博士論文

Molecular epidemiology of acute respiratory infections by influenza virus, respiratory syncytial virus, human bocavirus and rhinovirus in Vietnamese children

(ベトナムの小児におけるインフルエンザウイルス、

RS ウイルス、ヒトボカウイルス、ライノウイルスによる

急性呼吸器感染症の分子疫学**)**

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MOLECULAR EPIDEMIOLOGY OF ACUTE RESPIRATORY INFECTIONS BY INFLUENZA VIRUS, RESPIRATORY SYNCYTIAL VIRUS, HUMAN BOCAVIRUS AND RHINOVIRUS IN VIETNAMESE CHILDREN

ベトナムの小児におけるインフルエンザウイルス、RS ウイルス、ヒトボカウイルス、ライノウイルスによる急性呼吸器感染症の分子疫学

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ABBREVIATIONS

5'UTR	5' untranslated region
%	Percentage
^{o}C	Degree Celcius
µl	Microlitre
aa	Amino acid
AdV	Adenovirus
ARI	Acute respiratory infection
bp	Base pair
CBC	Complete blood count
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CRP	C-reactive protein
CXR	Chest X-ray
D	Day
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucletide triphosphate
FLU	Influenza virus
HA	Hemagglutinin
HBoV	Human bocavirus
HCoV	Human coronavirus
Hib	*Hemophilus influenzae* type b
hMPV	Human metapneumovirus
Hos	Hospitalization
hr	Hour
HRV	Human rhinovirus
IQR	Interquartile range
LED	Light-emitting diode
LRTI	Lower respiratory tract infection
M	Month
MEGA	Molecular evolutionary genetic analysis
Min	Minute
NA	Neuraminidase
Neg	Negative
NP	Nasopharyngeal
NPS	Nasopharyngeal swab
Nt	Nucleotide
OR	Odds ratio
PCR	Polymerase chain reaction
pdm	Pandemic
Pos	Positive
PIV	Parainfluenzae virus

RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RSV	Respiratory syncytial virus
SARS	Severe acute respiratory syndrome
sec	Second
ss	Seasonal
URTI	Upper respiratory tract infection
vs.	Versus
WBC	White blood cell
WHO	World Health Organization

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ABSTRACT

Information about viral etiologies of acute respiratory infections (ARIs) is essential for prevention, diagnosis and treatment but limited in developing tropical countries. This study described the clinical and epidemiological characteristics of viral ARIs in hospitalized children in Vietnam. Clinical data and nasopharyngeal samples were collected from children with ARIs at Ho Chi Minh City, Vietnam from April 2010 to May 2011. Four multiplex PCR assays were performed to detect 13 respiratory viruses. Viruses were detected in 64.6% of 1,082 cases, with 12% of co-detection. The leading detected viruses were rhinovirus (HRV) (30%), respiratory syncytial virus (RSV) (23.8%), and human bocavirus (HBoV) (7.2%). HRV was detected all year round, while RSV epidemic occurred mainly in the rainy season. Influenza A virus (FLUA) was found in both seasons with H3N2 in the rainy season and pandemic H1N1 2009 in the dry season. Other viruses were predominant in the dry season. HRV was detected in all age groups. RSV predominantly affected children < 6 months, parainfluenza virus (PIV) 1 6 – 12 months, PIV3 and HBoV 12 – 24 months, and FLUA > 24 months. PIV1 detection was associated with croup ($p<0.005$) and RSV detection was associated with bronchiolitis ($p<0.005$). HBoV and HRV were associated with hypoxia ($p<0.05$) and RSV was associated with chest retraction ($p<0.05$). RSV subgroup A had severity score higher than subgroup B ($p<0.05$). Pandemic H1N1 2009 virus and co-detection of other viruses did not lead to severer symptoms. In conclusion, HRV, RSV, and HBoV were the most commonly detected viruses and may increase the severity of ARIs in children.

CHAPTER 1

GENERAL INTRODUCTION

Acute respiratory infections

Acute respiratory infections (ARIs) are the most common illness in children that frequently lead to consultation in general practice and hospitalization. Young children experience about 4-8 respiratory tract infection episodes per year, and in older children the incidence is slightly lower with 2-6 episodes per year (1, 2). In developing countries, ARIs are still one of the leading causes of childhood mortality. Nearly 1 million children under five years old die from pneumonia in 2013, accounting for 15% of all deaths in this age group (3). In addition, ARIs cause a substantial burden on patients and their families, including the direct medical costs and the indirect costs of missed workdays or absences from school and day care (4).

Although *Streptococcus pneumoniae* and *Haemophilus influenzae* type b are the major bacterial causes of pneumonia worldwide, viruses also have a significant role (5). Either involving in the upper or the lower respiratory tract, respiratory syncytial virus (RSV), parainfluenza viruses (PIV), influenza (FLU) A and B viruses, and human rhinovirus (HRV) were being the most commonly identified (6). Other pathogens such as human coronavirus (HCoV), adenovirus (AdV), and enterovirus infected the respiratory tract at a lower frequency (7). Since 2001, five newly discovered respiratory viruses have emerged including human metapneumovirus (hMPV) (8), human bocavirus (HBoV) (9), HCoV NL63 (10), HCoV HKU1 (11), and severe acute respiratory syndrome (SARS) CoV (12).

The clinical significance of most respiratory viruses was well described. However, they usually have overlapping clinical manifestations so that it is difficult to distinguish the etiologic agents without a laboratory diagnosis. Most respiratory viral infections are confined

to the upper respiratory tract with symptoms such as coryza and cough. One third of ARIs in children develops lower respiratory tract symptoms such as wheezing, tachypnea and respiratory distress (13).

Respiratory viruses

Historically, viruses associated with ARIs were RSV, PIV, FLU, AdV, HCoV and HRV. Of these, the latter two viruses were primarily associated with upper respiratory tract infections (URTIs). The former viruses were mainly responsible for lower respiratory infections (LRTIs) (6, 7).

RSV is one of the most important pathogen of childhood and is associated with significant morbidity and mortality. RSV is generally considered as the main cause of bronchiolitis in young children. Most children will develop antibodies against RSV by the age of 2 years. Despite much effort, there is no safe vaccine available. Risk factors for severe RSV infections are young age, prematurity, chronic lung disease, congenital heart disease, Down syndrome, and immunodeficiency. Early infection with RSV during infancy is associated with increased development of asthma in children. RSV has a clear seasonal pattern, with peak of infection occurring annually in the winter season of temperate countries. RSV is a negative-sense single-stranded RNA virus belongs to the *Paramyxoviridae* family. There are two major antigenic subgroups, A and B, with a single RSV serotype. Strains of both subgroups often co-circulate, but usually one subgroup predominates (14).

FLU is a negative-sense single-stranded segmented RNA virus belonging to the family *Orthomyxoviridae.* The high rate of HA and NA gene mutation results in antigenic drift and causes the recurrent annual influenza epidemics seen in cold season in temperate climates. Since 1977, two FLU A subtypes H3N2 and H1N1 have been co-circulating with FLU B (15). In addition, the segmented nature of the influenza genome allows the re-assortment of genetic materials between two different influenza A viruses co-infecting the

single host cell (antigenic shift), which gives FLU A the potential for the development of global pandemics (7). In 2009, the WHO declared the influenza pandemic caused by the novel H1N1 influenza A virus which originated from the triple-reassortant swine influenza (H1) virus circulating in Northern America pigs (16). Influenza is a febrile illness characterized by respiratory and systemic symptoms that result in a significant number of hospitalizations in all age groups. Among children, those younger than 2 years of age or those with underlying diseases are most susceptible to severe consequences of influenza infection. Influenza activity peaks in the winter season of temperate zone but throughout the year in tropical countries (17). In the hospital setting, the identification of influenza virus is important, as appropriate infection control measures are crucial in preventing the outbreaks.

HRV is a positive-sense single-stranded RNA virus belonging to the family *Picornaviridae*. HRVs have been divided into 3 species A, B, and C, and further subdivided into more than 150 types. HRV C, which was described in recent years, has been associated with more severe diseases than the earlier identified ones. HRVs were long considered as only "the common cold" agent and were ignored by the medical community until recently, when their clinical spectrum of diseases expanded. Recent reports showed the infection rates higher than expected of HRVs among children hospitalized with LRTI. HRVs have gained more interests as the main cause of asthma exacerbations, severe LRTIs and wheezing in young children. HRV infections occur year round, but most frequently in late spring and early fall (18, 19).

PIV is a negative-sense single-stranded RNA virus and belongs to the family *Paramyxoviridae*. Four serotypes of PIV were known to infect humans. PIV1 mainly causes acute croup in infants and young children but also causes ARIs in all age groups. PIV1 outbreaks usually occur in fall in temperate climates. PIV2 is generally associated with mild URTI, croup in children, and occasionally, LRTI in fall months but less often than PIV1 and

PIV3. PIV3 is a common cause of severe LRTI in young children with a peak incidence in the first year of life. Infections with PIV3 occur all year round with high prevalence in winter and spring months in temperate climates. PIV4 is the least common of this group and is generally associated with mild URTI (7).

AdV is a double-stranded DNA virus belonging to the family *Adenoviridae*. More than 50 serotypes of adenovirus have been described and categorized into six subgenera (A to F). Serotypes 1-5, 7, 14, 19, and 37 infect the respiratory tract most often, and cause diseases vary from mild URTIs to severe LRTIs. Following infection, AdV can remain in the host for a long time. AdV can be shed from the upper respiratory tract for a week in adults and up to 6 weeks in children (7).

Over the past decade, several new viruses have been discovered as respiratory viruses. HBoV, a single-stranded DNA virus within the family *Parvoviridae*, was discovered for the first time in 2005 by Allander and colleagues in Sweden but then occurs globally (9, 20). HBoV has been detected in children with LRTI, mainly acute wheezing, and has been associated with abnormal chest radiographic findings. However, the causative role of HBoV was not clearly established since HBoV was often detected concurrently with other viral pathogens in one- to two-thirds of cases (20). In several studies, HBoV is rarely detected in asymptomatic individuals (20, 21). This is the evidence that HBoV can cause LRTI, in particular, acute wheezing.

Human metapneumovirus is a negative-sense single-stranded RNA virus that was discovered in the Netherlands in 2001 and belongs to the family *Paramyxoviridae*, and the genus *Pneumovirus* (8). hMPV causes both URTI and LRTI with the signs and symptoms are very similar to those caused by RSV. hMPV is also one of the common viruses detected in ARI patients with the prevalence has ranged from 3 to 25% in children <5 years old hospitalized with ARIs (7).

HCoV is a positive-sense single-stranded RNA virus within the family *Coronaviridae*. Up to now, five HCoVs have been identified including 229E, OC43, SARS-CoV, NL63, and HKU1. HCoV-OC43 and HCoV-229E, which were identified in the mid-1960s as a cause of one-third common cold cases, have associated with both URTI and LRTI (7). HCoV-NL63 which was discovered in the Netherlands in 2004 associated with croup and other symptoms of URTI and LRTI (10). HCoV-HKU1 was discovered in 2004 in Hong Kong and mostly associated with LRTIs (11).

Acute respiratory infections in Vietnam and rationale for this thesis

Vietnam is a developing country with the population of 90 million people as of 2012. Children under 15 years old accounted for 23% of the population (22). Together with the development of socioeconomics, the health care system of the country has been much improved. The under-five mortality rate which was 39.1 in 2000 had fallen rapidly to 24.1 per 1,000 live births in 2013 (3). According to the national health insurance policy, children <6 years of age receive the medical care free of charge in the public sector. The expanded program on immunization is also considered a successful child health care program, where children can receive free vaccination against tuberculosis, pertussis, measles, *Hemophilus influenzae* type b, etc. Pneumococcal and influenza vaccines are also available but on the fee-for-service basis.

However, ARIs are still a major burden on the families and the health care system in Vietnam. Pneumonia is the third leading cause of death among children aged less than 5 years in this country, representing 11.4% of all deaths in this age group (3). One third of hospital admissions among children are due to pneumonia. Furthermore, the average cost for childhood pneumonia treatment in Vietnam was estimated approximately one-tenth of the per-capita gross national product (GNP), in which the cost of medicament represented more than a half of the total treatment costs (23). Antibiotic resistance, usually as a consequence of

the unnecessary or inappropriate use of antibiotics in treatment of ARIs, is also a particular problem in Vietnam, which has one of the highest rates of penicillin and macrolide resistant pneumococci of all Asian countries (24, 25). Therefore, data on the causative agents of ARIs are extremely important and necessary in this country. Since it provides insight into the etiology, specific treatment can be applied and preventive measures can be implemented. As a result, the relative burden of disease caused by different respiratory pathogens can be better defined.

However, viral etiology data for ARIs in Vietnam and Southeast Asia, where recently occurred severe outbreaks of SARS-coronavirus as well as avian and H1N1 pandemic 2009 (H1N1pdm09) influenza, are scarce. During 2004-2008, Do *et al.* conducted a study to identify the viral etiology of ARIs among hospitalized children in Ho Chi Minh City (26). From 2007 to 2008, Yoshida *et al.* carried out another study in central Vietnam indicating the diversity of viruses causing ARIs in Vietnamese children (27). However, these studies did not describe the clinical impact between different kinds of viruses. Moreover, the temporal characteristics may influence the yearly epidemic of respiratory viruses. Therefore, this study was conducted to identify the common viral pathogens and describe their epidemiological and clinical features as well as evaluate their effect on disease severity of ARIs.

Previous studies and the primary result of this study showed that HRV, RSV, HBoV, PIV, and FLU A were the leading detected viruses among hospitalized children with ARIs in Vietnam (26, 27). However, these studies did not describe the molecular characteristics of respiratory viruses. During 2001 – 2006, Li et al. analyzed the evolutionary patterns of H1N1 and H3N2 viruses in Vietnam and found that the genetic evolution of HA, NA, M genes occurred independently and new strains in Vietnam may be appear earlier than in temperate zones (28). Vuong et al. analyzed the influenza viruses in Vietnam from 2001 to 2009 and found varying degrees of genetic similarity with vaccine strains of the Northern or Southern

Hemisphere (29). Since then, especially after the pandemic period, there is no updated information about the genetic characteristics of influenza viruses in Vietnam.

Being considered as the main causes of ARIs but having no or limited information on molecular characteristics in Vietnam, the four viruses mentioned above, HRV, RSV, HBoV and FLU A, were targeted in my studies. In addition to clinical features, the molecular epidemiology of these viruses was further characterized. Due to limit of time, PIVs and other minor viruses detected in this study were not analyzed further.

Aims of the thesis

While the specific research objectives were described in each of the research chapters from 2 through 6, the comprehensive objectives of this thesis were to describe the epidemiological and clinical features of common viruses detected from hospitalized children with ARIs at Ho Chi Minh City, Vietnam during 2010-2011, as well as to analyze the molecular characteristics of major viruses identified from this study.

Outline of the thesis

This thesis was organized into five major research chapters (chapters 2 through 6) together with the introductory and summary chapters. In chapter 2, the clinical and epidemiological characteristics of ARIs in Vietnamese children were described. Then, the typing, genetic characteristics and clinical features of FLU A, RSV, HBoV and HRV were described in the following chapters from 3 to 6.

Patient recruitment

Study site and patients

The study was conducted from April 1, 2010 to May 31, 2011 at the Respiratory Ward, the Children's Hospital 2, Ho Chi Minh City, Vietnam. The Children's Hospital 2 is a 1,400-bed tertiary referral and university-affiliated hospital, receiving pediatric patients from most parts of the city as well as other provinces in the south and southern central, Vietnam.

Since Children's Hospital 2 is one of the three national pediatric hospitals and usually in the patient overload situation, the inpatient admission criteria are strictly followed (Table 1).

Table 1. Admission criteria for ARIs at Children's Hospital 2 Ho Chi Minh City.

Indications for hospital admission
• Persistent SpO_2 ≤92% in room air • Cyanosis • Markedly elevated respiratory rate (>70 breaths/min) • Dyspnea and intercostal retractions • Risk factores: Chronic lung disease, Congenital heart disease (especially if associated with cyanosis or pulmonary hypertension), Immunocompromised diseases, Prematurity, Severe malnutrition • Age less than 3 months • Inability to maintain oral hydration in patients younger than 6 months • Difficulty in feeding as a consequence of respiratory distress • Parent unable to care for child at home

This area has a tropical climate with two distinct seasons: rainy season (May-October) and dry season (November-April). The temperature does not change much during the year, and varied between 24-32 ^{o}C during the time conducting this study.

Under-15-year-old children admitted for an ARI condition with an onset of illness less than 7 days before hospitalization were eligible for enrollment in the study. An ARI case was defined as any child presenting with cough and/or difficult breathing (30). Patients who had underlying chronic diseases (e.g. cystic fibrosis, bronchopulmonary dysplasia, congenital heart disease, immunodeficiency) or who were discharged from the hospital within the previous 7 days, or who had coexisting acute systemic illnesses (e.g. sepsis), or proven or suspected non-infectious respiratory symptoms (e.g. asthma), were all excluded from the study. Patients with previous respiratory infection within 3 weeks from the current hospitalization were also excluded to avoid prolonged viral shedding. Clinical samples and data were obtained on 2 fixed days (Monday and Thursday) each week from all the admitted children who satisfied the above criteria. During 14-month period from April 2010 to May

2011, a total of 11,777 patients admitted to Respiratory Ward of the Children's Hospital 2 and 1,082 cases of ARI were enrolled in this study.

Figure 1. Map of Vietnam with location of Ho Chi Minh City and the south and southern center of Vietnam [source from (31)]**.**

Clinical data

The research pediatricians explained and collected the signed consent from the participant's parent or guardian. Then, he or she examined the patient and recorded the demographic and clinical data on a standardized questionnaire (Appendix 7-8).

Demographic parameters included gender, age at diagnosis, birth weight, gestational age, personal history (day-care attendance, atopy, breastfeeding, previous wheezing episodes, exposure to tobacco smoke), family history (parental atopy, simultaneous respiratory infection in a family member), number of persons in household. Breastfeeding was defined as

being given mother milk up to the age of six months (32). Atopy was defined as a history of medical diagnosis of asthma, allergic rhinitis, eczema, or anaphylaxis. Exposure to tobacco smoke was considered positive if either parent smoked any number of cigarettes in the house. Clinical observations at admission included vital signs, symptoms and signs contributory to the diagnosis of ARIs, the presence of wheezing and chest retraction, and the trans-cutaneous oxygen saturation as measured by a portable pulse oxymeter (Siemens MicroO2, Siemens Medical Systems Inc., USA) using a pediatric sensor (Nellcor, CA, USA).

The laboratory workup included complete blood counts (CBC), C-reactive protein (CRP), and chest X-ray (CXR). In cases where more than one CBC had been affected, only the first count was recorded. Blood cultures were done only on patients with complications of pneumonia (pleural effusion, empyema, lung abscess, etc) or clinical deterioration after initial antibiotic therapy. Sputum samples for culture and Gram stain were only obtained from patients who could produce sputum, which was not common in young children. Treatment course (type and duration of O_2 support, antibiotic therapy) and outcome (duration of hospitalization and complications) were noted.

The diagnosis was made on the basis of clinical findings and CXR. ARI patients with the presence of an infiltrate on CXR were categorized as pneumonia (33). Bronchiolitis was defined as ARI patient under 2 years old presenting with wheezing or crackles and hyperaeration, or atelectasis, or peribronchial thickening on CXR (34). Laryngotracheobronchitis (croup) was characterized by hoarseness, cough, and stridor (35). Upper respiratory tract infection (URTI) was defined as ARI with no abnormalities on CXR. The disease severity was assessed by using the previously published severity score as described in Table 2 (36-39). Patients were managed and discharged according to routine clinical practice.

Table 2. Clinical severity score.

Symptom	Point score
Fever (>38^{o}C)	1
Cough	
Mild	1
Moderate	2
Severe	3
Rhinorrhea	
Mild[a]	1
Moderate to severe[b]	2
Hoarseness	1
Duration of illness >4 days	1
Apnea	3
Wheezing	5
Cyanosis	5
Chest retraction	5
Tachypnea	5
Severity score (sum)[c]	0-31

[a]Suction 0–4 times/day or wipe every 2 hr or less.
[b]Suction 5 times/day or 1 wipe/hr.
[c]A higher severity score indicates severer disease.

Clinical samples

Nasopharyngeal (NP) flocked swabs (MicroRheologics, Brescia, Italia) were obtained by trained personnel on 2 fixed days each week from all enrolled children within 24 hr after admission. The specimens were immediately placed in tubes containing 2 ml sterile physiological saline fluid and stored at - 20^{o}C until further analysis at the laboratory.

Ethical approval

This study was a collaborated project between Department of Developmental Medical Sciences, the University of Tokyo and Nihon University School of Medicine and Children's Hospital 2. The experiments were mainly taken place at Nihon University School of Medicine laboratory. The study was approved by the Scientific and Ethical Committee of the Children's Hospital 2, Ho Chi Minh City (#25A/QD-ND2) and Nihon University School of Medicine (#25-15-0). Written informed consent was obtained from parents or legal guardians

of all children enrolled in the study. Children under 6 years of age received treatment free of charge and this was not limited to study participants. Withdrawal of participants from the study did not affect the health care services they received.

CHAPTER 2

CLINICAL AND EPIDEMIOLOGICAL CHARACTERISTICS OF ACUTE RESPIRATORY INFECTIONS IN VIETNAMESE CHILDREN

1. INTRODUCTION

Acute respiratory infections (ARIs) are leading cause of morbidity and mortality among infant and young children worldwide. About 1 million children under 5 years of age died from pneumonia in 2013, accounting for 15% of all deaths in this age group and most of these deaths occurred in developing countries (3). In addition, ARIs cause a substantial burden on patients and their families, including the direct medical costs and the indirect costs of missed workdays or absences from school and day care (4). In Southeast Asia, several severe outbreaks such as SARS-coronavirus, avian and H1N1 pandemic 2009 influenza have recently occurred that makes ARIs become a more serious public health problem. Viruses are the most common cause of ARIs and the major reason for hospitalization among young children (7). However, due to limited resources and facilities, the role of individual respiratory virus in these settings has not been well documented. Better understanding of clinical and epidemiological characteristics of ARIs is essential for predicting the epidemics, estimating the etiologic agents, and establishing an effective prevention and treatment measures. Therefore, this study was conducted to identify the common viral pathogens among Vietnamese children hospitalized for ARIs, to describe the epidemiological and clinical features of ARIs, and to investigate the associations between detected specific viruses and the severity of ARIs.

2. MATERIALS AND METHODS

Patients and samples

The clinical samples and data collection was described in detail in chapter 1.

Virus detection

Nucleic acids extraction

Viral genomes were extracted directly from the respiratory specimens by using the QIAamp Viral RNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -80^{o}C.

Reverse transcription (RT)

For RT, 5 µl of extracted RNA was added to the reagent mixture consisting of 3 µl of 5× First-Strand Buffer (Invitrogen, Carlsbad, CA), 0.8 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.8 µl of 0.1 M DTT (Invitrogen), 0.8 µl of (200 U/µl) SuperScript Reverse Transcriptase III (Invitrogen), 0.8 µl of (1 µg/µl) random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 µl of (33 U/µl) RNase Inhibitor (Toyobo, Osaka, Japan), and distilled water. The total volume of reaction mixture was 15 µl. The reaction was carried out at 50^{o}C for 1 hr, followed by heating at 95^{o}C for 5 min to inactivate the enzyme and then rapidly cooled on ice. cDNAs were stored at -20^{o}C until further analysis.

Screening for target viruses by multiplex (semi)-nested PCR

Four separate sets of multiplex (semi)-nested PCR were used to detect 13 respiratory viruses in each NP sample (Table 3). Set 1 was to detect FLU A and B, RSV and hMPV. Set 2 was to detect PIV type 1, 2, 3 and 4. Set 3 was to detect HRV, HCoV OC43 and 229E. Set 4 was to detect AdV and HBoV. To increase the specificity, only samples positive on the first PCR assay were performed on the second PCR for confirmation. Samples positive for both

PCR assays were determined as positive. PCR assays for RNA pathogens (set 1-3) were performed as previously described (27). Test for DNA viruses was developed in this study using primers from published studies (40, 41). Briefly, 3 µl of (c)DNA was added to 22 µl the first-round PCR reaction mixture consisting of 5 µl of 5× Taq DNA polymerase buffer (Promega, Madison, WI, USA), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM each of the first-round primers, 1.25 U of (5 U/µl) Taq DNA polymerase (Promega, Madison, WI, USA), and distilled water. The PCR was performed at 94°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 57 °C for 30 sec, 72 °C for 60 sec, and a final extension at 72 °C for 7 min, and then held at 10 °C. For the second PCR, 1 µl of the first-round product was used as the template in the PCR mixture as described above containing the second round primers and the same PCR cycling program was followed. Positive templates and buffer sample as negative control were added in each test run.

Electrophoresis

The PCR products were run on 1.5% agarose gel, and the bands were visualized by SYBR Safe (Invitrogen, Tokyo, Japan) staining under blue LED (light-emitting diode). The results were recorded by photographer. The length of the PCR fragments was indicative of the putative pathogen.

Statistical analysis

Descriptive analyses for discrete variables were performed by using frequency distributions or rates. For continuous variables, medians with interquartile range (IQR) were used to describe the demographic and patients' characteristics.

Demographic and clinical characteristics of patients with virus positive were compared with those negative for virus. Patients with viral co-detection were also compared with those with mono-detection. Values were given as percentages for categorical variables, and as median with interquartile range (IQR) for continuous variables. Bivariate associations

were assessed by using χ^2 test or Fisher's exact test for categorical variables, and by using the Mann-Whitney *U* test for continuous variables. A two-sided value of $p<0.05$ was considered statistically significant.

Multivariate logistic regression analyses were performed to determine the association of specific type of detected virus (HRV, RSV, HBoV, PIV1, PIV3, and FLU A) with severe symptoms and diagnostic classification. The outcomes were tachypnea, chest retraction, SpO_2 ≤92% (for severe symptoms) and URTIs, croup, bronchiolitis, pneumonia (for diagnostic classification). Initially, univariate models were performed introducing as independent variables age, gender, prematurity, malnutrition, co-detection with other viruses, and type of detected virus. Subsequently, these variables (known as potential confounders) were introduced in a multivariate model with a step-wise approach, to eliminate the possibility of mutual confounding and interaction. Results were presented as odds ratio (OR) with 95% confidence interval (95% CI). ORs and 95% CIs that did not contain 1 were considered significant. The Hosmer-Lemeshow test was used to determine the goodness-of-fit of the model.

All analyses were conducted using the Statistical Package for Social Sciences version 16.0 (SPSS, Inc., Chicago, IL, USA).

Table 3. Primers and PCR assays for multiplex PCR and (semi)-nested multiplex PCR.

Assay	Virus[a]	Direction	Sequence (5' – 3')[b]	Target gene[c]	Amplicon (bp)
Set 1					
1st	FLU A	sense	CCTTCTAACCGAGGTCGAAACG	Matrix protein	241
		antisense	GCATTTTGGACAAAGCGTCTACG		
	FLU B	sense	AGACACAATTGCCTACCTGCTTTC	Matrix protein	352
		antisense	CTGAGCTTTCATGGCCTTCTGC		
	RSV	sense	CATGACTCTCCTGATTGTGGGATG	Nucleocapsid	271
		antisense	CCTTCAACTCTACTGCCACCTC		
	hMPV	sense	TGAAGTCAATGCGACTGTAGCAC	Matrix protein	371
		antisense	ATGCCTTTGGGATTGTTCATGGTC		
2nd	FLU A	antisense	ACAGGATTGGTCTTGTCTTTAGCC	Matrix protein	151
	FLU B	antisense	AGTCTAGGTCAAATTCTTTCCCACC	Matrix protein	106
	RSV	sense	AGCAGCAGGGGATAGATCTGGTC	Nucleocapsid	201
	Hmpv	antisense	TCACTGCTTATTGCAGCTTCAACAG	Matrix protein	287
Set 2					
1st	PIV1	sense	ATGATTTCTGGAGATGTCCCGTAGG	HA-NA	300
		antisense	TTCCTGTTGTCGTTGATGTCATAGG		
	PIV2	sense	CAATCAATCCTGCAGTCGGAAGC	HA-NA	386
		antisense	AAAGCGATGCAGACCACCAAG		
	PIV3	sense	GACACAACAAATGTCGGATCTTAGG	HA-NA	230
		antisense	ATACAGCCATCAACAGTCGTTGG		
	PIV4	sense	CTGAACGGTTGCATTCAGGT	Phosphoprotein	451
		antisense	TTGCATCAAGAATGAGTCCT		
2nd	PIV1	sense	CCACCACAATTTCAGGATGTGTTAG	HA-NA	210
	PIV2	antisense	TAACATAGAGCCTACCTTCTGCACC	HA-NA	329
	PIV3	antisense	GAAGACCAGACGTGCATCTCCA	HA-NA	148
	PIV4	antisense	GTCTGATCCCATAAGCAGC	Phosphoprotein	390

Table 3. Primers and PCR assays for multiplex PCR and (semi)-nested multiplex PCR (continued).

Assay	Virus[a]	Direction	Sequence (5' – 3')[b]	Target gene[c]	Amplicon (bp)
Set 3					
1st	HRV	sense	CCCACAGTAGACCTGGCAGATG	5'-noncoding region	254
		antisense	ACGGACACCCAAAGTAGTTGGT		
	HCoV 229E	sense	GGTTTTGACAAGCCTCAGGAAAAAGA	Membrane glycoprotein	573
		antisense	GTGACTATCAAACAGCATAGCAGCTGT		
	HCoV OC43	sense	GCTAGTCTTGTTCTGGCAAAACTTGGC	Membrane glycoprotein	335
		antisense	TGAATTGCGCTATAACGGCGC		
2nd	HRV	antisense	CAGGGTTAAGGTTAGCCGCATTC	5'-noncoding region	175
	HCoV 229E	antisense	CCATTGGCCACAACACCTGC	Membrane glycoprotein	230
	HCoV OC43	antisense	CTCAGCAAGTAACTAAGCATACTGCC	Membrane glycoprotein	170
Set 4					
1st	AdV	sense	TACATGCACATCKCSGGVCAGGA	Hexon gene	983
		antisense	CCRGCCARHACHCCCATRTTDCCHGT		
	HBoV	sense	CGCCGTGGCTCCTGCTCT	VP1/2	608
		antisense	TGTTCGCCATCACAAAAGATGTG		
2nd	AdV	sense	TGGCYWSCACNTWCTTTGACATYMG	Hexon gene	426
		antisense	GCRWAWGAHCCRTARCAKGGYTDCAT		
	HBoV	sense	GGCTCCTGCTCTAGGAAATAAAGAG	VP1/2	575
		antisense	CCTGCTGTTAGGTCGTTGTTGTATGT		

[a]FLU, influenza virus; RSV, respiratory syncytial virus; hMPV, human metapneumovirus; PIV, parainfluenzavirus; HRV, human rhinovirus; HCoV, human coronavirus; AdV, adenovirus; HBoV, human bocavirus.
[b]Degenerate primer abbreviations are as follows: M, A/C; R, A/G; W, A/T; S, C/G; Y, C/T; K, G/T; V, A/C/G; H, A/C/T; D, A/G/T; N, A/C/G/T.
[c]HA-NA, Hemagglutinin-Neuraminidase.

3. RESULTS

Characteristics of study population

The demographic characteristics and medical history data of the study population are shown in Table 4. Overall, the median age was 9 months (ranged from 0 to 161 months), 86% of patients were under 2 years old. Male were more commonly affected than female and the male to female ratio was 1.8:1. A half of patients (50.6%) were living in urban areas from the south of Vietnam. Fourteen of 446 (3%) patients over 1 year old had been received the seasonal influenza vaccine.

Table 4. Demographic characteristics and medical history data of study population and virus positive and negative groups.

