UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO IN SCIENZE
BIOMOLECOLARI E BIOTECNOLOGICHE

INDIRIZZO MICROBIOLOGIA MOLECOLARE E CLINICA

XXIV Ciclo

A STUDY ON VIRAL ETIOLOGIES OF LOWER RESPIRATORY INFECTIONS AND MOLECULAR CHARACTERIZATION OF INFLUENZA VIRUS H1N1 2009 CIRCULATING IN CENTRAL VIETNAM

Director : Prof. Bruno Masala

Tutor: Prof. Pietro Cappuccinelli

Doctorate thesis
Dr. Le Van An

ABBREVIATION

ARIs	= Acute respiratory infections
CPE	= Cytopathic effect
CT	= Cycle threshold
dATP, dCTP, dGTP and dTTP	
ddNTPs	= Dideoxynucleotide triphosphates
DNA/RNA	= Deoxyribonucleic acid / ribonucleic acid
dNTPs	= Deoxynucleotide triphosphates
dsDNA	= Double stranded DNA
ELISA	= Enzyme linked immunosorbant assay
FRET	= Fluorescence resonance energy transfer
NP	= Nucleoprotein
H or HA	= Hemagglutinin
N or NA	= Neuraminidase
HAI	= Hemagglutination inhibition
HBoV	= Human bocavirus
HCoV-HKU1	= Human coronavirus- HKU1
HCoV-OC43	= Human coronavirus- OC43
HCoV-NL63	= Human coronavirus- NL63
HCoV-229E	= Human coronavirus- 229E
HEK	= Human embryonic kidney cells
HMPV or hMPV	= Human Metapneumovirus
HPIV or PIV	= Human Parainfluenza Virus (Parainfluenza Virus)
IgG, IgA and IgM	= Immunoglobulin G (A, M) antibody molecule
IFA	= Immunofluorescent assay
LRTI (LRI)	= Lower respiratory tract infection
LLC-MK2	= A rhesus monkey cell line
MDCK	= Madin-Darby Canine Kidney cells
MGB	= Minor Groove Binding probes
PA	= Polymerase A protein
PB1	= Polymerase basic 1 protein
PB2	= Polymerase basic 2 protein
PCR	= Polymerase chain reaction
RSV	= Respiratory Syncytial Viruses
RT- PCR	= Reverse transcription- polymerase-chain-reaction
SARS	= Severe acute respiratory syndrome
SARS-CoV	= SARS-associated coronavirus.
URT	= Upper respiratory tract
WHO	= World Health Organization

TABLE OF CONTENTS

ABSTRACT

Respiratory infections are the most important cause of worldwide burden of disease in children and a major cause of mortality. In this study, viral etiologies of lower respiratory infections from children hospitalized in central Vietnam were detected by polymerase amplification assays. Total viral etiology was 46% and relative frequencies were 45% for influenza A virus, 42% for respiratory syncytial virus, 12% for adenoviruses, 8% for influenza B virus, and 5% both for parainfluenza type 1 and 3 virus. Influenza A (H1N1) 2009 virus was mainly responsible for infections in children from 3 months to 5 years of age. Detection of influenza A (H1N1) 2009 virus was performed both by isolation on MDCK cell culture and embryonated eggs and by amplification assays. In a group of 53 suspected patients A (H1N1) 2009 virus was detected in 32(60.4%) with a combination of both methods, and virus was isolated by cell culture in 24 patients. Characterization of HA and NA genes from representative isolates of influenza A (H1N1) 2009 virus was performed. Similarity of HA gene among Hue representative isolates are from 99.48% to 99.77% and similarity of NA sequences are from 99.57% to 99.86%. The sequences of HA and NA of Hue representative isolates were compared with 18 reference isolates from different countries showing similarity from 99.07% to 99.77% for HA and from 98.65% to 99.36% for NA sequences. Phylogenetic trees based on HA and NA sequences were constructed.

1. INTRODUCTION

1.1. Epidemiology and classification of lower respiratory infections

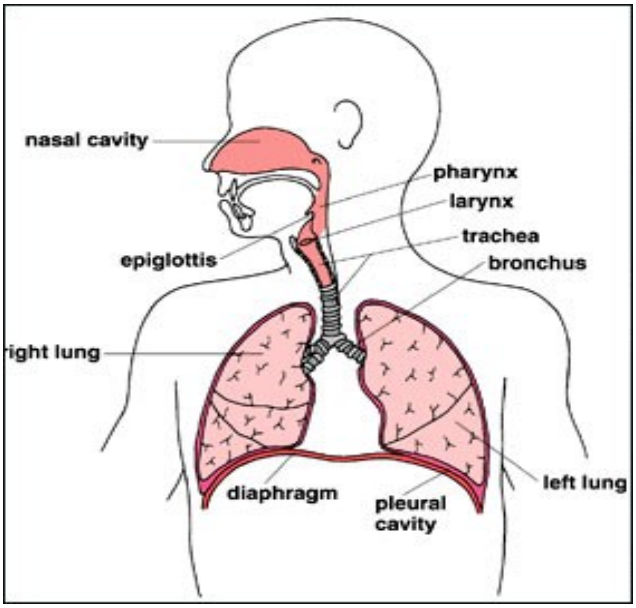
1.1.1. Epidemiology of lower respiratory infections

Respiratory infections are the most important cause of worldwide burden of disease in young children and a major cause of child mortality (62). Based on the data from the Global Burden Disease in 2004, the number of death caused by lower respiratory infections at all age worldwide was 4.2 million which contributed to 7.1% of total death. The number of death in low-income countries was 2.9 million which resulted in 11.2% of total death, while the number in high-income countries was 0.3 million corresponding to 3.8% of total death (127). Acute respiratory infections, mainly pneumonia, were responsible for 17% of total death in children younger than 5 years of age. Distribution of the world's child deaths due to pneumonia was 50% in Africa, 25% in Southeast Asia and 25% for the rest of the world (127). Each infected child had annually an average of three to six episodes of acute respiratory infections (ARIs) regardless of where they lived or what their economic condition was (102). Especially pneumonia is the leading cause of childhood mortality responsible for approximately 21% of deaths in children under 5 years of age in Africa each year (136). A survey on respiratory infections in children in USA from 1996 to 1998 showed that the incidence of lower respiratory infections hospitalized was 19% in children of preschool age, and the estimated percentage of children with acute lower respiratory infections hospitalized was 21,3% (40). In the Asia–Pacific area, the death incidence due to respiratory infections generally was 16,8%, and was mainly due to pneumonia (47). According the report of the Australian Lung Foundation in 2007, lower respiratory tract infections accounted for 3 million visits to the general doctors each year, and croup and bronchiolitis accounted for majority of winter hospitalizations in children (27).

1.1.2. Classification of lower respiratory infections

Acute respiratory infections from an anatomy point of view, are classified into upper respiratory infections, above the epiglottis, and lower respiratory infections, below the epiglottis (22). Although infection can cause injuries in more than one sites of the lower respiratory tract, most infected patients have a single site of major injury.

Figure 1
Schema of respiratory tract



The classification of lower respiratory infections based on anatomical location is useful because there is a close association between anatomical location of clinical syndrome and the causative agents as well as the severity of infections (22, 126). Thus anatomical classification of acute lower respiratory infections includes Bronchitis, Bronchiolitis and Pneumonia according to the site of major involvement (126).

1.1.3. Epidemiology of viral etiologies of acute lower respiratory tract infections

Among the numerous etiologic agents described, viruses are recognized as the predominant etiologic agents in ARI, in adults as well as children both in developing and industrialized countries (124). Several studies recently carried out worldwide to identify viral agents causing lower respiratory infections, showed that the most frequently detected virus was respiratory syncytial virus, which was annually varied from 30-80% in hospitalized infants and children younger than 5 years (40, 87, 104, 113, 117, 118), followed by influenza viruses, which accounted for between 20-30% (40), adenoviruses and parainfluenza viruses from 3-5% (118).

In Viet Nam, the dominant respiratory viruses causing acute respiratory infections are poorly understood, because of limited resources and laboratory facilities in most hospitals for detection of viruses. However, some studies on viral etiologies of acute respiratory infections carried out in recent years showed that respiratory viruses can be commonly detected in hospitalized Vietnamese

children. In these studies, the investigators included in their sample all the hospitalized pediatric patients with upper and lower respiratory infections. Specifically, in a recent study using multiplex polymerase chain reactions to identify respiratory viruses in 1766 hospitalized children with acute respiratory infections in a general hospital in Khanh Hoa province in the southern from February of 2007 through July of 2009, Yoshida and others showed that the commonest viruses were human rhinoviruses with rate from 23 to 28%, respiratory syncytial virus was encountered from 20 to 23%, influenza A viruses were from 13 to 15%, adenoviruses were 5% and parainfluenza virus type 3 was 4% (136). Another recently study carried out by investigators of the clinical research unit of Oxford University in Ho Chi Minh city for defining viral etiologies of acute respiratory infections among 306 hospitalized children younger than 5 years from November 2004 to January 2008 found that RSV also was the most frequently detected with rate of 24%, followed by influenza viruses with the percentage of 17%, while parainfluenza viruses were 7% and adenoviruses were 5% (4).

Besides the above-mentioned common respiratory viruses, other should be taken into consideration. In Hong Kong in 1997 eighteen cases of H5N1 infections in humans with severe respiratory symptoms occurred for the first time (20). This was a subtype of influenza A virus well known for causing the outbreaks in the poultry population.

Human cases of H5N1 infection with severe pneumonia have been reported in Viet Nam since 2003 and have continuously increased in the two consecutive years 2004 and 2005, with many fatal cases of severe pneumonia reported among the infected patients (20). Outbreaks of H5N1 infections in humans have spread in the following years in Asian countries such as China, Laos Cambodia, Indonesia and Philippines. The cumulative number of confirmed human influenza A (H5N1) infection reported to WHO on the 2nd August of 2011 was 563 human cases, with 330 fatal, among which 119 human cases and 59 deaths were from Viet Nam (129).

Another uncommon virus that responsible for a severe acute respiratory syndrome (SARS) in humans is the SARS-associated coronavirus (SARS-CoV). An outbreak of SARS started in late 2002, when cases of life-threatening respiratory disease with no identifiable cause were reported from Guangdong Province, China. The infections of SAR-CoV have spread out through the Asian region and other countries of the World in February-March of 2003 (56). SARS-CoV was isolated in April of 2004 and confirmed as a causative agent of this severe respiratory infection. In Viet Nam a total of 63 patients contracted SARS and five patients died of the disease (83).

1.2. Viral etiologies of acute lower respiratory tract infections

1.2.1. Respiratory Syncytial Viruses

1.2.1.1. Virology

RSV is an enveloped RNA virus of the genus *Pneumovirus* within the family *Paramyxoviridae*, this virus consists of two antigenic distinct groups, designated as A and B. Under electron microscopy, RSV has a pleomorphic spherical or filamentous form with the size of 80-350nm in diameter and up to 10μm in length. Glycoprotein spikes protrude from the lipid envelope which surrounds a tightly coiled nucleocapsid complex containing single stranded negative sense RNA (19, 22). RSV genome encodes 10 proteins, two of which are non-structural protein found only in infected cells and eight of the proteins are found in virions.

RSV grows well in many cell lines including HEp-2, HeLa, Vero and fibroblasts and replicates to a lesser degree in macrophages, RSV also replicates in primary human nasal and bronchial epithelial cells.

1.2.1.2. Epidemiology and pathogenesis

Humans are considered natural hosts of human RSV; infection results from direct contact with secretions from infected persons, other way of transmission may be through inoculation of virus into eye or nasal mucosa by contaminated hands. Once infected, virus replicates locally in nasopharynx and can persist in the infected child for up to three weeks. The virus can spread from the upper airway to the lower parts of respiratory tract through the respiratory epithelium or through the aspiration of infected secretions (124). The infected child may shed virus for several days and in the hospital environment, RSV can cause the outbreak of nosocomial infections (22, 124).

RSV causes annual epidemics during the cold months which began usually from late fall and continue throughout early spring. Fifty to 70% of infants are infected during the first year of life and up to three years of age all children are infected with RSV. Majority of primary infections are often mild, however, the virus can cause lower respiratory infections severe enough to require hospitalization such as bronchiolitis and pneumonia (104). Several studies worldwide in winter seasons identified the RSV as the most common causative agent in children younger than 3 years of age hospitalized with bronchiolitis. Approximately 70% to 89% of cases of bronchiolitis can be attributed to infection by RSV (87). A study by Shay et al showed that RSV may cause pneumonia from 30% to 60% of children younger than 5 years of age hospitalized in the winter time of 1994 and 1996 (104).

1.2.1.3. Virological diagnosis

Cell culture approaches are widely used for detection of RSV from clinical samples. RSV can infect many cell lines, but Hep-2 cells are commonly used for RSV isolation because of high sensitivity of viral recovery (63, 69). RSV is the most labile of the respiratory viruses, so the specimens should be inoculated into cell culture after collection. Cytopathic effect (CPE) in form of syncytial cells appears after 2 and 7 days of incubation at 36^0C.

At present, many commercially rapid antigen- detection assays are available for use in most hospitals and health care centres such as fluorescence assay, ELISA assay. The sensitivity and specificity of these tests are from 70-95% and 90-100% respectively (69).

Nucleic acid amplification techniques (PCR) in conventional and real-time platform have been developed since 2 decades for detection of this virus and this approach has become a standard assay for RSV diagnosis worldwide (30).

1.2.2. Adenoviruses

1.2.2.1. Virology

Adenoviruses constitute the family ***Adenoviridae***, which has been previously divided into two genera: *Mastadenovirus* and *Aviadenovirus*. Genus *Aviadenovirus* is limited to viruses of birds, whereas, all human adenoviruses are belonging to *Mastadenovirus* genus.

Human adenoviruses have so far had 51 serotypes divided into species (or subgroups) from A to F based on their capacity of agglutination of erythrocytes of human, rat and monkey as well as their oncogenicity in rodents (96, 133). Each species contains one or more serotypes, which were distinguished on the basis of their ability to be neutralized by type specific antisera.

In structure, adenoviruses are icosahedral particles (20 triangular surfaces and 12 vertices) that are 70 -100 nm in diameter. Particles contain a single linear, double stranded DNA molecule, proteins and no lipid envelop or outer membrane. Virion consists of a protein capsid surrounding a DNA containing core and capsid is composed of 252 capsomeres, of which 240 were hexons and 12 are pentons. Protruding from each of the penton base is an antenna like structure called the fiber. The hexon, penton and fibers constitute the major adenoviral antigens important in viral classification and diagnosis (22, 133).

All adenoviruses have the same organization of genomes, which means genes encoding specific functions are located at the same position on the viral chromosome (133). The DNA sequence coding for proteins with common functions among adenoviral serotypes can be divergent. This divergence can be used for analysis of adenoviral phylogeny and genetic diversity (22). Adenovirus can grow and produce CPE on continuous human cell lines and

replicate at very low or poor yield in non human cell cultures. In human cell lines, adenovirus can replicate in HeLa, KB, HEp-2, A549, primary human embryonic kidney (HEK). In nonhuman cell cultures, adenovirus can grow poorly in Cynomolgus Monkey kidney cells, Graham-293 cell line (41).

1.2.2.2. Transmission and pathogenesis

Adenoviral infections are common, and have a worldwide distribution, and no seasonal occurrences (26, 78). Adenoviral infections are frequent during childhood and there is a broad spectrum of adenovirus associated diseases due to the various serotypes and different tissue tropisms. Adenovirus can commonly infect and replicate at various sites of the respiratory tract, in the eye and gastrointestinal tract, although the virus can cause disease in many organs.

Adenoviruses are spread by human to human transmission, mainly by respiratory or fecal-oral contact (78), close interaction among people promotes spread of the viruses such as in classrooms and military barracks. The virus may be shed intermittently and over long periods from the pharynx and in feces.

1.2.2.3. Epidemiology and lower respiratory infections of adenoviruses

With respiratory diseases, adenovirus causes about 5% of upper respiratory tract infection in children younger than 5 years of age, these infections are frequently mild and self-limited. Adenoviruses are also causative agents of lower respiratory infections, they may be responsible for about 10% of the pneumonia in childhood, and most patients recover from these lower respiratory infections: however, some epidemics have resulted in a certain degree of mortality. Sequelae in those who recover can include bronchiectasis that can clinically manifest years after the primary infection (133). In a study from 1472 children hospitalized with lower respiratory infections from 1990 to 1998 in Korea, adenoviruses were found in 6% of patients, the main serotypes were type 2 (15%), type 3 (15%) and type 7 (41%). Mortality rate was 12% overall, and 19% was among the patients infected with type 7 (41). A surveillance for adenoviruses as causative agents in lower respiratory infections in children younger than 2 years of age in Santiago in Chile was carried out from 1989 to 2001, the virus was detected in 9.3% of the enrolled patients, and adenoviral cases were 13.7% in children over 6 months of age and in the younger than 6 months were 6.8%. Pneumonia and bronchitis were the most frequent diagnoses (86).

1.2.2.4. Virological diagnosis

Cell culture remains the gold standard for detection of adenoviruses in clinical laboratory (26, 72, 133), Cell line cultures of human origin, such as A549, Hep-2, and HeLa, can be used for the recovery of adenoviruses from all

clinical specimens. Cytopathic effect is usually visible in 2 to 7 days, although it may take up to 28 days (26).

Direct antigen detection is widely used for diagnosis of respiratory because it is rapid and reasonably sensitive, immunofluorescence is used for detection of adenovirus from respiratory samples although the sensitivities of adenovirus immunofluorescence assays with respiratory specimens are 40 to 60% compared to culture. Other antigen detections are also available such as ELISA, latex agglutination, but these assays are commonly used for detection of adenoviruses in stool samples and not for respiratory samples (26, 72).

Molecular methods are highly sensitive and are especially applicable when noninfectious virus is present, when the viral load is too low to be detected by culture, or when results are needed rapidly. The conventional and real-time PCR platforms are available in many clinical laboratories for diagnosis, these assays can be used with a variety of clinical samples (133), and some real-time protocols are also commercial for analysis of subtype of adenoviruses (30).

1.2.3. Human Parainfluenza Virus (HPIV)

1.2.3.1. Virology

Parainfluenza viruses belong to the family *Paramyxoviridae* comprising two genera: *Respirovirus* (PIV1 and PIV3) and *Rubulavirus* (PIV2 and PIV4). Parainfluenza viruses are similar in their appearance and in their biophysical characteristics. They have pleomorphic, spherical virions with average diameter from 150 to 200nm. HPIV 2 has been in some cases seen in filamentous forms (51). The virion consists of a nucleocapsid packaged in a lipid envelope spiking with projections of hemmaglutinin and neuraminidase glycoprotein or F transmembrane glycoprotein, the inner surface of the envelope is coated with nonglycosylated matrix protein. Nucleocapside contains a single strand of negative-sense RNA of 15500 nucleotides in length (39, 51).

HPIV can grow in primary rhesus monkey kidney cells or continuous cell line of LLC-MK2 (22). HPIV 1, 2 and 3 can replicate in embryonated chicken egg. However, eggs are not reliable and sensitive for the isolation of virus from patient samples (51).

1.2.3.2. Epidemiology and lower respiratory infections of HPIV

Similar to RSV, HPIV are causative agents of acute lower respiratory infections in young children. Serologic surveys indicate that at least 60% of children have been infected with HPIV3 by 2 years of age and that about 80% have been infected by 4 years of age (51). HPIV can also cause upper respiratory infections with cold-like symptoms. Approximately 25% of cases in which HPIV spread down to lower respiratory airway and cause severe lower respiratory infections that need to be hospitalized such as bronchiolitis, pneumonia (39, 78). A study of respiratory infections of parainfluenza viruses in

children younger than 5 years of age indicated that the frequency of HPIV infections was 12/1000 children/year, and children younger than 6 months of age represented 30% of the bronchiolitis cases (92). A study on 1347 children with acute lower respiratory infections in 1996-1998 reported that the rate of infections with HPIV 1 was 5%, HPIV 2 was 3% and HPIV 3 was 9% (40).

In Viet Nam recent studies showed that the rate of infections with HPIV in hospitalized children younger than 15 years of age were from 4 to 7% of all hospitalized children with acute respiratory infections (4, 136).

HPIV can cause nosocomial infections when pediatric patients are in the same wards. Immunity obtained after primary infection of HPIV can be short lived, thus reinfection of HPIV can occur throughout life, and disease is often milder (51, 78).

1.2.3.3. Laboratory diagnosis

Isolation of HPIV from clinical samples can be done on animal cell cultures such as primary rhesus monkey kidney cells or continuous monkey kidney cell line LLC-MK2. Cell cultures are observed for 10 days; HPIV clinical isolates rarely show CPE during primary isolation in tissue cultures, some HPIV cause varying degrees of CPE after 7 to 10 days of inoculation. Presence of HPIV is screened by hemadsorption of guinea pig red blood cells onto the tissue culture monolayer; immunofluorescence is more reliable and earlier to detect HPIV on cell cultures (38).

Antigen detection of HPIV directly in nasopharyngeal specimens can be performed with immunological assays such as ELISA and Immunofluorescence assays, the sensitivity of these assays was varying from 75-90% as reported by investigators (39).

Molecular amplification are widely used for detection of HPIV in clinical samples, especially RT-PCR can be used easily and efficiently to detect small numbers of virus in different fluids. Several laboratories have recently developed and used the multiplex RT-PCR assays to detect HPIV and also other respiratory viruses such as adenovirus, RSV, influenza A and B viruses (4, 61, 69).

1.2.4. Human Metapneumovirus (HMPV)

HMPV was first described in 2001 following its isolation from infants and children having a RSV-like disease and it is known as a member of family *Paramyxoviridae* like RSV. HMPV virions were visualized by electron microscope as pleomorphic spheres and filaments. The spherical particles had a reported diameter of 150 to 600nm with enveloped spike projections of 13 to 17 nm, Nucleocapsid contains a single strand of negative sense RNA molecule (19, 25). Based on genomic sequencing and phylogenetic analysis, there are two

major genotypes of HMPV, designated A and B and each genotype appears to have at least two distinct subgroups (49). HMPV can be isolated in rhesus LLC-MK2 cell line or African green monkey vero cells but replicates more slowly and its cytopathic affect is less prominent than with RSV (19).

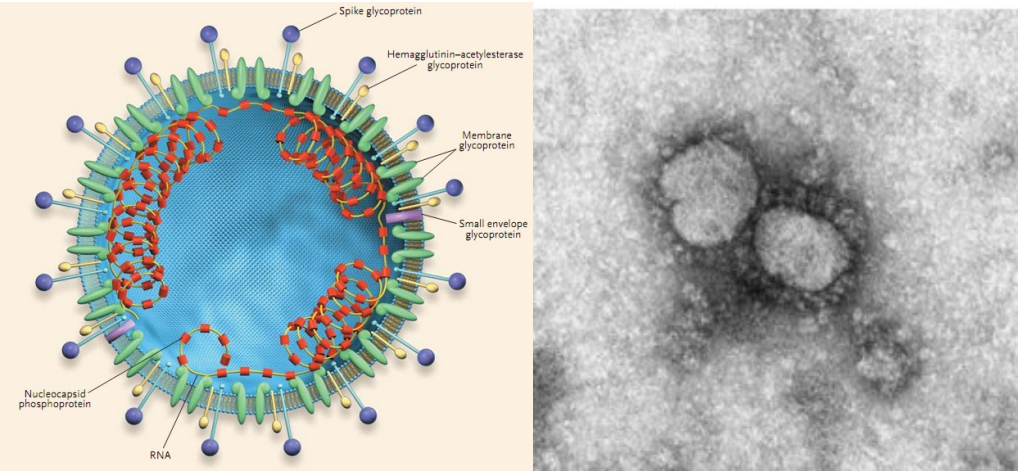
HMPV has a worldwide and a seasonal distribution, with most infections occurring during the winter and spring (19, 31). HMPV infection accounts for approximately 2% to 12% of pediatric lower respiratory illnesses and a lesser percentage in adults (31). In young children, the clinical symptoms associated with hMPV infection are virtually indistinguishable from those caused by RSV, varying from mild upper respiratory tract infections to severe pneumonia (31). HMPV can also cause severe and fetal infections in patients with malignancy and hematopoietic stem-cell transplant (19).

For laboratory diagnosis, HMPV can be isolated in cell cultures; however, this method is not used routinely in many laboratories. Immunofluorescent staining assays have been developed and used for identification of HMPV from nasopharyngeal samples with sensitivity and specificity of 73.9% and 94.1% respectively. But commercial diagnostic kits are not available yet (19). An ELISA to measure HMPV antibody has been described; however, antibody detection did not correlate well with RT-PCR results, limiting the usefulness of serology for diagnosing infections (69). RT-PCR assays in conventional and real-time formats have been developed and used in many laboratories for diagnostics because of their highly sensitivity and specificity (4, 19, 69). Currently, RT-PCR assays are the most common methods used to detect HMPV (49, 69).

1.2.5. Coronaviruses

Members of the *Coronaviridae* are known to cause respiratory or intestinal infections in humans and other animals. Coronaviruses are spherical enveloped particles about 100 to 160nm in diameter. Nucleocapsid has a long flexible, helical shape containing inside a single stranded, positive sense RNA 27 to 32 kb in size. The virus core is enclosed by a lipoprotein envelope, on which two types of prominent spikes project out. The long spikes about 20nm in length are present on all coronaviruses, whereas, the short spikes consisting of the hemagglutinin esterase glycoprotein are present in only some coronaviruses. The envelope also contains the transmembrane glycoprotein (M glycoprotein) and an envelope protein (E protein) (15, 59).

Figure 2
Structure of coronavirus and electron microscopic image of SARS-CoV (reproduced from 7, 43)



Coronaviruses can be divided into three serologically distinct groups. In present, group I and II have been isolated from mammals and group III from birds. Most coronaviruses naturally infect only one animal species or a limited number of closed related species (59). SARS-CoV is an exception, it has been shown to infect a wide range of mammals, including humans, nonhuman primates, Himalayan palm civets, raccoon dog, cats, dogs and rodents (59). Coronaviruses causing diseases in humans can be described as follows.

Table 1
Human coronaviruses and diseases (adapted from 7, 59).

Group	Virus	Host	Respiratory infection	Enteric infection
I	HCoV-229E	Human	X	
	HCoV-NL63	Human	X	
II	HCoV-OC43	Human	X	
	HCoV-HKU1	Human	X	
	SARS-Cov	Human	X	x

Natural infection in humans is through inoculation of infectious secretions from infected persons or fomites onto mucous membrane of the upper respiratory tract or inhalation of infectious droplets (7, 59).

Human infection due to HCoV-229E and HCoV-OC43 is often mild and limited in upper respiratory tract infections (59). The two recently identified human coronaviruses HCoV-NL63 and HCoV-HKU1 have been detected in persons with acute upper and lower respiratory tract infections (31, 59). SARS-CoV infection of humans nearly always resulted in a serious lower respiratory illness that required hospitalization. The overall fatality rate was around 10% but reached 50% in elderly patients and those with underlying illness (7, 59).

Etiological diagnosis and differentiation from other causes of atypical pneumonia can be made only by laboratory confirmation (15, 59). Isolation of SARS-CoV in cell culture must be carried in a biosafety level 3 laboratory, which is not available in most hospitals (15). Some serology assays have been developed to detect antibodies in the serum of SARS patients such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescent assay (IFA), these tests can produce positive results from 10 days to 21 days after the onset of illness. Varieties of molecular tests based on amplification techniques have been developed and used for detection of coronavirus infections in humans including SARS, and have become the standard for detecting coronavirus infections in humans and animals. Great care must be taken to prevent and recognize false positive test results due to contamination (7, 59, 69).

1.2.6. Rhinovirus and Bocavirus

1.2.6.1. Rhinovirus

Rhinoviruses are positive-sense single-stranded RNA viruses belonging to the family *Picornaviridae*. Rhinovirus infections occur year round, with peaks in late spring and early September in temperate climates (69, 120). Rhinovirus infections are spread from person to person by means of virus contaminated respiratory secretions. Nasal secretions contain a high concentration of virus, and infected persons readily contaminate their hands (120). Rhinovirus is transmitted either by direct contact with contaminated fomites followed by self-inoculation of the eye or nose or by aerosolized droplets (69, 120). Virus replicates primarily in nasal epithelial cells, and the shedding of virus coincides with acute rhinitis and may persist for 1 to 3 weeks (69).

Rhinovirus infections are mostly established in the upper respiratory tract, and cause upper respiratory tract (URT) infections, traditionally defined as common colds (120). Several studies recently using RT-PCR to detect respiratory viruses from respiratory samples showed that rhinoviruses were very common in lower respiratory infections, and in one study they were responsible for 21.8% of bronchiolitis in infants less than 12 months of age (69, 87). HRV-

infection is the most common virus associated with exacerbations of asthma of all ages and with COPD in adults and the elderly (69, 87).

Rhinoviruses have traditionally been isolated in cultures of human diploid fibroblast cell lines such as WI38 cells, HeLa cells; however, isolation was rarely done because use of more than one type of cell culture is required for optimal isolation (68, 69). RT-PCR assays become the methods of choice for diagnosis of rhinoviruses because of their high sensitivity in comparison to virus culture (68, 69, 87).

1.2.6.2. Bocavirus

Human bocavirus (HBoV) is a small, nonenveloped, single-stranded DNA virus within the family *Parvoviridae*, subfamily *Parvovirinae*, and genus Bocavirus (3, 99). HBoV was discovered in September 2005 by Allander and colleagues in Sweden. There have been numerous studies reporting HBoV prevalences ranging from 2 to 11% in respiratory tract specimens from over 12 countries on five continents including Europe, North America, Asia, and Australia (69), indicating that this virus is distributed worldwide (69). HBoV has been detected in children with LRTI and has been associated with abnormal chest radiographic findings in several studies, but the causative role was not clearly established (3, 69, 99).

Human bocavirus has not been replicated in vitro, and HBoV infections have been diagnosed almost exclusively using molecular methods (3, 69).

1.2.7. Influenza viruses

Influenza viruses belong to the family *Orthomyxoviridae*. AT present, the family *Orthomyxoviridae* has three genera or types: influenza A virus, influenza B virus and influenza C virus. They are distinguished by major antigenic differences in the nucleoprotein and matrix proteins, besides there are other differences in genetic organization, viral structure, host range, epidemiology and clinical characteristics between the three influenza virus types that are summarized in the table below

Table 2
Differences among influenza viruses (reproduced from Treanor J (115)

Influenza type	Genome	Host range	Epidemiology	Disease
Influenza A viruses	8 gene segments	Humans, avian, swine, equine, marine mammals	Antigenic shift and drift	May cause large pandemics with high mortality
Influenza B virus	8 gene segments	Humans only	Antigenic drift only	severe disease to older adults or high risk patients
Influenza C virus	7 gene segments	Humans and swine	Antigenic drift only	mild diseases, without seasonality

1.2.7.1. Influenza A viruses

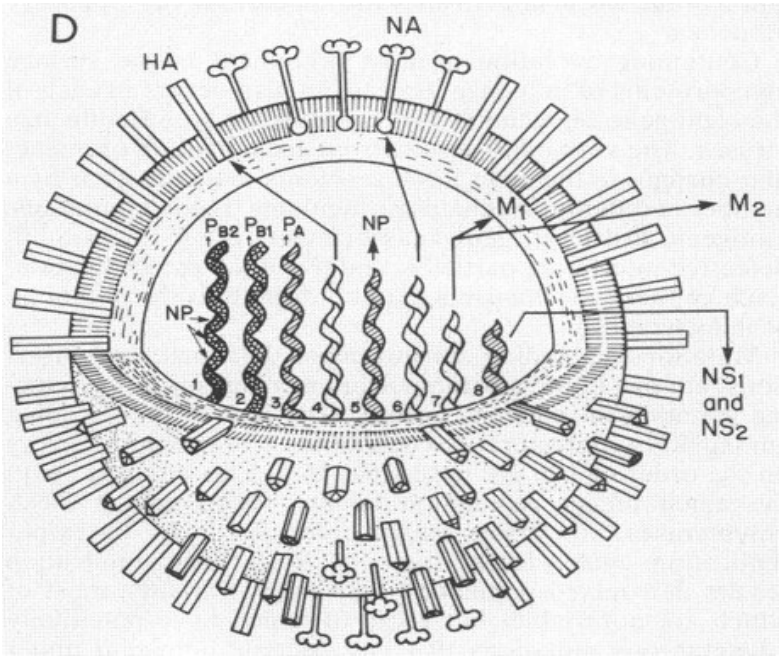
Influenza A viruses are small pleomorphic or spherical particles with diameter of 80-100nm, the typical virion consists of a host derived lipid bilayer envelope, which is internally lined with a matrix protein (M) and externally with spikes in rod shape of glycoprotein hemaglutinin (HA) and in mushroom shape of neuraminidase molecules (NA). In the core is a nucleocapsid of helical symmetry surrounding a segmented genome of negative-sense single stranded RNA. The genomic RNA is made of eight segments encoding for 11 known proteins: these gene segments, proteins and their functions are summarized in table 3 (35, 85, 103).

Influenza A viruses are further classified into subtypes based on two surface antigens, hemaglutinin (H) and neuraminidase (NA), and so far there are 16 hemaglutinin antigens (HA1 to HA16) and 9 neuraminidase antigens (NA1 to NA9). So each virus always contains one HA and one NA antigen in any combination.

Table 3
Genomic segments and their functions of influenza A viruses

Segment	Segment length	Protein	Functions
1	2340 nucleotides	PB2	polymerase B2 protein
2	2340 nucleotides	PB1	polymerase B1 protein
2	2230 nucleotides	PA	polymerase A protein
4	1775 nucleotides	HA	Hemaglutinin, viral attachment protein
5	1565 nucleotides	NP	Nucleoprotein
6	1410 nucleotides	NA	Neuraminidase, cleaves sialic acid and promote viral release
7	1025 nucleotides	M1	Matrix protein: viral structural protein
		M2	Membrane protein: form membrane channel
8	890 nucleotides	NS1	Nonstructural protein: inhibit cellular mRNA translation
		NS2	Nonstructural protein

Figure 3
Viral structure of influenza viruses (reproduced from Webster RG (123)



Influenza A viruses infect a variety of animals consisting of humans, swine, horse, sea animals and birds and establish a reservoir of influenza A viruses in nature.

- **Human influenza A viruses**

Influenza A viruses have been known to infect humans for very long time. However, there are only some defined subtypes of influenza A viruses causing infections in humans, and they have always been belonging to the HA subtypes 1, 2 and 3 and NA subtypes 1 and 2 (122). The first subtype of influenza virus known to cause pandemic in humans was H1N1 in 1918 and the sequence data of influenza strains obtained from patients who died during this outbreak showed that the sequences of HA of the H1N1 1918 were similar to the mammalian viruses and not to the avian viruses (93, 114). The major subtypes of influenza A viruses responsible for the consecutive pandemics were H2N2 (1957), H3N2 (1968) and H1N1 (1977).

New subtype H1N1 from swine origin occurred and caused pandemic in 2009 in humans (52, 138).

- **Avian Influenza A virus**

Aquatic birds are known to be natural reservoirs of influenza viruses and so far all of the known 16 HA and 9 NA subtypes of influenza A viruses have been isolated from birds, particularly in migrating water fowls (1, 115, 123). Infections of influenza A virus in aquatic birds are mostly asymptomatic, only few subtypes can cause infections with systemic involvement and high rate of death in some water birds such as H5N1 and H7N7 (58, 134). In water birds, influenza viruses replicate in the epithelial cells in intestinal tract and to lesser extent in cells of respiratory tract, consequently, influenza viruses can be excreted in high concentrations in feces. Contaminated water and feces serve as major routes of transmission of influenza viruses among wild water birds (134). International migration of aquatic birds can be responsible for transmission of avian influenza A viruses to other parts of the world.

- **Swine influenza A viruses**

Two subtypes of influenza A virus, H1N1 and H3N2, have been isolated from pigs (1, 123). These include classic swine H1N1, avian-like H1N1 and human- and avian-like H3N2 (123). Influenza A virus of H1N1 subtype (Hsw1N1) was first isolated from pigs in the USA in 1930 and subtype H3N2 was circulating in swine population in the USA since 1988, having its antigenic H3 similar to the human strain (134). The swine H3N2 subtype was also isolated in pig in other countries in Europe and Asia. Pigs have been known to serve as major reservoirs of HlNl and H3N2 influenza viruses and are frequently involved in interspecies transmission of influenza viruses (123, 134).

- **Influenza A viruses in other animals**

Besides three above mentioned species as reservoirs of influenza A virus, other animals are known to be infected with influenza A viruses.

Influenza A virus subtypes of H3N8, H7N7 can be commonly isolated from horse worldwide and other subtypes of H3N2, H2N2 and HINI have also been occasionally isolated in horse (123, 134).

H7N7 and H4N5 subtypes have been isolated from lung and brain of seal (123, 134).

H13N2 and H13N9, H1N3 have been isolated from whale and H10N4 from mink (123, 134).

1.2.7.2. Epidemiology and antigenic variations of influenza A virus

Transmission of human influenza viruses from person to person is primarily by airborne rout and secretions from infected individual, released by coughing as small virus containing particles of 1-5μm in diameter are inhaled by susceptible people. This mode of transmission is very efficient in winter time with high humidity and accounts for the abrupt onset and rapid spread of epidemic influenza in the community (66, 123). An influenza outbreak can be detected by the increase absenteeism in school and work and the increased number of emergency room visits (78).

The influenza outbreaks occur yearly among populations in temperate region throughout the world, normally one subtype become dominant in a given season. The main reason for annual occurrences of influenza epidemics is the emergence of a new strain of influenza A virus. When a new strain replaces the previous one, it can escape the host immunity generated by previously circulating strains and cause infection in humans. There are two different mechanisms of antigenic change: antigenic drift and antigenic shift.

Antigenic drift occurs when the genes encoding the viral surface antigens, the NA and the HA, undergo stepwise mutation each time the virus replicates. As a result of this, their proteins on the virus particle are so different enough that antibodies available in the host are unable to neutralize the virus, giving rise a variant capable of causing infection (1, 123, 134). This change happens every 2 to 3 years, producing local outbreaks of influenza A virus.

Antigenic shift occurs when two viruses from different host species co-infect an intermediate animal host. By reassortment of genetic segments from two viruses, a new virus arises that contains the necessary genes to enable infection in humans and also has surface antigens new to the host immune system. Antigenic shifts occur infrequently, taking place on average every 10 years (1, 115, 123, 134).

