-coded for 2016 (blue triangle) and 2017 (red square). C indicated the sample from the child (≤18 years) and A indicate the sample from an adult. Other genotypes for RSV-A (A) consist of GA1 to GA7, SAA1, NA1 to NA4, ON1 and for RSV-B (B) consist of GB1 to GB4, SAB1 to SAB4, URU1, URU2, THB, BA1 to BA10. The scale bar represents the number of nucleotide substitutions per site between close relatives. The number of strains was in parentheses.

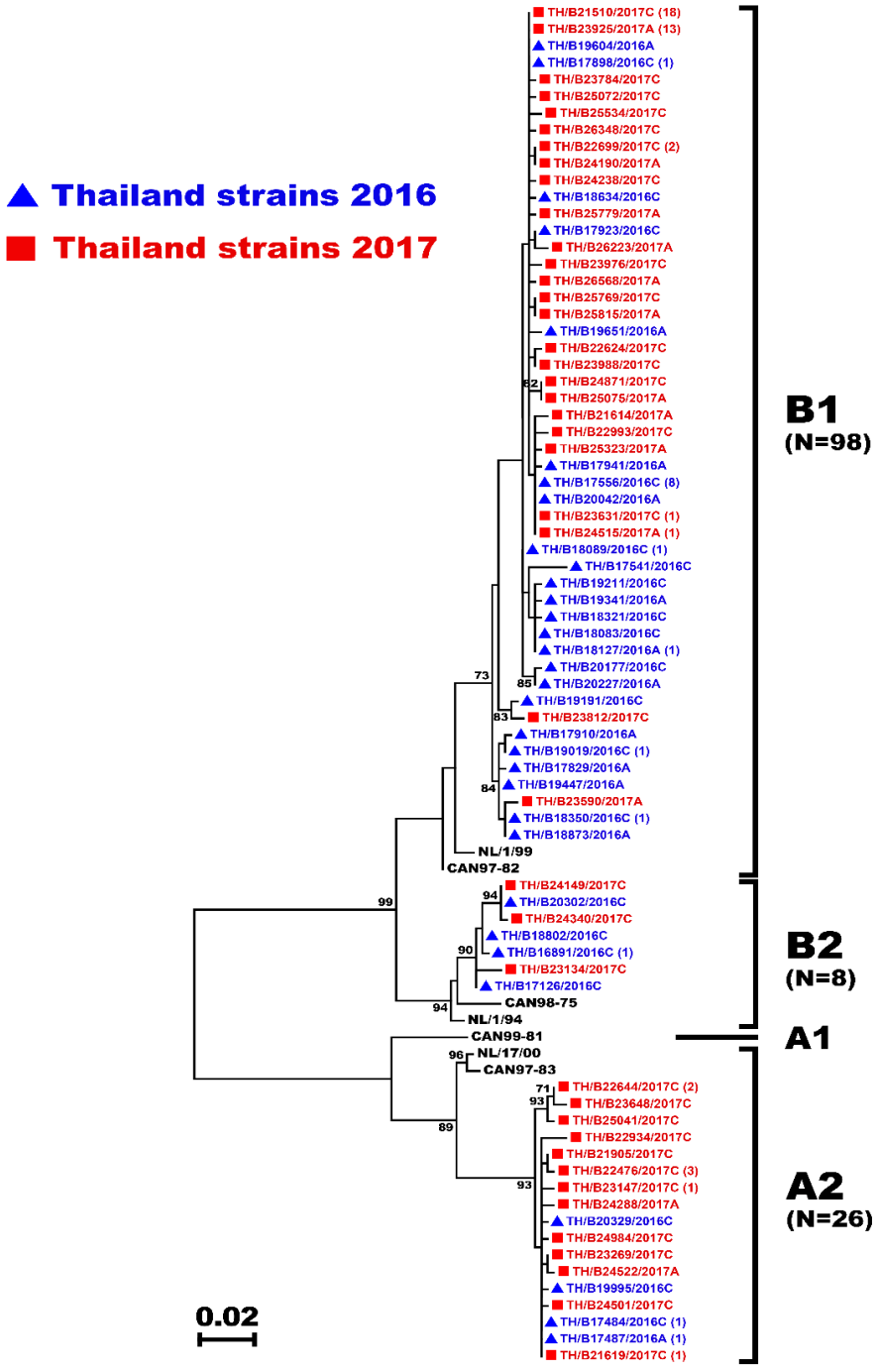

Figure 21 Phylogenetic tree of the hMPV-A and hMPV-B nucleotide sequences based on the partial F gene. Phylogenetic trees were constructed by ML method implemented in the MEGA program (v6). The reliability of the ML tree method based on the Tamura-Nei model was estimated using 1000 bootstrap pseudo-replications. Bootstrap values >70% are displayed at the branch nodes. Years of virus isolation are color-coded for 2016 (blue triangle) and 2017 (red square). C indicated the sample from the child (≤18 years) and A indicate the sample from an adult. Reference sequences for each genotype (A1, A2, B1, and B2). The scale bar represents the number of nucleotide substitutions per site between close relatives. The number of strains was in parentheses.

Co-infection rates of viral infections of RSV, hMPV, and influenza virus

Table 8 summarizes the number of occurrence of each virus in single and multiple infections as well as the number of co-occurrences of viruses for each possible virus combination (Table 8). The previously published influenza virus infections (January 2016 and June 2017 in Thailand) (121) was extended additional studies on the multiple infections in this study. The most common combination observed was RSV and the Influenza A H3N2 subtype. As a percentage, the virus most often found in co-infections was RSV (non-typed), which was found in more than 25.0% of cases, followed by hMPV (non-typed) (13.4%) and influenza (12.5%). Mixed infections of RSV, hMPV, and influenza virus in hospitalized patients were rare at 0.1% (3/2857).

Table 8 Rates of detection of three viral pathogens in patients with an influenza-like infection between January 2016 to December 2017, and the occurrence of multiple infections

Virus	Total	Single	Double	Triple	Mix infection (%)	RSV non-typed	RSV-A	RSV-B	hMPV non-typed	hMPV-A2	hMPV-B1	hMPV-B2	INF-H1N1	INF-H3N2	INF-B
RSV non-typed	521	391	128	2	25.0				16	1	1	1	23	67	19
RSV-A	177	160	18		10.2			13	1		1		1	2	
RSV-B	324	296	27	1	8.6		13		2	1	1		2	5	3
hMPV non-typed	186	161	22	3	13.4	16	1	2					1	1	1
hMPV-A2	26	24	2		7.7	1		1							
hMPV-B1	98	93	5		5.1	1	1	1					1		1
hMPV-B2	8	7	1		12.5	1									
INF-H1N1	349	324	25		7.2	23			1		1				
INF-H3N2	815	738	75	2	9.4	67	2	5	1						
INF-B	364	338	25	1	7.1	19		3	2		1				

Part 3: Evolutionary patterns of ON1 genotype derived from Whole-Genome Sequencing

(Manuscript in preparation)

Historically, most genetic studies of RSV are focused on the attachment glycoprotein (G) gene which is the most variable and has been commonly used for genotyping (122). For phylogenetic tree, previous study suggested that both the whole genome and full G gene CDS are suitable for evolutionary analyses, but the partial G CDS alone may produce misleading results (123). However, the studies of the sequence variability of RSV have required the stronger evolutionary signal provided by the full virus genome sequence (15,200 nucleotides [nt]: 11 open reading frames [ORFs] and noncoding regions). Interestingly, the considerable variation occurs in the other RSV proteins that beside the G protein can be used to provide understanding on the nature of the viral genome.

This study provided the first ON1 genome sequencing data of circulating RSV strains in Thailand (2011-2017). This genotype has spread so quickly across parts of worldwide (38), likely BA genotypes, and has also become predominant genotype in subgroup A in Thailand. The novel ON1 genomes can be used for comparative analysis with other previous studies on global and local RSV genomic diversity. In this study, all RSV genome sequences available in the GenBank database was collected to use primer design. The ON1 genotype samples were randomly selected for a full virus genome sequencing. The reference data sets were generated using the search term “txid11250 [Organism]) AND 13500[SLEN]: 17000[SLEN].” Phylogenetic tree of the genome sequences was constructed using the neighbor-joining method implemented in MEGA 6.0 with 1,000 bootstrap replicates. Nucleotide substitution rates and estimates for time to most recent common ancestor (tMRCA) was calculated in BEAST v1.8.0 both for a full genome and for the individual CDS.

Materials and Methods

Clinical Samples

Nasopharyngeal aspirates (NP) were determined ON1 positive by semi-nested RT-PCR during 2011 to 2017 (34, 124). The ON1 positive strains were randomly selected at least one strain per year for full virus genome sequence (Table 9).

Table 9 Details for samples used in this study.

Strain Name	Collection Date	Source	Age	Length of Genome (bp)	Accession number
TH_CU91	17/09/2011	NP Sample	1 y	15,219	MH447951
TH_C3208	17/09/2012	NP Sample	1 y	15,111	MH447952
TH_CB124	01/11/2013	NP Sample	9 m	15,221	MH447953
TH_CB135	01/11/2013	NP Sample	2 y	15,229	MH447954
TH_B10625	09/08/2014	NP Sample	1 y	15,225	MH447955
TH_B10806	27/08/2014	NP Sample	1 y	15,222	MH447956
TH_B18117	30/08/2016	NP Sample	1 y	15,209	MH447957
TH_B18247	04/09/2016	NP Sample	54 y	15,216	MH447958
TH_B23576	12/08/2017	NP Sample	3 y	15,220	MH447959
TH_B23964	23/08/2017	NP Sample	1 y	15,220	MH447960

RNA extraction and cDNA synthesis

RNA was extracted by using a commercially available Viral Nucleic Acid Extraction Kit (RBC Bioscience Co, Taipei, Taiwan). Samples from 200 µL were extracted following the manufacturer's protocol. RNA was eluted in 40 µL RNase free water from the column membrane. For cDNA synthesis, total RNA was first annealed with 6 µL random hexamers primer at 70 °C for 5 minutes and allowed on ice for 5 minutes. Then the viral RNA was performed in 15 µL reactions comprised of components of the ImProm-II

Reverse Transcription System (Promega, Madison, WI) and incubated at 42 °C for 2 hours. Finally, reverse transcriptase was inactivated by heating at 70 °C for 15 minutes.