Characteristics (%)	Total N=1,082	Virus Pos. N=699	Virus Neg. N=383	*p-value*[c]	OR (95% CI)
Male	64.7	66.7	61.1	0.073	1.27(0.98-1.65)
Age (m)[a]	9 (4-18)	9 (4-17)	10 (4-19)	0.250[d]	
Age group				**0.046**	
<6m	33.0	32.3	34.2		
6-<12m	23.9	26.6	19.1	**0.030**[e]	**1.48 (1.05-2.09)**
12-<24m	27.4	26.0	29.8	0.685	0.92(0.67-1.27)
≥24m	15.7	15.0	17.0	0.773	0.93(0.64-1.36)
Live in urban area	50.6	51.6	48.8	0.409	0.89(0.69-1.14)
Prematurity	9.2	10.0	7.8	0.273	1.31(0.83-2.04)
Breastfeeding	89.9	89.0	91.6	0.171	0.73(0.47-1.13)
Daycare	22.2	21.5	23.5	0.445	0.88(0.66-1.19)
Malnutrition	9.5	8.4	11.5	0.105	0.71(0.47-1.07)
Family size[a]	4 (3-5)	4 (3-5)	4 (3-5)	0.407[d]	
Siblings	47.8	46.2	50.7	0.181	0.83(0.65-1.07)
Passive smoking	58.7	59.8	56.7	0.333	1.13(0.88-1.46)
ARIs exposure	32.0	32.6	31.0	0.633	1.07(0.82-1.41)
Family atopy	42.1	41.3	43.3	0.502	0.92(0.71-1.18)
Days before hos.(d)[a]	3 (2-5)	3 (2-4)	3 (2-6)	**0.003**[d]	
Antibiotics before hos.	47.5	47.5	47.5	1.000	0.99(0.78-1.28)

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; ARI, acute respiratory infection;
Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[c]Comparison between virus positive and negative groups used chi-squared test except [d]Mann-Whitney-*U* test was applied for continuous variables.
[e]Age group <6m was used as a reference and compared to other age groups.

At admission, most patients had fever, cough, and runny noses (Table 5). On examination, the percentage of patients who had fast breathing, wheezing and chest retraction

were 44.5%, 58.9% and 55.3%, respectively. All patients were performed CBC and CXR. Among 110 patients who were tested for CRP, 16 (14.5%) had the level of CRP ≥ 40 mg/L. Diagnoses at admission were based on clinical and laboratory information and CXR interpretation. Nearly four-fifths of cases were diagnosed as lower respiratory tract infections (LRTIs). Laboratory investigation for bacterial pathogens was not systematically performed. However, 76% of patients received antibiotic treatment during hospitalization. No patients either required mechanical ventilation or had complications. All patients were fully recovered and discharged.

Table 5. Clinical features, diagnosis and treatment characteristics of study population and virus positive and negative groups.

Characteristics (%)	Total N=1,082	Virus Pos. N=699	Virus Neg. N=383	*p-value*[c]	OR (95% CI)
Fever	67.2	66.7	68.1	0.636	0.93(0.71-1.22)
High fever	6.7	6.9	6.5	0.899	1.05(0.64-1.74)
Cough	90.9	92.3	88.3	**0.036**	**1.59 (1.05-2.41)**
Runny noses	73.4	78.3	64.5	**<0.001**	**1.98 (1.50-2.61)**
$SpO_2 \leq 92\%$	8.5	8.4	8.6	0.910	0.97(0.62-1.52)
Tachypnea	44.5	42.5	48.3	0.073	0.79(0.61-1.01)
Chest retraction	55.3	58.9	48.3	**0.001**	**1.54 (1.20-1.97)**
Wheezing	58.9	60.7	55.6	0.121	1.23(0.95-1.58)
Rales	66.0	68.0	62.4	0.070	1.27(0.98-1.65)
Clinical Severity Score[a]	12 (8-13)	12 (8-13)	11 (7-13)	**0.029**[d]	
WBC ($\times 10^3/mm^3$)[a]	11.6 (9.392-15)	11.4 (9.39-14.6)	12.2 (9.40-15.5)	**0.037**[d]	
Elevated Neutrophil[b]	36.5	34.1	40.8	**0.034**	**1.33 (1.03-1.72)**
Abnormal chest X-ray	77.6	81.3	70.7	**<0.001**	**1.80 (1.35-2.42)**
Diagnosis				**0.007**	
URTIs	21.6	21.6	21.7	1.000	0.99(0.73-1.34)
Croup	6.1	4.7	8.6	**0.016**	**0.53 (0.32-0.87)**
Bronchiolitis	33.2	36.1	27.9	**0.007**	**1.45 (1.11-1.91)**
Pneumonia	39.1	37.6	41.8	0.193	0.84(0.65-1.08)
Oxygen	8.0	8.2	7.8	0.907	1.05(0.66-1.66)
Antibiotics	76.0	76.0	76.0	1.000	0.99(0.75-1.33)
Steroids	15.7	16.3	14.6	0.486	1.13(0.80-1.61)
Bronchodilator	51.8	56.9	42.6	**<0.001**	**1.79 (1.39-2.30)**
Hos. duration (d)[a]	6 (4-8)	6 (4-8)	5 (3-8)	0.203[d]	

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; URTI, upper respiratory infection; WBC, white blood cell.
Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Neutrophil >5,800/mm^3 according to Nelson Textbook of Pediatrics (42).
[c]Comparison between virus positive and negative groups used chi-squared test except [d]Mann-Whitney-*U* test was applied for continuous variables.

Viral detection and seasonal patterns

One or more respiratory viruses were detected in 64.6% (699/1,082) of patients (Table 6). Viruses could be found in all months and the monthly detection rate ranged from 47.4% to 90.2% during the studied period (Fig. 2). The ARI activity reached peak from August to October and the detection rate was also high during this time. HRV which used to be thought as a "mild common cold" agent was the most common identified virus in hospitalized patients with the overall detection rate of 30%. RSV was the second most in frequency, followed by HBoV, PIV3, PIV1, and FLU A. Other viruses such as hMPV, AdV, FLU B and HCoV were detected in a small proportion of patients. Neither PIV type 2 nor type 4 could be found in this study. Mixed detection of more than one respiratory virus was found in 129 patients (12%), in which, co-detection between HRV and RSV was the most frequent (48 cases) followed by the combinations of HRV and HBoV (16 cases). It is noted that 66.7% (52/78) of HBoV and 4 out of 7 (42.9%) HCoV OC43 found in this study were mixed detection with other viruses. Six patients had triple detection.

Regarding seasonal patterns, HRV was detected all year round with no distinct seasonality (Fig. 3). RSV epidemic occurred during the rainy season from May to October with the peak in October 2010. In contrast, PIV1, PIV3 and HBoV were predominant during the dry season. FLU A could be found in both rainy and dry seasons with the peaks usually lagged after the changing of seasons. The numbers of remaining viruses were insufficient to detect the seasonal patterns. However, 11 out of 13 hMPVs in this study were identified in the rainy season, 6 cases occurred in October 2010, and had a similar seasonal pattern with RSV.

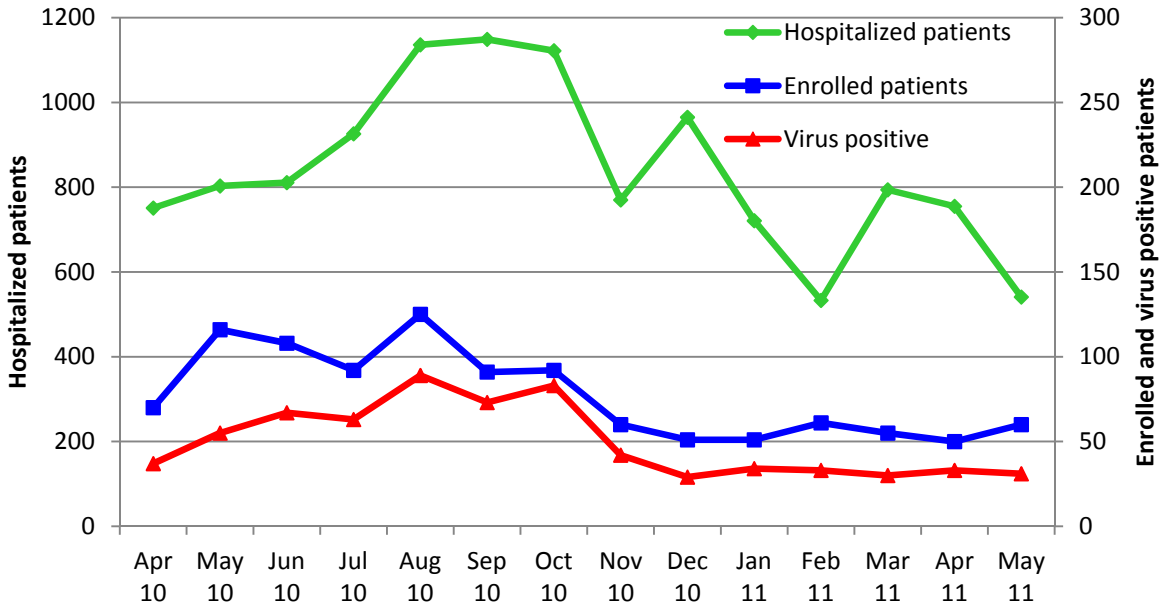


Figure 2. Number of hospitalized, enrolled and virus positive patients.

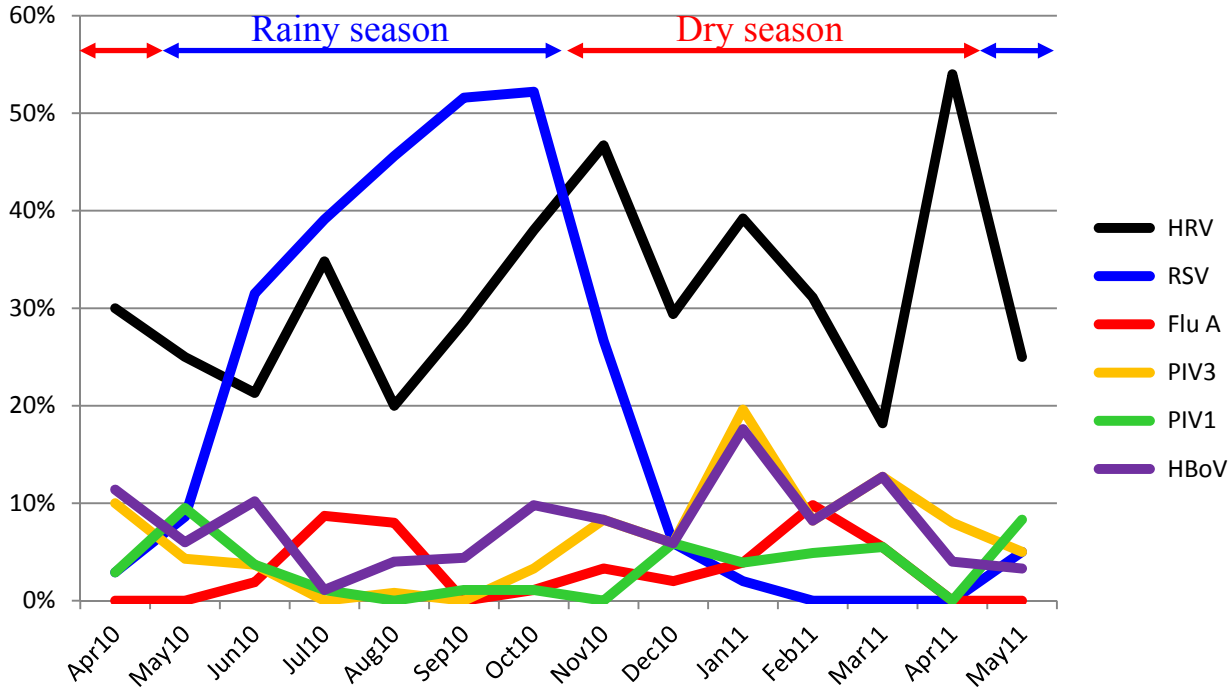


Figure 3. Monthly distribution of leading respiratory viruses among hospitalized children with ARIs from April 2010 to May 2011.

Abbreviation: HRV, human rhinovirus; RSV, respiratory syncytial virus; FLU, influenza virus; PIV, parainfluenza virus; HBoV, human bocavirus;

Table 6. Virus detected from 1,082 ARI patients.

Virus	HRV	RSV	HBoV	PIV3	PIV1	FLU A	hMPV	AdV	FLU B	HCoV 229E	HCoV OC43
HRV	234										
RSV	48	184									
HBoV	16	15	26								
PIV3	11	1	6	38							
PIV1	3	1	2		29						
FLU A	3	1	4			26					
hMPV	1		2		1		9				
AdV	2							9			
FLU B	1		2						4		
HCoV 229E										7	
HCoV OC43		3									4
HRV+RSV+HBoV	3										
HRV+RSV+AdV	1										
HRV+FLU A+HBoV	1										
HRV+PIV3+HBoV	1										
Total[a]	**325 (30%)**	**257 (23.8%)**	**78 (7.2%)**	**57 (5.3%)**	**36 (3.3%)**	**35 (3.2%)**	**13 (1.2%)**	**12 (1.1%)**	**7 (0.6%)**	**7 (0.6%)**	**7 (0.6%)**

Abbreviation: HRV, human rhinovirus; RSV, respiratory syncytial virus; PIV, parainfluenza virus; FLU, influenza virus; HBoV, human bocavirus; hMPV, human metapneumovirus; AdV, Adenovirus; HCoV, human coronavirus.
[a]Percentages are calculated based on the positive number of each virus.

Description and comparison of clinical features of the detected viruses

To figure out the difference between virus positive and negative groups, demographic and clinical data were compared (Table 4 and 5). With regard to age, infants aged from 6 to 12 months had a higher risk of viral detection compared with infants <6 months of age (OR=1.48, 95% CI: 1.05-2.09). Patients with virus positive were likely to admit to the hospital earlier in the course of their disease than those with virus negative (p=0.003). They also had the rates of cough (92.3 vs. 88.3%, p=0.036), runny noses (78.3 vs. 64.5%, p<0.001), chest retraction (58.9 vs. 48.3%, p=0.001), and most important, the clinical severity score (12 vs. 11, p=0.029) higher significantly than virus negative group. However, the latter group was likely to have higher number of WBC (12,200 vs. 11,400, p=0.037) and elevated neutrophil (40.8 vs. 34.1%, p=0.034). The radiograph was more abnormal in virus positive group than the virus negative group (81.3 vs. 70.7%, p<0.001). Regarding the diagnosis, virus positive patients were more likely to have bronchiolitis (36.1 vs. 27.9%, p=0.007) but less likely to have croup (4.7 vs. 8.6%, p=0.016). As a result, they received bronchiodilators more often than the latter group (56.9 vs. 42.6%, p<0.001). There were no significant differences in other characteristics as shown in Table 5.

To address the question of whether children with multiple virus detection have different characteristics to those with single virus detection, attempts were done to compare the two groups (Table 7 and 8). However, the differences were not statistically significant except that children with mono-detection have siblings with a significant higher percentage than those with co-detection (48.2 vs. 37.2%, p=0.025).

Table 7. Demographic characteristics and medical history associated with viral mono- and co-detection.

Characteristics (%)	Mono-detection N=570	Co-detection N=129	*p-value*[c]	OR (95% CI)
Male	67.4	63.6	0.410	0.85(0.56-1.26)
Age (m)[a]	9 (4-18)	9 (4-15)	0.429[d]	
Live in urban area	51.6	51.9	1.000	0.98(0.67-1.44)
Prematurity	9.5	12.4	0.330	1.35(0.74-2.45)
Breastfeeding	88.6	90.7	0.640	1.25(0.65-2.39)
Daycare	21.2	22.5	0.812	1.07(0.68-1.70)
Malnutrition	8.8	7.0	0.601	0.78(0.37-1.63)
Family size[a]	4 (3-5)	4 (3-6)	0.344[d]	
Siblings	48.2	37.2	**0.025**	**0.64 (0.43-0.94)**
Passive smoking	59.6	60.5	0.921	1.03(0.70-1.52)
ARIs exposure	31.5	37.2	0.214	1.28(0.86-1.91)
Family atopy	40.9	43.4	0.621	1.11(0.75-1.63)
Days before hos.[a]	3 (2-4)	2 (2-4)	0.303[d]	
Antibiotics before hos.	46.7	51.2	0.380	1.19(0.81-1.75)

Abbreviation: OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; ARI, acute respiratory infection;
Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Table 8. Clinical features, diagnosis and treatment characteristics associated with viral mono- and co-detection.

Characteristics (%)	Mono-detection N=570	Co-detection N=129	*p-value*[c]	OR (95% CI)
Fever	65.4	72.1	0.179	1.36(0.89-2.08)
High Fever	6.7	7.8	0.699	1.17(0.57-2.42)
Cough	92.1	93.0	0.856	1.14(0.54-2.40)
Runny noses	78.6	76.7	0.638	0.89(0.57-1.41)
SpO_2 ≤92%	7.5	12.4	0.080	1.73(0.94-3.19)
Tachypnea	42.8	41.1	0.768	0.93(0.63-1.37)
Chest retraction	58.4	61.2	0.620	1.12(0.76-1.66)
Wheezing	61.4	57.4	0.425	0.84(0.57-1.24)
Rales	67.0	72.1	0.297	1.27(0.83-1.94)
Clinical Severity Score[a]	12 (8-13)	12 (8-13)	0.801[d]	
WBC ($\times 10^3/mm^3$)[a]	11.4 (9.47-14.4)	11.5 (9.20-15.0)	0.898[d]	
Elevated Neutrophil[b]	33.7	36.2	0.605	0.89(0.59-1.33)
Abnormal chest X-ray	81.2	82.2	0.900	1.07(0.65-1.75)
Diagnosis			0.552	
URTIs	22.3	18.6	0.408	0.79(0.49-1.29)
Croup	5.1	3.1	0.490	0.59(0.20-1.72)
Bronchiolitis	35.8	37.2	0.762	1.06(0.71-1.58)
Pneumonia	36.8	41.1	0.367	1.19(0.81-1.76)
Oxygen	7.2	12.4	0.073	1.82(0.99-3.37)
Antibiotics	74.6	82.2	0.069	1.57(0.96-2.56)
Steroids	16.5	15.5	0.895	0.92(0.54-1.57)
Bronchodilator	56.8	57.4	0.922	1.02(0.69-1.50)
Hos. duration[a]	6 (4-8)	6 (4-8)	0.195[d]	

Abbreviation: OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; URTI, upper respiratory infection; WBC, white blood cell.
Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Neutrophil >5,800/mm^3 according to Nelson Textbook of Pediatrics (42).
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

The association of each type of respiratory virus with specific demographic and clinical features was also found. In terms of age distribution, most viral agents could be detected in all age groups. The age distributions of patients were specific for each type of viruses (Fig. 4). In particular, HRV was detected in all age groups. RSV was usually associated with diseases in children younger than 6 months while FLU A was mostly found in children over 2 years old. PIV3 developed ARIs frequently in children from 6 to 11 months, in contrast to PIV1 and HBoV being predominant in the second year of life.

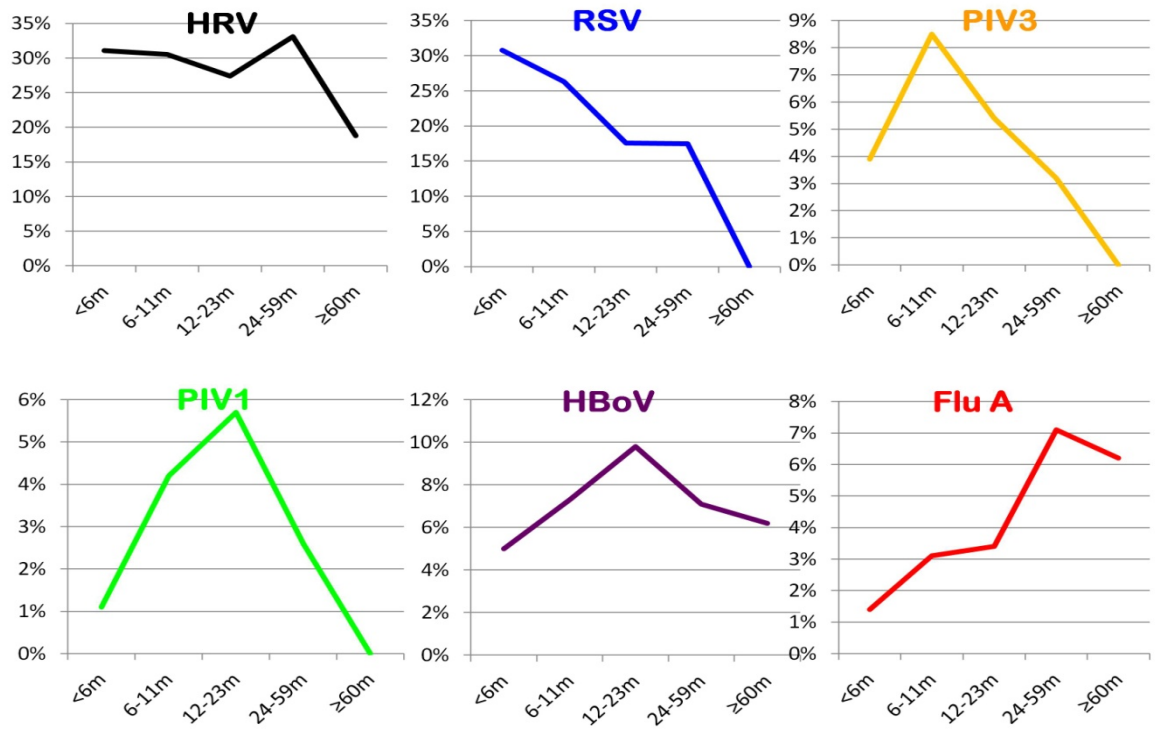


Figure 4. Age distribution of leading respiratory viruses.

Abbreviation: HRV, human rhinovirus; RSV, respiratory syncytial virus; FLU A, influenza A virus; PIV, parainfluenza virus; HBoV, human bocavirus;

The association between HRV, RSV, HBoV, PIV1, PIV3 and FLU A detection with demographic and clinical features is shown in Tables 9-20. The clinical features of FLU A, RSV, HBoV and HRV are further characterized in chapters 3-6. In this chapter, the symptoms and diagnostic classification associated with severe ARIs were focused.

In brief, patients with HRV positive were more likely to have hypoxia (12.3 vs. 6.9%, p=0.003), chest retraction (60.9 vs. 52.7%, p=0.013) and wheezing (63.7 vs. 56.8%, p=0.035) but less likely to have fever (56.0 vs. 72.0%, p<0.001) and to be diagnosed as croup (2.8 vs. 7.5%, p=0.002) than those negative for HRV (Table 9 and 10).

Table 9. Demographic characteristics and medical history associated with HRV detection.

Characteristics (%)	HRV(+) N=325	HRV(-) N=757	*p-value*[c]	OR (95% CI)
Male	69.8	62.5	**0.020**	**1.39(1.05-1.84)**
Age (m)[a]	9(4-18)	10(4-18)	0.392[d]	
Live in urban area	50.5	50.7	0.936	1.01(0.77-1.31)
Prematurity	11.1	8.5	0.172	1.34(0.87-2.07)
Breastfeeding	87.4	91.0	0.069	0.68(0.45-1.03)
Daycare	22.2	22.2	1.000	0.99(0.73-1.36)
Malnutrition	8.9	9.8	0.661	0.90(0.57-1.41)
Family size[a]	4(3-5)	4(3-6)	0.542[d]	
Siblings	46.5	48.3	0.569	0.92(0.71-1.20)
Passive smoking	56.3	59.7	0.298	0.87(0.66-1.13)
ARIs exposure	31.1	32.4	0.668	0.94(0.71-1.24)
Family atopy	40.0	42.9	0.370	0.88(0.68-1.15)
Days before hos.(d)[a]	3(1-4.5)	3(2-5)	0.209[d]	
Antibiotics before hos.	44.0	49.0	0.130	0.81(0.63-1.06)

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; ARI, acute respiratory infection; HRV, human rhinovirus. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Table 10. Clinical features, diagnosis and treatment characteristics associated with HRV detection.

Characteristics (%)	HRV(+) N=325	HRV(-) N=757	*p-value*[c]	OR (95% CI)
Fever	56.0	72.0	**<0.001**	**0.50(0.38-0.65)**
High fever	4.6	7.7	0.067	0.58(0.32-1.05)
Cough	91.1	90.8	0.865	1.04(0.66-1.63)
Runny noses	74.2	73.1	0.707	1.05(0.78-1.42)
$SpO_2 \leq 92\%$	12.3	6.9	**0.003**	**1.90(1.23-2.94)**
Tachypnea	40.9	46.1	0.116	0.81(0.62-1.05)
Chest retraction	60.9	52.7	**0.013**	**1.40(1.07-1.82)**
Wheezing	63.7	56.8	**0.035**	**1.33(1.02-1.74)**
Rales	65.5	66.2	0.838	0.97(0.73-1.27)
Clinical Severity Score[a]	12(8-13)	11(8-13)	0.341[d]	
WBC ($\times 10^3/mm^3$)[a]	11.7(9.8-15.1)	11.6(9.2-15.0)	0.236[d]	
Elevated Neutrophil[b]	37.7	36.0	0.595	0.92(0.70-1.21)
Abnormal chest X-ray	82.4	75.6	**0.014**	**1.52(1.09-2.11)**
Detection with other viruses	28.3	49.4	**<0.001**	**0.40(0.31-0.54)**
Diagnosis			**0.004**	
URTIs	24.9	20.2	0.084	1.31(0.96-1.78)
Croup	2.8	7.5	**0.002**	0.35(0.17-0.72)
Bronchiolitis	36.3	31.8	0.152	1.22(0.92-1.60)
Pneumonia	36.0	40.4	0.172	0.82(0.63-1.08)
Oxygen	12.0	6.3	**0.002**	**2.01(1.29-3.14)**
Antibiotics	68.9	79.0	**<0.001**	**0.59(0.44-0.79)**
Steroids	17.8	14.8	0.206	1.25(0.88-1.77)
Bronchodilator	56.9	49.7	**0.029**	**1.34(1.03-1.74)**
Hos. duration (d)[a]	5(3-8)	6(4-8)	0.784[d]	

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; URTI, upper respiratory infection; WBC, white blood cell; HRV, human rhinovirus. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Neutrophil >5,800/mm^3 according to Nelson Textbook of Pediatrics (42).
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Patients with RSV positive were younger (7 vs. 11 months, p<0.001) and were more likely to have chest retraction (68.5 vs. 51.0%, p<0.001) and bronchiolitis (47.1 vs. 28.8%, p<0.001) compared with RSV negative patients (Table 11 and 12).

Table 11. Demographic characteristics and medical history associated with RSV detection.

Characteristics (%)	RSV(+) N=257	RSV(-) N=825	*p-value*[c]	OR (95% CI)
Male	63.0	65.2	0.550	0.91(0.68-1.21)
Age (m)[a]	7(3-14)	11(4.5-19)	**<0.001**[d]	
Live in urban area	53.3	49.8	0.329	0.87(0.65-1.15)
Prematurity	11.3	8.6	0.265	1.35(0.85-2.13)
Breastfeeding	91.1	89.6	0.493	1.18(0.73-1.91)
Daycare	17.1	23.8	**0.025**	**0.66(0.46-0.95)**
Malnutrition	6.2	10.5	**0.039**	**0.56(0.32-0.98)**
Family size[a]	4(3-5)	4(3-6)	0.229[d]	
Siblings	44.4	48.8	0.208	0.83(0.63-1.11)
Passive smoking	65.0	56.7	**0.019**	**1.42(1.06-1.89)**
ARIs exposure	36.3	30.7	0.089	1.29(0.96-1.73)
Family atopy	40.9	42.4	0.657	0.93(0.70-1.24)
Days before hos.(d)[a]	2(2-4)	3(2-5)	**0.017**[d]	
Antibiotics before hos.	54.4	45.3	**0.010**	**1.44(1.09-1.91)**

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; ARI, acute respiratory infection; RSV, respiratory syncytial virus. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets. [c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Table 12. Clinical features, diagnosis and treatment characteristics associated with RSV detection.

Characteristics (%)	RSV(+) N=257	RSV(-) N=825	*p-value*[c]	OR (95% CI)
Fever	72.8	65.5	**0.033**	**1.41(1.03-1.92)**
High fever	8.2	6.3	0.297	1.32(0.78-2.24)
Cough	93.8	89.9	0.064	1.68(0.96-2.93)
Runny noses	82.5	70.5	**<0.001**	**1.97(1.38-2.81)**
$SpO_2 \leq 92\%$	5.8	9.3	0.095	0.60(0.34-1.06)
Tachypnea	41.6	45.5	0.314	0.85(0.64-1.13)
Chest retraction	68.5	51.0	**<0.001**	**2.09(1.55-2.81)**
Wheezing	59.9	58.5	0.717	1.05(0.79-1.40)
Rales	75.1	63.2	**<0.001**	**1.76(1.28-2.41)**
Clinical Severity Score[a]	12(8-13)	11(7-13)	**0.003**[d]	
WBC ($\times 10^3/mm^3$)[a]	10.9(8.7-13.8)	11.9(9.5-15.2)	**0.001**[d]	
Elevated Neutrophil[b]	25.1	40.0	**<0.001**	**1.99(1.45-2.73)**
Abnormal chest X-ray	83.2	75.9	**0.014**	**1.57(1.09-2.27)**
Detection with other viruses	28.4	53.6	**<0.001**	**0.34(0.25-0.47)**
Diagnosis			**<0.001**	
URTIs	16.0	23.4	**0.012**	**0.62(0.43-0.90)**
Croup	0.8	7.8	**<0.001**	**0.09(0.02-0.38)**
Bronchiolitis	47.1	28.8	**<0.001**	**2.19(1.65-2.93)**
Pneumonia	36.2	40.0	0.305	0.85(0.63-1.13)
Oxygen	5.1	9.0	**0.044**	**0.54(0.30-0.99)**
Antibiotics	79.8	74.8	0.103	1.32(0.94-1.87)
Steroids	10.9	17.2	**0.015**	**0.59(0.38-0.91)**
Bronchodilator	63.4	48.2	**<0.001**	**1.86(1.40-2.48)**
Hos. duration (d)[a]	6(4-8)	5(4-8)	0.067[d]	

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; URTI, upper respiratory infection; WBC, white blood cell; RSV, respiratory

syncytial virus. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Neutrophil >5,800/mm^3 according to Nelson Textbook of Pediatrics (42).
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Patients positive for HBoV were more likely to have hypoxia ($SpO_2 \leq 92\%$) (16.7 vs. 7.9%, p=0.007) and pneumonia (50.0 vs. 38.2%, p=0.04) compared with HBoV negative patients (Table 13 and 14).

Table 13. Demographic characteristics and medical history associated with HBoV detection.

Characteristics (%)	HBoV(+) N=78	HBoV(-) N=1004	*p-value*[c]	OR (95% CI)
Male	62.8	64.8	0.714	0.91(0.56-1.47)
Age (m)[a]	12(6-18)	9(4-18)	0.106[d]	
Live in urban area	57.7	50.1	0.196	0.73(0.46-1.17)
Prematurity	10.3	9.2	0.684	1.13(0.52-2.42)
Breastfeeding	88.5	90.0	0.656	0.84(0.41-1.75)
Daycare	26.8	21.8	0.295	1.32(0.78-2.22)
Malnutrition	12.8	9.3	0.314	1.44(0.71-2.89)
Family size[a]	4(3-6)	4(3-5)	0.809[d]	
Siblings	42.3	48.2	0.315	0.78(0.49-1.25)
Passive smoking	53.8	59.1	0.367	0.80(0.51-1.28)
ARIs exposure	30.8	32.1	0.808	0.94(0.57-1.54)
Family atopy	46.2	41.7	0.446	1.19(0.75-1.90)
Days before hos.(d)[a]	3(2-4)	3(2-5)	0.663[d]	
Antibiotics before hos.	46.2	47.6	0.804	0.94(0.59-1.49)

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; ARI, acute respiratory infection; HBoV, human bocavirus. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Table 14. Clinical features, diagnosis and treatment characteristics associated with HBoV detection.

