Università degli Studi di Pisa

Scuola di dottorato in Fisiopatologia Clinica e Scienze del Farmaco
Programma di Dottorato in Virologia Fondamentale e Clinica
Direttore: Prof. Luca Ceccherini-Nelli
SSD MED/07

Respiratory viral infections in ICU patients: comparison of upper and lower respiratory samples

Relatore:

Prof.ssa Alberta Azzi

Candidata:

Rosaria Arvia

ANNI ACCADEMICI 2012/2014

Index

Abbreviations:

ARDS: Acute Respiratory Distress Syndrome

ARI: Acute Respiratory Illness

ARTIs: Acute Respiratory Tract Infections

BAL: Broncho-Alveolar Lavage

COPD: Chronic Obstructive Pulmonary Disease

CoV: Coronavirus

DAD: Diffuse Alveolar Damage

EBV: Epstein-Barr Virus

EVs: Enteroviruses

HA: Hemagglutinin

HAdvs: Human Adenoviruses

HBoV: Human Bocavirus

HCMV: Human Cytomegalovirus

HE: Hemagglutinin-Esterase

hMPV: Human Metapneumovirus

HPIVs: Human Parainfluenza Viruses

HRVs: Human Rhinoviruses

HSV1: Herpes Simplex Virus Type 1

ICU: Intensive Care Unit

IGV: Integrative Genomic Viewer

LRTIs: Lower Respiratory Tract Infections

MERS: Middle East Respiratory Syndrome

NA: Neuraminidase

NGS: Next Generation Sequencing

NK: Natural Killer

RSV: Respiratory Syncytial Virus

RTIs: Respiratory Tract Infections

SARS: Severe Acute Respiratory Syndrome

TS: Throat Swab

URTIs: Upper Respiratory Tract Infections

Abstract:

Respiratory viruses cause many diseases, from mild to severe illnesses, and contribute significantly to morbidity and mortality worldwide. Different viruses can establish respiratory tract infections, they belong to the *Orthomyxoviridae, Coronaviridae, Picornaviridae, Paramyxoviridae, Adenoviridae, Parvoviridae* and in immunocompromised patients *Herpesviridae* families. Altogether, the etiologic diagnosis of respiratory viral infections has been underestimated so far. In patients with predisposing conditions the outcome of these infections can be more severe, sometimes requiring hospitalization, even in intensive care units (ICU), because of the development of pneumonia and acute respiratory distress syndrome (ARDS). For these patients, in particular, rapid diagnosis is essential. In addition, in ICU patients the significance of the detection of some members of *Herpesviridae* family, like HSV1, CMV and EBV, is controversial. The aim of this study was to clarify the prevalence of respiratory viruses and herpesviruses, and their role in ICU patients. Large part of this study was devoted to the development of diagnostic assays able to accurately characterise respiratory viruses quickly and at the lowest costs. Viral detection was performed in both upper and lower respiratory samples in order to compare the viral populations in these two compartments and, possibly, to draw informations concerning the role of the infection in severe cases.

Four duplex RT real-time PCRs, using EvaGreen fluorescent dye, were developed to identify and characterize the main respiratory RNA viruses directly from clinical samples. A duplex was performed to detect influenza A and influenza B viruses; a second duplex was performed to detect PIVs belonging to *Respirovirus* genera (PIV1 and PIV3) and RSV; the viruses target for the third duplex PCR were PIV type 2 and hMPV; and a fourth duplex was performed to detect CoV I and EV/RV.

The results of the duplex real-time PCRs were confirmed by sequencing positive samples and by comparison with other assays, including commercial, validated, assays, which gave similar results. A total of 156 clinical samples from upper and lower respiratory tract of 58 adult patients hospitalized in ICU

were analysed. In 80% of positive adult patients influenza A viruses were detected, in 8% influenza B viruses and rhinovirus/enterovirus and in 4% metapneumoviruses. In particular in all ICU adult patients positive for influenza A or B viruses, the virus was demonstrated in both upper or lower respiratory tract samples. For an adult patient positive for RV/EV, the virus was detected in both samples; while for another only the upper respiratory tract sample was positive. Only one upper respiratory tract sample from one patient was positive for hMPV. The results obtained in this study were in agreement with other published studies that showed influenza virus as the most common virus detected in ICU patients, followed by rhinoviruses.