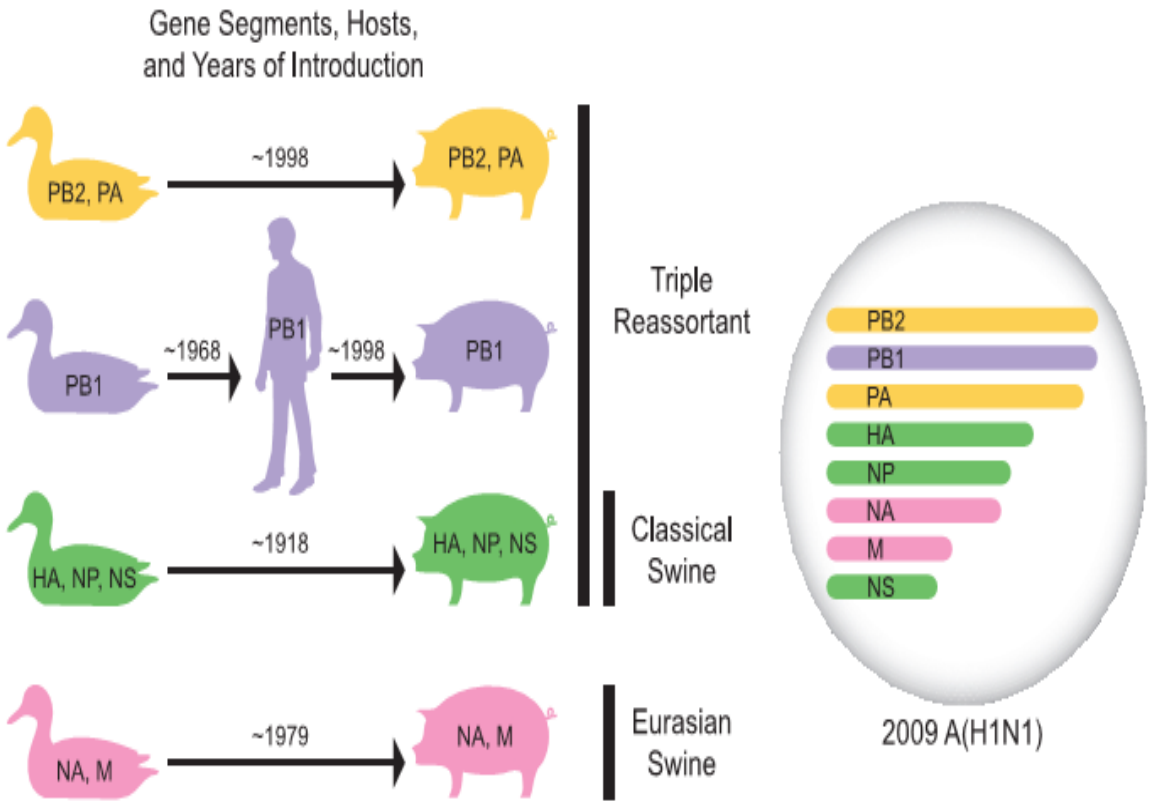
There have been several pandemics of influenza A viruses in humans associated with the reassortment of the current circulating strain of human influenza A virus.

In 1947 the prevalent strain was the H1N1 subtype and in 1957, there was a shift in both antigens of HA and NA, resulting in an H2N2 subtype (Asian influenza A strain). This Asian influenza A strain obtained its HA, NA and PB1 genes from an avian virus and the remain genes from human H1N1 subtype.

In 1968 when Hong Kong strain (H3N2) appeared, this strain was a reassortment of HA3, PB1 from avian virus and of NA2, plus five other genes, from the human H2N2 subtype circulating since 1957.

In April 2009, a human strain of novel H1N1 subtype appeared in Mexico, USA and Canada and then spread to other countries all over the world (79). The 2009 H1N1 virus contains a combination of gene segments that previously has not been reported in swine or human influenza viruses in the United States or elsewhere (33). The NA and M gene segments are from the Eurasian swine population and HA, NP, and NS gene segments are similar to those of classical swine viruses (33, 79, 109). The PB2 and PA gene segments are in triple reassortant lineage. The PB1 segment is from the swine triple reassortant viruses, this lineage of PB1 was seeded into swine from humans around 1998 (33).

Figure 4
Reassortment of novel H1N1 (2009) subtype of influenza A virus (adapted from Garten RJ (33).



Avian influenza viruses of the H5, H7 and H9 subtype do not currently possess the ability of sustained human-to-human transmission; this is associated with the difference in binding receptors and surface glycoproteins. Avian influenza viruses are binding preferentially to α 2,3-linked sialic acid (SA) residues existing in the ciliated cells of the respiratory tract and gut of aquatic birds and in the non-ciliated cuboidal bronchial cells at the junction between the respiratory bronchiole and type II cells lining the alveolar wall alveolus of human lower respiratory tract. In contrast, human influenza viruses preferentially bind to α 2,6-linked sialic acid (SA) residues, which are located predominantly on epithelial cells in nasal mucosa, paranasal sinuses, pharynx, tracheal, and bronchi of human respiratory tract (7, 58, 134). The different distribution of the viral specific binding receptors in human respiratory tract can explain for the facts that the recently reported infections of highly pathogenic avian H5N1 virus have had severe involvements of pneumonia and that the human to human transmission of avian H5N1 influenza viruses have been still limited because viral replication in upper respiratory tract is necessary for shedding out of virus containing droplets (20, 54, 134).

The hundreds of documented cases of human disease caused by viruses within the H5, H7 subtypes show the ability of influenza A viruses to infect humans by different routes. The majority of human cases of avian H5 and H7 virus infections have been associated with direct contact transmission from exposure to sick or dead poultry, often following slaughtering and preparing sick poultry for cooking. In a few cases of H5N1 subtype, transmission route might be inhalation of aerosolized infectious poultry feces or other material; consumption of uncooked blood from H5N1-infected ducks has been documented in some cases of H5N1 infection (7, 20).

1.2.7.3. Infections of influenza A viruses in humans

Human influenza viruses replicate almost exclusively in superficial cells of the respiratory tract, influenza virus is released from the apical surface of the cell, which may limit more systemic spread but facilitate accumulation of virus in the lumen of the respiratory tract for transmission to the new host susceptible host (134). Influenza virus replicates throughout the respiratory tract with virus being isolable from the upper and lower tracts of people naturally and experimentally infected with virus. The site of optimal replication in the respiratory tract for some strains may be regulated by the prevalence of the specific binding receptors of influenza virus. Majority of influenza virus infections result in clinical responses ranging from asymptomatic infection to primary viral pneumonia that progresses rapidly to a fatal outcome. The typical uncomplicated influenza syndrome is a tracheobronchitis with some involvements of small airway; the severe cases with primary viral pneumonia are seen in the patients with high risk factors such as elderly people, pregnant women, immunocompromised hosts (115, 134).

1.2.7.4. Laboratory diagnosis of influenza viruses

Isolation of influenza virus in cell culture or embryonated eggs is the most sensitive method for detection of virus in a clinical specimen of good quality; this method allows detection of virus relatively rapid around 3 days. The available system of cell cultures is commonly used and sufficiently sensitive to replace for embryonated eggs for isolation of currently circulating strains.

- Isolation of influenza virus on tissue culture or embryonated eggs

The most widely used cell culture for isolation of influenza viruses is Madin-Darby Canine Kidney (MDCK) tissue cultures. A combination of MDCK and LLC-CK2 continuous line of rhesus cells provides a sensitive system for influenza and parainfluenza viruses (21). Specimen is inoculated into the cell culture at the phase of confluence and incubated at temperature of 33-34^{0}C in 5% CO2 atmosphere. Growth of influenza virus can be seen with occurrence of cytopathic effect after 2-3 days of inoculation (128). Another approach for isolation of influenza viruses is inoculation of clinical samples into embryonated chicken 9-10 days of age. Three embryonated eggs are essentially inoculated with a specimen to ensure one good test, incubation of eggs for 2-3 days at 33-34^{0}C. In both cell cultures and embryos the presence of influenza virus in the harvested fluids can be detected by hemagglutination of chicken RBC (21, 128). Identification of influenza viruses can be done by immunoassays with available specific antiserum (21). Immunofluorescence is commonly used for this purpose, however, other assays as ELISA or HAI can be used, and especially HAI is useful for serotype identification of the circulating isolates (21, 115). Molecular amplifications are used commonly for detection and identification of the serotype of influenza virus with the specific subtype primers.

- Antigenic detection

Identification of influenza virus within a few hours as the cause of an acute respiratory infection is of considerable value to the clinical practice. Immunoassays as immunofluorescence (IF) and ELISA (enzyme linked immunosorbent assay) are most commonly employed for direct detection of viral antigen in clinical samples (115). Both direct and indirect immunofluorescent methods have been used for rapid detection of influenza viruses. Monoclonal antibodies are commercially available for these procedures, in which antibodies recognize the NP and M1 proteins of all type A and B. Sensitivity of IF assay is ranging from 70 to 100% in several studies (134). ELISA for antigen detection may have many formats, the most common of which uses a capture antibody bound to the solid phase. Incubation with a specimen results in the binding of viral antigen present in that specimen to the antibody. The bound viral antigen is then detected with another antibody using the direct or indirect method. The sensitivity of ELISA assay is similar to IF assay in many studies (115).

- Serology

A serum antibody response can be documented in 80% of human influenza infections, after infection; HA specific IgG, IgA and IgM antibodies are detected, whereas, in the secondary response, IgG and IgA antibodies are predominant (134). A variety of influenza-specific assays are available, including complement fixation, haemagglutination inhibition assay, single radial haemolysis, neutralisation, immunofluorescence and enzyme immunoassay. The traditional "gold standard" techniques for detecting influenza-specific antibodies are neutralization and the haemagglutination inhibition assay, as they can differentiate subtype-specific (including H5N1) and strain-specific serological responses (24**)**. Serology assays are often used for epidemiological studies, evaluating the immune response of a vaccine, and are not useful for diagnosis of an acute influenza infection because the confirmation can be made after 1-3 weeks and the double serum samples are needed for demonstration of increasing antibody titres (24, 40).

- Molecular techniques

Detection of specific viral nucleic acid has become a principle method for rapid viral diagnosis in clinical setting and in epidemiological surveillance because they are sensitive and specific, can be done with high input of specimen, and for typing and subtyping of influenza viruses, they can also detect other respiratory viruses with the multiplex format (4, 37, 61). Several different types of molecular diagnostic tests are available; the most commonly used are traditional reverse transcription-polymerase chain reaction (RT-PCR), RT-PCR with detection by enzyme-linked immunosorbent assay (ELISA RT-PCR), real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR) and nucleic acid sequence-based amplification (NASBA) (69, 110, 111). The traditional RT-PCR, ELISA RT-PCR, and Real-time RT-PCR are similar tests, except the PCR product is detected by different methods. These molecular methods can be described in more detail in the next section.

1.3. The role of molecular techniques for identification of respiratory viruses

1.3.1. Amplification

This enzymatic reaction allows in vitro amplification of specific DNA fragments from complex DNA samples and can generate microgram quantities of target DNA. Since its development in 1985, this technology has evolved into an increasingly powerful versatile tool in gene cloning, DNA sequencing, the study of molecular evolution, clinical diagnosis of microbial agents and genetic diseases veterinary medicine, food and agriculture, environment science, forensic medicine and analysis of human genome. Polymerase chain reaction is a powerful method with widespread applications in clinical microbiological

laboratory for research and diagnosis of bacterial and viral pathogens (65, 70, 88, 90, 108, 111).

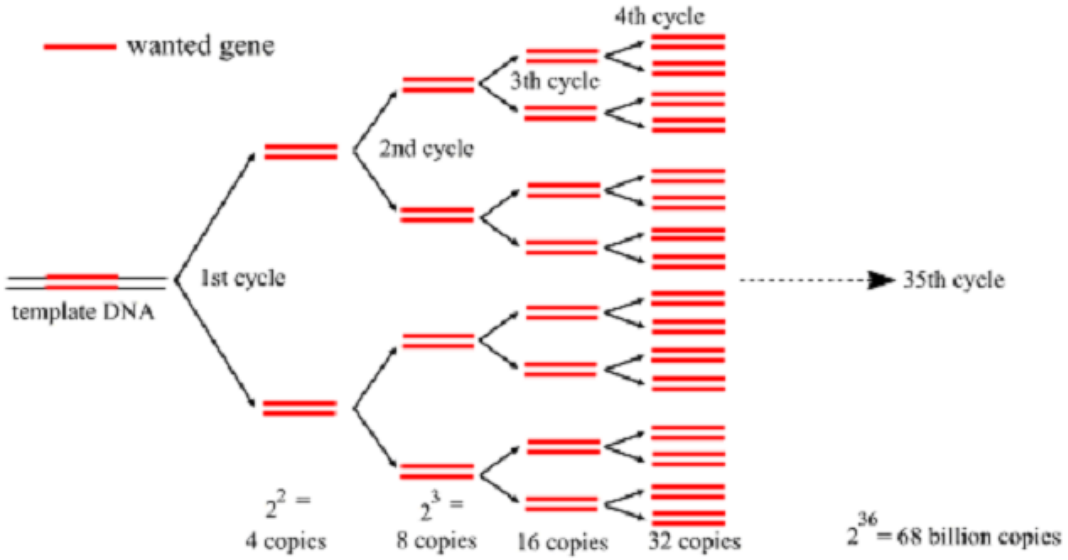
Principle of polymerase chain reaction

Polymerase chain reaction allows the exponential amplification of specific DNA fragment by in vitro DNA synthesis. A standard method requires a DNA template containing a target region to be amplified; two oligonucleotide primers flanking this target region; an enzyme thermostable DNA polymerase isolated from *Thermus aquaticus* is needed for new DNA fragment synthesis and other chemicals including deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. All the PCR components are prepared and processed in a procedure of repetition of three successive steps (53, 90, 108):
(i) Denaturation of double stranded DNA (dsDNA) into single stranded DNA (ssDNA) at high temperature of 90-96^{0}C for 30 to 90 seconds.
(ii) Annealing (hybridization) of two specific primers to each single stranded DNA at 5- 3' terminal at temperature of 50-60^{0}C for 1- 2 minutes.
(iii) Extension at temperature of 72^{0}C for 1- 2 minutes for synthesis of complementary DNA strands.

These three steps create a cycle of PCR and the number of the target DNA sequences doubles at the end of each cycle. In the next cycles, the newly synthesized DNA strands are separated again at the denaturation and each strand becomes a new template for annealing and synthesis. A PCR procedure is often carried out for 35 to 45 cycles (53, 108). The amplification procedure is automatically performed in a thermocycler with programmed heating and cooling.

Products of a PCR can be determined by a variety of methods, the most common method is to separate the products according to their sizes on agarose or polyacrylamide gels by electrophoresis, and they are visualized after staining with ethidium bromide. The standard DNA ladder with known size is analyzed in parallel with the samples to compare with the PCR products.

Figure 5
Exponential accumulation of target DNA products in PCR (reproduced from M. Somma M and Querci M (108)



1.3.2. Variants of PCR assays

1.3.2.1. Reverse transcription- polymerase chain reaction (RT-PCR)

PCR has been modified to amplify RNA molecules from living cells or viruses containing RNA. Numerous respiratory viruses with RNA genome such as influenza viruses, RSV, parainfluenza viruses, hMPV, rhinoviruses, enteroviruses and coronavirus especially SAR-CoV virus, the amplification in RT-PCR format is needed for molecular diagnostics (30). With this procedure, a step of synthesis of RNA molecule into a complementary DNA by reverse transcriptase is included before amplification of cDNA as the basic PCR protocol with Taq DNA polymerase. The RT-PCR is widely used for identification and quantification of RNA viruses and mRNA or rRNA in bacteria and fungi.

1.3.2.2. Nested PCR

A popular modification of PCR uses nested sets of primers to amplify a specific sequence by performing PCR procedure into two rounds. The first round uses a pair of external primers to amplify a long sequence, and the second round amplifies a shorter sequence from the first round sequence by using a pair of internal primers (70). Sensitivity of most nested PCR procedure is extremely high; the reamplification with the second set of internal primers serves to verify the specificity of the first round product. The transfer of reaction products from the first reaction will dilute out inhibitors that might be present in the sample (1, 69, 70).

1.3.2.3. Multiplex PCR

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more target DNA sequences are simultaneously amplified in the same reaction. Multiplex PCR is very cost-effective and useful in clinical microbiology for detection of multiple viral pathogens from clinical samples at the same time in one testing reaction (29, 29, 69).

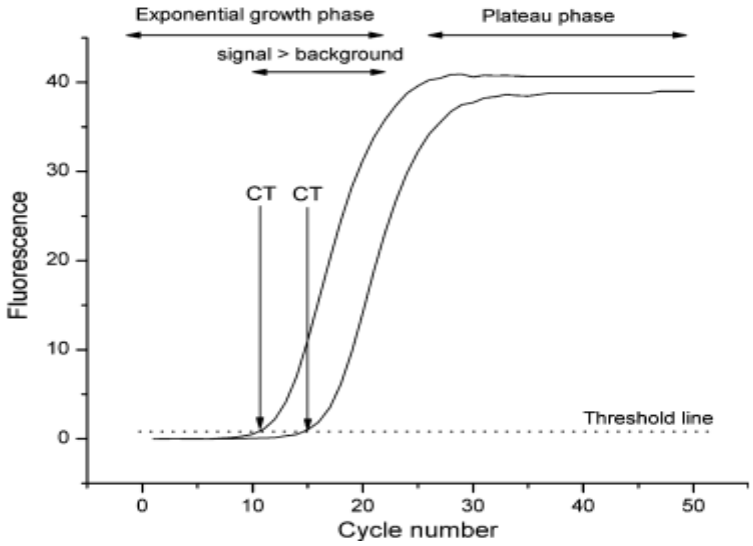
In acute respiratory infections, several studies have utilized multiplex PCR to both detect and type or subtype influenza viruses, PIVs, and RSV in clinical specimens (37, 69, 111). Lam and others used multiplex nested PCR to detect respiratory viruses from 304 clinical samples and showed that the overall positive rate as determined by multiplex nested PCR was 48.5%, which was significantly higher than those of virus isolation (20.1%), besides, their multiplex nested PCR assay also detected non cultivable viruses, particularly rhinoviruses, coronavirus OC43, and metapneumoviruses, contributed a major gain (15.6%) in the overall positive rate (61). Another study on developing and using a multiplex PCR for typing of influenza A and B viruses and subtyping of influenza A viruses from 206 clinical samples, the results showed that 179 was positive with influenza A virus, in which 110 samples were with swine origin influenza A H1N1 (2009), 62 with influenza A H1N1 (human) virus, 6 with H3N2 virus, 1 with untyped influenza A virus and 3 were positive for influenza B virus (37).

1.3.2.4. Real-time PCR

Real-time PCRs have many advantages in comparison with the conventional PCR in diagnosis of viral pathogenic pathogens from clinical samples; they can provide rapid diagnosis for clinical management and prevention; limit cross-contaminations; quantify DNA/RNA levels and more sensitivity. The real-time amplification reaction with the accumulation of the newly amplified products can be monitored by using specific fluorescent probes. This method combines PCR chemistry with fluorescent probe detection of amplified product in the same reaction vessel. Generally, both amplification and detection of amplified products are completed in an hour or less, which is considerably faster than conventional PCR and detection methods (30, 57, 121).
Real-time PCR needs a fluorescent reporter that binds to the product formed and generates a fluorescence signal that reflects the amount of product formed. During the initial cycles the signal is weak and cannot be distinguished from the background. As the amount of product accumulates a signal develops that initially increases exponentially. Thereafter the signal levels off and saturates (29, 68). Real-time PCR can be used for quantification of target DNA or RNA sequences in the human body fluid, which is done by measuring the number of cycles required for the signal to reach a threshold level which is called the CT value; the quantity of target DNA molecules of the sample is reciprocal to the

CT value, this means that the more amount of initial DNA molecules the sample has, the lesser number of cycles is to reach a threshold level (30, 57).

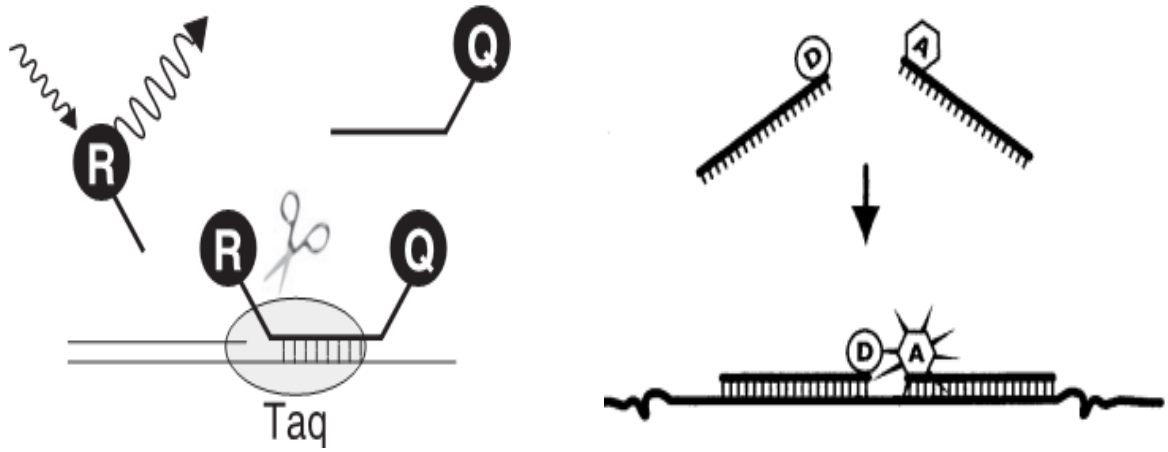
Figure 6
Kinetics of amplification - real-time PCR response curve (reproduced from Kubista et al. (57).



Many detection formats of PCR products have been developed for use in real-time PCR; the earliest and nonspecific format has been DNA binding dyes like ethidium bromide, SYBR® Green. These fluorescent dyes intercalate into double stranded DNA molecule and, in the bound state, emit fluorescent when excited by an appropriate light source (121). Detection formats by using fluorescence probes to hybridize to the target sequence during amplification are highly sensitive and extremely specific for diagnosis of viral pathogens on clinical samples. Three types of nucleic acid detection methods have been used most frequently with real-time PCR testing format: 5'nuclease (TaqMan probes), molecular beacons, and FRET hybridization probes.

In TaqMan probe format, a fluorescent reporter dye (FAM or VIC) covalently is attached to the 5'end of the probe, and a quencher dye (TAMRA) is covalently attached to the 3'end. When the reporter molecule on the TaqMan probe is stimulated by an appropriate light source to emit fluorescence, the energy is transferred to the quencher, thereby suppressing the emission of fluorescence by the reporter. During PCR, when the DNA polymerase extends the primers, the hybridized probes are cleaved by the 5'exonuclease activity of the enzyme and the corresponding quencher and reporter molecules are separated. The energy transfer to the quencher molecule is thus abrogated, and the reporter starts emitting fluorescence (63, 121).

Figure 7
TaqMan probe and FRET probes (reproduced from 67)



Another detection format used also very often is fluorescence resonance energy transfer (FRET). This method relies on the use of two probes that hybridize next to each other to a sequence located between the primers. One of these probes is labelled with a donor dye at the 3'end (Fluorescein, emitting green light), the other is labelled with an acceptor dye at the 5'end (LC Red 640 or LC Red 705, emitting red light). The probes are designed to hybridize during the annealing step to the same strand in a head-to-tail arrangement, at a distance of 1–5 nucleotides to bring the two dyes in close proximity (''kissing'' probes). The donor dye is stimulated by an appropriate light source to emit fluorescence. If both probes are bound to the specific target sequences, the fluorescence energy is transferred from the donor to the acceptor molecules (FRET), and the excited fluorophore emits a fluorescent signal (63, 121).

The third detection format is using molecular beacon, this format is less common used. In design, the probes have a fluorescent dye on the 5' end and a quencher dye on the 3'end of the probe. A region at each end of the molecular beacon probe is able to hybridize to itself to create a hairpin structure at low temperatures. The central region of the probe can be able to hybridize with a region of the target DNA product. At the annealing temperature in PCR cycle, the central region of the molecular beacon probe binds to the PCR product and forces the separation of the fluorescent reporter dye from the quenching dye. The effects of the quencher dye are obviated and a light signal from the reporter dye can be detected (30, 63, 121).

Other new probes can be used in real-time PCR for quantitative detection of viral infections such as TaqMan Minor Groove Binding probes (MGB probe), the term locked nucleic acid (LNA), and these probes can provide more accurate target discrimination and quantification (121).

During the last few years, several real-time PCR procedures have been developed and applied for diagnosis of viral pathogens of respiratory infections

such as influenza viruses, RSV, adenovirus and hMPV. Numerous real-time PCRs for diagnosis of these respiratory viruses can be summarized as following:

Table 4
The real-time PCR for diagnosis of respiratory viruses

Respiratory viruses	Format	Clinical sample	Reference
Influenza A viruses subtype H5N1	TaqMan	20	28
Pandemic H1N1/09	TaqMan	359	125
H1N1(09) and H3N2, H1N1, Flu B	TaqMan MBG	307	100
H1N1(2009), Influenza viruses A and B	TaqMan	317	44
RSV	TaqMan		74
Adenoviruses	TaqMan	2331	54
Parainfluenza viruses	TaqMan	2331	54

1.3.3. DNA Sequencing

Sequencing plays an important role in characterization of genome or DNA/RNA sequence of bacteria and viruses; typing bacteria and viruses, to confirm the identity of a mutation, check the fidelity of a newly created mutation or product of a PCR (97). In respiratory viruses, especially with some viral agents causing pandemics like influenza A viruses, characterization of genome sequences or gene fragment is essential to provide genetic sequence data for constructing phylogenetic tree for tracking the origin and viral genetic evolution, for monitoring and predicting emergence of a novel strain of influenza A viruses.

DNA sequencing includes several methods and technologies that are used for determining the order of the nucleotide bases in a molecule of DNA. The technology for sequencing has been advancing rapidly during recent years, and many approaches for DNA sequencing have been developed recently (105), some key approaches are shortly described as follows

1.3.3.1. The classical chain-termination method

The classical chain-termination method or Sanger sequencing, in which four separate primer extension reactions are carried out, each containing only one of the four chain-terminating dideoxynucleotide triphosphates (ddNTPs) including ddATP, ddGTP, ddCTP, ddTTP, along with template, polymerase, dNTPs, and a radioactively labeled primer. The result is a collection of many terminated strands of many different lengths within each reaction. The four

reactions are then electrophoresed in four lanes of a denaturing polyacrylamide gel to yield size separation with single-nucleotide resolution. The pattern of bands across the four lanes allows to be interpreted the primary sequence of the template under analysis (97, 105).

Dye-terminator sequencing is a modification of classical Sanger sequencing, in which fluorescent labeled ddNTPs are employed and a single primer extension reaction containing all four labeled ddNTPs is performed. The four ddNTPs are labeled with fluorescent dyes that have the same excitation wavelength but each of which emit different wavelength (105).

1.3.3.2. Chemical sequencing

Chemical sequencing was described by Maxam & Gilbert. In this method, end-labeled DNA fragments are subjected to random cleavage at adenine, cytosine, guanine, or thymine positions using specific chemical agents. The chemical attack is based on three steps: base modification, removal of the modified base from its sugar, and DNA strand breaking at that sugar position. The products of these four reactions are then separated using polyacrylamide gel electrophoresis. The sequence can be easily read from the four parallel lanes in the sequencing gel (32, 97).

1.3.3.3. Pyrosequencing method

Pyrosequencing method is based on the detection of the inorganic pirophosphate (PPi) released during the DNA polymerization reaction, each dNTP is incorporated into the chain extension by DNA polymerase and PPi is released. This liberated PPi is then converted into ATP by ATP sulphurylase and light is emitted via the firefly luciferase that catalyses luciferin into oxyluciferin (87). The average number of emitted photons per template chain in a given step is proportional to the number of deoxynucleotides incorporated per chain at that step. The sequence can then be determined by simply noting if incorporations occur and by counting the number of incorporations (by measuring the light intensity) in a given attempt (75).

1.3.3.4. Single-molecule sequencing with exonuclease

This method is based on the detection of individual fluorescent nucleotides in a flowing sample stream; this method is divided into steps: fluorescent labeling of the bases in a single fragment of DNA; attachment of this labeled DNA fragment onto a bead, movement of the labeled DNA bead into a flowing buffer steam, digestion of labeled DNA by an exonuclease that cleaves the 3'- end nucleotides, and identification of individual fluorescent labeled bases as they cross a focused laser beam (32).

1.4. Molecular epidemiology of influenza A virus

Molecular techniques have taken an important role in rapid diagnosis and epidemiological surveillance of influenza virus infections in humans and animal for the past several years (69, 88). Development of molecular diagnostic methods based on reverse transcription-polymerase chain reaction (RT-PCR), real-time RT-PCR, nucleic acid sequence-based amplification has made detection and genomic characterization of influenza virus isolates much easier and more rapid than the conventional methods such as cell culture and serological assays. They are particularly useful in rapid detection, surveillance of influenza virus outbreak, and also in monitoring for viral changes in their host specificity and pathogenicity (24, 69, 88, 91).

As described previously influenza A viruses change very often their antigenic characteristics over time, among their 8 genomic segments of genes, HA surface protein genes are always under host immune selective pressure, so they are evolving much more rapidly than the internal protein genes PB1, PB2, PA, NP, and M1 (123). The new strain of influenza A virus can emerge and cause the outbreak by antigenic drift or antigenic shift (60, 72).

Phylogenetic methods play an important role in the analysis of genomic sequence data of influenza virus (36, 60, 72, 84, 123). They are used to reconstruct the evolutionary relationships between different viral strains (72, 123). Three main approaches exist to phylogenetic tree reconstruction: distance-based methods, parsimony, and probabilistic methods based on maximum likelihood or Bayesian statistics (60, 80, 84).

Distance-based methods start by defining an evolutionary distance between sequences and then apply hierarchical clustering algorithms to obtain a phylogenetic tree (84). Neighbor-joining (NJ) is the most common distance method used to construct a distance tree. It is a clustering method that joins the two most closely related sequences (i.e. neighbor) according to the computed distance matrix to form subsets of the tree, which gradually build up the whole tree (60, 80). Distance-based methods are computationally efficient, but the reduction of the observed data to a distance matrix presents a loss of information (84). NJ trees are a useful starting point for estimating the minimum evolution (ME) tree, as well as for constructing trees using discrete methods, such as maximum likelihood and Bayesian approaches (60, 80).

Unlike distance-based methods, maximum parsimony is based on the minimum evolution principle and tries to find the tree that explains the data by the minimum number of mutations. The method has been applied successfully to the analysis of influenza virus sequence data. It is also computationally efficient, but lacks an explicit evolutionary model (other than minimum evolution), and it is not statistically consistent (84).

Probabilistic phylogenetic models have these desirable properties, Maximum Likelihood methods are currently the most commonly used ones in

phylogenetic tree construction, as long as sufficient computation power and time are available (60). Bayesian phylogenetic inference is based on the posterior probability of the tree. The posterior probability is calculated from the prior probability of the phylogeny and the tree likelihood by Bayesian theorem (60).
With influenza viruses, phylogenetic analysis can be helpful in

(i) Tracing the genetic origin of a particular virus strain through analysis of the viral genetic sequences (60, 72).

(ii) Monitoring genome reassortment and recombination (72, 106).

(iii) Predicting the evolution rate of influenza viruses (60).

(iv) Understanding the evolutionary dynamics and the geographical epidemiology of influenza A virus, which is essential in designing and optimal matching of vaccine strains (60, 84).

1.5. The aims of this study

Thua Thien Hue Province is located in the northern part of the central region of Viet Nam, and the presence of more than 1000 beds both in Hue Central Hospital and in the Hospital of Hue College of Medicine and Pharmacy makes Thua Thien Hue province the biggest health facility for the whole central Viet Nam. However, the limitation in clinical laboratories for diagnosis and surveillance for outbreak of viral infections has reduced the possibility of collecting and documenting data on viral etiologies of respiratory infections for many years.

Since 2008, the Carlo Urbani centre has been established in Hue College of Medicine and Pharmacy for improving capacity of training, research diagnosis and management of acute respiratory infections with the support of Italian Government and Sassari University. A microbiology laboratory with BSL3 facilities and equipments for molecular biology was included in this centre. Extensive training for reinforcing the existing expertise of the local staff in determining and identifying respiratory viruses in the region was carried out both in Vietnam and abroad.

In order to provide further knowledge on epidemiology of viral etiologies of acute respiratory infections in central Vietnam, needed for improving the strategies of prevention and patient management and also developing the capacity of laboratory diagnosis of respiratory viruses, we carried out this study on viral etiologies of lower respiratory infections and characterization of HA and NA gene segments of novel H1N1 subtype of influenza A virus causing respiratory infections in Hue with the following objectives:

1. To determine the viral etiology of lower respiratory infections in children hospitalized in Hue Central Hospital using molecular techniques (polymerase chain reaction).
2. To conduct a study for isolation and identification of the novel influenza A (H1N1) 2009 virus in the pandemic 2009 by virological methods (cell culture on MDCK) and reverse transcription-polymerase chain reactions.
3. To analyze and sequence relevant genes from swine-origin influenza A virus H1N1 isolated in Hue in 2009 in order to identify gene polymorphism and compare with referenced strains for molecular epidemiology purpose.

2. MATERIALS AND METHODS

2. 1. Study design

This study is a prospective, cross-sectional survey carried out on two population samples of patients hospitalized in Hue Central Hospital and Hue City Hospital.

Sample for the objective of determining the viral etiology of lower respiratory infections in ill children was constituted by pediatric patients with lower respiratory infections hospitalized in the section of lung and pulmonary diseases of the pediatric department of Hue Central Hospital from the middle of March 2010 to the sixth of April 2011.

Sample for the objective of isolating and identifying the novel influenza A (H1N1) 2009 virus was children and adult patients with suspected infections hospitalized in the isolation ward in Hue City Hospital from the beginnings of October to the middle of November 2009.

The study was conducted at the Department of Medical Microbiology and Microbiological Laboratory of Carlo Urbani Centre of Hue College of Medicine and Pharmacy and with close collaboration of medical doctors in the departments of Hue City Hospital and Hue Central Hospital.

Before implementing the study, a case-recorded form was constructed and a short discussion with collaborating clinical doctors was organized to introduce the procedure of selecting patients and collecting data and respiratory specimens from the selected patients. A complete package including all material and tools for collection of respiratory specimens was provided to the clinical staffs.

Ethics

Before carrying out the study, a written proposal was submitted for permission to the ethical boards of Hue College of Medicine and Pharmacy, Hue Central Hospital and Hue City Hospital for their approvals. The approvals for this study were also obtained from the heads of the clinical departments or units participating to the study.

Before collecting patient data and respiratory specimen, children parents or guardians were asked to give their oral consent to include the children into the study.

2.2. Definitions and selection of patients for study

2.2.1. Pediatric patients of viral lower respiratory infections

Children with symptoms of acute lower respiratory tract infections of suspected viral etiology hospitalized in the pediatric department of the section of lung and pulmonary diseases, Hue Central Hospital were chosen for collection of data and respiratory specimens. Acute viral lower respiratory infections were clinically defined as bronchitis, bronchiolitis and pneumonia (126).

- Acute bronchitis was diagnosed if patients have following symptoms: fever, sore throat, cough, wheezing (16).

- Acute bronchiolitis was defined as children have coryzal symptoms including rhinorrhea and fever and develop increased work of breathing with wheeze, and cough (17).

- Pneumonia was diagnosed when patients presented with symptoms of fever, coryza, cough, vomiting, refusal to feed, breathlessness. On clinical examination, respiratory rate, significant pallor, stridor, presence of crepitations and rhonchi were recorded (11).

The following patients were not eligible for this investigation:

- Suspected bacterial lower respiratory tract infections if patients have similar symptoms and signs of those above categories but the WBC, at the time of admission, were higher than 7.0 x 10^9 cells/L.

- Asthmatic patients.

2.2.2. Patients suspected with influenza A (H1N1)2009 infection

Patients suspected with influenza A (H1N1) 2009 infection had an abrupt onset with symptoms of fever, malaise, fatigue, headache, and muscular ache and signs of sore-throat, coryza, cough and/or presence of crepitations on clinical examination (79).

2.3. Collection of respiratory specimens and patient data

2.3.1. Collection of data and specimens from pediatric patients

The following data were collected from the patients by a clinical doctor in a case-recorded form.

- Demographic data (age, gender).
- Time of the onset of respiratory infections.
- Time of admission to the hospital.
- Clinical features and laboratory data.
- Clinical diagnosis.

Nasal pharyngeal swabs and or throat swabs from children patients with acute lower respiratory infections were collected and placed in the 3ml vial of DB universal viral transport (cat DB-220220).

The specimens were kept at minus 20^0C in freezer until transporting to the laboratory (normally 24 hours).

In the group of pediatric patients, a total of 216 patients were chosen and had their respiratory specimens collected for determination of viral etiologies.