Primer design

The whole genome sequence of the RSV-A retrieved from NCBI (National Center for Biotechnology Information) and then aligned using the multiple sequence alignment CLUSTAL W via BioEdit software version 7.0.9. The result from the alignment should reveal many conserved regions and details of each region. Then conserved regions were constructed as PCR amplification primer pairs. Before being used to design primers, the conserved regions were aligned to check the specificity of this region by using nucleotide BLAST. In this study, degenerate-base primers were designed to cover the variable RSV-A ON1 strains as possible. The RSV genome was consisted with 18 overlapping segments (Figure 22), each fragment ranging 630 to 1,500 nucleotides, for amplified and sequencing (Table 10).

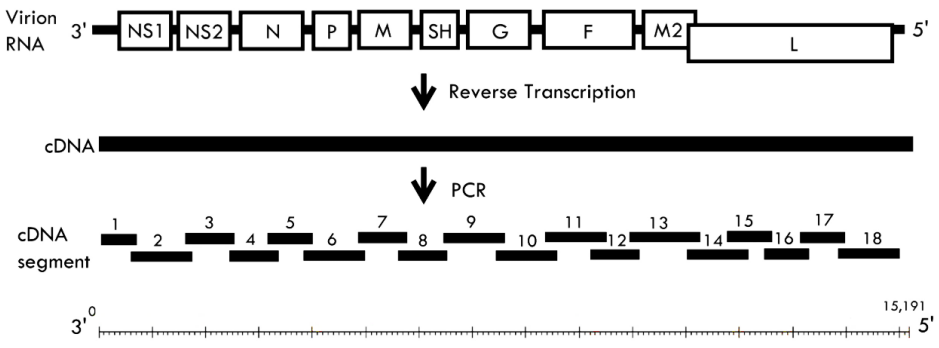

Figure 22 Design of PCR primer target sites in RSV.

Table 10 The primer used for the whole genome sequence.

Amplification primer				
Segment	Forward Primer	Reverse Primer	Locations	PCR product
1	CGAAAAAATGCGTACAACAAACTTGC	CTCATGTCTGTGATCATCAYTCTT	4-687	684 bp
2	CTAGCAAATCAATGTCACTAACACC	GGAGAGTCATGCCTGTATTCTG	536-1597	1062 bp
3	GGAGTGGATGTAACAACACATCG	GCTGATGTTTGATAGCCTCTAGTTC	1419-2283	865 bp
4	GGCATAATGGGAGARTAYAGAGG	CTCTTAYACCAACCATYGCATCTC	2133-2870	738 bp
5	GATCAGACAAACGATAATATAACAGC	GATGATTGGAACATGGGCACC	2720-3357	638 bp
6	GGCAAATATGGAAACATACGTGAAC	GGTAGCTCAAAGGTTTTGTTATGG	3226-4446	1221 bp
7	CAAGCAAATTCTGGCCTTACTTTAC	TGTTTTTGYTCTTGACTGTTGTGG	4305-5064	760 bp
8	TACAAGATGCAACAARCCAGATCA	CTAACTGCACTGCATGTTGATTGA	4892-5753	861 bp
9	CAAATAACAATGGAGTTGCCAATCC	GATAACGGAGCTGCTTACATCTG	5620-6852	1233 bp
10	GAGGATGGTACTGTGACAATGC	CTGTCCAACTCTGCAGCTCC	6647-7778	1132 bp
11	GGCAAATATGTCACGAAGGAATCC	GCCCRAGTTTATTCAATATAGCATAG	7569-8864	1296 bp
12	GACATACAAGAGTATGACCTCGT	GCATCTGTGATGTTGTTGAGCATA	8743-9567	825 bp
13	GCTTAGGCTTAAGATGYGGATTC	CACCCTTCGATACCACCCATATG	9360-10806	1447 bp
14	CTGGATGAACTGCATGGTGTAC	GCCCYTGAGGATATGTAGGTTC	10625-11831	1207 bp
15	GAGGTTTTGAGCACAGCTCCAAA	GGAATTCACATGGTCTACTACTGAC	11714-12515	802 bp
16	GGATTGGGTGTATGCATCTATAGA	GTTAACAACCCAAGGGCAAACTG	12344-13351	1007 bp
17	GACAGTAGTTATTGGAAGTCTATGTC	GGATGAAGTTCCACTACTGTACG	13178-14073	895 bp
18	CACTTTATTGCATGCTTCCTTGGC	AGTGTCAAAAACTAATCTCTCGTAA	13890-15180	1290 bp

PCR of the complete RSV-A ON1 genome

The 18 overlapping regions of the viral genome was amplified using PCR from cDNA to contain a nearly full-length genome of RSV. Briefly, each PCR reaction consisted of 2 µL cDNA was added to PCR mixture using the reaction mixture included SensiFAST Probe No-ROX One-Step Kit (Bioline USA Inc., Taunton, MA). Cycling conditions start an initial denaturation at 94°C for 3 minutes and then 40 cycles of PCR in each cycle of denaturation for 30 seconds at 94°C, then annealing for 30 seconds at 55°C and elongation for 1 minute at 72°C and ended with the final extension cycle at 72°C for 10 minutes. The PCR products were performed by electrophoresis using 2% agarose gel and purified using the GeneAll® ExpinTM gel extraction kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The purified PCR products were

randomly sequenced by First BASE Laboratories Sdn Bhd (Seri Kembangan, Selangor Darul Ehsan, Malaysia).

Sequence data analysis

The whole genome sequence was assembled and edited sequences into consensus sequences comparing with the reference in GenBank accession number JX627336 for ON1 genotypes by using Seqman software (DNASTAR lasergene 6). Then consensus sequences were exported and used for the phylogenetic analyses.

Reference data set

A comprehensive RSV genome dataset was generated from the GenBank database using as a starting set all reported RSV genomes. The search was conducted using the search term “txid11250 [Organism]) AND 13500[SLEN]: 17000[SLEN].” The collected whole genome sequences were aligned with the website name https://mafft.cbrc.jp/alignment/server/. We selected only the ON1 genotype from the sequences alignment. 163 sequences were used for the phylogenetic analyses with the Bayesian method of phylogenetic inference in BEAST v1.8.0 and the maximum likelihood method in MEGA 6.0.

Phylogenetic and Evolutionary analyses

The set of ON1 whole genome sequence (163 sequences) and ten ON1 whole genome sequences which were constructed in this study were combined for setting as ON1 WG dataset (n=173) and the alignments of the G gene second hypervariable region (HVR2) for ON1 which was found in Thailand (2011-2017) (n=99), as ON1 HVR2 G dataset were used for the phylogenetic analyses with the Bayesian method of phylogenetic inference in BEAST v1.8.0.

The nucleotide alignment file was used to generate xml files to set nucleotide substitution model, molecular clock model for use in BEAST v1.8.0. Name of sequences was tracked the data about collection date to compare with the retrieve sequences from GenBank database. A general-time reversible (GTR) model of nucleotide substitution and an uncorrelated lognormal (UCLN) relaxed molecular clock were used to predict the origin time of the most common ancestor, population dynamic and phylogeny. The MCMC chain length was 400 million for the ON1 WG dataset and 800 million for the ON1 HVR2 G dataset. The Markov chain was sampled 10,000 times for each run. BEAST results were analyzed to calculate the evolutionary rate using Tracer v1.5 and summary trees generated using TreeAnnotator v1.8.0. Trees were visualized and annotated in FigTree v1.4.0.

The same model was used for the full CDS analysis. BEAST was used to perform a Bayesian MCMC analysis for full CDS alignments. Bayesian MCMC analysis was performed with BEAST as described above for the ON1 WG dataset. A chain length of 400 million was used for each full CDS sampling a total of 10,000 times. Results were analyzed using Tracer v1.5.

Selection pressure analysis.

All ON1 whole genome sequences were the same of dataset which used for the phylogenetic analyses (173 sequences available in Genbank), their sequences were constructed for each RSV ON1 gene. All CDS individuals were aligned using ClustalW via BioEdit software version 7.0.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and were trimmed to only the coding sequence. Positive selection was determined for each alignment using the SLAC, FEL, and FUBAR algorithms available on the Datamonkey webserver. Sites were only considered positive if they met the cutoff criteria for at least

two of the algorithms, that is a p-value of less than 0.10 for SLAC and FEL and posterior probability of greater than 0.90 for FUBAR.

Results

ON1 global phylogenetic clustering and placement of Thailand genomes

The 10 Thailand genomes were compared with ON1 genomes from 10 countries from specimens collected between the years 2011 and 2017. Bayesian time-scaled MCMC phylogenetic tree was generated to investigate the global evolutionary dynamics and time-scaled phylogeny of the ON1 genotype (Figure 23). The analysis revealed that the ON1 viruses deviated for their ancestor during the year 2006.4. The clustering was more temporal than geographical. ON1 genomes from Thailand did not show a monophyletic grouping, but its clustering was dispersed with the contemporaneous genomes from the other countries.