Furthermore, three nested PCRs were developed to detect herpesviruses (CMV, EBV and HSV1). To understand better the role of these viruses in ICU patients all herpesviruses positive clinical samples obtained by nested-PCRs were further analyzed by quantitative real-time PCRs.

Altogether, 37 of 58 (about 64%) patients were positive for one or more herpesviruses.

EBV was detected in 25 patients of 58, either as single or mixed infection; CMV and HSV1 were detected in 15 patients either as single or mixed infection. Mixed infections were not rare.

In general, for EBV viral load in TS samples was higher than in BAL samples. These results could suggest a possible viral contamination of the lower respiratory tract from mouth or throat or both. In one case EBV DNA was detected in the BAL only in two successive specimens at a low viral load (10^3-10^4). Further monitoring should be performed to better understand these data.

In this study CMV was detected as single infection only in two patients. Regarding CMV association with pneumonia, the average values of viral load reported in literature vary; however a viral load in BAL samples, between $4{,}6x10^4$ and $5x10^5$ copies number/ml, has been proposed as a threshold for the diagnosis of pneumonia. None of the patients analysed in this study had a viral load within this range only in BAL sample.

HSV1 was detected in 15 patients of 58 as single or mixed infection. In general, all patients HSV1 positive had high viral load in TS and in BAL samples (average value 10^6). In one patient only the detection of a high viral

load in the BAL in absence of viral DNA in the TS could suggest an involvement of this virus in the lower respiratory tract disease.

The assays described could be particularly useful to screen a large number of patients for epidemiological studies and to assess the prevalence in the lower and upper respiratory tract of ICU patients with, regarding CMV, EBV and HSV1, the ultimate goal to understand the clinical significance of this phenomenon.

The possible contribute of the use of the NGS to the knowledges of the viruses involved in upper and lower respiratory tract infections was also studied preliminary in a small number of ICU patients. Nextera-XT protocol to MiSeq platform has been used.

1. Introduction

1.1. Human viral respiratory infections

Respiratory viruses cause many diseases and contribute significantly to morbidity and mortality worldwide [1]. Respiratory tract infections (RTIs) are divided into two categories, lower respiratory tract infections (LRTIs) and upper respiratory tract infections (URTIs) according to the localization of the infection. URTIs refer to infections of the nasopharynx, larynx, tonsils, sinuses, ears, including rhinosinusitis, tonsillitis, pharyngitis, laryngitis/laryngotracheitis and otitis media.

LRTIs refer to infections of the trachea, bronchus, and alveolus, including tracheitis, bronchitis, bronchiolitis, and pneumonia.

Respiratory infections are caused by various pathogens, but approximately 80% of cases are viral [2]. Different viruses can establish respiratory tract infections, most belong to the *Orthomyxoviridae, Coronaviridae, Picornaviridae, Paramyxoviridae, Adenoviridae, Parvoviridae* and *Herpesviridae* families.

Several respiratory viruses, previously unknown, have been discovered during the last years [3, 4], like human metapneumovirus [5], SARS coronavirus [6], HKU1 coronavirus [7], NL63 coronavirus [8], MERS coronavirus [9], bocavirus [10] and mimivirus [11]. Other viruses, like rhinoviruses, emerged as cause of lower respiratory tract infections. The identification of new viruses allowed to better define the viral aetiology in many respiratory diseases. The prevalence of respiratory viruses in adults is largely under explored, as most studies focus on children, while in severely ill or immunocompromised adults respiratory viruses can also lead to severe complications. The prevalence, clinical profile, and epidemiology of respiratory virus in adults are different from those in children. The prevalence of mixed respiratory viruses in adults is lower than that in children. However, the clinical significance of mixed respiratory virus remains unclear. Also, the relationship between geographical distribution, season, prevalence year, and respiratory viruses is not fully understood in adults.