2.3.2. Patients and specimens in the 2009 influenza outbreak

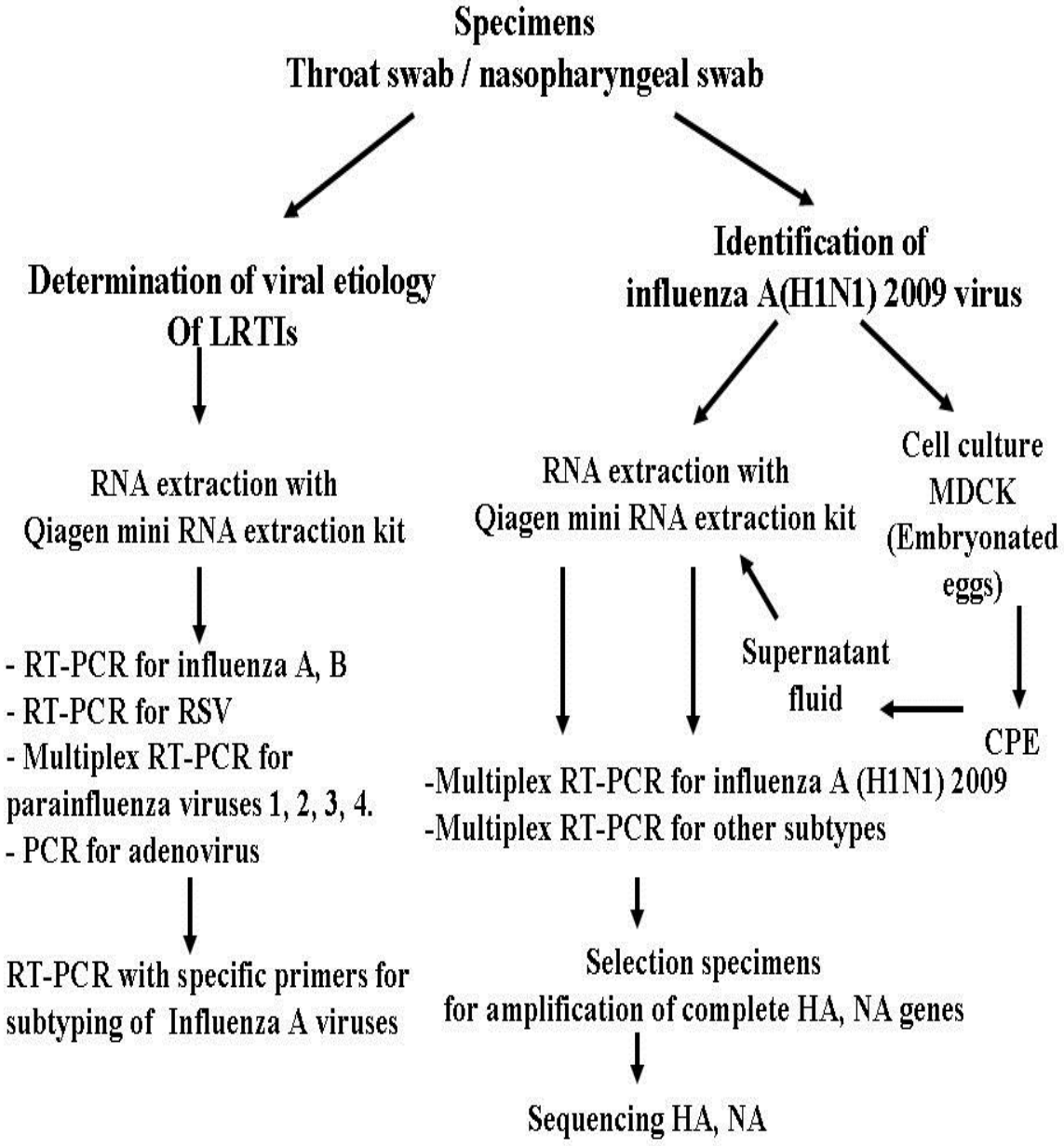
Patients suspected influenza virus infection with acute respiratory symptoms admitted to Hue City Hospital were chosen to collect the specimens for virus detection. The specimens were placed into 3ml of viral transport medium (VTM) and transferred immediately to the laboratory.

With this group of patients, nasal pharyngeal swabs or throat swabs were taken for identification of influenza A (H1N1)2009 virus from 53 suspected patients.

2.4. Preparation of respiratory specimens in the laboratory

Preparation of the respiratory specimens in both patients groups was carried out with the same procedure. However isolation on the cell culture was only carried out with the patient group for identification of the novel influenza A (H1N1) 2009 virus. The laboratory flow of working procedures for this study is shown in figure 8.

Figure 8
Laboratory procedures for this study



In the laboratory, 3ml VTM was mixed well and divided into four sterile cryogenic tubes, the first two tubes of 0.5ml each were for amplification assays, one 0.5ml tube was stored at minus 70^0C for later use and the other 0.5ml tube was used for RNA extraction by Qiagen viral RNA mini kit for RT-PCR as the manufacturer guidance; the other two tubes (1ml each) were used for isolation, which was carried out for diagnosis of influenza A (H1N1) 2009 virus in the influenza pandemic 2009. The isolation procedures on cell culture of MDCK and embryonated eggs are described in detail in the section for diagnosis of influenza A (H1N1)2009 virus.

2.5. Amplification assays for determination of viral etiologies of LRTI

2.5.1. Viral acid nucleic extraction from sample

Qiagen viral RNA mini purification kit (cat 52904) was used for extracting and purifying viral RNA/ DNA from all the specimens according to manufacturer s' specifications. Briefly, 140µl of specimens were added to a tube containing 560 µl of lysis buffer with RNA carrier, mixed well by vortex, incubated at room temperature for 10 min; applied all of this solution to the QIAamp mini spin column, centrifuged, washed twice with buffer AW1 and buffer AW2; viral RNA/DNAs was finally eluted in 60 µl of AVE solution and kept at - 20^0C. The RNA/DNA extraction from each specimen was used for all amplification assays for identification of viral etiologies of LRTI.

2.5.2. Amplification assays for detection of respiratory viruses

2.5.2.1. Amplification assays for identification of influenza viruses

In order to identify influenza viruses, three RT-PCR protocols were used. The first protocol was for amplification of matrix genes of influenza A virus and influenza B virus with the primers and protocol adapted from Boonsuk et al (10); the second protocol was for subtyping influenza A H1N1 (2009) with primer and the protocol adapted from WHO (128); the third protocol was for subtyping of subtypes seasonal H1N1 and H3N2 by the primers and protocol adapted from the national protocol for surveillance on influenza virus, Istituto Superiore di Sanita, Roma, Italia (13) and Percivalle et al (89).

For the positive controls of influenza viruses, the following isolates were used

- Influenza A H1N1 (2009) virus from the laboratory of respiratory viruses, Pasteur Institute, Ho Chi Minh city.
- H3N2/HongKong/8/68 from the laboratory of virology, San Raffaele Hospital, Milan, Prof. Massimo Clementi
- Seasonal H1N1/Puerto Rico/8/34 from the laboratory of virology, San Raffaele Hospital, Milan, Prof. Massimo Clementi.

- Flu B/ Lee/40 from the laboratory of virology, San Raffaele Hospital, Milan, Prof. Massimo Clementi.

The RT-PCR protocol for detecting influenza virus type A and type B

A multiplex RT-PCR for typing influenza A and B was carried out with the primers shown in table 5.

Table 5

The primers for amplification of matrix genes (10)

Type /subtype	Target gene	Primer	Primer Structure (5' - 3')
Influenza type A	Matrix (M)	FluA -M-F	TCAGGCCCCCTCAAAGCCG
		FluA -M-R	AGGGCATTTTGGACAAAKCGTCTA
Influenza type B	Matrix (M)	FluB -M-F	ATCTCGCTGTTTGGAGACACAAT
		FluB -M-R	TCAGCTAGAATCAGRCCYTTCTT

Synthesis of cDNA from RNA extraction and amplification were carried out with the one-step multiplex RT-PCR in one tube with the RT-PCR mixture. Components for one reaction were 2x One-Step RT-PCR buffer 12.5 µl [SuperScript III Platinum One-step RT-PCR kit (Invitrogen, cat 11732-020)], 4µl of primers mixture of Flu A-M-F/R (10µM) and Flu B-M-F/R (10µM) (0.4 nM for each primer), added 0.5 µl of RT/Taq DNA polymerase, 5µl of RNA extraction, then sterilized water of 3µl was added to reach the final volume of 25µl. The programme condition was reverse transcription at 50^0C for 30min, then initial denaturation of 94^oC for 3min, followed by 35 cycles with denaturation at 94^oC for 30 sec, annealing at 58^oC for 30sec, then extension at 72^oC for 1min, and final extension at 72^oC for 7min and storage at 4^oC. Positive controls with influenza A and B virus were run in parallel with the samples.

The amplified products were separated by electrophoresis on agarose gel of 1.5% (0.45g agarose + 30ml of 0.5 x TBE buffer, dissolved the agar by heating in microwave oven for 5min, cooled the melted agarose to about 60^0C and poured into the gel-casting tray and allowed the gel to solidify at room temperature, removed gently the comb and place the gel tray into the electrophoresis chamber containing 0.5 x TBE buffer, added the buffer into the chamber to cover the top of the gel, applied the electricity at 70 volt/ 30 -35 min for electrophoresis).

The gel tray was gently taken out and placed into a lidded chamber containing 0.5 x TBE buffer dissolved with ethidium bromide 0.5µg/ml for staining for 15 min and read under UV transilluminator (UVP). A 100bp DNA ladder was used as a molecular weight marker.

Influenza A was recorded as positive with the presence of 214 bp product for M gene of influenza A virus, and presence of 296 bp product for M gene of influenza B virus.

- **The RT-PCR protocol for identifying subtype (H1N1) 2009**

When the sample was positive for influenza A virus, further subtyping of was carried using primers and procedures as follows

Table 6
Primers of gene HA1 of (H1N1) 2009 subtype (128)

Type /subtype	Target gene	Primer	Primer Structure (5' - 3')
Influenza type A	HA1 (2009)	NIID-swH1 - F1	TGCATTTGGGTAAATGTAACATTG
		NIID-swH1 -R1	AATGTAGGATTTRCTGAKCTTTGG

The reverse transcription and amplification were carried out with the one-step RT-PCR in one tube with the RT-PCR mixture for one reaction. the components for one reaction were 2x One-Step RT-PCR buffer (superscript one step, invitrogen) of 12.5 µl, and 1µl (0.4 nM) of each primer NIID-swH1- F1 (10µM) and NIID-swH1 -R1(10µM), 0.5 µl of RT/Taq DNA polymerase (Invitrogen), 5 µl of RNA extraction and 5 µl of sterilized water was added to reach the final volume of 25µl. The cycling condition was reverse trancription at 50^{o}C for 30 min, then initial PCR activation at 95^{o}C for 15min, followed by 45 cycles with denaturation at 94 ^{o}C/ 30 sec, annealing at 50 ^{o}C/ 30 sec, and extension at 72 ^{o}C/ 1 min, completed amplification with final extension at 72 ^{o}C for 10 min. Positive control with RNA extraction of influenza A (H1N1) 2009 virus and negative control with H2O were run in parallel with the samples.

Amplified products were run on agarose 1.5% (preparation as described above) for electrophoresis for 30min, then staining in 0.5 x TBE buffer dissolved with ethidium bromide 0.5µg/ml for 15min.

The product band was visualized under UV light and the presence of 349 bp product of HA1 gene was recorded as positive for the subtype (H1N1) 2009 of influenza A virus.

- **Multiplex RT-PCR protocol for identifying subtypes of H3N2 and seasonal H1N1**

If the sample was positive with influenza A virus but negative for subtype (H1N1) 2009, the sample was analyzed further for the subtypes of H3N2 and seasonal H1N1 with a multiplex RT-PCR protocol with primers specific for these subtypes as follows

Table 7
Primers for amplification of H3N2 and seasonal H1N1 (13, 89)

H3N2	HA3	H3 F1	CCTTGATGGAGAAAACTGCACAC'
		H3 R1	TGTTTGGCATAGTCACGTTCA
Seasonal H1N1	HA1s	H1 F1	GAATCATGGTCCTACATTGTAGAAA
		H1 R1	ATCATTCCAGTACATCCCCCTTCAAT

The reverse transcription and amplification were carried out with the one-step multiplex RT-PCR in one tube with the RT-PCR mixture for one reaction. Components of one reaction were 12.5µl of 2x One Step RT-PCR buffer, and 0.5µl of each primer (10µM), 0.5µl of RT/Taq DNA polymerase (Invitrogen), and 5µl RNA extraction, and sterilized water was finally added to reach the final volume of 25µl.

The cycling condition was reverse transcription at 40^{o}C for 40 min, then amplification with activating denauration at 95^{o}C/10 min, followed by 40 cycles with initial denaturation at 94^{o}C/ 45 sec, annealing at 52^{o}C/ 1min 30 sec, extension 72^{o}C/ 2 min 30sec, completed amplification by final extension at 72^{o}C /5 min.

The samples were always run with positive controls with the above mentioned strains and negative control with sterilized water.

The amplicons were run in 2% agarose gel (0.60g agarose in 30ml of 0.5 x TBE buffer, and preparation as described) for electrophoresis, and stained with EtBr 0.5µg/ml in 0.5 x TBE buffer for 15 min and read under the UV transilluminator. A 100bp DNA ladder was used for molecular weight marker.

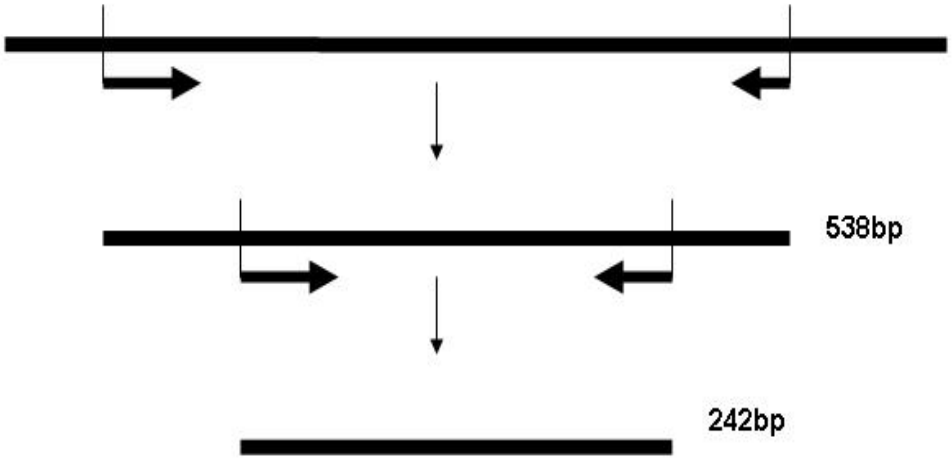
The testing samples were always run in parallel with positive control tubes with the specific subtypes described above and negative tube with sterilized water.

The presence of DNA band with the size of 814bp was positive for seasonal H1N1 subtype and with the band of 388bp was positive for H3N2 subtype.

2.5.2.2. Amplification assay for identification of syncytial respiratory virus (RSV)

Amplification for detection of RSV was carried out by a nested RT-PCR protocol described previously (74, 95) to amplify a 244 bp segment of the F gene as shown in figure 9.

Figure 9
Target products of nested amplification for RSV



- **Synthesis of complementary DNA**

Reverse transcription for synthesis of cDNA was carried out by a domestic kit (Viet Phat company, HMC) as the manufacturer 's specifications, Briefly, a volume of 12.5µl of RNA extraction was mixed with 6.5µl of reverse transcription buffer in a 0.2ml Eppendorf tube, added 1µl of standard RT enzyme, mixed well. Synthesis of complementary DNA was performed in thermocycler with the program at 25^0C for 5 min, then 42^0C for 30 min, and 85^0C for 5min.

- **Amplification**

The PCR protocol was implemented in two rounds, the first one was aiming at amplifying a segment of 538 bp, and the second round is to amplify an inn segment of 242 bp. The control positive was positive RSV sample identified by real-time RT-PCR provided by professor Drosten of the institute of Virology, in Bonn University. The primer pairs for nested amplification are in table 8.

Table 8
The primer pairs for nested amplification for identification of RSV (74, 95)

Primer name	RSV	Target gene
F1 Forward primer	GTTGGATCTGCAATCGCCAGTGGC	F
F2 Reverse primer	GTACATAGAGGGGATGTGTG	F
F3 Forward primer	TTAACCAGCAAAGTGTTAGA	F
F4 Reverse primer	TTTGTTATAGGCATATCATTG	F

The first round of PCR was performed with reagent components and their volumes presented in the table 9.

Table 9
Components and volumes of one reaction

Components	Volume in µl	Working concentration
PCR master mixture (Fermentas)	12.5 µl	
F1 forward (10µM)	1 µl	0.4nM
F2 reverse (10µM)	1 µl	0.4nM
Sterilized water	5.5 µl	
cDNA template	5 µl	
Total volume	25 µl	

The PCR mixture was prepared in a 0.2ml eppendorf PCR tube in a tray with ice. Amplification reaction was run in the Veriti thermocyler (Applied Biosystems) with the cycling condition as follows: initial activation at 95^0C for 15 min, followed by 40 cycles with denaturation at 95^0C for 1 min, annealing at 55^0C for 1 min, and extension at 72^0C for 1min.

The second round of PCR was with 3 µl of product from the first PCR and 0.4nM for each of primer F3 (10µM) and F4 (10µM); 12,5µl of PCR master mixture (Fermentas), and sterilized water finally added to reach the total volume of 25µl. The thermo-cycling condition was initial activation at 95^0C for 5min, followed by 40 cycles with denaturation at 95^0C for 1 min, annealing at 50^0C for 1 min and extension at 72^0C for 2 min, then final extension at 72^0C for 5min.

The amplified product was run on 1.5% agarose gel for electrophoresis; stained with ethidium bromide 0.5µg/ml in 0.5x TBE buffer for 15 min; and visualized the product bands under UV light as described above for the products of influenza viruses.

The testing samples were always performed in parallel with negative control with sterilized water and positive control with positive sample. The presence of product 242bp was positive for RSV as the band of the positive control.

2.5.2.3. Amplification for identification of adenovirus

This protocol was adopted from McDonough et al (71) to amplify a fragment of 134 bp of the hexon gene of adenoviral genome (71). The primers for this protocol are presented in table 10.

Table 10
Primers pairs for amplification of adenovirus

Name	Primer structure	size
ADE 1 forward	ATGACTTTTGAGGTGGATCCCATGGA	134bp
ADE 1 reverse	GCCGAGAAGGGCGTGCGCAGGTA'	

DNA plasmid for adenovirus control from Clinical Microbiological Laboratory of Sassari University, Prof. Caterina Serra

DNA of viruses was extracted from the patient sample with the same Mini Qiagen RNA purification kit as other RNA viruses in this study. Components of reagents for one reaction were 12.5µl of PCR master mixture (Fermentas), 1.25 µl (0.5µM for final concentration) of each primer ADE 1 F (10 µM) and ADE 1 R (10 µM), 0.25µl (0.5U) of thermo DNA polymerase (2U/µl), 5µl DNA extraction, and sterile, free DNA water was added to reach the final volume of 25µl.

The thermalcycling condition was heating at 94^0C for 5 minutes for the initial denaturation, followed by 30 cycles with denaturation at 94^0C for 30 seconds, annealing at 52^0C for 30 sec and extension at 72^0C for 60 sec. completed amplification with the final extension at 72^0C for 5 minutes.

The control positive with DNA plasmid and negative with sterile water were always run with the testing samples. PCR product was identified by electrophoresis with 1.5% agarose gel and stained in ethidium bromide 0.5µg/ml in 0.5x TBE buffer for 15min and visualized the DNA product under UV reader as described above.

The presence of product 134bp was recorded to be positive for adenovirus as the band of the positive control.

2.5.2.4. Multiplex RT-PCR procedures for parainfluenza viruses

For detection of human parainfluenza viruses, a modified multiplex nested RT-PCR protocol was used (61). All the amplification reactions were carried out in the Viriti thermocycler (Applied Biosystems). The primers for amplification of parainfluenza viruses were as follows

Table 11
Name and primer structure for parainfluenza virus (61)

Name	Parainfluenza viruses	Gene	Size (bp)
PIV-1-OF1	TCTGGATCCACCACAATTTCAG	HA-NA	848
PIV-1-OR1	WACCAGTTGCAGTCTKGGTTTC		
PIV-1-IF1	AATTGGTGATGCAATATATGCKTATTC	HA-NA	600
PIV-1-IR1	TCGACAACAATYTTTGGCCTATC		
PIV2-F1	CTTGCAGCATTTTCTGGGGAACTCC	HA-NA	716
PIV2-OR1	GCATCATCATCCTGGGAGCCTCTGT		
PIV2-F2	AGGACAGCAGAGGACCTCGGCATG	HA-NA	343
PIV2-R2	ACCTGATGTTCTTTGCGGTATGGGG		
PIV-3-OF1	GATTTTTGGAGATGCACGTCTG	HA-NA	1.118
PIV-3-OR1	GAGAGTGTTYTGTTTCGGATGG		
PIV-3-IF1	CAACTGTGTTCRACTCCCAAAG	HA-NA	717
PIV-3-IR1	TGGGTTYACTCTCGATTTTTGY		
PIV-4 AB-IF1	AYGGATGCATTCGAATTCCATCATTC	HA-NA	432
PIV-4AB-OR1	TCCRTRAGRCCYCCATACAARGG		
PIV-4AB-IF2	GACGGATGYYTRCKGWATTGTGT	HA-NA	231
PIV-4AB-IR2	CCRTRAGRCCYCCATACAARGGAA		

Reverse transcription for synthesis of cDNA was carried out by a domestic kit (Viet Phat company, HMC) as the protocol described in RSV.

Parainfluenza viruses for positive control were provided by Dr. Helmer and prof. Drosten, Medical Virology Institute of Bonn University.

Amplification was carried out in two rounds. The first round was run with the following PCR mixture.

PCR Master Mixture (Fermentas)	12.5µl
Primer solution	6.2µl
Sterilized water	1.3µl
cDNA Template	5.0µl
Total volume	25.0µl

In which the primers solution was a 6.2 µl primer mixture including 2.3 µl for each of PIV1 OF1 and OR1; 0.1 µl for each of PIV2 F1 and OR1; 0.5 µl for each of PIV3 OF1 and OR1; and 0.2 µl for each of PIV4-OF1 and OR1.

The thermal-cycling program for the first round was at 95^0C for 3min for activating denaturation, followed by 30 cycles with denaturation at 95^0C for 1min, annealing at 64^0C for 40 sec and extension at 72^0C for 5min, then final extension at 72^0C for 5min.

The second round was run with 2µl of the first round products and PCR reagent components per reaction as follows

Master Mix PCR (Fermentas)	12.5µl
Primer mixture of the second round	6.2µl
Sterilized water	4.3µl
DNA Template of the first round	2.0µl
Total volume	25.0µl

In which the primer mixture of the second round was a 6.2 µl primer solution including 1.5µl for each of PIV1-IF1 and IR1; 0.1 µl for each of PIV1-IF1 and IR1; 1.1 µl for each of PIV3-IF1 and IR1; and 0.4 µl for PIV4- AB IF2 and AB IR2.

The thermal-cycling program for the second round was initial denaturation at 95^0C for 3min, followed by 30 cycles with denaturation at 95^0C for 1 min, annealing at 64^0C for 40 min and extension at 72^0C for 3 min, and then final extension at 72^0C for 5 min.

The tubes with positive controls and negative with sterile water were run in parallel with the testing samples. The products of the second round was run on 2% agarose gel (0.6gr agarose + 30ml 0.5 x TBE buffer and preparation of gel was described in the section for influenza virus) for electrophoresis at 70 volt/ 30 -35 min.

The gel was stained with ethidium bromide 0.5 µg/ml in 0.5 x TBE buffer and visualized the presence of PCR product bands and marker under the UV light. The presence of the product bands with the specific sizes was recorded as positive by comparing to the DNA ladder of 100bp in length.

2.6. Diagnosis of influenza A (H1N1) 2009 virus by isolation on cell culture and amplification assay

2.6.1. Isolation of influenza virus on cell culture and embryonated eggs

Isolation of influenza virus was carried out according to a standard procedure (128). Briefly, 1ml portion of samples in VTM was pretreated by filtration with 0.4 µm size filters and then inoculated into the T-25 flash of Madin -Darby Canine Kidney (MDCK) cells grown at confluent phase in MEM (pH 7.2) containing 10% fetal bovine serum, penicillin [10U/ml], streptomycin [10mg/ml] and amphotericin B [0,25mg/ml]; cultures were incubated at 35.5^0C in a 5% CO2 incubator and followed daily for cytopathic effect (CPE) under the inverted microscope (10x and 20x) for 7 days. When the CPE was visualized,

the supernatant fluid was harvested for viral RNA extraction with the Qiagen viral RNA mini kit (Qiagen, USA) for typing and subtyping by RT-PCRs.

Embryonated eggs were recently put into use for isolation of in influenza virus in our laboratory in addition to MDCK cells method; chicken embryos 9-10 days old were used for inoculation of clinical samples. Three embryonated eggs were used for each clinical sample (128); briefly, 300µl of each sample was mixed well with 300µl antibiotic solution (gentamycin 40µg/ml, chloramphenicol 50µg/ml; penicillin 100UI/ml; streptomycin 100µg/ml; amphotericin B 5pg/ml), wiped the top of each egg with 70% ethanol and punched a small hole in the shell over the air sac, then inoculated with 200µl of antibiotic mixed sample, sealed the hole with paraffin and incubated the eggs at 34^0C for 2 days. Harvesting of allantoic and amniotic fluids was performed by chilling the eggs at 4^0C over night, then breaking the shell over to collect all the fluids. The presence of virus growth was tested by agglutination assay of chicken red blood cells. If hemagglutination was positive, confirmation was carried out by RT-PCR assays for H1N1 2009. If hemagglutination was negative, the harvested fluids were inoculated into the embryonated eggs two more times for negative confirmation of viral isolation.

2.6.2. Amplifications for identification of influenza A (H1N1) 2009 subtype

Besides isolation of the virus on cell culture, for detection and identification of influenza A (H1N1) 2009 subtype directly from respiratory specimens and also from cell culture for confirmation, two RT-PCR protocols were used.

(i) A modified multiplex RT-PCR for identification of influenza A (H1N1) 2009 subtype with protocol and primers adapted from WHO (128), this RT-PCR was also used to identify the virus from the cell culture.

(ii) When the specimen was positive with matrix gene but negative for HA1 (2009) gene, the specimen was analyzed further for detection of subtype H2N3 and seasonal H1N1 with the multiplex RT-PCR described above for identifying subtypes of H3N2 and seasonal H1N1 (13, 89).

A modified multiplex RT-PCR protocol for identification of influenza A (H1N1) 2009 virus

A multiplex RT-PCR was set up with the primers from WHO (128) described in the following table.

Table 12
Primers for amplification of influenza A (H1N1) 2009 virus (128)

Type /subtype	Target gene	Primer	Primer Structure (5' - 3')
Influenza type A	Matrix (M)	M30F2/08	ATGAGYCTTYTAACCGAGGTCGAAACG
		M264R3/08	TGGACAAANCGTCTACGCTGCAG
Subtype H1N1(2009)	HA1	NIID-swH1-F1	TGCATTTGGGTAAATGTAACATTG
		NIID-swH1-R1	AATGTAGGATTTRCTGAKCTTTGG

Synthesis of cDNA and amplification were performed with the one-step multiplex RT-PCR in one tube with the RT-PCR mixture. Reagent components for one reaction were 12.5µl of 2x One Step RT-PCR buffer (invitrogen), 1µl of each primer (10µM), 0.5µl RT/ Taq Polymerase, 5µl of RNA extraction, finally sterile water was added to reach the final volume of 25µl. Amplification was implemented in Veriti thermocycler with cycling programme: reverse transcription at 50^0C for 30min, initial PCR activation at 95^0C for 15 min, followed by 45 cycles with denaturation at 94^0C for 30 sec, annealing at 50^0C for 30sec, and extension at 72^0C for 1 min, completed amplification by final extension at 72^0C for 10 min.

Positive controls with RNA extraction of influenza A virus subtype H1N1(2009) and negative with sterile water were run with testing samples.

Amplified products were separated by electrophoresis in agarose gel 1.5%, then stained with EtBr 0.5 µg/ml in 0.5 x TBE buffer for 15min. the bands of DNA products were visualized with UV light. The presence of DNA bands with the size of 244bp was positive for matrix gene and with the size of 349bp for the gene of HA1 (2009), 100bp DNA ladder was used for marker. The presence of both DNA bands are essential for confirmation of influenza A (H1N1) 2009 subtype, presence of band with the 244bp size and without the 349bp band was recorded for influenza A virus.

2.6.3. Sequencing HA and NA genes of 2009 H1N1 subtype

Four strains of novel H1N1 subtype were randomly chosen for sequencing HA, NA genes. The supernatants of MDCK cell culture were collected and 140µl of each were processed for RNA extraction with Qiagen viral RNA mini purification kit as the above-described procedure. Synthesis of viral RNA into cDNA was implemented by the primer Uni 12(M) with the ThermoScript™ RT-PCR System (Invitrogen). Briefly 8µl of the RNA extraction was mixed with 2µl of of 5µM primer Uni 12(M) and 2 µl of 10 mM dNTPs to a volume of 12µl. The mixture was incubated at 65^0C for 5min. Then the following cDNA synthesis mixture containing 4 µl of 5x cDNA buffer, 1 µl of 0.1M DDT, 1 µl of RNaseOUT, 1 µl of sterilized, distilled water and 1 µl of Thermoscript^{TM} RT

were added into the tube. The reaction was carried out at 65^0C for 60min and was terminated by heating at 85^0C for 5min.

The primers and protocol from Chan et al (14) were used for amplification of the full lengths of HA and NA genes of (H1N1) 2009 subtype.

Table 13
Primers for sequencing HA and NA genes of (H1N1) 2009 subtype (14)

Target sequence	primer	Primer structure (5'-3')	
HA	HA-1	AGCAAAAGCAGGGGAAAATA	1778bp
	HA-1778	AGTAGAAACAAGGGTGTTTT	
NA	NA-1	AGCAAAAGCAGGAGTGAAAA	1413bp
	NA-1413	AGTAGAAACAAGGAGTTTTTT	
	Uni 12(M)	AGCRAAAGCAGG	

PCR amplification of entire HA and NA genes was performed by the protocol described previously (14) with the GoTaq PCR core systems (Promega, cat. 7660). Briefly, reaction mix including 5X green GoTaq flexi buffer of 10µl, 1µl of 10 mM dNTPs, 4 µl of 25 mM MgCl2, 0.5µl of GoTaq DNA polymerase (5U/µl), 5µl of each primer (10µM), 2 µl of cDNA and free- nuclease water to a final volume of 50µl. The amplification reaction was run in the Veriti thermocycler with the cycling conditions: initial denaturation at 94^oC for 4min, then followed by 30 cycles with denaturation at 94^oC for 20 sec, primer annealing at 43^oC for 30 sec and extension at 72^oC for 7 min, and terminated by final extension at 72^oC for 7 min.

The PCR products were purified by GenElute PCR Clean-up Kit (Sigma cat. NA1020). Briefly, 500 µl column preparation solution was added into to each mini spin column inserted in a collection tube, centrifuged at 12000g for 1 min, discarded the eluate. 500 µl of binding solution and 100 µl of PCR reaction were mixed well by vortex, and transferred into the binding column, the solution was centrifuged at 16,000g/m for 1 min and the eluate was discarded, the spin column was washed with 0.5 ml of diluted wash solution, and then centrifuged at maximum speed to remove excess ethanol. DNA bound in the spin column was incubated with 50 µl with elution solution or water for 1min and finally eluted by centrifuging the column at maximum speed for 1 minute. The amplification product is in the eluate which was stored at -20^0C.

Checking the purification of DNA product by running on 1% agarose gel for electrophoresis and stained with ethidium bromide, the DNA bands of 1778 bp for HA gene and 1410bp for NA gene were visualized on a UV transilluminator. The purified amplification products of HA and NA genes were sent for direct sequence (BMR Genomics, Padova, Italia).

2.6.4. Phylogenetic analysis

The complete sequence of HA and NA of the isolate 15 from our study was chosen for comparison with sequences of influenza A (H1N1) 2009 in the GenBank (45). The entire HA and NA sequences of influenza A (H1N1) 2009 viruses isolated from humans in neighboring countries of Asia (Cambodia, China, Hong Kong, India, Japan, Taiwan and Thailand); Europe (England, Germany, Italy and France); Australia; and America (USA, Mexico, Canada) from April 2009 to November 2009 were chosen and obtained from the GenBank at http://www.ncbi.nlm.nih.gov/genomes /FLU/Database/.

Table 14

Accession of HA sequences of influenza virus

	S-OIV A H1N1 (2009)	Region	segment	Accession No
1	Influenza A virus (A/Australia/6/2009(H1N1))	Australia	4 (HA)	CY055075
2	Influenza A virus (A/Vienna/INS291/2009(H1N1))	Austria	4 (HA)	CY066703.
3	Influenza A virus (A/Cambodia/NHRCC00011/2009(H1N1)	Cambodia	4 (HA)	CY080344.
4	Influenza A virus (A/Toronto/0462/2009(H1N1)	Canada	4 (HA)	GQ385300
5	Influenza A virus (A/Guangdong/06/2009(H1N1))	China	4 (HA)	HM780470
6	Influenza A virus (A/Paris/2580/2009(H1N1)	France	4 (HA)	GQ214144
7	Influenza A virus (A/Sachsen-Anhalt/101/2009(H1N1)	Germany	4 (HA)	CY045482
8	Influenza A virus (A/Hong Kong/01/2009(H1N1)	Hong Kong	4 (HA)	GQ168606
9	Influenza A virus (A/Delhi/NIV0951830/2009(H1N1))	India	4 (HA)	CY075884
10	Influenza A virus (A/Firenze/10/2009(H1N1))	Italy	4 (HA)	GQ351319
11	Influenza A virus (A/Japan/NHRC0001/2009(H1N1)	Japan	4 (HA)	CY065952
12	Influenza A virus (A/Mexico city/CIA2/2009(H1N1)	Mexico	4 (HA)	CY062498
13	Influenza A virus (A/Mexico/InDRE4487/2009(H1N1))	Mexico	4 (HA)	FJ998208
14	Influenza A virus (A/Netherlands/1039/2009(H1N1)	Netherland	4 (HA)	CY065792
15	Influenza A virus (A/Taiwan/526/2009(H1N1))	Taiwan	4 (HA)	CY047744.
16	Influenza A virus (A/Thailand/CU-H104/2009(H1N1))	Thailand	4 (HA)	CY075672
17	Influenza A virus (A/New York/3177/2009(H1N1))	New York	4 (HA)	CY041597
18	Influenza A virus (A/England/313/2009(H1N1)	England	4 (HA)	HM567600

Table 15
Accession of NA sequences of influenza virus

	S-OIV A H1N1 (2009)	Region	segment	Accession No
1	Influenza A virus (A/Australia/6/2009(H1N1)	Australia	6 (NA)	CY055077
2	Influenza A virus (A/Vienna/INS291/2009(H1N1)	Austria	6 (NA)	CY066705
3	Influenza A virus (A/Cambodia/NHRCC00011/2009(H1N1)	Cambodia	6 (NA)	CY080346
4	Influenza A virus (A/Toronto/0462/2009(H1N1)	Canada	6 (NA)	GQ385302
5	Influenza A virus (A/Guangdong/03/2009(H1N1)	China	6 (NA)	GQ250162
6	Influenza A virus (A/Paris/2573/2009(H1N1)	France	6 (NA)	GQ214152
7	Influenza A virus (A/Sachsen-Anhalt/101/2009(H1N1))	Germany	6 (NA)	CY045484
8	Influenza A virus (A/Hong Kong/415742/2009(H1N1)	Hong Kong	6 (NA)	GU931805
9	Influenza A virus (A/Delhi/NIV3610/2009(H1N1)	India	6 (NA)	CY088675
10	Influenza A virus (A/Firenze/10/2009(H1N1)	Italy	6 (NA)	GQ351320
11	Influenza A virus (A/Japan/NHRC0001/2009(H1N1))	Japan	6 (NA)	CY065954.
12	Influenza A virus (A/Mexico/InDRE4487/2009(H1N1)	Mexico	6 (NA)	FJ998214
13	Influenza A virus (A/Mexico city/CIA2/2009(H1N1)	Mexico	6 (NA)	CY062499
14	Influenza A virus (A/Netherlands/1039/2009(H1N1)	Netherland	6 (NA)	CY065794
15	Influenza A virus (A/Taiwan/526/2009(H1N1)	Taiwan	6 (NA)	CY047746
16	Influenza A virus (A/Thailand/CU-H106/2009(H1N1)	Thailand	6 (NA)	GQ866936
17	Influenza A virus (A/New York/3177/2009(H1N1)	USA	6 (NA)	CY041599
18	Influenza A virus (A/England/313/2009(H1N1)	United Kingdom	6 (NA)	HM567602

The MEGA5 software was used for phylogenetic analysis of HA and NA sequences. Following sequence alignment with CLC genomic workbench, the evolutionary distance was inferred by Maximum likelihood Method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap

test (100 replicates) are shown next to the branches. The MEGA5 automatically produce the phylogenetic tree diagram.

2.7. Data analysis

2.7.1. Viral etiologies on pediatric patients

- A positive case was defined as presence of any viral pathogen detected by PCR/ RT-PCRs in the samples of a given patient.
- Single infection was defined if only one viral pathogen was detected by PCR in the samples of a patient
- When more than one viral pathogen was detected in the samples of a patient, the case was classified as co-infection of respiratory viruses.

We categorized the age of patients by months /12 e.g 06 months = 0.5 year of age.

2.7.2. Diagnosis of influenza A (H1N1)2009 virus

- A positive case was defined as presence of any positive result produced by viral isolation and/ or RT-PCR.

2.7.3. Phylogenetic analysis

- The Geneious software, version 4.8.5 was used for reading the sequences
- MEGA5 software was used for phylogenetic analysis and phylogenetic tree construction (119).

2.7.4. Statistic calculation and comparison

Statistic calculation and comparison between proportions were performed with the MedCalc software - version 8.0.1.0.

3. RESULTS

3.1. Identification of viral etiology by polymerase chain reactions

3.1.1. Demographic data of the population

The study was carried out in 216 children with clinically diagnosed lower respiratory infections. The age of the patients ranged from 3 months to 15 years. The mean age was 2 years, and the median was 1.2 years, among the hospitalized patients, 78% of patients were from 3 months to 2 years of age, and 93% of patients were below 5 years of age.

Male were 127 (59%) and female 89 (41%). The rate of male patientswas significantly higher than that of the female ($p= 0.01$, $p< 0.5$).

Distribution of age and sex of the sample and children positive with viral respiratory viruses is presented in detail in table 16.