Characteristics (%)	HBoV(+) N=78	HBoV(-) N=1004	*p-value*[c]	OR (95% CI)
Fever	73.1	66.7	0.263	1.35(0.80-2.27)
High fever	6.4	6.8	0.902	0.94(0.36-2.41)
Cough	92.3	90.7	0.838	1.22(0.51-2.89)
Runny noses	75.6	73.2	0.692	1.13(0.66-1.94)
$SpO_2 \leq 92\%$	16.7	7.9	**0.007**	**2.43(1.24-4.43)**
Tachypnea	46.2	44.4	0.813	1.07(0.67-1.70)
Chest retraction	55.1	55.2	1.000	0.99(0.62-1.58)
Wheezing	62.8	58.6	0.477	1.19(0.74-1.92)
Rales	76.9	65.1	**0.034**	**1.78(1.04-3.07)**
Vomiting	55.1	57.4	0.723	0.91(0.57-1.45)
Diarrhea	30.8	27.0	0.509	1.20(0.72-1.98)
Clinical Severity Score[a]	12(8-13)	12(8-13)	0.359[d]	
WBC ($\times 10^3/mm^3$)[a]	12.7(10.4-15.0)	11.6(9.3-15.0)	0.093[d]	
Elevated Neutrophil[b]	45.5	35.8	0.090	0.66(0.42-1.06)
Abnormal chest X-ray	82.1	77.3	0.398	1.34(0.74-2.44)
Detection with other viruses	66.7	62.0	0.467	0.81(0.49-1.32)
Diagnosis			0.115	
URTIs	23.1	21.5	0.747	1.09(0.63-1.89)
Croup	3.8	6.3	0.620	0.59(1.18-1.94)
Bronchiolitis	23.1	34.0	0.060	0.58(0.33-1.01)
Pneumonia	50.0	38.2	**0.040**	**1.62(1.02-2.56)**
Oxygen	16.7	7.4	**0.004**	**2.51(1.32-4.77)**
Antibiotics	85.9	75.2	**0.033**	**2.01(1.05-3.86)**
Steroids	14.1	15.8	0.685	0.87(0.45-1.68)
Bronchodilator	52.6	51.8	0.896	1.03(0.65-1.63)
Hos. duration (d)[a]	6(4-9)	5(4-8)	**0.020**[d]	

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; URTI, upper respiratory infection; WBC, white blood cell; HBoV, human bocavirus. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Neutrophil >5,800/mm^3 according to Nelson Textbook of Pediatrics (42).
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

The detection of PIV3 had no significant association with severe symptoms such as hypoxia ($SpO_2 \leq 92\%$), retraction and tachypnea or specific diagnostic classification (Table 15 and 16).

Table 15. Demographic characteristics and medical history associated with PIV3 detection.

Characteristics (%)	PIV3(+) N=57	PIV3(-) N=1025	*p-value*[c]	OR (95% CI)
Male	71.9	64.3	0.258	1.42(0.78-2.57)
Age (m)[a]	9(6-15)	10(4-18)	0.759[d]	
Live in urban area	57.9	48.9	0.221	1.43(0.83-2.46)
Prematurity	8.8	9.3	1.000	0.94(0.36-2.41)
Breastfeeding	94.7	89.7	0.264	2.07(0.63-6.75)
Daycare	10.5	22.8	**0.032**	**0.40(0.17-0.94)**
Malnutrition	8.8	9.6	1.000	0.91(0.35-2.33)
Family size[a]	4(3-6)	4(3-5)	0.435[d]	
Siblings	42.1	48.1	0.415	0.78(0.45-1.34)
Passive smoking	59.6	58.6	1.000	1.04(0.60-1.79)
ARIs exposure	36.8	31.7	0.466	1.25(0.72-2.18)
Family atopy	42.1	42.0	1.000	1.01(0.58-1.72)
Days before hos.(d)[a]	2(1-3)	3(2-5)	**0.035**[d]	
Antibiotics before hos.	33.3	48.3	**0.029**	**0.54(0.31-0.94)**

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; ARI, acute respiratory infection; PIV3, parainfluenza virus 3. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Table 16. Clinical features, diagnosis and treatment characteristics associated with PIV3 detection.

Characteristics (%)	PIV3(+) N=57	PIV3(-) N=1025	*p-value*[c]	OR (95% CI)
Fever	68.4	67.1	0.886	1.06(0.59-1.88)
High fever	5.3	6.8	1.000	0.75(0.23-2.48)
Cough	96.5	90.5	0.158	2.87(0.69-11.96)
Runny noses	80.7	73.0	0.221	1.54(0.79-3.03)
$SpO_2 \leq 92\%$	3.5	8.8	0.223	0.37(0.09-1.57)
Tachypnea	33.3	45.2	0.100	0.60(0.34-1.06)
Chest retraction	45.6	55.7	0.171	0.66(0.39-1.13)
Wheezing	66.7	58.4	0.269	1.42(0.80-2.50)
Rales	64.9	66.0	0.886	0.95(0.54-1.66)
Clinical Severity Score[a]	8(7-13)	12(8-13)	0.192[d]	
WBC ($\times 10^3/mm^3$)[a]	11.2(8.9-14.1)	11.7(9.5-15.0)	0.182[d]	
Elevated Neutrophil[b]	33.3	36.7	0.673	1.15(0.65-2.04)
Abnormal chest X-ray	80.7	77.5	0.628	1.21(0.62-2.38)
Detection with other viruses	33.3	62.6	**<0.001**	**0.30(0.17-0.52)**
Diagnosis			0.667	
URTIs	15.8	22.0	0.323	0.66(0.32-1.37)
Croup	7.0	6.0	0.773	1.17(0.41-3.34)
Bronchiolitis	38.6	32.9	0.388	1.28(0.74-2.22)
Pneumonia	38.6	39.1	1.000	0.98(0.56-1.69)
Oxygen	3.5	8.3	0.312	0.40(0.09-1.67)
Antibiotics	75.4	76.0	0.875	0.97(0.52-1.80)
Steroids	21.1	15.4	0.262	1.46(0.75-2.82)
Bronchodilator	52.6	51.8	1.000	1.03(0.60-1.76)
Hos. duration (d)[a]	5(4-7)	6(4-8)	0.550[d]	

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; URTI, upper respiratory infection; WBC, white blood cell; PIV3, parainfluenza

virus 3. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Neutrophil >5,800/mm^3 according to Nelson Textbook of Pediatrics (42).
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Patients with PIV1 positive were older (13 vs. 9 months, p=0.038), had more fever (91.7 vs. 66.3%, p=0.001) but lower rate of chest retraction (36.1 vs. 55.8%, p=0.026) compared with those negative for PIV1. They were more likely to be diagnosed as croup (38.9 vs. 5.0%, p<0.001) compared with PIV1 negative patients (Table 17 and 18).

Table 17. Demographic characteristics and medical history associated with PIV1 detection.

Characteristics (%)	PIV1(+) N=36	PIV1(-) N=1046	*p-value*[c]	OR (95% CI)
Male	69.4	64.5	0.599	1.24(0.60-2.56)
Age (m)[a]	13(9-18)	9(4-18)	**0.038**[d]	
Live in urban area	52.8	50.6	0.866	0.91(0.47-1.78)
Prematurity	2.8	9.5	0.244	0.27(0.04-2.01)
Breastfeeding	91.7	89.9	1.000	1.24(0.37-4.11)
Daycare	22.2	22.2	1.000	1.01(0.45-2.22)
Malnutrition	2.8	9.8	0.245	0.26(0.04-1.95)
Family size[a]	4(3-5.5)	4(3-5)	0.753[d]	
Siblings	36.1	48.2	0.176	0.60(0.30-1.21)
Passive smoking	44.4	59.2	0.086	0.55(0.28-1.07)
ARIs exposure	25.0	32.2	0.468	0.70(0.32-1.50)
Family atopy	44.4	42.0	0.864	1.10(0.56-2.15)
Days before hos.(d)[a]	2.5(2-5.5)	3(2-5)	0.827[d]	
Antibiotics before hos.	61.1	47.0	0.126	1.76(0.89-3.49)

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; ARI, acute respiratory infection; PIV1, parainfluenza virus 1. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Table 18. Clinical features, diagnosis and treatment characteristics associated with PIV1 detection.

Characteristics (%)	PIV1(+) N=36	PIV1(-) N=1046	*p-value*[c]	OR (95% CI)
Fever	91.7	66.3	**0.001**	**5.58(1.70-18.32)**
High fever	19.4	6.3	**0.008**	**3.58(1.51-8.49)**
Cough	94.4	90.7	0.766	1.73(0.41-7.34)
Runny noses	77.8	73.2	0.702	1.27(0.57-2.84)
$SpO_2 \leq 92\%$	2.8	8.7	0.357	0.30(0.04-2.21)
Tachypnea	52.8	44.3	0.394	1.40(0.72-2.73)
Chest retraction	36.1	55.8	**0.026**	**0.45(0.22-0.89)**
Wheezing	50.0	59.2	0.303	0.69(0.35-1.34)
Rales	36.1	67.0	**<0.001**	**0.28(0.14-0.56)**
Clinical Severity Score[a]	10.5(7.5-13)	12(8-13)	0.625[d]	
WBC ($\times 10^3/mm^3$)[a]	10.4(8.9-12.9)	11.7(9.4-15.0)	0.099[d]	
Elevated Neutrophil[b]	44.4	36.2	0.379	0.71(0.36-1.38)
Abnormal chest X-ray	66.7	78.0	0.151	0.56(0.27-1.14)
Detection with other viruses	19.4	63.4	**<0.001**	**0.14(0.06-0.32)**
Diagnosis			**<0.001**	
URTIs	16.7	21.8	0.543	0.71(0.29-1.74)
Croup	38.9	5.0	**<0.001**	**12.16(5.89-25.14)**
Bronchiolitis	11.1	33.9	**0.003**	0.24(0.09-0.69)
Pneumonia	33.3	39.3	0.603	0.77(0.38-1.56)
Oxygen	5.6	8.1	0.762	0.66(0.15-2.81)
Antibiotics	86.1	75.6	0.169	1.99(0.76-5.19)
Steroids	38.9	14.9	**0.001**	**3.63(1.82-7.25)**
Bronchodilator	38.9	52.3	0.128	0.58(0.29-1.14)
Hos. duration (d)[a]	5(4-6.5)	6(4-8)	0.599[d]	

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; URTI, upper respiratory infection; WBC, white blood cell; PIV1, parainfluenza virus 1. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Neutrophil >5,800/mm^3 according to Nelson Textbook of Pediatrics (42).
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Patients positive for FLU A were older (15 vs. 9 months, p=0.002), were more likely to have fever (88.6 vs. 66.5%, p=0.005) but less wheezing (40.0 vs. 59.5%, p=0.024) compared with those without FLU A (Table 19 and 20). The detection of FLU A was less associated with bronchiolitis (14.3 vs. 33.8%, p=0.017).

Table 19. Demographic characteristics and medical history associated with FLU A detection.

Characteristics (%)	FLUA(+) N=35	FLUA(-) N=1048	*p-value*[c]	OR (95% CI)
Male	62.9	64.8	0.858	0.92(0.45-1.85)
Age (m)[a]	15(9-31.5)	9(4-18)	**0.002**[d]	
Live in urban area	51.4	50.6	1.000	0.96(0.49-1.89)
Prematurity	5.7	9.4	0.764	0.58(0.13-2.48)
Breastfeeding	88.6	90.0	0.773	0.86(0.29-2.49)
Daycare	34.3	21.8	0.096	1.87(0.91-3.82)
Malnutrition	8.6	9.6	1.000	0.88(0.26-2.95)
Family size[a]	4(3.5-6)	4(3-5)	0.384[d]	
Siblings	45.7	47.9	0.864	0.91(0.46-1.80)
Passive smoking	60.0	58.6	1.000	1.05(0.53-2.10)
ARIs exposure	34.3	31.9	0.854	1.11(0.54-2.26)
Family atopy	40.0	42.1	0.863	0.91(0.46-1.82)
Days before hos.(d)[a]	3(2-4)	3(2-5)	0.677[d]	
Antibiotics before hos.	54.3	47.3	0.492	1.32(0.67-2.60)

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; ARI, acute respiratory infection; FLU A, influenza A virus. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

Table 20. Clinical features, diagnosis and treatment characteristics associated with FLU A detection.

Characteristics (%)	FLUA(+) N=35	FLUA(-) N=1048	*p-value*[c]	OR (95% CI)
Fever	88.6	66.5	**0.005**	**3.91(1.37-11.16)**
High fever	5.7	6.8	1.000	0.83(0.19-3.54)
Cough	88.6	90.9	0.553	0.77(0.26-2.23)
Runny noses	80.0	73.2	0.441	1.46(0.63-3.39)
$SpO_2 \leq 92\%$	5.7	8.6	0.762	0.64(0.15-2.73)
Tachypnea	60.0	44.0	0.083	1.90(0.95-3.79)
Chest retraction	48.6	55.4	0.491	0.76(0.38-1.49)
Wheezing	40.0	59.5	**0.024**	**0.45(0.23-0.90)**
Rales	74.3	65.7	0.366	1.50(0.69-3.25)
Clinical Severity Score[a]	12(7-13)	12(8-13)	0.827[d]	
WBC ($\times 10^3/mm^3$)[a]	11.6(8.6-14.7)	11.6(9.4-15.0)	0.695[d]	
Elevated Neutrophil[b]	45.7	36.2	0.285	0.67(0.34-1.32)
Abnormal chest X-ray	82.9	77.5	0.541	1.40(0.57-3.42)
Detection with other viruses	25.7	63.4	**<0001**	**0.20(0.09-0.43)**
Diagnosis			0.593	
URTIs	22.9	21.6	0.836	1.07(0.48-2.40)
Croup	8.6	6.0	0.468	1.46(0.43-4.91)
Bronchiolitis	14.3	33.8	**0.017**	**0.33(0.13-0.85)**
Pneumonia	54.3	38.6	0.077	1.98(0.96-3.71)
Oxygen	5.7	8.1	1.000	0.68(0.16-2.90)
Antibiotics	94.3	75.4	**0.008**	**5.40(1.29-22.64)**
Steroids	17.1	15.7	0.813	1.11(0.45-2.72)
Bronchodilator	42.9	52.1	0.305	0.68(0.34-1.35)
Hos. duration (d)[a]	5(4-9)	6(4-8)	0.576[d]	

Abbreviation: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; d, day; m, month; hos, hospitalization; URTI, upper respiratory infection; WBC, white blood cell; FLU A, influenza A

virus. Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Neutrophil >5,800/mm^3 according to Nelson Textbook of Pediatrics (42).
[c]Chi-squared test was used except [d]Mann-Whitney-*U* test was applied for continuous variables.

The effect of respiratory virus detection on the risk of having severe clinical symptoms is shown in Table 21. In univariate analyses, HRV (Table 10) and RSV (Table 12) were positively associated with retraction, while HRV (Table 10) and HBoV (Table 14) were positively associated with hypoxia. In multivariate analyses adjusting for age, gender, prematurity, malnutrition and other viral co-detection (Table 21), HRV (AOR=1.49, 95% CI: 1.13-1.96, p=0.005) and RSV (AOR=1.96, 95% CI: 1.44-2.68, p<0.001) were the significant predictors of retraction. On the other hand, HRV (AOR=1.78, 95% CI: 1.14-2.79, p=0.012) and HBoV (AOR=2.32, 95% CI: 1.22-4.41, p=0.011) were the significant predictors for hypoxia. Detection of any specific type of viruses was not associated with tachypnea.

Regarding diagnosis, RSV positive was positively associated with bronchiolitis in both univariate (Table 12) and multivariate analyses (AOR=1.89, 95% CI: 1.37-2.61, p<0.001) (Table 22). Detection of PIV1 was less likely to have bronchiolitis (AOR=0.33, 95% CI: 0.11-0.97, p=0.045) but more associated with croup (AOR=8.31, 95% CI: 3.88-17.81, p<0.001) (Table 22). On the other hand, detection of HRV (AOR=0.31, 95% CI: 0.15-0.64, p=0.002) and RSV (AOR=0.08, 95% CI: 0.02-0.35, p=0.001) were less likely to be diagnosed as croup (Table 22). HBoV positive was positively associated with pneumonia in the univariate analysis (Table 14) but this association became borderlined in multivariate analysis (AOR: 1.59, 95% CI: 0.99 – 2.55, p=0.053) (Table 22).

Table 21. Association between specific respiratory virus and severe clinical signs.

Category	PCR	Tachypnea n(%)		AOR (95% CI)
		Yes	No	
HRV	Positive	133(40.9)	192(59.1)	0.81(0.61-1.08)
	Negative	349(46.1)	408(53.9)	1
RSV	Positive	107(41.6)	150(58.4)	1.07(0.78-1.46)
	Negative	375(45.5)	450(54.5)	1
HBoV	Positive	36(46.2)	42(53.8)	0.99(0.60-1.61)
	Negative	446(44.4)	558(55.6)	1
PIV3	Positive	19(33.3)	38(66.7)	0.61(0.33-1.12)
	Negative	463(45.2)	562(54.8)	1
PIV1	Positive	19(52.8)	17(47.2)	1.21(0.60-2.44)
	Negative	463(44.3)	583(55.7)	1
FLU A	Positive	21(60.0)	14(40.0)	1.28(0.60-2.73)
	Negative	461(44.0)	586(56.0)	1
Category	**PCR**	**Retraction n(%)**		**AOR (95% CI)**
		Yes	**No**	
HRV	Positive	198(60.9)	127(39.1)	**1.49(1.13-1.96)**
	Negative	399(52.7)	358(47.3)	1
RSV	Positive	176(68.5)	81(31.5)	**1.96(1.44-2.68)**
	Negative	421(51.0)	404(49.0)	1
HBoV	Positive	43(55.1)	35(44.9)	1.01(0.63-1.62)
	Negative	554(55.2)	450(44.8)	1
PIV3	Positive	26(45.6)	31(54.4)	0.70(0.40-1.21)
	Negative	571(55.7)	454(44.3)	1
PIV1	Positive	13(36.1)	23(63.9)	0.55(0.27-1.13)
	Negative	584(55.8)	462(44.2)	1
FLU A	Positive	17(48.6)	18(51.4)	1.05(0.52-2.13)
	Negative	580(55.4)	467(44.6)	1
Category	**PCR**	**SpO_2 ≤92% n(%)**		**AOR (95% CI)**
		Yes	**No**	
HRV	Positive	40(12.3)	285(87.7)	**1.78(1.14-2.79)**
	Negative	52(6.9)	705(93.1)	1
RSV	Positive	15(5.8)	242(94.2)	0.64(0.35-1.14)
	Negative	77(9.3)	748(90.7)	1
HBoV	Positive	13(16.7)	65(83.3)	**2.32(1.22-4.41)**
	Negative	79(7.9)	925(92.1)	1
PIV3	Positive	2(3.5)	55(96.5)	0.39(0.09-1.63)
	Negative	90(8.8)	935(91.2)	1
PIV1	Positive	1(2.8)	35(97.2)	0.33(0.04-2.48)
	Negative	91(8.7)	955(91.3)	1
FLU A	Positive	2(5.7)	33(94.3)	0.71(0.17-3.05)
	Negative	90(8.6)	957(91.4)	1

Abbreviation: HRV, human rhinovirus; RSV, respiratory syncytial virus; FLU A, influenza A virus; PIV, parainfluenza virus; HBoV, human bocavirus; AOR, adjusted odds ratio; CI, confidence interval;
The multivariate analyses included the virus status and all these variables known as potential confounders (Age, Gender, Prematurity, Malnutrition, and Co-detection with other viruses) to calculate adjusted ORs. The results of the Hosmer-Lemeshow test indicated high goodness-of-fit for the final model (p >0.05).

Table 22. Association between specific respiratory virus and diagnosis categories.

Category	PCR	URTIs n(%)		AOR (95% CI)
		Yes	No	
HRV	Positive	81(24.9)	244(75.1)	1.32(0.96-1.83)
	Negative	153(20.2)	604(79.8)	1
RSV	Positive	41(16.0)	216(84.0)	0.73(0.49-1.07)
	Negative	193(23.4)	632(76.6)	1
HBoV	Positive	18(23.1)	60(76.9)	1.08(0.62-1.90)
	Negative	216(21.5)	788(78.5)	1
PIV3	Positive	9(15.8)	48(84.2)	0.76(0.36-1.60)
	Negative	225(22.0)	800(78.0)	1
PIV1	Positive	6(16.7)	30(83.3)	0.73(0.29-1.81)
	Negative	228(21.8)	818(78.2)	1
FLU A	Positive	8(22.9)	27(77.1)	0.88(0.38-2.03)
	Negative	226(21.6)	821(78.4)	1
Category	**PCR**	**Croup n(%)**		**AOR (95% CI)**
		Yes	**No**	
HRV	Positive	9(2.8)	316(97.2)	**0.31(0.15-0.64)**
	Negative	57(7.5)	700(92.5)	1
RSV	Positive	2(0.8)	255(99.2)	**0.08(0.02-0.35)**
	Negative	64(7.8)	761(92.2)	1
HBoV	Positive	3(3.8)	75(96.2)	0.63(0.19-2.05)
	Negative	63(6.3)	941(93.7)	1
PIV3	Positive	4(7.0)	53(93.0)	0.92(1.32-2.67)
	Negative	62(6.0)	963(94.0)	1
PIV1	Positive	14(38.9)	22(61.1)	**8.31(3.88-17.81)**
	Negative	52(5.0)	994(95.0)	1
FLU A	Positive	3(8.6)	32(91.4)	1.16(0.34-3.97)
	Negative	63(6.0)	984(94.0)	1

Table 22. Association between specific respiratory virus and diagnosis categories (continued).

Category	PCR	Bronchiolitis n(%)		AOR (95% CI)
		Yes	No	
HRV	Positive	118(36.3)	207(63.7)	1.27(0.93-1.73)
	Negative	241(31.8)	516(68.2)	1
RSV	Positive	121(47.1)	136(52.9)	**1.89(1.37-2.61)**
	Negative	238(28.8)	587(71.2)	1
HBoV	Positive	18(23.1)	60(76.9)	0.64(0.36-1.14)
	Negative	341(34.0)	663(66.0)	1
PIV3	Positive	22(38.6)	35(61.4)	1.31(0.72-2.39)
	Negative	337(32.9)	688(67.1)	1
PIV1	Positive	4(11.1)	32(88.9)	**0.33(0.11-0.97)**
	Negative	355(33.9)	691(66.1)	1
FLU A	Positive	5(14.3)	30(85.7)	0.50(0.18-1.42)
	Negative	354(33.8)	693(66.2)	1
Category	**PCR**	**Pneumonia n(%)**		**AOR (95% CI)**
		Yes	**No**	
HRV	Positive	117(36.0)	208(64.0)	0.85(0.64-1.12)
	Negative	306(40.4)	451(59.6)	1
RSV	Positive	93(36.2)	164(63.8)	0.93(0.69-1.26)
	Negative	330(40.0)	495(60.0)	1
HBoV	Positive	39(50.0)	39(50.0)	1.59(0.99-2.55)
	Negative	384(38.2)	620(61.8)	1
PIV3	Positive	22(38.6)	35(61.4)	0.99(0.57-1.74)
	Negative	401(39.1)	624(60.9)	1
PIV1	Positive	12(33.3)	24(66.7)	0.76(0.37-1.57)
	Negative	411(39.3)	635(60.7)	1
FLU A	Positive	19(54.3)	16(45.7)	1.68(0.84-3.37)
	Negative	404(38.6)	643(61.4)	1

Abbreviation: HRV, human rhinovirus; RSV, respiratory syncytial virus; FLU A, influenza A virus; PIV, parainfluenza virus; HBoV, human bocavirus; AOR, adjusted odds ratio; CI, confidence interval;
The multivariate analyses included the virus status and all these variables known as potential confounders (Age, Gender, Prematurity, Malnutrition, and Co-detection with other viruses) to calculate adjusted ORs. The results of the Hosmer-Lemeshow test indicated high goodness-of-fit for the final model ($p > 0.05$).

The clinical severity score was higher in RSV, HRV, FLU A and HBoV single detection but this difference was not significant (Kruskal-Wallis test, $p>0.05$). Median duration of hospitalization of HBoV and RSV single detection was longer than that of other

viruses, however, the difference was not statistically significant (Kruskal-Wallis test, $p>0.05$). Other viruses such as FLU B, hMPV, HCoV, and AdV were not included in this analysis because of the small number of cases in each such category.

4. DISCUSSION

The contribution of disease associated with respiratory viruses in developing and tropical countries is currently poorly described. The present study focused on the contribution of 13 respiratory viruses to children hospitalized with ARIs in the south of Vietnam, and elucidated the impact of each viral detection on the disease severity and the burden of ARIs.

Viral detection and seasonal patterns

In this study, a wide variety of viral agents associated with ARIs in children in southern Vietnam was described. Identifying 11 different respiratory viruses associated with high positive percentage of hospitalized patients in this study was in concordance with the previous studies conducted in Vietnam (26, 27). Altogether, by using PCR methods, these studies confirmed the diversity of respiratory viruses and the burden of viral ARIs in this area.

One or more viruses were detected in 64.6% of ARI cases in this study. Since 2000, the introduction of PCR has increased the ability to detect respiratory viruses, including those are difficult to culture. Viruses accounted for 43-67% of ARI cases in studies during this time (43). The detection rate in the current study was higher than that in previous studies from surrounding countries such as Cambodia (44), Thailand (45), China (46), India (47), and Nepal (48). The differences in detection rates may be attributed to inclusive criteria, or the broader spectrum of studied viruses, or the more sensitive detection method such as PCR using in this study. It is obvious that a wider panel of target viruses is necessary to get the better possible yield of virus in hospitalized patients with ARIs.

HRV was the most common virus detected in this study with the rate of 30% of all ARI cases followed by RSV and HBoV. These 3 viruses accounted for 83.1% of viral positive patients, suggesting the significant burden of these viruses associated with ARIs among hospitalized children in this location. Using the high sensitivity method as PCR, HRV has been increasingly detected and shown to cause LRTIs among children (49, 50). This result is consistent with previous findings suggesting that HRV is one of the most frequent detected viruses of ARIs in children (37, 51-55).

The positive rates for virus detection in this study varied dramatically from month to month, ranging from 47.4% (May 2010) to 90.2% (October 2010) (Fig. 2). The viral detection rates were highest during months when there were the most hospitalized patients with ARI (from August to October). Simultaneously, RSV detection also reached the peak during this time. Therefore, it could be inferred that the ARIs epidemics to some extent were driven by the respiratory viruses, especially the RSV.

Although the detection rate was high, viruses were still not able to be identified in nearly 35% of enrolled children with ARIs. Several factors may account for this phenomenon. First, the utilization of nasopharyngeal flocked swab may have led to the poorer recovery of viruses compared with nasopharyngeal aspiration. However, the difference in yield rate was not significant (56). Moreover, the nasopharyngeal swab was less invasive for children and more acceptable for parents. Second, frozen specimens using for RT-PCR may have reduced the yield of viral detection since RNA may have degraded during freezing, thawing, and storing of the samples. Third, it is possible that some children may have had infection with bacteria or other viruses not included in the assays of this study (eg, enteroviruses, other types of HCoV such as NL63 and HKU1), or with agents yet to be discovered. Finally, despite the high sensitivity and specificity of PCR, the number of viral detection may have

been underestimated by using multiplex instead of monoplex assays, as some loss of sensitivity is an inherent limitation of multiplex PCR assays.

Since this study spanned only 14 months, this duration is short to have any conclusions regarding seasonal patterns of different respiratory viruses. However, some interesting points were found. First, the RSV epidemic peaked in relation to the rainy season. This seasonal pattern is distinct from that in temperate countries where RSV infections usually occur in the winter (57). Second, different from temperate zone, FLU A activity in Vietnam was found in both rainy and dry seasons. Unlike in many temperate countries, where PIV infections were prevalent during autumn or spring (7), and other tropic countries, where PIVs did not show significant annual seasonality (58), PIV in this study was detected mainly during the dry season and filled in for the weak RSV season. Although the reasons for the seasonality are almost unclear, determining the seasonality of respiratory viruses is very important in guiding appropriate preventive strategies and clinical management.

Clinical features of respiratory viruses and their association with severe disease

In the current study, statistical significance was achieved for the detection of virus and its association with clinical severity (Table 4). The virus-positive patients tended to have more severe symptoms and higher severity score so that they sought the hospital care earlier than the virus-negative patients. Although the WBC count was found higher in the virus-negative group, its sensitivity and specificity is not sufficient to differentiate bacterial from viral infection (59). Age from 6 to 12 months was a risk factor for virus detection, since maternal antibodies decrease dramatically but the immune system is not mature enough to protect the infants from infection at this age. Regarding diagnosis, potential viruses known to cause bronchiolitis such as RSV, HRV, and PIV3 accounted for a relatively large part in this study and explained why this diagnosis was seen significant more often in virus-positive group than in virus-negative group.

In this study, the relationship between overall co-detection and increasing disease severity could not be established. Recent studies have shown that co-detection are not uncommon events (43), however their effect on severity depends on the combination of different viruses (60).

Many viruses are responsible for ARIs and it is believed that the virus type may influence the clinical manifestations of infection. In this study, different age distributions of each virus type were found. This information may be useful in clinical practice. It is noted that more than one third of HRV positive cases occurred in infants under 6 months of age. This finding implies that HRV can cause a burden at a very early stage of life.

As in previous literature, RSV is always associated with bronchiolitis. The fact that most of RSV positive cases were diagnosed as either bronchiolitis or pneumonia and associated with chest retraction demonstrated that RSV by far the most important pathogen in children, which is comparable to the results of other studies (55, 61, 62). Croup was a clinical picture associated uniquely to PIV1 as seen in other study (63). In addition, RSV and HRV were less likely to be detected from patients diagnosed as croup in this study. Similarly, PIV1 was less likely to be detected from patients with bronchiolitis. This information is useful for physician in estimating the possible cause when the clinical diagnosis is made.

Of note, HRV was the most common virus detected from patients hospitalized for ARIs in this study and LRTIs also occupied two-thirds of HRV positive cases. In addition, HRV detection was also associated with the development of severe symptoms such as hypoxia and retraction. Moreover, majority of the patients with HRV detection (75%) had the onset of symptoms within 5 days before admission, suggesting HRV may be responsible for the symptom rather than bacterial superinfection in later presentation. HRV has also previously been demonstrated the ability to infect the lower respiratory tract (50, 64-66). Despite all these supporting data for the pathogenesis of HRV, caution must be considered

when interpreting the role of HRV since it could be found in approximately 15% asymptomatic individuals, compared with <5% of most other respiratory viruses (67).

The newly discovered HBoV were detected in a substantial number of cases and representing the third most common virus in this study. Although HBoV detection was significantly associated with hypoxia, HBoV was frequently detected in combination with other pathogens. HBoV was also often detected from asymptomatic individuals. However, several studies showed that identification of single HBoV was more common in sick patients than in control subjects (20). Therefore the role of HBoV as the true pathogen remains to be determined.

Although clinical signs and symptoms of various respiratory viruses are usually overlapping, this work found different clinical characteristics associated with common respiratory viruses. Taken together with the differences in seasonal patterns and the age distribution, it is possible to speculate the probability of diseases being caused by a specific virus. This may contribute to clinical benefits in developing country settings since facilities for virus detection are not available there. To document and analyze the demographic and clinical information is important, since it enables to put the results themselves into the practical context and allow them to be applied in clinical practice. On the other hand, knowledge of clinical information allows medical practitioners to link the potential risk factors and causes to the clinical outcomes, and helps to confirm the accuracy and reliability of the results.

Antibiotics were used in three quarters of children hospitalized with ARIs. This probably reflects the reluctance of physicians to manage sick patients while having no effective tools to support the diagnosis. The availability of viral diagnostic tests might decrease the number of subjects who receive antibiotics for viral infections or decrease the duration of initially empiric antibiotic administration in patients hospitalized with ARIs.

Strengths and limitations

The major strengths of this pediatric clinical study were the use of a highly sensitive multiplex (RT)-PCR for a wide range of respiratory viruses, the evaluation of important clinical characteristics, the high number of patients enrolled in the study, and the inclusion of whole spectrum of ARI disease severity, from mild URTI to severe cases of bronchiolitis and pneumonia, requiring oxygen supplementation.

