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DISSERTATION

Viral pathogens associated with lower respiratory tract infection

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Dedicated to .....

My land,

My family

Mona Embarek
2010

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1. Introduction

1.1. Disease burden and epidemiological facts

Lower respiratory tract infections are those affecting the trachea, bronchial tree or lung parenchyma (fig.1). Respiratory infections are the most common infections in humans (Huetas *et al*., 2005; Minosse *et al*., 2008) and a major cause of infant morbidity worldwide (Regamey *et al*., 2008). Although lower respiratory tract infections (LRTIs) are less frequent than upper respiratory tract infections (URTIs), the cost of LRTI is higher (between 1 and 13% of patients with LRTI are hospitalised) (Templeton, 2007). According to the WHO fact sheet (2008), LRTI is the first in rank of the top ten causes of death in low-income countries for the year 2004 (accounting for 11.2% of the total number of deaths) and the fourth cause of death in middle and high-income countries (accounts for 3.8%).

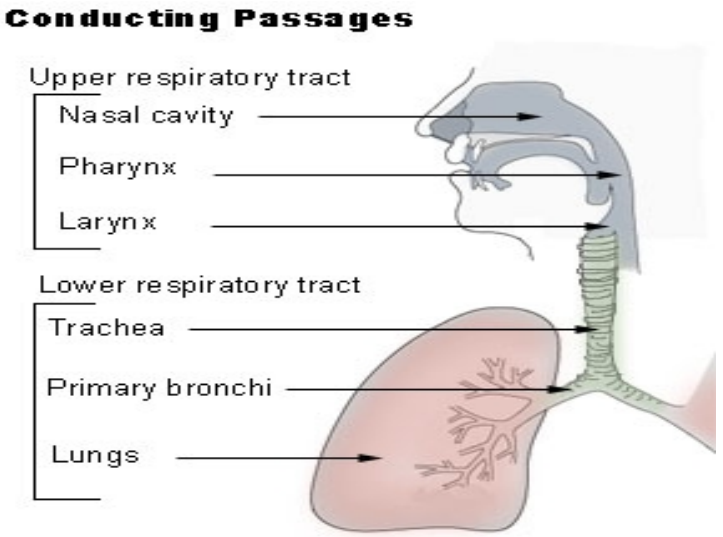

Fig .1: The respiratory system (adapted from http://en.wikipedia.org/wiki/Larynx)

Respiratory viruses are the most frequent cause of LRTIs in infants and young children (Artiles-Campelo *et al*., 2006; Calvo *et al*., 2008) and play an important role in LRTI affecting individuals of all age groups and immunocompromised patients (El-Mahallawy *et al*., 2005; Tsuchiya *et al*., 2005; Li *et al*., 2007). They are responsible for 60% or more of LRTIs (Elliott and Ray, 2004). Viruses accounted for 50 to 90% of LRTIs in children younger than five years in Brazil (Thomazelli *et al*., 2007). Creer *et al*. (2006) reported an identification rate of 63% for respiratory viruses associated with acute adult LRTI in contrast to 26% of bacteria in patients attending primary care. Viral etiology was identified in 31.8% of patients suffering from LRTI (Freymuth *et al*., 2004). Respiratory viruses were also associated with 50% of respiratory tract infections of lung transplant recipients with predominance of human metapneumovirus in bronchoalveolar lavage of those patients suggesting its causative role in LRTIs (Gerna *et al*.,

2006). Täger and colleagues (2006) aimed to estimate the frequency of respiratory viral infection in children with acute lymphoblastic leukemia in Chile; respiratory viruses were detected in 25% of those children during neutropenic episodes. Over 80% of identified LRTI in young children are attributed to six viruses: human respiratory syncytial virus, human parainfluenza viruses 1, 2, and 3; influenza A and B viruses and human metapneumovirus (Erdman *et al*., 2003).

Infections with respiratory syncytial virus (RSV), parainfluenza viruses (PIV), influenza viruses or rhinoviruses occurred at a rate of 4.5 % per year in bone marrow transplantation (BMT) recipients at the Fred Hutchinson Cancer Research Center in Seattle, USA, between 1990 and 1996 (Bowden, 1997). Healthy young adults are also more vulnerable to respiratory viruses than previously recognized. PCR testing of over 2000 nasopharyngeal swabs from adults presenting with influenza-like illness demonstrated that 30% were infected with influenza virus and 20% with RSV (Zambon *et al*., 2001). In 1994, Salih and others detected 83 viral infections in 213 children affected by LRTI in Sudan. The most affected group were children under five years old (79), most of whom were affected by RSV (28%), followed by parainfluenza (7%), adenovirus (5%) and influenza (2%). Influenza virus and RSV were found in 17 (34%) out of 47 patients affected by LRTI in a geriatric unit in France during 2005-2006 (Haber *et al*., 2009).

Accurate detection of respiratory viruses is important to guide antiviral therapy, prevent nosocomial spread, provide surveillance, and decrease hospital costs and lengths of stay (Kuypers *et al*., 2006).

1.2. Implications of respiratory viruses to LRTI

1.2.1. Pneumonia

Pneumonia is the infection of the distal lower respiratory tract, principally the alveolar space, including the small bronchi and bronchioles (Wunderink and Mutlu, 2006). About 150 million children around the world are affected by pneumonia on an annual basis (Bhutta, 2007) and more than two million deaths per year in children under 5 years of age are caused by pneumonia (Wardlaw *et al*., 2006). Pneumonia is a leading killer of children in developing countries (Ranganathan and Sonnappa, 2009) particularly in Africa, where the incidence of pneumonia parallels that of human immunodeficiency virus infection (Bhutta, 2007). In the developed world, pneumonia affects approximately 3% to 4% of children, a rate that is substantially higher than for adults (Colin, 2006). The role of viruses in community-acquired pneumonia (CAP) is very well documented (Roux *et al*., 2004; Angeles Marcos *et al*., 2006). Viral infection accounts for a substantial proportion of cases of acute pneumonia especially among young children and the elderly, immunocompromised, and those with co-morbidities. Influenza A and RSV is by far

the most common cause of viral pneumonia followed by adenovirus (AdV), human metapneumovirus (hMPV), parainfluenza virus (PIV) types 1, 2 and 3, and influenza B (Hui and Chan, 2006). RSV is a major pathogen in pneumonia studies accounting for between 14 and 40% of viral isolations in the United Kingdom **(**Farha and Thomson, 2005). Infants and children with congenital, acquired or chemotherapy-induced states of immunodeficiency are at high risk for the development of severe and prolonged RSV bronchopulmonary disease with a mortality rate between 15% and 78% (Chandwani *et al*., 1990; Harrington *et al*., 1992). Shedding of RSV for as long as 112 days has been documented in immunocompromised patients compared to 21 days in normal subjects (Chandwani *et al*., 1990). Pneumonia complicating RSV infection is almost exclusively viral in origin, whereas influenza virus may cause both a primary viral and a secondary bacterial or fungal pneumonia (Geretti, 2003). A case of fatal hemorrhagic pneumonia caused by human metapneumovirus in an immunocompetent 2-year-old girl was reported by Donoso and others (2008). Mathisen *et al*. (2009) investigated 2230 Nepalese children (from 2 to 35 months old) with CAP over a three-year period. Nine hundreds nineteen (919) viruses were isolated from 887 (40.0%) out of the 2,219 nasopharyngeal aspirates (NPA) with a valid PCR result, of which 334 (15.1%) yielded RSV, 164 (7.4%) influenza A, 129 (5.8%) PIV type 3, 98 (4.4%) PIV type 1, 93 (4.2%) hMPV, 84 (3.8%) influenza B, and 17 (0.8%) PIV type 2. The largest peaks of pneumonia occurrence coincided with peaks of RSV infection. In late March 2009, an outbreak of respiratory illness caused by novel swine-origin influenza A (H1N1) virus (S-OIV) was identified in Mexico (Perez-Padilla *et al*., 2009), where a total of 18 cases of pneumonia and confirmed S-OIV infection were identified among 98 patients hospitalized for acute respiratory illness at the National Institute of Respiratory Diseases in Mexico City from March 24 through April 24, 2009.

1.2.2. Bronchiolitis

Bronchiolitis is a potentially life-threatening infection affecting young children. The most common cause of bronchiolitis is RSV, which accounts for 2% to 3% of hospital admissions for infants aged less than one year. Other pathogens causing bronchiolitis include parainfluenza, influenza, and adenovirus. RSV bronchiolitis in infancy is severe enough to cause hospitalization and is a risk factor for allergic asthma in early adolescence (Sigurs *et al*., 2005). Co-infection with hMPV and RSV has been shown to cause a more severe bronchiolitis where it confers a ten-fold increase in relative risk (RR) of admission to a pediatric intensive-care unit for mechanical ventilation (Semple *et al*., 2005). Co-infection with hMPV was detected in 70% of 30 infants

suffering from severe respiratory syncytial virus bronchiolitis, such co-infection may be a factor influencing the severity of bronchiolitis in those infants (Greensill *et al*., 2003).

1.2.3. Acute exacerbations of bronchial asthma

Viral respiratory infections are the factor most frequently associated with asthma. There is a strong temporal association between viral respiratory infections and acute obstructive/asthmatic episodes (Castro-Rodríguez, 2007). Severe viral LRTI during the first years of life are associated with an increased risk of developing asthma in childhood (Illi *et al*., 2001; Sigurs *et al*., 2005). Gelfand (2000) estimated that more than 80% of acute exacerbations of asthma in children and at least 30% to 40% of exacerbations in adults with asthma are caused by viruses. Weinberger (2004) demonstrated that RSV in young children and rhinoviruses in older children and adults are the major causes of acute exacerbations of asthma.

1.2.4. Acute exacerbations of chronic obstructive pulmonary disease (COPD)

Exacerbation of COPD is defined according to the GOLD criteria (2006) as an event in the natural course of the disease characterized by a change in the patient's baseline dyspnea, cough, and/or sputum that is beyond normal day-to-day variations, which is acute in onset. Respiratory viruses are a common trigger for exacerbation of COPD (Rohde *et al*., 2003; Beckham *et al*., 2005; Martinello *et al*., 2006; Anzueto *et al*., 2007). While influenza virus appears to be the most frequently detected virus in acute exacerbations of COPD, RSV is also a leading cause of virus-induced exacerbation (De Serres *et al*., 2009). Greenberg (2002) stated that patients with COPD are prone to the development of significant lower respiratory tract symptoms from colds caused by viral respiratory pathogens. Viral infections with 10 influenza A (9%), 8 RSV (7%), 7 PIV-3 (6%), 4 hMPV (4%) and 3 rhinoviruses (3%) were detected in 34 (31%) out of 108 patients (De Serres *et al*., 2009). Respiratory viral infections were identified in 16 out of 34 (46.7%) exacerbations. Rhinovirus was cultured from four subjects, PIV type 3 from three subjects, and influenza A/H3N2 from one subject (Bandi *et al*., 2003). McManus *et al*. (2008) aimed to determine the incidence of viral infection in exacerbated and stable COPD patients in Ireland, respiratory viruses were detected in 37% of exacerbations, and 12% of stable COPD cases. Rhinovirus was the most common infecting agent identified and in two cases hMPV was detected.

1.2.5. Respiratory viruses and exacerbation of cystic fibrosis (CF)

Viral infections are often associated with severe respiratory symptoms in CF patients. In 13–52% of patients with an increase in lower respiratory tract symptoms, a viral pathogen was detected, with higher percentages in younger than in older patients (van Ewijk *et al*., 2005).

Twenty nine viral respiratory diseases were detected serologically by testing 275 sporadically collected sera from 75 patients with cystic fibrosis over a period of 30 months. Influenza A virus was the most frequent responsible viral pathogen (eleven times), followed by adenovirus (eight times), influenza B virus (five times), PIV type 3 (three times), PIV type 1 and RSV (one each) (Przyklenk *et al*., 1988). Whereas Hordvik *et al*. (1989) recorded that up to 77% of exacerbation in patients of cystic fibrosis were caused by influenza A and B viruses. Respiratory viruses were found in 16/31 (52%) among children with cystic fibrosis in Australia with predominance of RSV which was found in seven infants (Armstrong *et al*., 1998). The annual incidence of admissions per patient affected with cystic fibrosis that were associated with viral infection was 4.9% (Datta *et al*., 2008). Respiratory viruses were also detected in 63 (46%) out of 138 respiratory samples that obtained from 71 patients suffering from exacerbations of CF. In contrast, 23 out of 136 asymptomatic nasal swabs (16.9%) were positive for respiratory viruses. There were significantly more viruses being detected during respiratory exacerbations, in particular influenza A, influenza B and rhinovirus (Wat *et al*., 2008).

1.2.6. Predisposing for secondary bacterial infection

The majority of deaths in the 1918-1919 influenza pandemic likely resulted from secondary bacterial pneumonia (Morens *et al*., 2008; Fedson, 2009). Many factors are involved in the phenomenon of bacterial superinfection during viral respiratory disease. They include physical damage to the local respiratory physical barriers, up-regulation of genes that code for toxins, enhanced expression of receptors and many other factors participating in opportunistic adherence of bacteria (Brundage, 2006). Bacterial superinfection in influenza patients is the primary cause of increased mortality during influenza pandemics, with *Staph. aureus* having the highest fatality rate (Braun *et al*., 2007). Positive evidence (both epidemiological and experimental) for a crucial role of preceding viral infection in the etiology of bacterial respiratory infections was found. The etiology of community-acquired pneumonia in children with viral infection was assessed by serology. It was found that 39% of those children had a bacterial co-infection. Conversely, 20% of the children with a bacterial infection showed evidence of a prior viral infection. The most frequent combination for children under the age of five years was *Strept. pneumoniae* with RSV (Heiskanen-Kosma *et al*., 1998). The incidence of both viral and pneumococcal disease peaked in the winter months (Kim *et al*., 1996). An adherence assay to verify increased susceptibility of mammalian cells to bacterial adherence as a result of viral infection was first introduced in 1978. MDCK monolayer cells were exposed to various streptococcal strains and it was found that adherence of group B *Streptococcus* and various streptococcal species was only to the cells

infected with influenza virus and not to membranes of uninfected cells (Sanford *et al*., 1978). In subsequent adherence studies with *Staph. aureus*, it was found that pre-infection of MDCK cells with influenza A virus significantly enhanced bacterial adherence to monolayer cells. This effect varied depending on the virus and bacterial strain tested (Davison and Sanford, 1981). The synergy of a viral and bacterial infection compared to infection with a sole agent was studied in a cotton-rat model. The physiologic and pathologic changes in cotton rats infected with both *Staph. aureus* and influenza A virus were compared with those of animals infected with each pathogen alone. It was found that co-infected cotton rats demonstrated higher mortality (Braun *et al*., 2007). Incidence rates of invasive pneumococcal disease were shown to be associated with increased incidence of influenza viruses, RSV, adenovirus and parainfluenza virus 3 during a period of ten years in New Zealand (Murdoch and Jennings, 2009). A case of rapidly fatal necrotizing pneumonia in a 12 year old boy was caused by co-infection with parainfluenza virus type 1 and *Staphylococcus aureus* (Wenzel *et al*., 2009). Mixed viral-bacterial infections were associated with antibiotic treatment failure (Bakaletz, 1995).

1.3. Respiratory viruses

1.3.1. Influenza viruses

The influenza A, B, and C viruses represent three of the five genera of the family *Orthomyxoviridae* (Nelson and Holmes, 2007). Influenza A is antigenically highly variable and is responsible for most cases of epidemic influenza. Influenza B is also characterized by antigenic changes but causes epidemics only every 5-7 years. Influenza C is antigenically stable and causes only mild illness in sporadic cases (Brooks *et al*., 2007).

1.3.1.1. Structure of influenza viruses

The single-stranded, negative-sense RNA (ssRNA) genomes of influenza A and B viruses consist of eight separate segments while influenza C viruses contain seven segments of RNA, lacking a neuraminidase gene (Nelson and Holmes, 2007). Most of the segments code for a single protein as the PB2, PA, HA, NA, and NP genes (Brooks *et al*., 2007). Influenza viruses have a complex structure and possess a lipid membrane derived from the host cell. This envelope harbours the hemagglutinin (HA), the neuraminidase (NA), and the M2 proteins that project from the surface of the virus (Fig. 2). The matrix protein (M1) is located just beneath the envelope. The core of the virus particle is comprised of the ribonucleoprotein (RNP) complex, consisting of the viral RNA segments, the polymerase proteins (PB1, PB2 and PA), and the

nucleoprotein (NP) (Palese and Shaw, 2007). The nuclear export protein/non-structural protein 2 (NEP/NS2) is also present in purified viral preparations (Richardson and Akkina, 1999).

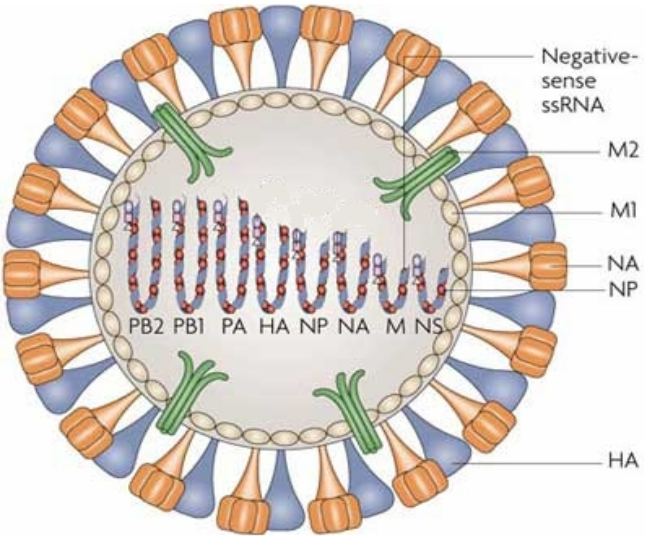

Fig. 2: Schematic structure of influenza virus
HA: hemagglutinin; M1: matrix protein 1; M2: matrix protein 2; NA: neuraminidase; NP: nucleocapsid protein; NS: non-structural protein; PA: polymerase; PB1: binding protein 1; PB2: binding protein 2. Adapted from Nelson and Holmes (2007).

The genome encodes eleven proteins, PB2, PB1, PB1-F2, PA, HA, NP, NA, M1, M2, NS1 and NS2 (Nelson and Holmes, 2007) (Fig. 2). The influenza virus hemagglutinin is a membrane-anchored, trimeric glycoprotein that mediates the attachment of virus to cell-surface receptors containing *α*-5-*N*-acetylneuraminic acid (sialic acid) thereby initiating viral entry and infection (Watowich *et al*., 1994). The antigenic determinants of HA are located in the 3´ portion (HA1) of the genomic RNA, which is approximately 1 kb in length (Zou, 1997). The neuraminidase is the second major glycoprotein of the influenza viruses. It specifically liberates sialic acid from those glycol-conjugates, thereby destroying virus-receptor-binding and facilitating release of newly formed virions from the cells (Colman, 1994). The two glycoproteins, HA and NA, exhibit substantial antigenic variation among influenza A viruses with sixteen HA subtypes (H1-H16) and nine NA (N1-N9) subtypes. Influenza B is not differentiated into subtypes (Kesson, 2007). Influenza virus strains are named according to their genus (type), the species from which the virus was isolated (omitted if human), location of isolate, the number of the isolate, the year of isolation, and in the case of the influenza A viruses, the hemagglutinin (HA) and neuraminidase (NA) subtypes (Bouvier and Palese, 2008). For example, the 921th isolate of an influenza A virus isolated from human in Nepal in 2006 is designed: A/Nepal/921/2006 (H3N2).

1.3.1.2. Evolution of influenza A viruses

There is a hypothesis that aquatic birds are the primordial source of all influenza viruses in other species. This is supported by the phylogenetic analysis together with the findings that viruses of all known HA and NA subtypes are maintained in avian species (Webster *et al*., 1992).

The two surface antigens of influenza viruses undergo antigenic variation independently of each other. Minor antigenic changes are termed antigenic drift; major antigenic changes in HA and/or NA are called antigenic shift. Antigenic shift is most likely to result in an epidemic (Brooks *et al*., 2007). Vaccines derived from inactivated influenza viruses have been used for prevention and control of influenza infection. However, changes in the envelope glycoproteins give rise to antigenic variants (Cox and Bender, 1995). Consequently, vaccines effective in previous seasons eventually may not be protective for future epidemics (Zou, 1997). Retrospective phylogenetic studies showed that viruses similar to vaccine strains circulated one or two years before a given strain was recommended as vaccine strain (Schweiger, 2006).

1.3.1.2.1. Antigen drift of influenza A viruses

Antigenic drift occurs as a result of point mutations in influenza viruses and refers to minor, gradual antigenic changes in the HA or NA proteins (Palese and Shaw, 2007). The HA protein is the major antigenic component of influenza viruses. Five antigenic domains (A-E) were identified for H3 viruses (Webster and Laver, 1980; Wiley *et al*., 1981). For H1 viruses, the antigenic sites are designated Ca1, Ca2, Cb, Sa, and Sb (Gerhard *et al*., 1981). Single point mutations in one HA antigenic site can be sufficient for antigenic variation (Wiley *et al*., 1981; Wilson *et al*., 1981; Wiley and Skehel, 1987). Antigenic drift has also been reported for the NA molecule (Paniker, 1968). Studies with monoclonal antibodies and amino acid sequence analysis have revealed two to three antigenic sites in NA molecule (Air and Laver, 1989). Two major antigenic sites are located on the outer surface of the molecule and a possible third antigenic site resides on the bottom of the head of the NA molecule (Palese and Shaw, 2007).

1.3.1.2.2. Reassortment of influenza A viruses

Because of the segmented nature of the genome of influenza viruses, when a cell is co-infected by two different viruses of a given type, mixtures of parental gene segments may be assembled into progeny virions. This phenomenon is called genetic reassortment (antigenic shift) and it may result in sudden changes in viral surface antigens (Brooks *et al*., 2007). There have been two pandemics the last 40 years, the Asian flu in 1957 (H2N2), and the Hong Kong flu in 1968 (H3N2) (Bragstad *et al*., 2008). The Asian flu in 1957 was a reassortant virus containing three segments from an avian strain (PB1, HA, and NA) and the other five from the virus that was

already circulating in the human population (H1N1) (Rabadan and Robins, 2007). Virological and molecular analysis revealed that these new pandemics emerge through reassortment with strains from the avian reservoir causing an antigenic shift. H3N2 and H1N1 influenza A viruses co-circulated in the human population since the re-emergence of H1N1 in 1977 (Bragstad *et al*., 2008). A reassortant H1N1 virus with the polymerase and NP genes derived from a H3N2 virus circulated in several countries from 1978 to 1980 (Young and Palese, 1979; Nakajima *et al*., 1981). The H3N2 has been the predominant influenza A strain during the last 20 years, with the exception of the 1988-1989 and 2000-2001 seasons where H1N1 infections dominated (Lin et al., 2004). New drift variants of H3N2 viruses with significantly changed antigenic features appeared during the seasons 1997/1998 and 2002/2003 (Schweiger, 2006). In the 2000-2001 season a new reassortant human strain, H1N2 emerged in Europe and became established in the autumn 2001 (Paget *et al*., 2002; Ellis *et al*., 2003). In 2002, a new lineage A/Fujian/411/02-like emerged in Asia and caused significant outbreaks on every continent (Barr *et al*., 2005; Chi *et al*., 2005).

1.3.1.3. Evolution and reassortment of influenza B viruses

Two antigenically distinct lineages of B viruses have established themselves in the early 1980s. The “Victoria-lineage” represented by B/Victoria/2/87 and the “Yamagata-lineage” represented by the strain B/Yamagata/16/88 (Yamashita *et al*., 1988; Kanegae *et al*., 1990; Rota *et al*., 1990). The two lineages co-circulated globally since the early 1980s for more than 10 years. The B/Victoria/2/87-lineage predominated during the 1980s and subsequently, the B/Yamagata/16/88 -lineage circulated during the 1990s with disappearance of the Victoria-lineage viruses from the northern hemisphere although they continued to circulate in the southern hemisphere, but did not play an important role (Yamashita *et al*., 1988; Nerome *et al*., 1998). During the 2001-2002 influenza season, virological surveillance highlighted the predominant circulation of B viruses with the re-emergence of B/Victoria/2/87-lineage viruses represented by B/Hong Kong/330/01, closely related to B viruses prevalent during the 1980s in the northern hemisphere and have become the predominant B viruses circulating worldwide for some years (Ansaldi *et al*., 2003).

Phylogenetic analysis of those viruses showed that they were reassortants of two influenza B lineages, the B/Victoria/2/87 and B/Yamagata/16/88 lineage. The HA gene derived from the B/Victoria/2/87 lineage whereas the NA gene derived from the B/Yamagata/16/88 lineage. An influenza B virus belonging to the B/Victoria/2/87 lineage was included in the 2002-2003 influenza vaccine (Paget *et al*., 2002). Both B lineages co-circulated in Europe during the season 2004-2005, but in different prevalence rates among different countries (EISS Weekly Electronic

Bulletin, 2005). Antigenic variants of influenza B viruses have been isolated continuously during periods of widespread influenza virus activity since its first isolation in 1940 and also the Yamagata and Victoria lineage viruses circulate since the 1980s (Rota *et al*., 1992). Estimates of the rate of evolution have shown that the HA and NA of B viruses evolve more slowly than those of A viruses (Yamashita *et al*., 1988; Nerome *et al*., 1998; Kanegae *et al*., 1990 and Hay *et al*., 2001).

1.3.2. Respiratory syncytial virus (RSV)

Human respiratory syncytial virus is a member of the *Pneumovirus* genus of the family *Paramyxoviridae* (Collins and Crowe, 2007). RSV is the most important pathogen causing upper and lower RTI in small children and is a major cause of morbidity in children under one year of age (Östlund *et al*., 2008).

1.3.2.1. Structure of respiratory syncytial virus

RSV is an enveloped virus with a negative-sense, nonsegmented, single-stranded RNA genome which encodes the synthesis of at least eleven viral proteins. There are three transmembrane glycoproteins: the attachment glycoprotein (G), the fusion glycoprotein (F) and the small hydrophobic protein (SH). There are two non-structural proteins, NS1 and NS2 (Sullender, 2000). The viral RNA is associated with four nucleocapsid/polymerase proteins: the nucleoprotein N, the phosphoprotein P, the transcription processivity factor M2-1, and the large polymerase subunit L. The virus appears as an irregular spherical particle of 100 to 350 nm in diameter when visualized with the electron microscope (Collins and Crowe, 2007) (Fig. 3).

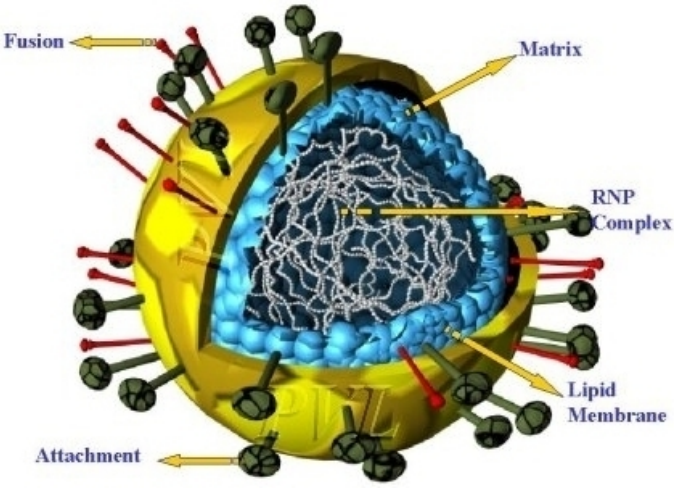

Fig. 3: Pneumovirus.
Adapted from department of Biological Sciences, University of Warwick (The group of respiratory syncytial virus & other pneumoviruses).

Two viral proteins, the attachment glycoprotein G and the surface glycoprotein F, are the main antigens responsible for inducing a neutralizing immune response and resistance to infection (Collins and Crowe, 2007). The G protein is a type II integral protein of 289 to 299 amino acids, depending on the viral strain (Martínez *et al*., 1999). The mature 90-kDa form of the G protein is obtained through extensive glycosylation of a 32-kDa polypeptide precursor by the addition of N-linked sugars to Asn residues and O-linked sugars to Ser and Thr residues (Wertz *et al*., 1985 and Wertz *et al*., 1989).