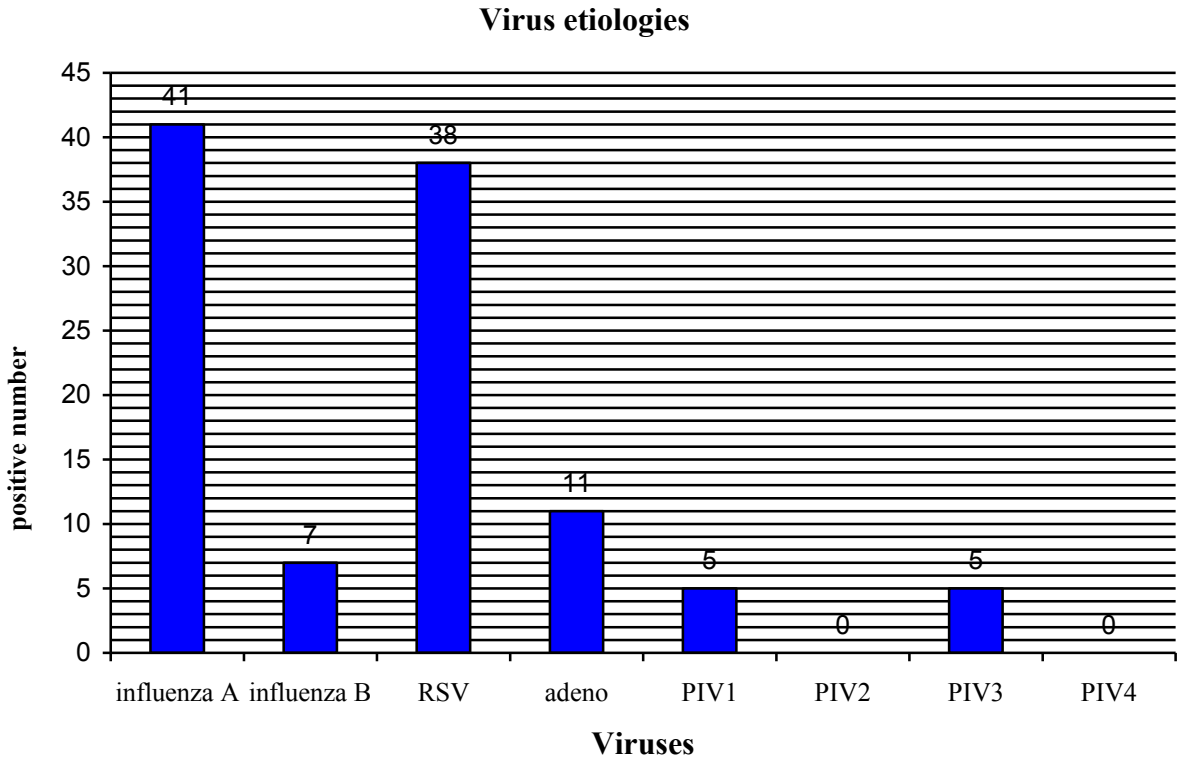
Table 16
Demographic data of the children population

Characteristics	Hospitalized children (N= 216)	Patients infected with respiratory viruses (N = 91)
Age	-	-
0.3- 1	99 (46%)	42 (46%)
>1- 2	64 (30%)	22 (24%)
>2- 5	38 (17%)	20 (22%)
> 5	16 (7%)	7 (8%)
Mean age	2	2.1 years
Median age	1.2	1.1
Male	127 (59%)	58 (64%)
Female	89 (41%)	33 (36%)

3.1.2. Viral etiology identified

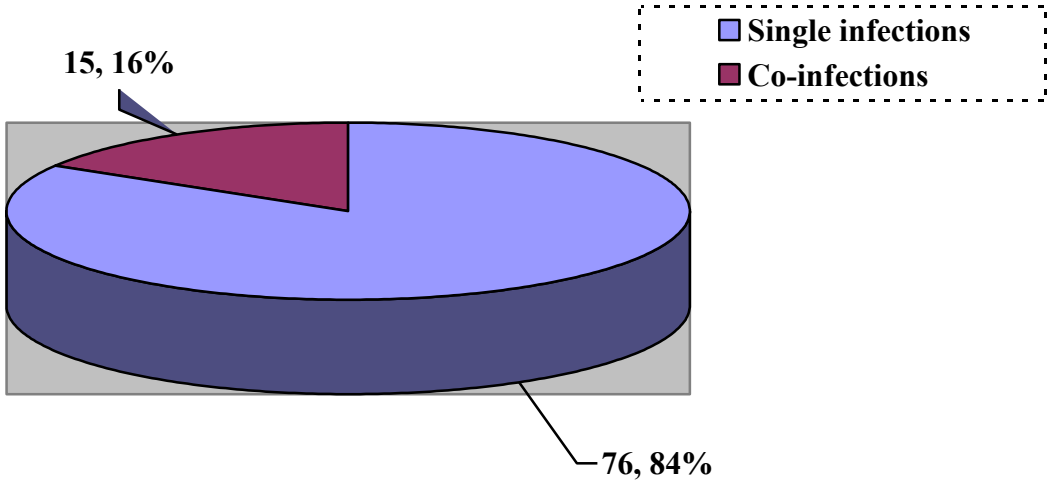
The number of positive cases for respiratory viruses was 91 with the rate of 42%; among which 15 cases (16%) were co-infections, and 76 cases (86%) were single infection.

Figure 10
Respiratory viruses identified from 216 patients



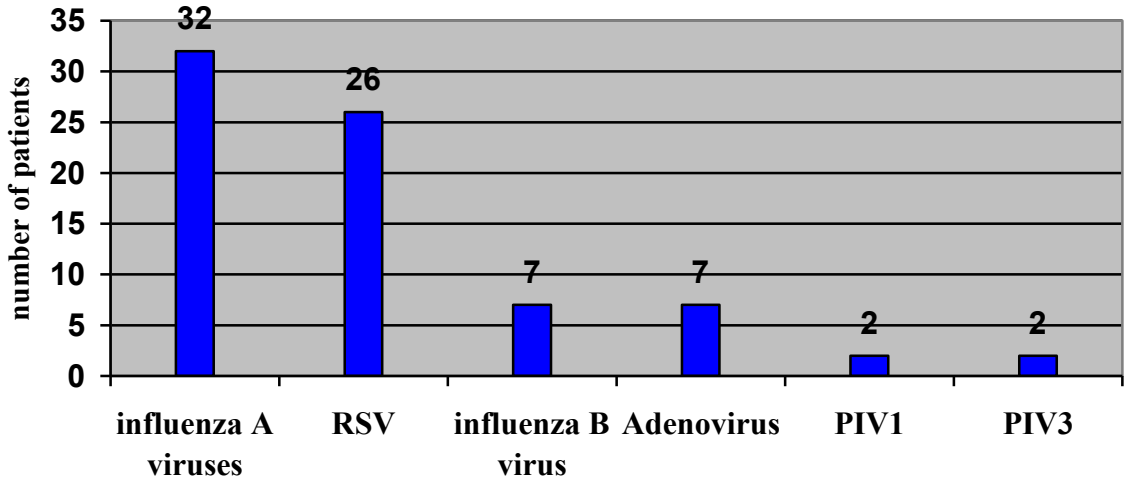
The total amount of 107 viruses was detected in 91 patients; of these influenza A viruses were detected in 41 cases with the rate 45%, Influenza B virus was in 7 with the rate of 8%. RSV was in 38 patient with the rate of 42%, adenoviruses were in 11 patients (12%), parainfluenza virus type 1 (PIV1) was detected in 5 cases (5%), and parainfluenza type 3 was found in 5 patients (5%). PIV2 and PIV4 were not detected in our samples.

Figure 11
Distribution of single infections and co-infections



In single infections, influenza A viruses was the most frequent with 32 cases (rate 42%), followed by RSV in 26 cases (rate of 34%), influenza B virus and adenovirus in 7 patients (9%) each, parainfluenza viruses 1 in 2 cases (3%) and parainfluenza virus 3 in 2 cases (3%). Respiratory viruses detected in single infection are presented in figure 12.

Figure 12
Respiratory viruses detected in single infections



In 15 patients with co-infection, 8 children were infected with both influenza A virus and RSV, 3 children with RSV and adenovirus, 2 with parainfluenza virus 1 and 3, one with RSV and parainfluenza virus and one children was infected with three viruses including RSV, adenovirus and influenza virus. The details of co-infection of viruses are shown in table 17.

Table 17
Co-infection of viruses

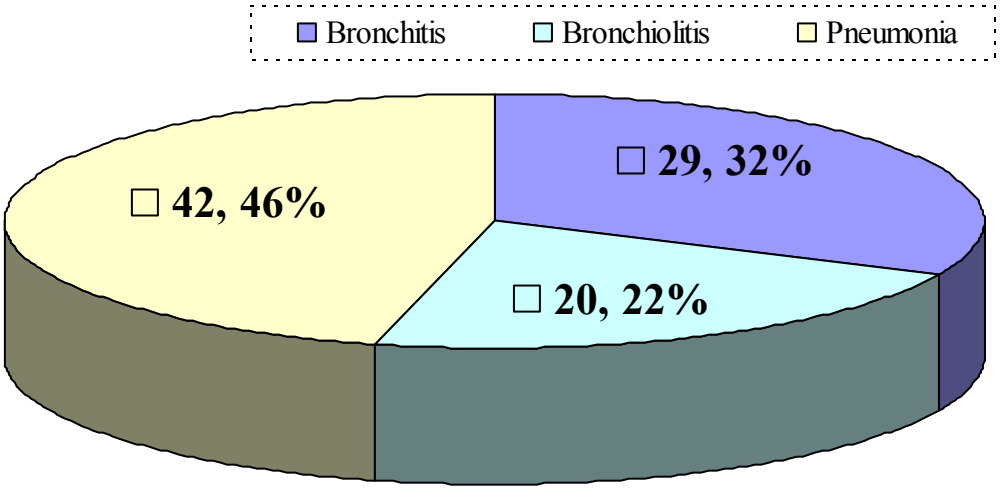
Co-infection of viruses	Quantity
RSV + influenza A virus	8
RSV + adenovirus	3
Parainfluenza virus 1 and 3	2
RSV + parainfluenza virus	1
RSV + adenovirus + influenza A virus	1
Total	15

3.1.3. Viral etiology distribution with different type of lower respiratory infections

3.1.3.1. Type of lower respiratory tract infections

In the 91 patients positive for virus, bronchitis was diagnosed in 29 patients with the rate of 32%, bronchiolitis was in 20 patients (22%) and pneumonia in 42 patients (46%). The differences between the locations of lower respiratory infections are not significant ($p > 0.05$). The frequency of the lower respiratory infections is described in figure 13.

Figure 13
Frequency of lower respiratory infections

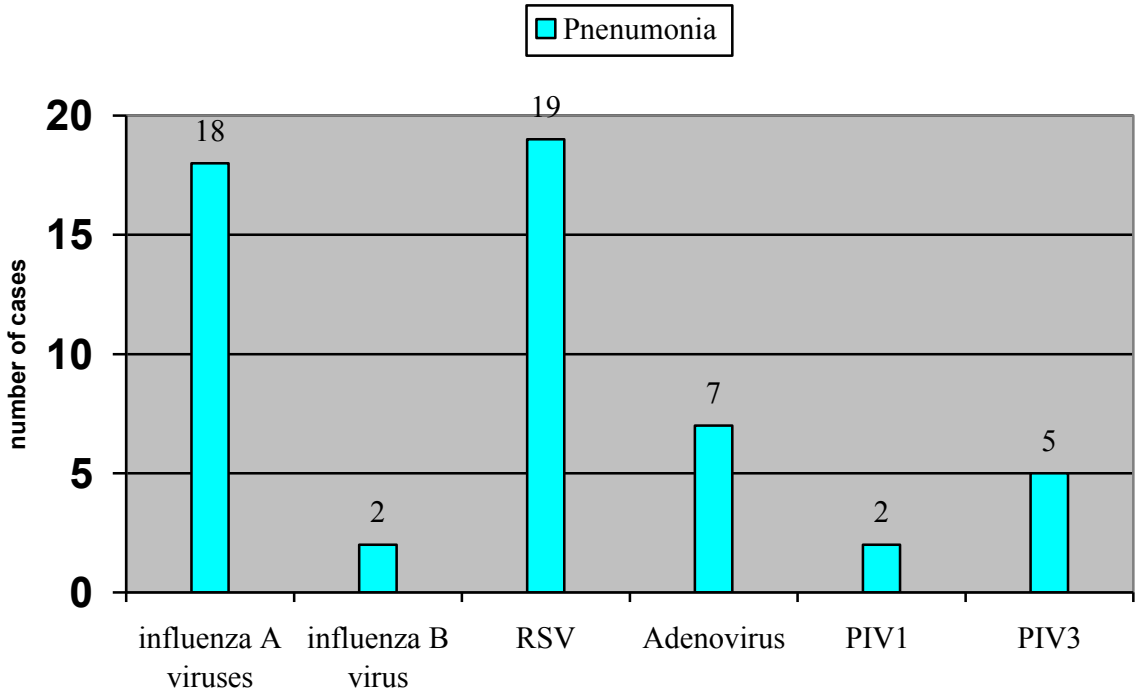


3.1.3.2. Viral etiology distribution and lower respiratory infections

- **Viral agent distribution in pneumonia**

RSV and Influenza A viruses were detected in 19 (21%) and 18 (20%) cases respectively, adenovirus was detected in 7 (8%), influenza B in 2 (2%), parainfluenza 3 encountered in 5 (5%) and PIV1 in 2 cases (2%). Distribution of viral agents in pneumonia is depicted as figure 14.

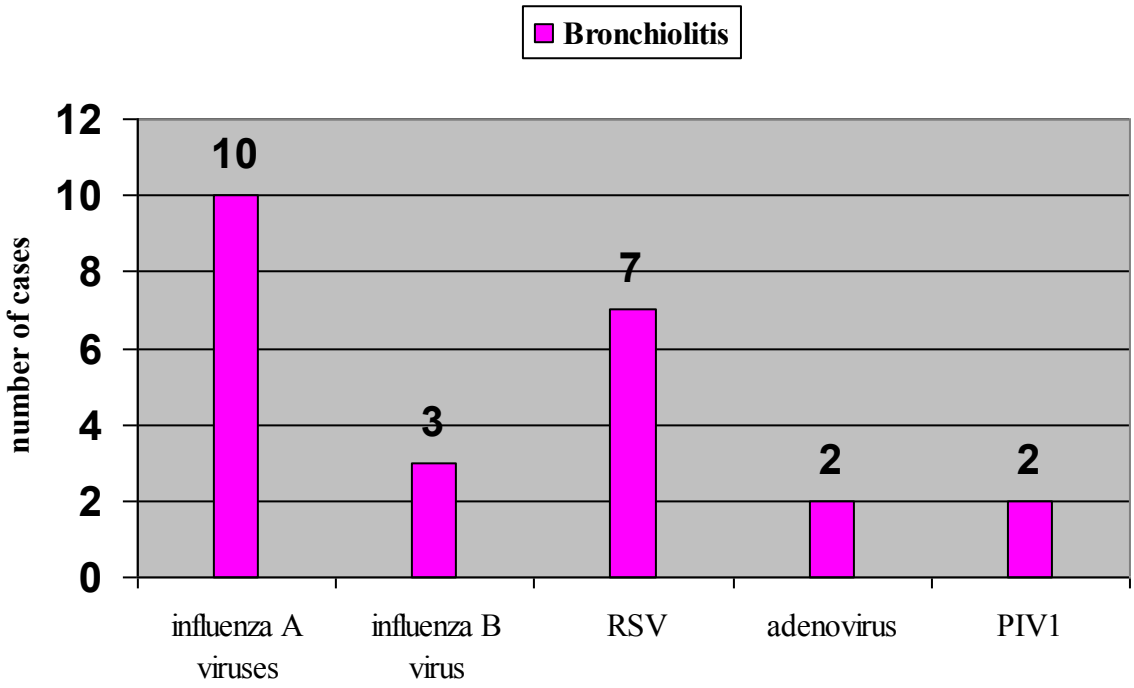
Figure 14
Viral agents in pneumonia



- **Viral agent distribution in bronchiolitis**

Influenza A viruses were detected in 10 cases (11%), followed by RSV in 7 (8%), influenza B virus in 3 (3%), adenovirus and PIV1 were equally in 2 (2%) for each. Viral agents in bronchiolitis are presented in the figure 15.

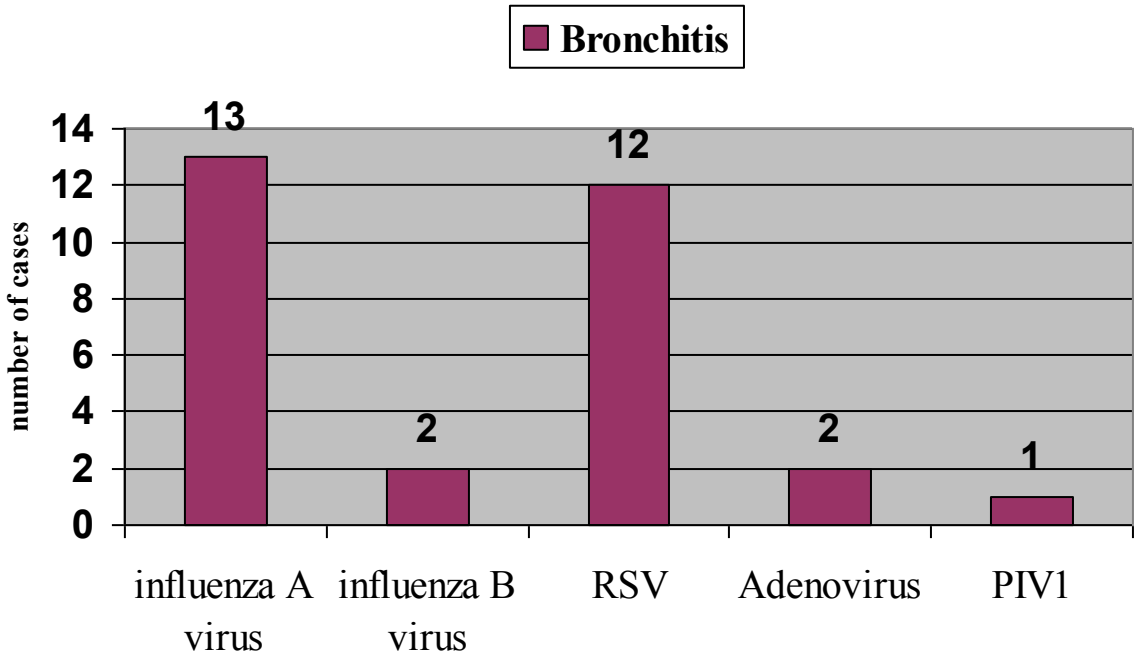
Figure 15
Viral agents in bronchiolitis



- **Viral agent distribution of bronchitis,**

Influenza A viruses were detected in 13 (14%), followed by RSV in 12 (13%), influenza B virus and adenovirus were equally responsible in 2 (2%) for each, and PIV1 in 1 case (1%). Viral agents in bronchitis are illustrated in figure 16.

Figure 16
Viral agent detected in bronchitis

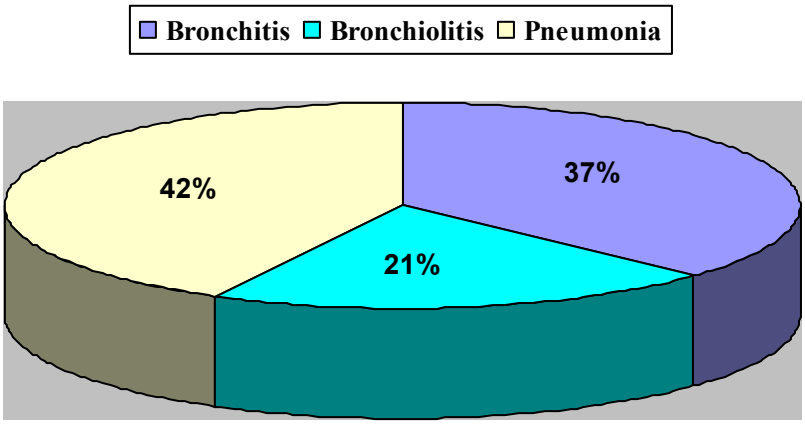


3.1.4. Status of viral infections and lower respiratory infections

3.1.4.1. Single virus infections of lower respiratory infections

In 76 patients with single infections, pneumonia was diagnosed in 32 patients (42%), followed by bronchitis in 28 patients (37%), and bronchiolitis in 16 patients (21%). The differences are not significant ($p > 0.05$). The frequencies of single virus infection of lower respiratory tract infections are shown in figure 17.

Figure 17
Frequency of lower respiratory infections of single infections



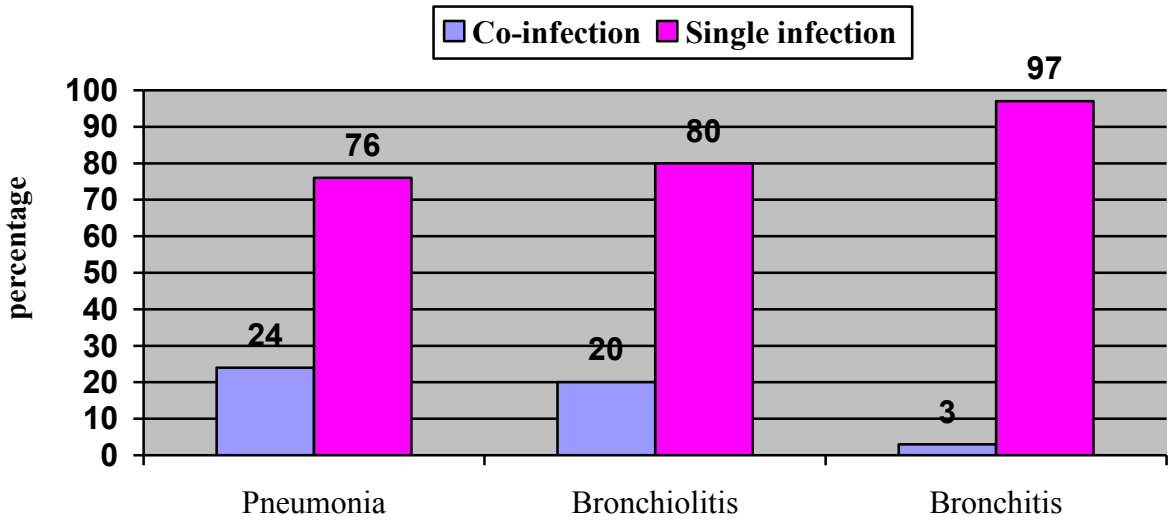
3.1.4.2. Co-infections of lower respiratory tract infections

- **Distribution of co-infections**

In 42 patients with pneumonia, 10 patients (24%) had co-infections of more than one respiratory virus, and 32 patients (76%) were single infections. Among 20 patients with bronchiolitis, 4 patients (20%) were recognized co-infections, and 16 patients (80%) were single infections; and among 29 patients with bronchitis, only one patient (3%) was co-infection and 28 (97%) were single infections. No difference was found in co-infections between pneumonia and bronchiolitis or between bronchiolitis and bronchitis. However, co-infection in pneumonia was slightly higher than in bronchitis ($p = 0.03$).

Percentage distribution of co-infections of lower respiratory tract infections is presented in figure 18.

Figure 18
Percentage distribution of co-infections of lower respiratory tract infections



3.1.4.3. Respiratory viruses of co-infections

- **Viral co-infections of pneumonia**

Co-infection of influenza A viruses and RSV was detected in 5 cases (30%), RSV and adenovirus was in 2 cases (13%), combination of PIV1 and PIV3 was detected in 2 cases (13%), and combination of three viruses including RSV, influenza A virus and adenovirus in 1 case (7%).

Table 18
Viral co-infections in pneumonia

Respiratory virus combinations	Pneumonia
RSV - influenza A viruses	5
RSV- adenoviruses	2
RSV-adenovirus- influenza virus	1
Parainfluenza virus 1 and 3	2
Total	10

- **Viral co-infections of bronchiolitis**

Co-infection of RSV and influenza virus was found in 3 cases and RSV and parainfluenza in 1 case.

Table 19
Co-infection of viral agents in bronchiolitis

Respiratory virus combinations	Bronchiolitis
RSV - influenza A viruses	3
RSV- parainfluenza virus 1	1
Total	4

- **Viral co-infection of bronchitis**

One patient was found to co-infected with RSV and adenovirus

3.1.5. Viral etiology distribution according to age

Among 91 infected with respiratory viruses, 42 children were from 3 months to 1 year of age, in this age group 34 patients had single infections and 8 children had co-infections. RSV and influenza A viruses were identified in 20 and 19 children, respectively; adenovirus was found in 5, influenza B virus in 3 and PIV1 and PIV3 in 3 children.

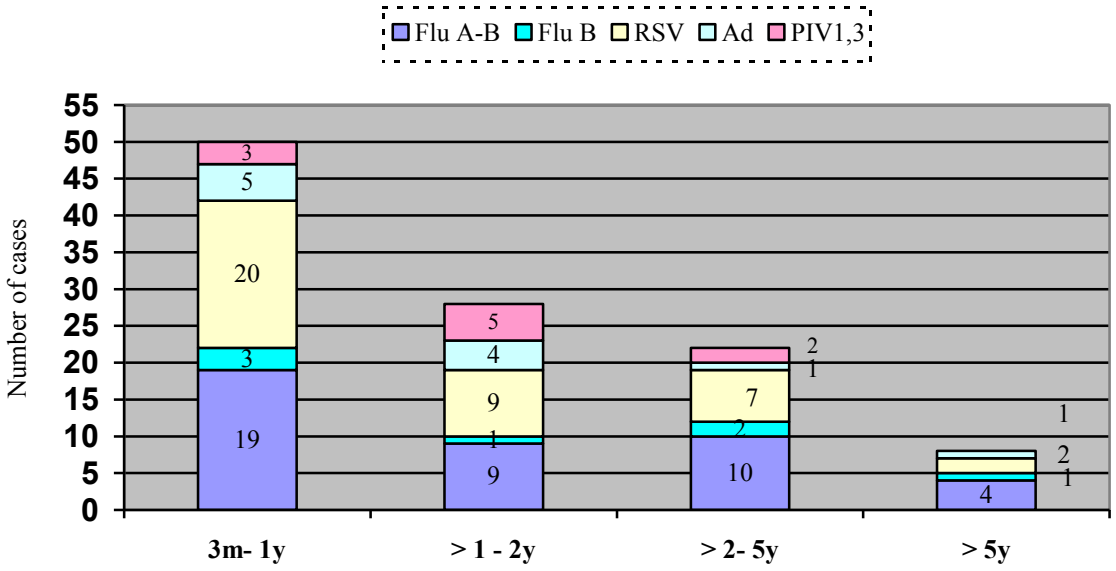
Among 22 children from 1 to 2 years of age, five children had co-infections and 17 children had single infections. RSV and influenza A virus were found in 9 children for each, followed by Parainfluenza virus 1 and 3 in 5 children, adenovirus in 4 children.

Among 20 children from 2 and 5 years of age, two children infected more than one viral agent, influenza A virus was dominant in 10 children, the second was RSV in 7, influenza B in 2, parainfluenza virus 1 and 3 in 2 and adenovirus in 1 children.

In the age group over 5 years of age, seven children had single virus infections, influenza A virus was identified in 4 children, RSV was also found in 2 children, adenovirus in 1 and influenza B virus in 1 children.

Distribution of age group and viral etiologies is presented in detail in figure 19.

Figure 19
Distribution of age group and viral etiologies



3.1.6. Seasonality of respiratory virus distribution

In 216 specimens collected in children hospitalized from the middle of March, 2010 to the sixth of April 2011, specimen numbers were different during the study time. The highest number was 49 in February of 2011, and the smallest number (5 specimens) was in April 2011. The mean of collected specimens was 22 per month.

From July to October of 2010, an outbreak of dengue fever occurred in Thua Thien Hue province and the section of lung and respiratory diseases was used for admission of children with dengue fever; no respiratory specimen was collected during this period of time.

The percentages of identified viruses varied in different months; in April of 2011, only 5 specimens were collected during 6 days and one positive case was identified (20%). Among the remaining months the rates of positive viral diagnosis were from 20 to 69% and the overall rate of positive viral detection was 42%.

The numbers of collected specimens and the rate of viral positive specimens during the study time are presented in table 20.

Table 20
Distribution of the rate of viral detection during study time

Month	Collected specimens	Viral positive specimens	Rate of viral positive specimens (%)
March-2010	19	8	42
April	30	12	40
May	37	9	24
June	9	4	44
November	16	11	69
December	22	11	50
January-2011	16	9	56
February	49	24	49
March	13	2	15
April	5	1	20
Total	216	91	42

3.1.7. Time distribution of specific viral etiologies

Among the respiratory viruses identified, influenza viruses and RSV were most frequently detected. Infections of these viruses were found throughout the study time; however, influenza viruses were higher during raining season with c 9 cases in December of 2010, and the highest number with 21 positive in February, 2011.

RSV infection was slightly higher in April of 2010 with 11 cases, but in the other months of this study the numbers of RSV infection were lower with a frequence from 2 to 6 cases/ month. The numbers of remaining viruses were insufficient to show time distribution. The distribution of infections of influenza viruses and RSV during time is presented in the figure 20 and figure 21.

Figure 20
Time distribution of influenza virus infections
No case was detected from July to October in 2010 because specimens were not available during this period.

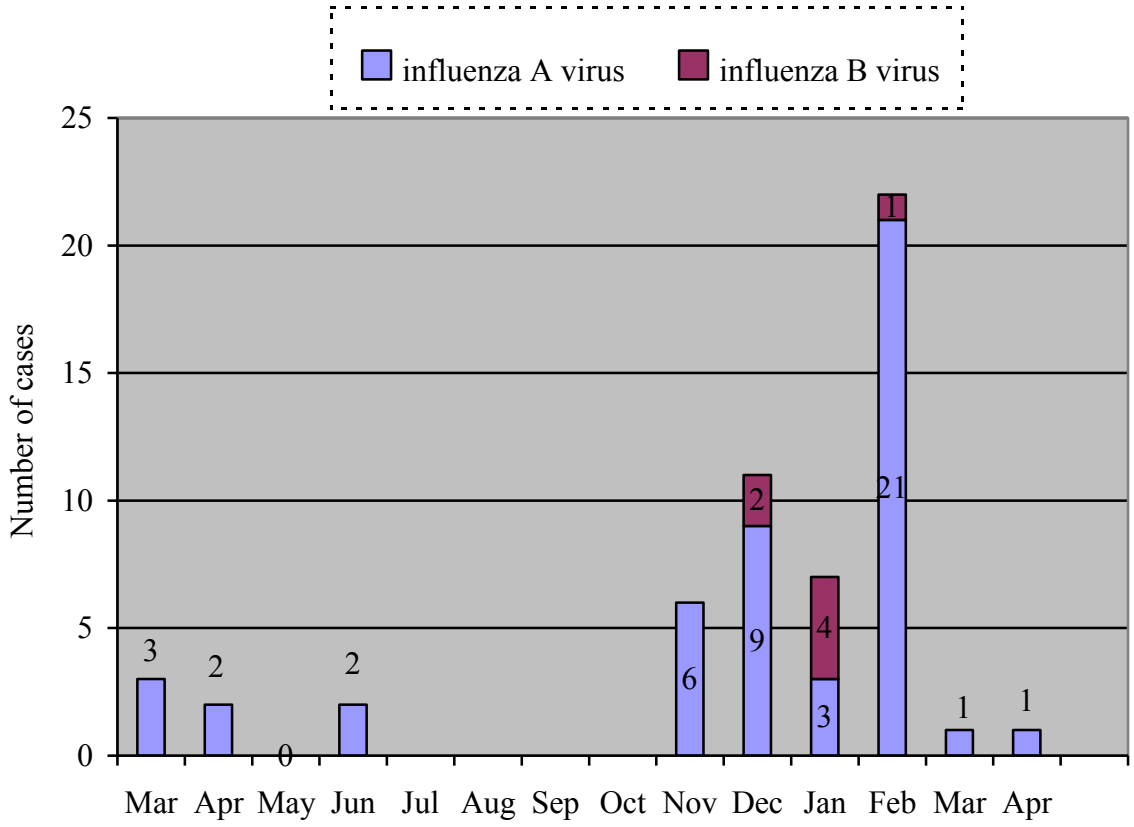
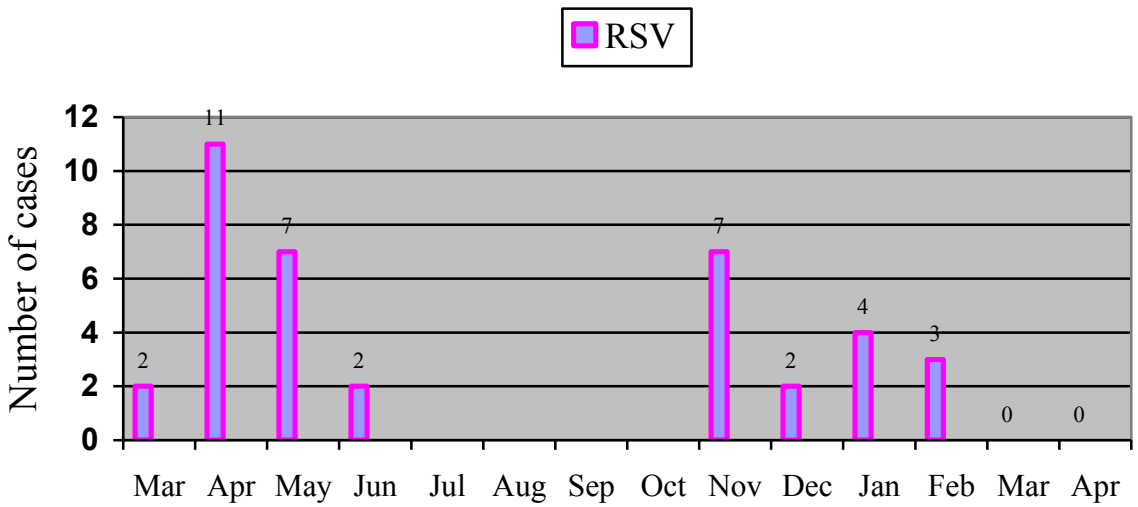


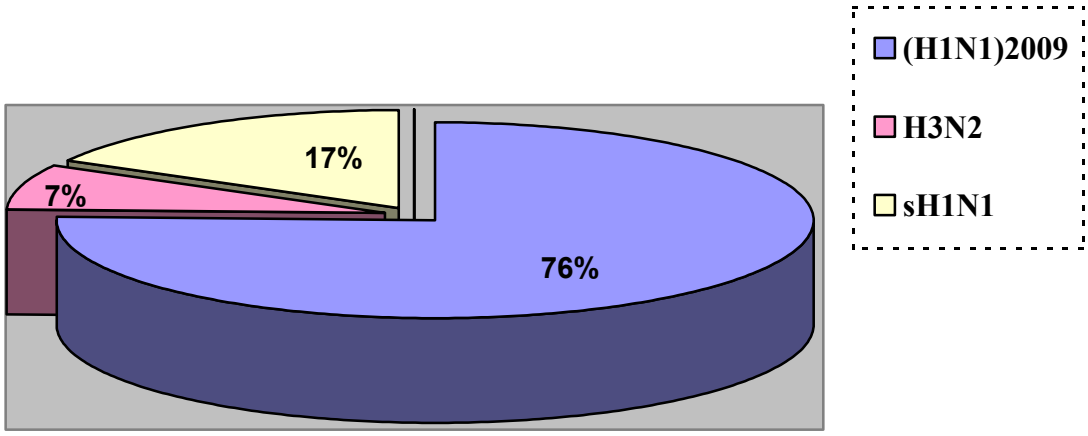
Figure 21
Time distribution of RSV infections
No case was detected from July to October in 2010 because specimens were not available during this period.



3.1.8. Influenza A viruses and subtype

Influenza A viruses were identified in 41 patients (45%), and influenza A (H1N1) 2009 virus was the prominent subtype in 31 cases (76%), followed by seasonal H1N1 in 7 cases (17%), and H3N2 subtype in 3 cases (7%). Distribution of influenza A virus subtypes is shown in figure 22.

Figure 22
Distribution of influenza A virus subtypes



3.2. Isolation and identification of the novel influenza A (H1N1) 2009 virus in the pandemic 2009 by virological methods (cell culture on MDCK/ embryonated chicken egg inoculation) and reverse transcription- polymerase chain reactions

3.2.1. Detection of influenza A virus

This study was carried out in 53 patients suspected influenza A (H1N1) 2009 virus infection admitted to the isolation department of Hue City Hospital in October and November 2009. Patients consisted of children and adults from 4 to 40 years of age; the mean of age was 14.3 years and median 14 years of age.

Both viral isolation and RT-PCR were carried out in 41 specimens and 12 specimens were tested only by RT-PCR. In the 41 samples tested by both methods, RT- PCR yielded positive result in 27, among which 5 were negative by isolation. Isolation yielded positive result in 24 specimens (58.5%), among which 22 were positive by both methods and 2 were positive only after isolation. Among 12 specimens tested only by RT-PCR, three specimens were positive for influenza A virus. Thus RT-PCR gave positive result in 30 specimens and two were positive after isolation.

According to the definition for a positive case of influenza A infection given in the previous chapter and the combination of results of the both methods

of detection, 32 cases (60.4%) were confirmed to be positive for influenza A virus, of these 8 (25%) were detected only by RT-PCR.

These results are summarized in table 21.

Table 21
Detection of influenza virus by isolation and RT-PCR in 53 specimens

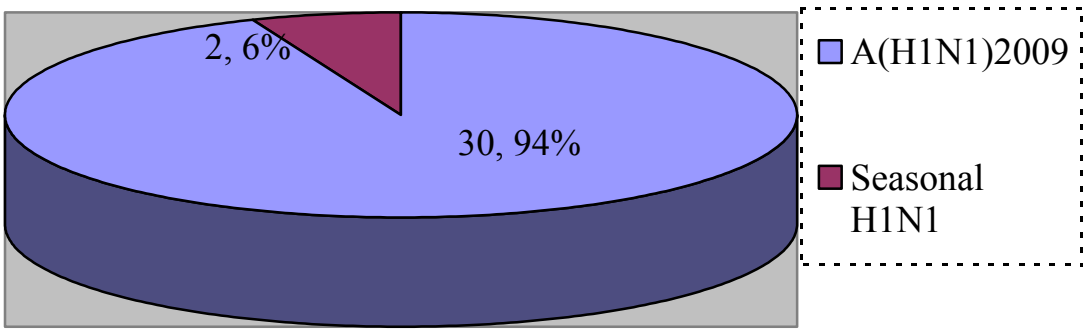
No of specimens	Viral isolation (%)		RT-PCR	
	Positive	Negative	Positive	Negative
41	24 (58.5)	17 (41.5)	27 (65.9)	14 (34.1)
12	-	-	3 (25)	9 (75)
53	24	17	30	23

In addition, in the group of 41 samples tested by both methods, a total of 29 yielded positive result. RT-PCR with 27 positive specimens show a sensitivity of 93.1%, and virus isolation with 24 positive results has a sensitivity of 82.7%.

3.2.2. Identification of subtypes of influenza A virus

In 32 positive specimens with influenza A viruses, influenza A (H1N1) 2009 virus was identified in 30 (93.7%) and seasonal H1N1 subtype of influenza A virus in 2 specimens (6.2%). Subtype (H3N2) of influenza A virus was not identified.

Figure 23
Influenza A virus subtypes



3.3. Characterization of the genes of HA and NA of influenza A (H1N1) 2009 virus and molecular epidemiology

3.3.1. Sequence analyses of the HA and NA in Hue isolates

Four sequences of the entire HA genes of isolates 6, 7, 10, 15 and three full length NA segments of isolates 6, 10 and 15 from Hue city were analyzed. The HA and NA sequences of three isolates 6, 10 and 15 were submitted to the GenBank database and are now available with the following access number:

Isolate 6: JN896300 for HA, JN896301 for NA;
Isolate 7: JN935017 for HA
Isolate 10: JN896302 for HA, JN896303 for NA;
Isolate 15: JN393307 for HA, JN656969 for NA (45).