In patients with predisposing conditions the outcome of these infections can be more severe, sometimes requiring hospitalization, even in intensive care units (ICU), because of the development of pneumonia and acute respiratory distress syndrome (ARDS). They frequently cause pneumonia in children, especially those younger than 2 years [12, 13]. For adult patients hospitalized in ICU, respiratory viruses account for about 30% of pneumonia cases, with mortality rates comparable to those of bacterial pneumonia [14]. So far, there are few studies concerning the detection of a large number of respiratory viruses in ICU patients [15-18]. Influenza A is the virus most frequently detected in these patients [18-20].

1.2. Influenza viruses

Influenza viruses belong to the *Orthomyxoviridae* family. Three types of influenza viruses have been recognized (types A, B, and C), on the basis of their type-specific nucleoprotein and matrix protein antigens. Influenza type A viruses can infect humans, birds, pigs, horses and other animals, but wild birds are their natural hosts [21]. However, the recent identification of two influenza-like virus genomes (designated H17N10 and H18N11) from bats has challenged this notion [22-26].

Influenza A viruses are classified into subtypes according to the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins. Until 2012, 16 HA subtypes and 9 NA subtypes were recognized.

In 2012, a new influenza virus genome (H17N10) was demonstrated in bats by next-generation sequencing (NGS). Studies on the two surface envelope proteins, HA and NA (NA-like) [22-27], demonstrate that neither protein has the corresponding canonical influenza virus functions or structures [25]. Therefore the new genome does not represent a ‘true’ influenza virus. Recently a similar virus genome, H18N11, was again identified by NGS, and neither HA nor NA have canonical structures or functions [26].

The genome of influenza virus type A and B consists of eight single-stranded negative-sense RNA segments, while influenza C virus has seven genome

segments. The three largest RNA segments of influenza A virus code for the polymerase proteins (PB2, PB1, PA), the fourth, fifth and sixth RNAs segments code for HA, NP and NA, respectively. The seventh RNA codes for the M1 and M2 proteins and the eighth RNA codes for NS1 protein. The influenza B virus genome is similar to that of influenza A virus. The HA, NA and M2 proteins are inserted into the host-derived lipid envelope. HA glycoprotein has the major antigenic determinants of influenza virus and plays an essential role in the initiation of infection. It is a trimer, composed of two structurally distinct regions: a triple-stranded, coiled-coil and a globular region that contains the receptors binding site. The HA is synthesized as a single polypeptide chain, which undergoes cleavage at three places. The N-terminal signal sequence is removed and, depending on the host cell and virus strain, the molecule is cleaved to give two polypeptide chains: HA1 and HA2. Cleavage of HA is essential for the fusing capacity and for the infectivity of the virus. The HA mediates attachment and entry of the virus on the cell surface by binding to sialic acid receptors. The binding affinity of the HA to the host sialic acid allows for the host specificity of influenza virus. In particular, avian influenza subtypes prefer to bind to sialic acid linked to galactose by α-2,3 linkages, frequent in avian respiratory and intestinal epithelium. On the contrary, human virus subtypes bind to α-2,6 linkages frequent in human respiratory epithelium. Swine has both α-2,3 and α-2,6 linkages in his respiratory epithelium, allowing for easy coinfection with both human and avian subtypes. Humans have been found to contain both α-2,3 and α-2,6 linkages in their lower respiratory tract and conjunctivae, which allows for human infections by avian subtypes [28].

The second antigenic determinant is NA protein. This protein is a sialidase responsible for cleavage of sialic acid from glycans on the host cell surface to release the emerging progeny virus, prevent virus aggregation, and help virus migration [29, 30].

The epidemiology of human influenza virus reflects the peculiar characteristics of the virus genome, segmented single-stranded RNA. The human influenza virus is able to elude host immunity and cause recurrent annual epidemics and, sometimes, major worldwide pandemics due to the introduction of antigenically novel viruses into an immunologically naïve human population.