1.3.2.2. Genetic diversity of RSV

RSV has a non-segmented RNA genome. Thus, it does not have the capacity for reassortment of genome segments, the process by which influenza viruses undergo antigenic shifts leading to influenza virus pandemics. However, as with other RNA viruses, RSV has a quite mutable genome by virtue of its dependence on an RNA polymerase that lacks the capacity of RNA proof -reading and editing (Sullender, 2000). RSV strains are separated into two major groups based on antigenic and genetic variability. The main differences between RSV groups A and B were found in the attachment glycoprotein G. Variability in this protein is greater than that in the other proteins, both between and within the major antigenic groups of RSV (Reiche and Schweiger, 2009) and thus contributing to the ability of the virus to cause re-infections and annual epidemics (Madhi *et al*., 2003). The predominance of RSV-A over RSV-B viruses has been attributed to the higher variability among the RSV-A strains. Differences in the extent of intragenetic diversity in the G protein between the two RSV subgroups may reflect differences in the evolutionary patterns of these viruses (Zlateva *et al*., 2005). Ten RSV group A genotypes have been described until now, eight of these genotypes are named GA1 to GA7, and South Africa A1 (SAA1) (Peret *et al*., 2000; Venter *et al*., 2001). The other two were novel genotypes, named NA1 and NA 2 and they were genetically close to GA2 strains (Shobugawa *et al*., 2009). RSV strains isolated in Chile were compared regarding their antigenic and genetic variability using a panel of monoclonal antibodies against G glycoprotein epitopes (EIA) and RFLP for N and G genes. No differences of RSV groups A/B in N gene patterns were observed. On the other hand, antigenic and genetic G gene patterns displayed a wide diversity of strains circulating during the year 2002 (Luchsinger *et al*., 2008). Comparisons among two of group A viruses, A2 and Long strain, and a group B virus, CH -18537 revealed that the G proteins of the two group A viruses were very similar (6% amino acid differences), whereas the group A and group B viruses had extensive differences (47% amino acid differences) (Johnson *et al*., 1987).

Comparative data were reported by Sullender and others (1990). Genetic similarity within the group was about 98%, whereas the group A and B G-protein gene sequences were quite divergent (44% amino acid differences). The prevalence and circulation of genotypes varied from country to country. In South Africa, some genotypes (GA5, GB3 and SAB3) were found over four consecutive seasons from 1997 to 2000 while the incidence of other genotypes (GA7, SAB1 and SAB2) was low in some seasons. Subgroup A showed a gradual build-up and then replacement of dominant genotypes, for example, GA5 (1997) was replaced by SAA1 (1998), which was then replaced by GA2 (1999). GA2 predominated for more than one season, increasing from 42% (1999) to 78% (2000) (Venter *et al*., 2001). In Germany, RSV group A was dominant in seven out of nine epidemic seasons. Phylogenetic analysis revealed that RSV group A genotypes GA2 and GA5 circulated from 1998 to 2007. Genotype GA7 was present in only two seasons (1999 to 2000 and 2002 to 2003). Comparison of the synonymous mutation/nonsynonymous mutation ratios showed greater evidence for selection pressure of genotype GA2 more than for GA5 (Reiche and Schweiger, 2009). An analysis of RSV circulating in Stockholm during the 2002-2003 season showed that 152 out of 234 viruses belonged to subgroup B and 82 to subgroup A. The subgroup A viruses could be further divided into genotypes GA2 (25) and GA5 (57) and the subgroup B viruses into GB3 and SAB1 strains. These strains clustered with subgroup A and subgroup B strains from Kenya within the same period, as well as with strains from Great Britain during 1995 to 1998. Only two genotypes of subgroup A, GA2 and GA5, were circulating during this time, and GA2 has been circulating in Sweden for more than 20 years (Östlund *et al*., 2008).

1.3.3. Human adenoviruses (hAdV)

Human adenovirus belongs to the genus *Mastadenovirus* of the family *Adenoviridae* (Berk, 2007). Adenoviruses are a common cause of respiratory tract infections especially in young children (Gu *et al*., 2003; Walls *et al*., 2003) and have been identified as a cause of outbreaks in institutional settings (Wong *et al*., 2008). Up to 10% of LRTIs in pediatric population are caused by adenoviruses (Moura *et al*., 2008).

1.3.3.1. Structure of adenoviruses

Adenoviruses are nonenveloped, icosahedral particles about 90 nm in diameter with fibers projecting from the vertices of the icosahedrons. The DNA is linear, double-stranded and non-segmented. The outer structure of the virus is comprised of 240 hexons and 12 pentons at

vertices of the icosahedron. Adenovirus fibers of species-specific lengths extend from the penton and are associated with hemagglutination properties (Berk, 2007).

The eleven structural proteins are designated II to XII in order of their decreasing apparent molecular mass, and the major soluble antigens characteristic of adenovirus infections are named hexon, penton, and fiber. Hexon, pentone and fiber are the major proteins of the viral coat (fig. 4). Twelve copies of the hexon trimer (polypeptide II) form each of the 20 triangular facets of the capsid. The pentameric penton base (polypeptide III) and trimeric fiber (polypeptide IV) form complexes called pentons at each of the 5-fold vertices. Polypeptide IIIa, polypeptide VI, polypeptide VIII, and Polypeptide XI are the minor coat proteins (Rux and Burnett, 2004).

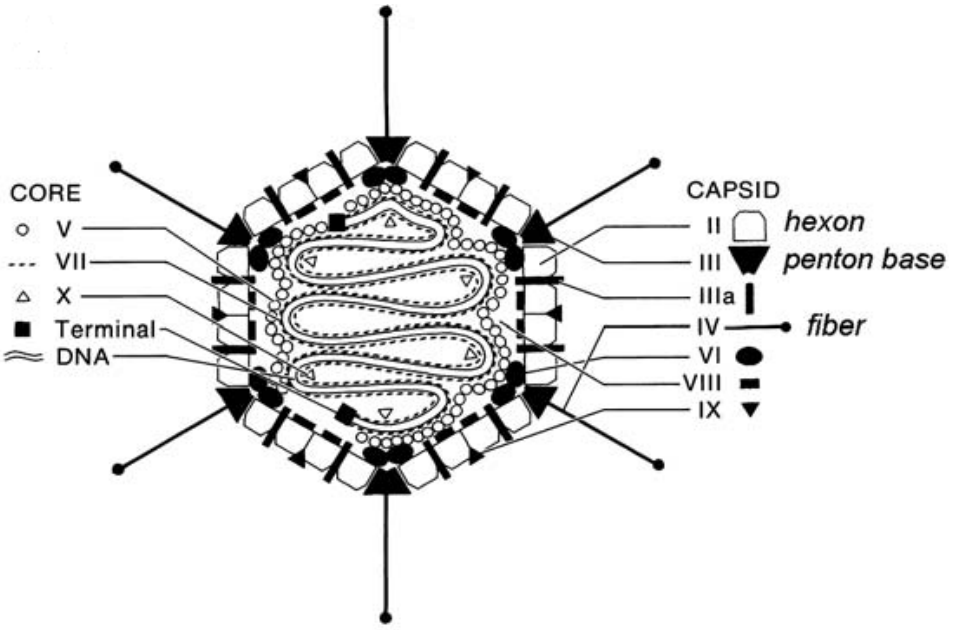

Fig. 4: Schematic representation of adenovirus structure
Adapted from Rux and Burnett, 2004, The Wister Institute, Philadelphia.

1.3.3.2. Typing of adenoviruses

Fifty-two serotypes of hAdV have been recognized based on their biological, physiochemical and genetic properties, and these are divided into seven species (Griesche *et al*., 2008; Zhu *et al*., 2009). AdV-B species have been divided further into two subgroups: B1, including AdV type 3 (AdV3), AdV7, AdV16, AdV21 and AdV50, and B2, including AdV11, AdV14, AdV34, and AdV35 (Stone *et al*., 2003). Species B (types 3, 7, 11, and 16), and species C (types 1, 2, 5, and 6) are found predominantly in children (Kidd *et al*., 1996; Kim *et al*., 2003; Adhikary *et al*.,2004). Species B and E (serotype 4) have been reported to cause acute respiratory tract infections in military camps (Erdman *et al*., 2002; Kolavic-Gray *et al*., 2002; Echavarria *et al*., 2003; Heim *et al*., 2003; Blasiole *et al*., 2004; Chmielewicz *et al*., 2005-b; Kajon *et al*., 2007). In

2007, serotype 52 was suggested as species G (Griesche *et al*., 2008). Respiratory disease is usually caused by viruses belonging to species B1, C, or E (Schmitz *et al*., 1983; Heim *et al*., 2003).

Genome analysis of eleven adenoviruses identified in nasopharyngeal aspirates obtained from 482 children in Brazil showed that species C was represented by serotypes 1, 2 and 5. Within species B, only serotype 7 (Ad7) was detected. Two genomic variants of Ad1, two variants of Ad2, one of Ad5, and one of Ad7 (7h) were identified (Moura *et al*., 2008). Louie *et al*. (2008) reported 3 cases of severe pneumonia associated with adenovirus serotype 14, a subspecies B2 serotype not previously associated with severe clinical illness, was confirmed by neutralization assay and sequencing of the hexon gene. Restriction enzyme showed all 3 viruses were identical and belonged to a new genome type that has been designated "Ad14a".

An outbreak of severe respiratory illnesses was associated with a rare hAdV serotype 14 in a US Air Force training facility. An estimated 551 (48%) of 1147 trainees with febrile respiratory illness were infected with Ad14; 23 were hospitalized with pneumonia, four required admission to an intensive care unit, and one died (Tate *et al*., 2009). A multiplex real-time PCR was used for epidemiological analysis of a broad range of hAdV serotypes in respiratory samples. The assay gave an hAdV positive rate of 7.1% (n = 811). Sequence analysis of hAdV positives showed that serotype 7 was the most prevalent followed by serotypes 2 and 3. Association of hAdVs with respiratory outbreaks was low at 2.3% (6 of 266 outbreaks tested) and no seasonal variation was observed for hAdV infections during the 2-year study period (2005-2006) (Wong *et al*., 2008). The most common identified serotypes among 96 AdV isolated from respiratory specimens in Toronto, Canada were serotype 3 which was identified throughout the duration of the study period from September, 2007 to June, 2008 (n = 44, 46%), serotype 2 (n = 25, 26%), serotype 1 (n = 17, 18%), and serotype 21 (n = 5, 5%) (Yeung *et al*., 2009).

1.3.4. Human metapneumovirus (hMPV)

Human metapneumovirus was isolated for the first time in 2001 by van den Hoogen *et al*. (2001) in previously virus-negative nasopharyngeal aspirates from children with respiratory tract infections in the Netherlands. HMPV has been classified in the *Metapneumovirus* genus of the *Paramyxovirus* family and is characterized by a non-segmented negative-strand RNA. HMPV has been isolated in several continents, suggesting a worldwide prevalence. It resembles human respiratory syncytial virus (RSV) with regard to disease signs and the ability to infect and cause disease in young infant as well as individuals of all ages (Boivin *et al*., 2002). Several studies

have demonstrated that hMPV accounts for a major proportion of hospitalizations for lower respiratory tract infections in infants and young children (Boivin *et al*., 2003; van den Hoogen *et al*., 2003; Mullins *et al*., 2004; Williams *et al*., 2004).

1.3.4.1. Structure of human metapneumovirus

HMPV virions were visualized by electron microscopy as pleomorphic spheres and filaments that were reported to have general similarity to those of RSV (Collins and Crowe, 2007) (Fig.3). HMPV is an enveloped, single stranded negative sense RNA virus that consists of 13,350 nucleotides (nt), comprising the N (nucleoprotein), P (phosphoprotein), M (matrix protein), F (fusion protein), M2 (matrix proteins M2-1 and M2-2), SH (small hydrophobic protein), G (glycoprotein) and L (RNA-dependent RNA polymerase) genes. The M2 gene contains two open reading frames and encodes the M2-1 and M2-2 proteins. Thus, hMPV consists of nine proteins. (Kaida *et al*., 2006).

1.3.4.2. Genetic variability of human metapneumoviruses

Sequence analysis of HMPV isolates has identified two main groups, A and B; each group is further subdivided into two subgroups, A1 and A2, and B1 and B2. Both virus groups were identified in various countries in the Americas, Europe, and Asia (Biacchesi *et al*., 2003; von den Hoogen *et al*., 2004; Huck *et al*., 2006). The HMPV F protein is a major antigenic determinant that mediates extensive cross-lineage neutralization and protection. Two distinct HMPV genetic groups represented by CAN98-75 (CAN75) and CAN97-83 (CAN83) exhibit 5 and 63% amino acid divergence in the fusion (F) and attachment (G) proteins, respectively. The two groups exhibited 48% antigenic relatedness based on reciprocal cross-neutralization assay with post-infection hamster sera. Infection with each strain provided a high level of resistance to re-infection with the homologous or heterologous strain. Hamsters immunized with a recombinant human parainfluenza virus type 1 expressing the fusion F protein of the CAN83 strain developed a serum antibody response that efficiently neutralized the virus from both lineages and were protected from challenge with either hMPV strain (Skiadopoulos *et al*., 2004).

The molecular epidemiology and genetic diversity of hMPV were analyzed for a 3-year period (2000-2002) from viruses that were identified in South Africa (Ludewick *et al*., 2005). Two major genetic groups (A and B) and 2 subgroups (1 and 2) of hMPV were identified, as well as 2-6 possible genotypes within the subgroups. A shift in the predominant group was documented in successive seasons. Whereas the F gene was relatively conserved between subgroups, a high degree of variation was observed in the extracellular domain of the G gene of the virus. The G protein homologies between groups A and B were 45.1%-53.1% at the nucleotide level and

22.4%-27.6% at the amino acid level. These results provided evidence for the diversity of both surface glycoproteins of hMPV in Africa. The shift in the viral genotype may not be accompanied with a change in the severity of illness caused by various hMPV. During 2002-2004, 3740 nasopharyngeal-wash specimens were investigated by Agapov and others (2006). HMPV was detected in 5% of all specimens compared with 28% for other respiratory viruses. Nucleotide sequence analysis of hMPV isolates revealed the predominant circulation of hMPV group A in the 2003 season but a switch to predominantly group B in 2004. Sequence analysis also revealed major differences in the hMPV G and SH genes but relative conservation of the F and N genes within each group. Ji *et al*. (2009) reported on the seasonal distribution of hMPV infection and the genetic variation of the virus in the Suzhou area of China. From November 2005 to October 2006, a total of 1932 children with acute respiratory infections were tested for the presence of hMPV using a RT-PCR. Of the 1932 collected samples, 128 (6.6%) tested positive for hMPV. Sequence analysis of the hMPV-N genes showed 99-100% homology with published sequences.

2. Materials

2.1. Clinical Samples

Respiratory samples were collected from 520 patients suffering from lower respiratory tract infections, admitted to Assiut University Hospitals, Assiut, Egypt during three consecutive winter-spring seasons from December 2005 until February 2008. Patients included in this study were of different age groups (40 days-80 years) and of both sexes (210 females and 310 males). Adults represented 86.5% (450 in number) of the total population enrolled in the study while children represented 13.5% (70 in number) (table 1).

Table 1: Patients' criteria

	Children		Adults	
Gender	Girls	Boys	Females	Males
Number	31	39	179	271
	70		450	
Percentage	13.5 %		86.5 %	
Total:	520			

Most of the patients involved in this study were admitted to the chest department (297 out of 520). All children that involved in the study presented to the pediatric clinic (table 2).

Table 2: Sites of admission

	Children	Adults		
Admission	Pediatric clinic	Chest clinic	Chest department	Intensive care unit
Number	70	80	297	73
Summation	70	450		
Total	520 Patients			

Clinical data were collected from the patients that included the personal data, respiratory manifestations, and associated risk factors (table 3).

Table 3: Clinical data collected from the patients

History	Respiratory diagnosis	Associated risk factors
1- Name, sex, age, occupation & residence. 2- Symptoms of the lower respiratory infection & time of their occurrence.	1-Site & time of admission. 2- Clinical diagnosis. 3- Mechanical ventilation.	1- Smoking habits. 2- Other system affection or immunosuppressive condition. 3- Associated cardiovascular condition. 4- Immunosuppressive medication. 5- Associated respiratory tract infection, bacterial or fungal.

Pneumonia was the most common clinical diagnosis made in the patients followed by COPD. Bronchitis and bronchial asthma were more or less equal in number (table 4).

Table 4: Number and percentage of patients according to the clinical diagnosis

Order	Clinical diagnosis	Number	Percentage
1-	Pneumonia (lobar-, broncho- or necrotizing pneumonia)	140	26.9%
2-	COPD with infection exacerbation	132	25.4%
3-	Bronchitis (acute or chronic)	79	15.2%
4-	Asthma (acute or chronic) with infection exacerbation	77	14.8%
5-	Bronchiolitis	28	5.4%
6-	Bronchiectasis	24	4.6%
7-	IPF associated with bronchopneumonia	23	4.42%
8-	Multiple pyemic abscesses (septic embolism)	6	1.15%
9-	Infected cystic lung	5	0.96%
10-	Infections affected both the lung and pleura (empyema; pyopneumothorax)	4	0.77%
11-	Lung abscess	2	0.4%
Total		520	100

2.2. Laboratory instruments

Apparatus	***Function***	***Source***
-3130xl Genetic Analyser	Capillary Sequencer	Applied Biosystems
-8520	Microwave	Privileg
-ALPS 50V	Thermosealer	ABgene
-Axiovert 40C	Microscope	Zeiss
-Eppendorf 5402	Cool-Tish Centrifuge	Eppendorf
-GenAmpl.PCR system 9700	Thermocycler	Applied Biosystem
-HERA cell 240	Incubator	Thermo scientific
-Light Cycler 2.0	Thermocycler	Roche
-Mastercycler epGradient	Thermocycler	Eppendorf
-Mx3000/3000P	RT-PCR cycler	Stratagene
-ND-1000Spectrophotometer	Photometer	Nanodrop
-REAX 2000	Vortexer	Heidolph
-T3000	Thermocycler	Biometra
-TS-100	Thermoshaker	Kisker-Biotechnology

2.3. Reagents

Gene Amplification reagents	***Source***
-5x/10xBuffer-MgCl2	Invitrogen
-dNTP(2´-deoxynucleoside5´triphosphates)	GE Healthcare
-DTT (Dithiothreitol)	Invitrogen
-H_2O RNase free	Sigma
-$MgCl^2$ (Magnesium Chloride)	Merck
-M-MLV(Moloney Murine Leukemia Virus) Reverse Transcriptase (RT)	Invitrogen
-PfuTurbo®Hotstart DNA Polymerase	Stratagene
-Platinum Taq DNA Polymerase	Invitrogen
-Random Primer	Invitrogen
-RNasin®RNase Inhibitor	Promega
-ROX Reference Dye	TIB MolBiol

2.4. Kits

Kit	***Source***
-Big Dye Terminator v3.1	Applied Biosystems
-Gel Extraction Spin kit, JETquick	GENOMED
- MSB®Spin PCRapace	Invitek
-QIAquick PCR Purification kit	Qiagen
- RTP®DNA/RNA Virus Mini Kit	Invitek

2.5. Chemicals, Culture Media and Solutions

Chemical	***Source***
-100/250 DNA-Ladder/Low Mass Ladder	Invitrogen
-Acetic acid (CH_3COOH) (100%)	Merck
-Agarose, ultra pure	Bio-Rad
-Avicel RC 581	FMC BioPolymer
-Bovine serum albumin	PAA
-Bromophenol blue	Bromma
-Calcium Chloride ($CaCl_2$)	Merck
-Crystal violet	Roth
-Diethylaminoethyl (DEAE)	Sigma
-Disodiumhydrogenphosphate (Na_2HPO_4)	Merck
-Eagle´s MEM-medium (EMEM-medium)	Gibco Brl.
-Ethanol, C2H5OH 96%	Merck
-Ethidium bromide 95%	Sigma
-Ethylendiamintetra acetic acid (EDTA)	Merck
-Turkey´s/Guinea pig erythrocytes	BFR (www.BFR.de)
-Fetal bovine serum (FKS)	PAN Biotech
-Ficoll 400	Pharmacia
-Formaldehyde 36%	Fluka
-GelRed Nucleic Acid Gel stain	Biotium
-Gentamycin (10mg/ml)	PAA
-H2O bidest	RKI
-L-Glutamin 200mM (100x)	PAA
-Magnesium Chloride $MgCl_2$	Merck
-Non-essential amino acids (100x)	PAA
-Potassium chloride (KCl)	Merck
-Potassium hydrogen phosphate(KH_2PO_4)	Merck
-Receptor destroying enzyme (RDE)	Sigma
-Sodium Chloride (NaCl)	Merck
-Sodium hydrogen carbonate (NaHCO3)	Merck
-Sodium citrate	Merck
-Sodium Pyruvate (100mM)	Sigma
-TPCK-Trypsin	Sigma
-Tris-HCL	Roth
-TrizmaBase	Sigma
-Trypsin-EDTA (1x)	PAA

Culture media & Solutions	***Preparation***
-Avicel-solution 2.5%	2.5g Avicel + 100ml H_2O,bidest.
-Avicel-Medium-solution (Overlay medium):12 ml/6 well plate	14ml: 2xEMEM / 0.4%BSA + 14ml: Avicel-solution (2.5%) + 280µl DEAE(1%)+ 280µl NaHCO3(5%) + 29µl TPCK-Trypsin(1mg/ml)
-EMEM growth culture medium (10%)	EMEM medium + 10% Fetal bovine serum (inactivated 30 min.by 56°C) + 1% L-Glutamine + 1% Non-essential amino acides (NEAA) + 0.5 ml Gentamycin/100 ml Medium + 1% Pyruvate. On 500ml medium (MEM/Hepes, or EMEM/Hepes) PH 7.2
-EMEM/Hepes	25MM Hepes
-EMEM maintenance medium (2%)	2% FBS (Fetal bovine serum) + 0.5 ml Gentamycin/100 ml Medium +1% L-Glutamine + 1% NEAA + 1% Pyruvate. On 500ml medium (MEM/Hepes, or EMEM/Hepes) PH 7.2
-Fixing solution 3%	450ml PBS „without" + 50ml Formaldehyde (36%)
-Gel -Loadingbuffer (6%)	25ml Bromophenol blue (1%) + 15ml Ficoll 400 + 1ml Tris-HCL (1M) + 0.2ml EDTA (0.5M) + 58.8ml H_2O,bidest.
-Crystal violet solution (staining solution)	1g Crystal violet + 44ml Ethanol + 1960ml H2O,bidest
-Phosphate buffered saline (PBS) „without"	8g/l NaCl + 0.2g/l KCl + 1.15g/l Na_2HPO_4 + 0.2g/l KH_2PO_4 + H_2O,bidest. PH:7.2
-PBS „with"	PBS „without" + 0.1g/l $CaCl_2$ + 0.1g/l $MgCl_2$
-PBS (viral transport medium)	PBS„without" + Benzylpenicillin(2x106IU/litre) +Gentamicin (250mg/liter)+ Nystatin (0.5x106 IU/liter)
-TAE-buffer (50%)	242g TrizmaBase + 57.1ml CH3COOH (100%) + 100ml EDTA (0.5M) + H2O,bidest.1L to be added.
-Trypsin/EDTA	0.05% trypsin (1:250) + 0.02% EDTA. 4Na in Hanks' balanced salt solution without CaCl2,MgCl2 • 6H2O, and MgSO4
-Trypsin-Medium (for Infection of MDCK/Prima cells with influenza virus)	0.2ml Trypsin (0.25 %/100 ml)+ 0.5 ml Gentamycin/100ml medium +1% Glutamine+1% NEAA+1% Pyruvate.On 500ml med.(MEM/Hepes) PH 7.2

2.6. Antisera used for hemagglutination inhibition (HI) test of influenza viruses

	Anti-serum	**Source**
Influenza A/H1	A/New Caledonia/20/99	Robert Koch Institute (RKI)
	A/Solomon Islands/3/06	RKI
	A/Brisbane/59/07	RKI
Influenza A/H3	A/Wisconsin/67/06	World Health Organization (WHO)
	A/Brisbane/10/07	RKI
Influenza B	B/Florida/4/06	RKI
	B/Malaysia/2506/04	RKI
	B/Jiangsu/10/03	RKI

2.7. Applied Oligonucleotides and Probes

The synthesis of the oligonucleotides and probes was conducted by both TIB MolBiol and Invitrogen companies. Sequences, melting temperatures (Tm) and the position in Genbank are shown in tables 5-13.

Table 5: Oligonucleotides for real time-PCR of Influenza A and B-M gene, HA and NA genes

Influenza A-M*	**Sequence**	**Ta**	**Position**(CY006148)**
AM+25	AGATGAGTCTTCTAACCGAGGTCG	67.2°C	24 - 47
AM-124 BB	CCTGCAAAAACATCTTCAAGTCTCTG	65.2°C	126 - 101
AM+64 MGB	VIC - TCAGGCCCCCTCAA NFQ	72.1°C	74 - 87
Influenza B-M	**Sequence**	**Ta**	**Position** (CY037400)
BMP+13	GAGACACAATTGCCTACCTGC	64.9°C	14 - 34
BMP-102	TTCCCACCGAACCAACAGTGTAAT	65.5°C	104 - 81
BMP-72 MGB	VIC-CTGCTTTGCCTTCTC	71.3°C	67 - 53
Influenza A-H1	**Sequence**	**Ta**	**Position** (AJ344014)
H1F-832	GGATCAGGAATCATCACCTCAAATGC	62.1°C	832 - 857
H1R-959	GGACACTCTCCTATTGTGACTGGGTG	62.1°C	959 - 934
H1-914 MGB	FAM –CTGCTGTTTATAGCTCC – MGB	69.0°C	914 - 898
Influenza A-H3	**Sequence**	**Ta**	**Position** (CY002056)
H3F-162	TCCTCATCAGATCCTTGATG	58.8°C	239 - 258
H3R-291	ACAGTTGCTGTAGGCTTTGC	64.7°C	367 - 348
H3-284 MGB	VIC-CTCTATTGGGRGACCC NFQ	67.0°C	283 – 299
Influenza A-N1	**Sequence**	**Ta**	**Position** (AJ518092)
N1-1078	ATGGTAATGGTGTTTGGATAGGAAG	66.3°C	1058 - 1082
N1-160 bp	ATCTCCCGCTATATCCTGACCACTCAGT	68.2 °C	1216 - 1189
N1 MGB	FAM-TCCATCCGTTCGGATCCCAAA NFQ	67.9°C	1141 - 1121
Influenza A-N2	**Sequence**	**Ta**	**Position** (CY008398)
N2-769	GATACTAAAATACTATTCATTGAGGAGG	61.2°C	768 - 795
N2-934	ATATCTACGATGGGCCTATTGGAGC	65.0 °C	931 - 907
N2-840 MGB	Vic-CACTCCTCGACATGCTG NFQ	72.1°C	850 - 834

Primers and probes were designed by the National Influenza Reference Centre, Robert Koch Institute.
***** *Primers modified according to Spackman et al., 2002/* ******Accession numbers at the Genbank.*
Ta: annealing temperature/ MGB: minor groove binding / NFQ: Non-fluorescent quencher.