However, this study still has some limitations. First of all, the clinical diagnosis was sometimes not accurate. Differentiating between bronchiolitis and pneumonia was sometimes difficult and tended to be subjective assessment since the signs shown in each condition were overlapped. Despite this limitation, these two categories reflect the severe condition of LRTI and represent of what happen in real clinical situations. Second, although a wide spectrum of diseases and severities were included, a large number of outpatients were not recruited. Therefore, the results of this study cannot be applied to the general pediatric population. Since the study included only hospitalized children, the results may have the selection bias towards more severe diseases. However, the comparison clinical features of specific type virus with each other may still be valid since the groups being compared share the same selection bias of being hospitalized. Third, the detection of viruses in nasopharyngeal samples is not enough to confirm the cause of ARIs. The samples were not examined the viral load by quantitative PCR. The high viral load may have association with the cause of ARIs (68). Information regarding bacterial infection was also not available in this study since obtaining appropriate sputum from the lower respiratory tract is not feasible in young children and blood culture usually reveals low positive rates due to high rate of antibiotic usage before hospitalization. Moreover, the study lacked a control group. Due to the sensitivity of PCR, it is able to detect the remnant material of old pathogens along with current active pathogens.

Using control samples to detect pathogens in asymptomatic children would help determine the likelihood of these viruses being a true pathogen.

Conclusions

The results of this study confirmed the diversity of viruses found among the children were associated with ARIs among them in the south of Vietnam. HRV, RSV and HBoV were the most commonly detected viruses and may increase the severity of ARIs in children. Specific viruses were frequently associated with specific clinical syndromes, specific seasonal patterns and age distributions. The ARIs epidemics to some extent were driven by the respiratory viruses, especially the RSV. The findings of this study emphasize the important role of viruses associated with ARIs in both aspects: magnitude and severity. Up to now, while bacterial ARIs is able to put under control by antibiotics or vaccine against *Streptococcus pneumoniae* and *Hemophilus influenzae* type b, there are no effective measures to control ARIs caused by viruses. Sooner or later, when bacterial ARIs are well controlled, viruses will become more fatal threat to child life. So far, only vaccine against influenza is available. In addition, the cost for treatment of viral ARIs is high. Therefore, to prevent viral ARIs in developing country setting, the ability of early diagnosis and treatment as well as basic preventive measures such as nutrition and hygiene should be improved. Similar surveillances on common viral agents should be continued in the future in order to know the fluctuation of these viruses. Especially, the control group should be added so that the causative role of each virus could be explained more accurately. Identification of bacteria causing ARIs should be done. More knowledge on viruses causing ARIs in children would help to decrease the disease burden, not only for Vietnam but also for other countries. Better understanding of the epidemiology of viral ARIs is essential to enable prediction of outbreaks and for planning of preventive and therapeutic control measures.

CHAPTER 3

MOLECULAR EPIDEMIOLOGY AND CLINICAL CHARACTERISTICS OF INFLUENZA A VIRUS IN VIETNAM DURING POST-PANDEMIC PERIOD

1. INTRODUCTION

Influenza virus infection is the major cause of morbidity and mortality in humans worldwide. The influenza epidemics lead to approximately 3 to 5 million severe illness cases and 250,000 to 500,000 deaths every year (69). The ability to acquire genetic changes makes the virus to overcome the immunity from previous infections and causes yearly epidemics. The hemagglutinin (HA) and neuraminidase (NA) genes of influenza A (FLU A) viruses usually mutate at high frequencies (70). As a result, the accumulation of these mutations at critical positions may lead to alter the antigenic characteristics of surface glycoproteins and the antiviral drug susceptibility. Therefore, monitoring these genetic changes of influenza virus is important for vaccine strain selection as well as identification of the drug resistant strains.

In addition, the influenza activity has been well described in temperate countries with outbreaks usually occurring during winter seasons (70). Thus, influenza vaccine is delivered annually to protect against the newly emerged influenza strains. In contrast, influenza virus circulates throughout the year with no clear seasonality in tropical regions. However, little epidemiological information about influenza makes it more difficult to develop the prevention and control programs in these settings (71). Moreover, it has been proposed that Southeast and East Asia are regions where influenza virus A/H3N2 first evolved before spreading worldwide (72, 73). Therefore, influenza virological surveillance in these areas is

necessary not only to understand the circulation of influenza viruses but also to identify the virus strains which may be included in the annual influenza vaccine composition.

In this study, the molecular, clinical, and epidemiological characteristics of influenza A viruses circulating during the post-pandemic season 2010 – 2011 in Vietnam were described. These data may improve the understanding of the temporal and geographic circulation of influenza viruses and provide important information to develop the public health vaccination policies throughout the region.

2. MATERIALS AND METHODS

Patients and samples

The clinical samples and data collection was described in detail in chapter 1.

Influenza virus detection and subtyping

Samples positive for FLU A by screening PCR as described in chapter 1 were further subtyped as seasonal H1N1, seasonal H3N2, or pandemic H1N1 2009 by multiplex nested PCR (74).

Sequencing and phylogenetic analysis

The HA, NA, and M genes of H1N1pdm09 and H3N2 viruses were amplified using gene-specific primers recommended by the World Health Organization (75). PCR products were then sequenced by the commercial company (Macrogen Japan Corp., Tokyo, Japan). Sequences were assembled by using Cap3 Sequence Assembly program (76). Vaccine strain and reference sequences used for the phylogenetic trees were obtained from the NCBI GenBank Database (www.ncbi.nlm.nih.gov/genbank) and GISAID EpiFlu™ (www.gisaid.org). The sequence data and the phylogenesis were analyzed using BioEdit v.7.0.5 (77). Phylogenetic analysis was performed using MEGA 5.0.5 (78). The neighbor-joining method (79) with maximum composite likelihood model (80) and 1,000 bootstrap

replicates was used to construct the phylogenetic trees. Deduced amino acid sequences were analyzed and compared with the vaccine strains A/California/7/2009 (H1N1pdm09) and A/Perth/16/2009 (H3N2). HA and NA numbering are accordant with the respective subtype.

The HA, NA and M sequences in this study have been deposited in the GenBank database under accession numbers KJ955501-KJ955605.

Antigenic site mapping of HA and NA

The HA and NA protein sequences of influenza virus in this study were compared with the vaccine strains. The amino acid changes were mapped to the previously reported HA (81-83) and NA antigenic sites (84, 85). Protein structures were rendered by PyMol software v1.3 (http://www.pymol.org) using the HA (PDB: 3LZG for H1, 1MQL for H3) and NA (PDB: 3NSS for N1, 1IVG for N2) structures from Protein Data Bank (RCSB PDB, http://www.pdb.org) (86).

Statistical analysis

Demographic and clinical characteristics of patients singly detected with H1N1pdm09 were compared to those with H3N2. Categorical variables between groups were compared by using χ^2 test or Fisher's exact test, and continuous variables were compared by using the Mann-Whitney *U* test. A two sided *P*-value <0.05 was considered statistically significant. All analyses were conducted using SPSS 16.0 software (Chicago, IL, USA).

3. RESULTS

Epidemiological characteristics of FLU A

Between April 2010 and May 2011, FLU A was detected in 35 of 1,082 samples (3.2%). Nine samples were found mixed detection with other respiratory viruses, such as human bocavirus (n=4), human rhinovirus (n=3), respiratory syncytial virus (n=1), and triple detection with human bocavirus and rhinovirus (n=1). FLU A subtyping was performed, and

14 samples were H1N1pdm09 and 21 were H3N2. Seasonal H1N1 subtype was not found in any samples. Co-detection was more common among H3N2 cases (7/21) than H1N1pdm09 cases (2/14). Male positive for FLU A was more often than female (male:female = 1.7). The median age of children detected with FLU A was 15 months (range from 1 to 60 months). Children younger than 6 months had the lowest FLU A detection rate (1.4%) while those aged from 24 to 59 months had the highest detection rate (7.1%). It is noted that among children less than 5 years old, there is a progressive increase in FLU A positive rate with increasing age (Fig. 4). Of note, FLU A was detected in both seasons of this tropical country (Fig. 5). Right after the pandemic period, only seasonal H3N2 subtype was detected in the rainy season (May – October) of 2010. This was also the time when the Southern Hemisphere vaccine was typically available. Then, the H3N2 virus was replaced completely by the H1N1pdm09 virus in the following dry season (November – April), the typical time for the Northern Hemisphere vaccine being available.

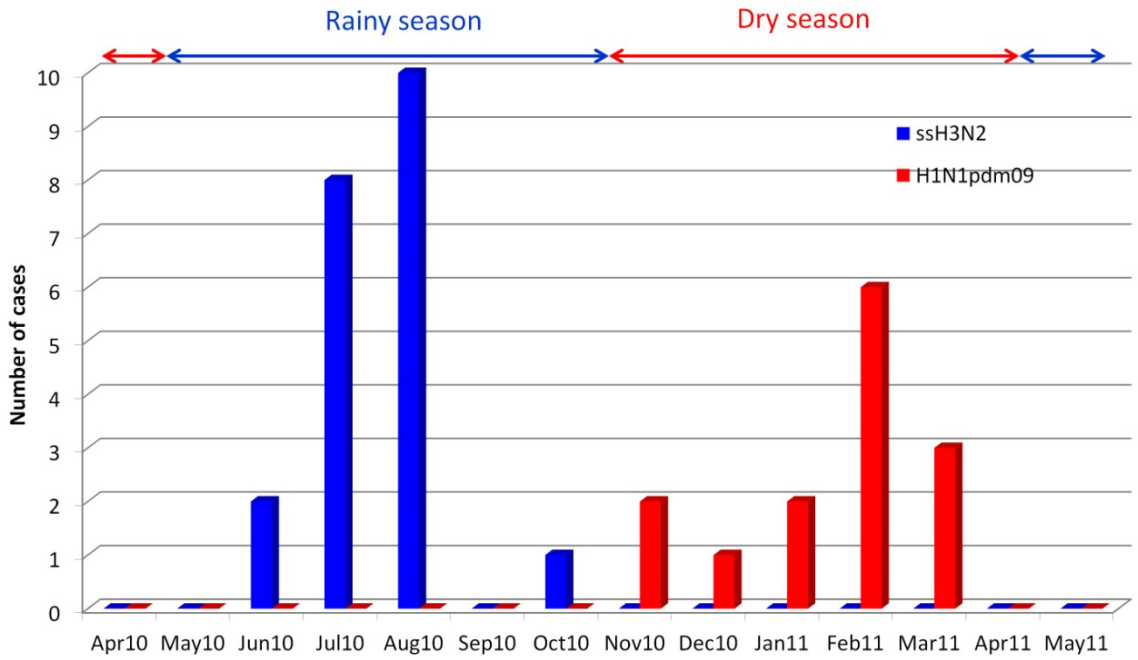


Figure 5. Seasonal pattern of FLU A from April 2010 to May 2011.

Abbreviation: ssH3N2, seasonal H3N2; H1N1pdm09, H1N1 pandemic 2009;

Clinical characteristics of FLU A

To exclude the confounding that co-detection may cause, only samples with single detection of H3N2 and H1N1pdm09 were compared (Table 23). However, there were no statistically significant differences between the two groups. The median age of patients detected with H1N1pdm09 was higher than those with H3N2 (26.5 vs. 20.5 months). H3N2 subtype was associated with LRTI more than H1N1pdm09 subtype (71.4 vs. 50%). One case of H1N1pdm09 (8.3%) had hypoxia and required oxygen supplementation. Patients with H1N1pdm09 detection had longer duration of hospitalization than those with H3N2 (5.5 vs. 4.5 days). No influenza-positive cases in this study were severe enough to require mechanical ventilation or become fatal. None of them had vaccinated against influenza before or received any antiviral treatment.

Table 23. Demographic and clinical characteristics of children detected with H1N1pdm09 and H3N2 viruses.

Characteristics (%)	H1N1pdm09 N=12	H3N2 N=14	*p-value*
Male	50.0	71.4	0.422
Age (m)(IQR)[a]	26.5(13.5-32)	20.5(10-33)	0.757
Flu vaccination	0.0	0.0	NA
Fever	83.3	100.0	0.203
Cough	100.0	92.9	1.000
Tachypnea	66.7	57.1	0.701
Retraction	50.0	50.0	1.000
$SpO_2 \leq 92\%$	8.3	0.0	0.462
Diagnosis			0.422
URTIs	50.0	28.6	
LRTIs	50.0	71.4	
Oxygen	8.3	0.0	0.462
Antiviral drugs	0.0	0.0	NA
Hospitalization length (d)[a]	5.5 (5-10)	4.5 (3-9)	0.090

Abbreviation: d, day; m, month; URTI, upper respiratory infection; LRTI, lower respiratory infection; NA, not applicable;
Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.

Molecular characterization and phylogenetic analysis

Sequences in this study were blast searched in the NCBI Influenza Virus Resource and the GISAID EpiFlu database in order to find the similar sequences. Other strains from Vietnam, reference and vaccine strains were also analyzed together. Genetic groups in this study were named according to the WHO Influenza Centre London classification (87).

a. H1N1pdm09 virus

Phylogenetic analysis showed the simultaneous co-circulation of three influenza virus groups in Vietnam (Fig. 6). In detail, one Vietnamese strain (A/HoChiMinh/962.11/2010) clustered with other strains from Vietnam, Cambodia, Singapore, and Australia in 2009-2010. These strains were characterized by the double mutations A197T and S203T and formed an additional genetic group which had not been named in the WHO Influenza Centre London classification (87). Another strain (A/HoChiMinh/1137.2/2011) belonged to genetic group 6, characterized by the mutations D97N, S185T in the HA1 region and E374K, S451N in the HA2 region. This strain clustered with the European and North American strains in 2011. Finally, the rest of Vietnamese strains in this study formed a distinct cluster within genetic group 7, characterized by the mutations S143G, S185T, A197T, E374K and S451N. They clustered with strains isolated in East and Southeast Asia, Australia, Europe in 2010-2011 and strains isolated in North and Central America in 2011-2012. Of note, all the Vietnamese strains in this study (except A/HoChiMinh/1137.2/2011 carried only L420V) carried all the substitutions L420V, D436E, and S539F that had not been reported in other influenza viruses before. These changes formed a unique signature for the 2010-2011 Vietnamese H1N1pdm09 sequences.

The HA protein of Vietnamese H1N1pdm09 showed some amino acid mutations with respect to the vaccine strain A/California/7/2009. Some of them located within the major antigenic sites Ca (H138Q, S203T), Cb (S71A), Sa (L161I), and Sb (S185T) of the HA

molecule (Fig. 8). Moreover, other mutations S143G and A197T were also found in the vicinity of the Ca and Sb antigenic sites.

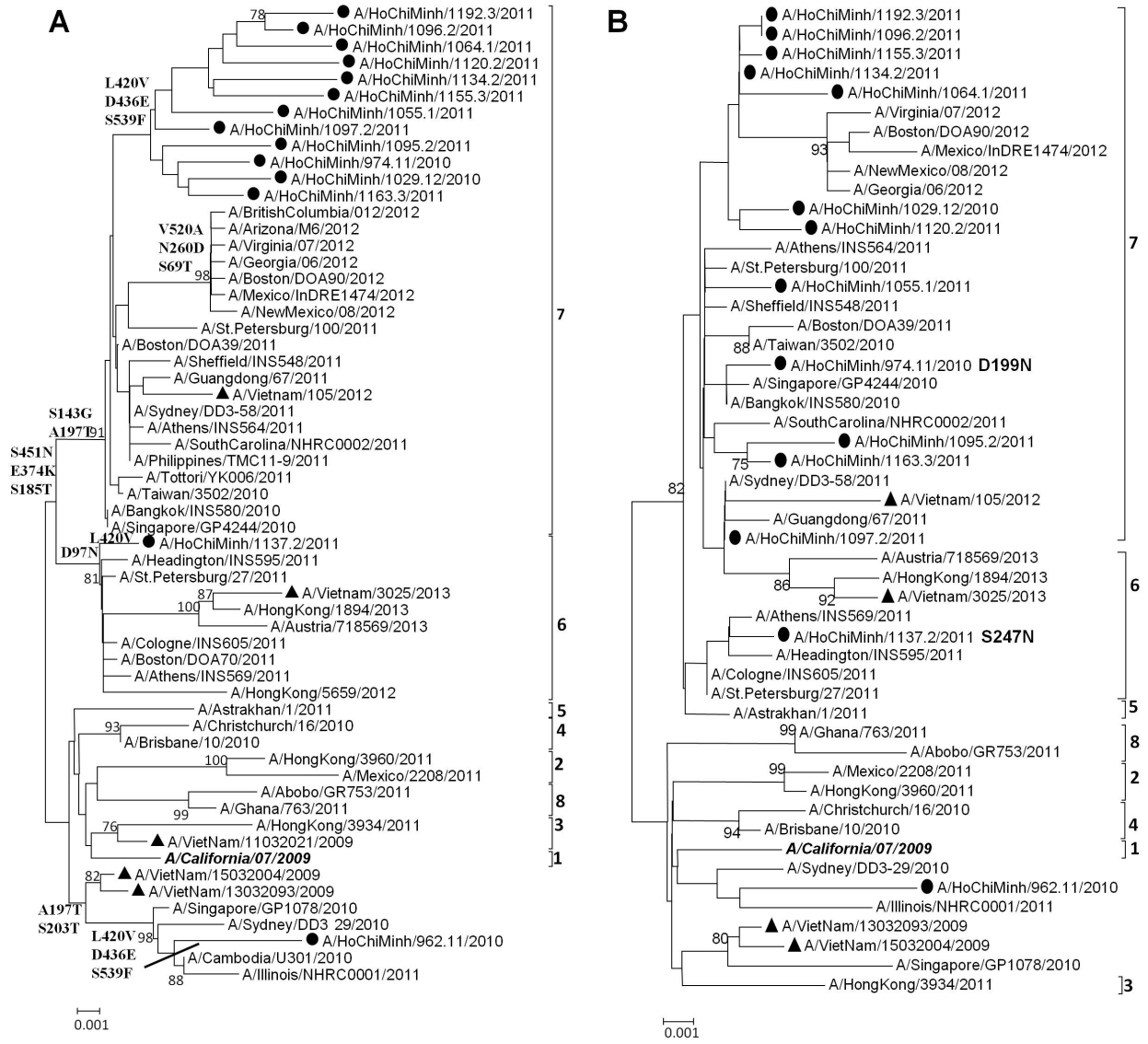


Figure 6. Phylogenetic analysis of HA (A) and NA (B) genes of influenza H1N1pdm09 viruses circulating in southern Vietnam during 2010-2011.

Phylogenetic trees were constructed with MEGA 5 software using the neighbor-joining method. Bootstrap values of greater than 70 % are shown at the branch nodes. Characteristic mutations of a particular branch are indicated on the left side nodes. Drug resistant mutations are shown in bold on the right of strains. The genetic group assignment is indicated by the brackets on the right. The influenza strains in this study are marked with solid round. The influenza strains from Vietnam are marked with solid triangles. Vaccine strain is shown in bold and italic.

The NA phylogenetic tree was generally compatible with the HA tree (Fig. 6). Compared with the vaccine strain, some of the amino acid substitutions of the NA protein involved in the antigenic sites J (S340F), L (S366N, N369K), and M (N386K). Additionally, none of the NA proteins contained the mutation H275Y, which are resistant to oseltamivir. However, two strains in this study carried the D199N and S247N mutations which were known to reduce the neuraminidase inhibitor susceptibility.

b. H3N2 virus

The phylogenetic analysis of the HA gene of H3N2 indicated that the 2010 Vietnamese sequences formed a distinct cluster, most closely related to the A/Perth/10/2010 subclade of the A/Victoria/208/2009 clade (Fig. 7). They did not group with the A/Perth/16/2009 clade (the vaccine strain). However, these two clades had similar antigenicity. These Vietnamese strains shared some similar key amino acid substitutions with the genetic groups 5 and 6 (D53N, Y94H, I230V, and E280A). They also carried the substitutions N389I, T485I, which had not been reported before. These mutations made the Vietnamese strains form the separate cluster from group 5 and 6.

When compared with the vaccine strain (A/Perth/16/2009), the Vietnamese strains had some mutations within the antigenic sites A (K144N, R150I), C (D53N, S54R, N278Y, E280A, N310H), D (R208K, T212A, S214I, I230V), and E (K62E, G78D, Y94H) on the HA protein (Fig. 8).

The NA phylogenetic tree was generally in agreement with that of the HA (Fig. 7). The NA protein also showed some mutations compared with the vaccine strain, in which 6 located at 3 antigenic sites F' (D339N), I' (S367N, K369T), L' (I464L, L466F, L466I) (Fig. 8).

The M gene analysis showed that all strains were resistant to Amantadine due to possession of the S31N substitution in the M2 ion-channel protein.

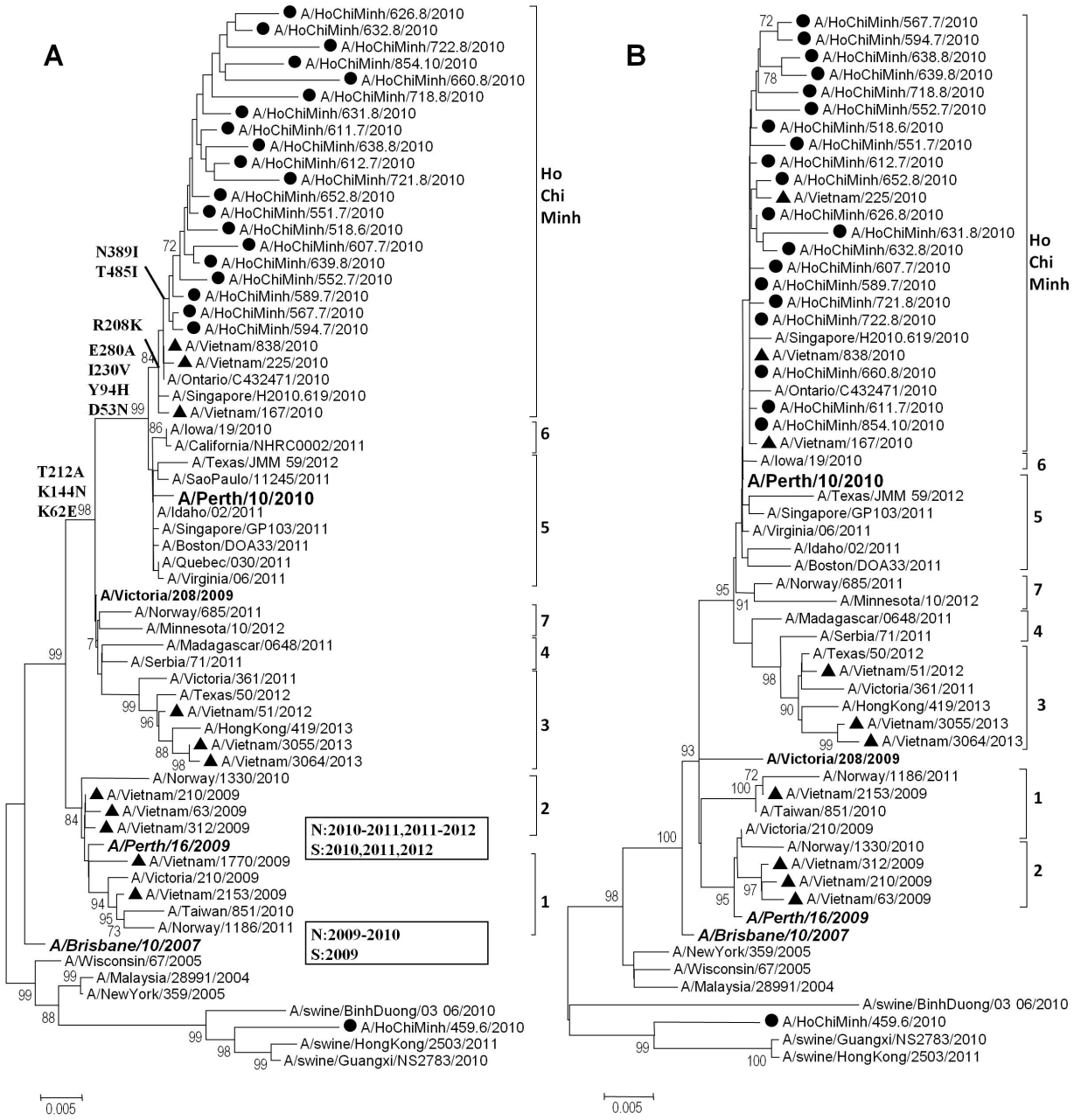


Figure 7. Phylogenetic analysis of HA (A) and NA (B) genes of influenza H3N2 viruses circulating in southern Vietnam during 2010-2011.

Phylogenetic trees were constructed with MEGA 5 software using the neighbor-joining method. Bootstrap values of greater than 70 % are shown at the branch nodes. Characteristic mutations of a particular branch are indicated on the left side nodes. The genetic group assignment is indicated by the brackets on the right. The influenza strains in this study are marked with solid round. The influenza strains from Vietnam are marked with solid triangles. The Perth/10/2010 sub-clade is boldfaced. Vaccine strains for the Northern (N) and Southern (S) Hemisphere are shown in bold and italic and corresponding years are enclosed in boxes.

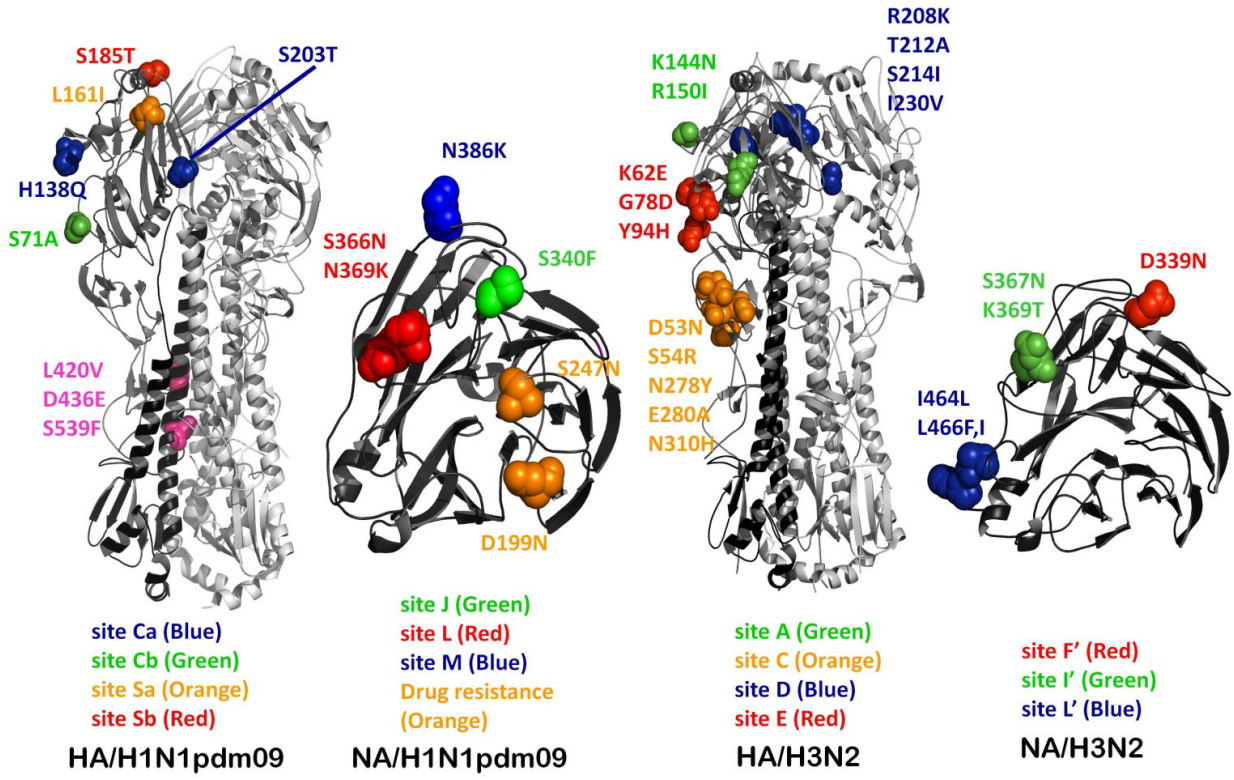


Figure 8. Mapping of mutations in antigenic sites of HA, NA proteins of influenza viruses in Vietnam during 2010-2011.

Three dimensional structures of HA and NA were downloaded from the Protein Data Bank (RCSB PDB, http://www.pdb.org) and visualized using PyMol (http:www.pymol.org). The amino acids in the antigenic sites of Vietnamese strains were compared with vaccine strains A/California/07/2009 (H1N1pdm09) and A/Perth/16/2009 (H3N2). Amino acid substitutions at each antigenic site are color-coded. The drug resistant mutations in NA of H1N1pdm09 are also shown. PDB entry: 3LZG (HA, H1N1pdm09), 3NSS (NA, H1N1pdm09), 1MQL (HA, H3N2), 1IVG (NA, H3N2).

Interestingly, one patient in this study was detected with the swine influenza H3N2 variant (A/HoChiMinh/459.6/2010). This variant clustered together with other swine influenza viruses from Binh Duong province (Vietnam), Hong Kong and China.

4. DISCUSSION

Influenza virus is a major pathogen that causes epidemics with thousands of human deaths every year. Worldwide influenza epidemics were considered to be seeded by viruses originated from Southeast and East Asia (73). The lack of information about temporal and geographic circulation of influenza virus in these areas can threaten human health and compromise the prevention and control strategies. In this study, the seasonal, clinical and molecular characteristics of influenza A viruses circulating in Vietnam during the post-pandemic period 2010-2011 were reported.

Unlike in temperate countries where a single peak of influenza infections is usually observed during winter seasons, in this study, influenza viruses were circulated throughout the year with 2 peaks occurring in the rainy and dry seasons. Not as expected, during the rainy season right after the pandemic period, no H1N1pdm09 virus was detected. Only H3N2 virus was detected in this rainy season (May – October), which coincides with the winter season in the Southern Hemisphere. During the following dry season (November – April), which coincides with the winter season in the Northern Hemisphere, H3N2 virus was completely replaced by H1N1pdm09. The national surveillance in Vietnam from 2006 to 2009 also revealed two peaks of seasonal influenza each year, one between June and October, and an additional minor peak during the winter time of the Northern Hemisphere (88, 89). Similar bimodal seasonal pattern of influenza activity was also reported in Thailand, Indonesia and India (90). Determining the onset and the duration of influenza epidemics in the community is critical in guiding the appropriate timing for influenza vaccination, especially for high risk individuals, as well as guiding the use of antiviral agents.

Vietnam has issued guidelines for the use of seasonal influenza vaccines and has been using the Northern Hemisphere formulation vaccine in the private sector (91). Due to

limitation of data on influenza seasonality, the usage of influenza vaccine composition was historically based on its hemispheric location. However, the phylogenetic analysis showed that the mid-2010 Vietnamese H3N2 viruses grouped with viruses from other countries in the winter 2010 – 2011 and belonged to the A/Perth/10/2010 subclade (Fig. 7). This subclade was antigenically similar to vaccine strain A/Perth/16/2009 in the 2010 winter season of the Southern Hemisphere and in the 2010 – 2011 winter season of the Northern Hemisphere. The Vietnamese H3N2 viruses did not group with the vaccine strain A/Brisbane/10/2007 in 2009 – 2010. These data proved that the influenza activity in Vietnam appeared earlier than in other temperate climate countries in the Northern Hemisphere, and the current Northern Hemisphere vaccine usage may be too late to provide the optimal protection. These findings are consistent with the recent genetic relatedness analysis of seasonal influenza A viruses circulating in Vietnam from 2001 to 2009, which showed the high match with the Southern Hemisphere vaccine strains (29). Therefore, these results, taken together with previous studies (29, 88-90), suggest that the Southern Hemisphere formulation and timing should be considered for use in Vietnam instead of the current Northern Hemisphere one. This is in accordance with reports from Brazil emphasizing that the adoption of seasonal influenza vaccine based on hemispheric location may not apply to tropical countries (92).