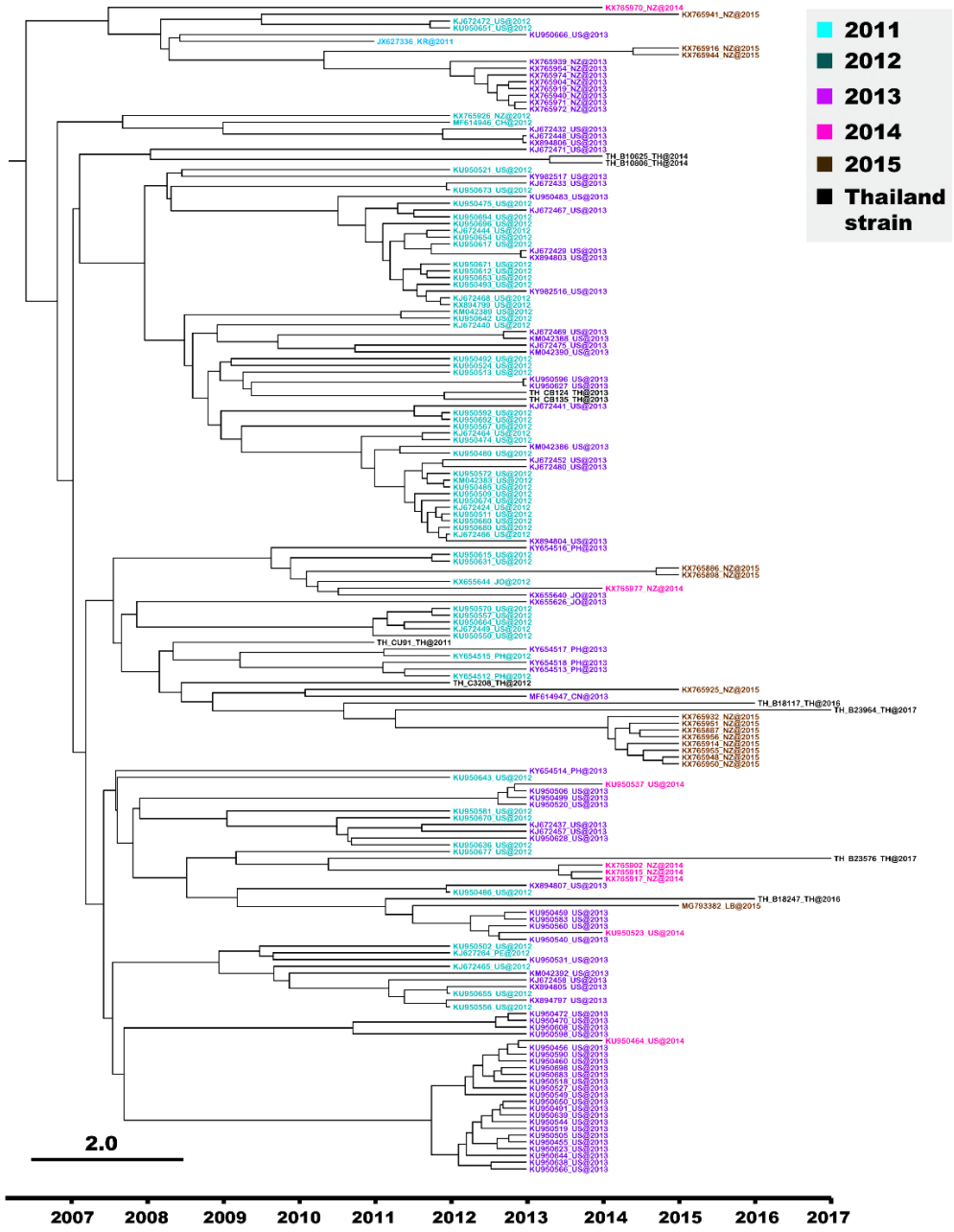

Figure 23 Tree of RSV-A ON1 Genome Sequences. This is a maximum clade credibility tree of ON1 genome sequences generated in this study and retrieved from GenBank. Tip times correspond to date of collection with the scale axis across the bottom showing the years. Tip labels show the accession number, country of isolation, and collection date. The labels are color coded with black for sequences from this study.

Estimation of RSV-A ON1 genome tMRCA and evolutionary rates

The full genomes generated in this study were combined to compare with the retrieve sequences from GenBank database, and these allowed an estimation of the global nucleotide substitution rates and tMRCA for all the recently sequenced RSV-A ON1 viruses. These estimates were calculated for the different ORFs and the whole-genome sequences (Figure 24 and Table 11). The evolutionary rates of the whole genome sequences were 3.91×10^{-4} substitutions/site/year [95% HPD 3.30×10^{-4} to 4.53×10^{-4}] for ON1 genotype. Evolutionary rates are given as the divergence among individual genes, with a confidence interval ranging from 3.36×10^{-4} to 1.56×10^{-3} substitutions/site/year (Table 12).

The whole genomes provided the accurate estimation substitution rates of the MRCA, as observed from the interval of lower and upper 95% highest posterior density (HPD) compared to individual ORF data for the same set of viruses. The highest changes in G and NS2 were observed using ON1 full genomes data set from this study (Figure 24). The whole-genome estimates for the evolutionary rates also provided a narrower confidence intervals than those from the individual ORFs.

The evolutionary rates based on the partial G CDS were 1.68×10^{-3} substitutions/site/year [95% HPD 1.63×10^{-3} to 1.73×10^{-3}] for ON1 Thailand strains (Table 11). For ON1 partial G in Thailand, tree showed the oldest strain in 2009.6 and its temporal clustering were detected within ON1 clade (Figure 25).

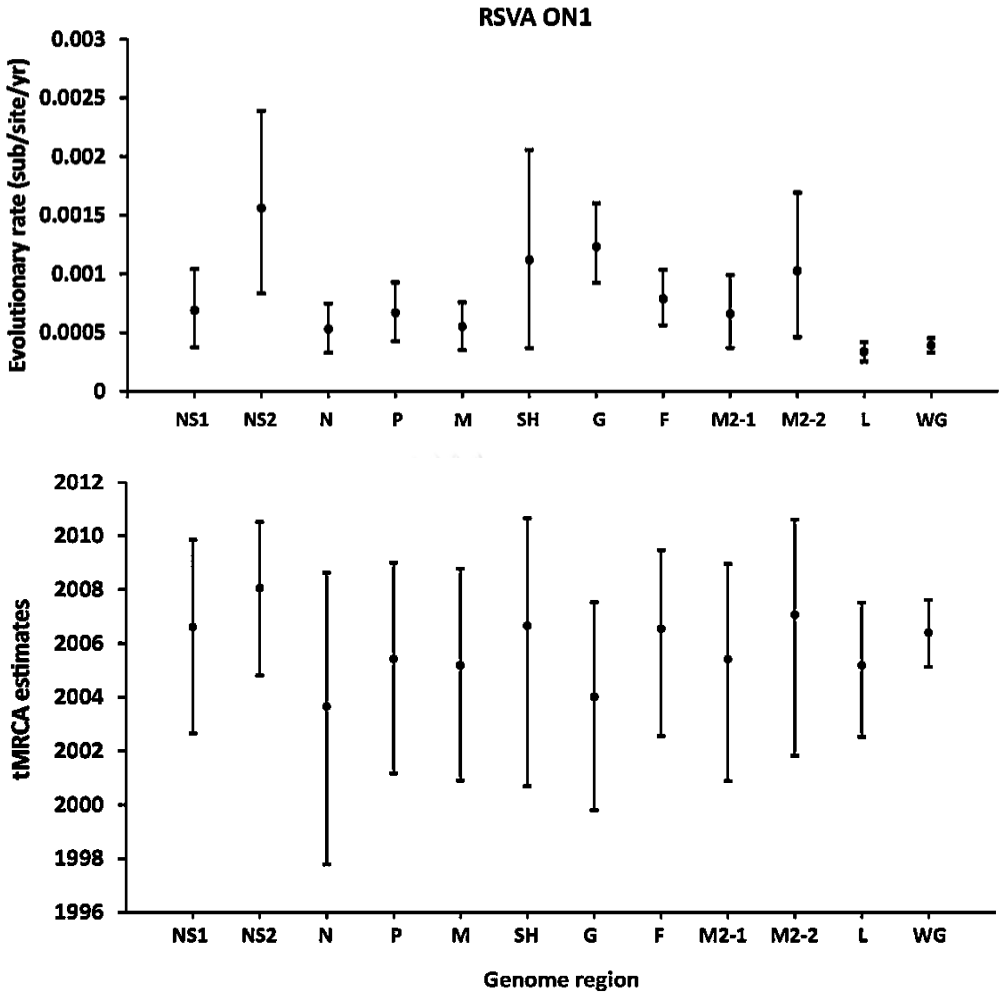

Figure 24 Estimates of the nucleotide substitution rates and tMRCA for RSV-A ON1 genotype in the individual ORFs and for the whole genome sequences.

Table 11 Mean evolutionary rates (substitutions/site/year) and times to most recent common ancestors (tMRCAs) as inferred by Bayesian analysis comparison between Thailand and global strains.