Table 6: Oligonucleotides***** for real time-PCR of RSV/N and G genes

RSV-N gene (A and B)	**Sequence**	**Position****
RSV-N15-F	GATGGCTCTTAGCAAAGTCAAGTT	15 - 38
RSV-184-R	CATCTTCAGTGATTAATARCATACCACATA	184 - 155
RSV-MGB FAM	ACAGGAGATAATATTGACACTC	100 - 121
RSV A-G gene	**Sequence**	**Position**
RSV-A-G 409	AAGACCAAAAACACAACAACAA	409 - 430
RSV-A-G 586	TTGGTATTCTTTTGCAGATAGTAGCC	586 - 564
RSV-A-G 556	6FAM-TTGGATTGTTGCTGCATATGCTGCTXTPH	532 - 556
RSV B-G gene	**Sequence**	**Position**
RSV-B-G 155	CAATGATAATCTCAACCTCTCTCA	155 - 178
RSV-B-G 303	GGTGAGACTTGAGTAAGGTAAGTG	302 - 279

*****Primers und probes were designed by Janine Reiche (Reiche and Schweiger, 2009).*
******Nucleotide positions are given according to the gene positions in RSV strain A2 (GenBank accession number U50362) and strain 8-60 (accession number M73545) for RSV groups A and B, respectively.*

Table 7: Oligonucleotides** for real time-PCR for hMPV-F (fusion) gene

Name	hMPV F Protein	Orientation*	Tm	Position
hMPV F S	GCTCCGTAATTTACATGGTGCA	S	58.0°C	14 - 35
hMPV F S1	GAAGCTCTGTGATTTACATGGTTCA	S	57.0°C	11 - 35
hMPV F AS	GACCCTGCATTCTGACAATACCA	A	58.7°C	167 - 145
hMPV F AS1	AGTAGATCCTGCATTTTTACAATACCA	A	58.9°C	167 - 141
hMPV F TMGB	6FAM-CCTTGCTGGATAGTAAAA	Probe	70.0°C	64 - 81
hMPV F TMGB1	6FAM-CCTTGTTGGATAATCAA	Probe	68.0°C	64 - 82

**S: Sense orientation, A: Antisense orientation according to GenBank (AB251561.1)*
***Primers und probes were designed by Andreas Nitsche (RKI, October 2007).*

Table 8: Oligonucleotides* used for real-time PCR of adenovirus polymerase gene

Type	Name	Sequence (5´-3´)		Ta**
C	P-025 P-026	CTGTATACGGAGACACTGACAG GGATTTTTCCTTTGGA	S*** AS	48°C
C	P-029	GCCCCAATGGGCATACATGCACATC CAGCACCCCCCGAATGTCAAA	S AS	55°C
C	P-030	GCCCGCGCCACCGAGACGTACTTC CCCACAGCCAGGGTAAACCGCGCCTTGTA	S AS	55°C
C	P-038	CTCCTTTGTCTTGGCGTGGAC GCGCGGAGCTTGCCCTTGGA	S AS	55°C
Q	P-033	GAGAAAGGACGCCGCCTTATGGA CAAACAGTTTCACATTCAACTGACCAGG	S AS	60°C
Q	P-034	GGGGACACCGGCTCATGGA GTTTCGCATTCCACGAGCCAGG	S AS	
Q	P-035	AGCCGGATACCGCCTCATGGA GGTTTCGCACTCCACTAACCAGG	S AS	
Q	P-039	GCGCGGACACAGACTCATGGA TCGCACTCGACGAGCCAGG	S AS	
Q	P-040	CTCAACGAGGACACGAACTCATGGA CAGTTTCACATTCCACCAGCCAGG	S AS	
Q	MGB033/1 MGB033/2	FAM - AGGTAAGAAACGCATCAAA – MGB/NFQ**** FAM - AGGTAAGAAGCGCATCAA – MGB/NFQ	S AS	

**Primers und Probes were designed by Chmielewicz et al. (2005-a).*
***Ta: Annealing temperature. ***S: sense orientation/ AS: antisense orientation. ****MGB/NFQ: Minor Groove Binder/non-fluorescent quencher. The MGB probes synthesized by Applied Biosystems; all other oligonucleotides synthesized by TIB MOLBIOL.*

Table 9: Oligonucleotides for fluorescence curve melting analysis of adenovirus

Name	Sequence (5´-3´)
LC033/A	CCGTACTTTTTGATGCGTTTC - FL LC RED 640 - TACCTTGCGACTCCATAA
LC033/B	AAAAACAAGTTTTCCGCCAT - FL LC RED 640 - TTTTTTGATGCGTTTCTTACCTTTGGT
LC033/C	GTGAGCTCTGGCCGTTCGG - FL LC RED 640 - GTCAAAAACCAGGTTTCCCC
LC033/D	GTAAGAAGCGCATCAAAAAGAACG - FL LC RED 640 - GGGAAAACTGGTTTTTGACCCCGA
LC033/E	CAAAAAGAACGGCGGAAAACTGGTTT - FL LC RED 640 - GATCCCAATCAGCCCGACC
LC033/F	CGTGTTTTTTGATGCG - FL LC RED 640 - TTCTTACCTCGGGTTTCCATGAG

**Primers designed by Chmielewicz et al. (2005-a). LC: Hybridization probes for FCMA/ FL: Fluorescein.*

Table 10: Oligonucleotides* for conventional-PCR and sequencing of influenza viruses

Influenza A-H1	**Sequence (5´– 3´)**	**Tm**	**Position** (CY002808)
H1 F1**	AGCAAAAGCAGGGGAAAATAAA	58.0°C	1 - 22
H1 F13	GGAAAATAAAAGCAACCAAAATGAA	55.3°C	13 - 37
H1 F21	AAAGCAACCAAAATGAAAGYAAAACT	55.0°C	21 - 46
H1 F1B	AGGCTACCATGCGAACAAC	55.7°C	98 - 116
H1 F555	CCAAACCTGAGCAAGTCCTATG	57.6°C	555 - 576
H1 F764	CTACTACTGGACTCTGCTGGAACC	57.5°C	764 - 787
H1 F832	GGATCAGGAATCATCACCTCAAATGC	59.8°C	864 - 889
H1 R467***	TTATGGGAGCATGATGCCGT	58.8°C	496 - 477
H1 RWH1-2	CACTACAGAGACATAAGCAT	44.2°C	686 - 667
H1 R724	GCTATTTCTGGGGTGAATCTTCTG	58.2°C	724 - 701
H1 R860	GCCTCTACTCAGTGCGAAAGC	58.3°C	860 - 840
H1 R1123	CAACCATCTACCATTCCAGTCCA	58.5°C	1123 - 1101
H1 R1144	TTCTGATGATGATAACCATACCAACC	56.3°C	1144 - 1119
Influenza A-H3	**Sequence (5´– 3´)**	**Tm**	**Position** (CY002056)
H3 F1	AGCAAAAGCAGGGGATAATTCTATTA	58.0°C	1 - 26
H3 F6	AAGCAGGGGATAATTCTATTAACCAT	57.1°C	6 - 31
H3 F26	AACCATGAAGACTATCATTGCTTTG	56.5°C	26 - 50
H3 F3A	CAAATTGAAGTGACTAATGC	47.0°C	174 – 193
H3 F3B	CGCAGCAAAGCTTACAGCAA	59.5°C	344 – 363
H3 FH3P	TACCCAGCGCTGAACGTGACTATGCC	67.2°C	557 - 582
H3 F519	TTTAGTAGATTGAATTGGTTGACCCA	57.6°C	519 - 544
H3 F755	CCCCAGCAGAATAAGCATCTATTG	58.9°C	755 - 778
H3 F785	AAACCGGGAGACATACTT	49.1°C	789 - 806
H3 R291	ACAGTTGCTGTAGGCTTTGC	55.9°C	367 - 348
H3 R380A	ATCCTGAGCGACTCCAGTCC	57.8°C	475 - 456
H3 R705	GGCTTCTTTTGGTAGAGACTGTGA	57.5°C	705 - 682
H3 R847	TGATGCATTCAGAATTGCAT	52.0 °C	924 - 905
H3 R3D	GTTTCTCTGGTACATTCCGC	53.6°C	1056 - 1037
H3 R1140	GCCTGAAACCGTACCAACC	57.5°C	1140 - 1122
H3 F1202	GTTGATTGCTGCTTGAGTGCT	57.2°C	1202 - 1182
Influenza A-N1	**Sequence (5´– 3´)**	**Tm**	**Position** (AJ518092)
N1 F1	AGCAAAAGCAGGAGTTTAAAATGAATC	57.6°C	1 - 27 (AB286001)
N1 F37	AAATAATAACCATTGGATCAATCAG	52.9°C	35 - 59
N1 F707	ATATTAAGAACACAAGAGTCTGAATGT	52.2°C	685 - 711
N1 F1075	ATGGTAATGGTGTTTGGATAGG	53.9°C	1076 - 1097
N1 R401	CAAGTGAGAACATGATATGAAAGG	53.0°C	399 - 376
N1 R791	CTTGAAGATTTTGTACGAGGC	53.1°C	789 - 769
N1 R1170	CGGTATCTGTCCATCCATTAG	52.4°C	1168 - 1148
N1 R1352	AATGCTGCTCCCACTAGTCCAG	59.6°C	1353 - 1332 (AB286001)
N1 R1440	CAACGAACTACTTGTCAATGGT	53.6°C	1441 - 1420 (AB286001)
Influenza A-N2	**Sequence (5´– 3´)**	**Tm**	**Position** (CY008398)
N2 F7	AGCAGGAGTGAAGATGAATCC	53.9°C	5 - 25
N2 F28	ATCAAAAGATAATAACGATTGGCTCTG	56.0°C	28 - 54
N2 F550	ATGGTCCAGCTCAAGTTGTCA	56.7°C	548 - 568
N2 F692	CCAGGAGTCAGAATGCGTTT	56.7°C	692 - 711

N2 F769	GATACTAAAATACTATTCATTGAGGAGG	43.0°C	768 - 795
N2 F965	TCCAGTTATGTGTGCTCAGG	53.3°C	957 - 976
N2 R488	TCCGATAAGGGGTCCTATCAT	51.2°C	486 - 466
N2 R645	CCATCGTAAATGAAGCTAGC	51.4°C	643- 624
N2 R807	GAACGATTTTCCCCTCCTCAA	58.0°C	807 - 787
N2 R877	ACCAGGATATCGAGGATAACAGGA	57.4°C	875 - 852
N2 R1060	CAAAGGCCCAGCCTTTCACT	61.0°C	1080 - 1061
N2 R1430	AGCTTATATAGGCATGAGATTGATGTCC	55.7°C	1430 - 1403
Influenza B-HA	**Sequence (5´– 3´)**	**Tm**	**Position** (AY504610)
HA F7	AGCAGAGCATTTTCTAATATCC	50.7°C	7 - 28
HA F78	AGATCGAATCTGCACTGGGAT	56.3°C	45 - 65 (AF129906)
HA FIB5	TGGAACCTCAGGATCTTGCCCT	62.3°C	457 - 478 (AF129906)
HA F800	AGACGGAGGGCTACCACAAA	58.9°C	786 - 805
HA R366	GAAAGCACCCGGATGTAACAG	58.2°C	364 - 344
HA R619	AAATGTATGGTACTTCTATTGTTAATG	50.9°C	619 - 593
HA RIB3	CTCCGTTGGCAGATGAGGTGAAC	63.1°C	701 - 679 (AF129906)
HA RIB2	CGCTTTGTGGTAGCCCTCCGT	65.0°C	773 - 753 (AF129906)
HA R961	GGCAATCTGCTTCACCAATTAAAGG	62.0°C	964 - 940
HA R1106	GGAGGTCTATATTTGGTTCCATTGG	58.7°C	1097 - 1073
Influenza B-NA	**Sequence (5´– 3´)**	**Tm**	**Position** (AY191499)
NA F48	AATGAACAATGCTACCTTCAAC	52.4°C	46 - 67
NA F111	TTATTATCACTATATGTGTCAGC	44.6°C	108 - 130
NA F500	CAGAAACAAGCTGAGGCA	52.6°C	500 - 517
NA F683	TGACACATACCATTCCTATGC	51.1°C	686 - 706
NA F1025	CACCCCCAGACCARATGA	52.9°C	1025 - 1042
NA F1105	TTGTCCATCAAAGAATGGCATC	56.6°C	1108 - 1129
NA R429	ATTCCTTTGGTCCACAAGCA	56.8°C	432 - 413
NA R580	GCTGCCATGTGGAAAAT	51.5°C	580 - 564
NA R747	CGATGCAATTGCAGGCACTT	60.6°C	750 - 731
NA R1129	TTGGATGCCATTCTTTGATG	54.1°C	1132 - 1113
NA R1480	GGTTTAGAACAGATTCAACCAT	51.2°C	1483 - 1462

**Primers were designed by NIC, RKI. **F: forward primer/ ***R: reverse primer*

Table 11: Oligonucleotides* for conventional, nested-PCR and sequencing of RSV-G gene

Primer	**RSV-A**	**Sequence(5´– 3´)**	**Tm**	**Position**
Sense	G-513-F	AGTGTTCAACTTTGTACCCTGC	58.0°C	513 - 534
Sense	RSVA-G-606-F	AACCACCACCAAGCCCACAA	62.9°C	606 - 625
Anti-sense	RSV-F-22-R	CAACTCCATTGTTATTTGCC	49.8°C	22 - 3
Anti-sense	F-131-R	CTGCACTGCATGTTGATTGAT	57.0°C	131 - 111
Primer	**RSV-B**	**Sequence(5´– 3´)**	**Tm**	**Position**
Sense	RSVB-G-524-F	TTGTTCCCTGTAGTATATGTG	49.2°C	524 - 544
Sense	RSVB-G-603-F	AAAACCAACCATCAAACCCAC	54.1°C	603 - 623
Anti-sense	RSV-F-55-R	AGTTAGGAAGATTGCACTTGA	51.6°C	55 - 35
Anti-sense	RSV-F-22-R	CAACTCCATTGTTATTTGCC	49.8°C	22 - 3

**Primers were designed by Janine Reiche, NIC, RKI.*

Table 12: Oligonucleotides* for conventional-PCR & sequencing of AdV species B and C

Primer/Conv.PCR	**Sequence(5´– 3´)**	**Tm**	**Position**
AdV HVR PCR B F1	GCATACATGCACATCGCCG	59.8°C	31 - 49
AdV HVR PCR B F2	GACAGGATGCTTCGGAGTACC	57.1°C	50 - 70
AdV HVR PCR B R1	AGAACGGTGTACGCAGGTAGAC	58.0°C	2812 - 2791
AdV HVR PCR B R2	GCTGATGCACTCTGACCACG	59.0°C	2767 - 2748
AdV HVR PCR C F1	ATGATGCCGCAGTGGTCTTAC	58.5°C	16 - 36
AdV HVR PCR C F2	ACGACGTAACCACAGACCG	57.0°C	161 - 179
AdV HVR PCR C R1	ATTAAAGGACTGGTCGTTGGTGTC	59.2°C	1980 - 1957
AdV HVR PCR C R2	GCCACCACTCGCTTGTTCAT	60.1°C	1577 - 1558
Primer/Sequencing	**Sequence(5´– 3´)**	**Tm**	**Position**
AdV HVR PCR B F571	CCAGAACCTCACGTGGGA	50.0°C	571 - 588
AdV HVR PCR B F1043	xTGAATGCGGTGGTTGACTTxx	51.4°C	1043 - 1061
AdV HVR PCR B R679	xxxACCCATAGCAGGGTTTCAT	51.4°C	679 - 661
AdV HVR PCR B R1116	xxxxGTCACCCAGAGAGTCAAGCx	50.6°C	1116 - 1098
AdV HVR PCR B R1662	xCACTTGAATGTGGAAAGGCAC	54.8°C	1662 - 1642
AdV HVR PCR C F652	GGCGAATCTCAGTGGAACGAA	50.3°C	652 - 672
AdV HVR PCR C R744	xxATAAGATCCATAGCATGGTTTCAT	50.5°C	744 - 721

**Primers described by Allard et al. (2001) which later on modified by Chmielewicz et al. (2005-a).*
x: abridged primer

Tab. 13: Oligonucleotides* for conventional, semi-nested PCR & sequencing of hMPV-F gene

Primer-Conventional	**Sequence(5´– 3´)**	**Ta**
3637-F	GTCAGCTTCAGTCAATTCAACAGAAG	64-65°C
4192-R1	CAGTGCAACCATACTGATAGGATG	64,5-66,7°C
4192-R2	TAGTGCAACCATACTGATGGGGTG	65,4-67,6°C
Semi-Nested	**Sequence(5´– 3´)**	**Ta**
3637-F	GTCAGCTTCAGTCAATTCAACAGAAG	64-65°C
4164-R	CCT GTG CTG ACT TTG CAT GGG	65-67°C

**Primers were designed by NIC, RKI according to Huck, 2006.*
F: forward primer/ R: reverse primer/ Ta: annealing temperature.

2.8. Software and Databank

Software	**Reference / Homepage**
BioEdit 7.0.9	Hall, 2007
ClustalW software	http://www.ebi.ac.uk/clustal
Influenza Sequence database	http://www.flu.lanl.gov
Lasergene 7.2.1	DNASTAR
LightCycler 2.0 software (ver. 3.5)	Roche
Mega 4.0	Tamura *et al*., 2007
NCBI Databank	http://www.ncbi.nlm.nih.gov/

3. Methods

3.1. Collection, transport and storage of the respiratory specimens

From the five hundreds twenty patients, 812 respiratory samples were obtained. Gargles, swabs and sputum specimens collected from patients in sterile plastic screw-capped containers. PBS was used as viral transport medium. Collected samples were transported in an ice tank filled with ice bags directly to the virology laboratory (Department of medical microbiology and immunology, Faculty of medicine, Assiut University, Assiut, Egypt). Filtration and aliquots from each sample were done and stored at -80°C until they were finally shipped to Germany. Types of respiratory samples collected are shown in table 14.

Table 14: Types of respiratory samples collected from the patients

Samples	Number	Percentage
1- Sputum samples	319	39%
2- Nasal swabs	215	26%
3- Throat swabs	122	15%
4- Throat gargles	105	12.9%
5- Tracheal aspirates	41	5%
6- Broncho-alveolar lavage	8	0.98%
7- Nasopharyngeal aspirates	2	0.2%
Total	812	100%

3.2. Detection of the causative viruses by real-time PCR

Real-time PCR was performed for the main respiratory viruses: influenza viruses (A and B), respiratory syncytial virus (RSV), adenovirus, and human metapneumovirus (hMPV).

3.2.1. Extraction of viral nucleic acids from respiratory samples

Extraction was performed from 400ul of original samples using RTP®DNA/RNA Virus Mini Kit according to manufactures' instructions.

3.2.2. Reverse transcription and cDNA synthesis (Influenza viruses, RSV and hMPV)

Reverse transcription was performed with 25ul of RNA and 15ul of a reaction mixture containing 200uM concentration of deoxynucleoside triphosphates (dNTPs), 5mM concentration of dithiothreitol (DTT), 0.4mM concentration of random primer, 20 units RNasin®RNase inhibitor, 100 units Moloney murine leukemia virus reverse transcriptase (M-MLV), and first-strand buffer. The reaction was carried out for 5 min. at 42ºC, followed by 30 min. at 37ºC and for 5 min. at 94ºC.

3.2.3. TaqMan real time-PCR

3.2.3.1. Real time-PCR for detection of influenza A and B viruses

Multiplex real-time PCR targeting the matrix (M) gene of influenza A and B viruses was performed in a 25µl of reaction mixture containing 3.0µl cDNA, PCR buffer 10x concentration [200mM Tris-HCL (pH 8.4), 500 mM KCl], 100µM dNTPs, 5mM MgCl2, 0.5U Taq DNA polymerase, 300nM concentrations of each of the primer pairs and 100nM of probes for both influenza A and B viruses (table 5). Viral strains A/Solomon Islands/3/06 and B/Malaysia/2506/04 were used as positive controls for influenza A and B viruses, respectively. The PCR thermal profile consisted of an initial step of 5 minutes (min.) at 95°C, followed by 45 cycles each consisting of 15 seconds (sec.) at 95°C, 30 sec. at 60°C. PCR was performed on the Stratagene MX3000P and MX3005P instruments. Following this, influenza A positive samples were subtyped by real-time PCR using primers targeting the hemagglutinin (HA) and neuraminidase (NA) genes. The reagents used were like those for the multiplex reaction with 250nM concentration of each of the primer pairs and 200nM of probes (table 5) in a final volume of 25µl. Influenza A/Solomon Islands/3/06 and A/Wisconsin/67/05 were used as positive controls of subtypes A/H1N1 and A/H3N2, respectively.

3.2.3.2. Real time-PCR for detection of respiratory syncytial virus (RSV)

For detection of RSV, cDNA was first amplified by a generic TaqMan PCR targeting the RSV nucleoprotein (N) gene. The real-time PCR mixture in a total volume of 25µl consisted of 1xPCR buffer, 100µM dNTPs, 5mM MgCl2, 0.5U Taq DNA polymerase, 300nM RSV-N15-F primer, 1200nM RSV-N184-R primer, 150nM RSV probe (table 6), and 3.0µl cDNA. The thermocycler conditions were as mentioned before for the multiplex real-time PCR of influenza viruses. RSV positive samples were differentiated into RSV groups A and B by group-specific real-time PCR targeting glycoprotein (G) gene. The reaction mixture contained 5µl cDNA, 250nM of each of the primer pairs, 200nM of the RSV probe (table 6), 1µM 6-carboxy-X-rhodamine and other reagents as those used for detection of the nucleoprotein gene. Amplification was carried out for 5min. at 95°C, followed by 45 cycles each consisting of 15 sec. at 95°C, 30 sec. at 60°C, and 15 sec. at 72°C.

3.2.3.3. Real time-PCR for detection of adenoviruses

Amplification was done for a highly conserved region of the adenoviral genome coding for the DNA polymerase (AdV DPol PCR) using a mix of ten primers and Minor Groove Binder (MGB) probes (table 8) that detect all the 51 serotypes with great sensitivity. Real-time PCR was set up in a total volume of 25µl containing 1xPCR buffer, 5mM MgCl2, 100µM dNTPs, 0.5U Taq DNA

polymerase, 100nM of each primer, 50nM of each probe, and 5µl of the DNA template. The temperature profile started with an initial step of 5min. at 95°C, followed by 40 cycles each consisting of 15 sec. at 95°C, and 30 sec. at 60°C.

3.2.3.4. Real time-PCR for hMPV detection

Amplification reactions were done for the hMPV fusion (F) gene because it is relatively conserved between subgroups. The reaction mixture consisted of 1xPCR buffer, 100µM dNTPs, 5mM MgCl2, 0.5U Taq DNA polymerase, 500nM of each primer, 100nM of each probe (tab. 7), and 3.0µl cDNA with a final volume of 25µl. The temperature profile was as mentioned before for the multiplex real-time PCR of influenza viruses.

3.3. Isolation of the detected viruses on tissue culture cells

Types of tissue culture cells and culture media used for each virus are shown in table 15

Table 15: Tissue culture cells and culture media

Virus	Tissue culture cells	Culture media	Propagation factor	Propagation rhythm
Influenza	MDCK cells	MEM/Hepes	1:4	every 3/4 days
RSV	Hep2 cells	MEM/Hepes	1:8	every 3/4 days
Adenovirus	Hep2 cells	MEM/Hepes	1:8	every 3/4 days
HMPV	-LLC-MK2 cells -Vero cells	MEM/Hepes EMEM/Hepes	1:3 1:5	every 3/4 days every 7 days

Conservation of the cell monolayer was performed in T-75 tissue culture flasks by discarding the old medium and washing 2-3 times with PBS7. About 5ml of trypsin-EDTA (0.25% for MDCK, LLC-MK2 and Vero cells), or trypsin (for Hep2 cells) was distributed over the entire cell sheet for 1min. and then removed. Another 5ml of trypsin-EDTA or trypsin solution was added and the flask was rocked for 1min. then removal of 4ml leaving only 1ml of trypsin-EDTA solution which was distributed over the entire cell sheet and the flask was incubated at 37°C until all cells detached from the plastic surface (about 10-15min. for MDCK; 5-7min. for Hep2 and Vero; 3 min. for LLC-MK2 cells). Gentle shaking sometimes was done to detach cells. To stop action of the trypsin, 10 ml of the growth medium was added. Pipetting up and down gently for 10 times was done to break up cell clumps. Distribution of the cell suspension was done in required number of new tissue culture flasks/tubes according to the propagation factor for each type of cells (tab. 15). Flasks and tubes were incubated at 37°C for 3-4 days. Medium change was done after 2 days from incubation. The cells were checked every month for the absence of Mycoplasma. Filtration of the respiratory samples and inoculation of 200µl of the filtrate on cell monolayer was performed in culture tubes

after discarding of the old growth medium and washing three times with fresh medium (trypsin-medium were used for washing of MDCK cells). The inoculum was allowed to adsorb for one hour at 37°C. For every tube, 2ml of the medium was added. Incubation at 33°C for MDCK; LLC-cells, and at 37°C for Hep2 cells was done. The trypsin-medium was used for propagation of influenza viruses. Positive and negative controls were included with each group. Exchange of medium with maintenance medium was done for the culture tubes every 3 days unless cytopathic effect (CPE) was observed. Tubes were monitored daily for CPE typical for each virus for 14 days (Influenza; RSV; Adenoviruses) or 21 (hMPV) before reporting inability to recover virus from the specimen. Positive viral cultures were passaged three times to obtain sufficient virus titers for further virus identification. Passaging was also done by day 6 or 7, even if no CPE was observed. Multiple aliquots were stored at -80°C

3.4. Titration of Influenza virus isolates

3.4.1. Plaque assay (Avicel) (*National Influenza Centre, RKI*)

The solutions used were listed before in section 3.5. The plaque test was performed for the first viral passages on MDCK-2 cells, using the 6 well plates. At the first day, MDCK-2 cells were inoculated (propagation factor 1:4) in EMEM and distributed as 3ml/well. Incubation was done for 3 days, at 37°C, in 5% CO^2 incubator (Cell lawns should be only 80% confluent after 3days). By the third day, the plates were washed twice with 2ml/well with EMEM (without additives) and viral dilutions (1:10) in a volume of 250µl/well were distributed on the plates in duplicates starting from a concentration of $1x10^{-2}$. PBS was used as a negative control. The plates were incubated for 45 min. at room temperature in a dark place and shaken every 15 min. was done. For each well, 2ml overlay medium was added and incubation was done at 37°C, in 5% CO^2 incubator for 3 days. At day 6, fixation was done for the plates with 2ml fixing solution/well and incubated for 30 min. at room temperature. The plates were washed with distilled water, stained with 1ml staining solution/well, and incubated for 10 min. at RT. The stain was then washed with distilled water.

Plaque forming unit (PFU)/ml calculation: after counting the plaques (the white dots on the monolayer with oblique light), the concentration of the initial viral suspension in PFU/ml was calculated using the dilution of the virus and the volume of virus solution placed on the single monolayer to determine the PFU/ml.

3.4.2. Hemagglutination assay (HA test) (*National Influenza Centre, RKI*)

The hemagglutinin protein agglutinates the erythrocytes, hence the derivative of its name. Then the test was read. Hemagglutination occurs when the RBCs are in suspension. In the absence of hemagglutination (as in the cell control wells), the RBCs form a compact button on the bottom of the wells. The titer is the highest dilution step in which hemagglutination is observed, the endpoint dilution is considered 1hemagglutination unit (HAU) and the number of HAUs/ 50µl is the reciprocal of the highest dilution.

3.5. Antigenic characterization of influenza virus isolates by hemagglutination inhibition (HI) assay

The ability of influenza viruses to agglutinate erythrocytes is inhibited by the presence of specific antibodies. Influenza virus isolates were subtyped by standard hemagglutination inhibition tests using a panel of the RKI and WHO reference antisera (section 3.6). The antisera were first treated with receptor destroying enzyme (RDE) for inactivation of non-specific inhibitors. The test was carried out in 96-well microtiter-plates. At first, 25µl of 0.9% NaCl solution was added to each well and 25µl antigen (influenza virus) were added to the first row of wells and mixed thoroughly. Serial dilutions were then done by transferring 25µl from each row to the next. From the last row 25µl were discarded. Another 25µl of 0.9% NaCl solution and then 50µl of 0.5% erythrocytes-suspension were added to each well. The plates were covered, shaken by a mechanical vibrator, and incubated at RT for 30min. (turkey RBCs) or 60min. (guinea pig RBCs). The highest dilution at which hemagglutination is still observed is 1HAU. The viral antigens should be standardized to a concentration of 4HAUs/25µl. Viral antigens were then diluted with 0.9% NaCl according to the dilution factor for each one of them. Each virus isolate was tested against the eight antisera provided. Each virus was tested in an individual plate which was labeled with the virus name and antisera. To wells B through H (B1-H12), 25µl of 0.9%NaCl solution were added then 50µl of each diluted antiserum were added to the first well of the appropriately numbered column. Serial two-fold dilutions of the treated sera were prepared by dispensing 25µl from the first well of numbered columns 1-8 to successive wells. The final 25µl were discarded from row H. The tested viral suspension (standardized to 4HAU) was added in a volume of 25µl to each well of the plate. The plates were then covered and shaken on a mechanical vibrator. After incubation for 30min. at RT, 50µl of 0.5% erythrocytes-suspension were added to all wells and mixed. The plates were covered and incubated at RT for 30min (turkey RBCs) or 60min. (guinea pig RBCs). Reaction of the tested viral suspension with its corresponding antibody results in antigen/antibody reaction, so

hemagglutination of the RBCs is inhibited. Viral isolates were identified as particular subtypes when they react with one reference antiserum to a four-fold or greater HI titer than to other antisera. Presence or absence of hemagglutination reaction was examined as mentioned before by the HA test. The HI titer is the reciprocal of the highest dilution of antiserum that inhibits hemagglutination.

3.6. Genotyping of adenovirus positive samples by fluorescence curve melting analysis (FCMA) (Chmielewicz *et al*., 2005-a)

FCMA for PCR products after AdV DPol PCR was performed in a Light Cycler 2.0 instrument using six pairs of hybridization probes, each specific for a single adenovirus species (A-F). FCMA is based on the fact that the sequences of serotypes of a particular species are highly homologous but differ from those of the other species. Consequently, a hybridization probe pair that is specific for one species has mismatches to the others, thus giving lower melting temperatures. The melting temperatures are characteristic for the target sequence/probe-pair combination, making them highly reproducible. Analysis of an amplicon with the different probe pairs will therefore give a characteristic melting pattern of one perfect match and five non-perfect matches, from which the species can be easily deduced. The PCR-products from each sample were added in a volume from 1-5µl to the melting mixture which consisted of 1xPCR buffer, 5mM MgCl2, and 150nM for each probe (table 9) to a final volume of 10µl. The samples were denatured at 85°C for 30 seconds, cooled to 35°C at maximum ramping rate (20°C/sec.), and reheated to 85°C at a ramping rate of 0.2°C/sec., during which the fluorescence data were acquired. Finally, the samples were cooled to 35 °C. The data were analyzed by the LC software (version 3.5). AdV18, AdV3, AdV2, AdV19, AdV4, and AdV41 were used as positive controls for AdV species A-F, respectively, and were included in each run in a volume of 1µl.