It is also noted that the sub-clade A/Perth/10/2010, to which the strains in this study belonged, emerged and spread worldwide in the winter season of 2010 – 2011 (93). Some of the amino acid changes of this sub-clade were located in the globular head of the HA, with Y94H being exposed at the surface of the trimer. The I230V substitution was located within the receptor binding pocket. These mutations may contribute to the lower reactivity of this sub-clade to antiserum raised against the vaccine strain A/Perth/16/2009 (93).

Regarding H1N1pdm09, phylogenetic analysis showed that there was co-circulation of different genetic groups in Vietnam during the post-pandemic period. None of these

genetic groups were considered antigenically distinct from the vaccine strain. Most of the Vietnamese strains belonged to group 7, clustered with contemporary strains from East and Southeast Asia, Australia during 2010 – 2011. Moreover, they also tended to group with the North and Central American strains during the 2011-2012 season. These observations suggest that the American influenza viruses in 2011 – 2012 may have an Asian origin.

Some of the amino acid substitutions were observed to locate within the major antigenic sites of the HA globular head. The S203T located within the Ca antigenic site but its buried position near the monomer-monomer interface makes its role unclear (82). The S185T located within the receptor binding site and the A197T located nearby may affect the binding of HA with its receptor (94). The E374K located in the HA oligomerization interface may have role in membrane fusion (95). The mutation D222G associated with more virulent genotype was not present in these samples (96). Recent studies have also found some important antigenic sites in the stem of HA, of which, the 2 unique mutations L420V and D436E in Vietnamese strains were located in the R1 and R2 antigenic sites (97). However, the antigenic properties of these viruses against the vaccine strain were not examined in this study. Therefore, whether these changes affect the vaccine effectiveness needs to be elucidated.

In addition, the mutation associated with oseltamivir-resistant H275Y was not detected in this study. Instead, 2 mutations D199N and S247N on the NA, which reduced the neuraminidase inhibitor susceptibility, were found. It should be noted that none of the patients in this study were treated with antiviral drugs. The S247N was observed in viruses from Australia and Southeast Asia with mild reduction in oseltamivir and zanamivir susceptibility (98). The D199N mutation has recently detected from patients with oseltamivir resistance (99, 100) and exhibited to reduce susceptibility to oseltamivir and zanamivir (101). Although the D199E/G/N mutations have respectively shown to reduce susceptibility to

oseltamivir in seasonal H1N1, H5N1, and B influenza viruses, the effect of D199N mutation among H1N1pdm09 needs further investigation (99). It is also important to note that the viruses in this study were not cultured to avoid the generation of adaptive mutations *in vitro*.

In this study, the detection rate was lowest in infant younger than 6 months, increased with increasing age and reached the highest rate among children 24 – 59 months. This phenomenon implies that maternal antibodies may provide protection against influenza during infancy. Previous research has demonstrated that this protection lasted up to one year after maternal influenza immunization (102). When the children become older, the involved influenza virus strains have drifted far apart and therefore the previous protection could not cross-protect against the new strains. Although the H1N1pdm09 caused significant concern and increased recognition of influenza as an important public health issue, H1N1pdm09 was not associated with more severe disease than seasonal influenza in this study. This result was also supported by similar reports from Australia (103), Canada (104), Netherlands (105), and United States (106).

The data reported in this study have several limitations. First, only 14 months of data were available. Additional surveillance data are needed to confirm the seasonality and the circulation of influenza viruses. Second, a small number of both H1N1pdm09 and H3N2 influenza cases may hamper the ability to reveal the significantly clinical differences among them. According to the hospital's triage process, patients with suspected influenza infections will be referred to Infectious Diseases ward for isolation and treatment. As only samples from Respiratory ward were collected, the number of influenza detection in this study may be underrepresented. However, this result was in line with the finding of recent systematic review indicating that influenza detection rate was 3% (2.2% - 4.0%) among children under 5 years of age hospitalized with acute respiratory infection (107). Moreover, the detection rate might depend on different types of influenza virus affecting individuals of different ages,

with school-aged children were the most commonly affected (89, 108). Most of patients in this study were < 2 years of age might explain for the low influenza detection rate. Third, the data were only collected on patients seeking hospital care, thus the results may not reflect episodes of milder disease occurring simultaneously in the community. Finally, the study was lack of the antigenic assay against vaccine strains and the antiviral drug susceptibility tests. Therefore, the inferences regarding the protection of vaccine strains or of antiviral drugs are limited. However, the genetic data are also useful since amino acid mutations in some important regions usually correlate with significant antigenic changes (109).

Although safe and effective influenza vaccines have been used for more than 60 years, they are still not available as a part of public health programs in many Asian tropical countries. Nevertheless, the recent pandemic influenza in 2009 as well as the ongoing outbreaks of emerging avian influenza H5N1 and H7N9 have increased the awareness about the public health importance of influenza and the need to control influenza in this area. Currently, Vietnam and some other countries are developing their domestic capacity to produce influenza vaccine in response to pandemic preparedness. The findings in this study may help to understand better the influenza seasonality and the viral circulation, which are essential for deciding the optimal time for vaccination as well as the suitable vaccine formulation. These data may help policy-makers to inform and develop the influenza prevention and control strategies, not only in Vietnam but may also be applied throughout the region with similar influenza seasonal pattern. Continuing surveillance and characterization of circulating viruses in order to identify the emergence of genetic, antigenic, and drug-sensitivity changes is critical to sustain the prevention and control policy.

CHAPTER 4

MOLECULAR EPIDEMIOLOGY AND DISEASE SEVERITY OF HUMAN RESPIRATORY SYNCYTIAL VIRUS IN VIETNAM

1. INTRODUCTION

Respiratory syncytial virus (RSV) is the major cause of ARIs among infants and young children worldwide (110). The clinical presentations can vary from mild URTIs to life threatening bronchiolitis and pneumonia which result in significant pediatric hospitalization and economic burden (111). Primary RSV infections occur during the first year of life in more than 50 % of infants, and by 2 years of age, almost all children have been infected at least once (112). RSV can cause re-infections throughout life with milder disease, indicating that either RSV infection induces an inadequate immune response or genetic variability of RSV is extensive (113). RSV is a negative-sense single-stranded RNA virus that belongs to the *Paramyxoviridae* family. RSV is divided into two major groups, A and B, initially based on the reaction of the virus with monoclonal antibodies against the major structural glycoproteins G and F (114) and later by genetic analysis (115). Each group can be further subdivided into genotypes by nucleotide sequence variability. The attachment glycoprotein G is the most divergent viral protein, both between and within the two groups, and a major target for neutralizing and protective antibody responses (116).

Along with the F protein, the second variable region at the C-terminal of the G protein that contains much of the G gene variability is commonly used in molecular epidemiological studies (117). So far, RSV group A is divided into 8 genotypes (GA1 to GA7, and SAA1), and so is RSV group B (GB1 to GB4, SAB1 to SAB3, and BA) (118). BA, which was first isolated in Buenos Aires in 1999, is a new genotype of group B with a 60-nucleotide duplication in the second variable region of the G protein gene (119). The two groups

circulate independently, but often at the same time, although group A viruses tend to predominate (112, 120). The presence of two groups has led to the speculation that there might be a relationship between the RSV-group infections and clinical severity. A number of studies were carried out but such a relationship has not been fully elucidated (121).

Although RSV has been recognized as an important pathogen in childhood, there is no published information regarding the molecular epidemiology and clinical characteristics of RSV in Vietnam. The aims of this study were to investigate the molecular epidemiology of RSV, as well as to compare the clinical characteristics of diseases associated with the detection of group A and B strains in hospitalized children in Ho Chi Minh City, Vietnam.

2. MATERIALS AND METHODS

Patients and samples

The clinical sample and data collection was described in detail in chapter 1.

Virus detection

All specimens were screened for RSV and other respiratory viruses such as influenza virus A and B, human metapneumovirus, parainfluenza virus types 1 to 4, human rhinoviruses (HRV), human coronaviruses (229E and OC43), adenovirus and human bocavirus by using multiplex semi-nested (RT)-PCR as described in chapter 1. The detection primers were targeted to the conserved region of nucleoprotein gene of RSV.

Genotyping of RSV

All samples positive for RSV by screening test were then subjected to grouping and genotyping by a semi-nested PCR as described previously (117). The second hypervariable region of the G protein gene of RSV was the target for the outer and inner PCRs. The final product sizes were possible for differentiating RSV group A, group B, and genotype BA with each other. The genotypes within each group were further identified by sequence analysis.

The products of nested PCR were sequenced bi-directionally by the commercial company (Macrogen Japan Corp., Tokyo, Japan). The nucleotide sequences were analyzed and compared with the reference strains available in the GenBank database. The sequence data and the phylogenesis were analyzed using BioEdit v.7.0.5 (77). A parsimony analysis was also conducted using MEGA version 3.1 (122). The method was performed using close-neighbor interchange with a random option and with 1,000 bootstrap repetitions.

The sequences of RSVs detected in this study have been submitted to GenBank and assigned accession numbers JX079948-JX079993.

Statistical analysis

Demographic and clinical characteristics were compared between patients with RSV positive and those negative for RSV, between patients with RSV mono-detection and those with RSV co-detection, and among patients with RSV A, RSV B and mixed detection of both RSV A and B. Values were given as percentages for categorical variables, and as median with interquartile range (IQR) for continuous variables. Bivariate associations were assessed by using χ^2 test or Fisher's exact test for categorical variables, and by using the Mann-Whitney *U* test or Kruskal-Wallis test for continuous variables, when appropriate. A two-sided value of $p<0.05$ was considered statistically significant. All analyses were conducted using the Statistical Package for Social Sciences version 16.0 (SPSS, Inc., Chicago, IL, USA).

3. RESULTS

RSV detection and seasonal pattern

RSV was identified in 23.8% (257/1,082) of patients, represented the second most common virus following HRV. Additionally, RSV was co-detected with other viruses in 28.4% (73/257) of all RSV positive samples. Co-detection between RSV and HRV was the most frequent (48 cases), followed by HBoV (15 cases). RSV A was the dominant subgroup

with 91.4% (235/257). RSV subgroup B had 5.1% (13/257). Of note, 9 cases (3.5%) contained both subgroup A and B. The RSV epidemic occurred during the rainy season, from May to October (Fig. 3).

Molecular epidemiology of RSV

Twenty-seven RSV A and 19 RSV B samples were selected randomly for sequencing. Twenty-seven of the group A strains clustered into one genotype, GA2 (Fig. 9). Of these, 9 sequences were found only once. Of the remaining 18 sequences, 5 sequence groups were found, with 2 to 9 isolates per group. The rates of divergence between prototype strain A2 and the Vietnamese strains were 12.2% to 14% at the nucleotide level and 24.2% to 28.8% at the amino acid level. Differences of up to 3.2% at the nucleotide level and 7.6% at the amino acid level were observed among the group A Vietnamese strains. The nucleotide and amino acid distances between the Vietnamese strains and the GA2 reference strains were up to 9% and 21.2%, respectively. The G protein gene of one of these strains, 1310-HCM/05.11-A, was identical to three sequences in GenBank, from Brazil (EU625712-SPIAL 1401/2006), Thailand (FJ489656-1359/BKK/07), and Scotland (HQ731741-R9061/07-08).

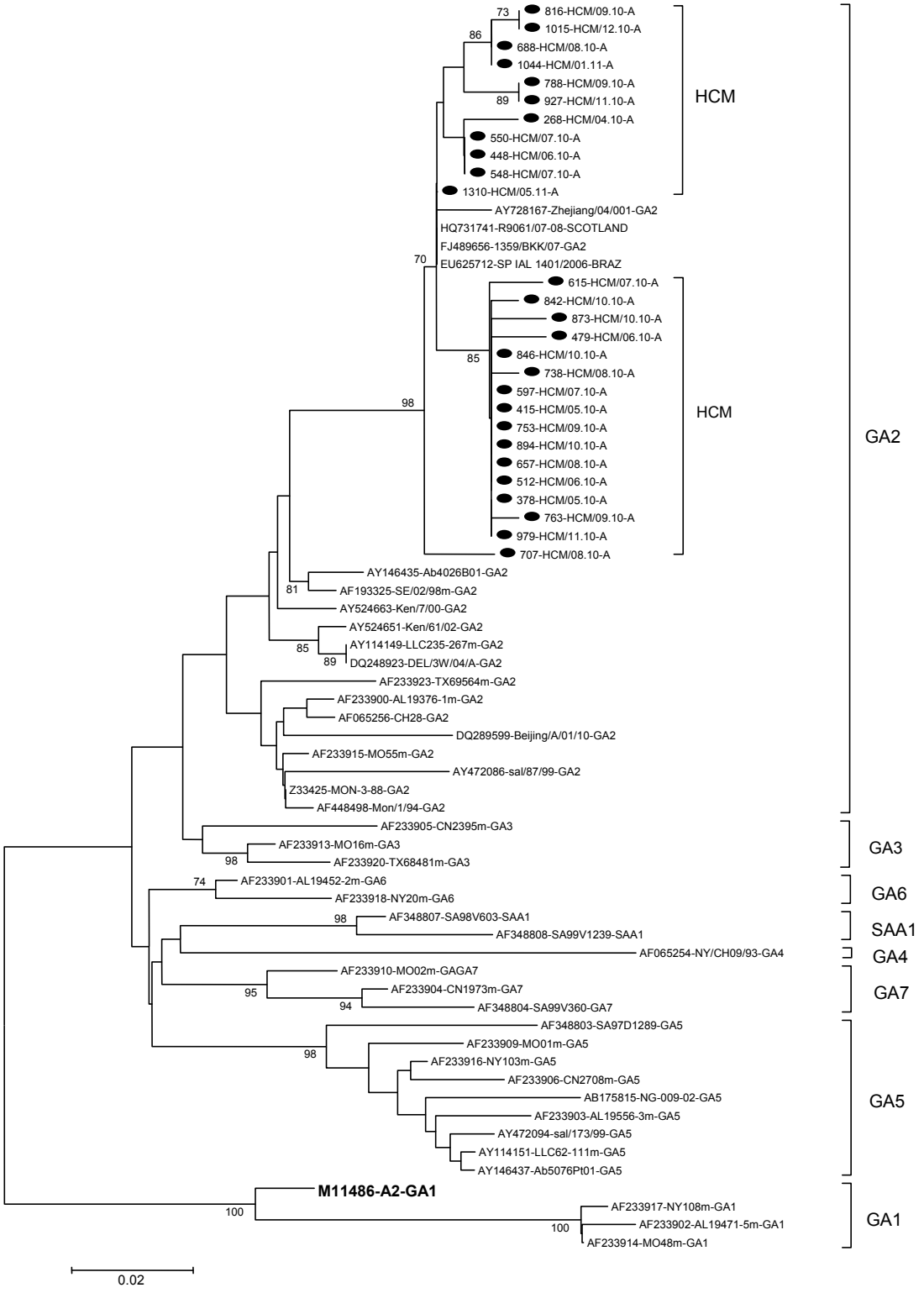


Figure 9. Phylogenetic tree for RSV-A nucleotide sequences based on 2nd variable region of G gene.

Phylogenetic tree was constructed with MEGA 3.1 software using the neighbor-joining method. Bootstrap values of greater than 70 % are shown at the branch nodes. The RSV strains in this study are marked with solid round. Prototype strain A2 (in bold face) for group A was also included. The genotype assignment is indicated by the brackets on the right.

All 19 of the group B strains belonged to the recently identified BA genotype, with a 60-nucleotide duplication in the second variable region of the G protein gene (119). These BA genotype strains were further clustered into 2 recently described subgenotypes BA9 and BA10 (123) (Fig. 10). The nucleotide and amino acid variations among the Vietnamese BA genotype strains were up to 7% and 12.9%, respectively. There was 2.5% to 6% divergence at the nucleotide level and 4.8% to 11.3% divergence at the amino acid level between the Vietnamese strains and the prototype genotype BA. Regarding the BA9 subgenotype, differences of up to 6% at the nucleotide level and 8.1% at the amino acid level were observed when comparing Vietnamese strains with reference strains. The nucleotide and amino acid distances between Vietnamese BA10 strains and BA10 reference strains were up to 5.5% and 11.2%, respectively.

The deduced amino acid sequences of the group A and group B isolates were compared to those of the prototype A2 and BA strains, respectively (Fig. 11). Twenty-seven of the Vietnamese group A viruses exhibited changes in the stop codon position compared with that of prototype strain A2, which had 298 amino acids in the deduced G-protein sequence. Among these, 26 Vietnamese group A strains had a predicted G protein of 297 amino acids, while 1 strain (763-HCM/09.10-A) had a premature stop codon that shortened the G polypeptide to 286 amino acids.

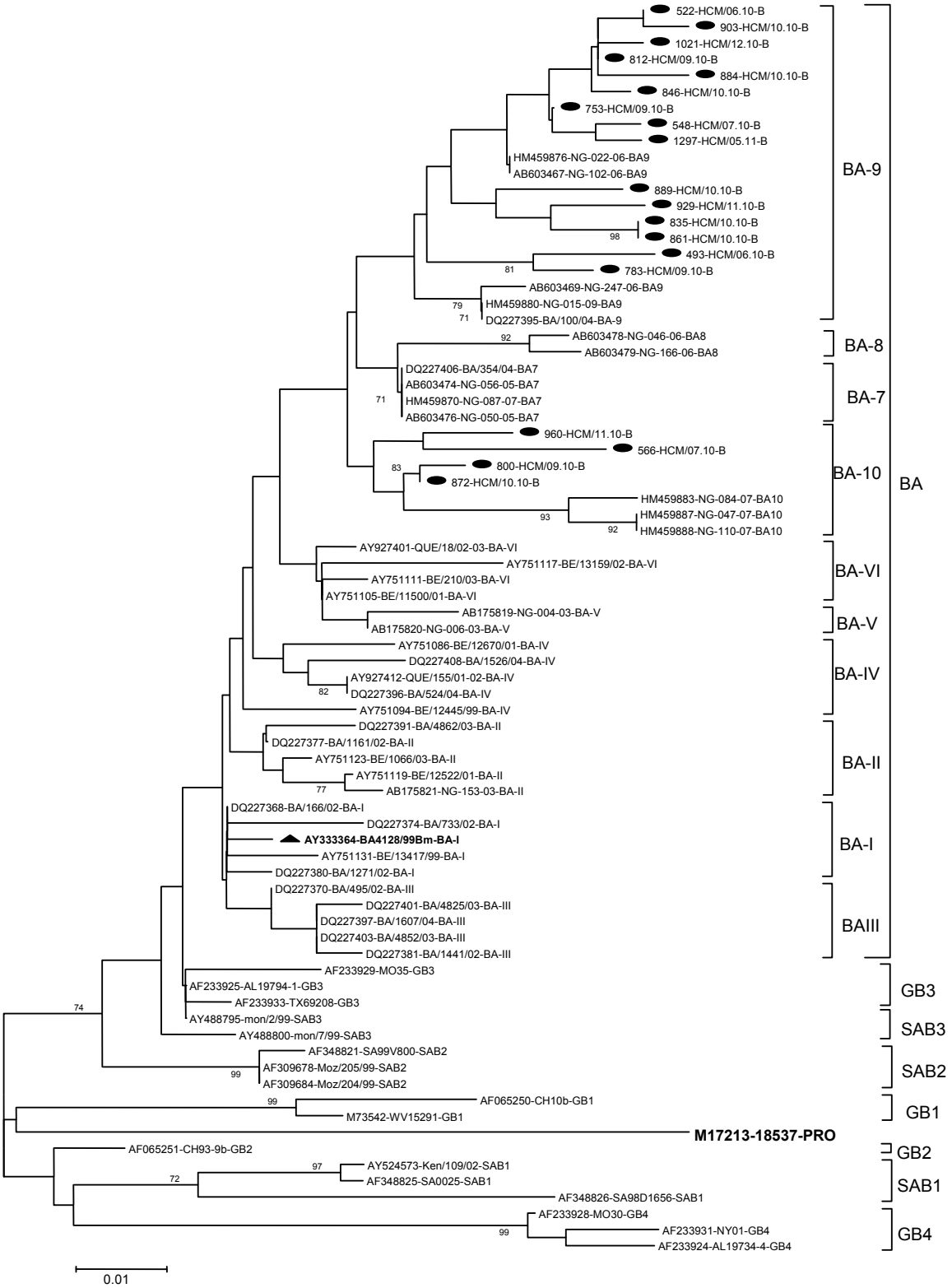


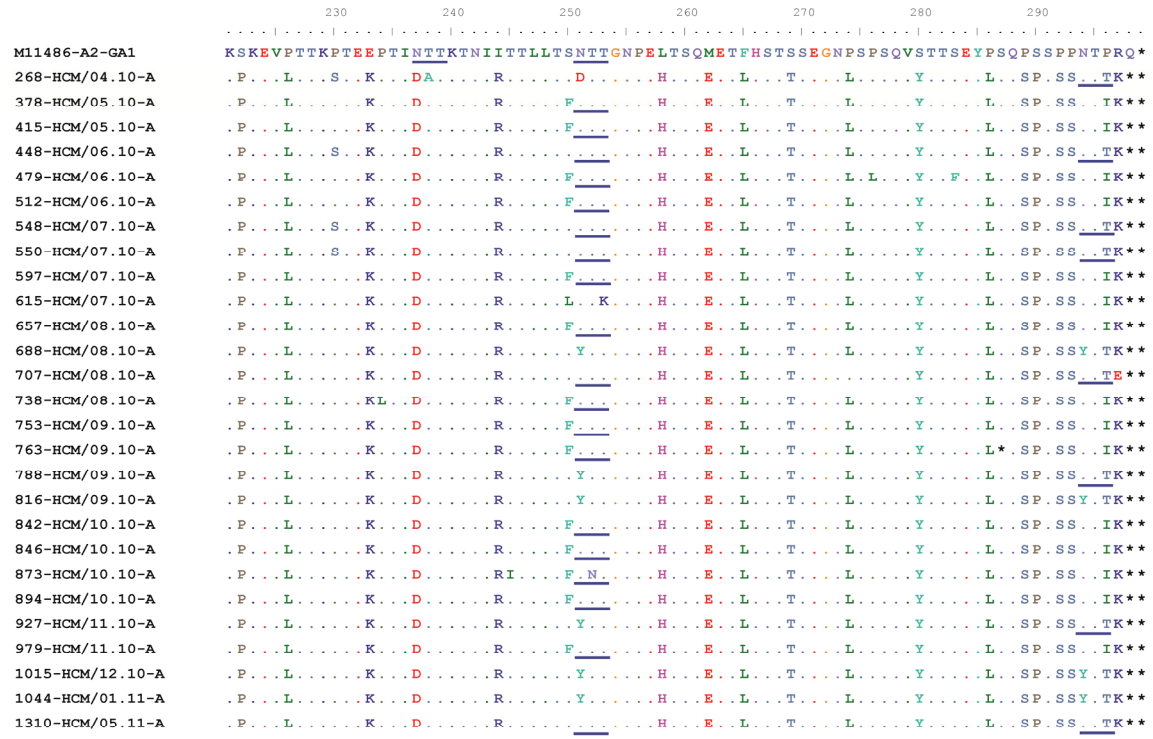
Figure 10. Phylogenetic tree for RSV-B nucleotide sequences based on 2nd variable region of G gene.

Phylogenetic tree was constructed with MEGA 3.1 software using the neighbor-joining method. Bootstrap values of greater than 70 % are shown at the branch nodes. The RSV strains in this study are marked with solid round. Prototype strain of BA genotype is marked with solid triangle. Prototype strain 18537 for group B was also included. The genotype assignment is indicated by the brackets on the right.

The G protein genes of the BA genotype were predicted to encode proteins of 2 different lengths, 312 (16/19 isolates) and 319 (3/19 isolates) amino acids. A serine (S) to proline (P) substitution was found within the 20-amino acid duplicated region at position 247 in all the Vietnamese BA strains. In addition, 14 of the 15 BA9 strains had a V271A substitution relative to the BA prototype strain, whereas all 4 BA10 strains had an E292G change. The majority (11/15) of the BA9 isolates also had amino acid substitutions at positions 270 (T270I) and 287 (H287Y).

The G protein is heavily glycosylated with both N-linked and O-linked sugars. Two putative N-glycosylation sites (NXT, where X is not proline) were identified in the second variable region of the G protein among the majority of the RSV A strains. The amino acid substitutions at some positions made these strains gain or lose the N-glycosylation sites. Similarly, two N-glycosylation sites were found to be conserved in all BA strains examined. Two of the BA10 isolates, 960-HCM/11.10-BA10 and 566-HCM/07.10-BA10, each acquired an additional N-glycosylation site as a result of amino acid substitutions at P231S and K258N, respectively.

A



B

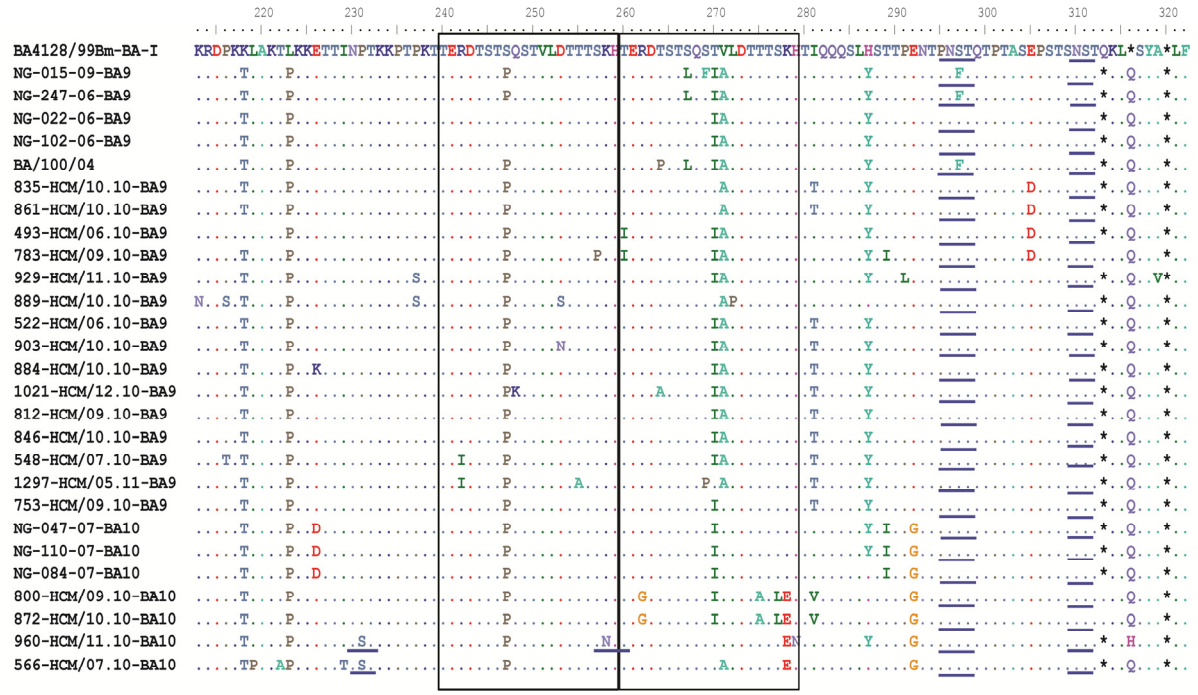


Figure 11. Amino acid alignments of 2nd variable region of G protein from RSV-A (A) and RSV-B (B).

Alignments are shown relative to the sequences of prototype strain A2 (GenBank accession number M11486) (A) and genotype BA strain BA4128/99B (GenBank accession number AY333364) (B). The amino acids shown correspond to strain A2 G protein positions 221 to 299 for the group A viruses or to strain BA4128/99B G protein positions 213 to 320 for the group B viruses. Identical residues are indicated by dashes. The two copies of the duplicated 20-amino-acid region in group B viruses are indicated by rectangles. Stop codons are indicated by asterisks. Potential N-glycosylation sites (NXT, where X is not proline) are underlined.

Disease severity associated with RSV detection

To examine the disease severity in the RSV positive and negative groups, clinical and demographic data were compared (Table 11 and 12). With regard to age, RSV positive children were significantly younger than RSV negative ones (7 vs. 11 months, $p<0.001$). RSV positive patients were admitted to the hospital earlier in the course of their disease than RSV negative patients (2 vs. 3 days, $p=0.017$). They also had higher rates of fever (72.8 vs. 65.5%, $p=0.033$), runny nose (82.5 vs. 70.5%, $p<0.001$), and chest retraction (68.5 vs. 51.0%, $p<0.001$). Most importantly, the clinical severity score of the RSV positive children was significantly higher than the RSV negative group (12 vs. 11, $p=0.003$). Regarding the diagnosis, RSV positive patients were more likely to have bronchiolitis (47.1 vs. 28.8%, $p<0.001$).

To address the question of whether children with RSV co-detection have different characteristics to those with RSV mono-detection, attempts were made to compare these two groups (Table 24 and 25). Patients with RSV co-detection were more likely to be hypoxia than those with RSV single detection (Table 25).

Table 24. Demographic characteristics and medical history associated with RSV- mono-, co-detection and subgroups.

Characteristics (%)	RSV mono N=184	RSV co N=73	p-value[b]	RSV A N=163	RSV B N=12	RSV A&B N=9	p-value[b]
Male	64.1	60.3	0.570	65.6	41.7	66.7	0.244
Age (m)[a]	7(3-13.5)	8(2-14)	0.914[c]	7 (3-13.5)	8.5 (3-16)	8 (2-11)	0.733[d]
Age group			0.266				0.836
<6m	41.8	45.2		42.3	33.3	44.4	
6-<12m	29.3	19.2		28.2	33.3	44.4	
12-<24m	17.9	26.0		17.8	25.0	11.1	
≥24m	10.9	9.6		11.7	8.3	0.0	
Prematurity (<37 wk)	10.3	13.7	0.512	9.8	16.7	11.1	0.751
Malnutrition	6.5	5.5	1.000	4.9	25.0	11.1	**0.021**
Days before hos.[a]	2(2-4)	3(2-4)	0.952[c]	2 (2-4)	2 (1-2)	3 (3-5)	0.065[d]

Abbreviation: d, day; m, month; wk, week; hos, hospitalization; RSV, respiratory syncytial virus; NA, not applicable; mono, mono-detection; co, co-detection;
Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets;
[b]Chi-squared test was used except [c]Mann-Whitney-*U* test and [d]Kruskal-Wallis test were applied for continuous variables.

Table 25. Clinical features, diagnosis and treatment characteristics associated with RSV- mono-, co-detection and subgroups.

Characteristics (%)	RSV mono N=184	RSV co N=73	p-value[b]	RSV A N=163	RSV B N=12	RSV A&B N=9	p-value[b]
Fever	73.9	69.9	0.536	73.0	66.7	100.0	0.168
Cough	93.5	94.5	1.000	92.6	100.0	100.0	0.437
Runny nose	85.3	75.3	0.069	84.7	83.3	100.0	0.440
$SpO_2 \leq 92\%$	3.8	11.0	**0.038**	3.7	8.3	0.0	0.596
Tachypnea	41.8	41.1	1.000	44.2	33.3	11.1	0.122
Chest retraction	69.0	67.1	0.786	69.9	50.0	77.8	0.299
Wheezing[d]	61.4	56.2	0.481	62.6	25.0	88.9	**0.008**
Clinical Severity Score[a]	12(8-13)	12(8-13)	0.326[c]	12 (8-13)	8 (7-8)	13 (8-14)	**0.005**[d]
Diagnosis			0.234				0.854
URTIs	18.5	9.6		19.6	16.7	0.0	
Croup	1.1	0.0		1.2	0.0	0.0	
Bronchiolitis	44.6	53.4		43.6	50.0	55.6	
Pneumonia	35.9	37.0		35.6	33.3	44.4	
Hos. duration[a]	6(4-8)	6(4-7)	0.983[c]	6 (4-8)	6.5 (3.5-8)	6 (3-6)	0.337[d]

Abbreviation: d, day; m, month; wk, week; hos, hospitalization; URTI, upper respiratory infection; RSV, respiratory syncytial virus; NA, not applicable; mono, mono-detection; co, co-detection;
Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets;
[b]Chi-squared test was used except [c]Mann-Whitney-*U* test and [d]Kruskal-Wallis test were applied for continuous variables.