Set data	Set data	tMRCA (95% HPD)	MeanRate (95% HPD)
ON1 (with global reference strains)	WG	2006.4 (2005.1 to 2007.6)	3.91×10^{-4} (3.30×10^{-4} to 4.53×10^{-4})
	NS1	2006.6 (2002.7 to 2009.8)	6.88×10^{-4} (3.76×10^{-4} to 1.04×10^{-3})
	NS2	2008.1 (2004.8 to 2010.5)	1.56×10^{-3} (8.34×10^{-4} to 2.39×10^{-3})
	N	2003.7 (1997.8 to 2008.6)	5.30×10^{-4} (3.30×10^{-4} to 7.50×10^{-4})
	P	2005.4 (2001.2 to 2009.0)	6.70×10^{-4} (4.30×10^{-4} to 9.30×10^{-4})
	M	2005.2 (2000.9 to 2008.8)	5.50×10^{-4} (3.50×10^{-4} to 7.60×10^{-4})
	SH	2006.7 (2000.7 to 2010.7)	1.12×10^{-3} (3.69×10^{-4} to 2.06×10^{-3})
	G	2004.0 (1999.8 to 2007.5)	1.23×10^{-3} (9.24×10^{-4} to 1.60×10^{-3})
	F	2006.5 (2002.5 to 2009.5)	7.89×10^{-4} (5.64×10^{-4} to 1.03×10^{-3})
	M2-1	2005.4 (2000.9 to 2008.9)	6.60×10^{-4} (3.70×10^{-4} to 9.90×10^{-4})
	M2-2	2007.1 (2001.8 to 2010.6)	1.03×10^{-3} (4.59×10^{-4} to 1.69×10^{-3})
	L	2005.2 (2002.5 to 2007.5)	3.36×10^{-4} (2.54×10^{-4} to 4.18×10^{-4})
ON1 (only from Thailand)	HVR2_G	2009.6 (2007.9 to 2010.7)	1.68×10^{-3} (1.63×10^{-3} to 1.73×10^{-3})

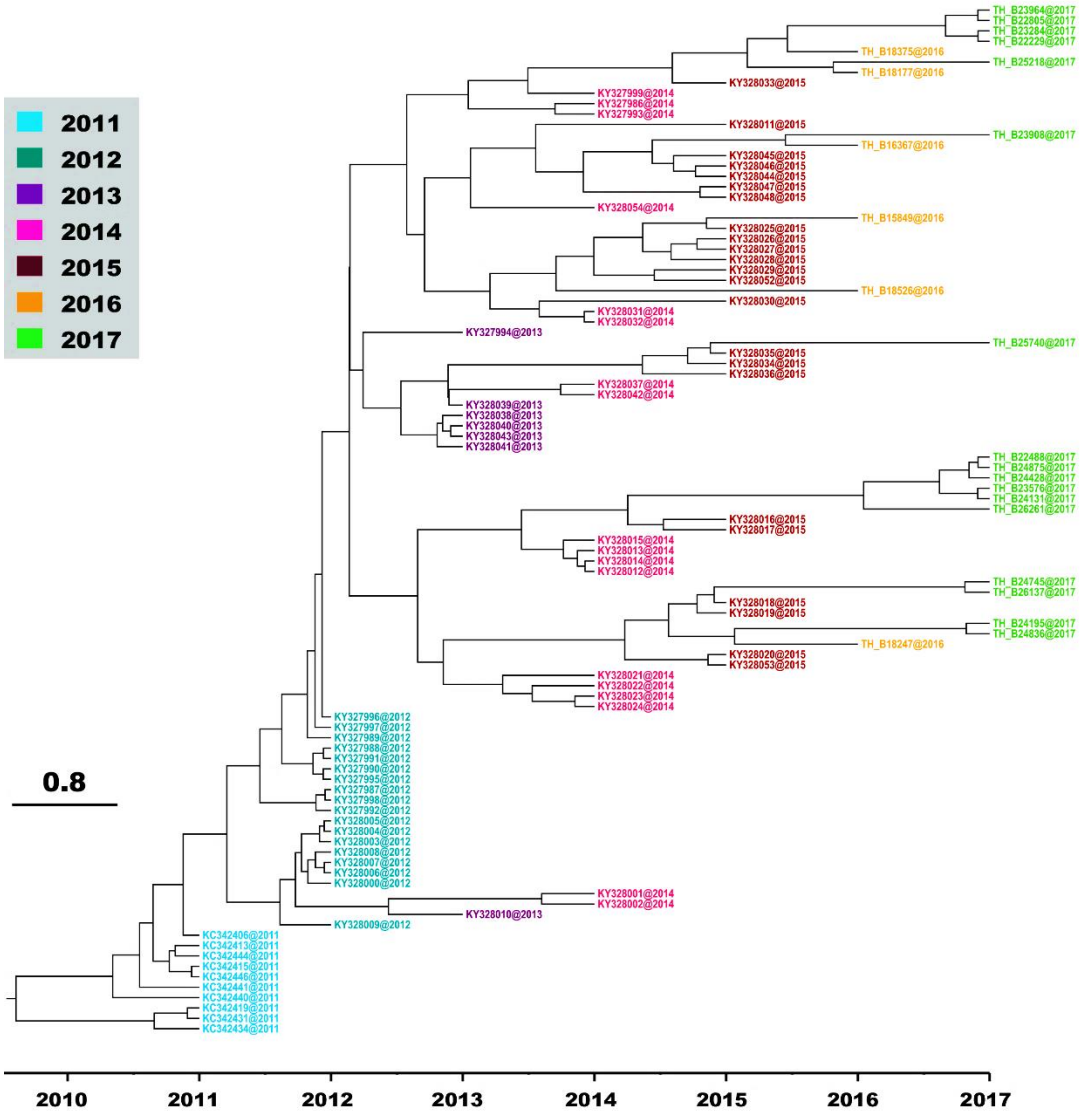

Figure 25 Tree of RSV-A (ON1) Partial G CDS sequences in Thailand (2011-2017). This is a maximum likelihood tree of ON1 attachment glycoprotein partial CDS sequences corresponding to HVR2 found in Thailand. Tip labels show the accession number and collection date.

Changes in G and F coding regions, comparing local and global viruses

For all known ON1 genomes, the amino acid changes encoded by the RSV coding sequences observed in Thailand were compared to the amino acid changes observed from other countries deposited into GenBank (Figure 26). A large percentage of the changes observed in the G protein were also observed globally, with 66% (16/24) of the

changes seen in Thailand G also observed in other parts of the world. The G protein was demonstrated the frequency of variable-region glycosylation (Figure 26). For the F protein, Thailand ON1 viruses 50% of the observed changes were also found global strains. Six of the protein specific are variable-region N-glycosylation. Many polymorphisms were observed in the F protein p27 domain and the signal peptide (Figure 26). NS2 protein have shown that high levels of the evolutionary rate (Figure 24), consistent with a protein interacting with polymorphic host target proteins. The majority of changes in the Thailand ON1 NS2 proteins were also observed in other parts of the world (Figure 26).

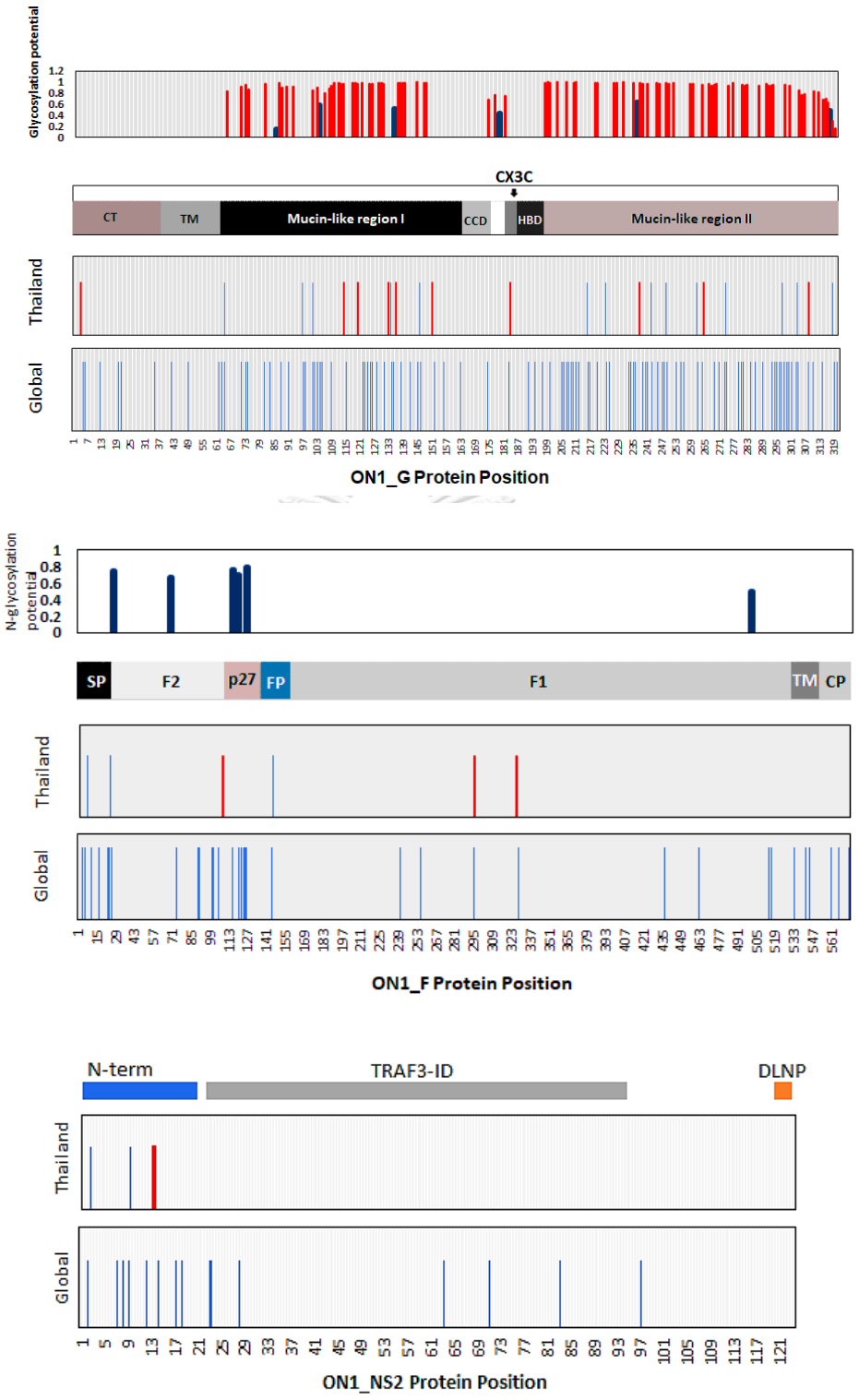

Figure 26 Thailand versus global changes in the G, F, and NS2 proteins. (A) The 321 amino acid long ON1 G protein showing the N-terminal location of its cytoplasmic domain (CD) and transmembrane domain (TM), two mucin-like regions with their N-linked (blue line) and O-linked (red line) glycans, and central cysteine noose which contains the 4 conserved cysteines, the central conserved domain (CCD), and the CX3C domain, followed by the heparin-binding domain (HBD). (B) The 574 amino acid long ON1 F protein showing signal peptide (SP), Fusion glycoprotein F2 (F2), p27, fusion peptide (FP), Fusion glycoprotein F1 (F1), transmembrane (TM) and CP. (C) The 123 amino acid long ON1 NS2 protein showing the N-terminal, TRAF3-ID, and DLNP.