Table 16: Melting temperature analysis of adenovirus serotypes

Species	Serotype	A	B	C	D	E	F
A	AdV12	58	47				
	AdV18	47	51				
	AdV31	57	50		49		52
B1	AdV3		59		49		52
	AdV7		59		49		52
	AdV16		59		49		52
	AdV21		59		49		52
	AdV50		59		49		46
B2	AdV11		59		49		46
	AdV14		59	63	49		46
	AdV34		59	63	49		46
	AdV35		59	63	53	43	
C	AdV1			63	53		
	AdV2				53		
	AdV5				53		
	AdV6				63		42
D	AdV8				63		42
	AdV9				63		42
	AdV10				63		42
	AdV13				63		
	AdV15				63		42
	AdV17				63		42
	AdV19				63		
	AdV20				63		
	AdV22				63		
	AdV23				63		
	AdV24				63		42
	AdV25				63		42
	AdV26				63		42
	AdV27				63		
	AdV28				63		42
	AdV29				63		42
	AdV30				63		
	AdV32				63		42
	AdV33				63		42
	AdV36				63		42
	AdV37				63		42
	AdV38				63		42
	AdV39				63		42
	AdV42				59		42
	AdV43				63		42
	AdV44				63		42
	AdV45				63	65	42
	AdV46				63		42
	AdV47				63		42
	AdV48				63		42
	AdV49				63		42
	AdV51				58		
E	AdV4		52				57
F	AdV40						57
	AdV41						

3.7. Conventional-PCR and nucleotide sequencing

To elucidate the evolutionary pathway of the established viruses demonstrated in this study, sequencing of the positive viral samples followed by phylogenetic analysis was done.

3.7.1. Conventional-PCR for Influenza viruses

PCR reactions targeting the hemagglutinin (HA) and neuraminidase (NA) genes of influenza A and B viruses were set up in a total volume of 50µl containing 1xPCR buffer, 200µM dNTPs, 2mM MgCl2, 0.5U Taq DNA polymerase, 250nM of each primer (table 10), and 5.0µl cDNA. Isolates A/H1N1/608/09, A/H3N2/469/09, and B/64/09 were used as positive controls for influenza A and B, respectively. Amplification was carried out in a GenAmp 9700 or Eppendorf epGradient instrument (section 3.2) for a total of 40 cycles after an initial denaturation step at 95°C for 5min. Reaction conditions are shown in table 17. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel and visualized with GelRed or Ethidium bromide under ultraviolet light (UV).

Table 17: Reaction conditions for PCR of HA and NA genes of influenza A and B viruses

Cycler	**Eppendorf / PE 9700**	**Time**	Gel: 1.5%
Program	95°C	5minutes	
40x	95°C	30seconds	Elpho: 95Volt → 45'
	59°C → A/H1/60°C → A/H3 54°C → A/N1/55°C → A/N2 51°C → B/HA/54°C → B/NA	30seconds	Marker:100bp → 5µl
	72°C	90sec. → A/H1 and A/H3 65sec. → A/N1/85sec. → A/N2 85sec. → B/HA/65sec. → B/NA	Stain: GelRed/ Ethidium bromide
	72°C	5min	
	4°C	∞	

3.7.2. Conventional-PCR for respiratory syncytial virus (RSV)

Conventional-PCR was performed for glycoprotein (G) gene of RSV typing the virus into two groups RSV-A and RSV-B. The reaction was conducted with 5µl DNA template and a mix volume of 45µl consisted of 1xPCR buffer, 100µM dNTPs, 4mM MgCl2, 0.5U Taq DNA polymerase, 250nM of each primer (table 11), and 5.0µl cDNA. Isolate RSV/1529/07 was used as RSV-A positive control and isolate RSV/591/07 as type B positive control. Amplification was carried out for 5min at 94°C followed by 40 cycles each consisted of denaturation step at 94°C for 30sec., annealing for 30sec. at 58°C for RSV A or 53°C for RSV B, and primer extension for 1min. at 72°C and finally, an extension step for 10min. at 72°C.

A nested-PCR was sometimes required using 1µl (for weak PCR-amplicons) or 5µl (negative result) of PCR-products obtained from the conventional reaction as DNA template for the nested reactions. The reaction mixture was performed in a final volume of 50µl with 250nM of each primer. The forward primers RSV A-G606-F and RSV B-G603-F and the reverse primer RSV-F22-R were used (table 11). The cycling profile was the same as for the conventional reaction except for the annealing temperature which was 55°C for RSV A and 53°C for RSV B. PCR products were electrophoresed by 2% agarose gel and visualized under fluorescence UV after staining with GelRed or Ethidium bromide.

3.7.3. Conventional-PCR for adenoviruses

Amplification of the adenovirus hexon gene was done with two rounds of PCR using PfuTurbo®Hotstart DNA Polymerase was used for the amplification of long PCR products as suggested by the manufacturer. The reaction mixture was set up in a total volume of 25µl containing 1xPCR buffer, 200µM dNTPs with dUTP, 2mM MgCl2, 0.5U Taq DNA polymerase, and 500nM of each primer (table 12), and 5.0µl DNA template. For the second round, PCR-products from the first round were used as DNA-template in a volume of 1µl. Amplification was done for 5min. at 95°C followed by 40 cycles each consisted of a denaturation step at 95°C for 30sec., annealing for 30sec. at 60°C and primer extension for 180sec. for species B AdV or 120sec. for species C AdV at 72°C and finally, an extension step for 10min. at 72°C. Amplified PCR products were analyzed by 2% agarose gel electrophoresis under UV light after staining with GelRed or Ethidium bromide.

3.7.4. Conventional-PCR for human metapneumovirus (hMPV)

PCR for hMPV was done for the fusion gene. The reaction mixture was set up in a total volume of 50µl containing 1xPCR buffer, 100µM dNTPs, 3mM MgCl2, 0.5U Taq DNA polymerase, and 250nM of each primer (table 13), and 5.0µl DNA template. For the first round, primers hMPV-3637-F, hMPV-4192-R1 and R2 were used. When weak products were produced, then the second round (semi-nested reaction) was done with 2µl of the first round product was served as a template for the second round PCR using primers 3637-F and 4164-R. Amplification was done for 5min. at 94°C followed by 40 cycles each consisted of a denaturation step at 94°C for 30sec., annealing for 30sec. at 60°C and primer extension for 45sec. at 72°C and finally, an extension step for 10min. at 72°C. The amplified products were examined with GelRed or Ethidium bromide under UV light.

3.7.5. DNA sequencing

Amplicons were purified either directly with MSB®Spin PCRapace kit or from agarose gels using JETquick Gel Extraction Spin kit (section 3.4) according to manufacturer's instructions. PCR products were sequenced directly after purification using the Big Dye Terminator v3.1 cycle sequencing kit (section 3.4) in an ABI-Prism 3130xl Genetic Analyzer (section 3.2). Reaction conditions for the sequencing are shown in table 18. Oligonucleotides used for the sequencing reactions for respiratory viruses are shown in tables (10-13).

Table 18: Conditions for the sequencing reaction

Reagent	**Concentration**	**Volume**	**Cycler**	**Eppendorf**	**Time**
Sequencing-Buffer	5x	1.5µl	Programm:	96°C	1min.
Primer F/R	2.5µM	0.5µl	25x	96°C	10sec.
PCR-H_2O	-	5.0µl		50°C	5sec.
Big Dye Terminator		1µl		60°C	4min.
Total volume: 8.0µl Master mix + 2µl purified PCR products				4°C	∞

3.8. Sequence alignment and phylogenetic analysis

Assembly of the sequencing contigs, translation of the nucleotide sequence into the protein sequence, and initial multiple sequence alignments were performed with the Lasergene 7.2.1 (DNASTAR, Madison, Wis.) group of programs. Phylogenetic trees for each gene were generated by using the maximum parsimony method with 1000 bootstrap replicates in a heuristic search with the Mega 4.0 (Tamura *et al*., 2007) software program. Unique representative sequences for each of the four respiratory viruses' genotypes were included in the phylogenetic analysis. Comparison between nucleotide or amino acid sequences were calculated using the DNADist or Protdist tool, respectively, of the Bioedit software, and the divergence values determined were described in terms of mean and standard deviation. Synonymous and nonsynonymous mutations were analyzed by the method of Nei and Gojobori (1986). The program SNAP (Synonymous/Nonsynonymous Analysis Program) that is provided by the human immunodeficiency virus sequence database website (http://www.hiv.lanl.gov/content/sequence/SNAP/.html) was used for analysis of synonymous mutations versus nonsynonymous mutations.

4. Results

About 1682 cases of lower respiratory tract infections (LRTIs) were admitted annually to the chest department , 280 cases to the chest intensive care unit (ICU), and more than 18 cases presented daily at the chest clinic of the Assiut University Hospital, Egypt during the winter-spring season 2007-2008 (Chest department database, Assiut University Hospitals, Egypt). At the pediatric clinic (Assiut University Hospitals, Egypt), more than 35 children presented daily with infections of the LRT during the winter-spring season 2007-2008. This study was performed to evaluate the role played by the main respiratory viruses, namely; influenza viruses A and B, respiratory syncytial virus (RSV), adenovirus, and human metapneumovirus (hMPV) to LRTIs at Assiut University Hospitals. Five hundred twenty patients (70 children and 450 adults) suffering from LRTI were included in the study. From them, 812 samples were obtained during three consecutive winter spring seasons from 2005 until 2008 (one or more samples were obtained from each patient). The epidemiological and demographic features (obtained by myself from the patients´ data sheets during collection of the samples) of the positive cases are shown in section 5.1 then followed consecutively in sections 5.2-5.8 with the complete characterization of the individual viruses detected.

4.1. Epidemiological findings according to real time-PCR

Seventy nine positive cases were detected with real-time PCR (representing a ratio of 15.2% of the total number of cases) for one or more of the main respiratory viruses (influenza viruses, RSV, adenovirus, and hMPV). Of those, 32 were children (accounting for 46% of the total number of children enrolled in the study) and 47 were adults (accounting for 10.4% of the adult group). For the children's group, 19 girls and 13 boys were found positive for viral LRTI but there was no significant difference between groups regarding the incidence of viral infections (P = 0.095). For the adults, 16 females versus 31 males were infected (P = 0.465) (tab. 19).

Table 19: Criteria of viral positive cases (total number of cases is 520)

Patient's groups		Number of positive cases		Percentage to the corresponding group	Percentage to total number of cases	
Children (70)	Girls	19	32	46%	3.65%	6.15%
	Boys	13			2.50%	
Adults (450)	Females	16	47	10.4%	3.07%	9.03%
	Males	31			5.96%	
Total	520	79			15.2%	

Because one or more samples were collected from each patient, the number of positive samples detected with real time-PCR was 104 which represented 12.8% of the total number of samples

collected. Of them, 46 samples were obtained from the children's group and 58 samples were collected from adults (tab. 20).

Table 20: Criteria of viral positive samples (total number of samples is 812)

Patient's groups		Number of positive samples		Percentage to the corresponding group	Percentage to total number of the samples	
Children	Girls	27	46	33%	3.32%	5.66%
	Boys	19			2.34%	
Adults	Females	24	58	8.6%	2.95%	7.14%
	Males	34			4.19%	
Total	812	104			12.8%	

4.1.1. Viral respiratory infection and age groups of patients

Of the 79 virus-positive patients, 22 were infected with influenza viruses, 16 with RSV, 20 with adenovirus, and 21 with hMPV. Analysis of the different age groups revealed that the majority of viruses were detected in the age group 0-4 years (30 out of 79 cases which accounted for 38% of the total number of positive cases). Patients between 35 and 60 years old (middle age group) accounted for 35% of all positive cases. Viral infection rate of the young adults (16-34 years) was 14% and that of the elderly (> 60 years) was 10% while only two patients of the adolescents' group (5-15 years) were found positive. There was a significant difference in the rate of detection of individual viruses among different age groups (P = 0.002). Most of the influenza viruses were detected in the middle age group, while RSV and hMPV were mostly identified in young children (0-4 years). Adenoviruses were more or less equally distributed among different age groups (tab. 21).

Table 21: Frequency distribution of viral respiratory infection according to age groups of patients

Respiratory Virus	Age groups (in years)						
	0-4	5-15	16-34	35- 60	> 60	Total	Percentage
Influenza	0	1	4	14	3	22	28%
RSV	12	0	1	3	0	16	20%
Adenovirus	7	0	3	6	4	20	25%
HMPV	11	1	3	5	1	21	27%
Total	30	2	11	28	8	79	100%

4.1.2. Viral LRTI and clinical diagnosis of patients

Pneumonia, the most common diagnosis among the positive cases, accounted for 27.85% of the total number of patients. Inflammation of the small bronchi (bronchiolitis) accounted for 20.25% while inflammation of the large ones (bronchitis) accounted for 12.66% of the positive cases.

Bronchial asthma and COPD with infection exacerbation represented 15.20% and 12.66% of the total number of positive cases, respectively. Interstitial pulmonary fibrosis with infection exacerbation was found in 6.33% of the patients. Less common LRTI include infected cystic lung, bronchiectasis and septic embolism (tab. 22).

Table 22: Incidence of viral respiratory infection according to clinical diagnosis of the patients

Clinical diagnosis	Number of cases	Percentage
1-Pneumonia (acute/chronic/necrotizing)	22	27.85%
2-Bronchiolitis	16	20.25%
3-Bronchial asthma with infection exacerbation	12	15.20%
4-COPD with infection exacerbation	10	12.66%
5-Bronchitis (acute/chronic)	10	12.66%
6-IPF with infection exacerbation	05	6.33%
7-Infected cystic lung	02	2.53%
8-Bronchiectasis	01	1.26%
9-Septic embolism (multiple pyemic abscesses)	01	1.26%
Total	79	100%

For the children's group, bronchiolitis was the most common diagnosis made in 50% of the positive cases followed by pneumonia which was detected in 28% of the positive cases. Cases of asthma represented 19% and that of bronchitis represented 3% of the positive cases in children group (fig. 5). Pneumonia was the most common diagnosis made among positive adults (28%). COPD was found in 21% of the positive adult group followed by bronchitis which was found in 19% of the positive cases. Cases of asthma represented 13% while IPF was detected in 10.6% of the positive adult group. The least frequently detected clinical cases were infected cystic lung, bronchiectasis, and septic embolism which were detected in 4.2%, 2.1%, and 2.1% in positive adult group, respectively (fig. 6).

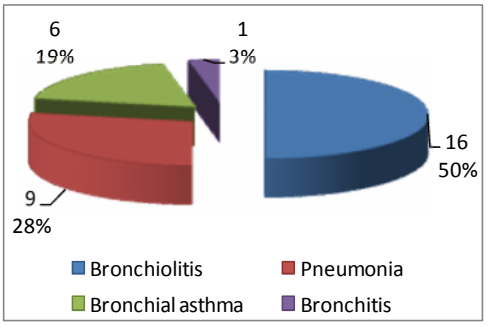

Figure 5: Clinical diagnosis of the positive children group

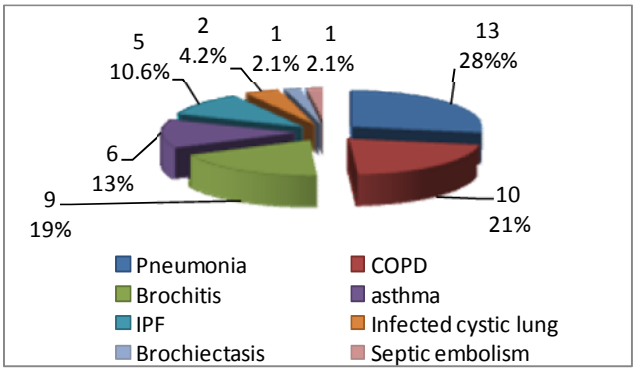

Figure 6: Clinical diagnosis of the positive adult group.

4.1.3. Relation of viral LRTI with residence and site of admission of patients

Viral LRTI was more prevalent in urban (53%) than rural areas (47%) (fig. 7). Most of the patients affected with viral LRTI were admitted to the chest department (45.5%) and the pediatric clinic (40.5%) (fig. 8).

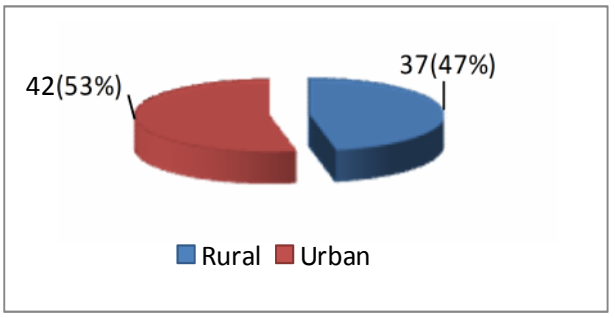

Figure 7: Viral LRTI and residence of the patients

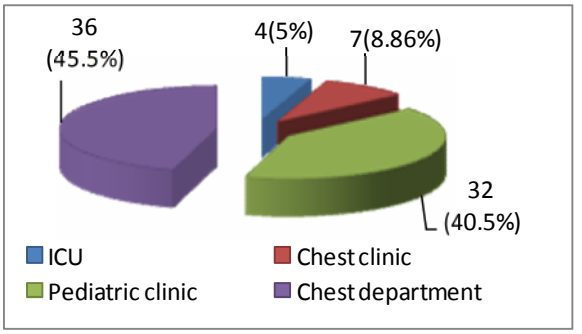

Figure 8: Viral LRTI and site of admission of the patients

4.1.4. Co-infection of the lower respiratory tract

Respiratory co-infection was detected in 44.3% of patients. Viral-bacterial co-infection was the most common type and found in 39.24% of cases. Viral-viral co-infection accounted for a ratio of about 5% of the total number of cases. One case was infected with both *Mycobacterium tuberculosis* and influenza B virus. For 44 cases (about 55.7% of patients), examination for bacterial or fungal infection was not performed (tab. 23).

Table 23: Co-infection of the lower respiratory tract

Co-infection	Type of affecting organisms	No. of patients affected		Percentage
1-Viral-viral infection	Adenovirus and RSV-A	3	4	5.06%
	Adenovirus and hMPV	1		
2-Viral-bacterial	1-*Staph.aureus*	11	31	39.24%
	2-Klebsiella species	5		
	3-*Streptococcus pneumoniae*	4		
	4-NLF Gram negative bacilli	1		
	5-*Mycobacteruim tuberculosis*	1		
	6-Mixed bacterial infection	9		
Total:		35		44.3%

4.1.5. Relation between viral LRTI and presence of one or more of the risk factors

Risk factors were divided into five major groups namely: smoking, associated pleura-pulmonary or cardiovascular condition, associated immune-suppressive condition, immunosuppressive medication, or other systemic affections (fig. 9).

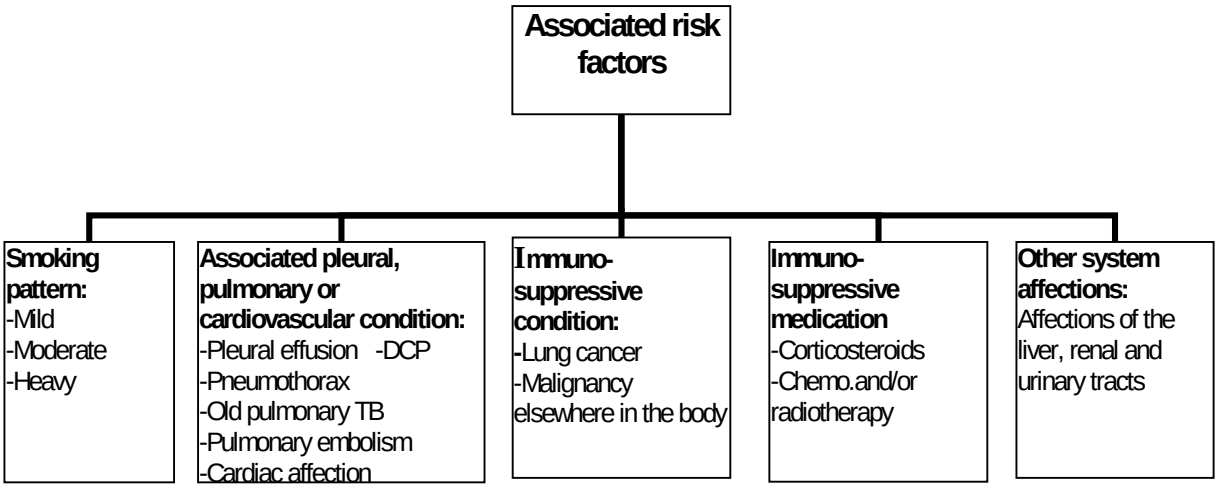

Figure 9: Relationship of risk factors with viral LRTI.

In adults, nine of the smoker group (mild, moderate and heavy smokers) and 12 in the ex-smoker group were found positive for viral LRTI (tab. 24). Accordingly, there was no significant difference between the smoker and non-smoker groups regarding the viral infection (P = 0.94).

Table 24: Incidence of viral LRTI and smoking pattern in adults

Smoking pattern		Number of positive cases		Number of the studied group		Percentage
1-Smoker group	a-Heavy smokers	2	9	26	86	7.7%
	b-Moderate smokers	3		20		15%
	c-Mild smokers	4		40		10%
2-Ex-smoker group		12		110		11%
3-Non-smoker group		26		254		10.2%
Total		47		450		

In the adult group, associated affections of the pleura or cardiovascular system were found in 22 out of the total 79 affected patients representing 28% of the total number of cases. Decompensated Core-pulmonale (DCP) was found in 13% of the positive cases. Pleural affections were detected in 5% of the cases. About 4% of the patients suffered from old pulmonary tuberculosis. Cardiac affections and pulmonary embolism were found in an equal number of patients with a ratio of 2.5% for each while pulmonary edema was the pleuro-pulmonary condition least frequently detected and was found in only one patient (1.26%). Corticosteroids were administered to 15 patients (acute exacerbations of asthma and COPD are routinely managed with corticosteroids). Two patients were suffering from lung carcinoma and thus managed with combined chemo- and radiotherapy. Diabetes mellitus (DM), the most common systemic affection was detected in 11.4% of the total number of positive cases, followed by hypertension which was found in 7.6% of the affected patients. Renal failure was detected in 4% of the positive cases. The least common associated systemic affection found was

hepatocellular failure. For children, Down´s syndrome was found in two infected children (2.5% of the positive cases).

4.2. Identification of viruses causing LRTI by real time-PCR

4.2.1. Influenza viruses

4.2.1.1. Detection of influenza A and B viruses by multiplex-PCR

Twenty two patients (25 samples) were found positive for influenza viruses of both types A and B. Most of the patients infected with influenza viruses were within the adult group (21 out of 22 patients) with a ratio of 4.6% of the total number of adults. One child was infected with influenza virus accounting for 1.4% of the total number of children (tab. 25). The amplification plots for influenza viruses are shown in fig. 10.

Table 25: Positive influenza virus cases and samples

	Number of positive samples	Types of positive samples	Number of positive cases	Percentage of the corresponding group	Percentage of total number of cases
Children	01	Nasal swabs: 1	01	1.4%	0.2%
Adults	24	Sputum: 19 Nasal swabs: 2 Gargle: 1 Tracheal aspirate: 1 Throat swab: 1	21	4.6%	4%
Total	25		22		4.2%

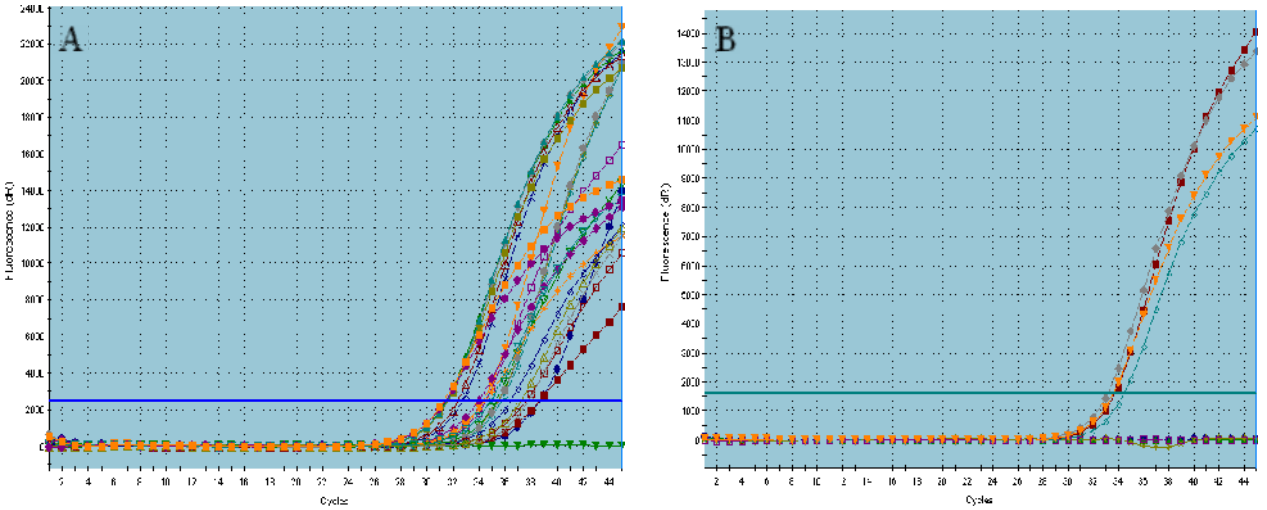

Figure 10: Amplification plots for influenza viruses
A: Amplification plots for influenza A viruses from different samples. The CT value ranged between 31 and 39. B: Amplification plots for influenza B virus case 370/08 (gargle and sputum samples) with CT value ranged between 32 and 35.

4.2.1.2. Typing of influenza viruses by real time-PCR

Influenza A viruses were further differentiated according to hemagglutinin and neuraminidase subtypes. During the 2006-2007 season, predominance of influenza A/H3N2 viruses was

observed. Influenza A/H1N1 viruses were predominant during the 2007/2008 season. The number of influenza B viruses varied from season to season. Typing and subtyping results of influenza positive samples are shown in tab. 26 and fig. 11.

Table 26: Typing and subtyping of influenza virus positive samples

		Season 2005/2006	Season 2006/2007	Season 2007/2008	Total		Percentage
Influenza A	H1N1	0	2	4	6	16	73%
	H3N2	0	8	0	8		
	Not differentiated (weak PCR-products)	0	2	0	2		
Influenza B		1	3	2	6		27%
Total		1	15	6	22		100%

Most of the subtypes of influenza A viruses that affected the adult group belonged to subtype H3N2. One child was infected with influenza B virus (fig. 11).

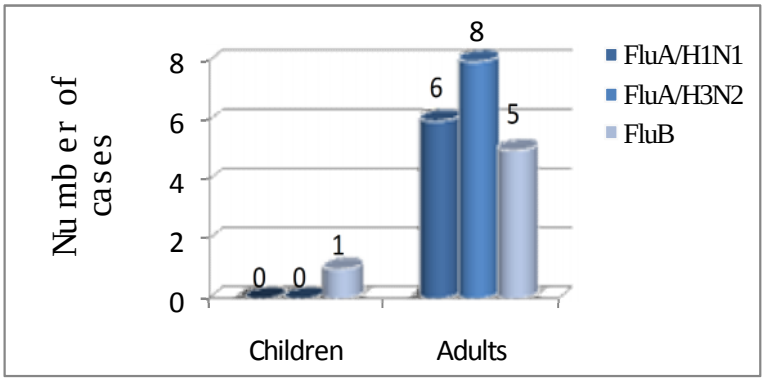

Figure 11: Types and subtypes of influenza viruses within children and adult groups

4.2.2. Respiratory syncytial virus (RSV)

4.2.2.1. Detection of RSV positive samples by real time-PCR

Sixteen patients (24 samples) were found positive for RSV. In contrast to influenza viruses which were more frequently detected in the adult population, most of the positive RSV cases belonged to the childrens' group (tab. 27). All RSV positive cases were detected for two seasons, 2006/2007 and 2007/2008. No case was detected in the season 2005/2006. The mean age of RSV-infected children was 15 (months) ± 14.76 [average mean ± standard deviations].

Table 27: Positive RSV cases

	Number of positive samples	Types of positive samples	Number of positive cases	Percentage to the corresponding group	Percentage to total number of cases
Children	19	Nasal swabs: 11 Throat swabs: 8	12	17%	2.3%
Adults	05	Sputum: 4 Gargle: 1	04	0.8%	0.77%
Total	24		16		3.07%

4.2.2.2. Differentiation of RSV using real time-PCR

RSV A was predominant in the positive RSV cases. Fourteen positive cases (eleven of them were children and three adults) had RSV group A representing a ratio of 63.6%. Only one patient (one sample) was found to be in the RSV group B representing 4.5% of the positive RSV cases. Two of the patients' samples could not be differentiated into type A or B due to weak PCR amplicons.