Influenza viruses have two different mechanisms that allow them to change and evade the immune response: antigenic drift (small changes in antigenicity) and antigenic shift (complete change in antigenic properties). Antigenic shift is derived from reassortment of gene segments between viruses, and occur only in influenza A viruses. In the twentieth century, there were three pandemics: in 1918 caused by H1N1 subtype (Spanish flu), in 1957 caused by H2N2 subtype (Asian flu) and in 1968 caused by H3N2 subtype (Hong Kong flu). The first pandemic of twenty-first century was caused by a new H1N1 subtype named swine flu because of its origin. The new reassortant influenza strain H1N1 was discovered in Mexico in March 2009 [31].

In adults, influenza A virus is the most common viral pathogen [18-20]. Influenza virus is the leading pathogen detected in adults with ARTIs (positive rate of 20.81) [18].

Influenza B viruses in circulation belong to two lineages distinct by their genetic and antigenic characteristics, which are referred to as the Yamagata and Victoria lineages, designated after their original isolates, B/Yamagata/16/88 and B/Victoria/2/87 [32, 33]. These two lineages have co-circulated since the late 1980s [33]. Since 2008, most B/Victoria/2/87 lineage viruses belong to the B/Brisbane/60/2008 genetic clade (Group 1) [34] based on the hemagglutinin (HA) gene sequences. Instead, since 2007, the majority of B/Yamagata lineage viruses have been distributed into two main groups, with distinct genetic and antigenic characteristics, Group 2, represented by B/Brisbane/3/2007, and Group 3, represented by B/Bangladesh/3333/2007 [35].

The characterization of influenza B viruses, and not only of influenza A viruses in circulation, is important, in order to select the virus to be included in influenza vaccines and to evaluate the efficacy of vaccination.

1.3. Coronaviruses

Coronaviruses (CoVs) belong to the *Coronaviridae* family. Coronaviruses are enveloped, single-stranded, positive-sense RNA.

CoVs are divided into three serological groups based on their natural hosts, nucleotide sequences and serological relationship. Group I and II viruses mainly infect mammals, while group III viruses are found in birds. The human coronaviruses are: 229E, OC43, NL63, HKU1, SARS, MERS, belonging to group I and group II. The first two human coronaviruses identified are CoV-229E (group I) and CoV-OC43 (group II). In 2003 it was identified SARS-CoV (group IIb) [6], and subsequently two other CoVs, CoV-NL63 (group I) [8] and CoV-HKU1 (group II) [7]. CoV-NL63 was first detected in 2004 in the Netherlands from a child with bronchiolitis by using a new method for virus discovery based on the cDNA-amplified restriction fragment-length polymorphism technique (cDNA-AFLP) [8], while CoV-HKU1 was first detected in Hong Kong in 2005 from an adult patient with chronic pulmonary disease [7]. In 2012 a new coronavirus, MERS-CoV, was identified in a 60-year-old Saudi man in Jeddah, Saudi Arabia [9].

CoV infections cause traditionally a low percentage of annual upper and lower respiratory infections, including severe disease outcomes in the elderly, immunocompromised and in infants [36]. CoV-229E, OC43, NL63 and HKU1 usually cause acute infection of the upper respiratory tract and less frequently are associated with lower respiratory tract diseases [37, 38]. Unlike, SARS-CoV caused an acute, atypical pneumonia and diffuse alveolar damage (DAD) in roughly 8,000 patients [39]. Those over 65 years of age often developed ARDS, resulting in mortality rates that exceeded 50%. Overall, SARS-CoV infection caused nearly 800 fatalities, representing a nearly 10% mortality rate [40]. Since 2003 no further cases of SARS-CoV infection occurred. It was a zoonosis, but the natural host of the virus was not identified. A palm civets was suggested [41].

Also MERS-CoV is associated with severe respiratory disease and with high lethality with over 857 official cases and 334 deaths, representing an approximately 35% case fatality rate to date in humans [42, 43]. MERS-CoV induced disease is particularly severe in patients with pre-existing co-morbidities. The occurrence of the majority of cases seems to follow a seasonal distribution: April 2012 (Zarqa public health hospital, Jordan) [44], April–May 2013 (Al-Hasa outbreak) [45], and April–May 2014 (Jeddah and United Arab Emirates outbreak) [46].