To assess the relationship between clinical severity and RSV subgroup, subjects that were co-detected with other viruses were excluded. One hundred and sixty-three cases with RSV A, 12 cases with RSV B, and 9 cases with mixed detection of both RSV A and B were compared (Table 24 and 25). No significant differences were found with respect to the presence of fever, cough, runny nose, tachypnea, chest retraction, and hypoxemia among the three groups. However, both wheezing and the clinical severity scores were greater in patients with group A and mixed A and B detection than those with group B detection ($p<0.01$).

4. DISCUSSION

RSV is one of the most important respiratory pathogens among infants worldwide. The identification of RSV in about one fourth of cases in this study confirmed that this virus is a dominant agent of respiratory disease in children. This finding is in line with previous reports from Vietnam and other countries (26, 27, 45, 46, 61, 124).

Regarding the seasonality in this study, the RSV epidemic peaked during the rainy season. RSV activity was completely absent for the 3 months of the dry season (February to April, 2011). The presence of RSV seasonality in relation to rainfall has been observed in India (125), Hong Kong (126), Thailand (62), the Philippines (62), Colombia (127), and Kenya (128). In tropical regions, children tend to be kept indoors during the rainy season, and the resultant crowding may account for the increased incidence of RSV infection. Another reason that has been suggested is that high humidity may help to prevent the virus from desiccation and loss of infectivity.

RSV is the most common viral pathogen causing LRTIs among infants and young children. However, there has been no information about the molecular epidemiology of RSV in Vietnam. This report provides data on the molecular characteristics of RSV from

hospitalized children in Ho Chi Minh City and represents the first such study in Vietnam. Information from this study will contribute to the growing database on the molecular diversity of RSV circulating worldwide. The results of this study indicated that RSV subgroup A and B co-existed in one epidemic and cases with RSV subgroup A predominated over those with subgroup B. These findings are in agreement with majority of studies in many countries around the world including Germany (129), Belgium (130), Argentina (111), Kenya (131), Japan (132), and India (117), with various patterns of subgroup predominance.

The identification of the GA2 as the predominant genotype in this study was consistent with the results of other reports. GA2 was the most common genotype of RSV group A found around the world and has persisted for many years (117, 129, 130, 132, 133). On the other hand, the G protein gene variable region of one Vietnamese strain was identical to strains from different parts of the world, including Thailand, Scotland, and Brazil.

In this study, 26 out of 27 GA2 genotype strains were predicted to encode a G protein of 297 amino acids in length, while one strain was shorter, of 286 amino acids. This mutant strain was sequenced directly from the clinical sample. To my knowledge, there has been one previous report of such a mutant strain, by Cane and Pringle (134). In that report, an isolate with the G protein of 289 amino acids was obtained from virus culture. My strain was genotype GA2. The previously reported strain was genotype GA1 (data not shown).

All Vietnamese subgroup B viruses fell into a new genotype BA, which was first detected in Buenos Aires, Argentina during 1999 (119). The major characteristic of genotype BA is that the G protein gene contains a 60-nucleotide duplication in the second variable region. The BA genotype strains in this study were further clustered into 2 recently described subgenotypes, BA9 and BA10. These subgenotypes were first described from RSV isolates in Japan in 2006 (123) but have not been reported elsewhere until now.

The Vietnamese BA strains had two different G protein lengths, 312 and 319 amino acids, which were reported in previous studies (135). In addition, alterations had occurred so that the duplicated region was no longer identical to the original one. Since its first appearance, the BA genotype has spread globally and was reported from many regions around the world. Some recent reports also showed that genotype BA has gradually replaced the other group B genotypes (119). It is possible that these changes in the G protein enhance the attachment of the virus to the host cell, or result in the antigenic modification which allows this virus to escape the immune response.

Potential N-glycosylation sites are thought to play an important role in helping viruses escape from the host immune response (136). The number and distribution pattern of glycosylation sites identified in this study were different between the 2 RSV subgroups. The group A strains in this study had 2 N-glycosylation sites within the second variable region of G protein while group B had 4 (Figure 11). Variations in the number and location of these sites can inhibit the recognition of RSV by antibodies to particular epitope (137).

The demographic and clinical information is important to put the results into a practical context and allow them to be applied in clinical practice. In the current study, statistical significance was achieved for the detection of RSV and its association with clinical severity. The RSV-positive patients tended to have more severe symptoms and a higher severity score that probably led them to seek hospital care earlier than the RSV-negative patients.

The fact that nearly half of the RSV detection (42.8%) occurred in children under 6 months showed that patients in this age group are the most vulnerable to RSV infections, despite the presence of maternal antibodies. Maternal antibodies are able to protect against severe RSV in children, however relatively high tilters are required (138). Unfortunately, with a half-life of 26 days antibodies received at birth quickly drop to unprotective levels

within the first month of life (138). The finding that RSV detection were mainly associated with bronchiolitis and pneumonia (47.1 and 36.2% of the cases, respectively), in which the diagnosis of bronchiolitis was significantly related to RSV, confirmed that this agent is important in LRTIs, as have other studies (139, 140).

The co-detection has led to the speculation that the presence of several types of virus in one respiratory specimen may affect the clinical presentation of ARIs. Viruses might interact indirectly or directly, and the effect may depend upon which viruses are co-detecting. The co-detection of RSV with other viruses increased the risk of LRTIs or severe LRTIs were also observed in other studies (141, 142). No study specified the association of RSV co-detection and hypoxia. In this study, most of RSV co-detections were in combination with HRV and HBoV, the 2 viruses that were associated with hypoxia in this population. That may explain for the increased rate of hypoxia in the RSV co-detection group.

Regarding clinical manifestation of each group, by using a composite severity score, detection of RSV group A (either alone or mixed with RSV group B) were associated with more severe disease than detection with RSV group B. Besides virus factors, the clinical severity of RSV infection is also associated with epidemiological and host factors, which include socioeconomic status, age, prematurity, and underlying heart and/or lung disease. To exclude these confounding factors, patients with underlying diseases or having co-detection with other viruses were not included.

Children with subgroup B were admitted to the hospital less frequently than those with subgroup A. Either subgroup B strains cause such a mild illness that there is no need to hospitalize the patient or the prevalence of subgroup B is truly low. However, in two studies, the proportions of children with subgroup A and subgroup B in the hospital and in the community were similar (143, 144). Although there was a small number of patients hospitalized with RSV B detection, 25% of them suffered from malnutrition. It could be

speculated that RSV B may cause mild diseases. Only on patients with risk factor such as malnutrition, RSV B may cause disease severe enough for hospitalization.

Several studies have examined the relationship between clinical severity and RSV subgroups. In approximately half of these studies, group A seemed to be associated with more severe clinical disease (143, 145-149), whereas no such difference was found in the others (120, 150-155). In only two studies have group B infections been reported to cause more severe disease (156, 157). This inconsistency could be attributed to difference in study design and population, definition of disease severity, the distribution of RSV subgroups, etc. In the recent study, Houben *et al.* reported that disease severity correlated positively with viral load during primary RSV infection (36).

The current study was limited to only one epidemic season. Because the predominant subgroups shift from year to year, the immunity against the previous circulating groups could have altered the severity of disease caused by specific subgroups. Therefore, continued observation and analysis of additional seasons is required to determine the association between subgroups and severity of disease.

In summary, RSV was found to be the agent most commonly and frequently associated with severe acute respiratory diseases in infants and children. Co-detection between RSV and other respiratory viruses were associated with hypoxia. The molecular characteristics of RSV were determined for the first time in Vietnam, and carried characteristics both similar to strains from other parts of the world and specific to Vietnam. Subgroup A and B of RSV were co-circulating and subgroup A was associated with more severe disease than subgroup B. Similar surveillance should be continued to follow the epidemiology of this virus. A better understanding of RSV epidemiology is essential to enable prediction of outbreaks and for planning preventive and therapeutic control measures.

CHAPTER 5

HUMAN BOCAVIRUS IN CHILDREN WITH ACUTE RESPIRATORY INFECTIONS IN VIETNAM

1. INTRODUCTION

Acute respiratory infections are the leading cause of morbidity and mortality among children worldwide. Viruses are responsible for most of acute respiratory infections. Up to now, many viruses have been identified as the main cause of acute respiratory infections, such as respiratory syncytial virus, human metapneumovirus, influenza virus, parainfluenza virus, coronavirus, and adenovirus. However, a rather high percentage of acute respiratory infections still remains unknown the etiology. In recent years, several newly discovered viruses were reported to have association with respiratory infections, including human bocavirus (HBoV) (7).

In 2005, HBoV was first identified in nasopharyngeal aspirate of children with acute respiratory infections in Sweden (9). This novel virus belongs to the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Bocavirus* with the single-stranded linear DNA genome of about 5.3 kb. The genome contains three open reading frames encoding 2 non-structural proteins, NS1 and NP1, as well as 2 structural capsid proteins, VP1 and VP2. Up to now, four species of HBoV have been identified, namely HBoV1, HBoV2, HBoV3, and HBoV4. HBoV1 is predominantly detected in respiratory samples. On the contrary, HBoV2 – 4 have been found mainly in stool samples (20).

Since its first discovery, HBoV has been widely detected in many countries. HBoV was mainly found in children with acute respiratory infections at the rate ranging from 1.5% to 19% (20, 158), indicating that HBoV is the third most common respiratory virus, after respiratory syncytial virus and human rhinovirus. However, in many epidemiological studies,

HBoV was co-detected frequently with other respiratory pathogens, as well as often seen in asymptomatic children, which raising the question about its causality. Despite of that, recent identification of HBoV in blood samples together with advances in serodiagnosis have provided the increasing evidence for a causal role of HBoV in respiratory illness (20). Most of the published studies so far emphasized on the prevalence and epidemiology of HBoV. Only a few reports provided data related to clinical characteristics of HBoV detection. Moreover, epidemiology of respiratory viruses seems to have the geographic and temporal characteristics.

Therefore, this study was conducted on respiratory specimens obtained from hospitalized children with acute respiratory infections during a 14-month period in Vietnam to investigate the epidemiological features and specify the clinical characteristics associated with HBoV detection. A phylogenetic analysis of HBoV was also carried out.

2. MATERIALS AND METHODS

Patients and samples

The clinical sample and data collection was described in detail in chapter 1.

Virus detection

HBoV was detected by PCR method using pan-bocavirus primers as described previously (40). Briefly, a pair of primers AK-VP-F1 (5'-CGCCGTGGCTCCTGCTCT-3') and AK-VP-R1 (5'-TGTTCGCCATCACAAAAGATGTG-3') was used in the first PCR. Another pair of primers AK-VP-F2 (5'-GGCTCCTGCTCTAGGAAATAAAGAG-3') and AK-VP-R2 (5'-CCTGCTGTTAGGTCGTTGTTGTATGT-3') was used in the nested-PCR to amplify the partial VP1 gene of HBoV (576 basepairs). In addition, each sample was also screened for other respiratory viruses by using multiplex semi-nested PCR as described in chapter 1.

Sequencing and phylogenetic analysis

All HBoV positive PCR products were sequenced by the commercial company (Macrogen Japan Corp., Tokyo, Japan). The nucleotide sequences were analyzed and compared with the reference strains available in the NCBI GenBank database. The sequence data and the phylogenesis were analyzed using BioEdit v.7.0.5 (77). A parsimony analysis was also conducted using MEGA 5 (78). The method was performed using close-neighbor interchange with a random option and with 1,000 bootstrap repetitions.

The sequences of HBoV detected in this study have been deposited in the GenBank database under accession numbers JX418234-JX418266, KF193582-KF193604.

Statistical analysis

Demographic and clinical characteristics were compared between patients with HBoV positive and those negative for HBoV, between patients with HBoV mono-detection and those with HBoV co-detection. Values were given as percentages for categorical variables, and as median with interquartile range (IQR) for continuous variables. Bivariate associations were assessed by using χ^2 test or Fisher's exact test for categorical variables, and by using the Mann-Whitney *U* test for continuous variables, when appropriate. A two-sided value of $p<0.05$ was considered statistically significant. All analyses were conducted using the Statistical Package for Social Sciences version 16.0 (SPSS, Inc., Chicago, IL, USA).

3. RESULTS

Epidemiological characteristics of HBoV

Between April 2010 and May 2011, HBoV was found in 78 samples (7.2%), representing the third most common agent after HRV (30%) and RSV (23.8%). Among these children positive with HBoV, 52 (66.7%) were mixed detection with other respiratory viruses, most frequently with HRV (n=16), followed by RSV (n=15). In addition, 5 cases had triple

detection of HBoV, HRV and RSV (n=3), FLU A (n=1), and PIV 3 (n=1). The male to female ratio of HBoV positive patients was similar to that of the enrolled patients (1.7 vs. 1.8). The children with HBoV had a median age of 12 months (range from 1 to 62 months). Children younger than 6 months had the lowest detection rate (5.0%) while those aged from 12 to 23 months had the highest HBoV detection rate (9.8%) (Fig. 4). HBoV was detected throughout the year. However, HBoV was detected more frequently during the dry season (December – April) than the rainy season (May – October) (9.8 vs. 5.7%, p=0.015, Fisher's exact test) (Fig. 3).

Clinical manifestations and disease severity

The demographic and clinical characteristics at presentation of all enrolled patients are shown in Table 13 and 14. Patients with HBoV positive tended to be older than those negative for HBoV but the difference did not reach significance (median age 12 vs. 9 months). The difference on prematurity and malnutrition rates between HBoV-positive and HBoV-negative children was also not significant. Fever, cough, runny nose, wheezing, and rales were the most frequently observed clinical signs among HBoV-positive patients. Patients with HBoV positive had a significantly higher rate of hypoxia (16.7 vs. 7.9%, p=0.007) which may result in longer hospitalized duration (median 6 vs. 5 days, p=0.02) than those were negative for HBoV. Rales were present in 76.9% of HBoV-positive patients, significantly higher than that of HBoV-negative patients, 65.1% (p=0.034). Vomiting and diarrhea were reported in 55.1% and 30.8% of patients with HBoV poitive, respectively. Abnormal findings on CXR were found in 82.1% of HBoV-positive patients. Regarding the diagnosis, HBoV was associated with diseases from the upper to the lower respiratory tract. In which, pneumonia was found more often in the children with HBoV positive than those negative (50.0 vs. 38.2%, p=0.04). No patient required mechanical ventilation. There was no fatal case.

Attempts were also made to compare the difference between HBoV mono- and co-detection groups (Table 26 and 27). The median age of children with HBoV mono-detection was higher than those with co-detection (14.5 vs. 10.5 months). However, this difference was not statistically significant (p>0.05). The demographic characteristics and clinical symptoms or signs in HBoV mono-detection and co-detection children were similar, except for prematurity which was less common in HBoV mono-detection group (0 vs. 15.4%, p=0.047, Fisher's exact test).

Table 26. Demographic characteristics and medical history associated with HBoV-mono- and co-detection groups.

Characteristics (%)	HBoV mono N=26	HBoV co N=52	*p-value*[b]
Male	65.4	61.5	0.807
Age (m)[a]	14.5(9-17)	10.5(5.5-19.5)	0.461[c]
Prematurity (<37 weeks)	0.0	15.4	**0.047**
Malnutrition	19.2	9.6	0.287
Days before hospitalization (d)[a]	3(2-5)	2(2-4)	0.596[c]

Abbreviation: d, day; m, month; HBoV, human bocavirus; mono, mono-detection; co, co-detection; Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Chi-squared test was used except [c]Mann-Whitney-*U* test was applied for continuous variables.

Table 27. Clinical features, diagnosis and treatment characteristics associated with HBoV-mono- and co-detection groups.

Characteristics (%)	HBoV mono N=26	HBoV co N=52	*p-value*[b]
Fever	65.4	76.9	0.293
Cough	92.3	92.3	1.000
Runny nose	80.8	73.1	0.580
Vomiting	46.2	59.6	0.336
Diarrhea	26.9	32.7	0.795
SpO_2≤92%	11.5	19.2	0.526
Tachypnea	53.8	42.3	0.349
Chest retraction	53.8	55.8	1.000
Wheezing	69.2	59.6	0.464
Rales	73.1	78.8	0.580
Abnormal chest X-ray	80.8	82.7	1.000
Diagnosis			0.947
URTIs	23.1	23.1	
Croup	3.8	3.8	
Bronchiolitis	19.2	25.0	
Pneumonia	53.8	48.1	
Hospitalization duration (d)[a]	6(4-9)	6(4.5-10)	0.713[c]

Abbreviation: d, day; m, month; URTI, upper respiratory infection; HBoV, human bocavirus; NA, mono, mono-detection; co, co-detection;

Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets.
[b]Chi-squared test was used except [c]Mann-Whitney-*U* test was applied for continuous variables.

Molecular characterization and phylogenetic analysis

The partial VP1 nucleotide sequences (454 basepairs) of all 78 positive specimens for HBoV were determined and compared with those of four established HBoV species 1 – 4 and the prototype strains ST1 and ST2 (DQ000495 and DQ000496). The phylogenetic analysis (Fig. 12) showed that all HBoV strains isolated in this study were clustered closely in the same branch with HBoV1 reference strains. This finding confirmed that HBoV1 was circulating in Vietnam. The sequence identity of 97.5 – 100% at the nucleotide level and 94.6 – 100% at the amino acid level were observed among the HBoV positive strains isolated. All Vietnamese strains showed high nucleotide sequence identity of 97.5 – 100% and amino acid identity of 95.9 – 100% with HBoV1 reference strains. These sequences also shared 97.7 – 99.6% and 98.0 – 100% nucleotide sequence identity as well as 95.9 – 99.3% and 96.6 – 100% amino acid identity with the HBoV prototype strain ST1 and ST2, respectively. The partial VP1 sequences of three Vietnamese strains were identical to the HBoV prototype strain ST2.

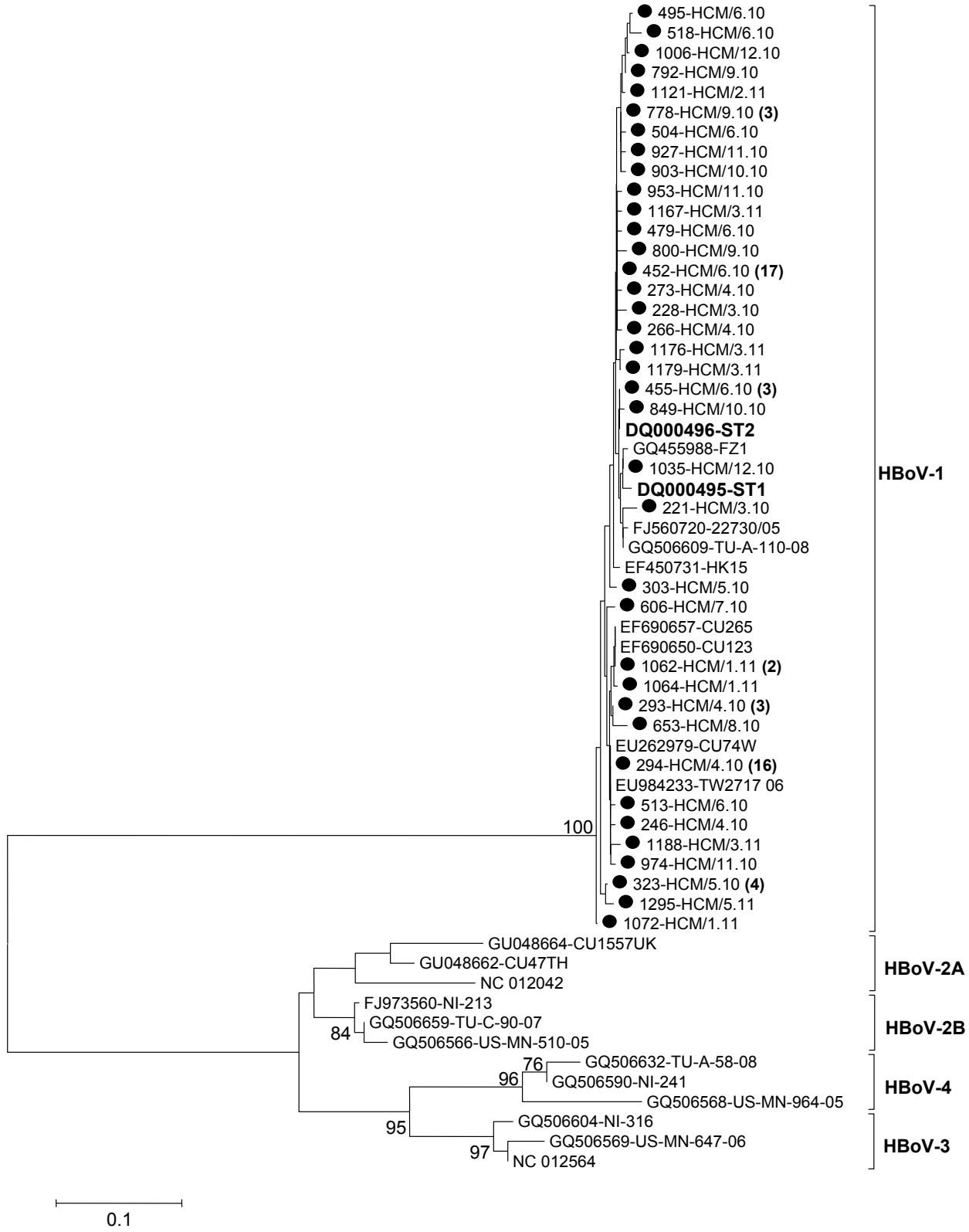


Figure 12. Phylogenetic analysis of the partial VP1 nucleotide sequences of HBoVs.
Phylogenetic tree was constructed with MEGA 5 software using the neighbor-joining method. Bootstrap values of greater than 70 % are shown at the branch nodes. The HBoV strains in this study are marked with solid round. Prototype strain ST1 and ST2 (in bold face) were also

included. Number of identical strains is indicated in the parentheses. The species assignment is indicated by the brackets on the right.

4. DISCUSSION

HBoV is a newly discovered human parvovirus that was first described in 2005 in nasopharyngeal aspirate of children with respiratory infection (9). The discovery of this virus also raised the concerns about its causative role, as well as its community and clinical impact. Since then, HBoV has been detected frequently worldwide not only in the respiratory samples (21, 27, 124, 159-162) but also in human feces (161, 163, 164), serum (165, 166), saliva (167) and urine (168). The epidemiological status and genetic characteristics of HBoV circulating in children with acute respiratory infections in Vietnam, however, remains unknown. To understand better the epidemiology of HBoV, the presence of HBoV and other common respiratory viruses in patients with acute respiratory infections in this region was investigated.

In this study, the rate of HBoV detection was 7.2% among hospitalized children with acute respiratory infections. This rate is in accordance with previously published rates, ranging from 1.5% to 19% (20). The discrepancy observed in the rate of HBoV detection between these studies may be explained by the differences in study design affecting age, recruitment criteria, study settings, study periods; the differences between hospital-based and community-based study; the differences in clinical specimen examination, diagnostic methods with different sensitivity and specificity, or regional and temporal differences. In this study, HBoV was the third most common respiratory agent after HRV and RSV, and it was more frequent than FLU A, FLU B, and PIVs. Since the evidence of HBoV as a true respiratory pathogen becomes more convincing (9, 169, 170), the high detection rate implies that HBoV is responsible for a large burden of illness in young children with regard to health care utilization.

Regarding the season, although HBoV was detected throughout the year, its peak was in the dry season from December to April of this tropical area. However, no consistent seasonal distribution of HBoV was found in many previous reports. Most studies demonstrated that HBoV was detected year round with high prevalence in the winter and spring (9, 21, 161, 168, 171), while some other reports showed the increased HBoV detection in late spring and early summer (124, 162). On the other hand, some authors did not observe the obvious seasonal activity of HBoV (172-174). Since this study spanned for about one year, in order to get a clear view on seasonality of HBoV, the continuing surveillance is necessary.

HBoV can be found in respiratory samples of all ages, but mainly in young children. In this study, the ages of HBoV-positive children ranged from 1 to 62 months, indicating that children over 5 years old were rarely detected with HBoV. The age group with the lowest detection rate was children younger than 6 months (5%). In contrast, the detection rate was highest among children 12 – 23 months (9.8%), the age at which most children have lost the maternal antibody protection, and decreased with the increasing age. These phenomena imply that maternal antibody against HBoV may protect the children during early infancy, and then, the immunity acquired from primary HBoV infection during early childhood may protect the children from getting later infection. The serological study in Japan revealed that nearly all individuals were seropositive for HBoV by the age of 5 years, and the seropositive rate was lowest in infants aged from 6 to 8 months and gradually increased with age (175).

The main symptoms of HBoV detection were cough, runny nose, fever and signs of respiratory distress such as tachypnea, lower chest retraction, wheezing, and hypoxia, which are also commonly seen among patients with respiratory infections. Therefore, it is impossible to differentiate clinically the respiratory infections caused by HBoV and other pathogens. However, in this study some clinical features which were distinct for HBoV were

also observed. Hypoxia was seen more often among children with HBoV-positive than those with HBoV-negative, which may lead to longer hospitalization duration. Moriyama et al. also observed that hypoxia was more severe in HBoV-positive patients than in RSV-positive ones (176). In this study, half of HBoV-positive patients were diagnosed as pneumonia. The existing literatures, in which HBoV infection was confirmed by serum PCR or serodiagnosis, also noted the significant association between HBoV infection and pneumonia (160, 166, 177, 178).

Co-detection of HBoV with other respiratory pathogens was found frequently, with the rate of up to 83% (20). However, HBoV viremia has been documented to be significantly more frequent in children with acute respiratory infections than in the control group, which supports the causative role of HBoV in respiratory diseases (21, 160, 165, 173). In this study, 66.7% of children with HBoV-positive were co-detected with other respiratory viruses. This result also confirmed that co-detection with other viruses was not an uncommon characteristic of HBoV. The overlapping seasonality of HBoV and other circulating viruses may explain for this phenomenon. Another explanation is the persistence, an interesting feature of HBoV (20). HBoV may persist in the airway rather long-lasting and the prolonged viral shedding may explain for the high co-detection rate observed in many studies.

Unexpectedly, no correlation was found between co-detection and clinical symptoms. The rates of severe symptoms and lower respiratory tract infections did not increase in co-detection group. Co-detection of HBoV and other respiratory viruses did not increase the disease severity. A quantitative PCR analysis may be helpful to clarify the role of HBoV to the respiratory diseases when being detected alone or together with other viruses.

The phylogenetic analysis of HBoV strains in this study and the reference strains revealed that HBoV species 1 was circulating in pediatric patients with acute respiratory infections in Vietnam. The recent review summarized the current knowledge on HBoV

demonstrated that HBoV1 is predominantly found in respiratory samples, while the rest of HBoV species, including HBoV2, HBoV3, and HBoV4, have been found mainly in human stool (20). However, two studies of children with acute respiratory infections in Korea (179) and China (180) reported that the enteric HBoV2 was also identified in nasopharyngeal samples. Recently, the research from Japan reported the identification of all four HBoV species in respiratory samples from children with respiratory tract infections (181). To determine whether all HBoV species are involved with respiratory diseases, the pan-bocavirus primers were used in this study. However, only HBoV1 was found in children with acute respiratory infections in Vietnam and is in line with the above review's findings (20). The geographical differences may explain for the different distribution of HBoV species worldwide. The absence of HBoV2 – 4 in this study suggests these viruses may not directly involve in respiratory illnesses. The sequence analysis also revealed that the circulating HBoVs were closely related to the original strains. Most polymorphisms identified in this study did not result in amino acid changes at the corresponding protein. The high similarity suggests that HBoV1 genes may be highly conserved, and serological tests should be universal application and interpretation worldwide.

In conclusion, this study provides the first data on the molecular background of HBoV among children with respiratory infections in Vietnam. Information from this study will contribute to the growing database on the molecular diversity of HBoV circulating worldwide. Moreover, these data indicated that HBoV seems to be an important and frequent pathogen in respiratory tract infection in children. Further surveillance and molecular characterization of HBoV including the healthy control group and using serology and/or PCR detection of HBoV in blood is essential to clarify the clinical impact as well as to provide further genetic information of HBoV.

CHAPTER 6

HUMAN RHINOVIRUS IN HOSPITALIZED CHILDREN: CLINICAL, EPIDEMIOLOGICAL AND VIROLOGICAL FEATURES

1. INTRODUCTION

Human rhinovirus (HRV) is the most common cause of URTIs in humans and frequently causes a mild, self-limiting illness often known as common cold. The development of PCR methods has markedly increased the detection of HRV and revealed greater association of HRV with more severe diseases in children such as bronchiolitis, pneumonia and asthma exacerbation (19, 49, 182). Therefore, their impact on overall morbidity and substantial cost for health care is thought to be considerable (183). HRV belongs to the genus *Enterovirus*, family *Picornaviridae* with highly genetic diversity. More than 150 serotypes/ genotypes of HRV have been described so far and classified into 3 main species: HRV-A, HRV-B, and the recently recognized HRV-C (184). However, the association between HRV species and clinical outcomes are still controversial. Initially, the new HRV-C species was reported to cause more severe diseases than HRV-A and HRV-B (184, 185), but recent data exhibited similar clinical syndromes across all species (186).

Moreover, HRV is not diagnosed routinely in clinical practice, and the clinical impact of HRV compared with other viruses such as influenza, RSV is still not known much. Therefore, the role of HRV in ARIs need hospitalization is also not well established, especially in tropical countries.

In this study, prospective viral surveillance among hospitalized children was performed to assess the incidence, clinical and epidemiological characteristics associated with HRV detection as well as genetic diversity of HRV.

2. MATERIALS AND METHODS

Patients and samples

The clinical sample and data collection was described in detail in chapter 1.

Virus detection and Sequencing

Multiplex nested PCR assays were used to screen for 13 respiratory viruses including HRV as described previously (187). HRV was identified by using PCR primers, targeting the highly conserved 5' untranslated region (5'UTR), that can detect all known serotypes as well as the novel HRV-C species (188). For species and type determination, another RT-PCR was performed to amplify the VP4/VP2 region on every 5th HRV-positive sample (n=58) (189). Both strains of PCR products were then sequenced by the commercial company (Macrogen Japan Corp., Tokyo, Japan).

Phylogenetic Analysis

Nucleotide sequences of the VP4/VP2 coding region (616 – 1004, numbered by EF582385) (190) were aligned along with reference sequences (obtained from http://www.picornaviridae.com). Phylogenetic trees were constructed by maximum-likelihood analysis using the MEGA5 software, with the most suitable model for nucleotide substitution estimated by MEGA5, and bootstrap analysis of 1000 replicates (78). Types were assigned when the sequences in this study had ≥90 % nucleotide similarity to a known prototype sequence or clustered with a reference sequence in phylogenetic analysis with a bootstrap value above 70%. VP4/VP2 sequences in this study are available at GenBank under accession numbers KM676336-KM676393.

Statistical analysis

Demographic and clinical characteristics of patients with HRV mono-detection were compared with those negative for any virus, those positive for other viruses than HRV, and those with HRV co-detection. Patients with HRV-A mono-detection were also compared with those with HRV-C mono-detection. Values were given as percentages for categorical variables, and as median with interquartile range (IQR) for continuous variables. Bivariate associations were assessed by using χ^2 test or Fisher's exact test for categorical variables, and by using the Mann-Whitney *U* test for continuous variables. A two-sided value of $p<0.05$ was considered statistically significant. A two sided value of $p<0.05$ was considered statistically significant. All analyses were conducted using SPSS 16.0 (Chicago, IL, USA).

3. RESULTS

Epidemiological and clinical characteristics of HRV

HRV was found in 325 patients (30%), with 91 (28%) were mixed detection with other viruses. Of these, RSV was the most commonly co-detected agent recorded (48/91, 52.7%). HRV could be detected throughout the year with several peaks occurred in July, November (2010) and January, April (2011). HRV-A and C were detected in almost every month with no clear seasonal distribution (Fig. 13).