Alignment of 4 HA gene sequences of isolates 6, 7, 10, 15 from Hue showed high similarity and percent identities of nucleotides were ranging from 99.48% to 99.77%, with nucleotide differences from 9 to 4 nucleotides as presented in table 22.

Table 22
Identity of HA sequences and nucleotide difference of 4 isolates from Hue city

HA sequence - isolate code	Identity (%)	Nucleotide difference
HA -10 / HA-15, HA- 7	99.77	4
HA-15 / HA-7	99.65	6
HA-10 / HA- 6	99.59	7
HA- 6 / HA -15, HA - 7	99.48	9

Comparison of NA gene of three isolates 6, 10, 15 was carried out similarly, their identity percentages were from 99.57% to 99.86%. Similarities among three NA sequences and the number of nucleotide difference are shown in table 23.

Table 23
Similarities of three NA sequences and number of nucleotide difference

NA sequence - isolate code	Identity (%)	Nucleotide difference
NA-10 / NA-15	99.86	2
NA-6 / NA-15	99.72	4
NA-10 / NA- 6	99.57	6

3.3.2. Phylogenies of the HA and NA segments with referenced isolates

Comparison between the HA sequences of 4 representative isolates 6, 7, 10, 15 with reference HA sequences by using Maximum Likelihood method shows very close similarities with percentages from 99.77% (4 different nucleotides) to 99.07% (16 different nucleotides). The HA sequences of Hue influenza A (H1N1) 2009 viruses are more similar in HA gene sequence to the following referenced isolates: A/Japan/NHRC0001/2009(H1N1), A/Firenze/10/2009(H1N1), A/Cambodia/NHRCC00011/2009(H1N1). The representative isolates of Hue influenza A (H1N1) 2009 virus cluster together in the phylogenetic tree of HA gene.

Comparison between the NA gene sequences of 3 representative isolates with 18 reference NA sequences similarily, percentages of similarities are from 99.36% (9 different nucleotides) to 98.65% (19 different nucleotides). In phylogenetic tree pattern of NA gene sequences, 3 Hue representative isolates cluster together in a separated cluster, and they are similar to the following referenced isolates: A/Vienna/INS291/2009(H1N1), A/Firenze/10/2009(H1N1), A/Japan/NHRC0001/2009(H1N1), A/Taiwan/526/2009(H1N1), and A/ England/313/2009(H1N1).

Differences in detail between Hue representative sequences in HA gene and 18 reference sequences are summarized in table 24 and percentage similarities of Hue HA sequences and 18 reference sequences are in table 25.

Differences between of the NA gene sequences of 3 Hue representative isolates of influenza A (H1N1) 2009 virus and 18 referenced NA sequences are shown in detail in table 26 and table 27.

Table 24

Different locations of nucleotide sequences of HA gene between Hue representative isolates and 18 reference HA sequences

	Differences at nucleotide sequences in HA gene																																																		
	36	54	81	155	174	177	197	221	233	317	339	340	440	445	464	495	539	551	635	690	698	701	734	771	786	802	861	962	965	966	1055	1088	1181	1190	1193	1246	1292	1313	1362	1403	1440	1503	1554	1590	1717	1737	1738	1739	1740	1741	1747
CY062498[a]	A	C	G	A	A	C	T	G	G	T	G	A	G	G	C	C	T	A	T	T	A	C	A	T	G	A	A	G	T	A	T	T	G	G	T	C	A	A	G	A	T	C	C	G	T	T	A	G	G	A	G
JN393307	–	–	–	–	–	–	**C**	–	**A**	**C**	–	–	**A**	–	–	–	–	**T**	–	**A**	–	–	**G**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**G**	–	–	–	–	–	–	–	–	–	–	–	–	–
HUE/6/2009	–	–	–	–	–	–	C	–	–	C	**T**	–	–	–	–	–	–	T	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**G**	–	–	–	–	–	–	–	**A**	**G**	**A**	**T**	**C**	–
HUE/7/2009	–	–	–	–	–	–	C	–	–	C	–	–	–	**A**	–	–	–	T	G	A	–	–	–	–	–	–	–	–	–	–	–	–	**A**	–	–	–	–	**G**	–	–	–	–	–	–	–	–	–	–	–	–	–
HUE/10/2009	–	–	–	**C**	–	–	C	–	–	C	–	–	–	–	–	–	–	T	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**G**	–	–	–	–	–	–	–	–	–	–	–	–	–
CY075672	–	–	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CY066703	–	–	–	–	G	–	C	–	–	C	–	–	–	–	–	A	–	–	–	A	G	–	–	–	–	–	–	–	–	–	C	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GQ385300	–	–	–	–	–	–	C	–	–	C	–	–	–	–	T	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–
CY047744	–	–	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A
CY045482	–	A	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–
GQ214144	–	–	–	–	–	A	C	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–
CY041597	–	–	–	–	–	A	C	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–
CY065792	–	–	A	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–
FJ998208	–	–	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–
CY065952	–	–	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	–	–	–
GQ168606	–	–	–	–	–	–	C	A	–	C	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	G	C	–	–	–	–	–	–	–	–	–	–
HM780470	–	–	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	A	A	–	–	–	–	–	–
GQ351319	–	–	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
HM567600	G	–	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	T	T	–	–	–	–	–	–	–	–
CY075884	–	–	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–	–	–	A	–	–	–	–	G	–	–	–	A	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CY080344	–	–	–	–	–	–	C	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CY055075	–	–	–	–	–	–	C	–	–	C	–	G	–	–	–	–	–	C	–	A	–	–	–	C	–	–	–	–	C	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Complete HA sequence of Influenza A virus (A/Mexico city/CIA2/2009(H1N1); .Hue isolates are in red

Table 25
Identity percentages of Hue HA gene sequences and reference sequences

Acession No.	A/ Hue/ 6/2009 (H1N1)		A/ Hue/ 7/2009 (H1N1)		A/ Hue/ 10/2009 (H1N1)		A/ Hue/ 15/2009 (H1N1)	
	Different nucleotides	Identity (%)	Different nucleotides	Identity (%)	Different nucleotides	Identity (%)	Different nucleotides	Identity (%)
CY062498	11	99.38	8	99.55	6	99.66	8	99.55
CY075672	14	99.19	7	99.59	6	99.65	5	99.71
CY066703	16	99.07	11	99.36	10	99.42	9	99.48
GQ385300	13	99.24	8	99.54	7	99.59	8	99.54
CY047744	12	99.30	8	99.54	7	99.59	6	99.65
CY045482	12	99.30	7	99.59	6	99.65	6	99.65
GQ214144	12	99.30	7	99.59	6	99.65	6	99.65
CY041597	12	99.30	7	99.59	6	99.65	7	99.59
CY065792	13	99.24	8	99.54	7	99.59	6	99.65
FJ998208	12	99.30	6	99.65	5	99.71	5	99.71
CY065952	10	99.42	6	99.65	5	99.71	4	99.77
GQ168606	15	99.13	10	99.42	9	99.48	9	99.48
HM780470	14	99.19	8	99.54	8	99.54	7	99.59
GQ351319	11	99.36	6	99.65	5	99.71	4	99.77
HM567600	14	99.19	9	99.48	8	99.54	8	99.54
CY075884	15	99.13	10	99.42	9	99.48	8	99.54
CY080344	11	99.36	6	99.65	5	99.71	4	99.77
CY055075	16	99.07	11	99.36	10	99.42	9	99.48

Table 26

Different locations of nucleotide sequences of NA gene between Hue representative isolates and the reference isolates

	Difference of nucleotide sequences of NA gene																																				
	27	104	126	144	160	163	192	217	255	256	283	284	294	297	307	316	254	389	411	456	467	529	532	541	595	714	742	820	894	954	990	1113	1115	1176	1179	1221	1357	1408
CY062499	C	G	T	C	A	A	G	A	G	G	A	G	T	G	G	A	G	G	G	G	G	G	G	G	G	C	G	C	C	T	T	T	A	T	C	T	A	G
HUE/15/2009	–	**A**	–	–	**C**	–	**A**	–	**A**	**A**	–	**A**	–	**A**	**A**	–	–	**A**	–	–	**C**	**A**	**C**	**A**	**A**	–	–	T	–	**C**	–	–	–	–	–	–	**G**	–
HUE/6/2009	–	**A**	–	–	**C**	–	**A**	–	**A**	**A**	–	**A**	–	**A**	**A**	–	–	**A**	–	–	**C**	–	–	–	–	–	–	T	–	**C**	–	–	–	–	–	–	–	–
HUE/10/2009	–	**A**	–	–	**C**	–	**A**	–	**A**	**A**	–	**A**	–	**A**	**A**	–	–	**A**	–	**A**	**C**	**A**	**C**	**A**	**A**	–	–	T	–	**C**	–	–	–	–	–	–	–	–
CY041599	–	–	C	–	–	–	–	–	A	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	A	T	–	C	–	–	–	–	–	–	G	–
CY045484	–	–	–	T	–	–	–	–	A	–	G	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	A	T	–	C	–	–	–	–	–	–	G	–
CY047746	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C		–	–	–	–	–	G	–
CY055077	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C	C	–	–	–	–	–	G	–
CY065794	T	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C	–	–	–	–	–	–	G	–
CY065954	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C	–	–	–	–	–	–	G	–
CY066705	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C	–	–	–	–	–	–	G	–
CY080346	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	G	T	–	C	–	–	–	–	–	–	G	–
CY088675	–	–	–	–	–	–	–	–	A	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C	–	–	–	–	T	–	G	–
FJ998214	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	A	T	–	C	–	–	–	–	–	–	G	–
GQ214152	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	G	–	–	A	–	–	–	–	–	–	–	A	T	G	C	–	–	–	–	–	–	G	–
GQ250162	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C	–	–	–	–	–	C	G	–
GQ351320	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C	–	–	–	–	–	–	G	–
GQ385302	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	G	A	–	–	–	–	–	–	–	–	–	A	T	–	C	–	–	–	C	–	–	G	–
GQ866936	–	–	–	–	–	G	–	G	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C	–	G	G	–	–	–	G	T
GU931805	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	A	T	–	C	–	–	–	–	–	–	G	–
HM567602	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	T	–	C	–	–	–	–	–	–	G	–

CY062499: complete NA sequence of influenza A virus (A/Mexico city/CIA2/2009(H1N1).
Hue isolates are in red

Table 27
Percentages of similarity between of NA gene sequences of 3 Hue representative isolates and the reference NA sequences

Acession No.	A/ Hue/ 6/2009 (H1N1)		A/ Hue/ 10/2009 (H1N1)		A/ Hue/ 15/2009 (H1N1)	
	Different nucleotides	Identity (%)	Different nucleotides	Identity (%)	Different nucleotides	Identity (%)
CY062499	12	99.15	17	98.79	17	98.79
CY041599	13	99.08	18	98.72	16	98.87
CY045484	13	99.08	19	98.65	17	98.79
CY047746	9	99.36	15	98.94	14	99.01
CY055077	10	99.29	16	98.87	15	98.94
CY065794	10	99.29	16	98.87	15	98.94
CY065954	9	99.36	15	98.94	14	99.01
CY066705	9	99.36	15	98.94	13	99.08
CY080346	10	99.29	16	98.87	14	99.01
CY088675	11	99.22	17	98.79	15	98.94
FJ998214	11	99.22	17	98.79	14	99.01
GQ214152	13	99.08	19	98.65	15	98.94
GQ250162	10	99.29	16	98.87	14	99.01
GQ351320	9	99.36	15	98.94	13	99.08
GQ385302	11	99.22	18	98.72	16	98.87
GQ866936	13	99.08	19	98.65	17	98.79
GU931805	11	99.22	17	98.79	14	99.01
HM567602	9	99.36	15	98.94	13	99.08

Figure 25

Phylogeny of HA genes of 4 influenza A (H1N1) 2009 viruses in Thua Thien Hue and 18 reference sequences by using Maximum Likelihood method, the length scale measured the number of substitutions per site

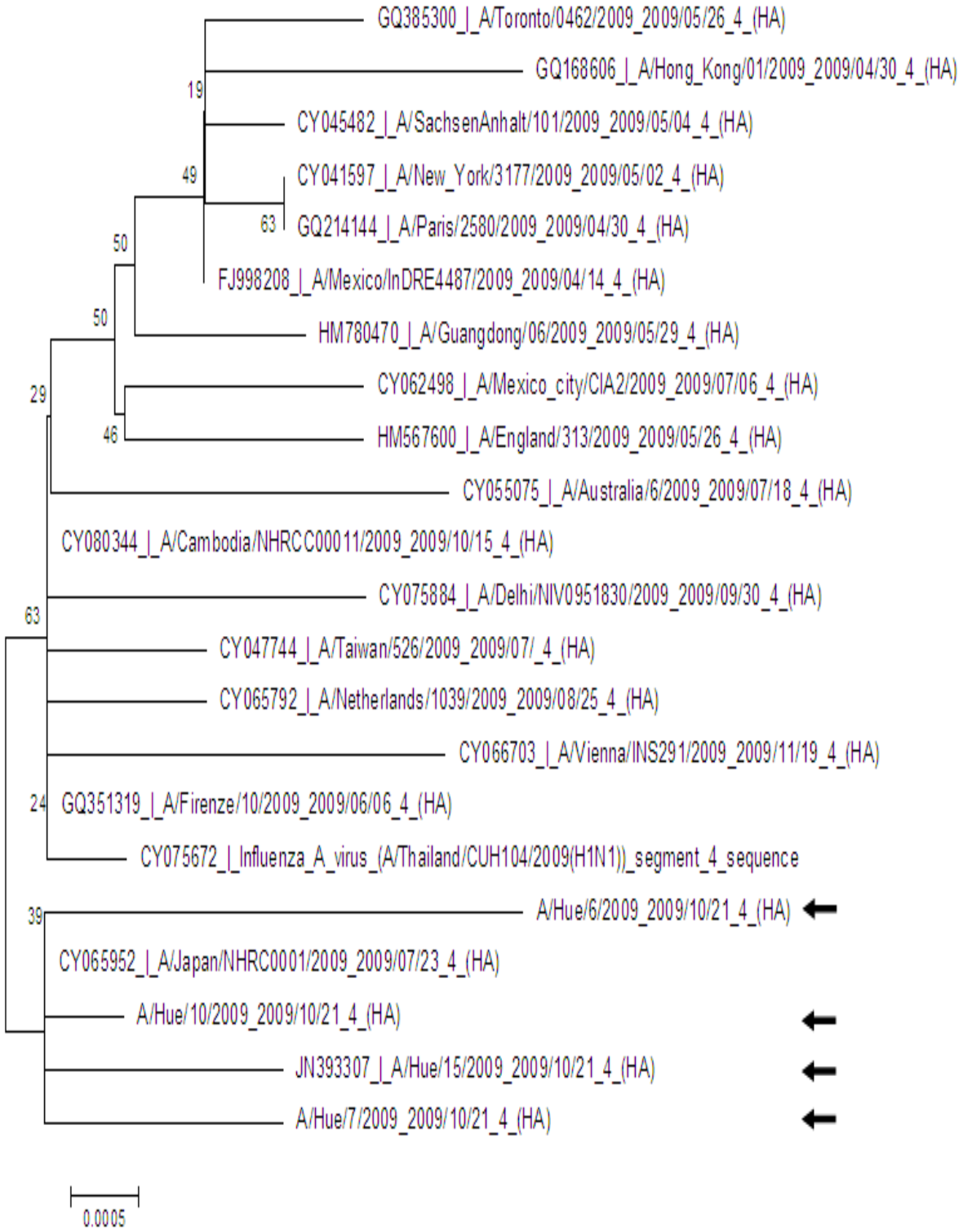
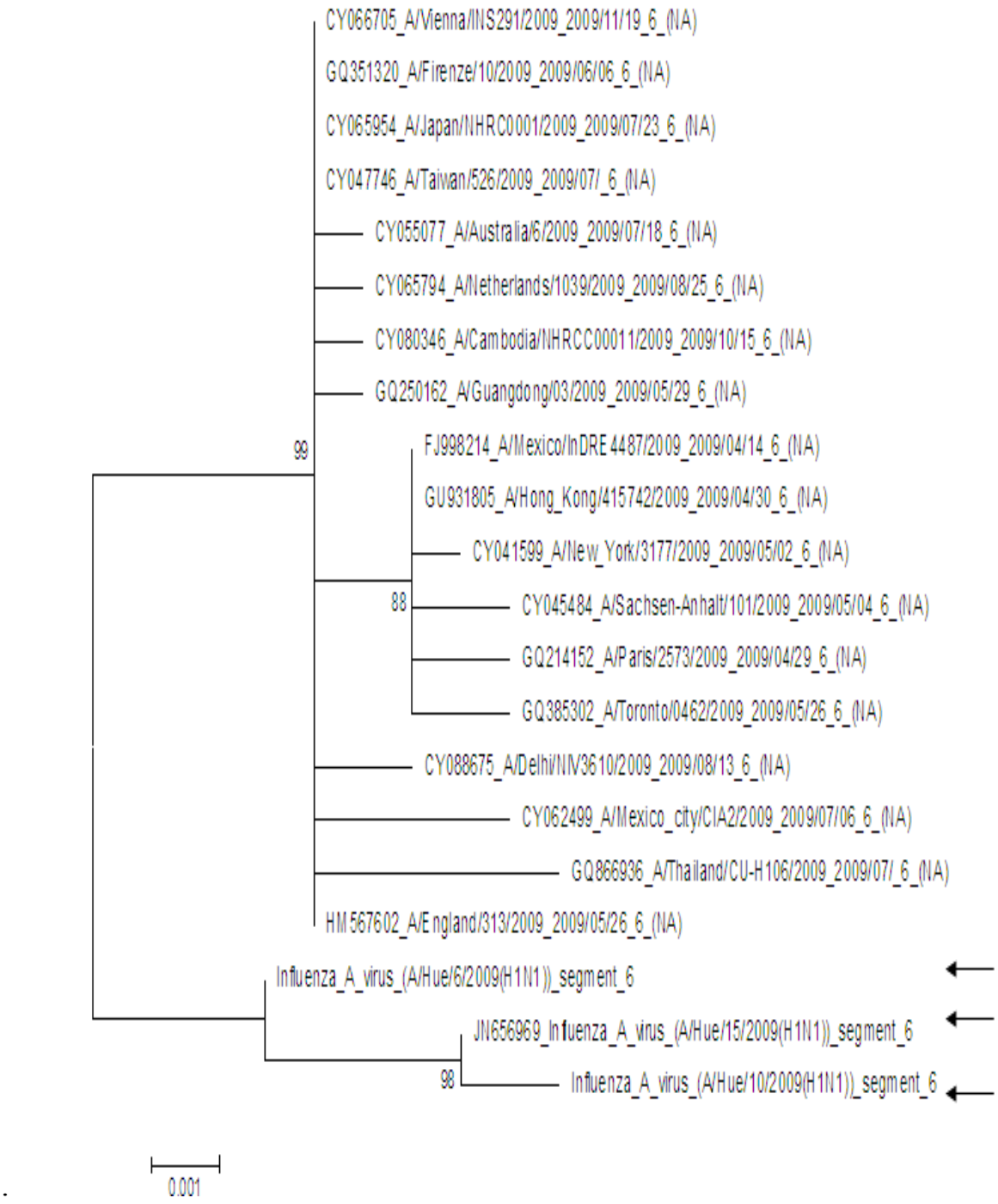


Figure 26

The phylogeny of NA genes of three influenza A H1N1(2009) viruses in Thua Thien Hue and 18 reference sequences by using Maximum Likelihood method, the length scale measured the number of substitutions per site.



4. DISCUSSION

4.1. Identification of viral etiology by polymerase chain reactions.

Our study carried out in 216 children hospitalized with clinical diagnosis of lower respiratory infections during 10 months from the middle of March of 2010 to the sixth of April, 2011, forty two percents (42%) of the patients with lower respiratory tract infections were confirmed to be infected by respiratory viruses, among them 86% were single infections and 16% were co-infected with more than one respiratory virus. Sixty nine percent (69%) of the infected children were from 3 months to 2 years of age and 91% were less than or 5 years of age.

Influenza A virus was the most common detected virus, and accounted for 45% of children with viral lower respiratory tract infections. RSV (42%) was the second common virus detected, adenovirus was in 12% and other viruses including influenza B virus and parainfluenza viruses 1 and 3 were detected at lower frequencies of 8%, 5%, and 5 %, respectively.

Our results on viral etiologies can be compared with other reports from recent studies on viral etiologies of lower respiratory tract infections in Asia (9, 112, 113, 137). Their findings indicated that viral agents was identified from 32.2% to 73.92% of acute lower respiratory tract infections and that RSV was the most frequently detected virus. Specifically, a study carried out by Tang et al in Hangzhou, China on 34,885 children with LRTI from January 2001 to December 2006 found viral pathogens in 11,297 patients (32.3%). RSV was found in 40.7%, followed by parainfluenza viruses 3 in 4.3%, influenza A virus in 2% and adenovirus in 1.7% (113). In another study by Zhang et al in Harbin, China, respiratory viruses were found in 63.1% (260/412) of patients hospitalized with lower respiratory tract infections and RSV was the most frequent detected virus in 25% of 260 children, followed by influenza A viruses in 14.4%, and PIV3 in 10.9%, flu B in 4.9% and adenovirus in 4.1%(137). A study from India by Bharaj et al using PCR to detected viral etiologies in children hospitalized with lower respiratory tract infections from 2005 to 2007 in Delhi also indicated that respiratory viruses were detected in 35.2% of LRTI and RSV was the prominent virus in 61 of 130 positive cases, the second detected virus was parainfluenza virus 3 in 22, parainfluenza virus 2 in 17, hMPV in 11, parainfluenza virus 1 in 10 and influenza A in 9 cases (9). A study of multiple centers in Hokkaido, in the north of Japan on etiologies of lower respiratory tract infection from 2000 to 2001 on 921 patients with pneumonia and bronchitis showed that among viral etiologies, RSV, influenza A virus and adenovirus were the most common virus detected in 20.4%, 11.9% and 2.9% of all the causative pathogens respectively (81).

As reported in the introduction, recent studies in southern city of Viet Nam on viral etiology of acute respiratory tract infections indicated that RSV and influenza viruses were dominant causes of acute respiratory infections in

young children hospitalized with respiratory infections (4, 136). The results from our study also provide further information on viral etiologies of lower respiratory tract infections in children population, especially in the central region of Viet Nam. Influenza A virus was prominent in our study may be due to the local epidemiological situation during the study time, where a mild outbreak of influenza A virus infection possibly occurred in rainy season from November of 2010 to March in 2011(137), and the trend of increasing number of influenza virus infections was shown in the seasonal distribution of identified viruses in Figure 20.

In some of the above-mentioned studies, a wide spectrum of respiratory viruses was included in their testing assays and some novel described viruses were found with high frequencies in lower respiratory infections such as hMPV, HBoV, hCoV-NL63, hCoV-HKU1 (9, 48). These respiratory viruses were not included in our panel of viruses for detection because this study was in starting years of establishment of molecular techniques for detection of respiratory viruses, setting-up of more molecular techniques for the novel respiratory viruses is necessary in the coming time.

Our results showed that 16% of LRTI were co-infection of more than one respiratory virus and RSV was the most commonly found in co-infection with 80% (12/15), and influenza A virus in 60% (9/15), adenovirus in 27% (4/15). In other recent reports, the rate of co-infection in Asian countries varied from very low number with 27 co-infections among 11270 specimens positive for respiratory viruses to a high frequency of 46.59% (4, 48, 113). Specifically, in a study in which novel viruses were not included, co-infection with RSV was very high at 85.2% (23/27), followed by parainfluenza virus 3 in 33.3% (9/27) and influenza A virus in 29.6% (8/27) (113). Whereas, in a recent study (48), in which, novel respiratory viruses were included, HBoV and influenza A virus were present in co-infection at high rate (79.8% and 75.86 respectively). In another recent study co-infection was found in 23.5% of 34 children with identified viruses and hMPV showed high rate of co-infection at 69% and RSV in 30% (112). All studies show a great variability of the co-infection rate probably due both to the methodology used and the sample characteristic and our study is in agreement with this behavior.

In our results, pneumonia was the most frequent diagnosis of lower respiratory infections in hospitalized children with 46%, followed by bronchitis in 32% and bronchiolitis in 22%. In pneumonia RSV and influenza A virus were commonly detected, followed by adenovirus, PIV3, influenza B virus and PIV1. In bronchiolitis and bronchitis influenza A virus was the most common viral agent, followed in descending order of frequencies by RSV, influenza B virus and adenovirus and PIV1. The distribution of lower respiratory infections and viral etiologies in our study were similar to the recent reports (9, 48, 87). Especially, in the report by Zhang et al in Harbin China, among 260 virus positive samples, pneumonia was diagnosed in 53.1% and RSV, parainfluenza viruses 1, 3 were commonly detected in children less than 6 months and in those

2-4 years of age; bronchiolitis was found in 25.8% and RSV was the most frequent cause; and tracheobronchitis in 21.2% and influenza A virus was associated with that infection (137).

Our results showed co-infections in 24% of patients with pneumonia, while only 3% patients with bronchitis had co-infections. Our dominant co-infection was influenza A virus and RSV in pneumonia (in 5 patients) also in bronchiolitis (in 3 patients), this suggest that co-infections in our study may be associated with the epidemic situation of influenza A virus infection in the region as mentioned above and higher co-infection in lower respiratory infections may be a cause for hospitalization of children younger than 1 years of age (8 children with co-infections among 42 patients in this age group). Severity of respiratory infections is increased by co-infection of multiple viral pathogens as reported in certain recent studies (94, 101). However, several studies found no connection between co-infection of viral pathogens with severity of respiratory infections (18, 33, 48, 50). Additional studies are needed to understand better the role of co-infection in severity of respiratory infections.

The interruption of patient availability from June to October in 2010 led to the lack of data showing the distribution of respiratory viruses during these months of the year. However, during the remaining months, our results showed lower respiratory tract infections due to RSV and influenza A virus occurred throughout several months of the year. Several cases of RSV infections were found in April and May, suggesting that RSV infection occurs also in hot and high humidity season. A recent study on ARI in Ho Chi Minh city showed that RSV infection occurred during rainy season from May to October (4); however, the climate in the southern region is rather different from that of the northern part of the central region with raining season from September to the end of March (115). Additional study for a longer period of time lasting 3 to 4 years is needed to define a specifically seasonal pattern of RSV infections in this region.
Influenza A virus infections occurred also in several months of the year; however, the majority of influenza A infections were detected during the winter time from November and up to the peak in February. Seven influenza B virus infections were found only during winter time from December to February. Occurrence of influenza virus infections in our study was also in agreement with Vietnam national data on influenza epidemic in 2010 to August 2011 reported by WHO, in which the number of positive specimens for influenza A virus was increasing from the 46th week (November) of 2010, approached the peak in the 9th week of 2011, and the positive number was descending at a minimun in the end of April (the week 15-16) of 2011 (129). Our results also showed that the subtype (H1N1) 2009 of influenza A virus was currently responsible for majority of human infections (76% of total influenza A virus) in the central region, the previously subtypes causing seasonal influenza epidemic in humans including H1N1 and H3N2 were detected in low rate 17% and 7% respectively. Our finding is consistent with the recent report on Influenza Laboratory Surveillance Information from WHO, subtype (H1N1) 2009 has prevalently

circulated in many countries during the last winter all over the world, subtype (H1N1)2009 was the most frequent identified among all the subtypes of influenza A virus and influenza B virus (4, 112, 129).

4. 2. A study for isolation and identification of the novel influenza A (H1N1) 2009 virus in the pandemic 2009

This study was aimed at setting up the two virological methods of viral isolation in cell culture and amplification assay to detect the novel H1N1 swine-origin influenza virus during the flu pandemic 2009 in Thua Thien Hue province. Viral isolation of influenza virus on MDCK was set up and implemented on 41 specimens from patients with flu symptoms, and yielded positive result in 24 specimens (58.5%), RT-PCR was used in the same time to detected influenza virus directly from the same specimens and produced 27 positive (65.9%). A total of 12 other specimens were collected in the end of 2009 pandemic in the province and were transferred to our laboratory irregularly, that explained why RT-PCR was only used for those specimens, among which 3 positive specimens were detected.

Isolation of influenza virus on culture cells is considered a traditional and standard method for diagnosis of virus, this method is difficult to use in most clinical laboratories for diagnostic purposes, because it is time consuming, and of low sensitivity in comparison to other rapid assays. Furthermore it requires that laboratory has facilities for cell culture, and well trained staffs in cell culture (24, 69, 111). However isolation is still needed in reference and research laboratories, since it is necessary for standardizing other utilized assays, and confirmation of the positive isolates for reference purpose; isolation of virus on cell cultures also produces a large amount of virus for biological and genetic characterization of virus and vaccine development (24, 40, 111).

Isolation of influenza virus on embryonated chicken eggs was classically employed for detection of influenza virus from clinical samples; this isolation method was found to be equal in sensitivity to cell cultures for detection of influenza A virus H3N2 (21). In developing countries, the embryonated eggs can be easy to be available from the local suppliers; it is especially useful for production of influenza vaccine because inoculation of influenza virus into embryonated eggs yields higher viral titres (24, 130). However, virus isolation in embryonated chicken eggs is labour intensive and time consuming and has a very low sensitivity for detection of influenza B virus (21, 76).

In our study, embryonated chicken eggs were employed as an additional isolation method with the specimens positive on MDCK cells isolation and positive results were confirmed. Isolation of influenza virus on embryonated chicken eggs from clinical specimens will be implemented in our laboratory in the coming months.

Although isolation of influenza virus on cell culture was a new technique for our laboratory, it showed positive results in 58.5% of the samples and, if compared with RT-PCR (65.9%), has no significant difference ($p=0.45$) using

McNemar's test. Our results were similar to the previous report of Lam et al. when they compared their RT-PCR results with those of viral isolation and immunofluorescent assay. From 303 clinical samples, influenza A virus was isolated in 15 (5%) specimens and multiplex RT-PCR yielded positive in 19 (6.3%) specimens and the difference was not significant (61). In another study by Percivalle et al during the winter-spring season 2006-2007, 38 influenza virus strains were detected in respiratory specimens from patients hospitalized in a pediatric department in Pavia (Italy). A total of 35 (80.4%) were detected and typed by RT-PCR, 34 (80.1%) by direct immunofluorescent antibody test and 27 (71%) by conventional virus isolation (89). In our results, multiplex RT-PCR was able to detect more cases (27 or 65.9%) than viral isolation did (24 or 58.5%), in agreements with the results of the above mentioned studies. Several studies indicated that sensitivity of PCR is higher than the conventional methods including viral isolation and antigenic detection assays (69, 61, 89).

Multiplex RT-PCR was very useful in detection of influenza virus because it can identify type and subtypes and also give result from 8 to 12 hours after receiving specimens (128). In this study we used the multiplex RT-PCR with primers recommended from WHO for identification of swine origin influenza A virus. This standard protocol was set up for use not only in our study but also for training of laboratory staffs working in clinical microbiological laboratories in the central region for diagnosis of influenza A (H1N1) 2009 virus during the pandemic 2009 as recommended by the Vietnamese national reference centre.

4. 3. Molecular epidemiology of influenza A (H1N1) 2009 viruses isolated from Thua Thien Hue

The term of molecular epidemiology was firstly used by Kilbourne in 1973 for describing epidemiology of influenza virus infection and, since that time, the analysis of gene sequence variation, with the advent of sophisticated molecular techniques, especially high-throughput sequencing technologies, has become a standard practice for molecular epidemiology (77). Molecular methods have produced an enormous amount of data on molecular typing (subtyping) of specific viruses and datasets of complete genome sequences of viral isolates. The integration of epidemiological analysis with phylogeny and genealogy data can provide valuable information on the evolution of viral pathogens (36 ,42), on the origin of new viruses and the source of outbreaks of infection (42, 77, 107), on pattern of transmission and spread, and on the evolutionary genetics of viral emergence and virulence (72, 77).

Influenza viruses can rapidly acquire genetic diversity because of high replication rates in infected hosts, the presence of an error-prone RNA polymerase (which introduces mutations during genome replication), and segment reassortment. The rates of change are higher in HA and NA genes than the other internal genes. In particular, the accumulation of mutations of HA gene can result in changes in amino acid residues on the viral surface glycoproteins or

hemagglutinin, especially in regions recognized by antibodies. These changes can reduce the protective effect of antibodies produced previously against the currently circulating viral subtype. The segment reassortment can result in major changes in antigenicity (antigenic shift) that can lead to emergence of a novel virus, which has a hemagglutinin segment of influenza A virus from another host species, and can be transmitted efficiently among humans (72). Change in the genetic structure of NA gene may result in reduced sensitivity or resistance to antiviral drugs (135).

The WHO Global Influenza Surveillance Network (GISN) was established in 1952 to monitor for novel emerging strains, the global surveillance programme is still operating and a panel of experts meets twice a year to review antigenic, genetic and epidemiological data on the evolution of influenza viruses and provides recommendations in areas including laboratory diagnostic, vaccines, antiviral susceptibility and risk assessment (72, 132, 135).
The pandemic influenza A (H1N1) 2009 was firstly reported in spring 2009 in Mexico and the US and the virus spread rapidly to many countries and regions of the world (33, 79). This emerging influenza A virus (H1N1)`2009 subtype is a new reassortment of six gene segments from the known triple reassortant swine virus, and two gene segments (NA and matrix protein) from the Eurasian influenza A (H1N1) swine virus lineage (139). In Viet Nam the pandemic influenza A H1N1 virus infection was firstly reported on 30th of May 2009 in a student returning from the US, and in a short period of time from May to the middle of July, 309 confirmed cases of influenza A (H1N1) 2009 infection were reported. Most of the cases (87%) were imported to Viet Nam through airline passengers arriving in Ho Chi Minh City (81) and infected people were coming from Australia, the United State, Thailand, Singapore, Germany, Hong Kong where the pandemic influenza A H1N1 infection was in active phase. So far there has not been any genetic sequence of the influenza A (H1N1) 2009 virus isolated in Vietnam available in the influenza virus resource database for sequence comparison. The representative isolates of influenza A (H1N1) 2009 virus causing influenza infection in Thua Thien Hue were highly similar in HA gene sequences and they cluster together in a phylogenetic tree. Phylogenetic tree pattern of HA showed that our influenza A virus isolates were closely related to the HA sequences of worldwide circulating isolates, and especially to Influenza A/Japan/NHRC0001/2009(H1N1) followed by Influenza A/Cambodia/NHRCC00011/2009(H1N1 and Influenza A/Firenze/10/2009(H1N1). Comparison of the NA genetic sequences among 3 representative isolates in Thua Thien Hue also shows high similarities (from 99.57% to 99.86%) and clustering together in phylogenetic tree. In the phylogenetic pattern comparison of our NA sequences with 18 reference worldwide isolates of influenza A (H1N1) 2009 virus, the isolates in Thua Thien Hue are splitted out in a separated cluster althought they are highly similar to the referenced isolates. In particular, the representative isolates in Thua Thien Hue are more similar to the following isolates: A/Vienna/INS291/2009(H1N1, A/Japan/NHRC0001/2009 (H1N1),

A/Firenze/10/2009(H1N1), A/Taiwan/526/2009(H1N1) and A/ England/313/2009(H1N1). Point mutation in gene can frequently occur resulting in increased diversity of the pandemic isolates of influenza A (H1N1) 2009 virus; strains isolated in the end of the epidemic are often more diversified from the initial isolates due to several passages and replications in human hosts. The outbreak in Vietnam was starting at the end of May, 2009; unfortunately we were not able, up to now, to obtain the isolates of the initial cases of the outbreak in the province of South Vietnam for further analysis and comparison. Hopefully, this could be done in the future if the national collection of influenza A (H1N1) 2009 virus isolated from the provinces will be available for analysis.

CONCLUSIONS

Respiratory viruses were common in lower respiratory infections and most of viral lower respiratory infections were in children from 3 months to 5 years of age. Viral etiologies including Influenza A virus and RSV, adenovirus, influenza B virus and parainfluenza virus 1 and 3 were identified in hospitalized children with clinically diagnosed lower respiratory infections. Influenza A (H1N1) 2009 subtype were mainly responsible for most of influenza virus infections, and found highly in raining season from November to February. Viral isolation and polymerase chain reaction are useful and sensitive for identification of influenza A (H1N1) 2009 virus in 2009 pandemic. Characterization and comparison of HA and NA sequences among Hue representative isolates of influenza A (H1N1) 2009 was done and showed their high similarities; the representative isolates are also highly similar to reference isolates worldwide and cluster together in phylogenetic trees based on HA and NA sequences.