4.2.3. Adenoviruses (AdV)

Adenoviruses were detected with real time-PCR in 23 samples collected from 20 patients. Within the adult group, 13 cases were diagnosed positive for AdV representing 2.9% of the total number of adults. The number of positive AdV cases in children was seven which represented 10% of the total number of children (tab. 28). The mean age of AdV-infected children was 1.54 (years) ± 1.47 [average mean ± standard deviations].

Table 28: Positive adenovirus cases

	Number of positive samples	Types of positive samples	Number of positive cases	Percentage to the corresponding group	Percentage to total number of cases
Children	9	Nasal swabs: 7 Throat swabs: 2	7	10%	1.34%
Adults	14	Sputum: 12 Gargle: 1 Nasal swab: 1	13	2.9%	2.5%
Total	23		20		3.84%

4.2.4. Human Metapneumovirus (hMPV)

For hMPV, 21 positive cases were diagnosed with real time-PCR. Infection with hMPV was detected more frequently in children than adults. Infected children with hMPV were 12 (with a percentage of 17% of the total number of children) while the number of adults infected with hMPV was nine representing a ratio of 2% of the total number of adults involved in the study (tab. 29). All hMPV positive cases were detected in the season 2007/2008. The mean age of hMPV-infected children was 2.3 (years) ± 1.67 [average mean ± standard deviations]. In fig. 12, the amplification plots for patient 349 are shown.

Table 29: Positive hMPV cases

	Number of positive samples	Types of positive samples	Number of positive cases	Percentage to the corresponding group	Percentage to total number of cases
Children	17	Nasal swabs: 12 Throat swabs: 5	12	17%	2.3%
Adults	15	Gargle: 7 Sputum: 4 Nasal swab: 2 Nasal aspirate: 1 Throat swab: 1	9	2%	1.73%
Total	32		21		4%

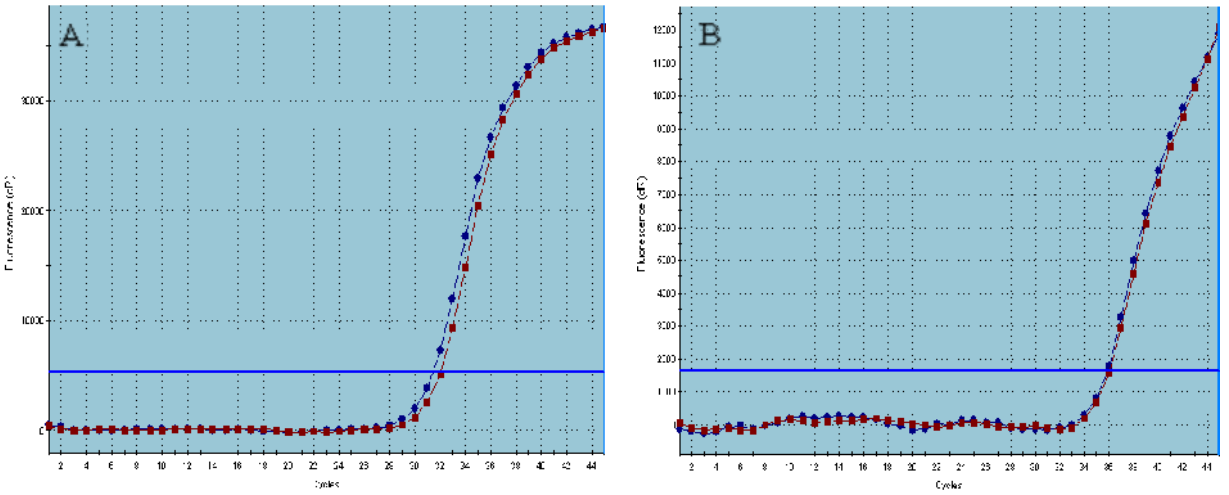

Figure 12: Amplification plots for samples obtained from the nose and throat of a positive hMPV case.

A: 349(NS) with a CT value of 32. B: 349(TS) with a CT value of 36.

*Difference in CT between different samples taken from the same patient.

4.3. Isolation of viruses on tissue culture cells

For the respiratory viruses included in this research, six influenza-positive viruses and two adenovirus-positive viruses could be isolated on tissue culture cells. Trials were done to isolate hMPV on LLC-MK2 and Vero cells but no virus could be isolated on both types of tissue culture cells. Difficulties in growing RSV and hMPV from clinical samples have been recorded. For either, no positive samples could be isolated (tab. 30). A characteristic cytopathic effect for both adeno and influenza viruses is shown in fig. 13 and 14, respectively.

Table 30: Isolation of viruses on tissue culture cells

Virus	Tissue culture cells	Number of isolates/ total number of positive samples (percentage)	Types of positive samples	Number of isolates/ total number of positive cases (percentage)
Influenza	MDCK	6/25 (24%)	Sputum: 5 Tracheal aspirate: 1	6/22 (27.3%)
RSV	Hep2	0/24 (0%)	0	0/16 (0%)
Adenovirus	Hep2	2/23 (8.7%)	Nasal swab: 1 Throat swab: 1	1/20 (5%)
HMPV	LLC-MK2 Vero cells	0/32 (0%)	0	0/21 (0%)

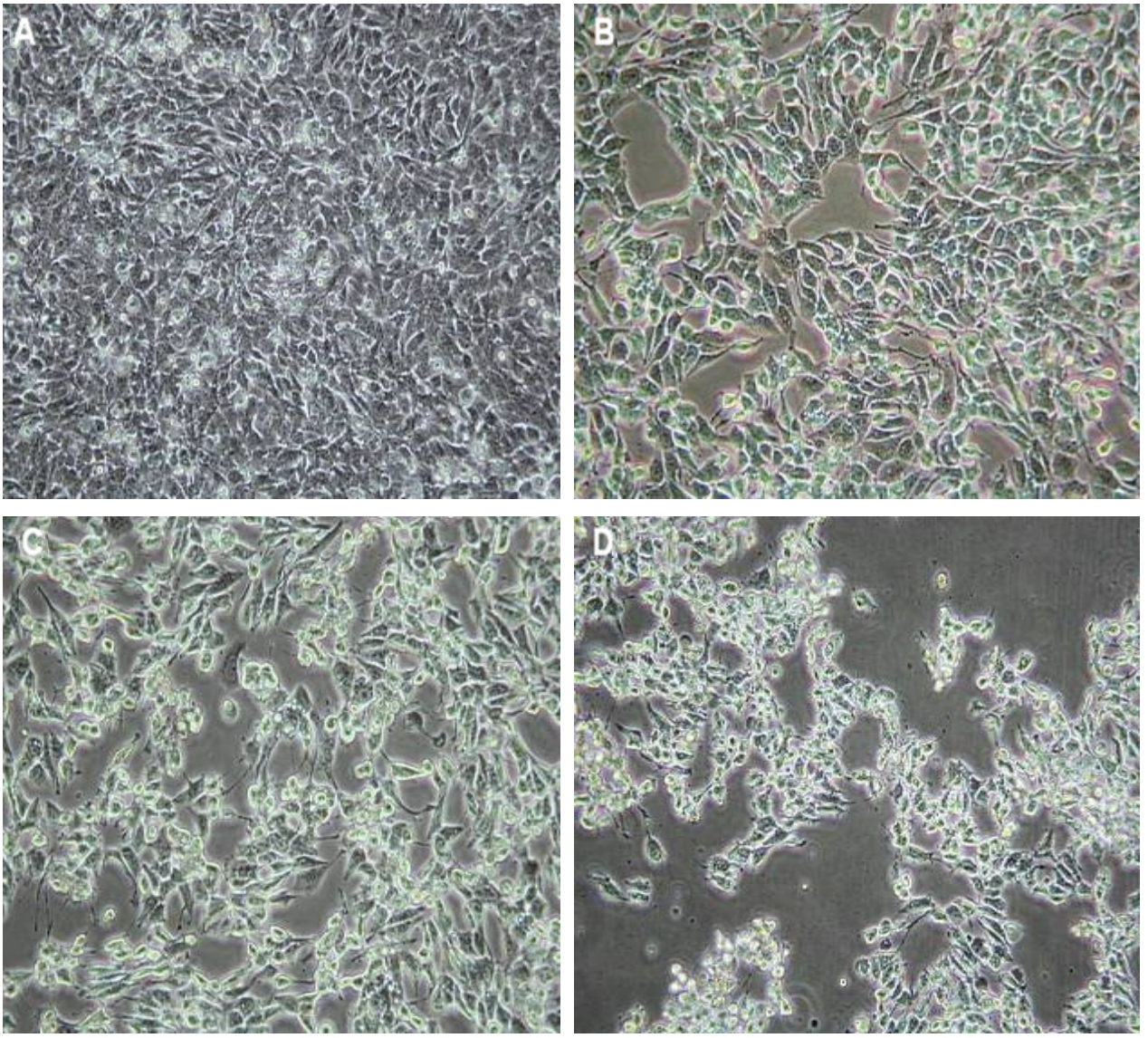

Figure 13: Unstained Hep2 cells after inoculation with adenovirus (sample 394NS/08)
(A) Non-inoculated cells.
(B) Three days post-infection: focal cell rounding occurred.
(C) Four days post-infection: increased rounding and clustering of the cells in grape-like clusters.
(D) Five days post infection: generalized clumping of the cells and detachment from the vial surface occurred. The cells were observed using 40X magnification.

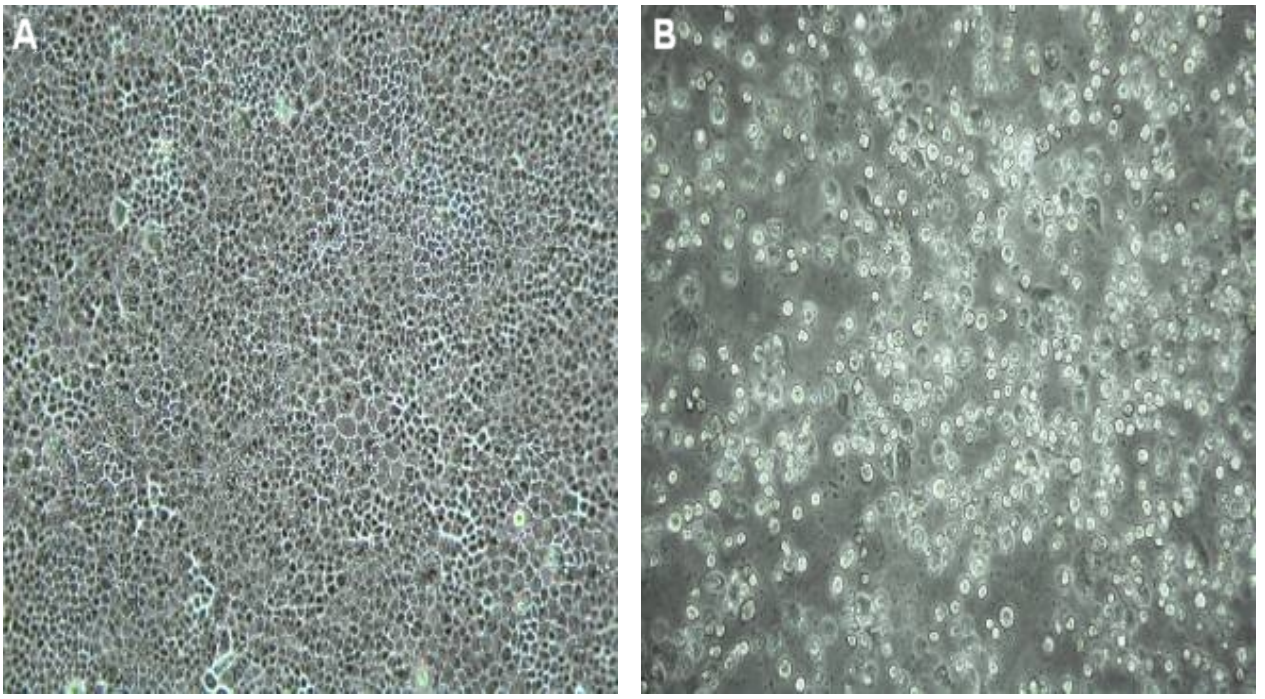

Figure 14: Unstained MDCK cells after inoculation with influenza virus (sample 370S/08) Rounding and detachment of the cells occurred one day post-infection (B) which was followed rapidly by detachment of the whole monolayer in comparison with non-infected cells (A). The cells were observed using 40X magnification.

4.4. Titration of influenza virus isolates

4.4.1. Titration of influenza virus isolates using the plaque assay

The plaque test was performed for determination of the virus titre of five influenza A and one influenza B isolates. Plaque-forming units were calculated for one millilitre (ml) of the tissue culture fluid. The results of the plaque test are shown in table 31. Plaque test for isolate 428(S)/08 is shown in fig. 15.

Table 31: Titration of influenza virus isolates by the plaque assay

Isolate		Plaque assay
Influenza A	Isolate 57(S)/06	1.3×10^{3} PFU/ml
	Isolate 62(S)/07	1.2×10^{6} PFU/ml
	Isolate 358(TA)/08	1.9×10^{3} PFU/ml
	Isolate 425(S)/08	1.6×10^{3} PFU/ml
	Isolate 428(S)/08	1.8×10^{5} PFU/ml
Influenza B	Isolate 370(S)/08	1.4×10^{6} PFU/ml

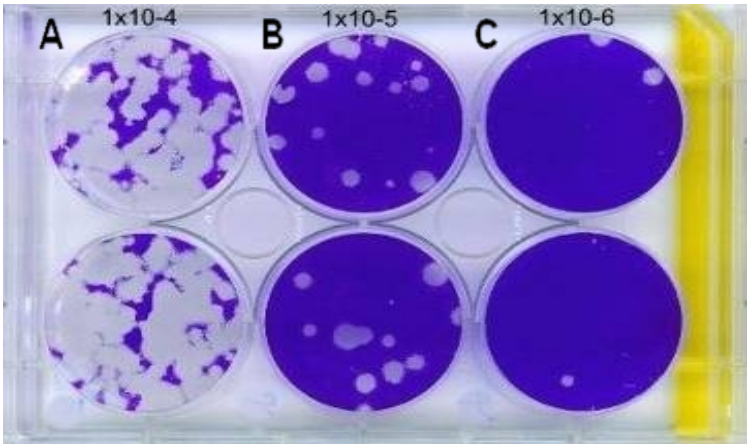

Figure 15: Plaques produced on MDCK cells by isolate 428(S)/08 (influenza A virus)
A, B, and C were infected monolayers with 10^{-4}, 10^{-5}, and 10^{-6} dilutions of the supernatant from the first passage of the isolate, respectively.

4.4.2. Titration of influenza virus isolates by the hemagglutination (HA) assay

Agglutination of the RBCs in suspension is recorded using a "+" symbol while the absence of hemagglutination is recorded with the symbol "-" (as in the cell control wells where RBCs form a compact button at the bottom of the wells). When a portion of the RBCs is partially agglutinated or partially settled, a "+/-" symbol is used. Influenza A/ H1N1 and influenza B reacted very well with turkey (T) RBCs, while those of influenza A subtype H3N2 react better with Guinea pig (GP) RBCs. Influenza B isolate 370(S)/08 showed the highest HA titer (+/- 1280). The titres of influenza A/H1N1 isolates 358(TA)/08, 425(S)/08, and 428(S)/08 were +/- 40, +/- 160, and +/- 320, respectively. For influenza A/H3N2 isolates 57(S)/06 and 62(S)/07, HA titers were 80 and +/- 160, respectively (tab. 32).

Table 32: Hemagglutination titres of influenza virus isolates

Blood	Isolate	Titer							
		10	20	40	80	160	320	640	1280
GP	57(S)(A/H3N2)/06	+	+	+	+	-	-	-	-
GP	62(S)(A/H3N2)/07	+	+	+	+	+/-	-	-	-
T	358(TA)(A/H1N1)/08	+	+	+/-	-	-	-	-	-
T	425(S)(A/H1N1)/08	+	+	+	+	+/-	-	-	-
T	428(S)(A/H1N1)/08	+	+	+	+	+	+/-	-	-
T	370(S)(B)/08	+	+	+	+	+	+	+	+/-

4.5. Antigenic characterization of influenza virus isolates by the hemagglutination inhibition (HI) assay

Influenza A/H1N1 virus isolates reacted well with the immune serum against both the vaccine strains A/Solomon Islands/3/2006 and A/Brisbane/59/07, and to a lesser extent with antiserum to the reference strain A/New Caledonia/20/99. The highest titre was that for isolate 425(S)/08. Isolate 428(S)/08 reacted with the antisera to the three reference strains with titres of 1:40, 1:160, and 1:10, respectively (fig. 16). For influenza A/H3N2 virus isolates, reaction occurred with antisera to the reference strains A/Wisconsin/67/05 and A/Brisbane/10/07. Only one influenza B virus isolate reacted well with the immune serum to the reference strain B/Malaysia/25/06 with a titre of 1:40 (tab. 33).

Table 33: Hemagglutination inhibition titres of influenza virus isolates

Blood	Isolates	Virus titer	Antisera							
			H1			H3		B		
			A/New Caledo. /20/99	A/Sol. Islands /3/2006	A/Bris. /59/07	A/Wisc. /67/05	A/Bris. /10/07	B/Flor. /4/06	B/Mal. /2506/ 04	B/Jian. /10/03
T	A/New Cal. /20/99		320	160	80	Not tested	Not tested	Not tested	Not tested	Not tested
T	A/Sol. Islands /3/2006		40	640	640	Not tested	Not tested	Not tested	Not tested	Not tested
T	A/Bris./59/07		<20	160	640	Not tested	Not tested	Not tested	Not tested	Not tested
GP	A/Wisc./67/ 05		Not tested	Not tested	Not tested	640	160	Not tested	Not tested	Not tested
GP	A/Brisbane /10/07		Not tested	Not tested	Not tested	1280	1280	Not tested	Not tested	Not tested
T	B/Florida /4/06		Not tested	Not tested	Not tested	Not tested	Not tested	640	<20	320
T	B/Malaysia /2506/04		Not tested	Not tested	Not tested	Not tested	Not tested	<20	1280	<20
T	B/Jiangsu /10/03		Not tested	Not tested	Not tested	Not tested	Not tested	160	<20	640
GP	57(S)/06 (A/H3N2)	8	<10	<10	<80	**20**	**80**	<10	<10	<10
GP	62(S)/07 (A/H3N2)	10	<10	<10	<80	**80**	**80**	<10	<10	<10
T	358(TA)/08 (A/H1N1)	64	<10	**80**	**160**	<10	<80	<10	<10	<10
T	425(S)/08 (A/H1N1)	64	**40**	**320**	**320**	<10	<80	<10	<10	<10
T	428(S)/08 (A/H1N1)	64	**10**	**40**	**160**	<10	<80	<10	<10	<10
T	370(S)/08 (B)	128	<10	<10	<80	<10	<80	<10	**40**	<10

Figure 16: Hemagglutination inhibition assay of isolate 428(S)/08 (influenza A/H1)
The test was performed in a microtiter plate using turkey erythrocytes. When reaction between the antigen (virus) and antibody occur, erythrocytes settle to the bottom of the well as a spherical red button. The characteristic pattern of complete inhibition of hemagglutination is noted by the tear-shaped streaming of erythrocytes. HI titers are listed in table 33.

4.6. Typing of adenovirus with fluorescence curve melting analysis

Seven species of AdV are known (A-G). For the 23 adenovirus positive samples that were detected with real time-PCR, 19 could be typed with the fluorescent melting curve analysis. Species B AdV was the most frequently detected one comprising seven samples (30%) followed by species C and D (each was 21.75%). Species A or F was positive only in one patient. No samples belonged to species E or species G (figure 17). Four samples had weak PCR-amplicons and thus could not be typed into specific adenovirus species. Example for the melting peaks for AdV species B and A are shown in figure 18.

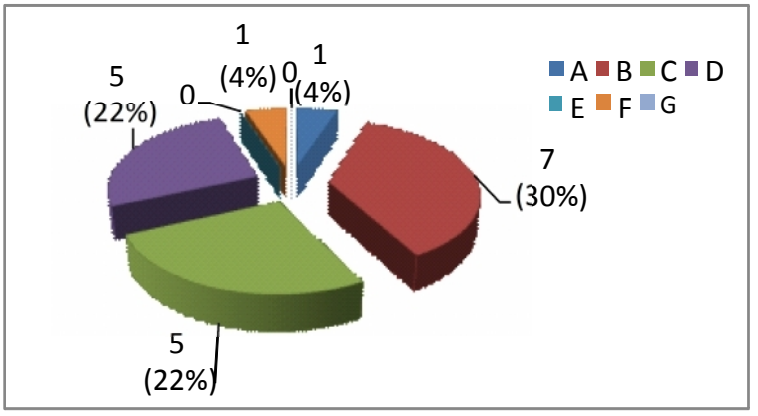

Figure 17: Melting curve analysis of adenovirus positive samples
Species A Species B Species C Species D
Species E Species F Species G

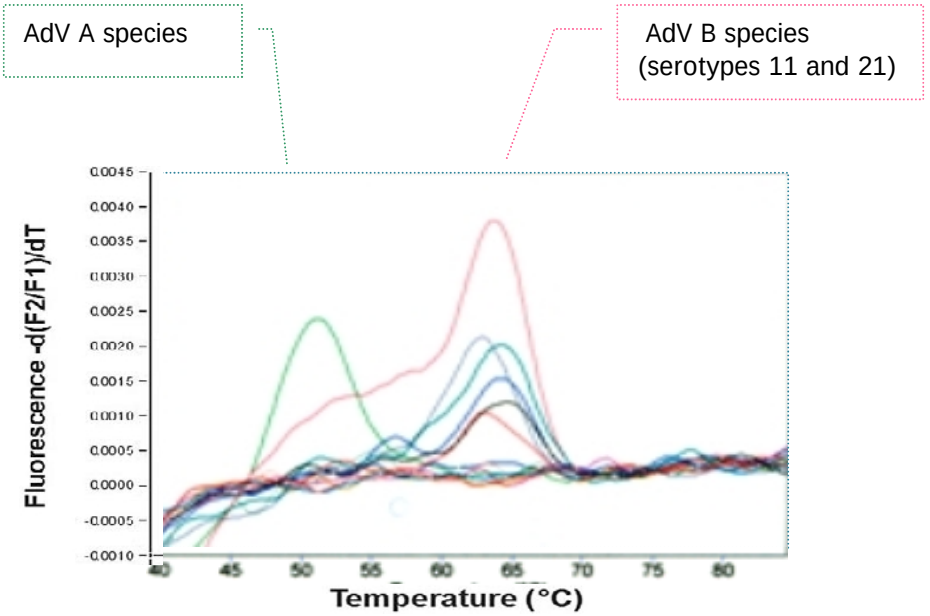

Figure 18: FMCA with hybridization samples for AdV species B and A-PCR products
Seven samples of adenovirus were examined by FMCA with probes specific for AdV species B. The six serotypes of species B AdV have the highest melting temperature (~ 63ºC) while serotypes of species A AdV have a lower melting temperature (~ 50ºC).

4.7. Sequencing and phylogenetic analysis of respiratory viruses

4.7.1. Influenza viruses

4.7.1.1. Influenza A/H1N1 viruses

4.7.1.1.1. Phylogenetic analyses of the hemagglutinin (HA) gene

Hemagglutinin gene sequences of H1N1 strains were compared with those from German isolates (obtained from the NIC-RKI) and published HA gene sequences available through Influenza Sequence Database (http://www.flu.lanl.gov/). The Brisbane group divided into two sublineages A and B and is characterised by amino acid substitution at positions 35 (D35N), 140 (K140E), 188 (R188K), and 273 (E273K) compared to recent A/H1N1 viruses. Group B showed amino acid substitution of alanine by threonine at position 189 (A189T). The German strains of 2009 clustered in A/Brisbane/59/2007 group B while those of 2008 clustered in A/Brisbane/59/2007 group A. The phylogenetic analysis showed that viruses from Egypt clustered inside the reference strain A/Brisbane/59/2007 group A. They show phylogenetic distance to the vaccine strain A/Solomon Islands/3/2006. The three isolates are closely related to each other and share also similarity to isolates from Germany isolated also in 2008 (bootstrap value 79). They showed amino acid mutation at position 270 where the amino acid proline was substituted by serine (P270S). The other Egyptian influenza strain 306(NS)/08 showed some similarity to the strain THR/1/08 from Germany (bootstrap value 73) (fig. 19).

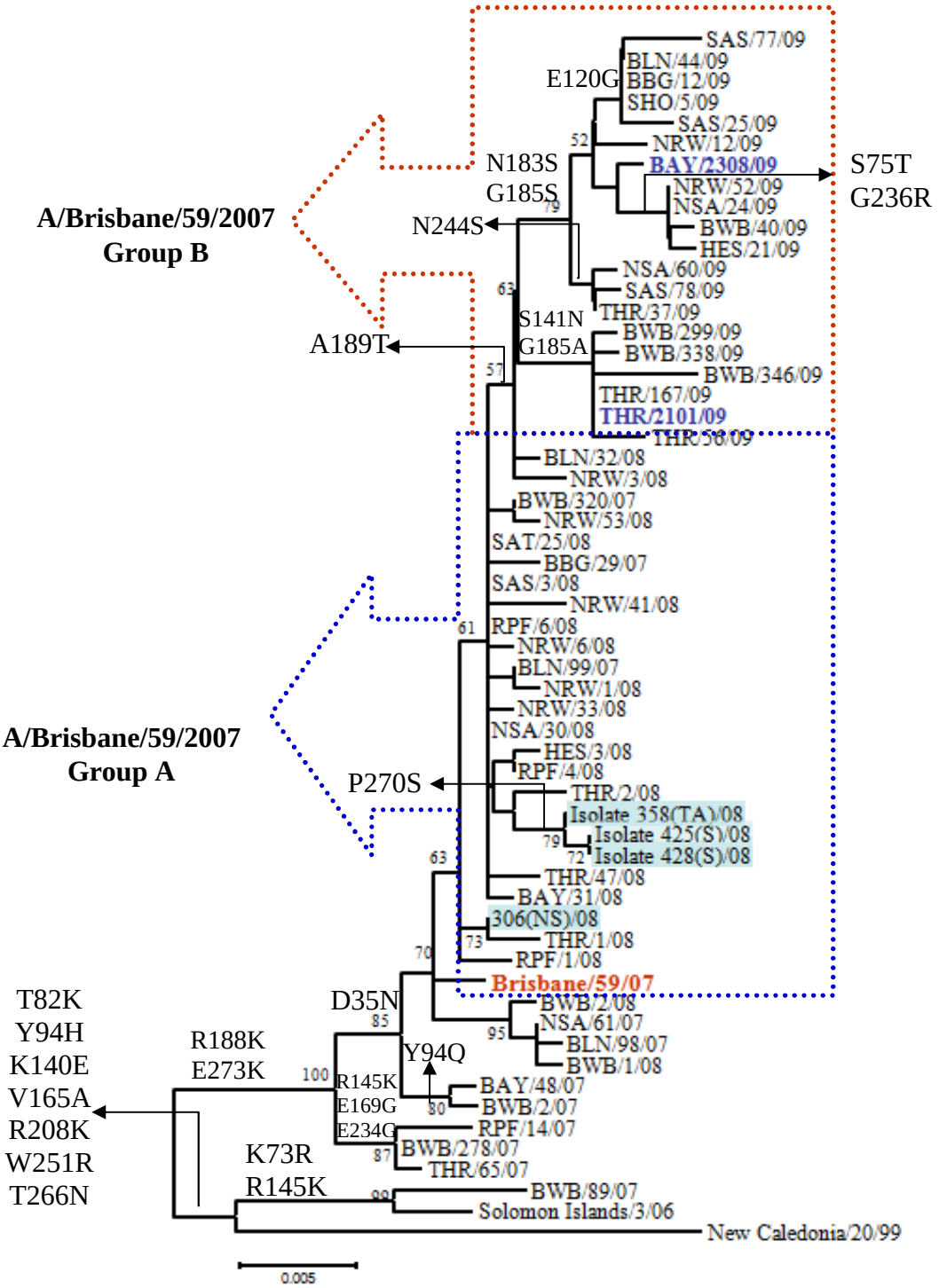

Figure 19: Phylogenetic analysis of hemagglutinin gene of influenza A/H1N1 viruses
Four viruses identified in the study group (shaded in light blue) were included in this analysis. Three viruses could be isolated whereas sample 306(NS)/08 was sequenced directly from the patient´s specimen. Two isolates from Germany belonging to A/Brisbane/59/2007 group B were highlighted in dark blue and used as reference strains for the group. The tree was rooted to New Caledonia/20/99. The phylogenetic trees were generated with Neighbour-Joining method and bootstrapped with 1000 replicates. The percentage of bootstrap frequencies at the major branches are indicated. Viruses are identified by the geographic location, number, and year of isolation. The lengths of the horizontal lines are proportional to the number of nucleotide differences as indicated by the bar.