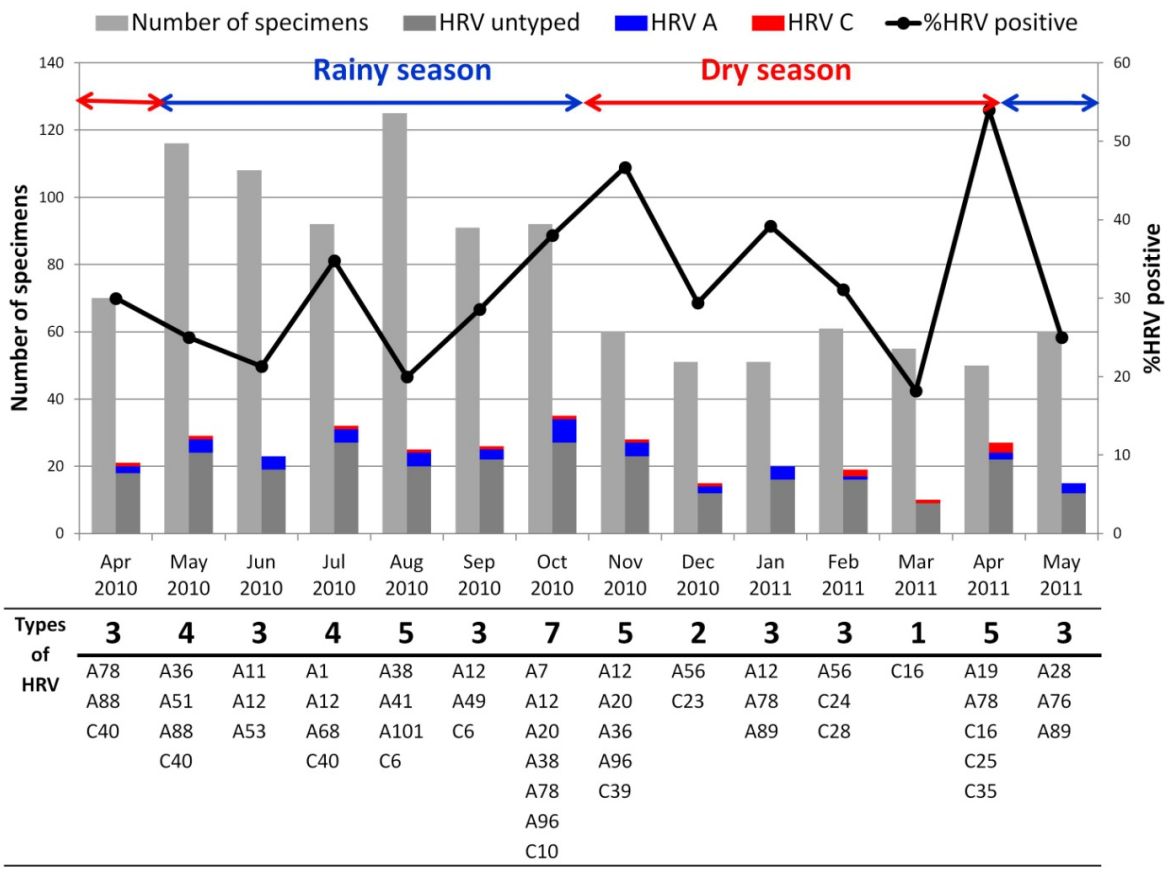


Figure 13. Monthly distribution of HRV types from April 2010 to May 2011

Abbreviation: HRV, human rhinovirus

To determine the clinical findings associated with the presence of HRV, demographic and clinical characteristics of patients with HRV mono-detection were compared with those were negative for all viruses, those positive with viruses other than HRV, and those with HRV co-detection (Table 28 and 29). The median age of HRV mono-detection patients was 9 months and comparable to other groups. The difference on prematurity and malnutrition prevalence between HRV mono-detection children and other groups was not significant. Regarding clinical symptoms, fever occurred less often in HRV mono-detection than in other groups (p<0.001). In terms of severe symptoms, children with HRV mono-detection were

more likely to have chest retraction (57.3% vs. 48.3%, p=0.031) and abnormal findings in CXR (80.5% vs. 70.7%, p=0.003) than those with PCR negative.

Of note, mono-detection with HRV had more hypoxia than other viruses significantly (12.4% vs. 5.1%, p=0.002). However, the former also had higher white blood cell and neutrophil counts than the latter (11900 vs. 11300, p=0.006 and 4895 vs. 4020, p=0.004, respectively). Compared with HRV co-detection, HRV mono-detection was less likely to have chest retraction (57.3% vs. 70.3%, p=0.032). Finally, the blood eosinophil count was significantly elevated among patients with HRV mono-detection as compared with those with PCR-negative, or detection with other virus, or HRV co-detection (p<0.001). Pneumonia was the most common diagnosis among HRV patients followed by bronchiolitis and URTIs. During the rainy season, HRV was the second most common detected virus among patients with pneumonia and bronchiolitis after RSV. However, during the dry season when the activity of RSV was low, HRV emerged as the most common virus associated with pneumonia and bronchiolitis that needed hospitalized (Fig. 14).

Regarding the association of HRV species and clinical characteristics, the difference between HRV-A and HRV-C mono-detection was compared. The median age of cases with HRV-C detection was higher than those with HRV-A detection but not significant (17.5 vs. 10, p=0.192). No significant differences were observed between HRV-A and HRV-C with regard to epidemiological and clinical characteristics (Table 28 and 29).

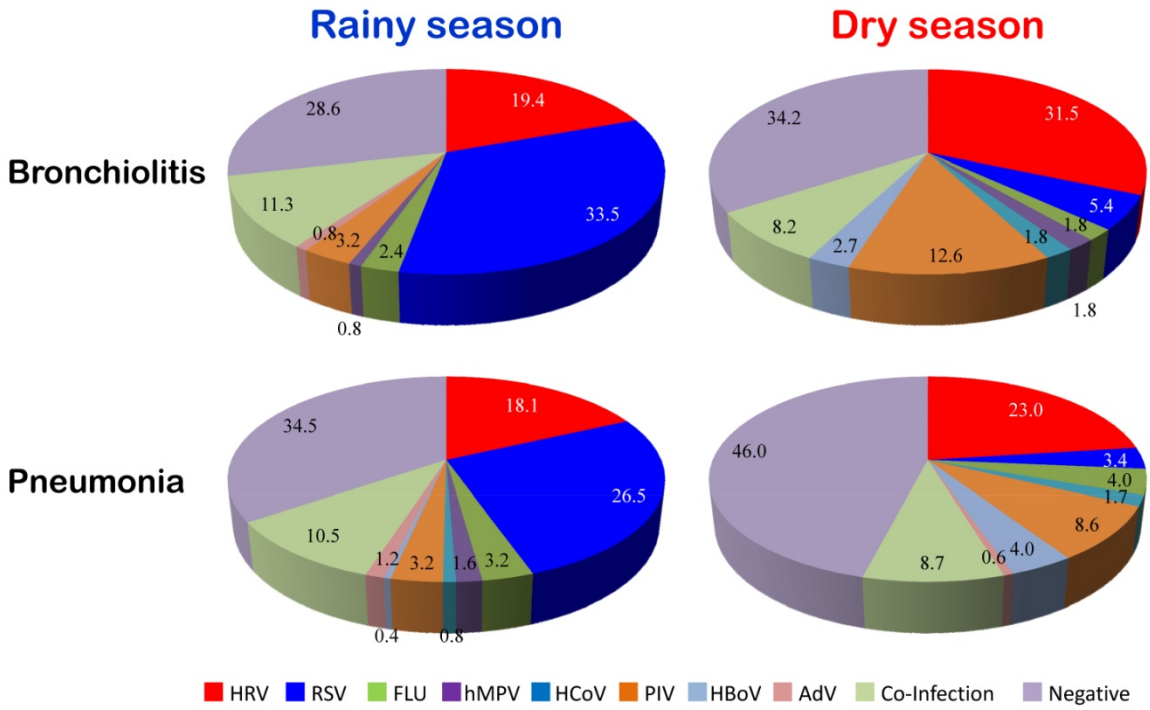


Figure 14. Proportions of pneumonia and bronchiolitis associated with HRV and RSV detection during rainy and dry seasons.

Abbreviation: HRV, human rhinovirus; RSV, respiratory syncytial virus; FLU, influenza virus; hMPV, human metapneumovirus; HCoV, human coronavirus; PIV, parainfluenza virus; HBoV, human bocavirus; AdV, adenovirus;

Table 28. Demographic characteristics and medical history of patients with HRV mono-detection compare to those with no detected respiratory virus, or with respiratory viruses other than HRV, and HRV co-detection, and HRV-A compare to HRV-C.

Characteristics (%)	HRV mono-detection N=234	PCR negative N=383	*p-value*	Other viruses N=374	*p-value*	HRV co-detection N=91	*p-value*	HRV-A (mono) N=28	HRV-C (mono) N=12	*p-value*
Male	71.8	61.1	**0.007**	63.9	**0.044**	64.8	0.228	64.3	75.0	0.716
Age (m)[a]	9(4-19)	10(4-19)	0.425	9(4-17)	0.913	9(3-14.5)	0.628	10(6-20)	17.5(8-31.5)	0.192
Prematurity	12.0	7.8	0.090	8.8	0.215	8.8	0.555	17.9	0.0	0.298
Malnutrition	9.8	11.5	0.594	8.0	0.426	6.6	0.516	7.1	8.3	1.000
Days before hos(d).[a]	3(1-5)	3(2-6)	0.127	3(2-4)	0.534	2(2-4)	0.232	3(2-6)	2.5(1-4.5)	0.342

Abbreviation: d, day; m, month; hos, hospitalization; HRV, human rhinovirus;
Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets. Categorical variables were compared by using Fisher's exact test, and continuous variables were compared by using Mann-Whitney *U* test.

Table 29. Clinical features, diagnosis and treatment characteristics of patients with HRV mono-detection compare to those with no respiratory virus detected, or with respiratory viruses other than HRV, and HRV co-detection, and HRV-A compare to HRV-C.

Characteristics (%)	HRV mono-detection N=234	PCR negative N=383	*p-value*	Other viruses N=374	*p-value*	HRV co-detection N=91	*p-value*	HRV-A (mono) N=28	HRV-C (mono) N=12	*p-value*
Fever	50.0	68.1	**<0.001**	75.9	**<0.001**	71.4	**<0.001**	64.3	50.0	0.490
Cough	90.2	88.3	0.509	93.3	0.167	93.4	0.516	89.3	91.7	1.000
Runny nose	72.6	64.5	**0.041**	81.8	**0.009**	78.0	0.397	71.4	83.3	0.693
$SpO_2 \leq 92\%$	12.4	8.6	0.132	5.1	**0.002**	12.1	1.000	7.1	0.0	1.000
Tachypnea	40.6	48.3	0.067	43.9	0.449	41.8	0.900	35.7	50.0	0.490
Chest retraction	57.3	48.3	**0.031**	57.2	1.000	70.3	**0.032**	57.1	50.0	0.738
Wheezing	63.2	55.6	0.064	58.0	0.203	64.8	0.898	57.1	75.0	0.477
Rales	64.5	62.4	0.607	70.1	0.180	68.1	0.604	60.7	75.0	0.484
Abnormal CXR	80.5	70.7	**0.003**	80.4	0.751	84.6	0.627	85.7	91.7	1.000
WBC[a] (cells/mm^3)	11900(10000-15200)	12200(9400-15500)	0.933	11300(8990-13700)	**0.006**	11400(9170-15500)	0.242	12500(10500-14700)	13900(10500-16300)	0.738
Neutrophil[a] (cells/mm^3)	4895(2883-7560)	4748(2872-7434)	0.933	4020(2409-6377)	**0.004**	3860(2526-8048)	0.322	4334(2859-7805)	6036(5328-8935)	0.457
Eosinophil[a] (cells/mm^3)	239(109-482)	118(41-303)	**<0001**	99(38-197)	**<0.001**	127(62-268)	**<0.001**	195(105-317)	383(187-437)	0.086
Diagnosis			**0.006**		**0.040**		0.529			0.557
URTIs	26.9	21.7	0.144	18.7	**0.020**	19.8	0.201	17.9	33.3	
Croup	3.0	8.6	**0.006**	6.4	0.087	2.2	1.000	3.6	0.0	
Bronchiolitis	34.6	27.9	0.087	35.8	0.794	40.7	0.369	32.1	16.7	
Pneumonia	35.5	41.8	0.127	39.0	0.391	37.4	0.797	46.4	50.0	
Hos. Duration(d)[a]	5(3-8)	5(3-8)	0.776	6(4-8)	0.084	6(4-8)	0.078	5(3.5-7.5)	4.5(3.5-7)	0.850

Abbreviation: d, day; hos, hospitalization; URTI, upper respiratory infection; HRV, human rhinovirus;
Note: All results are expressed in percentages except for ([a]) in median with interquartile range between brackets. Categorical variables were compared by using Fisher's exact test, and continuous variables were compared by using Mann-Whitney *U* test.

Molecular characterization and phylogenetic analysis

In all, 58 out of 325 samples were randomly selected for sequencing. By VP4/VP2 sequence analysis, HRV-A was the major detection (44/58, 75.9%) followed by HRV-C (14/58, 24.1%). No HRV-B was found. There were 21 different HRV-A types and 10 different C types. The most commonly detected types were A12 (n=9) and C40 (n=3) (Fig. 15). Although partial samples were typed, it seems that the circulation of more types may be responsible for the peaks of HRV activity (Jul 2010: 4 types; Oct 2010: 7 types, Nov 2010: 5 types; Apr 2011: 5 types). Some types were found across several months, such as A12, A78, and C40, suggesting that these types may circulate in the population over long time periods. On the other hand, the almost distinct type distributions from month to month have proved the rapid turnover and diversity nature of HRV populations. The mean nucleotide difference between HRV-A and HRV-C was 37.2%. The mean nucleotide variability within HRV-C was greater than that within HRV-A (23.9% vs. 19.7%).

One HRV-A sequence (VNM308-MAY.2010) did not cluster with any previously known types and showed 13% nucleotide difference to the nearest reference types (A88, A89), suggesting the new type. By BLAST searching, only 3 sequences with 97 – 99% similarity were found in GenBank (as of 24 Sep 2014), and all were from neighbor countries (Thailand, Cambodia). These strains showed the pairwise-distances of at least 11.3% with all available A88, A89 sequences and formed a separate cluster with high bootstrap value (Fig. 15). Following the criteria proposed by McIntyre et al. (pairwise-distance >10.5%), these strains could be assigned as new type. However, their VP1 sequences which are required for type assignment are not available, they may be designated as provisionally assigned type (numbered sequentially as HRV Apat5) (190).

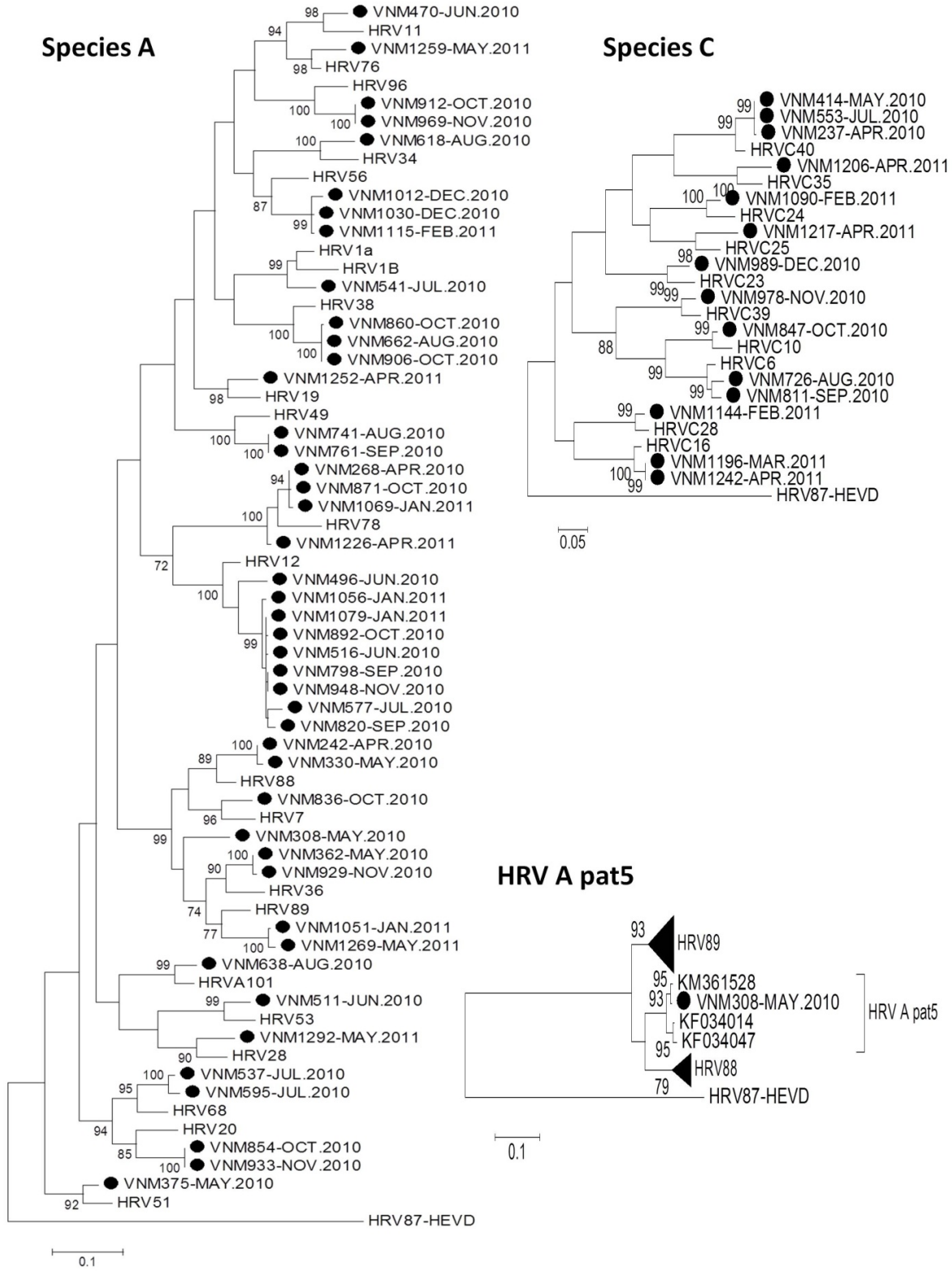


Figure 15. Diversity of HRV types detected in the study.

Phylogeny of the VP4/VP2 sequences of HRV-A, HRV-C, HRV-Apat5 detected in this study and reference strains were constructed with MEGA 5 software using the maximum-likelihood method. Data were bootstrap re-sampled 1000 times to assess the robustness of branches,

values of greater than 70 % are shown at the branch nodes. The HRV strains in this study are marked with solid round. HEV-D (HRV-87) (GenBank number AY040243) sequence was used to root the trees.

4. DISCUSSION

This study documented a significant burden of HRV in children with ARIs. HRV was by far the most frequently detected, in the majority as mono-detection, among children ill enough to be hospitalized. HRV was also more common than RSV, which is well-known to be the major causes of hospitalization in children. Consistent with the findings of previous studies worldwide, where HRV detection rates varied from 26% to 33% in hospitalized children (51, 191-196), HRV was found in 30% of patients in this study. Although the clinical value of HRV detection was arguable due to the identification of HRV from asymptomatic individuals, there was still strong association of HRV detection with true infection, especially in children less than 2 years old (192, 197, 198).

In addition, HRV has been shown experimentally the ability to infect the bronchial epithelium of immunocompetent individuals and replicate in the lower airways (50, 64-66). This contradicts previous assertion considered HRV as a “common cold” agent and causes mild diseases. When compared to children positive with other viruses, those hospitalized with HRV were equally to have predisposing underlying medical condition (prematurity and malnutrition). That means HRV detection is associated with severe diseases requiring hospitalization even in previously healthy children.

Despite traditionally associated with mild URTIs, HRV in this study was responsible for up to three-quarters of serious LRTIs in hospitalized children. Moreover, HRV detection was more likely to associated with severe findings such as difficult breathing, abnormal CXR. When compared with other viruses, in which RSV was the most prevalent, HRV also had more hypoxia significantly. The WBC and neutrophil counts were found higher in the HRV

mono-detection group. However, their sensitivity and specificity are not sufficient to differentiate bacterial from viral infection (59). Moreover, HRV infection has been proved to stimulate neutrophil chemoattractants which result in increasing blood neutrophil circulation (50, 199-201). Therefore, neutrophil elevation in this study may not reflect secondary bacterial infection in HRV. In addition, eosinophil count was significantly increased in HRV-detected children as compared with other groups. In some conditions, HRV can stimulate cytokine production such as eotaxin, eotaxin-2 to attract and stimulate eosinophils (50). Since blood eosinophil count is the predictor of reactive airway disease, this finding is consistent with previous reports indicating early infection of HRV as an important risk factor for asthma development (182, 202).

The most common co-detection was found between HRV and RSV. The high incidence and overlapping of seasonal distribution of both agents might explain for this phenomenon. No significant differences were seen in clinical symptoms related to HRV co-detection, except for the more prevalence of chest retraction, reflecting the decrease of lung compliance and severe difficult breathing. Similar findings were found in some studies (194) but not in others (198, 203). Since HRV is known to have long shedding period, therefore the co-detection may be due to successive infections. However, patients with HRV co-detection in this study admitted to hospital earlier than those with HRV mono-detection or those positive with other viruses (2 vs. 3 days), suggesting the true co-detection is more likely.

The present study indicates that HRV-A predominated over HRV-C while HRV-B was not detected. Similar findings were also reported with HRV-A being the most prevalent (191, 193). The absence of HRV-B may be due to either it is a minor species or its milder nature which does not lead to hospitalization. In the recent study, HRV-A was reported more common during infancy, while HRV-C was the most common species in older age group (186). Since the enrolled patients were almost young infants, it may explain why HRV-A was

found more frequent than HRV-C. The clinical features of children with HRV-A were similar to those with HRV-C. Moreover, there were no significant differences between both species in terms of severity, comparable with previous studies (186, 198). In contrast, other authors have suggested HRV-C might be responsible for more severe disease (184, 185, 191). The variations could be attributed to the difference either in viral load or in studied population and study designs. Therefore, these data should be treated with caution.

Consistent with other studies, HRV was circulating throughout the year with several peaks of activity in this study (184, 193, 196, 197). Although seasonal variation is a common feature of many respiratory viruses, its causes are largely unknown. To some extent, the year-round circulation of HRV may be explained by the great genetic diversity of HRVs. This speculation was supported by the rapid changing of HRV types from month to month. Even in some types that lasted for several months (e.g. HRV A12), the strains of the same type were often different (Fig. 15). Moreover, the peaks of HRV activity are likely due to the introduction of more HRV types into the community at the same time. These findings imply that HRV strains may circulate in the time-limited manner. On the other hand, the transmission of HRV may explain for the sustainability of HRV epidemics. First, HRV has long shedding period, typically 2-3 weeks (201). Second, the mild or asymptomatic HRV infection may be more likely to transmit the virus from one to others (204). Third, the non-enveloped virion structure of HRV may increase the indirect transmission through surfaces (205).

The using of VP4/VP2 sequences for classification and typing has been shown to agree well with the more reliable typing method using VP1 sequences. However, it is not sufficient for the identification of new types. Therefore, sequencing the VP1 of the novel type HRV Apat5 identified here is required for confirmation (190).

In summary, this study indicated HRV was associated with significant morbidity and severe LRTIs in hospitalized children. There were no differences between HRV-A and HRV-C in terms of disease severity. The genetic diversity and changing of HRV types over time were observed and may be responsible for the year-round circulation of HRV. However, additional studies with healthy controls using quantitative PCR methods are needed to understand better the role of HRV in LRTIs. Moreover, as the impact of HRV becomes better defined, the preventive and therapeutic interventions for HRV infections should be implicated in clinical practice.

CHAPTER 7

SUMMARY AND FUTURE WORK

Acute respiratory infections remain a leading cause of morbidity, mortality among children worldwide. While epidemiological data is available from industrialized nations, limited information about etiologies of ARIs makes it difficult to improve the prevention, diagnosis and treatment in developing and tropical countries. Therefore, it is necessary to know the epidemiological and clinical features of agents associated with ARIs in these areas.

By using PCR method to screen for respiratory viruses in nasopharyngeal swabs from hospitalized children in Vietnam, this study demonstrated that viruses were detected in 64.6% of cases. HRV (30%), RSV (23.8%), and HBoV (7.2%) were the most frequently detected viruses, followed by other viruses. HRV was detected all year round, while RSV epidemic occurred mainly in the rainy season. FLU A was found in both the rainy and dry seasons with seasonal H3N2 in the former and H1N1pdm09 in the latter. Other viruses were predominant in the dry season. HRV was identified in all age groups. RSV predominantly affected children < 6 months, while the highest FLU A detection was found among children > 24 months. PIV3 was detected mostly in the 6 – 12 month-old group, whereas the highest peak of PIV1 and HBoV detection was in the 12 – 24 month-old group. PIV1 was associated with croup and RSV was associated with bronchiolitis ($p<0.005$). Detection of HBoV and HRV was associated with hypoxia and RSV detection with chest retraction ($p<0.05$). These data show that a high incidence of virus-associated ARIs was found among hospitalized children in Vietnam. HRV, RSV, and HBoV were the most commonly detected agents and may increase the severity of ARIs in children. This demographic, epidemiological and clinical information is very useful to improve the diagnosis, prevention and treatment activities especially in resource-limited countries.

The genetic characteristics of leading viruses were further analyzed. Phylogenetic analyses showed that Vietnamese H1N1pdm09 sequences in 2010-2011 formed the distinct cluster, with other contemporary Asian and 2012-American sequences, suggesting a possible common ancestor. The Vietnamese H3N2 viruses in mid-2010 belonged to the emerging subclade Perth10/2010, which then spread worldwide in 2011. These results confirm that Southeast Asia may be the hot spot for influenza viruses to evolve before spreading worldwide. Although Vietnam locate in the Northern Hemisphere and are using the Northern Hemisphere vaccine formulation in private sectors, the Vietnamese influenza viruses in this study were well matched with the Southern Hemisphere vaccine formulation. Together with the bimodal seasonality of influenza found in this study, this information is useful for policy-makers in development of the national vaccination program. Two strains carrying S247N and D199N in NA which reduced the neuraminidase inhibitor susceptibility were found. Mutations at antigenic sites were also identified in these viruses. One case was detected with the swine H3N2 variant. Surveillance of influenza viruses in tropical countries is important not only for the development of their prevention and control strategies but also for earlier identification of the emerging new strains that may be selected for future vaccine.

RSV is a major cause of ARIs in children but information on the clinical and molecular characteristics of RSV infection in developing countries is limited. In this study, RSV A was the dominant subgroup, accounting for 91.4% (235/257), followed by RSV B, 5.1% (13/257), and 3.5% (9 cases) had a mixed detection of these subgroups. The phylogenetic analysis revealed that all group A strains belonged to the GA2 genotype. All group B strains belonged to the recently identified BA genotype, and further clustered into 2 new subgenotypes BA9 and BA10, firstly described in Japan. One GA2 strain had a premature stop codon which shortened the G protein length. This is the first time this mutant strain was identified directly from a clinical sample. RSV detection was significantly

associated with younger age and higher severity score than those without ($p<0.005$). Patients with RSV co-detection were more likely to be hypoxia than those with RSV single detection ($p=0.038$). RSV A detection was associated with more severe disease than RSV B detection, implying that determination of the subgroup at presentation would be useful for appropriate management and group A may have more priority than group B in choosing candidate strains for vaccine development. This is the first time the molecular features and disease severity of RSV were described in Vietnam. These results will not only contribute to the growing database on the molecular diversity of RSV circulating worldwide but may also be useful in clinical management and vaccine development.

HBoV, a novel virus, is recognized to increasingly associate with respiratory infections of previously unknown etiology in young children. In this study, HBoV was the third most common detected virus (7.2%). HBoV was associated with severe diseases, as the rates of hypoxia and pneumonia were higher, and hospitalization duration was longer in patients with HBoV detection than in those without ($p<0.05$). The phylogenetic analysis of partial VP1 gene showed minor variations and all HBoV sequences belonged to species 1 (HBoV1). These results imply that HBoV1 may have an important role in ARIs among children.

HRV has been recently identified as the leading cause in asthma exacerbation, severe bronchiolitis and pneumonia. In this study, HRV was the most frequently detected virus (30%). Among HRV mono-detected children, 70% were diagnosed as either pneumonia or bronchiolitis, and 12.4% had hypoxia that needed oxygen supplementation. Children with HRV were more likely to have difficult breathing and abnormal chest X-ray ($p<0.05$). Children hospitalized with HRV had less fever but more hypoxia and high blood eosinophil count than those positive with other viruses ($p<0.001$). Co-detection of HRV had more difficult breathing than mono-detection ($p<0.05$). During the weak season of RSV (dry

season), HRV replaced RSV to become the most common virus associated with bronchiolitis and pneumonia. Among 58 randomly selected HRVs for sequencing, only 2 species were detected with HRV-A being more common (44/58) than HRV-C (14/58). The clinical symptoms of HRV-C were similar to those of HRV-A. Phylogenetic analysis revealed diversity of circulating HRV types including 21 types of HRV-A and 10 types of HRV-C. Of note, one strain of HRV-A showed a high nucleotide difference exceeding the defined threshold and proved to be new type. These results provide new insights into the burden and severity of HRV and highlighted the need for its routine diagnosis in hospital settings.

In summary, this study confirmed the diversity and the importance of respiratory viruses associated with ARIs required hospitalization in Vietnamese children. Specific viruses were frequently associated with specific clinical syndromes, specific seasonal patterns and age distributions. The demographic, epidemiological and clinical information from this study is useful to improve the diagnosis, prevention and treatment activities especially in resource-limited countries. The molecular characterization of common respiratory viruses was determined for the first time in Vietnam, and some interesting results were found such as new type of HRV, new strain of RSV, and drug resistant strain of FLU A.

However, many challenges still remain to be done in the future. First, new strains of RSV, abnormal swine H3N2 variant in humans, and new type of HRV were identified. Research on the full genome of these new strains is obviously necessary. Second, HRV and HBoV are among the most commonly detected viruses in patients with ARIs. Recently, increasing evidence shows a strong association of HRV and HBoV detection with severe LRTIs. Thus, identifying these viruses at presentation is very useful for physician to give appropriate treatment, prognosis, and prevention. Although a PCR assay used for the detection of HRV and HBoV has been developed, it requires skilled technicians and specialized instrumentation and is not suitable for the detection of these viruses in the field.

Therefore, a simpler, more rapid, and more sensitive diagnostic assay is needed. Since HRV has more than 100 serotypes, develop the rapid detection method such as Loop-mediated Isothermal Amplification assay (LAMP) is required. Also, develop a LAMP or an immunochromatography (IC) assay to detect HBoV is necessary. Third, more studies should be done to acquire the better information on bacterial ARIs. Bacteria and viruses are the most common pathogens causing pneumonia in children in developing countries. Laboratory confirmation of bacterial or viral pneumonia is often ether time-consuming or not available in some clinical settings. The management of pneumonia is often based on physician judgment using clinical, radiographic, and laboratory data. Using the combination of these data is possible to distinguish bacterial from viral pneumonia. However, the lack of validated clinical prediction rules in children has led to overprescribe of antibiotics in clinical practice, especially in developing countries. Therefore, there is the need to develop and validate a clinical prediction rule to distinguish bacterial from viral pneumonia in children. This project is in progress and has finished collecting samples (blood and naso-tracheal aspirate samples).

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Appendix 1: Information sheet (English)

Children's Hospital 2
Ho Chi Minh City

INFORMATION FOR PATIENTS

Tel: 84-8-38295723
Fax: 84-8-38232137

The study: Epidemiology and identification of causative agents among infants and children admitted to Respiratory department of Children's hospital 2 with acute respiratory infections.

Your child is invited to survey research on the epidemiology and identification of causative agents among infants and children with acute respiratory infections. First, it should be clear that:

It is up to your own willingness to join the research.

Even after joining, it is also your right to withdraw from the research at any time. And your child will still receive standard health care. No action will be taken against you.

Please read this information sheet or ask a reliable person help to read. A copy of this information sheet will be given to you. Before agreeing to join the research, please take the time to ask for any further questions and discuss with your relatives, friends, doctor or the medical staff about this research.