REFERENCES

1. Abravaya K, Hu HY, Khalil O (1997) Strategies to Avoid Amplicon Contamination. In Lee H, Morse S and Olsvik O, editors. Nucleci Acid Amplification Technologies: Application to Disease Diagnosis. Boston: Eaton Publishing. 125-133
2. Alexander DJ (1982) Ecology aspects of influenza A viruses in animals and their relationship to human influenza: a review. J. Roy Soc. Med. 75: 799-809.
3. Allander T (2008) Human Bocavirus. J. Clin. Virol. 41: 29–33.
4. Anh Ha LD, H. van Doorn HR, Ngoc MN, Juliet E. Bryant, Thanh Hang TH, Ha DQ, Tan LV, Tan TT, Wills B, Chau VVN, Hien MV, Khanh CV, Dung NM, Farrar J, Hien TT, de Jong MD (2011) Viral Etiologies of Acute Respiratory Infections among Hospitalized Vietnamese Children in Ho Chi Minh City, 2004–2008, PLoS ONE. 6(3): e18176.
5. Australian Department of Health and Aging (2011), Australian Influenza Surveillance Report. No. 10, 2011, Reporting Period: 23 July to 5 August 2011.
6. Bellau-Pujol S, Vabret A, Legrand L, Dina J, Gouarin S, Petitjean-Lecherbonnier J, Pozzetto B, Ginevra C, Freymuth F (2005). Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses, J. Virol. Methods 126: 53–63
7. Belser JA, Maines TR, Tumpey TM, and Katz JM (2010) Influenza A virus transmission: contributing factors and clinical implications, expert reviews in molecular medicine. 12. e39:
8. Berger A, Drosten Ch, Doerr HW, Stürmer M, Preiser W (2004) Severe acute respiratory syndrome (SARS)—paradigm of an emerging viral infection. J. Clin. Virol. 29: 13–22.
9. Bharaj P, Sullender WM, Kabra SK, Mani K, Cherian J, Tyagi V, Chahar HS, Kaushik S, Dar L and Broor S (2009) Respiratory viral infections detected by multiplex PCR among pediatric patients with lower respiratory tract infections seen at an urban hospital in Delhi from 2005 to 2007. Virol J. 6: 89 available: http://www.virologyj.com/content/6/1/89.
10. Boonsuk P, Payungporn S, Cheochansin T, Samransamruajkit R, Amonsin A, Songserm T, Chaisingh A, Chamnanpood P, Cgitinimitkul S, Theamboonlers A, Poovorawan Y (2008) Detection of influenza virus types A and and type A subtypes (H1, H3, H3 and H5) by multiplex polymerase chain reation. Tohoku J. Exp. Med. 215: 247-255.
11. Boyer KM, and Cherry JD (1981). Nonbacterial pneumonia. In Feigin & Cherry, editors. Textbook of Pediatric infectious diseases. Philadelphia: W.B Saunders Company. 186-196.
12. CDC. Weekly influenza surveillance report, seasonal influenza 2010-2011

influenza season week 32 ending August 13 2011- US. Virologic surveillance. Availabe http://www.cdc.gov/flu/weekly/weeklyarchives 2010-2011/data/whoAllregt31.htm.
13. Centro Nazionale OMS per la Sorvrglianza dell' influenza (NIC) (2007). Protocolli operativi per la diagnostica di laboratorio dell'influenza, Dipartimento di malattie infettive, parassitarie e immunomediate, Istituto Superiore di Sanità, Roma.
14. Chan CH, Lin KL, Chan Y, Wang YL, Chi YT, Tu HL, Shieh HK, Liu WT (2006) Amplification of the entire genome of influenza A virus H1N1 and H3N2 Subtypes by Reverse-Transcription Polymerase Chain Reaction. J. Virol. Methods 136: 38–43
15. Cheng VC, Lau SP, Woo PY, and Yuen KY (2007) Severe Acute Respiratory Syndrome Coronavirus as an Agent of Emerging and Reemerging Infection. Clin. Microbiol. Rev. 20: 660–694.
16. Cherry JD (1981). Acute Bronchitis. In Feigin & Cherry, editors, Textbook of Pediatric Infectious Diseases. Philadelphia: W.B Saunders Company.1: 174-177.
17. Cherry JD (1981) Bronchiolitis and Asthmatic Bronchitis. In Feigin & Cherry, editors. Textbook of Pediatric infectious diseases. Philadelphia: W.B Saunders Company. 1: 178-186.
18. Choi EH, Lee HJ, Kim SJ, Eun BW, Kim NH, Lee JA, Lee JH, Song EK, Kim SH, Park JY, Sung JY (2006) The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000–2005. Clin. Infect. Dis. 43: 585-92.
19. Collins PL and Crowe JE (2007) Respiratory Syncytial Virus and Metapneumovirus. In Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: LippincottWilliams & Wilkins. 1601- 1646
20. Committee of the World Health Organization Consultation on Human Influenza A/H5. (2005) Avian Influenza A (H5N1) Infection in Humans. N Engl J Med 353: 1374-85.
21. Couch RB, Kasel JA (1995) Influenza, In Lennette EH, Lennette DA and Lennette ET, editors. Diagnostic procedures for Viral, Rickettsial and Chlamydia infections. Washington: American Public health Association. 431-446.
22. Denny FW (1999) Acute Viral Lower Respiratory Infection in Children. In Dolin R and Wright PF, editors. Viral Infections of the Respiratory Tract, New York: Marcel Derker. 3-23.
23. Drazen JM (2003) SARS — Looking Back over the First 100 Days, N Engl J Med. 349: 319-20
24. Dwyer DE, Smith DW, Catton MG and Barr IG (2006) Laboratory diagnosis of human seasonal and pandemic influenza virus infection. MJA 185: S48–S53.
25. Easton AJ, Domachowske JB and Rosenberg HF (2004) Animal Pneumoviruses: Molecular Genetics and Pathogenesis. Clin. Microbiol.

Rev. 17: 390–412
26. Echavarrıa M (2008) Adenoviruses in Immunocompromised Hosts, Clin. Microbiol. Rev. 21: 704-15.
27. Edward B & Jenkins C (2007) Acute Respiratory Infection Syndromes. Respiratory Infectious Disease Burden in Australia, 1st edition, Brisbane, the Australian Lung Foundation: 11-41.
28. Ellis J, Smith J, Braham S, Lock M, Barlow K, and Zambon M (2007) Design and Validation of an H5 TaqMan Real-Time One-Step Reverse Transcription-PCR and Confirmatory Assays for Diagnosis and verification of Influenza A Virus H5 Infections in Humans, J. Clin. Microbiol. 45: 1535–1543.
29. Elnifro EM, Ashshi AM, Cooper RJ, and Klapper PE (2000) Multiplex PCR: Optimization and Application in Diagnostic Virology, Clin. Microbiol. Rev. 13: 559–570
30. Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JDC, Wengenack NL, Rosenblatt JE, Cockerill FR, and Smith TF (2006) Real-Time PCR in Clinical Microbiology: Applications for Routine Laboratory Testing, Clin. Microbiol. Rev. 19(1): 165-256.
31. Fouchier RM, Rimmelzwaan GF, Kuiken T and Osterhaus ADE (2005) Newer Respiratory Virus Infections: Human Metapneumovirus, Avian Influenza Virus, and Human Coronaviruses. Curr .Opin. Infect. Dis 18: 141–146.
32. Franca LTC, Carrilho E and Kist TBL (2002) A Review of DNA Sequencing Techniques. Q. Rev. Biophys. 35: 169–200.
33. Fujitsuka A, Tsukagoshi H, Arakawa M, Goto-Sugai K, Ryo A, Okayama Y, Mizuta K, Nishina A, Yoshizumi M, Kaburagi Y, Noda M, Tashiro M, Okabe N, Mori M, Yokota S, and Kimura H (2011) A Molecular Epidemiological Study of Respiratory Viruses Detected in Japanese Children with Acute Wheezing Illness. BMC Infect. Dis. 11:168.
34. Garten RJ. et al (2009) Influenza Viruses Circulating in Humans Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H1N1). Science 325: 197-201.
35. Guertler L, Virology of Human Influenza, in Kamps BS, Hoffmann C, Preiser W, editors. Influenza Report 2006. Paris. Flying Publisher. 87-91.
36. Hall BG and Barlow M (2006) Phylogenetic Analysis as a Tool in Molecular Epidemiology of Infectious Diseases. Ann Epidemiol. 16: 157–169.
37. He J, Bose ME, Beck ET, Fan J, Tiwari S, Metallo J, Jurgens LA, Kehl SC, Ledeboer N, Kumar S, Weisburg W, and Henrickson KJ (2009) Rapid Multiplex Reverse Transcription-PCR Typing of Influenza A and B Virus, and Subtyping of Influenza A Virus into H1, 2, 3, 5, 7, 9, N1 (Human), N1 (Animal), N2, and N7, Including Typing of Novel Swine Origin Influenza A (H1N1) Virus, during the 2009 Outbreak in Milwaukee, Wisconsin. J. Clin, Microbiol. 47: 2772–2778.

38. Henrickson KJ, Human parainfluenza viruses, In Lennette EH, Lennette DA and Lennette ET, editors. Diagnostic procedures for Viral, Rickettsial and Chlamydia infections. Washington: American Public health Association. 481-494.
39. Henrickson KJ (2003) Parainfluenza Viruses. Clin Microbiol. Rev. 16: 242–264
40. Henrickson KJ, Hoover S, Kehl KS, and Hua WM (2004) National Disease Burden of Respiratory Viruses Detected in Children by Polymerase Chain Reaction. Pediatr. Infect Dis. J. 23: S11–18.
41. Hierholzer JC (1995) Adenoviruses. In Lennette EH, Lennette DA and Lennette ET, editors. Diagnostic procedures for Viral, Rickettsial and Chlamydia infections. Washington: American Public health Association. 169-188.
42. Holmes EC (1998) Molecular epidemiology and evolution of emerging infectious diseases. Brit. Med. Bull. 54: 533-543.
43. Holmes KV (2003) SARS-Associated Coronavirus. N Engl J Med. 348: 1948-1952.
44. Hong JY, Lee HJ, Piedra PA, Choi EH, Park KH, Koh YY, Kim WS (2001) Lower Respiratory Tract Infections due to Adenovirus in Hospitalized Korean Children: Epidemiology, Clinical Features, and Prognosis. Clin. Infect. Dis. 32: 1423-9.
45. Huber I, Campe H, Sebah D, Hartberger C, Konrad R, Bayer M, Busch U, Sing A (2011) A Multiplex One-Step Real-Time RT-PCR Assay for Influenza Surveillance, Euro Surveill. Feb17:16(7).
46. Influenza virus resource. Available: http://www.ncbi.nlm.nih.gov/genomes/FLU/Database.
47. Jamrozik E, Musk A.W (2011) Respiratory Health Issues in The Asia-Pacific Region: an overview. Respirology 16: 3–12.
48. Jin Y, Zhang RF, Xie ZP, Yan KL, Gao HC, Song JR, Yuan XH, Cheng WX, Hou YD, Duan ZJ (2011) Newly identified respiratory viruses associated with acute lower respiratory tract infections in children in Lanzou, China, from 2006 to 2009. Clin Microbiol Infect. 10.1111/j.1469-0691.
49. Kahn JS (2006) Epidemiology of Human Metapneumovirus. Clin. Microbiol. Rev. 19: 546–557.
50. Kaplan NM, Dove W, Abd-Eldayem SA, Abu-Zeid AF, Shamoon HE, and Hart CA (2008) Molecular Epidemiology and Disease Severity of Respiratory Syncytial Virus in Relation to Other Potential Pathogens in Children Hospitalized With Acute Respiratory Infection in Jordan. J. Med. Virol. 80: 168–174.
51. Karron RA, Collins PL (2007) Parainfluenza Viruses. In Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: Lippincott Williams & Wilkins. 1497-1526.
52. Khanna M, Gupta N, Gupta A, and Vijayan VK (2009) Influenza A

(H1N1) 2009: A Pandemic Alarm. J. Biosci. 34: 481–489.
53. Kidd KK, Ruano G (1995) Optimizing PCR, in McPherson MJ, Hames BD, and Taylor GR, editors, PCR2: A Practical Approach, Oxford. Oxford University Press. 1- 22
54. Kim C, Ahmed JA, Eidex RB, Nyoka R, Waiboci LW, Erdman D, Tepo A, Mahamud AS, Kabura W, Nguhi M, Muthoka P, Burton W, Breiman RF, Njenga MK, Katz MA (2011) Comparison of Nasopharyngeal and Oropharyngeal Swabs for the Diagnosis of Eight Respiratory Viruses by Real-Time Reverse Transcription-PCR Assays. PLoS One.6(6): e21610.
55. Korteweg C and Jiang Gu (2008) Pathology, Molecular Biology, and Pathogenesis of Avian Influenza A (H5N1) Infection in Humans. Am. J. Pathol 172:1155–1170
56. Ksiazek TG, Cynthia S, Goldsmith M S, et al (2003) A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome, N Engl J Med 348: 1947-1958.
57. Kubista M, Andrade J, Bengtsson M, Forootan A, Jona J, Lind K, Sindelka R, Sjoback R, Sjogreen B, Strombom L, Stahlberg A, Zoric N (2006) The real-time polymerase chain reaction, Mol. Asp. Med. 27: 95–125.
58. Kuiken T, Holmes EC, McCauley J, Rimmelzwaan GF, Williams CS, Grenfell BT (2006) Host Species Barriers to Influenza Virus Infections, Science. 312: 394-397.
59. Lai MC, Perlman S, Anderson LJ (2007) Coronaviridae. In Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: Lippincott Williams & Wilkins. 1305-1335.
60. Lam TTY, Hon CC, and Tang JW (2010) Use of Phylogenetics in the Molecular Epidemiology and Evolutionary Studies of Viral Infections, Crit. Rev. Clin. Lab. Sci. 47: 5–49.
61. Lam WY, Yeung ACM, Tang JW, Ip M, Chan EWC, Hui M and Chan PKS (2007) Rapid Multiplex Nested PCR for Detection of Respiratory Viruses, J. Clin Microbiol. 45: 3631–3640
62. Lanata Cl, Rudan I, Boschi-Pinto, Tosmaskovic L, Cherian T, Weber M, and Campbell H (2004) Methodological and Quality Issues in Epidemiological Studies of Acute Lower Respiratory Infections in Children in Developing Countries, Int. J. epidemiol. 33: 1362-1372.
63. Lee M, Squirrell D, Leslie D and Brown T (2009) Homogeneous Fluorescent Chemistries for Real-Time PCR, in Logan J, Edwards K, and Saunders N, editors. Real-Time PCR: Current Technology and Applications, London: Caister Academic Press. 23-45.
64. Leland D.S and Ginocchio CC (2007) Role of Cell Culture for Virus Detection in the Age of Technology, Clin. Microbiol. Rev. 20: 49–78.
65. Logan J and Edwards K (2009) An Overview of PCR Platforms, in Logan J, Edwards K, and Saunders N, editors. Real-Time PCR: Current Technology and Applications, London: Caister Academic Press. 8-22.

66. Lowen AC, Mubareka S, Steel J and Palese1 P (2007) Influenza Virus Transmission is Dependent on Relative Humidity and Temperature. PLoS Pathogens 3 (10): e151.
67. Mackay IM, Arden KE and Nitsche A (2002) Real-time PCR in virology. Nucleic Acids Res. 30: 1292–1305.
68. Mackay IM (2008) Human Rhinoviruses: The cold Wars Resume. J. Clin. Virol. 42: 297–320
69. Mahony JB (2008) Detection of Respiratory Viruses by Molecular Methods, Clin Microbiol Rev. 21: 716-47.
70. Martin GP and Timmers E (1997) PCR and Its Modifications for the Detection of Infectious Diseases. In Lee H, Morse S and Olsvik O, editors. Nucleci Acid Amplification Technologies: Application to Disease Diagnosis. Boston: Eaton Publishing. 79-99.
71. McDonough M, Kew O, Hierholzer J (1993) PCR detection of human adenoviruses. In: Persing DH, Smith TF, Tenover FC, White TJ, editors. Diagnostic molecular microbiology: principles and applications. Washingtion DC: American Society for Microbiology. 389-93.
72. McHardy AC, Adams B (2009) The Role of Genomics in Tracking the Evolution of Influenza A Virus. PLoS Pathog 5(10): e1000566.
73. Menegus MA (1999) Laboratory Diagnosis of Respiratory Infection with Respiratory Viruses, in Dolin R and Wright P.F, editors. Viral Infections of the Respiratory Tract, New York: Marcel Derker. 307-325.
74. Mentel R, Wegner U, Bruns R and Guertler L (2003) Real-time PCR to Improve the Diagnosis of Respiratory Syncytial Virus Infection. J. Med. Microbiol. 52: 893–896.
75. Metzker M (2010) Sequencing technologies — the next generation. Nature Rev. Genet 11: 31-45.
76. Monto AS, Maassab HF nd Bryan ER (1981) Relative Efficacy of Embryonated Eggs and Cell Culture for Isolation of Contemporary Influenza Viruses. J. Clin. Microbiol. 13: 233-235
77. Moya A, Holmes EC and González-Candelas F (2004) The Population Genetics and Evolutionary Epidemiology of RNA Viruses. Nat. Rev. Microbiol. 2: 279-288.
78. Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA (1998). Orthomyxoviruses, in Medical Microbiology, 3rd edition. Mosby. 472-478.
79. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team (2009) Emergence of a Novel Swine-Origin Influenza A (H1N1) Virus in Humans. N Engl J Med. 360: 2605-15.
80. Nei M (1996) Phylogenetic Analysis in Molecular Evolutonary Genetics. Annu. Rev. Genet. 30: 371- 403.
81. Nguyen Van Binh, Lan PT, Tinh PT, Nguyen TMN (2010) Description of the First Pandemic Influenza A (H1N1) Cases in Vietnam, June-July 2009. OSIR. 3(1): 10-13.

82. Numazaki K, Chiba S, Umetsu M, Tanaka T, Yoshimura H, Kuniya Y, Miura J, Adachi N, Ukae S, Mori T, Ueda D, Hotsubo T and Sato T (2004). Etiological Agents of Lower Respiratory Tract Infections in Japanese Children. In vivo 18: 67-72
83. Ohara H (2004) Experience and review of SARS control in Viet Nam and China, Trop. Med. Health 32: 235-240
84. Ojosnegros S, Beerenwinkel N (2010) Models of RNA Virus Evolution and Their Roles in Vaccine Design, Immunome Research. 6 (Suppl 2): S5
85. Palese P and Shaw ML (2007) Orthomyxoviridae: The Viruses and Their Replication, In. Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: Lippincott Williams & Wilkins. 1647- 1689
86. Palomino MA, Larranaga C, Villagra E, Camacho J and Avendano LF (2004) Adenovirus and respiratory syncytial virus-adenovirus mixed acute lower respiratory infections in Chilean infants. Pediatr Infect Dis J. 23(4):337-41.
87. Paranhos-Baccala G Komurian-Pradel F, Richard N, Vernet *G*, Lina B, Floret D (2008) Mixed respiratory virus infections, J. Clin. Virol. 43: 407-410
88. Pasick J (2008) Advances in the Molecular Based Techniques for the Diagnosis and Characterization of Avian Influenza Virus Infections, Transbound Emerg Dis. 55: 329–338.
89. Percivalle E, Rovida F, Piralla A, Rognoni V, Zavattoni M, Baldanti F, Marchi A, Gerna G (2008). Rapid typing, subtyping and RNA quantification of influenza virus type A strains in respiratory secretions. New Microbiol, 31: 319-327.
90. Powledge TM (2004) The polymerase Chain Reaction. Advan in Physiol Edu. 28: 44-50.
91. Pybus OG and Rambaut A (2009) Evolutionary analysis of the dynamics of viral infectious disease. Nat. Rev. Genet 10: 540-550.
92. Reed G, Jewett HP, Thompson J, Tollefson S, and Wright PT (1997) Epidemiology and Clinical Impact of Parainfluenza Virus Infections in Otherwise Healthy Infants and Young Children › 5 Years Old, The J. Infect. Diseases.175: 807–13.
93. Reid AH and Taubenberger JK (2003) The Origin of the 1918 Pandemic influenza virus: a continuing enigma, J. Gen. Virol. 84: 2285–2292.
94. Richard N, Komurian-Pradel F, Javouhey E, Perret M, Rajoharison A, Bagnaud A, Billaud G, Vernet G, Lina B, Floret D, Paranhos-Baccalà G (2008) The Impact of Dual Viral Infection in Infants Admitted to a Pediatric Intensive Care Unit Associated with Severe Bronchiolitis. Pediatr. Infect. Dis. J. 27: 213-7.
95. Rohwedder A, Keminer O, Forster J, Schneider K, Schneider E and Werchau H (1998). Detection of Respiratory Syncytial Virus RNA in Blood of Neonates by Polymerase Chain Reaction, J. Med. Virol. 54: 320–327.

96. Russell W.C (2009) Adenoviruses: Update on Structure and Function, J Gen Virol. 90: 1–20.
97. Sambrook J, Russell DW (2001) DNA sequencing, in Sambrook J and Russell DW, editors. Molecular Cloning: A Laboratory Manual, 3rded, Vol. 2. Cold Spring Harbor Laboratory Press, NY. 12.1–12.120.
98. Sails AD (2009) Applications in Clinical Microbiology. In Logan J, Edwards K, and Saunders N, editors. Real-Time PCR: Current Technology and Applications. London: Caister Academic Press. 178-219.
99. Schildgen O, Muller A, Allander T, Mackay IM, Voelz S, Kupfer B, and Simon A (2008) Human Bocavirus: Passenger or Pathogen in Acute Respiratory Tract Infections?. Clin. Microbiol. Rev. 21: 291–304.
100. Selvaraju SB, Selvarangan R (2010) Evaluation of Three Influenza A and B Real-Time Reverse Transcription-PCR Assays and a New 2009 H1N1 Assay for Detection of Influenza Viruses. J. Clin. Microbiol. 48: 3870-3875.
101. Semple MG, Cowell A, Dove W, Greensill J, McNamara PS, Halfhide C, Shears P, Smyth RL, Hart CA (2005) Dual infection in infants by humanmetapneumovirus and human respiratory syncytial virus is associated with severe bronchiolitis. J. Infect. Dis. 191: 382–386.
102. Simoes EF, Cherian T, Chow J, Shahid-Salles S, Laxminarayan R, and John TJ (2006) Acute Respiratory Infections in Children, In Jamison DT, Bremen JG, Measham AR, Alleyne G, Cleason M, Evans DB, Jha P, Mills A, Musgrove P, editors. Disease Control Priorities in Developing Countries. New York: Oxford University Press. 483-497.
103. Shaw MW, Arden NH, & Maassab HF (1992) New Aspects of Influenza Viruses, Clin. Microbiol. Rev. 5: 74-92.
104. Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ (1999) Bronchiolitis-Associated Hospitalizations Among US Children, 1980-1996. JAMA. 282: 1440-1446
105. Shendure JA, Porreca GJ, and Church GM (2008) Overview of DNA Sequencing Strategies, in Curr. Protoc. Mol. Biol. John Wiley & Sons. 81:7.1.1-7.1.11.
106. Smith GJD, Bahl J, Vijaykrishn D, Zhang J, Poona LLM, Chen H, Webster RG, Peiris JSM, and Guan Y (2009) Dating the emergence of pandemic influenza viruses, PNAS. 106: 11709–11712.
107. Snow M (2011) the contribution of molecular epidemiology to the understanding and control of viral diseases of salmonid aquaculture. Vet. Res. 42:56. Available: http://www.veterinaryresearch.org/content/42/1/56.
108. Somma M, Querci M (2006) Polymerase Chain Reaction (PCR), in Querci M, Jermini M, Van Den Eede G, editors, The Analysis of Food Samples for the Presence of Genetically modified organisms, WHO, Regional office for Europe, 6 (2-29). Available: http://mbg.jrc.ec .europa.eu/capacitybuilding/documentation.htm. Accessed 20 July 2011.
109. Staats CB, Webster RG, Webby RJ (2009) Diversity of Influenza Viruses

in Swine and the Emergence of a Novel Human Pandemic Influenza A. Influenza Other Respi Viruses. 3(5): 207–213.
110. Stockton J, Ellis JS, Saville M, Clewley JP and Zambon MC (1998) Multiplex PCR for Typing and Subtyping Influenza and Respiratory Syncytial Viruses, J. Clin. Microbiol. 36: 2990–2995.
111. Suarez DL, Das A, Ellis E(2007) Review of Rapid Molecular Diagnostic Tools for Avian Influenza Virus, Avian Diseases. 51: 201–208.
112. Sung CC, Chi H, Chiu NC, Huang DTN, Weng LC, Wang NY, Huang FY (2011) Viral etiology of acute lower respiratory tract infections in hospitalized young children in Northern Taiwan. J. Microbiol. Immunol. Infect. 44: 184-190.
113. Tang LF, Wang TL, Tang HF, Chen ZM (2008) Viral pathogens of Acute Lower Respiratory Infection in China, Indian Pediatrics. 45: 971-975.
114. Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE, Fanning TG (1997). Initial Genetic Characterization of the 1918 "Spanish" Influenza Virus. Science 275: 1793-1796
115. Thua Thien Hue. The Climate in Thua Thien Hue. Available at http://www3.thuathienhue.gov.vn/GeographyBook/.
116. Treanor JJ (1999). Influenza A and B viruses in Viral Infections of the Respiratory Tract, in Dolin R, and Wright PF editors. Viral Infections of the Respiratory Tract. Marcel Dekker. NY. 105-160.
117. Tregoning JS and Schwarze J (2010) Respiratory Viral Infections in Infants: Causes, Clinical Symptoms, Virology, and Immunology, Clin. Microbiol. Rev. 23: 74–98
118. Tsai HP, Kuo PH, Liu CC and Wang JR (2001). Respiratory Viral Infections among Pediatric Inpatients and Outpatients in Taiwan from 1997 to 1999. J. Clin. Microbiol. 39: 111–118.
119. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol. (10):2731-9
120. Turner RB, Couch RB (2007) Rhinoviruses. In Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: Lippincott Williams & Wilkins. 895-909.
121. Watzinger F, Ebner K, Lion T (2006) Detection and Monitoring of Virus Infections by real-time PCR. Mol. Asp. Med. 27: 254–298.
122. Webby RJ and Webster RG (2001) Emergence of influenza A viruses, Philos. Trans. R. Soc. Lond. B. Biol. Sci. 356: 1817- 28.
123. Webster RG, Bean WJ, Gorman OT, Chambers TM, and Kawaoka Y (1992) Evolution and Ecology of Influenza A Viruses. Clin. Microbiol. Rev. 56: 152-179.
124. Weissenbacher M.C and Ávila MM (1999) Viruses as The Cause of Upper and Lower ARI in Children: General Characteristics and Diagnosis, in Respiratory Infections in Children edited by Benguigui et

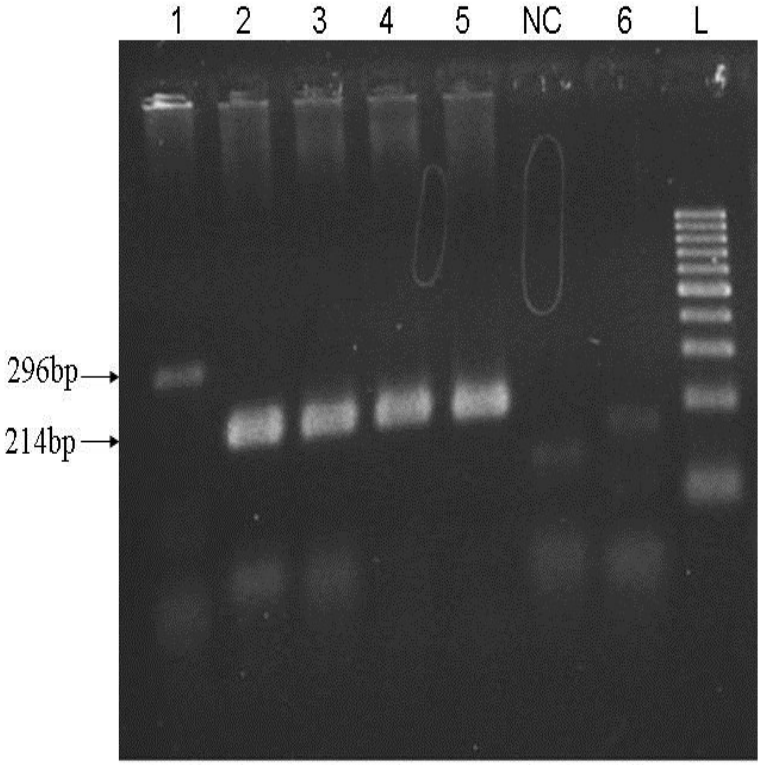
others, Pan American Health Organization, WHO offcice in Washington D.C: 87-103.
125. Wenzel JJ, Panning M, Kaul KL, Mangold KA, Revell PA, Luna RA, Zepeda H, Perea L, Vazquez-Perez JA, Young S, Rodic-Polic B, Eickmann M, Drosten C, Jilg W, Reischl U (2010). Analytical Performance Determination and Clinical Validation of the novel Roche Real-time Ready Influenza A/H1N1 Detection Set. J. Clin. Microbiol. 48: 3088-94.
126. WHO, Proposal for the Classification of Acute Respiratory Infections and tuberculosis for the Tenth Revision of the International Clasification of Diseases (1985). WHO/RSD/85.25.
127. World Health Organization (2008) The Global Burden of Disease 2004 Update. 1-146.
128. World Health Organization (2002) WHO Manual on Animal Influenza Diagnosis and Surveillance. Available: www.who.int/vaccine_research/diseases/influenza/.
129. World Health Organization (2009). WHO information for laboratory Diagnosis of pandemic (H1N1) 2009 virus in humans -revised. Available: www.who.int/csr/resources/publications/swineflu/.
130. World Health Organization (2011) Influenza Laboratory Surveillance Information, number of specimens positive for influenza virus by subtypes in Viet Nam. Available: http://www.who.int/influenza/surveillance_monitoring.
131. World Health Organization (2009) Global pandemic influenza action plan to increase vaccine supply: progress report 2008. www.who.int/vaccines-documents/.
132. World Health Organization (2011, WHO global technical consultation: global standards and tools for influenza surveillance. WHO/HSE/GIP/2011.1.
133. Wold WM, Horwitz MS (2007). Adenoviruses. In Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: Lippincott Williams & Wilkins. 2395-2436.
134. Wright PF, Neumann G, Kawaoka Y (2007). Orthomyxoviruses. In Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: LippincottWilliams & Wilkins. 1691- 1740.
135. Writing Committee for the Joint Technical Consultation on Avian Influenza at the Human-Animal Interface. FAO-OIE-WHO Joint Technical Consultation on Avian Influenza at the Human-Animal Interface. 7–9 October, 2008, Verona, Italy. Consultation Summary (2010) Influenza and Other Respiratory Viruses. 4 (Suppl. 1): 1–29.
136. Yoshida L, Yoshino H, Nguyen HAT, Suzuki M, Vu TD, Le TH, Yanai H, Kilgore P, Moriuchi H, Dang DA and Ariyoshi K (2010) Viral Pathogens Associated With Acute Respiratory Infection In Vietnamese Children. Pediatr. Infect. Dis. J. 29: 75–77.

137. Zhang HY, Li ZM, Zhang GL, Diao TT, Cao CX, Sun HQ (2009) Respiratory viruses in hospitalized children with acute lower respiratory tract infections in Harbin, China. Jpn. J. Infect. Dis. 62: 458-460.
138. Zar HJ (2006) Global paediatric pulmonology: Out of Africa. Paediatr Resp. Rev. 7 Suppl 1: S226-8.
139. Zimmer SM, and Burke DS (2009) Historical Perspective- Emergence of Influenza A (H1N1) Viruses. N Engl J Med. 361: 279-85.

APPENDIX

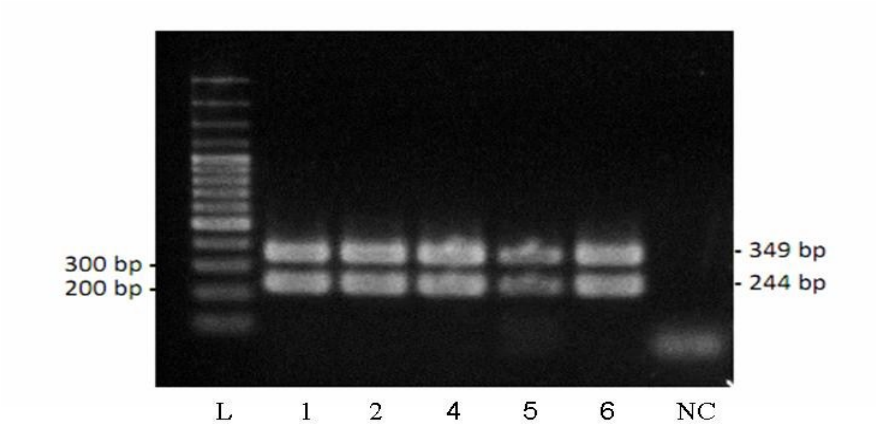
Appendix 1
Results of PCR / RT-PCR assays and viral isolation on cell culture

Picture A1-1
Multiplex RT-PCR result for diagnosis of influenza A virus and influenza B virus



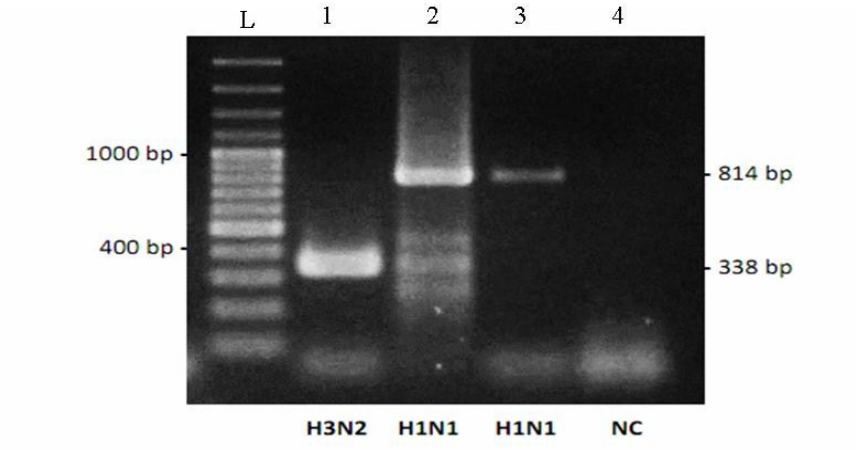
L: 100bp DNA ladder; sample 1: positive for gene M(296bp)/FluB;
sample 2, 3, 4, 5 positive for gene M (214bp)/ FluA; NC: negative control;
sample 6 negative for FluA and FluB

Picture A1-2
The results of multiplex RT-PCR for S-OIV H1N1 (2009)



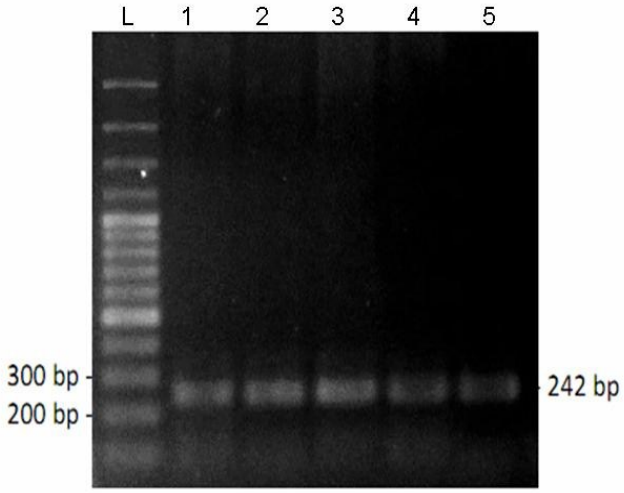
L: 100bp DNA ladder; 1, 2, 3, 4, 5, 6 positive samples for gene M (244bp) and gene HA1(349bp); NC: negative control

Picture A1-3
The results of Multiplex RT-PCR for H3N2 and seasonal H1N1



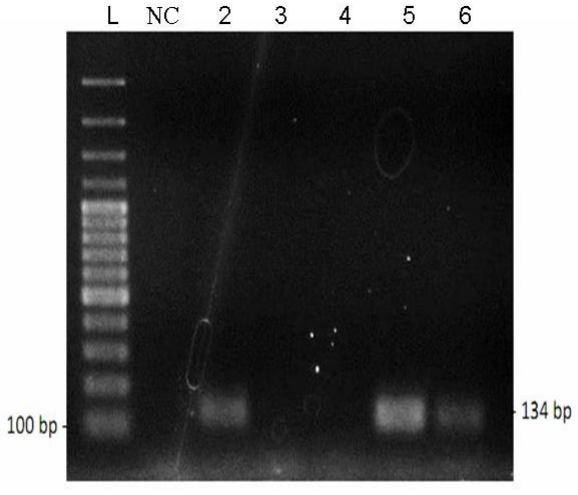
L: 100bp DNA Ladder; sample 1 positive for gene HA3 (338 bp), sample 2 and 3 positive for gene HA1 (814bp) of seasonal H1N1; NC: negative control

Picture A1-4
The result of Nested RT-PCR for RSV



L: 100bp DNA ladder; sample 1, 2, 3, 4, 5 positive for RSV

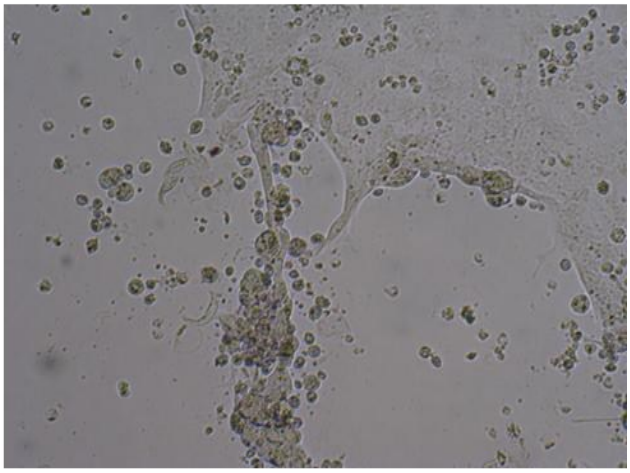
Picture A1-5
PCR result for adenovirus



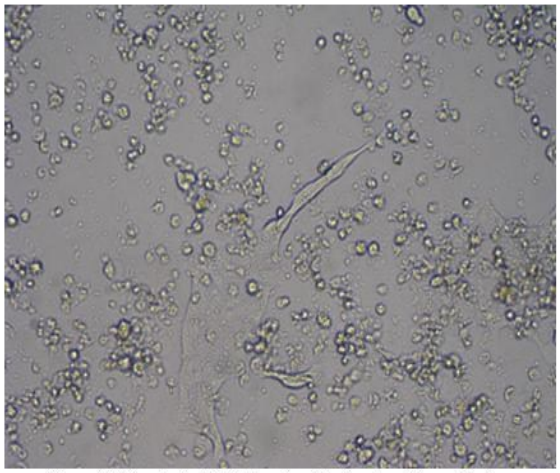
L: 100bp DNA ladder; NC: negative control, sample 2, 5, 6 positive for adenovirus; sample 3 and 4 negative for adenovirus.