The initial cases of MERS-CoV were detected among patients admitted with severe community-acquired pneumonia and the majority of them required intensive care unit admission [45, 47]. Subsequently, individuals with mild or no symptoms were reported [47, 48]. Also MERS-CoV infection is zoonotic. Camels and bats have been hypothesized as viral reservoir.

All coronaviruses contain three main structural proteins, spike (S), membrane (M) and envelope (E) proteins in the viral envelope. S proteins are the major antigenic determinants of coronavirus and are highly glycosylated. The S protein mediate receptor attachment, viral-host cell membrane fusion, cell-mediated immunity, and pathogenesis of coronavirus infection. This protein is a type I membrane protein that contains N-terminal receptor-binding (S1) and C-terminal membrane fusion (S2) domain. The S1 subunit contains a receptor binding domain (RBD). S2 is the transmembrane subunit containing two amphipathic heptad repeats (HR1 and HR2) and the transmembrane domain. The binding of S1 protein to the receptor induces the S2 domain to reorganize into coiled-coil formation during cell-virus fusion. The M protein is the most abundant constituent of coronaviruses, it is conserved within each group but divergent across the three groups. The E protein is a small integral membrane protein with a short hydrophilic N-terminal domain, followed by a hydrophobic region, then a hydrophilic C-terminal domain. It is extremely divergent across the three groups and in some cases among members in the same group. For group II coronaviruses, with the exception of SARS-CoV, an additional hemagglutinin-esterase glycoprotein (HE) is present. HE protein of coronaviruses is related to influenza C HA, this suggests that there was a recombination between influenza C virus and the genomic RNA of an ancestral coronavirus. Inside the virion, a coronavirus possesses a helically symmetric ribonucleocapsids core formed by the association of nucleocapsid (N) protein with genomic RNA. The ribonucleoprotein core is enclosed by a lipoprotein envelope composed of membrane (M) glycoprotein.

CoV-229E, OC43, NL63 and HKU1 can be detected in individuals of all ages, including elderly patients with fatal outcome [49] and those with underlying diseases of the respiratory tract [7]. CoV-NL63 and OC43 are less common then other coronaviruses.

The most common symptoms due to coronavirus infection are rhinorrhea, fever, and abdominal breath sounds [50]. Coinfection of coronaviruses with RSV has been described in children [51, 52].

In a study conducted in UK in patients of all age, both dual and single infections associated with respiratory outcomes were observed for HKU1 as well as for NL63 and OC43 coronaviruses [53].

In this study a high number of coinfections was observed for HKU1, NL63 as well as for OC43, mostly with RSV. Similar rates of lower and upper infections were observed in single HKU1 or OC43 infection compared with coinfection, whereas both URTI and LRTI were observed more frequently in single compared to mixed infection with NL63. No differences in clinical outcome were observed between single and dual infections with RSV and Coronaviruses NL63, HKU1 or OC43 indicating that RSV may presumably facilitate coronavirus infection without increasing disease severity.

1.4. Enteroviruses

Enteroviruses (EVs) are a common cause of respiratory tract infections and are classified into seven species (EVA-D and rhinoviruses [HRVs] A-C) with more than 200 different serotypes.

EVs of the family *Picornaviridae*, genus Enterovirus are small, non-enveloped, and possess a single-stranded positive (messenger)-sense RNA genome of ~7.4 kb.

The genome is organized into a long noncoding region, designed the 5' untranslated region (5'UTR), which precedes a single open reading frame (ORF). The ORF is subdivided into three regions, P1 to P3. The P1 region codes for the 4 structural proteins (VP1-VP4) that comprise the viral capsid. These are organized from 5' to 3' as VP4 (1A), VP2, (1B), VP3 (1C) and VP1 (1D). The P2 and P3 regions code for 7 non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) that are essential for the viral life cycle. Downstream of the ORF is a short noncoding region (3'UTR) and a terminal polyadenylated tail.