Overview of Research

Acute respiratory infections (ARIs) are leading cause of morbidity and mortality among children worldwide. About 2 million children under 5 years of age died from pneumonia each year, and most of these deaths occurred in developing countries. Viruses are the most common cause of ARIs and the major reason for hospitalization of young children. However, due to limited resources and facilities, the role of individual respiratory virus in developing countries has not been well studied.

Purposes

To establish an effective prevention and treatment measures, better understanding of clinical and epidemiological characteristics of ARIs is needed. Moreover epidemiological study including molecular level is needed. For these purposes, clinical samples and data are examined.

Methods

Nasopharyngeal samples are collected from patients. Disinfected samples are transported to Nihon University for further molecular epidemiological studies. Other clinical information are recorded and sent to Nihon University. They are analyzed anonymously. The results are sent to your hospital after examined.

Protection of privacy

Clinical data and examined data are analyzed and reported to meetings and submitted to journals. Your personal data are anonymously recorded. Your private information will never leak to outside.

Risk
There is very small risk for your child in this research. Obtaining nasopharyngeal swab sometimes may cause nausea or vomit, and no other risk.

About bearing the expenses
If you join the research, it will not cost you anything at all. There is also no payment for your nasopharyngeal swab donation.

Withdraw of joining research
Although you agree to join the research, you can withdraw from the research at any time. Your child will still receive the standard health care and no action will be taken against you.

Enquiries
Should you need any information, please do not hesitate to contact your doctor or the following address as given below:

Please read the Informed Consents and if you agree, then sign the document. If you feel comfortable and allow us, we can start taking sample. We will highly appreciate your support.

Thank you for your cooperation

Dr. Tran Dinh Nguyen
Cardiology department, Children's Hospital 2
14 Ly Tu Trong St., Ward Ben Nghe, District 1, Ho Chi Minh City
Tel: 84 0908659102
Email: dinhnguyentran@yahoo.com
The project leader: Prof. Satoshi Hayakawa, Nihon University, Japan

Appendix 2: Information sheet (Vietnamese)

Bệnh viện Nhi Đồng 2
Thành phố Hồ Chí Minh

THÔNG TIN CHO BỆNH NHÂN

Tel: 84-8-38295723
Fax: 84-8-38232137

Nghiên cứu: Dịch tễ học và xác định các tác nhân gây bệnh ở em nhập khoa hô hấp Bệnh viện Nhi Đồng 2 với bệnh nhiễm trùng đường hô hấp cấp tính.

Con của Ông/Bà được mời vào nghiên cứu về đặc điểm dịch tễ học và tác nhân gây nhiễm trùng hô hấp cấp. Trước tiên, chúng tôi muốn Ông/Bà hiểu rằng:

Việc tham gia vào nghiên cứu này là hòan tòan tự nguyện.

Ông/Bà có quyền từ chối không cho con của Ông/Bà tham gia vào nghiên cứu hoặc rút ra khỏi nghiên cứu bất kỳ lúc nào Ông/Bà muốn. Và con của ông bà vẫn được chăm sóc theo tiêu chuẩn của bệnh viện dù không tham gia vào nghiên cứu.

Xin Ông/Bà đọc kỹ phiếu thông tin này hoặc nhờ một người tin cậy của Ông/Bà đọc giúp. Một bản sao của phiếu thông tin này sẽ được đưa cho Ông/Bà. Trước khi Ông/Bà chấp thuận đồng ý cho con của Ông/Bà tham gia vào nghiên cứu này, xin hãy dành thời gian để đặt câu hỏi cho bất kỳ thắc mắc nào và bàn bạc cùng người thân, bạn bè hoặc bác sỹ riêng hoặc các nhân viên y tế về nghiên cứu này.

Tổng quan về nghiên cứu

Nhiễm trùng đường hô hấp cấp tính là nguyên nhân hàng đầu gây bệnh tật và tử vong ở trẻ em trên toàn thế giới. Khoảng 2 triệu trẻ em dưới 5 tuổi chết vì viêm phổi mỗi năm, và hầu hết các trường hợp tử vong xảy ra ở các nước đang phát triển. Virus là nguyên nhân phổ biến nhất của nhiễm trùng đường hô hấp cấp và là lý do nhập viện chính của trẻ nhỏ. Tuy nhiên, do nguồn lực và cơ sở vật chất hạn chế, vai trò của các vi rút đường hô hấp ở các nước đang phát triển đã không được nghiên cứu kỹ.

Mục đích

Để thiết lập một biện pháp điều trị dự phòng hiệu quả, cần hiểu biết tốt hơn về đặc điểm lâm sàng và dịch tễ học của nhiễm trùng đường hô hấp cấp. Hơn nữa, nghiên cứu dịch tễ học ở cấp độ phân tử là cần thiết. Đối với mục đích này, các mẫu bệnh phẩm và dữ liệu lâm sang được khảo sát.

Phương pháp

Mẫu mũi họng được thu thập từ các bệnh nhân. Mẫu khử trùng được vận chuyển đến Đại học Nihon để nghiên cứu sâu hơn về dịch tễ học phân tử. Thông tin lâm sàng khác được ghi lại và gửi đến Đại học Nihon. Thông tin này được phân tích ẩn danh. Các kết quả được gửi lại bệnh viện sau khi kiểm tra.

Bảo vệ sự riêng tư

Dữ liệu lâm sàng và dữ liệu sau khảo sát được phân tích và báo cáo tại các hội nghị và gửi đến các tạp chí. Dữ liệu cá nhân của con Ông/Bà được ẩn danh. Thông tin cá nhân của con Ông/Bà không bao giờ bị rò rỉ ra bên ngoài .

Rủi ro
Có rất ít nguy cơ xảy ra cho con Ông/Bà khi vào nghiên cứu này. Lấy phết mũi họng gây nhợn, đôi khi có thể gây nôn. Ngòai ra không có một nguy cơ nào khác.

Về chi phí
Nếu con Ông/Bà tham gia vào nghiên cứu, Ông/Bà sẽ không phải trả bất kỳ chi phí nào cho nghiên cứu này. Cũng không có thanh toán cho phết mũi họng Ông/Bà tặng cho chúng tôi.

Từ chối tham gia nghiên cứu

Mặc dù đã đồng ý tham gia nghiên cứu, Ông/Bà có thể rút ra khỏi nghiên cứu bất kỳ lúc nào. Cho dù con Ông/Bà không tham gia vào nghiên cứu, con Ông/Bà vẫn được điều trị và chăm sóc y tế theo đúng tiêu chuẩn.

Thắc mắc

Nếu Ông/Bà có bất kỳ thắc mắc nào về nghiên cứu này, xin vui lòng gặp bác sỹ điều trị hoặc bác sỹ có tên dưới trang thông tin này để được giải đáp.

Xin vui lòng đọc các văn bản chấp thuận và nếu Ông/Bà đồng ý, sau đó ký tên vào tài liệu. Nếu Ông/Bà cảm thấy thoải mái và cho phép, chúng tôi có thể bắt đầu lấy mẫu. Chúng tôi rất cảm ơn sự hỗ trợ của Ông/Bà.

Cảm ơn Ông/Bà đã hợp tác.

BS. Trần Đình Nguyên
Khoa Tim mạch, Bệnh viện Nhi Đồng 2
14 Lý Tự Trọng, Phường Bến Nghé, Quận 1, Thành phố Hồ Chí Minh
Tel: 84 0908659102
Email: dinhnguyentran@yahoo.com

Chủ nhiệm dự án: Giáo sư Satoshi Hayakawa, Đại học Nihon, Nhật Bản

Appendix 3: Informed consent (English)

Children's Hospital 2
Ho Chi Minh City

INFORMED CONSENT

Tel: 84-8-38295723
Fax: 84-8-38232137

The study: Epidemiology and identification of causative agents among infants and children admitted to Respiratory department of Children's hospital 2 with acute respiratory infections.

Consent from parents or relatives:

I was explained information about research and understood the points given below and I agree to join this research.

I was explained the following points (check box).
□Overview of the research
□Purposes
□Methods
□Protection of privacy
□Risk
□About expenses which will be covered by the research project
□Voluntariness and option to withdraw from research
□Enquiries

I agree to join the research and that sample is used for the research.
Name of patient: _____
Name of Relative: _____ Signature: _____
Relationship to patient: _____ Date: _____
Name of doctor who explained: _____ Signature: _____
Date: _____

Withdrawal of joining research

Although I agreed to join the research, at this time I would like to withdraw.

The study: Epidemiology and identification of causative agents among infants and children admitted to Respiratory department of Children's hospital 2 with acute respiratory infections.

Name of patient: _____
Name of Relative: _____ Signature: _____
Relationship to patient: _____ Date: _____

Appendix 4: Informed consent (Vietnamese)

Bệnh viện Nhi Đồng 2
Thành phố Hồ Chí Minh

ĐỒNG Ý THAM GIA NGHIÊN CỨU

Tel: 84-8-38295723
Fax: 84-8-38232137

Nghiên cứu: Dịch tễ học và xác định các tác nhân gây bệnh ở em nhập khoa hô hấp Bệnh viện Nhi Đồng 2 với bệnh nhiễm trùng đường hô hấp cấp tính.

Đồng ý của cha mẹ hoặc người thân:

Tôi đã được giải thích thông tin về nghiên cứu và hiểu rõ những điểm dưới đây và tôi đồng ý tham gia nghiên cứu này.

Tôi đã được giải thích những điểm sau đây:
□ Tổng quan về nghiên cứu
□ Mục đích
□ Phương pháp
□ Bảo vệ sự riêng tư
□ Rủi ro
□ Về chi phí sẽ được chi trả bởi các dự án nghiên cứu
□ Tự nguyện và tự do rút khỏi nghiên cứu
□ Thắc mắc

Tôi đồng ý tham gia nghiên cứu và mẫu được sử dụng cho các nghiên cứu.
Tên bệnh nhân: _____
Tên của người nhà: _____ Chữ ký: _____
Mối quan hệ với bệnh nhân: _____ Ngày: _____
Tên của bác sĩ giải thích: _____ Chữ ký: _____
Ngày: _____

<u>Rút khỏi nghiên cứu</u>

Mặc dù tôi đã đồng ý tham gia nghiên cứu, vào thời điểm này, tôi muốn rút lui.

Nghiên cứu: Dịch tễ học và xác định các tác nhân gây bệnh ở trẻ em nhập khoa hô hấp Bệnh viện Nhi Đồng 2 với bệnh nhiễm trùng đường hô hấp cấp tính.

Tên bệnh nhân: _____
Tên của người nhà: _____ Chữ ký: _____
Mối quan hệ với bệnh nhân: _____ Ngày: _____

Appendix 5: Ethical approval (Children's Hospital 2)

DEPT. of HEALTH HCMC
CHILDREN'S HOSPITAL 2

SOCIALIST REPUBLIC of VIETNAM
Independence – Freedom – Happiness

THE ETHICS COMMITTEE'S APPROVAL

The Ethics Committee of Children's Hospital 2
14 Ly Tu Trong, District 1
Ho Chi Minh City, Vietnam

Investigator: Dr. Tran Đinh Nguyen

Study site: Children's Hospital 2 – HCMC

Study documents in Vietnamese:

- The protocol: "Epidemiology and identification of causative agents among infants and children admitted to Respiratory Department of Children's Hospital 2 with acute respiratory infections from February 2010 to February 2011".
- Patient Information Sheet for Children.
- Informed Consent Form for Children.

After:

- Reviewing all documents listed above
- Discussing between Committee members

The Ethics Committee of Children's Hospital 2 has approved for this study to be conducted by Dr. Tran Đinh Nguyen at Children's Hospital 2 from February 2010 to February 2011.

HCMC, January 26, 2010
Chairman of Ethics Committee

SỞ Y TẾ BỆNH VIỆN NHI ĐỒNG 2 THÀNH PHỐ HỒ

HA MANH TUAN, M.D.,PhD.

HEALTH SERVICE OF HO CHI MINH CITY
CHILDREN'S HOSPITAL 2

SOCIALIST REPUBLIC OF VIETNAM
Independence-Freedom-Happiness

Ho Chi Minh City, January 19th, 2010

APPROVAL LETTER
SCIENCE RESEARCH PROJECTS YEAR 2010

The Committee approves Science research projects year 2010 is established by decision No ..25A/QĐ-NĐ

Approval date	: 19/1/2010.
Place	: PIC room-Children's Hospital 2.
Chairman	: Hà Mạnh Tuấn, MD., PhD.
Board of Committee	: Trương Quang Định, MD.PhD. ; Nguyễn Thị Hạnh Lê, MD.; Hồ Lữ Việt, MD; Trịnh Hữu Tùng, MD.; Võ Phương Khanh, MD.; Nguyễn Huy Luân, MD. MS.
Reviewer	: Nguyễn Huy Luân, MD. MS.; Nguyễn Thị Hạnh Lê, MD.

The Committee approved

Project : "Epidemiology and identification of causative agents among infants and children admitted to Respiratory department of Children's Hospital 2 with acute respiratory infections from February 2010 to February 2011".

Author : Trần Đình Nguyên, MD.

Project No. : CS/CH/NĐ2/010/05

Review Committee:

- Reviewer 1 (Nguyễn Huy Luân, MD.):
  - ✓ Author need to modify more details about project: patient's consent, protocol to collect data.
  - ✓ Need of collecting sample procedure and research protocol.
- Reviewer 2 (Nguyễn Thị Hạnh Lê, MD.) :
  - ✓ The feasibility of sample collecting.
  - ✓ Due to the project which may be presented outside Vietnam: need the copyright of the statistical analysis software (SPSS 15.0).
  - ✓ Author need to follow the Government's law and policy.

THE ETHICS COMMITTEE APPRVOVED THIS PROJECT

Secretary

Hồ Lữ Việt, MD.

Chairman

BỆNH VIỆN NHI ĐỒNG 2

Hà Mạnh Tuấn, MD., PhD.

Appendix 6: Ethical approval (Nihon University School of Medicine)

平成２６年４月１０日

病態病理学系微生物学分野
教授　早川　智　殿

医学部倫理委員会
委員長　杉　谷　雅　彦

「ウィルスを中心とした呼吸器感染症の診断、分子疫学、
病態に関する研究」研究計画申請に関する件（通知）

平成２６年２月２６日付けで申請のありました標記のことについて，当委員会で慎重に審議した結果，下記のとおり許可されましたので，通知いたします。

記

１　許可年月日　　平成２６年４月１０日

２　承認番号　　２５－１５－０

以　　上

平成２６年４月１６日

病態病理学系微生物学分野
教授　　早川　智　　殿

倫 理 委 員 会 幹 事

倫理委員会審査申請結果の通知について

標記のことについて，別紙のとおり審査申請結果に関する通知をお送りいたしますので，ご査収ください。

以　　上

Appendix 7: Questionnaire (English)

ACUTE RESPIRATORY INFECTIONS QUESTIONNAIRE

No. ……. Record No: …….....

I. GENERAL INFORMATION

1. Name :…….....…….. Male ☐ Female ☐
2. Date of birth :…….....… Age: …… year …….. month
3. Address :…….....…… Rural ☐ Urban ☐
4. Date of Examination :….....-……..-201
5. Date of Admission :...……-…..…-201 Date of Discharge: ……..…-…….-201
6. Name of Mother/ Father :……..

II. CURRENT ILLNESS

1. Chief complaint:…….
2. Duration from onset of symptoms to admission:……

3. Symptom	Yes	No	Unknown	How many days?
Fever				
Cough				
Rhinorrhea				
Sore throat				
Dyspnea				
Wheezing				
Red eyes				
Rash				
Headache				
Myalgia				
Nausea/Vomiting				
Diarrhea				
Others				

4. Antibiotics before admission ☐.
Antibiotic names:…….……How many days……

III. PAST MEDICAL HISTORY

1. Birth history:

- Number of babies in family: ……/……..
- Gestational age: …… weeks < 37 weeks ☐ ≥ 37 weeks ☐
- Birth weight: …… g < 2500 g ☐ ≥ 2500 g ☐
- Vaginal delivery ☐ Vacuum extraction ☐ Forceps ☐ Cesarean delivery ☐ Other ☐ ……
- Birth Asphyxia ☐ Breast-feeding ☐. When to stop breast-feeding.....
- Vaccination Diphtheria-Pertussis ☐. How many shots and When……
  Measles ☐. How many shots and When……
  Flu ☐. How many shots and When……

2. Diseases:

- Congenital heart defect ☐. Diagnosis of Congenital heart defect: ……
- Pulmonary hypertension ☐ -Heart failure ☐ - Ever needed ventilatory support ☐
- Bronchopulmonary dysplasia ☐ - Neuromuscular disorders ☐
- Immunodeficiency disorders ☐
- Wheezing episodes ☐. How many times: …….
- Respiratory infections ☐. How many times/year…….When was the last episode:.....

- Other disorders (Down's syndrome, cystic fibrosis, etc): ……
- Exposure to ARIs within 3 weeks ☐: At home ☐ At school ☐ Other:……

3. Family:

- Who is caregiver: ……
- Attending day care center ☐
- Number of siblings at home: …… - House's surface area: ……m^2
- Any smoker at home ☐
- Wood-burn stove ☐ Oil-burn stove ☐ Gas-burn stove ☐ Other: ……
- Father: Occupation: …… - Educational level: ……
- Mother: Occupation: …… - Educational level: …….
- Family Atopy ☐ Type of Atopy: Allergic Rhino-conjunctivitis ☐ Allergic Sinusitis ☐ Food allergy ☐ Drug allergy ☐ Asthma ☐ Eczema ☐ Other allergy ☐
- Tuberculosis ☐ -Genetic disorders ☐ - Immunodeficiency disorders ☐

IV. SYSTEMS EXAMINATION

1. Vital signs:

- Consciousness: Awake and Alert ☐ Irritable ☐ Lethargy ☐ Other ☐
- Central cyanosis ☐
- Pulse: ……bpm – Axillary temperature: ……^oC – Respiratory rate: ……rpm
- Weight: ……Kg - Height: …… cm - SpO_2 = ……..% /FiO_2 =…….%
- Malnutrition ☐ mild ☐ moderate ☐ severe ☐

2. Respiratory system:

- Cough ☐ - Rhinorrhea ☐ - Sneezing ☐
- Rash ☐ - Wheezing ☐ - Stridor (when in rest) ☐
- Tachypnea ☐ - Lower chest retraction ☐ - Nasal flaring ☐
- Abdominal and accessory muscles retraction ☐ - Apnea >15 second ☐

Signs	Left lung	Right lung	Signs	Left lung	Right lung
Rales			Decrease breath sounds		
Ronchi			Dull sound		
Stridor			Overinflated		
Other			Other		

Other systems	Normal	Abnormal	Abnormal signs
ENT			
Cardiovascular			
GI			
Orthopedic			
Neurological			
Others			
Severity score			

V. INVESTIGATIONS

1. CBC:

-WBC = ... Neu/Gran=.....%. L=.....% Mono=.....% E= .....% Baso=.....%.
- RBC = …...….M/mm^3 Hb = ...……..g% Hct = ...…..%
-MCV = ……...fl MCH = …..pg MCHC = ……...g% PLT = ..…..K/mm^3
- Anemia mild ☐ moderate ☐ severe ☐

CRP		mg/L	Na^+		mmol/L
Urea		g/L	K^+		mmol/L

Creatinin		mg/L	Ca^{++}		mmol/L
ALT		U/L	Cl^{-}		mmol/L
AST		U/L	Other		

2. Blood gas:

pH			SaO_2		%
$PaCO_2$		mmHg	HCO_3		meq/L
PaO_2		mmHg	Base excess (BE)		meq/L

3. Chest X-ray: Date: ……../……/201

Normal ☐ Abnormal ☐ Not done ☐

Abnormal signs	**Upper R**	**Middle R**	**Lower R**	**Upper L**	**Lingular**	**Lower L**
Interstitial opacity						
Brochial wall thickening						
Airspace opacity						
Consolidation						
Cavity syndrome						
Pleural effusion						
Pneumothorax						
Focal hyperinflation						
Diffused hyperinflation						
Collapse						
Bronchiectasis						
Pneumomediastinum						
Other						
Other						

Cardiomegaly ☐, Cardio-thoracic rate =…….

Chest X-ray diagnosis:……

4. Sputum bacterial culture: Done ☐ Neg ☐ Pos ☐. Bacteria name: ……

5. Blood bacterial culture: Done ☐ Neg ☐ Pos ☐. Bacteria name: ……..

6. PPD test: Done ☐ Pos ≥10mm ☐ Neg ☐

7. HIV: Done ☐ Neg ☐ Pos ☐

8. Pleural fluid: Transudative ☐ Exudative ☐ Pus ☐

9. CSF: Done ☐ Normal ☐ Abnormal ☐

10. Urinalysis: Done ☐ Normal ☐ Abnormal ☐

11. Urine bacterial culture: Done ☐ Neg ☐ Pos ☐. Bacteria name: ……

12. Stool bacterial culture: Done ☐ Neg ☐ Pos ☐. Bacteria name: ……

13. ECG: Done ☐ Nornal ☐ Abnormal ☐

14. Echocardiogram: ……

15. Other: ……

16. Nasopharyngeal PCR result: …….

VI. DIAGNOSTIC CATEGORIES

Upper respiratory infections ☐

Croup ☐

Bronchiolitis ☐

Pneumonia ☐

Other diagnosis along with: ……..

Other note: ……..

VII. TREATMENT

1. Oxygen: Canullas☐ mask☐ NCPAP☐ Ventilator☐ Max FiO_2=...% Max PEEP=…cmH_2O
2. IV fluid ☐. Type:…….
3. Bronchodilator: Not use ☐
   - Nebulizer ☐ Name:…….. Duration (d):……
   - Oral ☐ Name:…….. Duration (d):……
   - IV ☐ Name:…….. Duration (d):……
4. Antibiotics: Not use ☐
   - Oral ☐ Name:…….. Duration (d):……
   - IV ☐ Name:…….. Duration (d):……
   - IV ☐ Name:…….. Duration (d):……
   - IM ☐ Name:…….. Duration (d):……
5. Corticosteroids: Not use ☐
   - Nebulizer ☐ Name:…….. Duration (d):……
   - Oral ☐ Name:…….. Duration (d):……
   - IV ☐ Name:…….. Duration (d):……
6. Mucolytic ☐. Name:…….
7. Other ☐ Name: ……..
8. Other:…….
9. Physiotherapy ☐

VII. COMPLICATIONS: ☐

Respiratory failure ☐ Pulmonary superinfection ☐ Sepsis ☐
Pleural effusion ☐ SIADH ☐ ARDS ☐
Baro/Volutrauma of lung ☐ Pulmonary fibrosis ☐ Pulmonary hypertension episode ☐
Pneumothorax/mediastinum ☐ Death ☐

Appendix 8: Questionnaire (Vietnamese)

PHIẾU THU THẬP SỐ LIỆU ARI

STT ……. Số hồ sơ: …….....

I. PHẦN HÀNH CHÁNH

1. Họ và tên :…….....……. Nam ☐ Nữ ☐
2. Ngày sinh :…….....… Tuổi: …… tuổi …….. tháng
3. Địa chỉ :…….....…… Nông thôn ☐ Thành thị ☐
4. Ngày khám :…....-……..-201
5. Ngày nhập viện :...……-…..…-201 Ngày XV: ……..…-…….-201
6. Họ tên cha/mẹ :……..

II. BỆNH SỬ

1. Lý do nhập viện:…….
2. Ngày bệnh trước khi nhập viện:……

3. Triệu chứng	Có	Không	Không thông tin	Mấy ngày?
Nóng/ Sốt				
Ho				
Chảy mũi				
Đau họng				
Khó thở				
Khò khè				
Đỏ mắt				
Nổi ban				
Nhức đầu				
Đau nhức cơ				
Buồn nôn/nôn				
Tiêu chảy				
Triệu chứng khác				

4. Điều trị KS trước nhập viện ☐.
Loại KS:……..……Số ngày…….

III. TIỀN SỬ

1. Sản khoa:

- Con thứ mấy: ……/……..
- Tuổi thai: …….. tuần < 37 tuần ☐ ≥ 37 tuần ☐
- Cân nặng lúc sinh:…… Kgram < 2500 gram ☐ ≥ 2500 gram ☐
- Sanh thường ☐ Sanh hút ☐ Sanh kềm ☐ Sanh mổ ☐ Khác ☐ ……
- Ngạt sau sanh ☐ Bú sữa mẹ ☐. Ngưng sữa mẹ lúc.....
- Tiêm ngừa Bạch hầu-Ho gà ☐. Cách nay bao lâu……
  Sởi ☐. Cách nay bao lâu……
  Cúm ☐. Cách nay bao lâu……

2. Bệnh lý của trẻ:

- Tim bẩm sinh ☐. Tật tim bẩm sinh: ……
- Cao áp phổi ☐ -Suy tim ☐ - Có giúp thở ☐
- Loạn sản phế quản phổi ☐ - Bệnh lý thần kinh cơ ☐
- Suy giảm miễn dịch ☐
- Khò khè ☐. Số lần khò khè: …….
- Nhiễm trùng hô hấp ☐. Bao nhiêu lần ……/năm. Lần cuối cách nay bao lâu:.....tháng
- Bệnh lý khác: ……

- Tiếp xúc ai bị bệnh hô hấp trong 3 tuần qua ☐: Ở nhà ☐ Trường học ☐ Khác:……

3. Gia đình:

- Người chăm sóc: ……
- Nơi sinh hoạt: Gia đình ☐ Trường học ☐
- Số người trong gia đình: …… - Diện tích nhà: …….
- Có ai trong nhà hút thuốc lá ☐
- Nấu bếp củi ☐ dầu hỏa ☐ gas ☐ Khác: ……
- Cha: Nghề nghiệp: …… - Trình độ học vấn: ……
- Mẹ: Nghề nghiệp: …… - Trình độ học vấn: ……
- (Gia đình) Tạng dị ứng ☐ Dạng dị ứng: Viêm mũi dị ứng ☐ Viêm xoang ☐
  Dị ứng thức ăn ☐ Dị ứng thời tiết ☐
  Dị ứng thuốc ☐ Suyễn ☐ Chàm ☐
- Lao ☐ -Bệnh di truyền ☐ - Suy giảm miễn dịch ☐

IV. KHÁM

1. Sinh hiệu:

- Tri giác: Tỉnh ☐ Kích thích ☐ Lơ mơ ☐
- Tím trung ương ☐
- Mạch: ……l/p . - Nhiệt độ nách: ……^{o}C – Nhịp thở: ……l/p
- Cân nặng: …… Kg - Chiều cao: …… cm - SpO_2 = ……..% /FiO_2 =…….%
- Suy dinh dưỡng ☐ nhẹ ☐ trung bình ☐ nặng ☐

2. Triệu chứng hô hấp:

- Ho ☐ - Chảy mũi ☐ - Hắt hơi ☐
- Nổi ban ☐ - Khò khè ☐ - Thở rít khi nằm yên ☐
- Thở nhanh ☐ - Co lõm ngực ☐
- Co kéo gian sườn ☐ - Phập phồng cánh mũi ☐ - Cơn ngưng thở >15 giây ☐

Triệu chứng	Phổi Trái	Phổi Phải	Triệu chứng	Phổi Trái	Phổi Phải
Ran ẩm/nổ			Phế âm giảm		
Ran ngáy			Gõ đục		
Ran rít			Ngực căng phồng		
Khác			Khác		

Cơ quan	Bình thường	Bất thường	Dấu hiệu bất thường
Tai mũi họng			
Tim mạch			
Tiêu hóa			
Da cơ xương khớp			
Thần kinh			
Khác			
Điểm LS			

V. CẬN LÂM SÀNG

1. Máu:

- BC = ...Neu/Gran=.....%. L=.....% Mono=.....% E= .....% Baso=.....%.
- HC = ….....….triệu/mm^3 Hb = ...……..g% Hct = ...…..%
- MCV = ……...fl MCH = …..pg MCHC = ……...g% TC = ..…..K/mm^3
- Thiếu máu nhẹ ☐ trung bình ☐ nặng ☐

CRP		mg/L	Na^+		mmol/L
Ure		g/L	K^+		mmol/L
Creatinin		mg/L	Ca^{++}		mmol/L

SGPT		U/L	Cl^-		mmol/L
SGOT		U/L	Khác		

2. Khí máu động mạch:

pH			SaO_2		%
$PaCO_2$		mmHg	HCO_3		meq/L
PaO_2		mmHg	Base excess (BE)		meq/L

3. X quang phổi: Ngày làm: ……../……/201

Bình thường ☐ Bất thường ☐ Không làm ☐, lý do:……

Bất thường	**Trên P**	**Giữa P**	**Dưới P**	**Trên T**	**Lưỡi**	**Dưới T**
Thâm nhiễm mô kẽ						
Dày thành phế quản						
Thâm nhiễm phế nang						
Đông đặc						
Hình ảnh hang						
Tràn dịch màng phổi						
Tràn khí màng phổi						
Ứ khí khu trú						
Ứ khí lan tỏa						
Xẹp phổi						
Dãn phế quản						
Tràn khí trung thất						
Khác						
Khác						

Bóng tim to ☐, chỉ số Tim/Lồng ngực=…….

Chẩn đoán Xquang:……

4. Soi cấy đàm: có làm ☐ Am tính ☐ Dương tính ☐. Loại vi trùng: ……

5. Cấy máu: có làm ☐ Am tính ☐ Dương tính ☐. Loại vi trùng: ……..…

6. IDR: có làm ☐ Dương tính ≥10mm ☐ Am tính ☐

7. HIV: có làm ☐ Dương tính ☐ Am tính ☐

8. Dịch màng phổi: Thấm ☐ Tiết ☐ Mủ ☐

9. Dịch não tủy: có làm ☐ Bình thường ☐ Bất thường ☐

10. TPTNT: có làm ☐ Bình thường ☐ Bất thường ☐

11. Cấy nước tiểu: có làm ☐ Am tính ☐ Dương tính ☐. Loại vi trùng: ……

12. Cấy phân: có làm ☐ Am tính ☐ Dương tính ☐. Loại vi trùng: ……

13. ECG: có làm ☐ Bình thường ☐ Bất thường ☐

14. Siêu âm tim: ……

15. Khác: ……

16. PCR phết mũi họng: …….

VI. PHÂN LOẠI CHẨN ĐOÁN

Các loại viêm hô hấp trên ☐

Viêm thanh quản ☐

Viêm tiểu phế quản ☐

Viêm phổi ☐

Chẩn đoán khác đi kèm: ……..

Ghi chú khác về chẩn đoán: ……..

VII. ĐIỀU TRỊ

1. Thở oxy: canulla mũi☐ mask☐ NCPAP☐ thở máy☐ Max FiO_2=..%Max PEEP=...cmH_2O
2. Dịch truyền tĩnh mạch ☐. Tên:…….
3. Dãn phế quản: không dùng ☐
   - Khí dung ☐ Tên:…….. Số ngày:……
   - Uống ☐ Tên:…….. Số ngày:…….
   - TM ☐ Tên:…….. Số ngày:……
4. Kháng sinh: không dùng ☐
   - Uống ☐ Tên:…… Số ngày:……
   - TM ☐ Tên:……..…….. Số ngày:……
   - TM ☐ Tên:…….. Số ngày:……
   - TB ☐ Tên:…….…….. Số ngày:……
5. Corticosteroids: không dùng ☐
   - Khí dung ☐ Tên:…… Số ngày:……
   - Uống ☐ Tên:…….. Số ngày:……
   - TM ☐ Tên:……. Số ngày:……
6. Loãng đàm ☐. Tên:…….
7. Thuốc tim mạch ☐ Loại thuốc: ……..
8. Khác:…….
9. VLTL hô hấp ☐

VII. BIẾN CHỨNG: ☐

Suy hô hấp	☐	Bội nhiễm phổi	☐	Nhiễm trùng huyết	☐
Tràn dịch màng phổi	☐	Tăng tiết ADH không thích hợp	☐	ARDS	☐
Chấn thương áp suất, thể tích	☐	Xơ hóa phổi	☐	Cơn CAP	☐
Tràn khí màng phổi, trung thất, dưới da	☐			Tử vong	☐