Picture A1-6
Isolation of Influenza virus on MDCK

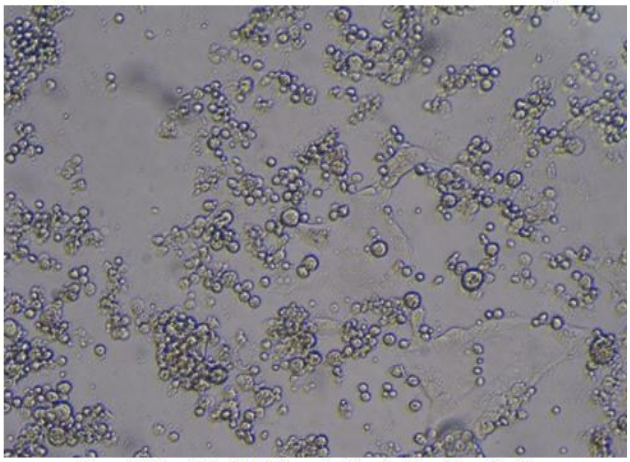
Cell culture MCDK - non-infected



patient No 32- CPE – infected after 2 days



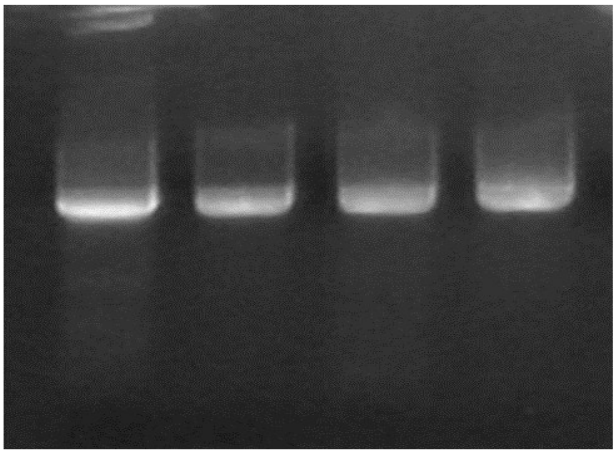
patient No 14-CPE – infected after 3 days



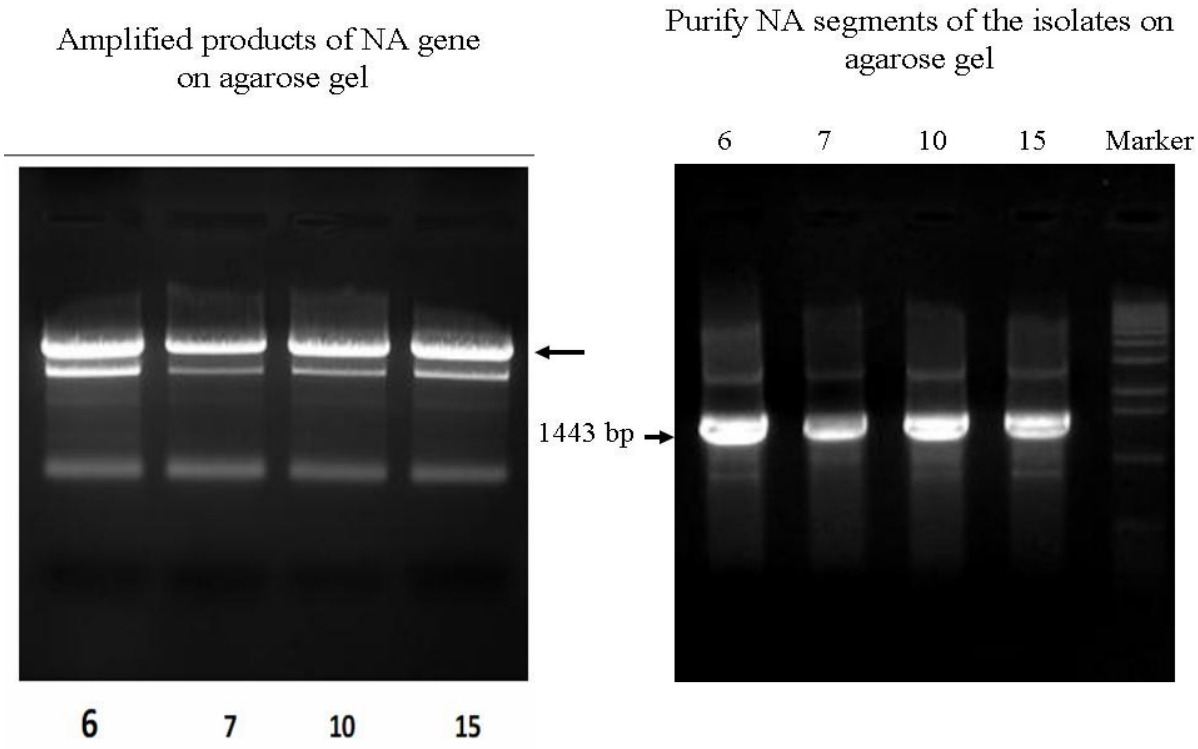
patient No 17- CPE – infected after 3 days

Picture A1-7
Amplification and purification of HA genes of the isolates for sequencing

HA gene on agarose gel after amplified with primers HA-1 and HA-1178, and purified by GenElute PCR Clean-up kit



Picture A1-8
Amplification and purification of NA genes of the isolates for sequencing



Appendix 2
Results of sequencing HA and NA genes of the representative isolates of influenza A virus H1N1 (2009) in Hue

HA Sequences of the Representative Isolates 6, 7, 10, 15 in Hue

HA sequence of the isolate 6 in Hue (HA_6)
(A/Hue/6/2009(H1N1) - Accession number: JN896300

TAGTTCTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGTTATCATGC
GAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACT
CTGTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCC
CCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAA
TCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACATCTAGTTCATACAATGGA
ACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCAATTGAGCTCAGTG
TCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGGCCCAATCATGACTCG
AACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTA
ATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCCTACATTAATGAT
AAAGGGAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTAGTGCTGACCAA
CAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACATCAAGATACAGCAAG
AAGTTCAAGCCGGAAATAGCAATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATGA
ACTATTACTGGACACTAGTAGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAAT
CTAGTGGTACCGAGATATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATT
TCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCCAAGGGTGCTATAAAC
ACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGGAAAATGTCCAAAATATGTA
AAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGA
GGCCTATTTGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGA
TGGTACGGTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAG
CACACAGAATGCCATTGACGAGATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAA
TACACAGTTCACAGCAGTAGGTAAAGAGTTCAACCACCTGGAAAAAAGAATAGAGAATT
TAAATAAAAAGGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTGTTGG
TTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTATATG
AAAAGGTAAGAAGCCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAA
TTTTACCACAAATGCGATAACACGTGCATGGAAAGTGTCAAAAATGGGACTTATGACTAC
CCAAAATACTCAGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGGGGTAAAGCTGG
AATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACT
GGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGGTCTCTACAGTGTAG
AATATGTATTTAACATAGATC

HA sequence of the isolate 7 in Hue (HA_7)
(A/Hue/7/2009(H1N1) - Accession number: JN935017

TAGTTCTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGTTATCATGC
GAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACT
CTGTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCC
CCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAA
TCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACATCTAGTTCAGACAATGGA
ACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCAATTGAGCTCAGTG
TCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAATTCATGGCCCAATCATGACTCG
AACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTA
ATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCCTACATTAATGAT
AAAGGGAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACGAGTGCTGACCAA
CAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACATCAAGATACAGCAAG
AAGTTCAAGCCGGAAATAGCAATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATGA
ACTATTACTGGACACTAGTAGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAAT
CTAGTGGTACCGAGATATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATT
TCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCCAAGGGTGCTATAAAC
ACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGGAAAATGTCCAAAATATGTA
AAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGA
GGCCTATTTGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGA
TGGTACGGTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAAAG
CACACAGAATGCCATTGACGAGATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAA
TACACAGTTCACAGCAGTAGGTAAAGAGTTCAACCACCTGGAAAAAAGAATAGAGAATT
TAAATAAAAAGGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTGTTGG
TTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTATATG
AAAAGGTAAGAAGCCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAA
TTTTACCACAAATGCGATAACACGTGCATGGAAAGTGTCAAAAATGGGACTTATGACTAC
CCAAAATACTCAGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGGGGTAAAGCTGG
AATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACT
GGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGGTCTCTACAGTGTAG
AATAT

HA sequence of the isolate 10 in Hue (HA_10)
(A/Hue/10/2009(H1N1) - Accession number: JN896302

TAGTTCTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGTTATCATGC
GAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTCACAGTAACACACT
CTGTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCC
CCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAA
TCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACATCTAGTTCAGACAATGGA
ACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCAATTGAGCTCAGTG
TCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGGCCCAATCATGACTCG
AACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTA
ATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCCTACATTAATGAT
AAAGGGAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTAGTGCTGACCAA
CAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACATCAAGATACAGCAAG
AAGTTCAAGCCGGAAATAGCAATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATGA
ACTATTACTGGACACTAGTAGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAAT
CTAGTGGTACCGAGATATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATT
TCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCCAAGGGTGCTATAAAC
ACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGGAAAATGTCCAAAATATGTA
AAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGA
GGCCTATTTGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGA
TGGTACGGTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAG
CACACAGAATGCCATTGACGAGATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAA
TACACAGTTCACAGCAGTAGGTAAAGAGTTCAACCACCTGGAAAAAAGAATAGAGAATT
TAAATAAAAAGGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTGTTGG
TTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTATATG
AAAAGGTAAGAAGCCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAA
TTTTACCACAAATGCGATAACACGTGCATGGAAAGTGTCAAAAATGGGACTTATGACTAC
CCAAAATACTCAGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGGGGTAAAGCTGG
AATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACT
GGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGGTCTCTACAGTGTAG
AATATGTATTTAA

HA sequence of the isolate 15 in Hue - Influenza A virus (A/Hue/15/2009(H1N1) - Accession number JN393307 (HA_15)

TAGTTCTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGTTATCATGC
GAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACT
CTGTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCC
CCATTACATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAA
TCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACATCTAGTTCAGACAATGGA
ACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCAATTGAGCTCAGTG
TCATCATTTGAAAGGTTTGAGATATTCCCCAAAACAAGTTCATGGCCCAATCATGACTCG
AACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTA
ATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCCTACATTAATGAT
AAAGGGAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTAGTGCTGACCAA
CAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACATCAAGATACAGCAAG
AAGTTCAAGCCGGAAATAGCAATAAGGCCCAAAGTGAGGGATCAAGAAGGGAGAATGA
ACTATTACTGGACACTAGTAGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAAT
CTAGTGGTACCGAGATATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATT
TCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCCAAGGGTGCTATAAAC
ACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGGAAAATGTCCAAAATATGTA
AAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGA
GGCCTATTTGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGA
TGGTACGGTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAG
CACACAGAATGCCATTGACGAGATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAA
TACACAGTTCACAGCAGTAGGTAAAGAGTTCAACCACCTGGAAAAAAGAATAGAGAATT
TAAATAAAAAGGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTGTTGG
TTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTATATG
AAAAGGTAAGAAGCCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAA
TTTTACCACAAATGCGATAACACGTGCATGGAAAGTGTCAAAAATGGGACTTATGACTAC
CCAAAATACTCAGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGGGGTAAAGCTGG
AATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACT
GGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGGTCTCTACAGTGTAG
AATATGTATTTAA

Na Sequences of the Representative Isolates in Hue

NA sequence of the isolate 6 in Hue (6_NA)
(A/Hue/6/2009(H1N1) - Accession number: JN896301

TTGGTTCGGTCTGTATGACAATTGGAATGGCTAACTTAATATTACAAATTGGAAACATAA
TCTCAATATGGATTAACCACTCAATTCAACTTGGGAATCAAAATCAGATTGAAACATGCA
ATCAAAGCGTCCTTACTTATGAAAACAACACTTGGGTAAATCAAACATATGTTAACATCA
GCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAACGGGCAATTCCT
CTCTCTGCCCTGTTAATGGATGGGCTATATACAGTAAAAACAACAGTATAAGAATCGGTT
CCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCA
AAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAATGACAAACATTCCAATGGAACCATTA
AAGACAGGAGCCCATATCCAACCCTAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCAT
ACAACTCAAGATTTGAGTCAGTCGCTTGGTCAGCAAGTGCTTGTCATGATGGCATCAATT
GGCTAACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGTACAACG
GCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTGAGAACACAAGAGTCT
GAATGTGCATGTGTAAATGGTTCTTGCTTTACTGTAATGACCGATGGACCAAGTGATGGA
CAGGCCTCATACAAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGTCGAAAT
GAATGCCCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACA
TGTGTGTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACCAGAAT
CTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGACAATCCACGCCCTAAT
GATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGAGTAAAAGGATT
TTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTCAAGAAA
CGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAAT
AAAGCAAGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGC
ATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTTGAACTAATCAGAG
GGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAGCAGCATATCCTTTTGTGGTGTA
AACAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGAG

NA sequence of the isolate 10 in Hue (10_NA)
(A/Hue/10/2009(H1N1) - Accession number: JN896303

TTGGTTCGGTCTGTATGACAATTGGAATGGCTAACTTAATATTACAAATTGGAAACATAA
TCTCAATATGGATTAACCACTCAATTCAACTTGGGAATCAAAATCAGATTGAAACATGCA
ATCAAAGCGTCCTTACTTATGAAAACAACACTTGGGTAAATCAAACATATGTTAACATCA
GCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAACGGGCAATTCCT
CTCTCTGCCCTGTTAATGGATGGGCTATATACAGTAAAAACAACAGTATAAGAATCGGTT
CCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCA
AAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAATGACAAACATTCCAATGGAACCATTA
AAGACAGAAGCCCATATCCAACCCTAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCAT
ACAACTCAAGATTTGAGTCAATCCCTTGGTCAACAAGTGCTTGTCATGATGGCATCAATT
GGCTAACAATTGGAATTTCTGGCCCAAACAATGGGGCAGTGGCTGTGTTAAAGTACAACG
GCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTGAGAACACAAGAGTCT
GAATGTGCATGTGTAAATGGTTCTTGCTTTACTGTAATGACCGATGGACCAAGTGATGGA
CAGGCCTCATACAAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGTCGAAAT
GAATGCCCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACA
TGTGTGTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACCAGAAT
CTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGACAATCCACGCCCTAAT
GATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGAGTAAAAGGATT
TTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTCAAGAAA
CGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAAT
AAAGCAAGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGC
ATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTTGAACTAATCAGAG
GGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAGCAGCATATCCTTTTGTGGTGTA
AACAGTGACACTGGGGGTTGGTCTTGGCCAGACGGTGCTGAG

NA sequence of the isolate 15 in Hue - Influenza A virus (A/Hue/15/2009(H1N1)) - Accession number JN656969 (15_NA)

TTGGTTCGGTCTGTATGACAATTGGAATGGCTAACTTAATATTACAAATTGGAAACATAA
TCTCAATATGGATTAACCACTCAATTCAACTTGGGAATCAAAATCAGATTGAAACATGCA
ATCAAAGCGTCCTTACTTATGAAAACAACACTTGGGTAAATCAAACATATGTTAACATCA
GCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAACGGGCAATTCCT
CTCTCTGCCCTGTTAATGGATGGGCTATATACAGTAAAAACAACAGTATAAGAATCGGTT
CCAAGGGGGATGTGTTTGTCATAAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCA
AAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAATGACAAACATTCCAATGGAACCATTA
AAGACAGGAGCCCATATCCAACCCTAATGAGCTGTCCTATTGGTGAAGTTCCCTCTCCAT
ACAACTCAAGATTTGAGTCAATCCCTTGGTCAACAAGTGCTTGTCATGATGGCATCAATT
GGCTAACAATTGGAATTTCTGGCCCAAACAATGGGGCAGTGGCTGTGTTAAAGTACAACG
GCATAATAACAGACACTATCAAGAGTTGGAGAAACAATATATTGAGAACACAAGAGTCT
GAATGTGCATGTGTAAATGGTTCTTGCTTTACTGTAATGACCGATGGACCAAGTGATGGA
CAGGCCTCATACAAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGTCGAAAT
GAATGCCCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACA
TGTGTGTGCAGGGATAACTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAACCAGAAT
CTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGACAATCCACGCCCTAAT
GATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGAGTAAAAGGATT
TTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTCAAGAAA
CGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAAT
AAAGCAAGATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGC
ATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTTCTGGGTTGAACTAATCAGAG
GGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAGCAGCATATCCTTTTGTGGTGTA
AACAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGAG

Appendix 3

A3-1: The ethic permission of Hue College of Medicine and Pharmacy

To:

The Chairman

The Scientific Committee of Hue College of Medicine and Pharmacy

Hue College of Medicine and Pharmacy

APPLICATION FOR ETHIC APPROVAL FOR SCIENTIFIC RESEARCH

Name of investigator: Le Van An

The title of study project:

A Study on Viral Etiologies of Lower Respiratory Infections in Children Hospitalized in Thua Thien Hue

This study is a part of my study project for my Ph. D thesis of the doctorate programme in Biomolecular and Biotechnological Sciences in Sassari University.

Supervisor: Prof. Pietro Cappuccinelli, University of Sassari and honorable professor of Hue College of Medicine and Pharmacy

Objectives of the project: to develop the molecular techniques for diagnosis of respiratory viruses and find out the frequency of etiological viruses causing lower respiratory tract infections in children hospitalized in Thua Thien Hue province.

The intended period of time for the study project:

One year from January, 2010 to January of 2011

The participants will be enrolled: This study will be carried out in children with viral lower respiratory infections hospitalized in Hue Central Hospital.

The sample size of patients: 200 -300 children with viral lower respiratory infections

The procedures will be taken:

The study will be carried out in closed collaboration with an experienced clinical doctor of the Paediatric Department of Hue Central Hospital in order to implement the following steps:

- Selecting the patients, giving consultations to the patients and the patient s' supervisors to obtain the agreement of their involvements in the study.
- Performing clinical examinations and completing the patient recording form.

- Collecting the respiratory samples of throat swab or nasal pharyngeal swabs.

Declaration of responsibility:

- Agreement (in writing or oral) from patients or through patient s' guardians will be taken before entering them into study.
- I am aware that any medical procedure, which can be used on patient, may bring all the potential risks to patient s' health and I also know with certainty that the procedure performed for collection of respiratory specimen will make no harm to ill children.
- The standard and certified safe tools for specimen collection will be used for this purpose and the sampling procedure will be safely performed by an experienced doctor.
- The patient data, record and results of examination and laboratory analysis will be kept only for scientific purpose and not for anything else.

I confirm that the information contained in this application is correct and true.

Hue 20th, December, 2009
Investigator Signature

Le Van An

Approval

The Scientific Committee of Hue College of Medicine and Pharmacy

Hue College of Medicine and Pharmacy

Chairman and Rector

Prof. Dr. Cao Ngoc Thanh

A3-2: The ethic permission of Hue Central Hospital
Vietnamese language version

ĐẠI HỌC HUẾ
TRƯỜNG ĐẠI HỌC Y - DƯỢC HUẾ

CỘNG HOÀ XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập- Tự do- Hạnh phúc

ĐƠN XIN PHÉP THỰC HIỆN NGHIÊN CỨU

Kính gởi:
- Ban Giám Đốc Bệnh Viện Trung Ương Huế
- Trưởng Khoa Nhi Bệnh viện Trung Ương Huế

Tôi tên là Lê Văn An
Đơn vị công tác: Th.S Giảng viên bộ môn Vi sinh Đại Học Y Dược Huế
Hiện tôi đang làm nghiên cứu về đề tài nghiên cứu về « **Bệnh nguyên virus gây nhiễm trùng hô hấp dưới ở trẻ em nhập viện** »
Mục tiêu của nghiên cứu nhằm phát triển các kỹ thuật sinh học phân tử để xác định bệnh nguyên virus và xác định tần suất các virus hô hấp gây nhiễm trùng hô hấp dưới ở trẻ em.
Nghiên cứu dự định thực hiện lấy mẫu từ bệnh nhi tại bệnh nhi thuộc Nhoa Nhi hô hấp, Bệnh Viện Trung Ương Huế
Thực hiện nghiên cứu này chúng tôi tiến hành lấy bệnh phẩm ngoáy họng hay ngoáy tỵ hầu trên số 200 trẻ em có chẩn đoán nhiễm trùng hô hấp dưới gồm viêm phế quản, viêm tiểu phế quản và viêm phổi
Thời gian dự kiến: 1 năm từ tháng 01 năm 2010 đến tháng 01 năm 2011.
Trong thời gian nghiên cứu chúng tôi sẽ tuân thủ các bước sau
- Hợp tác chặt chẽ với bác sĩ điều trị ở khoa Nhi trong việc tư vấn cho bệnh nhân và người nhà, theo dõi, chẩn đoán và lấy mẫu bệnh nhân nhằm đảm bảo tuyệt đối an toàn cho người bệnh.
- Cung cấp các dụng cụ lấy mẫu chuẩn và thực hiện kỹ thuật lấy mẫu không ảnh hưởng đến sức khỏe của người bệnh.
- Cung cấp kết quả xét nghiệm kịp thời theo yêu cầu cho bệnh phòng để đáp ứng yêu cầu theo dõi và điều trị người bệnh.

Chúng tôi xin thực hiện đúng các quy định về chăm sóc sức khỏe cho người bệnh của Khoa và của Bệnh Viện.
Mong sự chấp thuận của Khoa và của Ban Giám Đốc bệnh viện
Xin trân trọng cám ơn

Huế ngày 15 tháng 12 năm 2009
Cán bộ nghiên cứu

Lê Văn An

Khoa Nhi
Bệnh Viện TW Huế

BS.CKII. Đinh Quang Tuấn

Ban Giám đốc
Bệnh Viện TW Huế

BỆNH VIỆN TRUNG ƯƠNG HUẾ

GS.TS. Bùi Đức Phú

English translation

HUE UNIVERSITY
COLLEGE OF
MEDICINE AND PHARMACY

SOCIALIST REPUBLIC OF VIETNAM
Independence-Freedom-Happiness

PETITION FOR RESEARCH PERFORMANCE

For the attention of:

- The Board of Directors of Hue Central Hospital
- The Head of Department of Pediatrics

I, the undersigned, am Le Van An
Workplace: MA. Lecturer of Department of Microbiology, Hue College of Medicine and Pharmacy
I am doing the research on topic **"The viral etiology caused lower respiratory infections in hospitalized children"**
The research aims to develop the molecular biology techniques to identify viral etiology and the frequency that those viruses caused lower respiratory infections in children. We plan to take samples from the children patients at Respiratory Division of Department of Pediatrics under directly Hue Central Hospital.
We shall take the samples from 200 children who diagnosed with lower respiratory infections including bronchitis, bronchiolitis and pneumonia by swabbing their throats and fauces.
The proposed time: one year from January 2010 to January 2011

During the researching time, we will abide by the followings:

- To cooperate closely with the doctors in Department of Pediatrics in giving advice to patients and their families, monitoring, diagnosing and taking samples from the patients with the guarantee for absolute safety to patients.
- To provide standard sample taking tools and techniques which do not affect the health of the patients.
- To provide timely test results to meet the requirements of the monitor and treatment to patients.

We shall perform our research in accordance with regulations on health care for patients of the Department and Hospital.

Whilst awaiting for your acceptance, we would like to express our sincerest thanks to you all.

Hue, December 15, 2009
Researcher
(signed)
Le Van An

Department of Pediatrics
Hue Central Hospital
(signed)
Dr. 2nd degree specialist. Dinh Quang Tuan

The Board of Directors of Hue Central Hospital
(signed and sealed)
Prof. PhD.MD. Bui Duc Phu

THUA THIEN HUE

DEPARTMENT OF FOREIGN AFFAIRS

I, Ton Nu Dieu Hanh, a translator of Thua Thien Hue Foreign Relations Service Center, do solemnly declare that, to the best of my knowledge, this is a full and faithful English translation made by me from the attached copy of Vietnamese original.

Hue, September 26, 2011

Translator

TON NU DIEU HANH

Foreign Relations Service Center hereby certifies that the affixed signature is the true signature of Ms. Ton Nu Dieu Hanh, ID card No.191 594 686 issued on July 5, 2002 at Thua Thien Hue Public Security.

Hue, September 26, 2011

DEPUTY DIRECTOR

SỞ NGOẠI VỤ TỈNH THỪA THIÊN HUẾ TRUNG TÂM DỊCH VỤ ĐỐI NGOẠI

NGUYENTHANH BINH

Appendix 4
Patient recording sheet

PATIENT RECORDING SHEET
(FOR LOWER RESPIRATORY TRACT INFECTIONS)

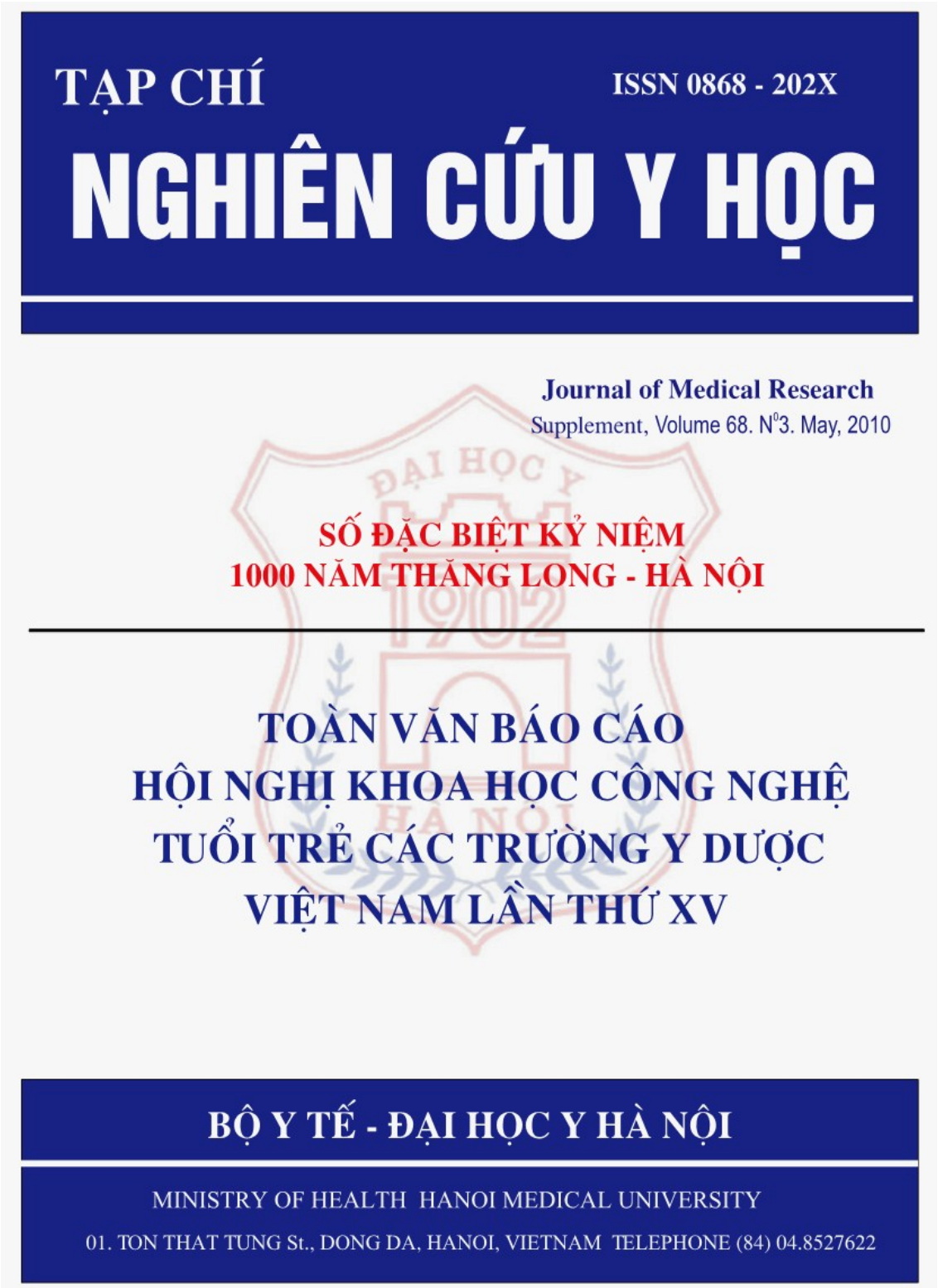
Patient full name.....sex.....age.....
Home address.....
Date of onset.....
Date of admission.....
Clinical diagnosis Bronchitis □ Bronchiolitis □ Pneumonia □
Other diagnosis.....
Symptoms and signs at the time of collection

Symptoms and signs	**Yes**	**No**	**Notes**
Fever			
Dry cough			
Productive cough			
Coryza			
Sore throat			
Dyspnea			
Rales (or wheezes) in lungs			
Lung consolidation			
Pleural effusion			
Stupor / coma			
X ray			

Severity estimation mild □ moderate □ severe □ very severe □
Sort of specimens
Nasal pharyngeal swab □ throat swab □ bronchial aspirate □
Other specimens.....

Date.....month.....year.....
Responsible Doctor

Appendix 5
Publications and presentation in scientific meeting
A5-1: Publications in Vietnamese Journals



CHẨN ĐOÁN NHIỄM VIRUS CÚM A Ở ĐƯỜNG HÔ HẤP BẰNG PHÂN LẬP VIRUS VÀ KỸ THUẬT KHUẾCH ĐẠI GEN RT - PCR

Lê Văn An, Lê Thị Bảo Chi, Huỳnh Hải Đường
Đại Học Y Dược Huế

Mục tiêu: *Chẩn đoán virus cúm A và các dưới týp bằng dùng kết hợp phân lập virus cúm trên nuôi cấy tế bào và xét nghiệm khuếch đại gen.* ***Phương pháp:*** *Phân lập virus cúm từ các mẫu bệnh phẩm hô hấp trên ở nuôi cấy tế bào MDCK và đồng thời dùng các kỹ thuật khuếch đại gen để xác định trực tiếp virus cúm A và các phân týp của cúm A từ mẫu bệnh phẩm.* ***Kết quả:*** *Bốn mươi bệnh phẩm lấy ở đường hô hấp của 40 bệnh nhân nghi nhiễm cúm được chẩn đoán với 2 xét nghiệm trên, có 26 bệnh nhân (tỷ lệ 65%) dương tính với virus cúm A, trong đó virus cúm A lợn đang gây dịch (H1N1sw) được phát hiện ở 20 bệnh nhân (có tỷ lệ 77%), virus cúm A mùa xác định ở 6 bệnh nhân (tỷ lệ 23%) dương tính còn lại, không có trường hợp nào xác định nhiễm H3N2 và H5N1 trong nhóm bệnh nhân khảo sát. Kỹ thuật khuếch đại gen RT - PCR cho kết quả dương tính ở tất cả 26 mẫu (tỷ lệ 100%) và cấy phân lập virus chỉ cho dương tính ở 20 bệnh nhân (tỷ lệ 77%) đã được xác định bởi RT - PCR.* ***Kết luận:*** *Dùng cả hai phương pháp chẩn đoán virus học đặc hiệu để xác định virus cúm A và định các dưới týp ở mẫu bệnh phẩm hô hấp. Kết quả cho thấy virus cúm A được chẩn đoán với tỷ lệ cao và xác định rõ các phân týp gây bệnh.*

I. ĐẶT VẤN ĐỀ

Virus cúm có 3 týp A, B và C, là tác nhân gây nhiễm trùng đường hô hấp ở người, trong đó virus cúm týp A thường gây nên các vụ dịch cúm ở người. Về mặt cấu trúc virus học, virus cúm có bao bên ngoài bằng lipid, trên bề mặt của bao ngoài có nhiều gai ngưng kết hồng cầu mang kháng nguyên H và gai neuraminidase mang kháng nguyên N. Dựa vào hai kháng nguyên này virus cúm týp A còn được chia thành nhiều dưới týp và hiện nay có 16 dưới týp với kháng nguyên H và 9 dưới týp với kháng nguyên N [1], [2].

Trong thiên nhiên virus cúm A có túc chủ là các loài động vật gồm các loài chim nước gọi là virus cúm A của chim; một số dưới týp có túc chủ là lợn (virus cúm A lợn); virus cúm A ngựa; và chỉ một vài dưới týp virus cúm A phân bố ở người. Virus cúm A ở chim có thể thay đổi do đột biến gen hay do sự tổ hợp gen để trở thành các dưới týp mới và gây nên những vụ dịch cúm ở các loài động vật túc chủ mới như lợn và người.

Bệnh do virus cúm A ở người thường do một số dưới týp lưu hành là H2N2 (1957), H1N1 mùa, và H3N2. Tuy nhiên trong những năm gần đây một số dưới týp virus cúm A có nguồn gốc động vật có thể gây nhiễm trùng ở người gồm virus cúm chim H5N1 và rất gần đây là virus cúm A dưới týp H1N1 có nguồn gốc từ lợn (swine H1N1) hiện đang gây nên đại dịch ở khắp nơi trên thế giới và đang lây lan mạnh ở Việt Nam [2].

Xác định sớm nhiễm virus cúm A giúp dùng thuốc chống virus sớm, đề ra biện pháp phòng và chống lây lan, đặc biệt với các dưới týp virus cúm có độc lực cao gây nhiễm trùng nặng như H5N1. Hiện nay có nhiều phương pháp chẩn đoán virus cúm bao gồm phân lập virus; xác định kháng nguyên virus cúm ở mẫu nghiệm hô hấp; xác định RNA của virus cúm bằng kỹ thuật phân tử và xác định kháng thể trong máu bệnh nhân. Chẩn đoán bằng phân lập virus hiện là phương pháp chuẩn, phương pháp này cần có phương tiện nuôi cấy tế bào và phải được thực hiện với những phòng thí nghiệm có mức an toàn sinh học cấp 3. Xác định kháng nguyên virus cúm trong mẫu nghiệm hô hấp bằng xét nghiệm nhanh có độ nhạy thấp (10 - 70%) và không phân biệt được các dưới týp của virus cúm A, do vậy hiện không được Tổ Chức Y Tế Thế Giới (TCYTTG) khuyên dùng để theo dõi dịch cúm [3]. Xác định RNA của virus trong bệnh phẩm bằng kỹ thuật RT - PCR là phương pháp chẩn đoán nhanh và đặc hiệu, có thể thực hiện được ở các phòng thí nghiệm cấp 2 và phương pháp này hiện được TCYTTG khuyên dùng ở nhiều phòng thí nghiệm để chẩn đoán nhanh virus cúm [1]; xác định kháng thể chống virus cúm A trong huyết thanh người bệnh ít hữu ích vì cho kết quả chậm, thường chỉ dùng trong điều tra dịch tễ học.

Để tham gia vào hoạt động giám sát nhiễm trùng virus cúm A trong những năm tới ở địa bàn Thừa Thiên Huế và các tỉnh miền Trung. Ở phòng thí nghiệm Vi sinh thuộc trung tâm Carlo Urbani và bộ môn Vi sinh trường Đại Học Y Dược Huế, chúng tôi đã xây dựng có sẵn và đưa vào áp dụng hai phương pháp chẩn đoán virus cúm A gồm phân lập virus cúm trên nuôi cấy tế bào và các kỹ thuật khuếch đại gen RT - PCR để xác định virus cúm A và các dưới týp gây bệnh ở người. Bài báo này chúng tôi trình bày kết quả bước đầu áp dụng hai phương pháp chẩn đoán nêu trên trong xác định virus cúm A và định danh các dưới týp gây bệnh ở bệnh nhân có biểu hiện lâm sàng nghi ngờ bị nhiễm virus cúm A mới trong thời gian vừa qua tại Thừa Thiên Huế.

II. ĐỐI TƯỢNG - VẬT LIỆU VÀ PHƯƠNG PHÁP NGHIÊN CỨU

2. 1. Đối tượng nghiên cứu

Chúng tôi thực hiện chẩn đoán virus cúm A ở các bệnh nhân tuổi từ 3 đến 18 tuổi có triệu chứng hô hấp với lâm sàng nghi ngờ nhiễm H1N1 hoặc VPQ cấp do virus nhập viện tại bệnh viện thành phố Huế từ tháng 5/ 10 đến 15/ 11 năm 2009.

2. 2. Vật liệu nghiên cứu

2. 2. 1. Sinh phẩm dùng phân lập virus cúm:

» Môi trường vận chuyển virus (viral transport medium: VTM) của hảng Becton Dickinson

» Tế bào MDCK (Madin - Darby Canine Kidney cells) (ATCC CCL34)

» Môi trường nuôi cấy tế bào MEM (pH 7. 2) + 10% fetal bovine serum + dung dịch kháng sinh gồm penicillin [10 U/ml], streptomycin [10 mg/ml], and amphotericin B [0. 25 mg/ml]), đệm HEPES (25mM).

» Dung dịch Trypsin 0, 05% (1x), với EDTA, trypsin, TCPK

2. 2. 2. Sinh phẩm cho kỹ thuật khuếch đại gen

» Bộ sinh phẩm tách RNA của virus của hảng Qiagen (Cat No: 52904),

» Sinh phẩm cho kỹ thật khuếch đại là RT - PCR Master mix (2x), superscript III RT/Platinum Taq DNA polymerase của hảng Invitrogen.

» Các chứng dương RNA của các virus H1N1sw (viện Pasteur TP HCM), H1N1s (mùa), H3N2 (Viện San Raffaele, Milan)

» Các gen mồi khuếch đại gen M của virus cúm A, và các gen mồi khuếch đại gen H1 của virus H1N1 (2009), gen mồi khuếch đại gen H1N1 mùa, gen mồi khuếch đại H3 của virus H3N2 và H5 của H5N1 theo khuyến cáo của TCYTTG như ở bảng 1. Tất cả gen mồi này được chọn từ các tài liệu [1, 2, 5].

Bảng 1. Chi tiết về các gen mồi dùng để chẩn đoán virus cúm A và định các dưới týp

Týp/ subtýp	Gene đích	Tên mồi	Cấu trúc chuỗi	Kích thước đoạn khuếch đại
Influenza type A	Matrix (M)	M30F2/08	ATGAGYCTTYTAACCGAGGTCGAAACG	244bp
		M264R3/08	TGGACAAANCGTCTACGCTGCAG	
Influenza A H1N1(sw) swine 2009 virus	HA1sw	NIID - swH1 Conv - F1	TGCATTTGGGTAAATGTAACATTG	349bp
		NIID - swH1 Conv - R1	AATGTAGGATTTRCTGAKCTTTGG	
H3N2	HA3	H3 P1	5′ - CCTTGATGGAGAAAACTGCACAC - 3′	338bp
		H3 P2	5′ - TGTTTGGCATAGTCACGTTCA - 3′	
H1N1 mùa (s)	HA1s	H1 P1	5′ - GAATCATGGTCCTACATTGTAGAA A - 3′	814bp
		H1 P2	5′ - ATCATTCCAGTACATCCCCCTTCAAT - 3′	
H5N1	HA5	H5 - 1	5′ - GCCATTCCACAACATACACCC - 3′	219 bp
		H5 - 3	5′ - CTCCCCTGCTCATTGCTATG - 3′	

2. 2. 3. Phương tiện và máy móc cần:

Phương tiện của BSL3, máy ly tâm, tủ ấm CO_2, Veriti Gradient PCR (Applied Biosystems, USA), máy lắc, điện di, máy đọc gel.