The three tested isolates 358(TA)/08, 425(S)/08, and 428(S)/08 showed eleven, eight, and two amino acids changes relative to A/New Caledonia/20/99, A/Solomon Islands/3/06, and A/Brisbane/59/07, respectively, while sample 306(NS)/08 showed ten, seven, and one amino acid mutations relative to A/New Caledonia/20/99, A/Solomon Islands/3/06, and A/Brisbane/59/07, respectively (tab. 34).

Table 34: Predicted amino acid substitutions of H1 region of the HA of recent influenza A/H1N1 strains

Strain	Amino acid residues								
	35	73	82	94	128	140	145	165	186
A/New Caledonia/20/99	D	K	T	Y	V	K	R	V	D
A/Solomon Islands/3/06	D	R	K	H	T	E	K	A	D
A/Brisbane/59/07	N	K	K	H	V	E	R	A	I
306(NS)/08	N	K	K	H	V	E	R	A	D
Isolate 358(TA)/08	N	K	K	H	V	E	R	A	D
Isolate 425(S)/08	N	K	K	H	V	E	R	A	D
Isolate 428(S)/08	N	K	K	H	V	E	R	A	D

Strain	Amino acid residues						
	188	208	222	251	266	270	273
New Caledonia/20/99	R	R	Q	W	T	P	E
Solomon Islands/3/06	R	K	R	R	N	P	E
Brisbane/59/07	K	K	Q	R	N	P	K
306(NS)/08	K	K	Q	R	N	P	K
Isolate 358(TA)/08	K	K	Q	R	N	S	K
Isolate 425(S)/08	K	K	Q	R	N	S	K
Isolate 428(S)/08	K	K	Q	R	N	S	K

The numbers correspond the amino acids´ position in the HA molecule. The letters represent the predicted amino acid residues: A, alanine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; K, lysine; N, asparagines; Q, glutamine; P, proline; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

4.7.1.1.2. Phylogenetic analyses of the neuraminidase (NA) gene

All the tested viruses clustered inside the A/Brisbane/59/2007 group which is characterized by three main amino acid substitutions compared to A/Solomon Islands/3/06 at positions 45 (asparagine instead of histidine), 78 (glutamic acid instead of lysine), and 249 (lysine instead of glycine). The three isolates 358(TA)/08, 425(S)/08, and 428(S)/08 were similar to each other as well as to German strains for the season 2008/2009. They showed substitution of histidine by tyrosine at position 275 (H275Y) which is characteristic for oseltamivir-resistant viruses. Sample 306(NS)/08 was closely related to the German isolate A/THR/1/08 and revealed no resistance to neuraminidase inhibitors oseltamivir and zanamivir (fig. 20).

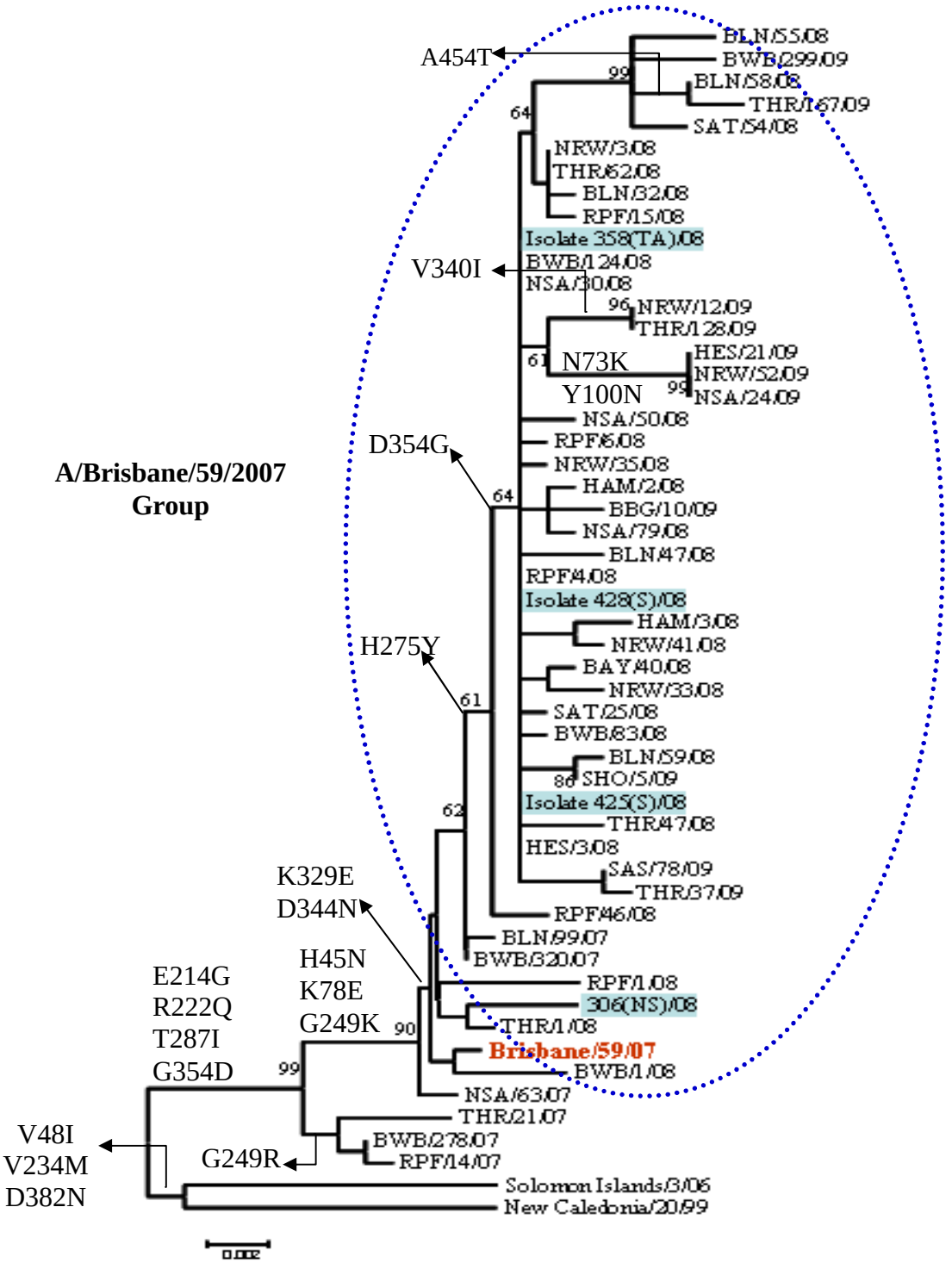

Figure 20: Phylogenetic analysis of neuraminidase gene of influenza A/H1N1 viruses
The tested viruses are shaded in light blue. The reference vaccine strain A/Brisbane/59/2007 is shown in bold and coloured dark red. The tree was generated with Neighbor-Joining method with 1000 bootstrap replicates and rooted to New Caledonia/20/99. The scale bar represents 0.2% of nucleotide changes between close relatives.

4.7.1.2. Influenza A/H3N2 viruses

4.7.1.2.1. Phylogenetic analyses of the hemagglutinin (HA) gene

Hemagglutinin gene sequences from H3N2 strains were compared with each other as well as with strains from Germany and reference strains available from GenBank. Five influenza A/H3N2 viruses were available for sequencing. The season 2006/2007 was characterized by co-circulation of two different variants of A/H3N2 viruses. A/Nepal/921/06-like viruses possessed three amino acid substitutions compared to A/Wisconsin/67/05 (N6I, R142G, and K173E).

However, typical of the A/Brisbane/10/07 group were the amino acid substitutions G50E and K140I. The Egyptian viruses showed amino acid substitutions at position 193 (S193E) and 225 (D225N) which confer resistance to amantadine.

Three strains from Egypt clustered within the vaccine strain A/Brisbane/10/2007-group A (bootstrap value 89), while the other two strains clustered in the A/Nepal/921/2006 group and were genetically very close to each other (fig. 21).

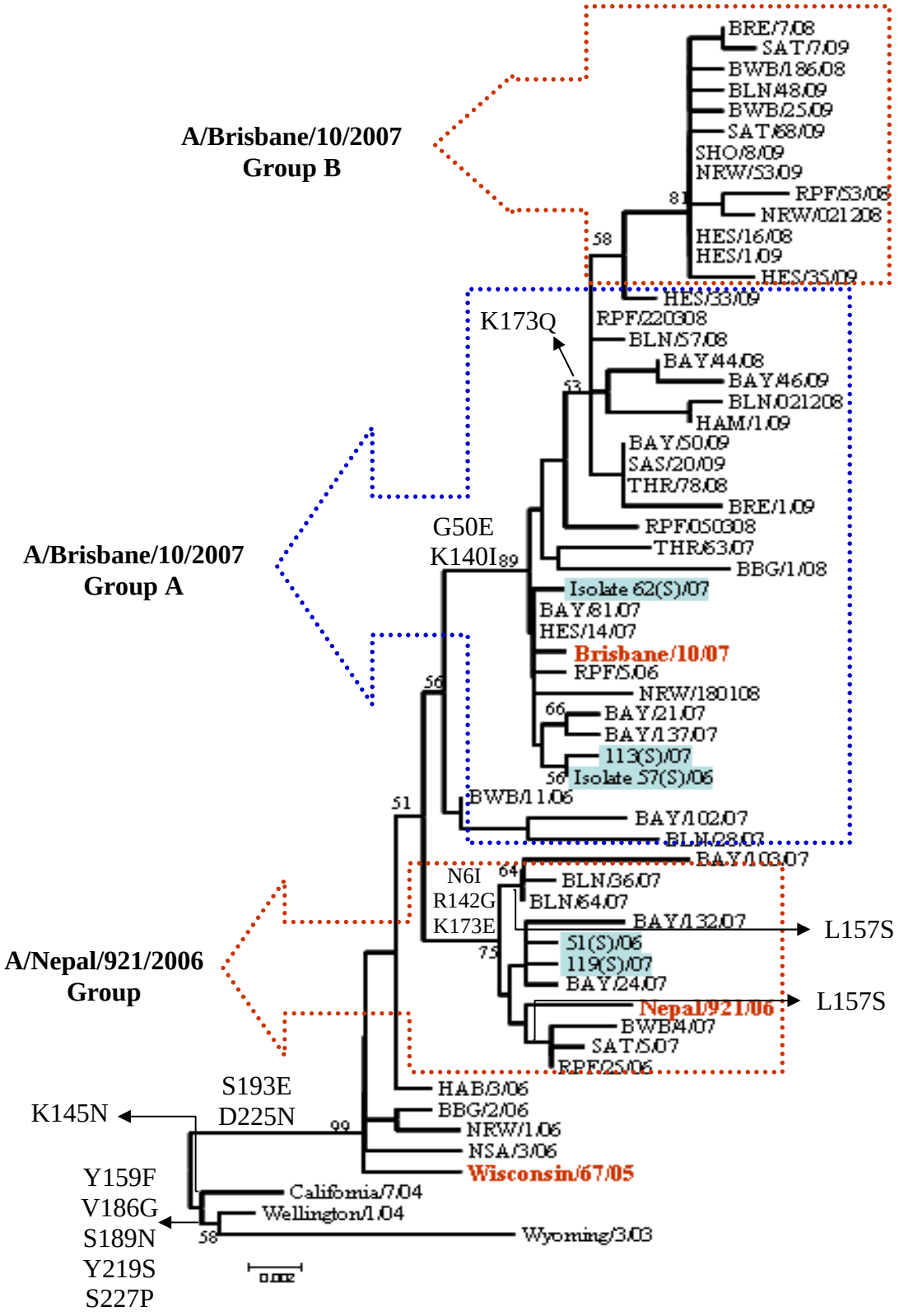

Figure 21: Phylogenetic analysis of hemagglutinin gene of influenza A/H3N2 viruses
The tested viruses are shaded in light blue. Reference strains are in bold and coloured dark red. The tree was generated with Neighbor-Joining method with 1000 bootstrap replicates and rooted to Wyoming/3/03.

Isolate 57(S)/06 shared amino acid identities of 98.3%, 99.7%, and 97.4% and nucleic acid homologies of 98.96%, 99.8%, and 98.3% relative to the reference strains A/Wisconsin/67/05, A/Brisbane/10/07, and A/Nepal/921/06, respectively. The amino acid homologies of isolate 62(S)/07 were 98.6%, 100%, and 97.7% while the nucleic acid homologies were 99%, 100%, and 98.6% relative to the three reference strains, respectively. Sample 51(S)/06 showed amino acid homologies of 97.7%, 98%, and 98.6% and nucleotide sequence homologies of 98.6%, 98.77%, and 99% relative to the three reference strains, respectively. The amino acid sequence identities of sample 113(S)/07 were 98.3%, 99.7%, and 98.5% and the nucleotide sequence homologies were 99%, 99.9%, and 98.5% relative to the reference strains, respectively. For sample 119(S)/07, the amino acid sequence identities were 98%, 98.3%, and 98.9% and the nucleic acid sequence homologies were 98.87%, 98.96%, and 99.24% relative to the three reference strains, respectively.

Isolates 57(S)/06, 62(S)/07 and sample 113(S)/07 possessed five and seven amino acid substitutions compared to A/Wisconsin/67/05 and A/Nepal/921/06, respectively, while they had no amino acid changes relative to A/Brisbane/10/07. Sample 51(S)/06 exhibited seven, five, and five amino acid substitutions relative to the three reference strains, respectively. For sample 119(S)/07, in the amino acid there were six, five, and four changes relative to the reference strains A/Wisconsin/67/05, A/Brisbane/10/07, and A/Nepal/921/06, respectively (tab. 35).

Table 35: Amino acid substitutions of the studied influenza A viruses/H3 gene

Isolate designation	Residue number											
	59	66	138	144	156	158	172	173	189	202	212	239
Wisconsin/67/05	V	G	D	T	K	R	H	L	K	G	H	I
Brisbane/10/07	V	E	N	T	I	R	H	L	K	G	Y	V
Nepal/921/06	V	E	N	A	K	G	Q	S	E	V	Y	V
Isolate 57(S)/06	V	E	N	T	I	R	H	L	K	G	Y	V
Isolate 62(S)/07	V	E	N	T	I	R	H	L	K	G	Y	V
51(S)/06	I	G	N	A	K	G	H	L	E	G	Y	V
113(S)/07	V	E	N	T	I	R	H	L	K	G	Y	V
119(S)/07	V	G	N	A	K	G	H	L	E	G	Y	V

The numbers correspond to the amino acids' position in the HA gene of influenza A/H3N2 viruses. The letters represent the predicted amino acid residues as mentioned in table 52.

4.7.1.2.2. Phylogenetic analyses of the neuraminidase (NA) gene

Two isolates could be phylogenetically characterized in this study. They clustered within the A/Brisbane/10/2007 group which characterized by the amino acid substitutions H150R, V194I, Y310H, and S372L relative to A/Wisconsin/67/05. They showed phylogenetic distance to the A/Nepal/921/06 group which is characterized by the amino acid changes N43S and S315R relative to A/Wisconsin/67/05 (fig. 22).

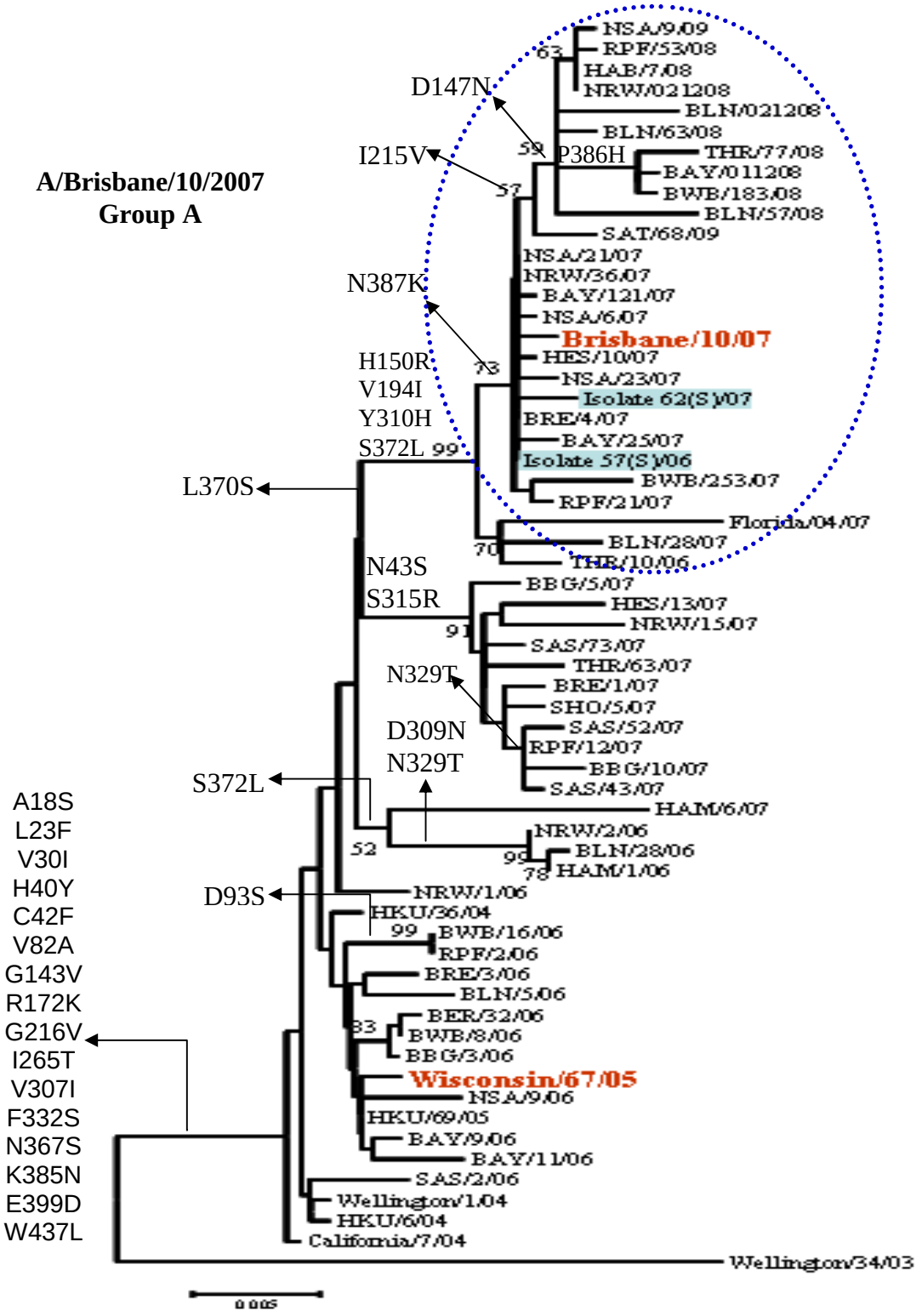

Figure 22: Phylogenetic analysis of neuraminidase gene of influenza A/H3N2 viruses
The viruses studied are shaded in light blue. Reference strains are shown in bold and coloured dark red. The tree was generated with Neighbor-Joining method with 1000 bootstrap replicates.

4.7.1.3. Influenza B viruses

4.7.1.3.1. Phylogenetic analyses of the hemagglutinin (HA) gene

The phylogenetic tree describes the two lineages of influenza B viruses, the Yamagata and Victoria lineage which established itself at the beginning of the 1980s and circulated with varying intensity. The viruses that circulated in Germany and in other European countries during the season 2007/2008 belonged to the Yamagata lineage represented by three different variants as shown in fig. 23. However, the next season 2008/2009, Victoria lineage viruses predominated and Yamagata lineage viruses were rarely isolated in Europe and the northern hemisphere. The isolate 370(S)/08 that could be sequenced and analysed in this study clustered within B/Malaysia/2506/04 group (Victoria lineage) which showed amino acid substitutions K48E, K80R, and K129N with regard to the former reference strain B/Shandong/7/97. It shared 100% nucleotide identity with isolate 38/08 from Berlin (fig. 23).

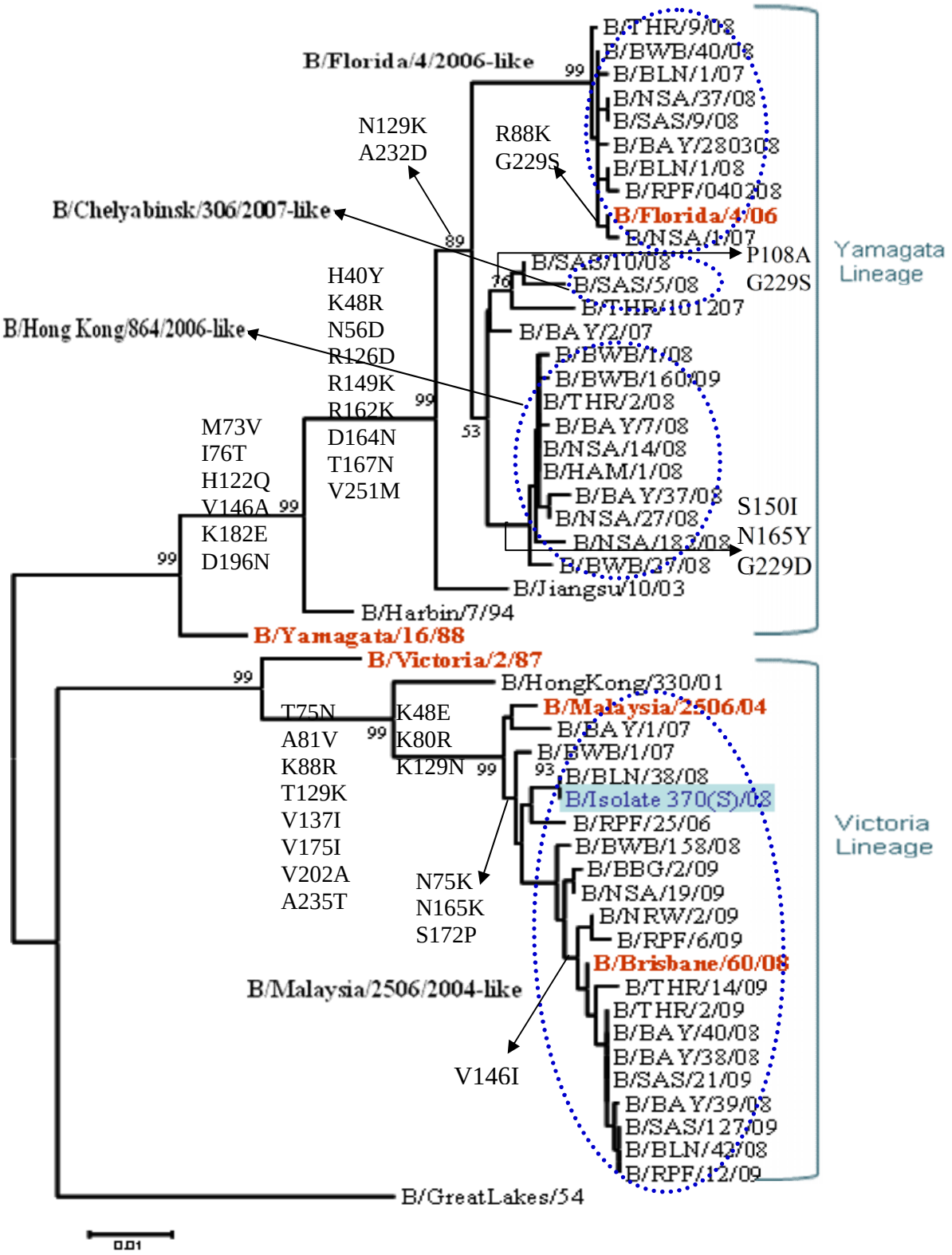

Figure 23: Phylogenetic analysis of hemagglutinin gene of influenza B viruses
The viruses tested are shaded in light blue. Reference strains are red coloured and shown in bold. The tree was generated with Neighbor-Joining method with 1000 bootstrap replicates. The scale bar represents 0.1% of nucleotide changes between close relatives.

Isolate 370(S)/08 showed amino acid homologies of 99% and 98.3% and nucleotide sequence homologies of 99.4% and 99.2% relative to the reference strains B/Malaysia/2506/04 and B/Brisbane/60/08, respectively. It had two amino acid homologies relative to B/Malaysia/2506/04 and four amino acid substitutions relative to B/Brisbane/60/08 (tab. 36).

Table 36: Amino acid substitutions of the studied influenza B viruses HA gene

Isolate designation	Amino acid residues					
	75	134	146	165	172	199
B/Malaysia/2506/04	N	S	V	N	S	A
B/Brisbane/60/08	K	P	I	K	P	T
B/Isolate 370(S)/08	N	P	V	N	S	T

The numbers correspond to the amino acid position in the HA molecule. For amino acids' abbreviations see table 34.

4.7.1.3.2. Phylogenetic analyses of the neuraminidase (NA) gene

Influenza B viruses circulating during the season 2007/2008 were found to belong to Yamagata lineage. These viruses are subdivided into two subgroups according to their HA gene. B/Florida/4/2006-like viruses possessed a Yamagata lineage HA whereas B/Malaysia/2506/2004-like viruses had a HA typical of recent Victoria lineage viruses. As a consequence, all these HA-Victoria lineage viruses were reassortants with an NA retained from Yamagata lineage viruses. This was also the case with the Egyptian isolate 370(S)/08. The NA gene of this virus clustered with B/Malaysia/2506/04-like viruses and showed close similarity to the German strain B/NSA/15/07 which clustered also in the B/Malaysia/2506/2004 group (fig. 24).

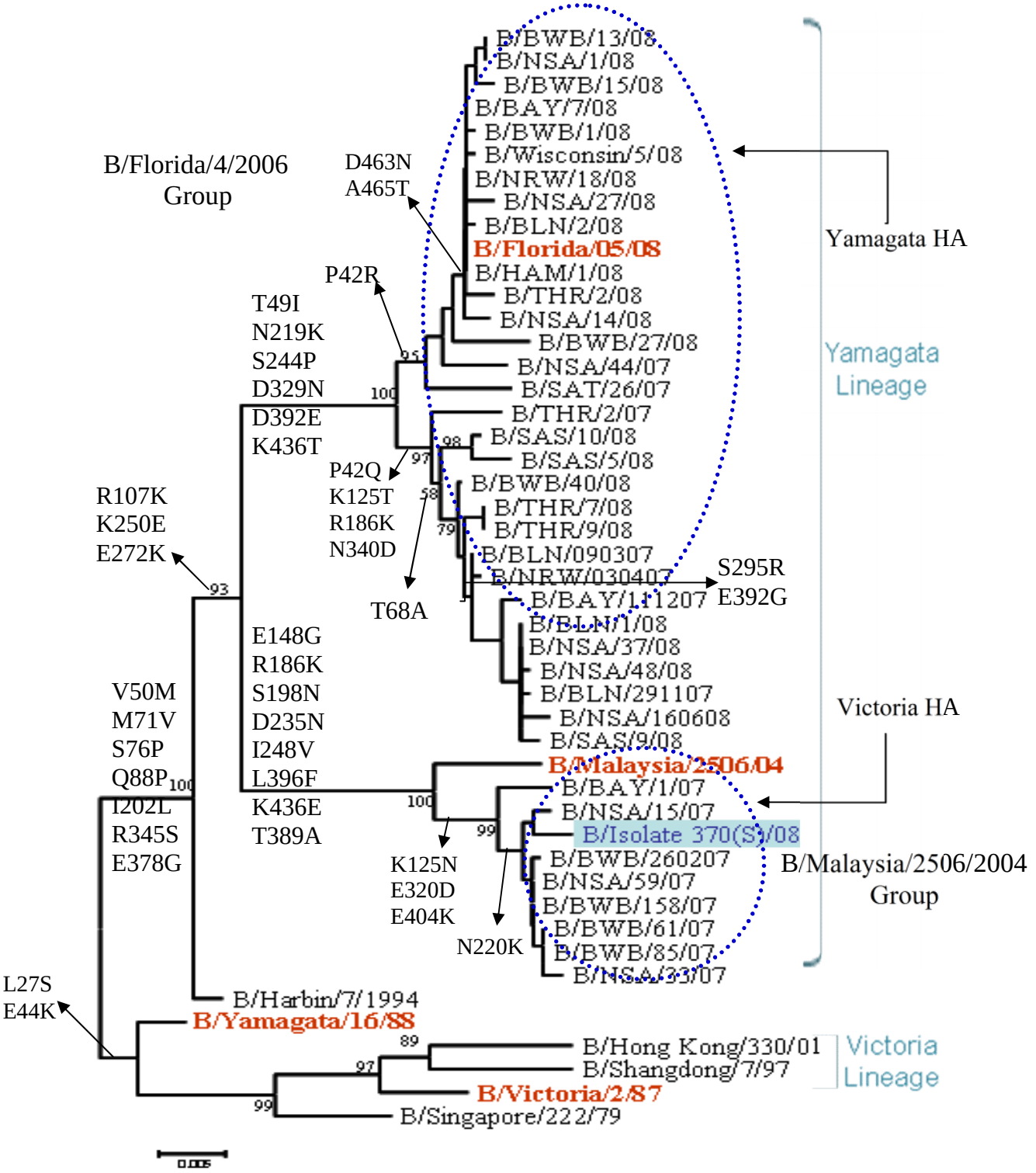

Figure 24: Phylogenetic analysis of neuraminidase gene of influenza B viruses
The tested viruses are shaded in light blue. Reference strains are red coloured and shown in bold. The tree was generated with Neighbor-Joining method with 1000 bootstrap replicates. The scale bar represents 0.5% of nucleotide changes between close relatives.