Selection pressure analysis

The whole genome was used to identify potential positively selected sites for each of the coding regions in RSV by the SLAC, FEL, and FUBAR algorithms on the Datamonkey webserver. There were multiple positively selected sites predicted (241, 247, 298, 310, and 319) in the G coding region (Table 12). These predicted positively selected sites were located only in the mucin-like regions. Positive selection was notably absent in the other RSV coding regions with the cutoffs used in this study.

Table 12 Sites under positive selection in RSV-A ON1 genotype.

Gene	Site	SLAC		FEL		FUBAR	
		dN-dS	p-value	dN-dS	p-value	dN-dS	Post. Pr.
G	241	1.528	0.198	18.603	0.113	1.637	0.938
	274*	1.944	0.075	15.113	0.073	0.967	0.922
	298*	2.314	0.05	19.326	0.05	1.562	0.966
	310*	1.691	0.092	11.231	0.096	0.502	0.852
	319	2.098	0.162	32.617	0.078	3.662	0.977

*Indicate strong site-specific substitutions with at least three selection models.

Population dynamics

Since its origin in the 2006.4 (Table 11), the BSP of the whole genome of ON1 genotype (Figure 27) showed a continues at a rapid pace between 2008 and 2010, followed by a peak in the 2010. The ON1 global showed a relatively constant population size during 2011 to 2013, before the population growth size continued to fall until 2015 and growth to shape stable population in the ending 2017.

We also assessed the time-course trend of the relative genetic diversity of HVR2 G based on the effective population size (EPS) estimated for overall ON1 strains circulating in Thailand during the seven-years investigation period (2011-2017) (Figure

28). The BSP presented that EPS remained relatively steady in the first year of the investigation in which NA1 had been circulating as the predominated RSV-A genotype (Figure 11). However, starting from 2012 until 2014, the rapid increase in the size of EPS was observed, which was followed by a short and relatively stable stage of EPS until the late-2016. In the last year of investigation (2017), the size of EPS showed a small decrease ending point, which is consistent with the fact that RSV-B have been replacing the groups of the RSV in the current time (Figure 29).

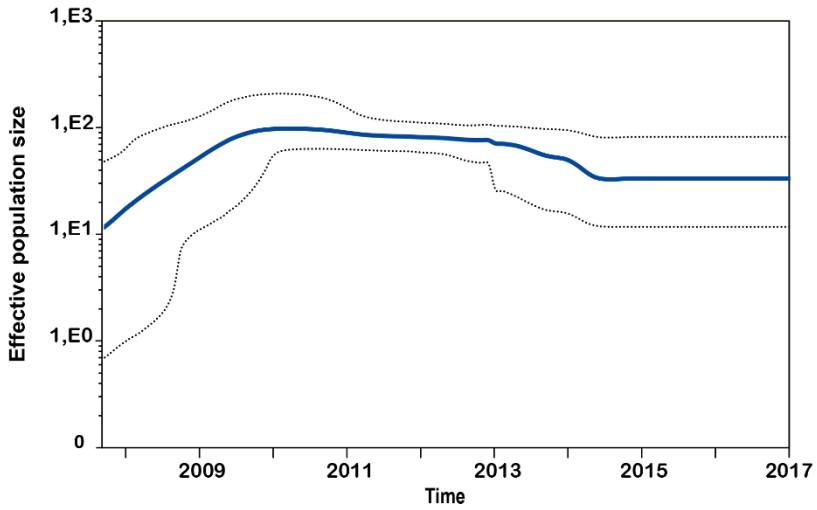

Figure 27 The whole genome of RSV-A ON1 Bayesian skyline plots (BSP). The estimated change in effective population size (Y axis) over time (X axis) for whole genome dataset is indicated. The thick line represents the mean estimates, whereas the dot line represented the 95% highest-posterior-density intervals.

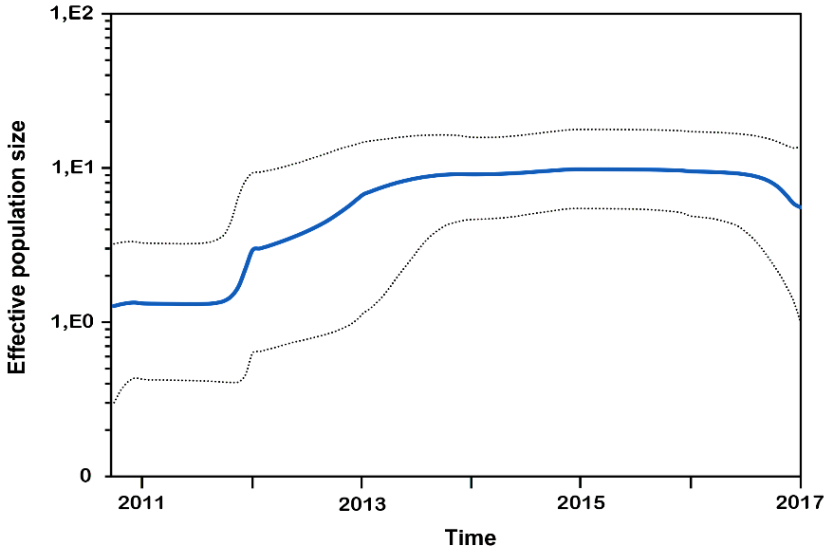

Figure 28 The partial G gene of RSV-A ON1 genotype Bayesian skyline plots (BSP) in Thailand. The estimated change in effective population size (Y axis) over time (X axis) for G gene dataset is indicated. The thick line represents the mean estimates, whereas the dot line represented the 95% highest-posterior-density intervals.

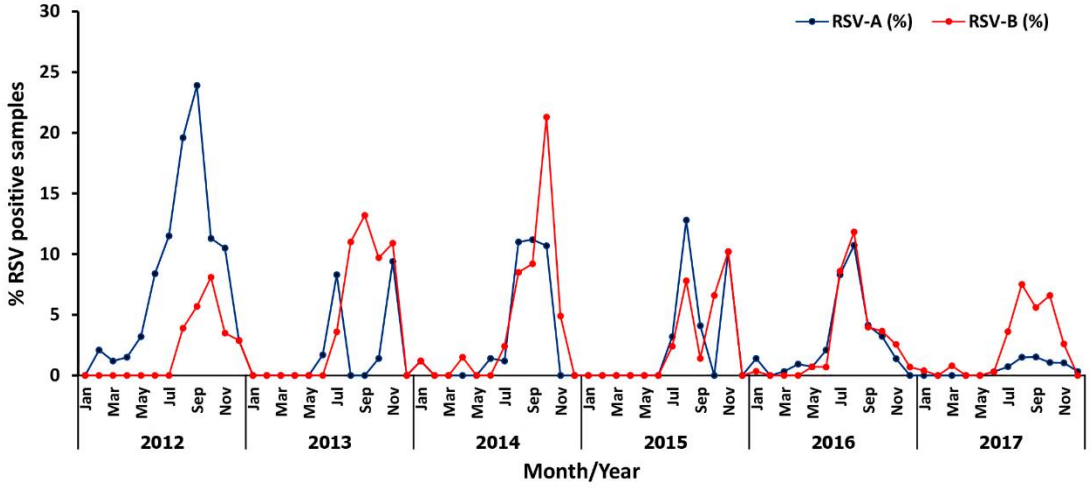

Figure 29 Temporal distribution of RSV positive rates in acute respiratory tract infection samples collected in Thailand, from 2012 to 2017.

Part 4: Prevalence and phylogenetic characterization of enterovirus D68 in pediatric patients with acute respiratory tract infection in Thailand

(Published in Jpn. J. Infect. Dis. 2016; 69;426–430)

Enterovirus D68 (EV-D68) belongs to the family *Picornaviridae* and genus *Enterovirus* in the species *Enterovirus D*. It was first isolated in the United States in 1962 from respiratory specimens obtained from four pediatric patients with pneumonia and bronchiolitis (72). Since then, EV-D68 infections have been reported in small numbers and accounted for only 26 reports among enteroviruses detected in the United States during 36 years surveillance period from1970-2005 (73). Between 2008-2010, clusters of EV-D68 were reported in the Philippines, Japan, the Netherlands, and the United States (125). In recent years, EV-D68 infections have emerged in Italy (2010-2012), France (2009-2010), China (2009-2012), New Zealand (2010), Great Britain (2009-2010), Kenya (2008-2011), Japan (2005-2010), the Netherlands (2010), and Thailand (2009-2011) (74-82). The large outbreak in the United States and Canada in 2014 likely affected many more than the 1,100 laboratory-confirmed cases (83). Thereafter, reports of EV-D68 occurred in Europe including Norway (95), Denmark (85) and the Netherlands (86).