2. 3. Phương pháp nghiên cứu

2. 3. 1. Lấy bệnh phẩm:

Mỗi bệnh nhân có triệu chứng hô hấp chúng tôi chỉ thực hiện lấy 1 mẫu bệnh phẩm bằng ngoáy họng hay ngoáy mũi hầu vào thời gian đầu lúc bệnh nhân nhập viện tương ứng với thời gian từ 2 đến 4 ngày sau khi có triệu chứng. Cho que ngoáy vào 2ml môi trường vận chuyển virus (VTM: viral transport medium) chuyển về phòng thí nghiệm và đặt vào tủ lạnh ở 40C, thực hiện tách chiết RNA và nuôi cấy virus trong vòng 24 giờ sau khi lấy mẫu nghiệm [4].

2. 3. 2. Nuôi cấy để phân lập virus

Nuôi cấy và phân lập virus được thực hiện trong phòng thí nghiệm an toàn sinh học cấp 3 (BSL3) tại trung tâm Carlo Urbani thuộc bộ môn Vi sinh, ĐHY Dược Huế. Với bệnh phẩm của bệnh nhân đã cho vào môi trường VTM, lắc đều và lấy 1ml cho vào bình nuôi cấy tế bào MDCK đã phát triển trong môi trường MEM (pH 7. 2) + 10% fetal bovine serum, penicillin [10 U/ml], streptomycin [10mg/ml], và amphotericin B [0. 25 mg/ml]), rồi ủ ở nhiệt độ 35, 50C trong tủ ấm 5% CO_2. và theo dõi hiệu ứng tế bào bệnh lý (CPE) ở kính hiển vi đảo ngược (độ phóng đại 10X và 20X) hàng ngày trong 7 ngày liên tục. Khi có hiệu ứng tế bào bệnh lý (CPE), lấy dịch nổi của môi trường cấy để xác định virus cúm A bằng kỹ thuật RT - PCR với gen M, và định dưới týp virus bằng các cặp gen mồi

H1sw, H3, H1mùa và H5.

2. 3. 3. Kỹ thuật RT - PCR

Bệnh phẩm từ môi trường VTM sau khi lắc đều lấy 140µl để tách chiết RNA với bộ sinh phẩm của Qiagen và quy trình tách được thực hiện theo hướng dẫn của hảng Qiagen.

Chúng tôi dùng 2 quy trình khuếch đại gen có bước đầu chuyển đổi ngược (RT - PCR): quy trình (1) dùng cặp gen mồi M30F2/08/ M264R3/08 để khuếch đại đoạn gen M có ở tất cả các týp virus cúm A nên có giá trị xác định virus influenza A và cặp mồi khuếch đại đoạn gen HA1 của virus H1N1sw để xác định týp virus H1N1sw (2009) gây dịch hiện nay (Bảng 1). Quy trình 1 đã được phát triển do viện quốc gia về bệnh nhiễm trùng Nhật Bản và được TCYTTG khuyến cáo sử dụng để chẩn đoán nhiễm virus H1N1sw hiện nay ở nhiều nước [1]. Quy trình này hiện cũng được sử dụng để xác định virus H1N1sw ở viện Pasteur thành phố HCM và viện VSDTTW. Quy trình (2) để xác định dưới týp H3N2 và H1N1 mùa, H5N1, quy trình này được sử dụng để chẩn đoán các dưới týp của virus cúm A được sử dụng ở phòng thí nghiệm chẩn đoán tham chiếu virus cúm của WHO ở Roma và vùng Sardegna của Italia [5]. Hai quy trình này chúng tôi đã thử với các mẫu chứng dương tương ứng và cho kết quả hoạt động tốt trên các mẫu chứng dương tính trước khi ứng dụng để chẩn đoán virus từ bệnh phẩm của bệnh nhân.

Quy trình (1) để khuếch đại gen M và HA1sw với kỹ thuật khuếch đại RT - PCR một bước trong một ống nghiệm với tổng thể tích 25µl, trong đó 12, 5µl dung dịch master mix RT - PCR (invitrogen), nồng độ cuối cùng cho mỗi gen mồi là 0. 6µM và 0. 5µl RT/platinium Taq DNA polymerase và 5µl bệnh phẩm và nước cất 2 lần. Phản ứng được chạy trên máy Veriti Gradient PCR (Applied Biosystems, USA) với chương trình. RT 50^{0}C/ 30phút, tách cDNA thành sợi đơn đầu tiên ở 95^{0}C/15phút, tiếp đó phản ứng được lập lại 45 chu kỳ với 95^{0}C/30giây, cặp đôi 50^{0}C/30giây, tổng hợp chuỗi 72^{0}C/1phút. Cuối cùng kéo dài chuỗi ở 72^{0}C/10phút [1].

Quy trình (2) cũng thực hiện kỹ thuật khuếch đại RT - PCR một bước trong một ống nghiệm với tổng thể tích 25µl, trong đó 12, 5µl dung dịch master mix RT - PCR (invitrogen), nồng độ cuối cùng cho mỗi gen mồi HA3 là 0, 12µM, đối với gen mồi HA1s là 0, 32µM cho mỗi gen mồi, 0. 5µl RT/platinium Taq DNA polymerase và 5µl bệnh phẩm và nước cất 2 lần. Phản ứng được chạy trên máy Veriti Gradient PCR (Applied Biosystems, USA) với chương trình. chuyển đổi ngược (RT) từ RNA thành cDNA ở 40^{0}C/ 40phút, rồi tách cDNA thành sợi đơn đầu tiên ở 95^{0}C/10phút, tiếp đó phản ứng được lập lại 40 chu kỳ với 94^{0}C/45giây, cặp đôi 52^{0}C/1phút 30giây, tổng hợp chuỗi 72^{0}C/2 phút 30giây. Cuối cùng kéo dài chuỗi ở 72^{0}C/5phút [5].

Tất cả các mẫu nghiệm được thử luôn luôn có thực hiện kèm theo 1 mẫu chứng dương và chứng âm để kiểm tra hoạt động của phản ứng và kiểm tra sự nhiễm chéo của mẫu nghiệm.

Sau khi hoàn tất khuếch đại trong hai quy trình trên, sản phẩm của phản ứng được điện di trên thạch agarose có nồng độ 1, 5% trong dung dịch đệm TBE (0, 5Mx), nhuộm màu với Ethidium bromide (EtBr) và đọc dưới máy đọc huỳnh quang. sự hiện diện của các đoạn gen với kích thước xác định (trong bảng 1 về gen mồi) được kết luận là dương tính.

2. 4. Xử lý số liệu và đánh giá

Kết quả của các mẫu nghiệm thực hiện với các phương pháp trên được nhập vào máy vi tính với phần mềm excel và tính toán bằng phương pháp thống kê thông thường.

Những bệnh nhân có 1 trong 2 xét nghiệm hoặc phân lập virus dương tính hoặc/ và RT - PCR dương tính được chẩn đoán dương tính với cúm A. Những mẫu phân lập có hiệu ứng tế bào bệnh lý (CPE) được kiểm tra bằng thực hiện RT - PCR với dịch nuôi cấy tế bào, và có gen M (cúm A) dương tính và phải có mặt các gen týp virus đặc hiệu (gen H1sw với H1N1sw (2009), gen H3 của H3N2, gen H1mùa của H1N1 mùa, và H5 của virus H5N1.

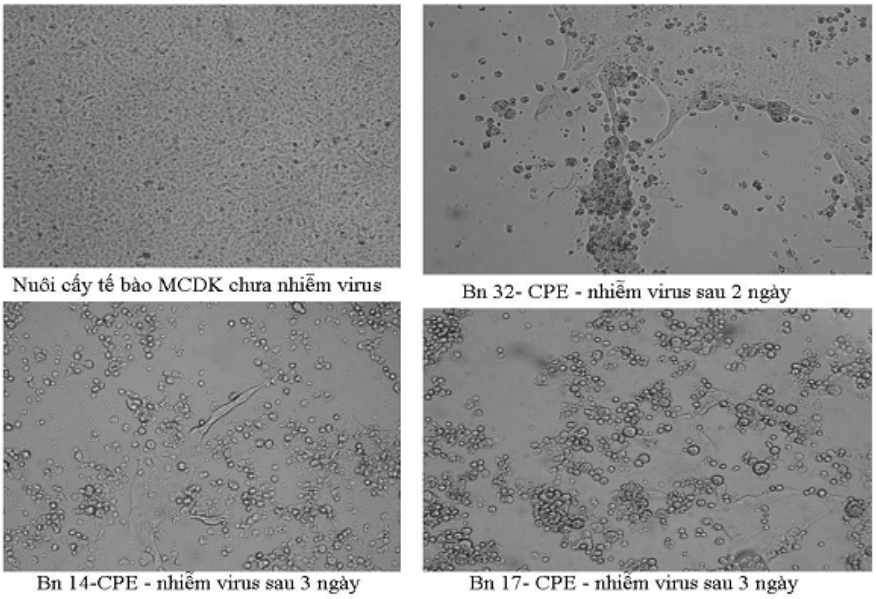
III. KẾT QUẢ

Bốn mươi (40) bệnh nhân có triệu chứng nhiễm trùng hô hấp được lấy bệnh phẩm và xét nghiệm virus học xác định virus cúm A và các dưới týp thường gặp. Trong đó 30 bệnh nhân được chẩn đoán lâm sàng là viêm phế quản nghi do cúm, 10 bệnh nhân nhiễm trùng hô hấp trên gồm viêm họng, nhiễm siêu vi. Bằng cả hai phương pháp chẩn đoán phân lập virus cúm và khuếch đại gen có 26 bệnh nhân (tỷ lệ 65%) dương tính với virus cúm A, trong số đó có 20 bệnh nhân (tỷ lệ 77%) bị nhiễm virus cúm A dưới týp H1N1sw và 06 bệnh nhân (tỷ lệ 23%) bị nhiễm virus H1N1 mùa. Không có bệnh nhân nào xác định nhiễm H3N2 và H5N1.

Bảng 2. Kết quả xác định virus cúm A và các dưới týp

	Dưới týp	Số bệnh nhân	RT - PCR	Phân lập virus
	H1N1sw	20	20	17
	H3N2	0	0	0
	H1N1 mùa	06	06	03
	H5N1	0	0	0
Âm tính với virus cúm A	-	14	-	-
Tổng số và tỷ lệ		40	26 (65%)	20 (50%)

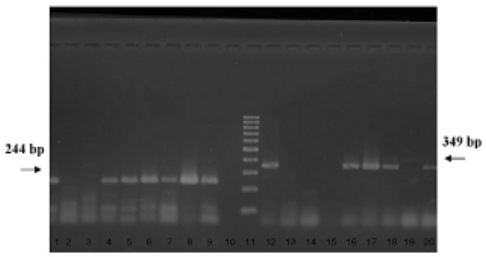
3. 1. Phân lập virus: Trong 40 mẫu nghiệm của bệnh nhân có 20 mẫu nghiệm cấy phân lập virus dương tính chiếm tỷ lệ dương tính 50% và trong số mẫu xác định dương tính với cúm A thì tỷ lệ phân lập được là 77%, trong đó 17 bệnh nhân nhiễm trùng H1N1sw và 03 bệnh nhân nhiễm trùng với H1N1 mùa. Tất cả các mẫu phân lập có hiệu ứng tế bào dương tính đều được xác định lại với RT - PCR với gen M và các gen mồi định dưới týp đều cho kết quả phù hợp với kết quả xét nghiệm RT - PCR trực tiếp từ mẫu nghiệm.



Hình 1. Hiệu ứng tế bào bệnh lý của một số mẫu nghiệm

3. 2. Kỹ thuật khuếch đại gen RT - PCR

Bốn mươi mẫu nghiệm được thực hiện xét nghiệm RT - PCR xác định virus cúm A và các dưới týp virus cúm có 26 mẫu dương tính như trình bày ở **bảng 2**.



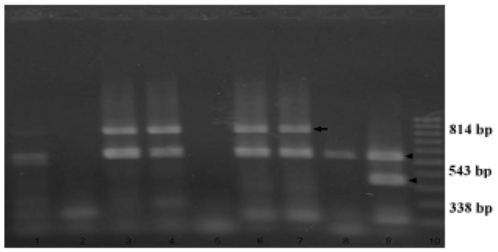
Hình 2. Kết quả quy trình RT - PCR (1) xác định virus cúm A (gen M) và gen H1sw của H1N1sw

Xác định gen M

- Mẫu 1. chứng dương với gen M (244bp), mẫu 10 chứng âm.
- Các mẫu nghiệm số 2, 3 là mẫu nghiệm âm tính.
- Các mẫu số 4, 5, 6, 7, 8, 9 là các mẫu dương tính với gen M

Xác định gen H1sw

- Mẫu 12 chứng dương với gen H1sw (349bp), mẫu số 13 chứng âm với gen H1sw
- Các mẫu số 14, 15, 19 âm tính với H1sw.



Hình 3. Kết quả quy trình PCR (2) xác định gen NP (543bp), gen H1 (mùa) 814 bp, gen H3 (338 bp). Mẫu nghiệm 1, 3, 4, 6 dương tính với H1N1 mùa (có gen NP và gen H1), mẫu 2 và 5 âm tính với virus cúm A.

» Mẫu 7 chứng dương H1N1 mùa,

» Mẫu 8 chứng dương với virus cúm A (gen NP)

» Mẫu 9 chứng dương tính H3N2 (gen NP và gen H3)

» Mẫu 10 thang DNA mẫu kích thước vạch 100 bp

IV. BÀN LUẬN

Trong nghiên cứu này chúng tôi chỉ thực hiện lấy mẫu nghiệm 1 lần từ ngày thứ 2 - 4 sau khi bệnh nhân bị sốt và có triệu chứng đường hô hấp, và ở mỗi bệnh nhân chỉ lấy 1 mẫu nghiệm và tỷ lệ xác định virus cúm là 65% bệnh nhân nhiễm virus cúm A trong tổng số 40 bệnh nhân có chẩn đoán lâm sàng nghi ngờ nhiễm trùng virus cúm. Trong số 14 bệnh nhân (35%) không tìm thấy virus cúm A trong khảo sát này không có nghĩa là bệnh nhân không bị nhiễm virus cúm A, tỷ lệ chẩn đoán dương tính do virus cúm A có thể cao hơn khi thực hiện lấy nhiều mẫu nghiệm hơn (2 mẫu) một mẫu ngoáy tỵ hầu và một mẫu ngoáy hong trên mỗi bệnh nhân, thêm vào đó mỗi bệnh nhân có thể thực hiện lấy mẫu nhiều lần như nhiều nghiên cứu khác ở nước ngoài khi muốn tìm căn nguyên virus gây nhiễm trùng đường hô hấp [4]. Rõ ràng khi thực hiện lấy nhiều mẫu một cách chặt chẽ trên một người bệnh thì chi phí xét nghiệm cho một người bệnh phải tăng lên gấp nhiều lần.

Về phương pháp chẩn đoán virus học, phân lập virus là một xét nghiệm chuẩn vàng để chẩn đoán virus, khi cần có virus sống để làm kháng nguyên, nghiên cứu về đặc tính di truyền học của virus. Phương pháp này chỉ được thực hiện ở các trung tâm chuyên sâu vì cần phòng thí nghiệm có mức an toàn cấp 3, quy trình kỹ thuật qua nhiều bước công phu cần nhiều kỹ năng, kiến thức và kinh nghiệm nuôi cấy tế bào, ủ bệnh phẩm vào tế bào, theo dõi hiệu hiệu ứng và định danh virus phát triển. Trong nghiên cứu này tỷ lệ phân lập có virus cúm trên tổng số mẫu dương tính là 77%, tỷ lệ nuôi cấy dương tính thấp so với tỷ lệ xác định dương tính khi xét nghiệm với kỹ thuật RT - PCR. Tuy nhiên tỷ lệ này cũng tương tự với nhiều nghiên cứu khác ở nhiều nơi trên thế giới khi chẩn đoán virus cúm trên mẫu nghiệm lâm sàng bằng nuôi cấy và RT - PCR, thì nuôi cấy chỉ đạt được với tỷ lệ thấp từ 60% đến 92% [6], [7]. Theo tài liệu của TCYTTG thì kỹ thuật RT - PCR thường có độ nhạy cao hơn nuôi cấy phân lập virus từ 2 - 13% [3]. Chúng tôi cho rằng phân lập virus có tỷ lệ này rất có ý nghĩa trong điều kiện của phòng thí nghiệm Vi sinh tại ĐHYDược Huế bước đầu thực hiện nuôi cấy virus. Để đạt được tỷ lệ phân lập virus cao đòi hỏi nhiều bước từ khâu lấy bệnh phẩm, trong quá trình vận chuyển mẫu lấy phải bảo quản trong dây chuyền lạnh và phải nuôi cấy ngay khi đến phòng thí nghiệm để tránh virus khỏi bị bất hoạt.

Kỹ thuật khuếch đại gen với những cặp gen mồi định týp virus cúm A và các dưới týp được sử dụng trong khảo sát này đã được thử nghiệm ở nhiều trung tâm lớn của TCYTTG về tính đặc hiệu và độ nhạy, do vậy kết quả dương tính của xét nghiệm này với các dưới týp ở mẫu nghiệm cho tính khẳng định tin cậy. Trong bối cảnh dịch H1N1sw như hiện nay, chúng tôi sử dụng quy trình 1 để xác định virus cúm A và H1N1sw, những trường hợp cúm A dương tính (có gen M) nhưng H1N1sw âm tính chúng tôi dùng quy trình 2 để xác định các dưới týp khác.

Kết quả của chúng tôi cho thấy rằng dịch cúm đang xảy ra hiện nay ở người không chỉ do virus cúm A lợn H1N1sw (77%) mà có nhiều trường hợp do dưới týp H1N1 cúm mùa (23%) thường gặp ở người. Trong khảo sát này chúng

tôi không phát hiện trường hợp nào dương tính với dưới týp H3N2 gây bệnh thường gặp ở người và dưới týp H5N1, khi khảo sát với số bệnh nhân lớn hơn, chúng tôi hy vọng sẽ có các trường hợp nhiễm trùng với các dưới týp như vừa đề cập.

V. KẾT LUẬN

Sử dụng kỹ thuật nuôi cấy phân lập virus và kỹ thuật khuếch đại gen RT - PCR xác định virus cúm týp A và các dưới týp H1N1sw, H3N2, H1N1mùa, H5N1 để chẩn đoán nguyên nhân nhiễm cúm A trên 40 bệnh nhân nghi ngờ nhiễm cúm chúng tôi phát hiện được 65% bệnh nhân bị nhiễm virus cúm A, trong đó 77% bị nhiễm virus cúm lợn H1N1sw và 23% nhiễm H1N1 mùa, không tìm thấy trường hợp nào bị nhiễm H3N2 và H5N1. Nuôi cấy phân lập virus và kỹ thuật khuếch đại gen RT - PCR hữu ích và xác định chính xác virus cúm A và các dưới týp virus gây nhiễm trùng ở người.

TÀI LIỆU THAM KHẢO

[1]. WHO information for laboratory diagnosis of pandemic (H1N1) 2009 virus in humans - update

[2]. Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from suspected human cases, Revised August 2007

[3]. WHO recommendations on the use of rapid testing for influenza diagnosis, July, 2005.

[4]. WHO/CDS/EPR/ARO/2006. Collecting, preserving and shipping specimens for the diagnosis of avian influenza A(H5N1) virus infection Guide for field operations, October 2006.

[5]. Donatelli Isabella (2007), Protocolli Opertivi Per La Diagnostica Di Laboratorio Dell ' Influenza, Centro Nazionale OMS Per La Sorveglianza Dell' Influenza (NIC) Dipartimento Di Malattie Infettive, Parassaitarie e Immunomediate, Instituto Superiore De Sanita, Roma, .

[6]. Ellis JS, Fleming DM, and Zambon MC(1997), Multiplex Reverse Transcription - PCR for Surveillance of Influenza A and B Viruses in England and Wales in 1995 and 1996, Journal of Clinical Microbiology, p. 2076 - 2082

[7]. Claas ECJ, Van Milaan AJ, Sprenger M J W, Ruiten - Stuiver M, Arron G, Rothbarth PH, and Masure N (1993), Prospective Application of Reverse Transcriptase Polymerase Chain Reaction for Diagnosing Influenza Infections in Respiratory Samples from a Children's Hospital, Journal of Clinical Microbiology, p. 2218 - 2221

Summary

DIAGNOSIS OF INFLUENZA A VIRUS AND ITS SUBTYPES IN RESPIRATORY INFECTIONS BY VIRUS ISOLATION AND REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTIONS

Objectives: *to detect influenza A virus and its subtypes by ultilizing combinatively isolation of influenza virus and reverse transcription - polymerase chain reactions.* ***Methods:*** *the combination of isolation of influenza virus on MDCK cell culture and RT - PCRs for diagnosis of type A and subtypes of influenza virus were performed on respiratory samples of patients suspected with flu.* ***Results:*** *Samples collected from 40 patients were processed for viral detection and 26 patients (65%) were positive with influenza A virus by isolation and / or RT - PCRs; pandemic H1N1 swine virus was detected in 20 patients (77%) and seasonal H1N1 influenza A virus was positive in other 06 patients (23%), no case of H3N2 and H5N1 was identified in this patients group. RT - PCRs was positive in all the 26 positive samples (100%), and virus isolation was positive only in 20 of the RT - PCR positive group (77%).* ***Conclusion:*** *both cultural and molecular methods are useful for detection of influenza A virus and its subtypes from respiratory samples. These results showed that influenza A virus was present at high rate in patients with acute respiratory infections and the causative subtypes were clearly identified.*

TRƯỜNG ĐẠI HỌC Y DƯỢC HUẾ
HUE COLLEGE OF MEDICINE AND PHARMACY

TẠP CHÍ Y DƯỢC HỌC

JOURNAL OF MEDICINE AND PHARMACY

Số 03
2011

Huế, 6-2011

CHẨN ĐOÁN VIRUS HỢP BÀO ĐƯỜNG HÔ HẤP GÂY NHIỄM TRÙNG HÔ HẤP DƯỚI BẰNG KỸ THUẬT RT-PCR TỔ

Lê Văn An[1], Huỳnh Hải Đường[1],
Nguyễn Chiến Thắng [1], Piero Cappuccinelli[2]
(1) Bộ môn Vi sinh, phòng thí nghiệm Carlo Urbani, Đại học Y Dược Huế,
(2) Viện Vi sinh thực nghiệm và lâm sàng, Đại học Sassari

Tóm tắt

Mục tiêu: Phát triển và áp dụng quy trình kỹ thuật RT-PCR tổ để chẩn đoán virus RSV gây nhiễm trùng hô hấp ở bệnh nhân nhi. **Phương pháp và đối tượng nghiên cứu:** Xây dựng và hiệu chỉnh quy trình kỹ thuật RT-PCR tổ (nested RT-PCR) để khuếch đại vùng gen F của genome RSV, thực hiện thực nghiệm với các mẫu chứng và áp dụng để chẩn đoán với các bệnh phẩm lâm sàng. **Kết quả nghiên cứu:** Kết quả thực hiện với các mẫu chứng cho thấy kỹ thuật khuếch đại đặc hiệu RNA của virus RSV mà không khuếch đại các genome của các tác nhân virus và vi khuẩn gây nhiễm trùng hô hấp thường gặp, ngưỡng xác định của quy trình là 10^2 bản sao. Áp dụng chẩn đoán trên 109 mẫu bệnh phẩm ngoáy họng của bệnh nhi nhiễm trùng hô hấp cho kết quả 27 (tỷ lệ 24,8%) bệnh nhi nhiễm trùng với RSV, trong đó viêm phế quản có 6/30 (20%), viêm tiểu phế quản có 7/26(27%) và viêm phổi là 14/53 (26,4%). **Kết luận:** Kỹ thuật RT-PCR tổ này là một phương pháp chẩn đoán hữu ích và tin cậy để chẩn đoán nhiễm trùng do virus RSV ở đường hô hấp.

Abstract

DETECTION OF RESPIRATORY SYNCITIAL VIRUS (RSV) IN LOWER ACUTE RESPIRATORY INFECTIONS BY NESTED REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Le Van An, Huynh Hai Duong,
Nguyen Chien Thang, Cappuccinelli P

Objective: To develop and apply a nested reverse transcription- polymerase chain reaction (nested RT-PCR) for detection of RSV in lower acute respiratory infections.

Materials and methods: A nested reverse transcription- polymerase chain reaction was used to amplify a sequence of the F gene in the RSV genomic RNA, optimized and compared the sensitivity and specificity of this assay with the control samples and then applied this procedure for diagnosing RSV from clinical samples. **Results:** This nested RT-PCR assay amplified the specific target fragment of RSV RNA and did not amplify any sequence of genomes of the tested common viruses and bacteria causing respiratory infections. The minimal level of detection of this procedure was 10^2 copies/ml. Results for detection of RSV on 109 samples of throat swabs or nasopharyngeal swabs from children with lower respiratory infections showed that twenty seven patients were positive with RSV (24.8%), among which six out of 30 (20%) were with bronchitis, seven out of 26 (27%) were with bronchiolitis and fourteen out of 53 (26.4%) were with pneumonia. **Conclusion:** This nested RT-PCR was found to be useful and reliable for detection of RSV in respiratory infections.

1. ĐẶT VẤN ĐỀ

Nhiễm trùng hô hấp do virus RSV rất thường gặp ở trẻ em, virus này gây nhiễm trùng hô hấp trên, rồi lan rộng xuống đường hô hấp dưới. Ở các trẻ em nhiễm trùng virus hô hấp dưới phải nhập viện thì RSV gây viêm tiểu phế quản ở 50 đến 90% trẻ em, viêm phổi từ 5 đến 40% và viêm thanh phế quản từ 10 đến 30% [2]. Trong một nghiên cứu theo dõi ở Hoa Kỳ cho thấy có đến 68,8% trẻ em nhiễm trùng trong năm đầu tiên, và tỷ lệ này đạt đến 82,6% trẻ nhiễm trùng vào năm thứ 2 và đến 3 tuổi thì 100% có nhiễm virus này [1]. RSV cũng là tác nhân virus gây nhiễm trùng xảy ra trong môi trường bệnh viện, nhiễm trùng thường xảy ra trong mùa dịch với tỷ lệ thay đổi từ 20% đến gần 50% [1], [2]. Các nhiễm trùng hô hấp nặng do RSV có thể đưa đến tử vong ở trẻ em nhỏ, nó còn gây nhiều biến chứng khác ở đường hô hấp. Chẩn đoán sớm nguyên nhân cho phép cách ly sớm để tránh lây lan ở trong môi trường bệnh viện.

Các phương pháp chẩn đoán virus học nhiễm trùng RSV gồm phân lập virus trên một số nuôi cấy tế bào đặc hiệu như Mv1Lu, Hep-2, MRC-5, tế bào Vero [1]. Phương pháp này chỉ làm ở những phòng thí nghiệm có trang bị, tỷ lệ dương tính không cao, kết quả thường chậm sau 5-7 ngày hoặc xác định kháng nguyên virus RSV trong dịch rửa hô hấp [5]. Gần đây nhiều phòng thí nghiệm đã phát triển các kỹ thuật RT-PCR để chẩn đoán virus học RSV ở bệnh phẩm lâm sàng, kỹ thuật này có độ nhạy và độ đặc hiệu cao trên 90% khi so sánh với nuôi cấy hay xét nghiệm tìm kháng nguyên virus. Hiện nay nhiều quy trình RT-PCR để chẩn đoán RSV được thương mãi hóa [1], [5], [9].

Để có được một xét nghiệm về virus học tin cậy để xác định các tác nhân virus gây nhiễm trùng hô hấp chúng tôi thực hiện xây dựng quy trình RT-PCR nhằm chẩn đoán virus RSV và sử dụng nó trong chẩn đoán bệnh phẩm ở lâm sàng. Bài báo này chúng tôi báo cáo kết quả việc áp dụng kỹ thuật này trong chẩn đoán nhiễm trùng RSV ở bệnh nhân nhi có nhiễm trùng hô hấp dưới tại Huế từ tháng 3 năm 2010 đến tháng 1 năm 2011.

2. VẬT LIỆU VÀ PHƯƠNG PHÁP NGHIÊN CỨU

2.1 Vật liệu nghiên cứu

Các mồi

Các cặp mồi được chọn nhằm khuếch đại vùng gen F của genome cả hai type virus RSV theo tác giả Rohwedder A và Mentel, theo kỹ thuật RT-PCR tổ (nested RT-PCR) với cấu trúc các mồi như dưới đây [6], [7].

Bảng 2.1. Cấu trúc các đoạn mồi được dùng

Tên đoạn mồi	Cấu trúc đoạn mồi	Vị trí đoạn mồi ở vùng gen F	Kích thước vùng khuếch đại
F1	GTTGGATCTGCAATCGCCAGTGGC	6090–6113	539 bp
F2	GTACATAGAGGGGATGTGTG	6609–6628	
F3	TTAACCAGCAAAGTGTTAGA	6222–6241	242 bp
F4	TTTGTTATAGGCATATCATTG	6443–6463	

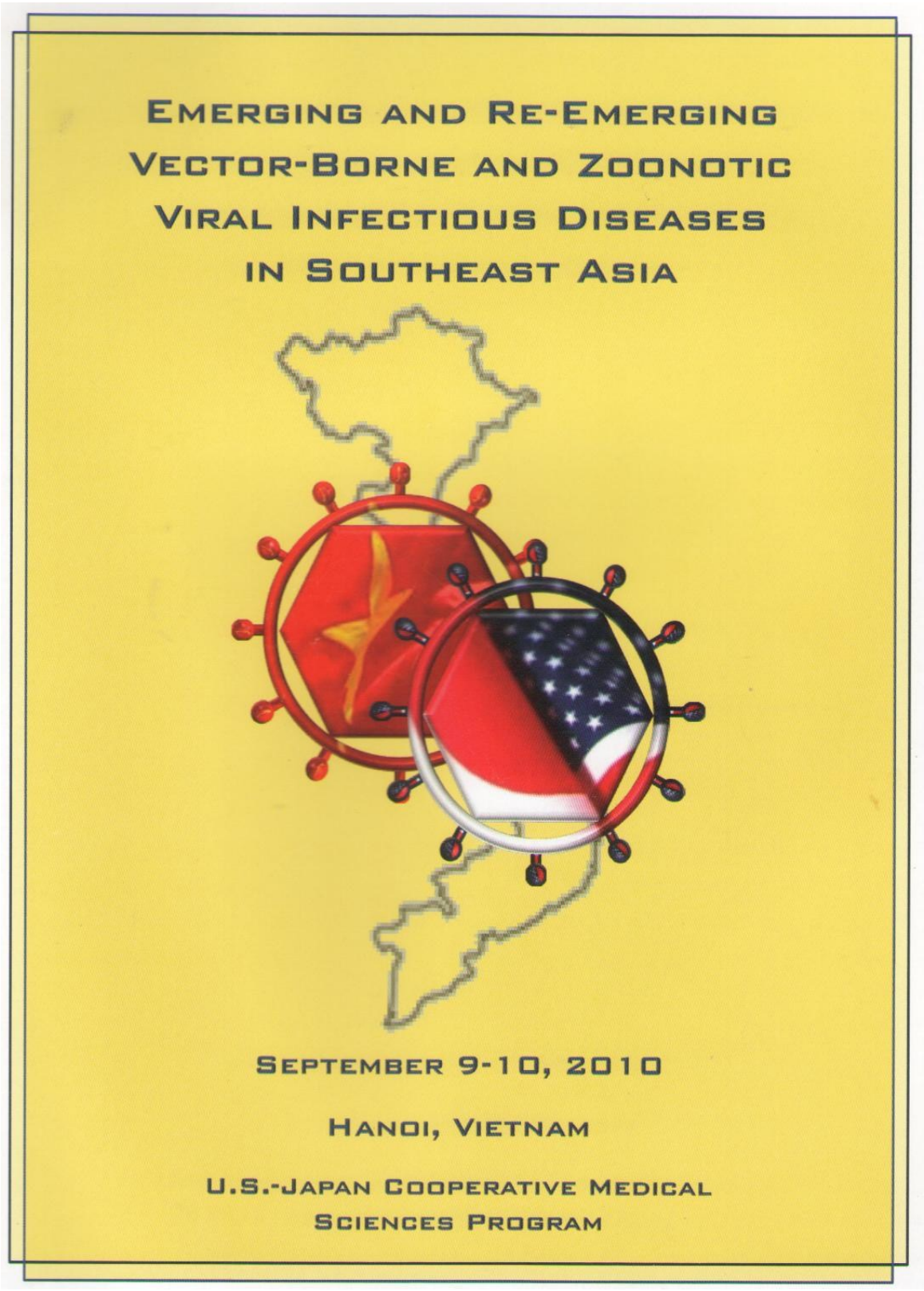
Sinh phẩm

Bộ sinh phẩm chuyển đổi ngược từ RNA sang cDNA của hãng Việt Phát, HCM (chứa RT, dNTPs, các mồi hexamer), Bộ sinh phẩm PCR master mix của hãng promega.

Chủng virus chứng

Các virus cúm A gồm các dưới type H1N1 mới, H1N1 mùa, H3N2, adenovirus, parainfluenza virus; các vi khuẩn *Staphylococcus aureus*, *Streptococcus*

A5-2: Poster presentation in the scientific meeting



P4. Diagnosis of influenza A virus and its subtypes in respiratory infections by virus isolation and reverse transcription – polymerase chain reactions

Le Van An, Le Thi Bao Chi, Huynh Thi Hai Duong
Hue College of Medicine and Pharmacy, Vietnam

Background: Influenza virus consists of three genera or types: influenza A virus, influenza B virus, and influenza C virus, they all cause acute respiratory infections in humans. Among them influenza A virus can frequently cause flu epidemics and pandemics in Humans. Influenza A viruses are classified into subtypes based on two surface antigens, haemaglutinin (H) and neuraminidase (N). there are 16 subtypes of hemaglutinin H and 9 subtypes of neuraminidase N. Only have some limited subtypes caused epidedmics and circulated in humans, namely H2N2, H3N2 and seasonal H1N1. However, some subtypes of influenza A virus of animal origin have recently caused infection in humans such as avian influenza virus (H5N1) and swine – origin influenza virus (H1N1). In this report, we present the preliminary results of virological diagnosis of influenza A virus and its subtypes by virus isolation and reverse transcription-polymearse chain reactions in patients suspected with swine-origin flu in Hue city in 2009.

Materials and Methods Patients: Forty samples of throat swab or nasal swab from 40 patients with acute respiratory infections suspected swine origin flu hospitalized in Hue city hospital from the beginings of October to the end of November, 2009 were enrolled in this study.

Methods: Two virological methods for detection of influenza viruses were set up in laboratory of microbiology of the Carlo Urbani centre of department of microbiology, HCMP- these are isolation of influenza virus on cell culture of MDCK and reverse transcription- polymerase chain reactions, in the later two multiplex RT-PCR procedures were set up, one procedure was carried out to amplify M gene of influenza A virus and H1 gene of swine origin influenza A virus (H1N1), the other procedure was to amplify H1 gene of seasonal H1N1 subtype; H3 gene of H3N2 subtype and H5 gene of H5N1 subtype. Each sample of nasal or throat swabs in viral transport medium was partly inoculated into MDCK in D-MEM growth medium and followed for cytopathogenic effect, virus growth was analysed further by the RT-PCRs for confirmation and subtype identification. The rest part of sample was extracted for virus RNA and amplified by both of the RT-PCR procedures to identify influenza A virus and subtypes.

Results: Forty patients were for viral detection and 26 patients (65%) were positive with influenza A virus by isolation and /or RT-PCRs; RT-PCRs were positive in all the 26 positive samples (100%), and virus isolation was positive only in 20 of the RT-PCR positive group (77%). Pandemic H1N1 swine virus was detected in 20 patients (77%) and seasonal H1N1 influenza A virus was positive in other 06 patients (23%), no case of H3N2 and H5N1 was identified in this patient group.

Conclusion: both cultural and molecular methods are useful for detection of influenza A virus and its subtypes from respiratory samples. These results showed that influenza A virus was present at high rate in patients with acute respiratory infections and the causative subtypes were clearly identified.

Acknowledgements

I am very thankful to my supervisor, Prof. Pietro Cappuccinelli, who for many years has been working with me as a highly respected colleague in medical microbiology, an advisor in academic environment, a kind professor and supervisor for my scientific research for Ph. D education in Sassari University. During ten years of collaboration with Hue College of Medicine and Pharmacy in Viet Nam, he has always found all the ways to support for the development and improvement of the facilities of our microbiological laboratory, the capacity and qualification in scientific research and training of the scientist staffs of Hue College of Medicine and Pharmacy and especially of the Department of Medical Microbiology.

I would like to express my gratitude to Prof. Bruno Masala for his kind support to my study in the Ph.D School of Sassari University.

I would like to acknowledge the scholarship and the generous financial support from the Government of Italy that granted through the project of Carlo Urbani, my research is impossible without all the supports of the project; I am grateful to the project managers, namely, Dr. Stefano Ferroni, Dr. Chiodi Daelli Federico in that they always provided facility support for my research and all conditions for travels and stay in Sassari University.

I would like to thank the Rector, Prof. Dr. Cao Ngoc Thanh, and the board of directors of Hue College of Medicine and Pharmacy and Hue University for creating favorable conditions for me to pursue the Ph. D study in Sassari University.

My acknowledgements also go to my staffs in the Department of Medical Microbiology in Hue College of Medicine and Pharmacy for their close collaborations in laboratory works, namely, Dr. Le Thi Bao Chi, B.Sc. Huynh Thi Hai Duong, M.Sc. Nguyen Hoang Bach, B.Sc. Nguyen Chien Thang.

Many thanks go in particular to clinical doctors in the Department of Pediatric of Hue Central Hospital, namely Dr. Dinh Quang Tuan, Dr. Le Thi Ngoc Viet, Dr. Phan Thi Bich Chi for their collaboration in clinical work and collection of respiratory specimens. My acknowledgements also go to Dr. Tran Bui, Dr. Nguyen Thanh, Hue City Hospital for their collaboration in collection of respiratory

specimens of the outbreak of S-OIV H1N1 2009 infection.

I take this opportunity to express my gratitude to prof. Dr. Rubino Salvatore, prof. Dr. David Kelvin, Dr. Alberto Arberti, Dr. Maura, Piero s‘ wife Mrs. Scilla and his children for their Generosities, friendship and hospitality during my stay in Sassari of Sardegna.

I would like to thank all my closed friends and people who have contributed their roles to the successful implementation of thesis, as well as expressing my apology that I could not mention personally one by one.

Finally, I am deeply indebted to my family: my parents, brothers and sisters they are always an immense encouragement source throughout my entire life. My wife and daughters, their love and support without any complaint or regret has enabled me to complete my Ph. D research, I owe them my every achievement.

Sassari, October 2011