4.7.2. Phylogenetic analysis of respiratory syncytial virus

To date, ten genotypes are known for RSV A based on the G gene. Genotypes GA1-GA7 and SAA1 were assigned by Peret *et al*. (1998, 2000) and Venter *et al*. (2001), respectively. The other novel genotypes NA1 and NA2 were assigned by Shobugawa *et al*. (2009) represented by the two strains NG-016-04 and NG-082-05, respectively. Reference sequences for each genotype were obtained from GenBank.

To accurately define the extend of genetic variability within and between groups, the nucleotide sequence and the predicted amino acid sequence of the second variable region of all the Egyptian viruses (blue coloured) were determined and then compared to the A2 prototype strain.

The deduced amino acid sequence of the Egyptian viruses indicated that all of them shared an expected G protein gene length of 297 amino acids (aa). It was found that eleven of the Egyptian RSVA viruses clustered inside the new genotype NA1 (supported by the high bootstrap value) along with the Japanese strain NG-016-04 (fig. 25) while only one RSVA virus belonged to genotype GA2 along with other sequences retrieved from GenBank and one German strain. None of the Egyptian viruses clustered inside the genotype NA2 group. Among the genotype NA1, the nucleotide divergence (%) detected was 0.91 ± 0.19 [average mean ± standard deviations], while the amino acid divergence (%) was 4.1 ± 0.1 [average mean ± standard deviations] within the Egyptian RSVs. All sequences revealed a higher degree of amino acid divergence compared to the degree of nucleotide divergence.

To study the evolutionary divergence of the RSVA sequences, the number of synonymous and non-synonymous nucleotide substitutions was estimated. On average, the synonymous mutation/non-synonymous mutation (ds/dn) ratio was 6.68 for the Egyptian genotype NA1 viruses. This implicates a neutral selection pressure on the variable region for genotype NA1, for which more synonymous mutations have been observed.

The amino acid substitutions at different positions along the deduced amino acid sequence were identified in the Egyptian RSVA genotype NA1 viruses relative to the prototype A2. They included substitutions at 16 amino acids: S222P, P226L, E233K, N237D, I244R, L258H, M262E, F265L, S269T, S280Y, P286L, P289S, S290P, P292S, P293S, P296T, and R297K.

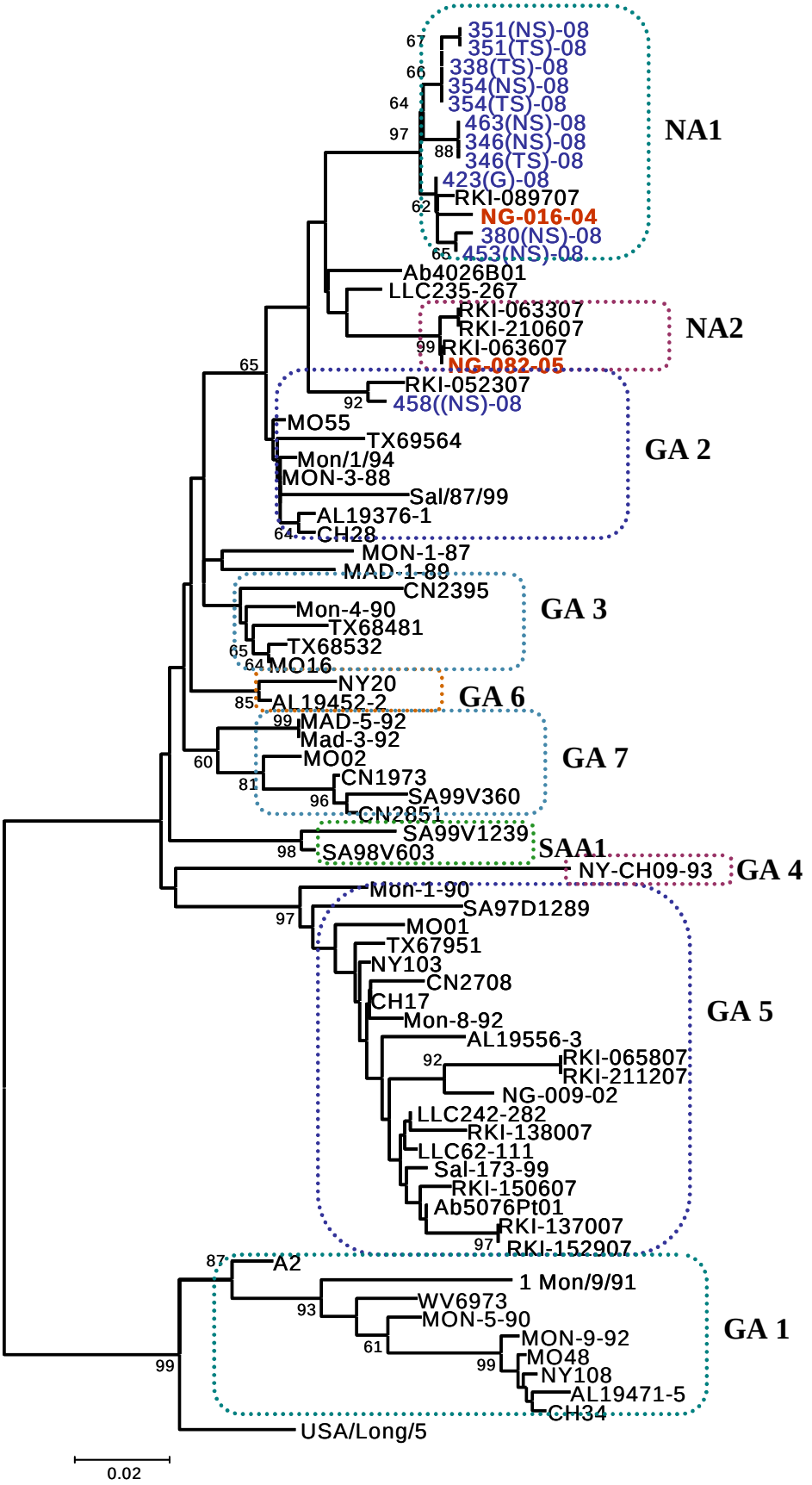

Figure 25: Phylogenetic analysis of RSVA-G gene

Tested viruses are coloured blue. The Japanese strains NG-016-04 and NG-082-05 are shown red in bold and were used as reference strains for the novel two RSV genotypes. Only bootstrap values more than 60 are shown.

4.7.3. Phylogenetic analysis of adenoviruses

Adenoviruses are differentiated into seven species which are named A-G (Griesche *et al*., 2008; Zhu *et al*., 2009).

The Egyptian viruses included in this study clustered within AdV species B and C (fig. 26).

One virus of the season 2005/2006 was closely related to reference strains of serotype 21. It showed six amino acid substitutions according to the reference strain hAdV21-AV1645 (G145A; T208A; P256L; E272Q; S424T; Q434E).

Viruses of the season 2006/2007 were found to represent and cluster within AdV11 serotype reference strain. They were identical to each other. They showed 12 amino acid substitutions along the deduced amino acid alignment in comparison to the reference strain hAdV-11p-Slobitski (H119R; T125N; V150I; E153K; N212T; Q220P; G273E; T274S; D386N; P400S; G420R; F425S).

The two Egyptian AdV-C isolates of the season 2007/2008 were identified in two specimens from the same patient. These viruses were identified as AdV serotype 2 because of their close clustering with AdV2 reference strain. They showed deletion of the glutamic acid at position 131 along the deduced amino acid alignment compared to hAdV serotype 2. Amino acid substitutions were also observed (D133E; E137D; D176N; G269D; N426S; G427D; D433N) among these two isolates.

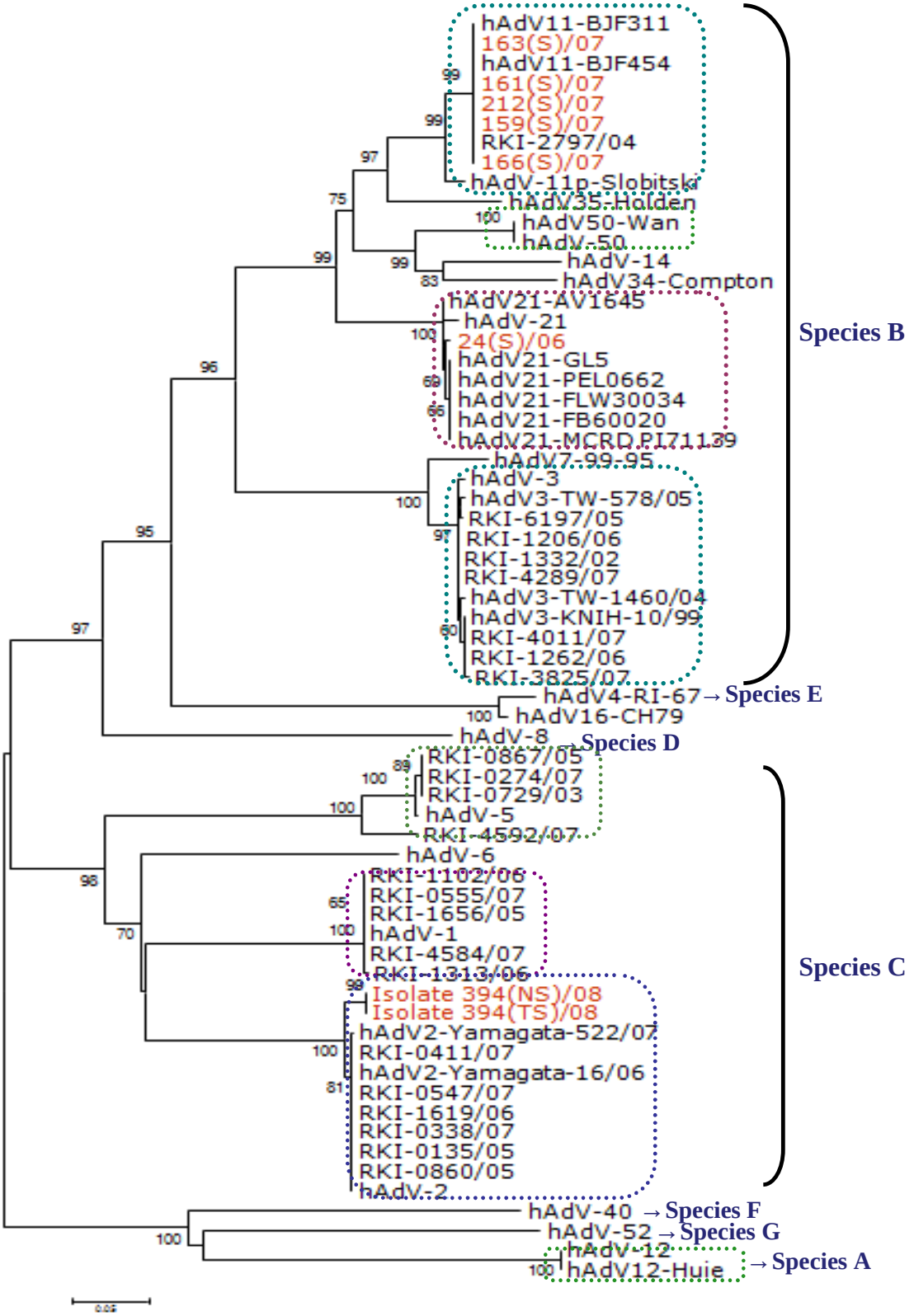

Figure 26: Phylogenetic analysis of Adenovirus hexon gene

Egyptian viruses included in the study are coloured dark red. The tree was generated with Neighbor-Joining method with 1000 bootstrap replicates. The scale bar represents 0.6% of nucleotide changes between close relatives.

4.7.4. Phylogenetic analysis of human Metapneumovirus

The F gene of hMPV is used for molecular characterization of hMPV. At present, these viruses can be differentiated into two main groups (A and B) which are further divided into subgroups A1, A2, B1, and B2. Reference for the subgroups were: CAN-14-00 (accession number AY145299) for subgroup A1, NL-1-00 (accession number AF371337) for subgroup A2, NL-99-01 (accession number AY304361) for subgroup B1, and NL-94-01 (accession number AY304362) for subgroup B2.

Phylogenetic analysis including 24 hMPV-positive specimens from Egypt as well as other sequences obtained from the GenBank and RKI showed clearly the differentiation into group A and B viruses as well as further subgroups supported by high bootstrap values (A1=98; A2=98; B1=94; B2=73). All the Egyptian hMPV viruses clustered in B1 and B2 groups (fig. 27). Among the B1 group, sequences shared 98.8% of nucleotide homology and 99.3% of amino acid homology. For the B2 group, the nucleotide homology within the Egyptian hMPV viruses was 97.7% while the amino acid homology was 98%. The nucleotide and amino acid homologies between B1 and B2 groups were 94% and 97%, respectively. The nucleotide and amino acid homologies between the Egyptian hMPV (group B) and group A viruses were 93.3% [average mean] and 96% [average mean], respectively.

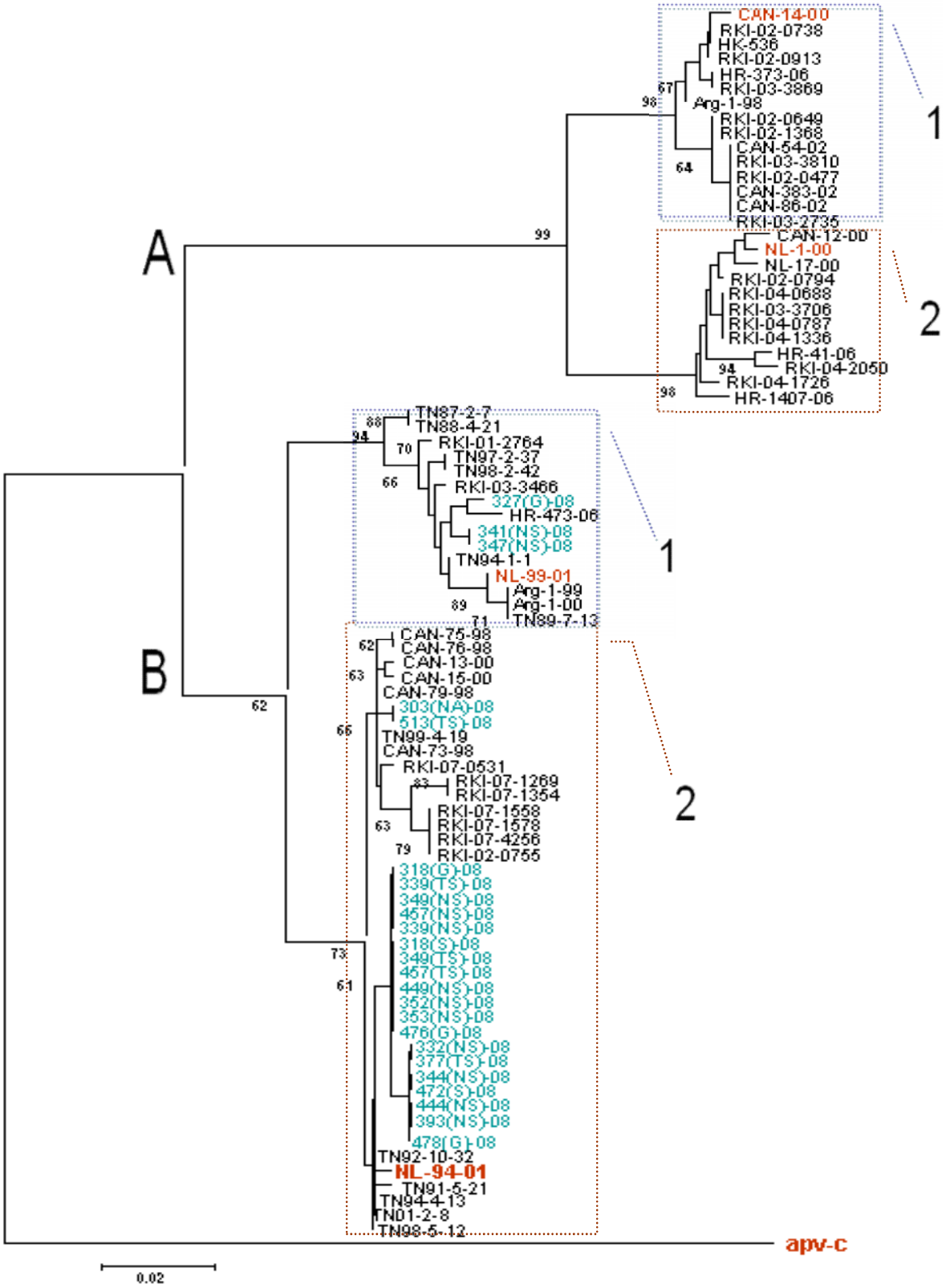

Figure 27: Phylogenetic analysis of hMPV fusion gene

Egyptian viruses included within the study are coloured blue. Reference strains are shown red in bold. The tree was generated with Neighbor-Joining method with 1000 bootstrap replicates and rooted to avian pneumovirus-C. The scale bar represents 2% of nucleotide changes between close relatives.

5. Discussion

Infections of the lower respiratory tract cause significant morbidity at Assiut University Hospitals in Assiut, Egypt among both children and adult groups to determine the role played by viruses as causative agents in these LRTIs at Assiut University Hospitals was the aim of this study. Most of the previous research that was conducted at Assiut University Hospitals concerned bacterial infections of the LRT and few data were available regarding the viral causes of LRTIs which depended only on conventional tools for isolation of the causative viruses. This is the first study at Assiut University Hospitals (which provides medical service not only for Assiut Governorate but also for the whole of Upper Egypt), to use molecular methods for detection and characterization of the main respiratory viruses that infected the lower respiratory tract. A two-year study was conducted from 2007 to 2009 at the National Influenza Centre in Germany (Robert Koch Institute, Berlin). Respiratory samples (812 in number) obtained from 520 patients (presented at Assiut University Hospitals, Assiut, Egypt) suffering from LRTIs were collected from December 2005 to February 2008 and were analyzed for the main respiratory viruses (influenza viruses A and B; respiratory syncytial virus; human metapneumovirus; human adenoviruses). Detection and characterization of these viruses was done using both the molecular and conventional diagnostic methods. As the rapid detection of respiratory viruses is only possible by molecular methods (Templeton, 2007), detection of viral infection was done at first with the real time-PCR.

5.1. Incidence of respiratory viruses among the groups studied

Using real time-PCR, 79 positive cases (104 positive samples) for one or more of the main respiratory viruses were detected. The overall infection rate among the population studied was 15.2%. In the adult group, the rate of viral LRTI was 10.4%. This rate is comparable to a previous report from the United Kingdom (Macfarlane *et al*., 1993) where 19 respiratory viruses were detected out of 113 pathogens in patients who suffered from community-acquired LRTI (16.8%). This infection rate is lower than those reported in some studies from Switzerland (29%) (Garbino *et al*., 2004), and Italy (42.2%) (Minosse *et al*., 2008). Most of the adults were recruited into this study several days after the onset of respiratory infection (delay in seeking medical advice). Late presentation is accompanied with a decrease in viral replication and thus, reduced detection rate of respiratory viruses. This led to an underestimation of the rate of viral infection in those patients. The difference in viral affection rate between population included in this study and previous reports from other parts of the world could be also attributed to different demographic and ecological factors between different populations.

For the children's group studied here, the situation was to some extent different. Mothers usually presented their children at the pediatric clinic within 8-24 hours after appearance of respiratory infection symptoms which enhanced a high detection rate for respiratory viruses among children by real-time PCR. Moreover, infants have a high rate of respiratory viral infection because of decreasing maternally-acquired antibodies. The rate of viral LRTI in the children's group was 46% for one or more of the main respiratory viruses. This is in agreement with previous findings in Korea, Italy, Hong Kong, Japan, and Rome. On the other hand, studies of viral infections among children from Sudan, China, India, and Korea reported a lower incidence (tab. 38).

Table 37: Rate of viral LRTI among children in different regions

Study	Country	Target population	viral LRTI rate
This study	Egypt	Children with LRTI	46%
Salih *et al*., 1994	Sudan	Children with acute LRTI	39%
Yun *et al*., 1995	Korea	Children with acute LRTI	45.9%
Ahn *et al*., 1999	Korea	Hospitalized Children with acute LRTI	22.1%
Sonoda *et al*., 1999	Japan	Hospitalized children with LRTI	43.9%
Pierangeli *et al*., 2007	Rome	Children with acute LRTI	42.7%
Canducci *et al*., 2008	Italy	Pediatric population with LRTI	46.6%
Tang *et al*., 2008	China	Children with acute LRTI	32.3%
Bharaj *et al*., 2009	India	Pediatric patients with LRTI	35.2%
Sung *et al*., 2009	Hong Kong	Children with acute LRTI	47%
Wan *et al*., 2009	China	Hospitalized Children with LRTI	35.8%

Among the different age groups involved in our study, the age group 0-4 years was the group most affected by LRTI viral infections (30 out of 79 cases which represented a ratio of 38%). This is in line with many previous reports (Salih *et al*., 1994; Sonoda *et al*., 1999; Izurieta *et al*., 2000; Cabelo *et al*., 2006; Thomazelli *et al*., 2007; Pierangeli *et al*., 2007; Bharaj *et al*., 2009). Increased rates of viral respiratory infections in this age bracket may be attributed to the decrease in the level of maternal antibodies (McIntosh *et al*., 1978; Watt *et al*., 1986). Influenza viruses and hMPV were detected in 28% and 27% of the positive cases, respectively, while adenoviruses and RSV were found in 25% and 20% of the positive cases.

In this study, influenza viruses were the dominant agents detected in infected adults with a ratio of 4.6% which is consistent with the rate of 5% reported by Tsuchiya *et al*. (2005). Influenza viruses were the most common in infected adults during seven seasons in Sweden (Östlund *et al*., 2004) and during 2005-2007 in China (Ren *et al*., 2009). On the other hand, influenza viruses were least frequently detected in the infected children, with a ratio of 1.4% of the total number of

children enrolled in the study. The same ratio (1.4%) was also reported by Ordás *et al*. (2006) in Spain. Similar influenza virus infection rates were also reported by others (Salih *et al*., 1994; Yun *et al*., 1995; Carballal *et al*., 2001; Noyola *et al*., 2005; Bharaj *et al*., 2009). Influenza A was the most frequently detected virus of the influenza viruses. This is in agreement with other records worldwide (Arkema *et al*., 2008; Pariani *et al*., 2008; Vidal *et al*., 2008; Falchi *et al.*, 2009). All influenza A viruses of the season 2006/2007 were A/H3N2 viruses. Predominance of influenza A/H3N2 during the season 2006/2007 had been also recorded in Switzerland (NCI, Switzerland, Report 2006-2007); Germany (Biere and Schweiger, 2008); France (Falchi *et al*., 2009); Greece (Melidou *et al*., 2009) and many other parts of the world. It was circulating during every season from 1993 to 2007 apart from 2000 to 2001 (Bragstad *et al*., 2008). Influenza A/H3N2 viruses co-circulated with influenza A/H1N1 viruses during 2004-2005 and with influenza B viruses during 2005-2006 (Pariani *et al*., 2008). Influenza A/H1N1 viruses detected in this study predominated during the season 2007/2008. In Turkey, predominance of influenza A/H1N1 viruses during the season 2007/2008 was also observed (Ciblak *et al*., 2009). In this research, one case of influenza B virus was detected during 2005/2006, three cases during 2006/2007, and two cases during 2007/2008. According to EISS (European Influenza Surveillance Scheme, 2009), the prevalence of influenza A (both H1N1 and H3N2 subtypes) and B viruses was comparable between different seasons with predominance of influenza A subtype H3N2. The seasonal report (AGI 2008/2009) stated that mild influenza activity was detected in Germany during the season 2005/2006 which was followed by strong activity during 2006/2007 season. Season 2007/2008 was characterized by moderate influenza activity while during 2008/2009 strong influenza activity was observed worldwide.

In contrast to the low detection rate of influenza viruses in the children's group, RSV was detected in 17% of the children involved in this study which is consistent with Wan *et al*. (2009) who found RSV in 17.6% of the affected children and Sarasini *et al*. (2006) detected RSV in 18.9% of the children in Italy. RSV was found to be the most common respiratory virus detected in infants and young children in many former reports (Ekalaksananan *et al*., 2001; Garcia-Garcia *et al*., 2001; Cabello *et al*., 2006; Ordás *et al*., 2006; Pierangeli *et al*., 2007; Thomazelli *et al*., 2007) supporting its role as the main virus associated with lower respiratory tract infections in young children. The frequency of RSV in young children might be caused by their immune status, where the magnitude of the immune response (acquired from maternal antibodies) in the early years is low (McIntosh *et al*., 1978; Watt *et al*., 1986).

RSV group A strains were detected in this study at higher rates (14 out of 22 patients) than group B strains (only one case). This was similar to other findings from South Africa (Madhi *et al*., 2003), Kenya (Scott *et al*., 2004), Argentina (Viegas and Mistchenko, 2005), Japan (Sato *et al*., 2005), Northern India (Parveen *et al*., 2006), Belgium (Zlateva *et al*., 2007), and Germany (Reiche and Schweiger, 2009). In contrast to the high rate of RSV found in the children's group, the rate of RSV found in the adult group in this study was only 0.8%. As demonstrated before, RSV is an important respiratory virus in childhood (Gökalp *et al*., 2009).

Our data indicated also that hMPV is an important respiratory pathogen along with RSV as a cause of LRTI in infants and young children. HMPV was detected in 17% of children's group. This is in agreement with Ordás *et al*. (2006) who found hMPV in 16.2% of infected children in Spain and Thomazelli *et al*. (2007) who detected hMPV in 17.8% of the studied children's group in Brazil. The detection rates of 13.1% reported from Italy (Sarasini *et al*., 2006), 6.6% from China (Ji *et al*., 2009), 6.4% from the United States of America (Esper *et al*., 2003), and 3.5% from Finland (Heikkinen *et al*., 2008) were lower. HMPV incidence can vary from year to year, sometimes equaling or exceeding RSV incidence (Falsey *et al*., 2003; Maggi *et al*., 2003). A difference in average ages was observed in this study between children infected with hMPV and those infected with RSV. A relatively high mean age of hMPV-infected children was observed in this study [2.3 ± 1.67 years (mean ± standard deviation)] while the mean age for RSV-infected children was lower (15 ± 14.76 months) (P = 0.09). This was similar to that reported by other authors (Parrott, *et al.*, 1973; García García *et al*., 2004). The existence of a longer-lasting maternal immunity to hMPV than to RSV and the finding that the pathogenesis of hMPV disease favors older children have been proposed as potential explanations (Peiris *et al.*, 2003; Mullins *et al*., 2004). HMPV was found in 27% of the positive cases (in a ratio of 4% of the total cases). This is coincident with rates of hMPV reported in Georgia (4.5%) (Falsey *et al*., 2003) and in Italy (4.8%) (Gerna *et al*., 2007). Regarding adenoviruses, the rate of detection was 10% in the children's group studied. This is in concordance with the infection rate of AdV detected by Freymuth *et al*. (1997) which was 10% in the children's group studied in France and also that reported by Ahn *et al*. (1999) in Korea (12.7%). Adenoviruses were found in 2.9% in the adult group of this study. This is comparable to the findings of Tsuchiya *et al*. (2005) in Brazil (0.9%) and Nikonova *et al*. (2009) in Russia (3.4%).

5.2. Respiratory viral infection and clinical diagnosis of the patients

In the children's group, bronchiolitis was the most common diagnosis made in 50% of the children. This is in agreement with the findings of Døllner *et al*. (2004), Garcia-Garcia *et al*.

(2006-a), Garcia-Garcia *et al*. (2006-b), Calvo *et al*. (2008), and Caracciolo *et al*. (2008) where bronchiolitis was the most common clinical diagnosis in 48%, 49.5%, 46.4%, 52%, and 57.1% of the total number of children, respectively. Many reports ascertain the association of RSV and bronchiolitis in their results (Choi *et al*., 2006; Mansbach *et al*., 2008; Mlinaric-Galinovic *et al*., 2009). Pneumonia was the second most common clinical diagnosis demonstrated in positive children (28%) described here. This is in agreement with the findings of Døllner *et al*. (2004), where pneumonia was the second most common one (after bronchiolitis) in the children's group studied. In Korean children, pneumonia was the most common diagnosis (Yun *et al*., 1995). Pneumonia was the most common diagnosis in all patients in this study. It was predominant in the adult group (28%) and constituted 27.85% of all cases. The critical role of respiratory viruses in the etiology of community-acquired pneumonia has been recorded previously (Greenberg, 1991; Dowell *et al*., 1996; Angeles Marcos *et al*., 2006). Respiratory viral infections constitute the second etiology of community-acquired pneumonia after *Streptococcus pneumonia* (Mayaud *et al*., 1992; Glezen *et al*., 2000; de Roux *et al*., 2004).