The spectrum of diseases caused by EV-D68 ranged from mild to severe respiratory tract infection, and fatalities have been reported (87). EV-D68 is unique among enteroviruses because it shares some biological features with rhinovirus. While most enteroviruses tolerate low pH and grow well at 37 C, EV-D68 is acid-labile and thrives at 33 C (88). Clinically, EV-D68 infection affects the upper and lower respiratory tract and the mainly children (83). EV-D68 infection has also been associated with acute severe myelitis (84) and cranial nerve dysfunction (89, 90). To date, there is neither an effective treatment nor a prophylactic vaccine specific for EV-D68.

We initially conducted surveillance of EV-D68 infection in Thailand between 2006-2011 and found that 25 nasopharyngeal or nasal swab samples were positive for EV-D68 (76). However, data on EV-D68 activity in Thailand after 2011 are lacking.

To determine whether EV-D68 was under diagnosed regionally, we retrospectively tested specimens previously obtained from pediatric patients with acute respiratory infections during 2012-2014 and evaluated data on epidemiological, clinical, and EV-D68 strains found in Thailand.

Materials and Methods

Clinical samples

During 2012-2014, nasopharyngeal and nasal swabs specimens from patients diagnosed with acute respiratory tract infection from three tertiary care hospitals in Bangkok and one tertiary care center in Chon Buri Province, the east of Thailand was collected in viral transport media and sent to the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University in order to screen for common respiratory viruses (influenza A and B, RSV, human metapneumovirus, parainfluenzavirus, coronavirus and adenovirus).

RNA extraction and cDNA synthesis

We randomly selected the samples during 2012-2014 from children less than five years of age to test for EV-D68. Reverse transcription-polymerase chain reaction (RT-PCR) was performed according to the following procedures. Total RNA was extracted using a commercially available Viral Nucleic Acid Extraction Kit (RBC Bioscience Co, Taipei, Taiwan). Five µL of total viral RNA was used to synthesize cDNA using random hexamer primers following the manufacturer's protocol. The cDNA synthesized was used as the template for PCR amplification.

Semi-nested PCR

For Rhinovirus/enteroviruses screening, semi-nested PCR using a primer set covering the 5'UTR/VP2 region was performed as previously published (76). Positive samples were selected for sequencing (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia).

Sequence data analysis

All sequences were assembled by Seqman program of DNASTAR Software (v5.0). The nucleotide sequences of the 5'UTR/VP2 were aligned with other sequence references in GenBank using the multiple sequence alignment CLUSTAL W via BioEdit software version 7.0.9. Sequences that matched EV-D68 were further analyzed and reported.

Phylogenetic analysis

The phylogenetic tree was constructed from VP4/VP2 regions using the Neighbor-joining (NJ) method implemented in the MEGA 6.0 with a bootstrap of 1,000 replicates. Also, we demonstrated the epidemiological pattern of EV-D68 since 2009 when it was first identified in Thailand and listed the clinical manifestations of children with confirmed EV-D68 infection during 2012-2014.

Results

Five out of 837 samples (0.6%) were positive for EV-D68. The review of the clinical charts showed that the patients in which the samples were derived were hospitalized for viral pneumonia (Table 14). One patient (TH-CB103) required mechanical ventilation and one patient (TH-TU45) was afebrile. Three patients had no prior episodes of wheezing while two had recurrent wheezing. None had neurological symptoms and no co-infections with other respiratory viruses were detected. All patients recovered from their illness.

Table 13 Clinical characteristics of the five hospitalized cases due to EV-D68 infection in Thailand during 2012-2014.

Patient	Strain	Age	Sex	Sample	Date of Collection	Symptoms	Diagnosis	Severity	Accession number (5'UTR/VP2)
1	TH-CB103	4	M	Tracheal suction	27-Sep-13	Fever, Cough, Runny Nose, Dyspnea, Wheezing, respiratory failure	Viral pneumonia	Required intubation and mechanical ventilation	KR080363
2	TH-CB108	4	M	NP suction	27-Sep-13	Fever, Cough, Runny Nose, Dyspnea, Wheezing	Viral pneumonia	Required hospitalization	KR080364
3	TH-TU44	3	M	NP suction	11-Dec-14	Fever, Cough, Vomiting, Wheezing, Chest Retractions	Viral pneumonia	Required hospitalization	KR080360
4	TH-TU45	2	F	NP suction	13-Nov-14	Cough, Runny nose, Wheezing (third episode)	Viral pneumonia	Required hospitalization	KR080361
5	TH-TU48	1	F	NP suction	13-Dec-14	Low grade fever, Cough, Runny Nose, Vomiting, Wheezing (fourth episode), Dyspnea, Chest Retraction	Viral pneumonia	Required hospitalization	KR080362

We also summarized the epidemiological data of EV-D68 in Thailand since 2009 (76). The prevalence of EV-D68 was 0.9% in 2009 (5/584), 1.6% in 2010 (10/611), 4.3% in 2011 (10/232), 0% in 2012 (0/238), 0.9% in 2013 (2/232) and 0.8% in 2014 (3/367). The highest prevalence was in 2011, and seasonal pattern of EV-D68 infection was similar to influenza virus as it generally peaked during the rainy seasons (Figure 30). Interestingly, infections also appeared in the drier months of February and March.

Phylogenetic analysis of VP4/VP2 region showed that all EV-D68 TH-strains clustered into group 1 along with most of the EV-D68 strains found in 2014 in the United States, Canada, Sweden and China (Figure 31). Furthermore, EV-D68 TH-strains found in 2013 and 2014 were very closely related to the EV-D68 identified in China in 2014 (GenBank accession number KP240936). Whole genome sequences of EV-D68 identified in Thailand in 2011 confirmed that it closely resembled strains in the 2014 US outbreak (data not shown) (126). In addition, alignment of the nucleotide sequence of 5'UTR among TH-strains showed 23 nucleotide deletions at positions 682 to 704 and 12 nucleotide deletions at positions 721 to 732 (data not shown).

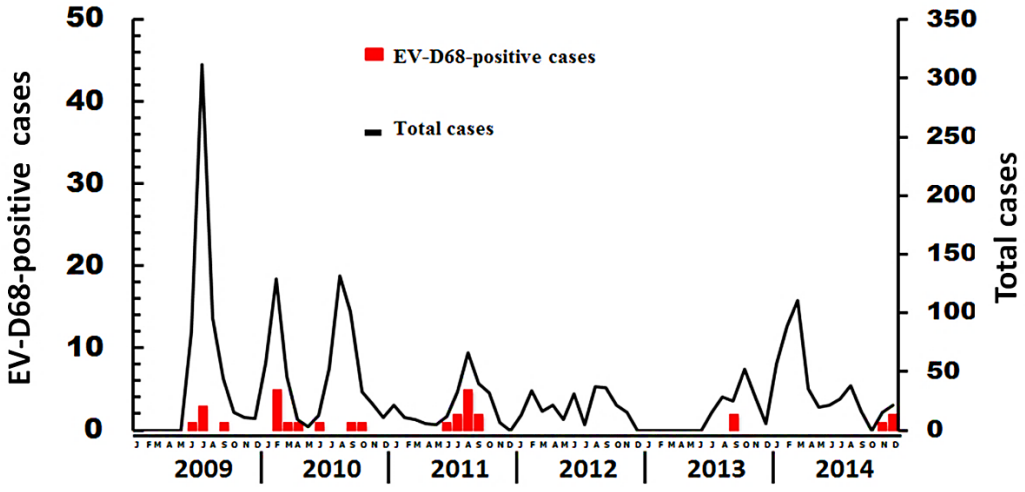

Figure 30 Seasonal distribution of EV-D68 in Thailand between 2009-2014 (76). Bar graphs showed the number of cases with confirmed EV-D68 infection.

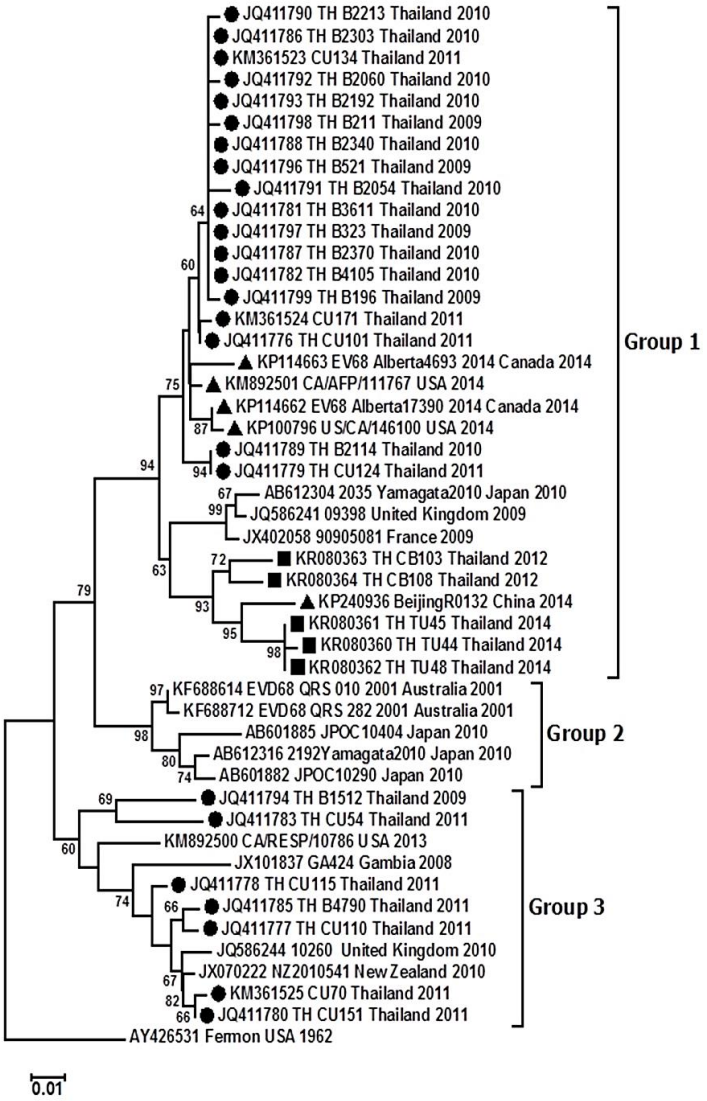

Figure 31 Phylogenetic relationship of EV-D68 detected among Thai pediatric patients during 2009–2014. The trees were constructed from nucleotide alignments of VP4/VP2 using neighbor-joining method and MEGA program (v 6.0). The genetic distances were calculated according to the Kimura-parameter model. Bootstrap support values of 1000 pseudo-replicates are indicated at the branch nodes. Squares denote samples from this study, while circles indicate strains previously reported from Thailand between 2009 and 2011. Triangles denote strains from the US 2014 outbreak.

CHAPTER IV
DISCUSSION AND CONCLUSION

We carried out a large-scale surveillance study of RSV to investigate the circulation patterns and genetic diversity in Thai children over four consecutive years from January 2012 to December 2015. Overall, RSV was identified in 8.4% of the respiratory samples using semi-nested RT-PCR. This finding is consistent with that of studies in several other countries, including Senegal (11.4%) (127), Colombia (8.9%) (128), and the USA (7.7%) (129). Other studies on RSV detection rates have reported low prevalence rates in North Korea (2.7%) (130) and Brazil (2.4%) (131). In contrast, a study conducted in West China revealed the highest RSV detection rate of 23.7% (132). The reason for the differences in the RSV detection rate may be based on differences in technical approaches, the number of patients tested, and even the duration of the study.

In 2012-2015, RSV-A (57.6%) occurred more frequently than RSV-B, which is consistent with the finding in multiple other geographic regions (36, 133, 134). However, several studies have reported that patterns involving alternating epidemics of RSV-A and RSV-B have been observed during annual RSV epidemics (135). The periodic shifts in the predominant RSV group have been driven by the dynamics of population immunity and subgroup-specific herd immunity. Research showed that RSV-A has been rapidly replaced by RSV-B in the course of two consecutive seasons (136). Moreover, others studies have also reported that the periodic subgroup shift has been observed every 2 (50) or 3 years (100). Using the findings of this study along with those of our previous study (34), we can see that there has been an alternate pattern involving the two groups of Thai RSV strains, with subgroup shifts occurring every two years (i.e., BAABBA). Only one sample (0.3%) was infected with both RSV groups. Co-infection with RSV-A and RSV-B has previously been described in multiple geographic regions (137-139).

Regarding the seasonality of RSV infection in Thailand, the data indicated clear seasonal peaks in the incidence of RSV during the rainy season (July to November). In contrast, low activity occurred during the hot and dry seasons (Figure 10 and Figure 29).

This finding seems to be consistent with findings from neighboring countries such as Vietnam (140) and Cambodia (28). Other studies in temperate climates identified seasonal patterns of RSV infections, with more infections in the winter (134, 136, 141). These observations suggest that the seasonal variability of RSV depends on the geographic location, season, and year.

The phylogenetic tree revealed that the RSV-A strains mainly belonged to two genotypes, NA1 and ON1 (Figure 11), and almost all of the RSV-B strains belonged to genotype BA9 (Figure 12), which is consistent with our previous findings in Thailand (34). The patterns of circulating RSV genotypes indicated that the previously predominant NA1 strains were replaced with ON1 strains. In 2012, the majority of RSV-A strains belonged to genotype NA1; afterward, a rapid change in the circulating RSV-A strains occurred, leading to genotype ON1 replacing the previously circulating NA1 genotype from 2014. It was reported that the ON1 virus initially appeared in Asia in 2011 and rapidly spread across the world (32, 36, 38, 106, 108, 142). Interestingly, the emergence and spread of new ON1 strains followed the pattern associated with the BA genotype, which replaced all other RSV-B strains. The molecular epidemiological surveillance of RSV has indicated that ON1 strains successfully replaced the NA1 strains (108, 142, 143). The causes of the epidemiological predominance of ON1 strains remain unknown, but there is no evidence that they cause more severe disease than other strains (141, 144). Additionally, the NA3 genotype that was first identified in Beijing (37) was also found in Thailand in 2012.

From 2012 to 2015, we found that RSV-B strains were clustered into three genotypes: BA9, BA10, and BA-C. The BA9 strains were first detected in Thailand in 2010. Infections involving this strain increased within a short period, and it is now the predominant genotype. In contrast, other RSV-B genotypes were detected only sporadically. Research (145) suggests that the duplication in the G gene HVR2 of genotypes BA and ON1 might affect transmission by changing an antigenically-related epitope. The antigenic changes caused by gene duplication may assist in immune evasion, thus providing a selective advantage to viruses with gene duplications. Alternatively, the function of the protein produced from the duplicated G gene (which is

involved in attachment to target cells) may render these viruses fitter than the RSV without the gene duplication (138). This may explain why these genotypes were able to spread globally in a short period as well as why they have almost completely replaced the other genotypes in group B (32, 37, 109, 143).

The high levels of genetic variability associated with the G protein HVR2 was confirmed by the alignment of the predicted amino acid sequences of strains from both RSV groups. N-glycosylation sites can alter the antigenic characteristics of the virus and enable specific epitopes to be masked (146). There is a potential N-glycosylated site located at amino acid position 237 of the G protein (outside the duplicated region) that is highly conserved in almost all ON1 strains. In contrast, only some NA1 strains had an N-glycosylation site at this residue, which was also reported by *Esposito et al.* (2015) (134). Studies on changes in the conformation of the G protein related to N-glycosylation have shown that N-glycosylation sites may help the virus to evade the host immune system (122). In contrast, research has indicated that N-glycosylation of proteins does not affect their conformation but does increase their stability (147). However, the observed variations in the potential N-glycosylation sites represent RSV genotype-specific characteristics. Furthermore, most of the positively selected sites in the G protein were in close proximity to and, in some cases, overlapped with, predicted glycosylation motifs (i.e., at amino acid positions 237 and 274, as shown in Table 2). This suggests that positive selection based on N-glycosylation sites may play an important role in driving viral diversity (148). For example, amino acid position 274 is located within an antigenic site, and it has been reported to be positively selected (8, 149).

This study revealed that RSV predominantly circulates in the rainy season (July to November) in Thailand. The strains that co-circulated in 2012-2015 were RSV-A (genotypes NA1, ON1, and NA3) and RSV-B (genotypes BA-C, BA9, and BA10). RSV is a continually evolving virus, the persistence of which in the community is driven by basic evolutionary factors: variations in amino acid sequences, potential N-glycosylation site patterns, and sites that are positively selected. Our findings will help the authorities to

understand RSV epidemic patterns, predict potential outbreaks, and develop preventive and treatment strategies.

Our study investigated the substantial increasing of RSV infection among Thai children with ILI between 2010 and 2015 (34, 124), using semi nested RT-PCR. In 2016-2017, RSV has been interested as causative agents in adult since it has been reported its circulation in hospitalized elderly individuals. However, antigen-based tests in general often have reduced sensitivity in adults due to low levels of viral shedding. Molecular methods offer high sensitivity in diagnosis is essential not only for more sensitivity and timely therapeutic intervention but also for the identification more accurate detected these viruses in populations, especially in older adults, and the avoidance of unnecessary antibiotic treatment. The current study, we developed and validated multiplex (one-step) real-time RT-PCR assay for rapid detection of RSV and hMPV.

The diagnostic tool for clinical tests need for rapid results with high enough sensitivity to determine the infectious agent coverage all infection stages to allow in the early management and treatment of patients. With the multiplex real-time RT-PCR developed in the study, the amount of RNA transcript standard can be detected at least 100 copies of RSV and 10 copies of hMPV per reaction. In conclusion, this assay provides the highly sensitivity detection and rapid diagnostic thus rendering it feasible and attractive for large scale surveillance of RSV and hMPV infections.

Further the studied RSV prevalence in 2012-2015, we conducted over a 2-year study period (2016-2017) among 8,842 patients hospitalized who presented with ILI in Thailand, the multiplex (one-step) real-time RT-PCR assays was used to detect RSV and hMPV pathogens in a fast and cost-effective way. Overall, RSV was detected in 11.4% and hMPV was detected in 3.6% of cases associated with influenza-like respiratory illness in the two-year study period. RSV and hMPV in individuals were highest among children aged <5 years with rates of infection at between 15.4–21.2% and 5.5–5.7%, respectively. A similar rate of these viral infections has previously been reported, with infections of 31.1% and 5.7% of RSV and hMPV infections among children (<5 years of age) with acute respiratory tract infection (150). On the other hand, RSV had a low burden of symptomatic

respiratory illness among older children and adults, and the opposite trend was observed for influenza virus infection (151). These findings are supported by previously reported studies on the epidemiology of respiratory virus infection (3, 4, 152).

In the present study, the seasonal distribution of hMPV infections overlapped with RSV infection, which was similar to data from previous studies (3, 150, 153). Although Thailand is located geographically in the northern hemisphere, the seasonality of respiratory infection is similar to that of several nearby tropical setting, which peaks with the rainy season and declines during the hot and dry months, such as Indonesia, Malaysia, the Philippines and the Southern hemisphere countries that include Australia and New Zealand (154, 155). In Australia, the seasonality of RSV infection in temperate Australia is similar to that in temperate zones in the Northern Hemisphere, with a peak incidence of RSV infection being in the winter. This tropical and temperate difference in the seasonality of RSV infection in Australia suggests that meteorological factors may be important in driving RSV seasonality in Australia (154). Also, a study from Bangladesh found an increased risk of respiratory infection following rainy days only in households with three or more people per room, suggesting a link between rainfall and population crowding or proximity (155). In Thailand, the school learning period overlaps with the rainy season, so it is possible that host behavior is associated with an increased risk of respiratory infection.