Asthma was found in 19% of the positive children's group. Many previous reports proved the role of viruses in the acute exacerbation of bronchial asthma (Wolf *et al*., 2006; Maffey *et al*., 2008). Among the different respiratory viruses, RSV and hMPV (in addition to rhinovirus) could possibly induce exacerbation among children with reactive airway disease (Gern and Busse, 1999; Tuffaha *et al*., 2000; Peiris *et al*., 2003).

COPD was the second most common diagnosis after pneumonia; it was detected in 21% of the affected adults in this study. In the case of exacerbation of COPD, a respiratory virus could be detected in 37% to 56% of the cases (Seemungal *et al*., 2001; Bandi *et al*., 2003; Rohde *et al*., 2003; Beckham *et al*., 2005; McManus *et al*., 2008). In a case-control study performed in Germany, it was found that acute exacerbations of COPD requiring admission to hospital are associated with the presence of respiratory viruses in respiratory specimens and a higher viral recovery was observed in specimens of the lower respiratory tract than those of the upper respiratory tract (Rohde *et al*., 2003).

It can be summarized that in the adult population of this study, the rate of viral LRTI was lower in comparison with data of other investigators. Influenza viruses were the most common viruses detected, and pneumonia was the most common clinical diagnosis. Concerning the children's group, a high viral LRTI rate was recorded which is consistent with many previous reports. RSV and hMPV were the most common viruses found in the positive children, and bronchiolitis was the most common clinical diagnosis.

5.3. Co-infection of the lower respiratory tract

It should be emphasized that most of the respiratory viruses are subject to a seasonal pattern; hence, viral-viral co-infection might be possible (Samransamruajkit *et al*., 2006). Co-infection with more than one of the respiratory viruses was detected in 5% of the positive cases in this study. Three patients were co-infected with adenoviruses and RSV-A, and one patient was co-infected with adenovirus and hMPV. In line with these findings, co-infection was observed in 4.8% of patients in Italy (Pierangeli *et al*., 2007), 6.7% in Italy (Minosse *et al*., 2008), and 3.4% of patients in Russia (Nikonova *et al*., 2009). However, a lower rate (1.6%) was found in China (Ren *et al*., 2009). Contribution of viral co-infection to the severity of LRTI has been investigated in Spain (Calvo *et al*., 2008) and it was found that fever was significantly more frequent, hospital stays were longer, and antibiotic treatment was used more in infants with multiple viral infections than in the RSV-infected group. Dual infection by more than one respiratory pathogen was also associated with increased severity of bronchiolitis in infants (Semple *et al*., 2005). The frequent occurrence of mixed viral and bacterial infections was proved in this study and was supported by previous data (Lehtinen *et al*., 2006; Cheng *et al*., 2009). In our study viral-bacterial co-infection was found in 39.24% of the positive cases. Also, it should be taken in consideration that examination for bacterial pathogens was not performed in 55.7% of the cases, therefore bacterial co-infection cannot be excluded for them.

5.4. Comparison between conventional methods and real time-PCR for the diagnosis of viral respiratory infection

The shipment of samples from Egypt to Germany rendered the isolation of respiratory viruses on tissue culture cells difficult. This explains the isolation of eight viruses (six influenza viruses and two adenoviruses) out of the 104 positive samples (obtained from 79 positive cases) that were diagnosed by real time-PCR.

The transportation of samples affected the detection rate of viruses by isolation but not their detection by real-time PCR. The role of real time-PCR as a diagnostic method for rapid, sensitive, and specific detection of viruses from the respiratory specimens, particularly those that fail to propagate successfully in tissue culture cells, was proven (Pozo *et al*., 2008). Also, the detection of more than one pathogen in a single reaction (multiplex PCR) saves time and costs (Boivin *et al*., 2004). Viral isolation was for decades the gold standard for diagnosis. It takes a long time to get positive cultures, ranging from 7-12 days for influenza viruses and more than 2 weeks for hMPV (van Elden *et al*., 2002). Some adenovirus serotypes require up to 3 weeks to show a cytopathic effect (Lin B *et al*., 2004).

5.5. Characterization of respiratory viruses

5.5.1. Influenza viruses

In the present study, influenza A/H1N1 viruses were mainly detected during the season 2007/2008. Hemagglutination inhibition (HI) data showed that the three influenza A/H1N1 viruses of the season 2007/2008 that could be isolated on MDCK cells reacted with high titers with antisera to A/Solomon Islands/3/2006 (the A/H1N1 vaccine strain recommended by the WHO during 2007/2008) and A/Brisbane/59/2007 (the recent antigenic variant). Two isolates showed low hemagglutination inhibition titers with antisera to A/New Caledonia/20/99 (which was the recommended WHO A/H1N1 vaccine strain until 2006/2007), suggesting that antigenic drift was occurring in the HA gene of these strains. Similar HI results were observed for influenza A/H1N1 viruses isolated in 35 countries during 2008 (WHO influenza center in London, 2008), indicating that the Egyptian H1N1 viruses were antigenically similar to the H1N1 viruses circulating early in Europe and other regions.

Phylogenetic analysis of the hemagglutinin gene of the Egyptian influenza A/H1N1 viruses of the season 2007/2008 showed that they were genetically close to the vaccine strain A/Brisbane/59/2007. Clustering of influenza A/H1N1 viruses of the season 2007/2008 inside the A/Brisbane/59/2007 group was also observed in previous reports (AGI Seasonal Report 2008/2009; Eshaghi *et al*., 2009). Replacement of A/Solomon Islands/3/2006-like viruses (influenza A/H1N1 viruses) by A/Brisbane/59/2007-like viruses was reported in the season 2006/2007 in Japan (Saito *et al*., 2008) and in the season 2007/2008 in Lebanon (Zaraket *et al*., 2009), Canada (Reyes *et al*., 2008) and other parts of the world (CDC, weekly report, 2007-2008 season; WHO collaborative Centre, London, Annual Report, 2008). The three Egyptian influenza isolates also clustered in a subgroup characterized by an additional substitution at position 270 (P270S). The amino acid substitutions at positions 35, 140, 188, and 273 (compared to A/New Caledonia/20/99) were also observed in previous reports from National Influenza Center in London (Annual Report, 2008) and Germany (AGI Seasonal Report, 2008/2009) and characteristic of Brisbane/59/07-like viruses.

The neuraminidase gene analysis of the Egyptian influenza A/H1N1 viruses of the season 2007/2008 revealed that they clustered in the A/Brisbane/59/2007 group and showed phylogenetic distance to the vaccine strain A/Solomon Islands/3/2006 which was similar to the German strains circulating during the same season 2007/2008. The substitution of histidine by tyrosine at position 275 (H274Y in N2 numbering) relative to A/Solomon Islands/3/2006 in the neuraminidase gene was detected among the three Egyptian influenza A/H1N1 isolates of the season 2007/2008. This amino acid modification is linked to oseltamivir resistance and was also

reported by many European countries (Meijer *et al*., 2009), Turkey (Ciblak *et al*., 2009), Japan (Tamura *et al*., 2009), Canada (Eshaghi *et al*., 2009), also from Oceania, South East Asia and South Africa (Hurt *et al*., 2009) and many other countries of the world. The average prevalence of oseltamivir-resistant viruses (ORVs) in Europe was 20.1% for winter 2007-2008 with an overall average prevalence of ORVs by country ranged from 8.3% to 65% in Norway (Meijer *et al*., 2009). The NA sequences of three Egyptian influenza A/H1N1 viruses form a cluster which was characterized by a difference in amino acid residue 354 (D354G). This amino acid modification has also been reported for strains circulating in Europe during 2007/2008 (Meijer *et al*., 2009). To summarize: the phylogenetic comparisons of the HA and NA genes showed that the Egyptian influenza A/H1N1 viruses are represented by A/Brisbane/59/2007, the recently recommended vaccine virus for 2008/2009.

All the Egyptian influenza A/H3N2 viruses were detected during the season 2006/2007. Two influenza isolates could be antigenically characterized as A/Brisbane/10/2007-like. A/Brisbane/10/2007 is the recommended WHO A/H3N2 vaccine strain for the year 2008/2009. The Egyptian influenza A/H3N2 isolate of the year 2006 reacted with low HI titers with antiserum to A/Wisconsin/67/2005 (the A/H3N2 vaccine strain recommended during 2007-2008 for the northern hemisphere). The Egyptian influenza A/H3N2 isolate of the year 2007 reacted with high titer with antiserum to A/Wisconsin/67/2005. Influenza A/H3N2 German (AGI Seasonal Report, 2008/2009) and American (CDC weekly report, 2007-2008 season) isolates from the season 2007/2008 also reacted well with antiserum to A/Brisbane/10/2007. Analysis of the hemagglutinin sequences of the Egyptian influenza A/H3N2 viruses showed that they had amino acid modifications with regard to A/Wisconsin/67/2005. They clustered in two different groups (A/Brisbane/10/2007 and A/Nepal/921/2006 groups). This was similar to the German influenza A/H3N2 viruses isolated during the same season 2006/2007 (AGI Seasonal Report, 2008/2009). Two Egyptian viruses of the year 2007 and one of the year 2006 clustered inside A/Brisbane/10/2007 group A. The characteristic amino acid substitutions at positions 50 and 140 (G50E and K140I) relative to A/Wisconsin/67/2005 among the A/Brisbane/10/2007-like Egyptian viruses were also reported for influenza A/H3N2 viruses isolated from other countries (WHO collaborative Centre, London, Annual Report, 2008). The other two Egyptian strains of the season 2006/2007 clustered in the A/Nepal/921/2006 group and possessed three amino acid substitutions N6I, R142G, and K173E relative to A/Wisconsin/67/2005.

A/Nepal/921/2006-like viruses were also identified during the same season in France (Falchi *et al*., 2009), Korea (Baek *et al*., 2009), Switzerland (NCI; Switzerland, winter season 2006-2007), Germany (AGI Seasonal Report, 2008/2009), Greece (Melidou *et al*., 2009) and other countries

(WHO collaborative Centre, London, Annual Report, 2008). The amino acid substitutions at positions 193 (S193E) and 225 (D225N) relative to the reference strain A/Wisconsin/67/2005 detected in the Egyptian influenza A/H3N2 viruses were also recorded by Lin *et al*. (2008) in Taiwan, Dapat *et al*. (2009) in Myanmar, and Melidou *et al*. (2009) in Greece.

Analysis of the neuraminidase gene sequences of the Egyptian influenza A/H3N2 viruses could be performed for one isolate from the season 2005/2006 and one from the season 2006/2007. They clustered in the A/Brisbane/10/2007 group possessing the amino acid substitutions H150R, V194I, Y310H, S372L, and N387K relative to A/Wisconsin/67/05 strain. These amino acid substitutions were also reported by Dapat *et al*. (2009) and the WHO Influenza Reference Center in London (WHO collaborative Centre, London, Annual Report, 2008) and are characteristic of the NA gene of Brisbane/10/2007 like viruses.

To summarize: the phylogenetic comparisons of the HA gene showed that the Egyptian influenza A/H3N2 viruses represented two groups: the A/Brisbane/10/2007 and A/Nepal/921/2006 group, while the NA genes of two strains were closely related to that of A/Brisbane/10/2007, the recently recommended A/H3N2 vaccine virus for 2008/2009.

For influenza B viruses, the phylogenetic analysis of HA gene of the Egyptian influenza B virus isolate and also viruses isolated in Germany and recent reference strains showed in coincidence with previous findings the divergence of influenza B viruses into two lineages, the Yamagata and the Victoria lineage (Rota *et al*., 1990; Nerome *et al*., 1998; Jian *et al*., 2008). A high variability was observed for influenza B viruses of the Yamagata lineage for the time period 2006-2008 with co-circulation of three sublineages: B/Florida/4/06-like, B/Chelyabinsk/306/07-like, and B/Hong Kong/864/06-like viruses. However, antigenic and genetic data revealed that the Egyptian isolate from 2007/2008 which was the unique influenza B virus that could be sequenced in this study represented the Victoria lineage. The Egyptian isolate showed high HI titer with antiserum to B/Malaysia/2506/2004 strain. It shared the same modified amino acids (K48E, K80R, and K129N) with the vaccine strain B/Malaysia/2506/2004 (the influenza B component of the influenza vaccine during 2007/2008) relative to the former reference strain B/Hong Kong/330/2001. Other influenza B viruses analyzed during the same period in different parts of the world represented B/Malaysia/2506/2004. Like Influenza B viruses with a similar antigenic profile circulated in Russia (Ivanova *et al*., 2008), Taiwan (Lin YP *et al*., 2008), Germany (AGI Seasonal Report, 2008/2009), and Myanmar (Dapat *et al*., 2009). The phylogenetic analysis of the NA gene of the Egyptian influenza B virus isolate represented B/Malaysia/2506/2004 (Yamagata lineage) and showed the amino acid substitutions E320D, K404K, and N220K relative to B/Malaysia/2506/2004 strain. These amino acid modifications

have been reported in Myamar, Thailand, Vietnam, China (Dapat *et al*., 2009), Europe and other parts of the world (WHO collaborative Centre, London, Annual Report, 2008). Analysis of sequences of the HA and NA genes of influenza B Egyptian strain showed that it was a reassortant with a B/Hong Kong/330/2001-like HA gene (Victoria/87 lineage) and a B/Malaysia/2506/2004-like NA gene (Yamagata NA lineage). Reassortant influenza B viruses with a Victoria/87-like HA and the Yamagata/88-like NA were identified in many countries since 2001 (Chi *et al*., 2003; Lin YP *et al*., 2004; Xu *et al*., 2004).

5.5.2. Respiratory syncytial virus

RSV group A viruses were the most frequently detected ones during the two seasons 2006/2007 and 2007/2008. Predominance of RSV A during seven out of nine seasons in Germany from 1998 to 2007 (Reiche and Schweiger, 2009) and during the season 2006/2007 in Japan (Shobugawa *et al*., 2009) and in Hungary (Pankovics *et al*., 2009) was reported. RSV group A comprises ten different genotypes: GA1 to GA7 (Peret *et al*., 1998; Peret *et al*., 2000), SAA1 (Venter *et al*., 2001; Frabasile *et al*., 2003), and the two new genotypes NA1 and NA2 (Shobugawa *et al*., 2009) on the basis of classification of the C-terminal variable region of the G protein. The higher diversity of the RSV subgroup A is attributed to the gradual build-up and replacement of the dominant genotypes among subgroup A in comparison to subgroup B (Choi and Lee, 2000). All but one of the Egyptian RSV A viruses of the season 2007/2008 represented the genotype NA1. The nucleotide divergence determined for genotype NA1 was 0.91%. Since the amino acid divergence of genotype NA1 (4.1%) was greater than the nucleotide divergence, mutations in nucleotides resulted in amino acid changes. Selection pressure for NA1 is neutral due to the existence of more synonymous mutations. The amino acid substitutions that have been observed in the Egyptian NA1 genotype were also found in the Japanese NA1 strains (Shobugawa *et al*., 2009) as a consequence of global circulation of viruses. The amino acid sequences of the Egyptian NA1 subtype (like RSV A genotype GA2) predicted to encode G proteins of 297 amino acids in length which is in agreement with RSV A GA2 strains that were detected in Germany (Reiche and Schweiger, 2009), and Japan (Shobugawa *et al*., 2009) during the same period or RSVA GA2 strains detected during previous periods in Argentina (Viegas and Mistchenko, 2005), Belgium (Zlateva *et al*., 2007), and Brazil (Botosso *et al*., 2009).

So after analysis of the sequencing data of the Egyptian RSV strains it was found that most of them represented one of two new genotypes that have been described recently. These genotypes were genetically related to genotype GA2 but distinguished from GA2 in the phylogenetic tree and pairwise nucleotide distances.

5.5.3. Adenoviruses

Only two quantitative PCR assays were available for the detection of all 52 serotypes of AdV. The first one (Heim *et al*., 2003) was a generic assay which provides no further information on the amplified virus species; and the second one (Allard *et al*., 2001) composed of 6 single assays, each specific for one of the six species, so that every sample has to be examined in six separate reactions (Chmielewicz *et al*., 2005-a). Therefore, a real time-PCR developed by a colleague of the NRC Berlin (Chmielewicz and others, 2005-a) at RKI, Germany was used here. The amplification reaction was performed for genes involved in the DNA replication because these genes are highly conserved in all AdV serotypes (Liu *et al*., 2000; Botting and Hay, 2001). The amplified sequences of that part of the genome were nearly identical within each species but differ from the other species. Five primer pairs have been chosen that flanked the selected probe target sequence. Subsequent fluorescence curve melting analysis (FCMA) of the amplified products enabled identification of the adenoviral species. FCMA applied for single-nucleotide polymorphism analysis after amplification in a Light Cycler instrument (takes about 10 minutes), was adapted to differentiate the AdV DPol PCR amplicons with regard to the AdV species, based on the fact that the sequences of serotypes that are grouped into a species are highly homologous but differ from those of the other species. So, a hybridization probe pair that is specific for one species has mismatches to the others, giving lower melting temperatures. These melting temperatures are characteristic for the target sequence/probe-pair combination, making them highly reproducible (Chmielewicz *et al*., 2005-a). The Egyptian AdV positive samples that could be sequenced and characterized in the study were eight in number. Adenoviruses of the season 2006/2007 clustered inside the AdV B serotype 11 group. Phylogenetic analysis of these viruses showed that they were identical to each other and also were exactly identical to the partial hexon gene sequence of the German isolate RKI-2797/04 that was collected in Turkey. AdV-11 strains were classified into at least two genome types, designated AdV-11p and AdV-11a. These two types exhibit different tropisms: AdV-11a infects the respiratory epithelial cells while AdV-11p infects the renal epithelial cells (Li *et al*., 1991). AdV-11a strains were more frequently isolated in China from 1965 to 1985 (Li *et al*., 1991). An AdV-11a strain was also circulating in a military camp in Turkey (Chmielewicz *et al*., 2005-b) and was also associated with an outbreak of acute respiratory disease in 2006 in China (Zhu *et al*., 2009). The Egyptian AdV strain of the season 2005/2006 belonged to the AdV B serotype 21 group. Infection with AdV-21 was also detected in Canada (Yeung *et al*., 2009). The other two viruses (two samples from the same patient) could be isolated on tissue culture cells and were found to be AdV species C serotype 2. AdV serotype 2 was associated with respiratory infections in Brazil (Moura *et al*.,

2008) and Canada (Wong *et al*., 2008; Yeung *et al*., 2009). The three serotypes (serotype 11, 21 and 2) were circulating (with other AdV serotypes) also in Germany during 2001-2007 (NIC, RKI, 2009).

5.5.4. Human Metapneumovirus

Amplification and sequencing of a 500 base pair fragment of the F gene of the Egyptian hMPV strains and data obtained from other viruses isolated in Germany and other reference strains showed, in correspondence with previous findings, the presence of two major groups A and B, each consisting of two subgroups (1 and 2) (van den Hoogen *et al*., 2004; Boivin *et al*., 2004; Huck *et al*., 2006; Kaida *et al*., 2006; Oliveira *et al*., 2009). The viruses studied clustered in hMPV group B. Subgroup B1 that was detected among the Egyptian viruses in this study has previously been detected in Australia (Mackay *et al*., 2006), and in Brazil (Riccetto *et al*., 2009). In South Africa, subgroup B2 was dominant during 2000 and cocirculated with subgroups A1 and A2 during 2001 (Ludewick *et al*., 2005). In Brazil, 74% of hMPV strains that were detected in infected children belonged to group B in contrast to 26% belonging to group A during a 4-year-study from 2003 to 2006 (Oliveira *et al*., 2009). In contrast to our findings, hMPV strains detected in infants with acute respiratory disease in Italy were 71.7% subtype A and 28.3% subtype B (Canducci *et al*., 2008). High prevalence rate for hMPV subgroup A1 (72%) were recorded also in France (Foulongne *et al*., 2006). In Iran, 94.1% of positive cases for hMPV in children belonged to genotype A (Arabpour *et al*., 2008). In this study, a high degree of homology was observed among the Egyptian hMPV F gene at both the nucleotide and amino acid levels. The nucleotide homology between the Egyptian hMPV subgroups B1-B2 was 94% and the amino acid homology was 97%. Within clusters B1 and B2, nucleotide homology of 98.8% and 97.7% was detected while the amino acid homologies were 99.3% and 98%, respectively. In line with these findings, the hMPV F gene was relatively well conserved in previous reports in Canada (Bastien *et al*., 2003), the Netherlands (van den Hoogen *et al*., 2004), USA (Agapov *et al*., 2006), France (Foulongne *et al*., 2006), Germany (Huck *et al*., 2006), and in other parts of the world (tab. 38).

Phylogenetic analysis based on the hMPV F genes of strains in Thailand showed that the sequence similarities within each group exceeded 97% and those between groups were 87-88% and 81-84%, respectively (Samransamruajkit *et al*., 2006). Some other data showed more variability between hMPV subgroups. In Japan, the nucleotide homology within groups was about 92%, which was lower than the nucleotide homology found in the Egyptian viruses and the amino acid homology was about 98% (Ishiguro *et al*., 2004). Nucleotide homology of 91.6%-

95.3% and amino acid homology of 92.0%-94.1% were detected between hMPV B1 and B2 strains from Canada and other countries (Boivin *et al*., 2004).

Table 38: Genetic diversity among human metapneumovirus F (fusion) gene

Study	Country	Nucleotide sequence homology				Amino acid sequence homology			
		B1	B2	B1-B2	A-B	B1	B2	B1-B2	A-B
This study	Egypt	98.8%	97.7%	94%	93.3%	99.3%	98%	97%	96%
Bastien *et al*., 2003	Canada	-	-	94.3-99.9%	83.0-83.6%	-	-	98.3-99.8%	94.1-95.4%
Boivin *et al*., 2004	Many countries	96.0-99.9%	97.2-99.4%	92.0-94.1%	81.5-85.3%	99.5-100%	99.1-100%	98.1-99.1%	93.1-96.3%
van den Hoogen *et al*., 2004	The Netherlands	97-100%	97-100%	94-96%	84-86%	99-100%	99-100%	97-99%	94-97%
Ludewick *et al*., 2005	USA	98-100%	96-100%	93-95%	-	100%	99.4%	98.4%	-
Foulongne *et al*., 2006	France	97.2-100%	97.2-100%	92.6-94.4%	82.7-86.5%	-	-	-	-
Huck *et al*., 2006	Germany	97.1-99.8%	98.3-99.5%	94.0-95.7%	83.6-87.4%	-	-	-	-

For the hMPV-F gene, amino acid sequence identity between subgroups is high which has a substantial contribution to cross-neutralization and cross-protection between the HMPV subgroups (Biacchesi *et al*., 2003).

Variability in the hMPV F gene between groups A and B among the nucleotide and amino acid sequences differed in many previous reports. Among the South African strains, the estimated homologies for the F gene between groups A and B were 83-85% at the nucleotide level and 93.2%-95.8% at the amino acid level (Ludewick *et al*., 2005). In Canada, the nucleotide and amino acid homologies between group A and B hMPV were 81.5%-85.3% and 93.1%-96.3%, respectively (Boivin *et al*., 2004). The nucleotide homology between hMPV group A and B for the German strains was 83.65%-87.4% (Huck *et al*., 2006). On the other hand, Ishiguro *et al*. (2004) detected much higher variability between hMPV group A and B in the F gene among the nucleotide sequences (60%) comparable to more conserved sequences among the amino acid (94%). Briefly, the Egyptian hMPV strains in this study belonged to group B. Sequence analysis of the fusion gene showed, in correspondence with previous reports, a high degree of homology worldwide at both the nucleotide and amino acid levels.

The data described above lead to a conclusion that respiratory viruses detected in this study were similar to circulating viruses elsewhere in the world.

6. Summary

Infections of the lower respiratory tract (LRTI) cause significant morbidity at Assiut University Hospitals, Egypt, among both children and adults. Determining the contribution of viruses as causative agents to these LRTIs was the aim of this study. The present study is the first one using both conventional and molecular methods for detection and characterization of the main respiratory viruses at Assiut University Hospitals.

A total of 520 patients were enrolled in the study, 70 of them (13.5%) were children and 450 (86.5%) were adults. From these patients, 812 samples were obtained during three consecutive winter-spring seasons from 2005 until 2008. The research was conducted over a two-year period from 2007 to 2009 at the National Reference Center for Influenza (Robert Koch Institute, Berlin, Germany). Detection of the corresponding viruses was performed first by real-time PCR. Seventy nine patients (104 samples) were diagnosed positive for one or more of the main respiratory viruses with a viral infection rate of 15.2%. Infected children were 32 in number (46% of the children group) and infected adults were 47 (10.4% of adults). Of the 79 virus-positive patients, 22 were infected with influenza viruses, 21 with human Metapneumovirus (hMPV), 20 with adenoviruses, and 16 with respiratory syncytial virus (RSV). The age group 0-4 years was the most affected by respiratory viruses (30 out of 79 cases, which accounted for 38% of the total number of positive cases). Most of the influenza viruses were detected in the middle age group (35-60), while RSV and hMPV were mostly identified in young children (0-4 years). Adenoviruses were more or less equally distributed among different age groups. Pneumonia was the most common diagnosis, accounting for 27.85% of the total number of positive cases. Isolation of respiratory viruses on tissue culture cells turned out to be very difficult especially for viruses like hMPV and RSV. Only eight viruses (six influenza viruses and two adenoviruses) out of the 104 positive samples diagnosed by real-time PCR could be isolated on tissue culture cells. Nucleotide and amino acid sequences of the detected respiratory viruses were compared with sequences from Germany and those deposited at GenBank.

It could be concluded that viral isolation on tissue culture cells should not be used as the primary method for diagnostic purposes as it is time-consuming and not as sensitive as polymerase chain reaction. The phylogenetic characterization of the detected viruses revealed that they were similar to those reported from other parts of the world during the same period. This indicates a worldwide spread of these viruses during winter-spring seasons. Diagnostic tests for respiratory viruses should be incorporated in the routine diagnostic study of patients with lower respiratory tract infection, and further active respiratory viral surveillance will be required in the future.

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List of abbreviations

%	Percent
°C	Grade Celsius
aa	Amino acid
BFR	Bundesfirmenregister
bidest	Bidestilled (distilled two times)
BMT	Bone marrow transplantation
Bp	Base pairs
BSA	Bovine serum albumin
CAP	Community acquired pneumonia
cDNA	Complementary Deoxyribonucleic acid
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disease
CPE	Cytopathic effect
CT	Threshold curve
DCP	Decompensated core-pulmonale
ddNTP	Dideoxyribonucleoside triphosphate
DEAE	Diethylaminoethyle
DM	Diabetes mellitus
DMSO	Dimethylsufoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DPol	DNA Polymerase
ds	Double stranded
DTT	Ditiothreitol
EDTA	Ethylendiamintetraacetic acid
EISS	European Influenza Surveillance Scheme
EMEM	Eagle's minimum essential medium
Et al.	Et alii
F (primer)	Forward
F (gene/protein)	Fusion (gene / protein)
FAM	6`carboxyfluorescein
FCS	Fetal calve serum
G	Gargle
G protein	Glycoprotein
HA	Hemagglutinin
HAdV	Human adenovirus
HCl	Hydrochloric acid
Hep2	Human laryngeal carcinoma
hMPV	Human metapneumovirus
IPF	Interstitial pulmonary fibrosis
LLC-MK2	Kidney Rhesus monkey-*Macaca mulatta*
LRTI	Lower respiratory tract infection

M	Matrix
MDCK	Madin-Darby canine kidney
MEM	Minimal essential medium
mg	Milligram
Min.	Minute
MGB	Minor Groove Binder
µl	Microliter
µM	Micromole
mM	Millimole
N (gene)	Nucleoprotein (gene)
NA	Neuraminidase
NaCl	Sodium chloride
NS	Nasal swab
nt	Nucleotide
ORVs	Oseltamivir resistance viruses
P	Phosphoprotein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PIV	Parainfluenza virus
RBCs	Red blood cells
RF	Respiratory failure
RKI	Robert Koch Institute
RNA	Ribonucleic acid
ROX	6-carboxy-X rhodamine
RSV	Respiratory syncytial virus
S	Sputum
SH protein	Small hydrophobic protein
S-OIV	Swine-origin influenza virus
StD	Standard deviation
TA	Tracheal aspirate
TB	Tuberculosis
TBE	Tris, Boric acid, EDTA Buffer
TPCK	Tosyl-L-Phenylalanine-chloromethyl ketone)
TS	Throat swab
UK	United Kingdom
USA	United States of America
UV	Ultraviolet irradiation
Vero cells	African green monkey kidney cells

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Mona Embarek
2010

Eigenständigkeitserklärung

„Ich, Mona Embarek, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: [Viren als Erreger unterer Atemwegsinfektionen] selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die unzulässige Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.