Our study in 2016-2017, RSV-B (66.4%) occurred more frequently than RSV-A, which is consistent with previous reports from other countries (127, 156), but was in contrast with our previous studies on the prevalence of RSV infection in Thailand (34) and other countries (133, 156). Several previous studies, including from our group, have reported the alternating antigenic pattern of RSV infection over time (127, 133, 156). It has been hypothesized that the periodic shifts in the predominant RSV subgroup are driven by the dynamics of population immunity and subgroup-specific herd immunity (157), which is analogous to periodic lineage shifts of the influenza B virus over time (158). Regarding the relationship between clinical severity of infection and RSV types and subtypes, some studies have observed that RSV group A infection was related to

increased illness severity (159, 160), whereas other studies observed that RSV group B infection resulted in more severe disease (161, 162). The present study confirmed that genotypes of ON1 and BA9 completely replaced the previous genotypes, such NA1, and other BA genotypes, which have been reported in other countries (33, 134), although they do not appear to cause more severe disease than other genotypes (141, 145).

Phylogenetic analysis of the hMPV F gene in the present study showed that both A and B types co-circulated in Thailand over the two-year study period. Similar to our findings, all three subtypes of hMPV (A2, B1, and B2) co-circulated each year in other studies, including South Korea, Italy, Australia, and Norway (163-166). Ludewick et al. (167) reported that a maximum three subtypes circulated in any 1-year, which is consistent with our study and past research in Thailand in 2011 (119). Several studies have indicated that hMPV genotype A might be more virulent than genotype B (168, 169). However, few studies have reported that no clinical differences were found between hMPV genotypes (170, 171). Until recently, the relationship between clinical severity of respiratory infection and genetic variability of hMPV has remained unclear.

Regarding multiple infections, the results of this study showed a small but significant level of multiple infections between age groups (p=0.0313), which were most frequently observed in the secondary school age group (3.0%) (Table 7). A reason for this finding could be that these children have increased contact with other children making co-infection in this age group more likely. RSV was co-detected mainly with influenza virus infection (Table 8), indicating that overlapping of the seasonal co-infection with RSV and influenza infections occurred (121). There have been reports showing no relationship between disease severity and multiple virus infections [48], while other studies have shown that viral co-infection was significantly associated with longer duration of symptoms, especially in RSV, and that this may increase the clinical severity of acute respiratory infection among children infected with RSV (172-174). In addition, several studies in hMPV-infected children found that such viral co-infections were associated with increased disease severity (116, 175). However, this was not confirmed in other studies (176, 177).

Data from the present study showed that the prevalence of infection with RSV and hMPV were the primary causes of respiratory illness during early childhood and were important during later childhood and adulthood, but the hMPV infection was less frequent than RSV infection, consistent with the previously reported findings. The second main finding of this study was that age distribution analysis showed that RSV and hMPV were the most common viral pathogens affecting the respiratory tract of young children, while influenza was more common in older patients (121). Thirdly, the seasonal occurrence of virus infections showed some overlap, and the seasonality of certain viral infections in the area may help in diagnosis. The data presented expand our understanding of the epidemiology of RSV and hMPV causing respiratory illness in Thai people and may be helpful to clinicians and researchers interested in the treatment and control of viral respiratory tract infections.

The 10 Thailand ON1 genomes generated in this study were used to assess local versus global ON1 variety. Phylogenetic clustering appeared to be more influenced by time of virus sample collection than by geographical location, suggesting that this novel virus spreads rapidly in a short period of time. Important for vaccine design, the RSV-A variants observed to be in equilibrium with global variants, similar to the present study. Future work will help clarify this phenomenon, which might be useful for the development of any RSV vaccine used locally.

Thailand ON1 (1.68×10^{-3} substitutions/site/year) was slightly lower in comparison with previously reported substitution rates (6.03×10^{-3} substitutions/site/year) (104). Our findings support the notion that evolutionary rates for viruses are highly context-specific and decrease when calculated from long-term sampling data (178). This may reflect that a short-term transmission is acting to eliminate deleterious mutations that are purified from the virus population in the longer term.

The observed sites of change in the G and F proteins were frequently in exposed regions of the proteins which have multiple potential N-glycosylation sites for immune evasion. The RSV envelope proteins are heavily glycosylated (179), and the potential O-linked glycosylation sites (serine or threonine) comprise up to 30% of the G protein amino

acid sequence (180). Changes toward or away from asparagine can be associated with a change of overall glycosylation of the protein to be driven by pressure from the host immune system (181). The RSV F protein contains N-glycosidic bonds only 10 to 20% of its mass as carbohydrate (179). Many polymorphisms observed in the F protein p27 domain which is not found in the mature protein is likely to serve as a spacer that is freed by cleavage during F maturation (182). The NS2 protein appear to modulate the host innate immune responses (183-185) and may influence movement of infected cells (186). The NS2 showed an elevated level of evolutionary rate (Figure 24), consistent with a pathogen-host protein interaction. Monitoring the local and global protein changes in NS2 in the N-term domain, NS2 can interact with host cellular proteins such as TRAF3 interactions (185).

We use algorithms to predict the codon sites are under positive selection sites. There were multiple positively selected sites predicted in the G coding region (L274P, L298P, and L310P). These predicted positively selected sites were located in the N-terminal cytoplasmic domain and the mucin-like regions. Haider et al. (187) noticed mutation at position 274, which is located within an antigenic site, selected positively having high entropy value suggesting a variation at this position. This mutation was also identified in earlier studies (188, 189).

The rapid increase in the size of the global ON1 was observed in 2010. In fact, the timing of rapid EPS growth detected in this study was consistent with the first appearance of ON1 in Ontario, Canada (8). ON1 genotype has spread widely in a short period of time, notably in 2011–2012, and was reported as the dominant RSV-A strain in Europe in 2012–2013 (108, 190), in Africa in 2012 (110) and in Asia in 2011 (102). Including, the previous studies from Korea and Philippine demonstrated similar findings (143, 188). In Thailand, the EPS increased gradually until 2013 and then peak in 2014, this phenomenon was consistent with the evidence which ON1 viruses have either partly or fully replaced the previously circulating NA1 in 2014. However, the EPS showed a decline in 2017, it is possible explanation for this slight down in EPS may be viral interference with other circulating group at that time, such as RSV-B group (Figure 29).

This study described RSV-A ON1 genome evolution and supported previous conclusions on patterns of local RSV-A variation relative to global RSV diversity (54). One motivation for studying full-genome was to increase the sensitivity for tracking RSV across short-term transmission chains. Agoti et al (54) asked if viruses identical in their G gene regions had differences elsewhere in their genomes. All RSV genomes revealed 7 sets of viruses with identical G region. This increased resolution will be important in future studies examining RSV household transmission patterns to identify who acquires infection from whom. The outcomes of these studies can help with infection control.

In recent years, the circulation of EV-D68 has risen worldwide as exemplified by the outbreak in United States. Our study examined the prevalence of EV-D68 in Thailand in children less than five years of age as they appeared to be most vulnerable to infection (83). We found a prevalence of EV-D68 of 0.6% between 2012-2014 and 0.8% overall, which is reportedly lower than countries in Europe in recent years such as 7.7% in Germany (93), 2% in the Netherlands (86), 17% in Norway (94), and 11% in Denmark (85). Prior to 2014, the prevalence of EV-D68 in Thailand ranged from 0-4.3%, averaging 1.5% and slightly above the 0.45% found in China (77) and 0.87% in Japan (80).

EV-D68 infection has consistently been associated with severe respiratory tract and occasionally severe bronchospasm (83, 191). Several Thai children with EV-D68 had wheezing and all presented severe bronchospasm at the time of their first hospital visit. These children, however, had no neurological symptoms unlike reports from Colorado and California (89, 90). EV-D68 infection in Thailand was mostly observed during the rainy seasons and the circulation pattern was similar to influenza virus (192). This was different from other studies in which the seasonality of EV-D68 appeared to be in summer and autumn (74).

The phylogenetic analysis of the VP4/VP2 region found that EV-D68 TH strains detected in 2013-2014 are clustered into the same group of a virus circulating in United States in 2014 (193). The viral genome revealed two deletions at the 5'UTR spacer region between the end of the internal ribosome entry site (IRES) and the beginning of the polyprotein open reading frame. These deletions have been found in EV-D68 isolated in

South Africa (194), China (195) and the United States (193). Whether these deletions affect the initiation of translation and virulence will require further investigation.

In conclusion, our study demonstrated the epidemiological data of EV-D68 detected in Thailand during 2009-2014. We also provided the clinical data of pediatric patients hospitalized due to severe EV-D68 infection, which supported the pathogenic burden of this virus.

Conclusion

This informative data provided the prevalence of RSV in Thailand over during 2012-2017 seasons. Including the evidence indicates that epidemiology may play a crucial role to help prevention of RSV infections associated with acute RTIs in Thai children. In addition, the multiplex (one-step) real-time RT-PCR assay was successfully implemented for diagnosis of RSV and hMPV infections in a routine diagnostic laboratory. It is easily to detect the sample, a high sensitivity and specificity approach for the diagnosis of RSV and hMPV infections that is simple and rapid. Moreover, a better understanding of the interplay between the virus epidemiology, the genetic variability, and evolutionary dynamics in the new RSV community epidemics may contribute to better cause rapid emergence design and develop the new therapeutic approaches for the treatment of the common respiratory viruses associated with acute RTIs.

The epidemiological data of EV-D68 detected in Thailand during 2009-2014 supported the pathogenic burden of this virus. Continued monitoring for severe respiratory tract infection especially in children may help control EV-D68 and others emerging enterovirus.

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APPENDIX

VITA

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