As already mentioned, HEVs are currently divided into four species (HEV-A to HEV-D) depending on their sequence similarities and biological properties. HEVs typing is based on comparing the sequences encoding the VP1 capsid protein: viruses of different genotypes have 75% nucleotide identity and 85% amino acid identity [54, 55]. New HEV genotypes have been described in the last few years and associated with respiratory diseases, other have been reclassified. Individual serotypes have different temporal pattern of circulation and can be associated with different clinical manifestations. HEVs are responsible for a wide range URTIs and LRTIs occurring in adults and children. Although the majority of HEVs infections remains asymptomatic. More than 100 serotypes have been identified. Twenty-three types of HEV-C have so far been identified. In 2010 in Switzerland a new genotype of EV, EV-C104, was identified from respiratory samples collected during 2004-2007 in 8 children with respiratory signs and symptoms and acute otitis media [56]. In a following epidemiologic study conducted in Italy, five strains of the new EV104 genotype were detected in patients (age 2 to 62) with respiratory diseases [57]. Recently EV104 has been associated with LRTIs. EV-C109 was identified in Nicaragua in September 2010 [58]. In 2010, EV-C105 and EV-C116 were detected in patients with gastroenteritis in Congo and Sakhalin Island [59], and other EV-C105 strain was detected in subjects with respiratory disease in Peru [60]. In Italy, during the course of the Community-Acquired Pneumonia Pediatric Research Initiative (CAP-PRI) study in winter 2010-2011, two HEV-C strains, EV-C117 and EV-C118 were detected [61-64]. Several clinical case studies reported the etiological role of CoxA16, the HEV-71 and, as already mentioned, a newly discovered genotypes HEV-C104 in the development of acute or fatal pneumonia indicating the HEVs belonging to species A to C can be responsible for severe LRTIs.

HRVs include 3 species, HRV-A, HRV-B, and HRV-C, containing over 100 serotypes. HRV-C has been identified using molecular methods and it was first associated with severe clinical presentations in infants and immunocompromised adults [65].

HRVs are well known for causing the common cold, although they have been implicated also in bronchitis and asthma attacks.

Several recent epidemiological studies suggest that HRV-A and HRV-C are the predominant species associated with acute respiratory illnesses in hospitalized children and adults [65, 66].

HRV-C could be associated with more severe clinical illnesses, including lower respiratory tract infections and asthmatic exacerbations, than HRV-A and HRV-B [67, 68]. HRV-B was detected in a relatively small number of patients with ARIs [69-71]. However the association between HRV type and disease severity is not fully understood. There may be important differences in the susceptibility of individuals to the replication of HRV in lower airway tissues.

Some studies suggested that HRVs can propagate in lower airway tissues and this may be an important factor in the development of airway obstruction, coughing, and wheezing that can lead to bronchiolitis and pneumonia [72].

Many studies showed that HRV was the most prevalent pathogen in upper respiratory tract, both in children than in adults [73]. HRV was, also, the most common co-infecting virus [74].

Some reports have associated co-infection with a second respiratory virus with a more severe disease [75, 76], whereas others have not [77, 78].

1.5. Metapneumoviruses

Human metapneumovirus (hMPV) was discovered in the Netherlands in 2001 from nasopharyngeal aspirates collected during a 20-year period from hospitalized children and infants with acute respiratory tract infection (RTI) having signs and symptoms similar to that of RSV infection [5]. The virus genomic sequence was identified by using a randomly primed PCR protocol [5].

hMPV belongs to the *Pneumovirinae* subfamily (genus Metapneumovirus) and *Paramyxoviridae* family. It is an enveloped, non segmented, negative-stranded RNA virus. There are two genotypes of hMPV (A and B) and subsequent genetic analysis of these viruses, based upon the sequence variability of the attachment (G) and fusion (F) surface glycoproteins, have subdivided the

genotypes into the subgroups A1, A2, B1 and B2. Subgroup A2 is again subdivided into A2a and A2b.

The hMPV genome contains eight genes that code for nine proteins. The order of the genes in the genome (from 3' to 5' end) is N–P–M–F–M2–SH–G–L. The proteins are: the nucleoprotein (N protein), the phosphoprotein (P protein), the matrix protein (M protein), the fusion glycoprotein (F protein), the putative transcription factor (M2-1 protein), the RNA synthesis regulatory factor (the M2-2 protein), the small hydrophobic glycoprotein (SH protein), the attachment glycoprotein (G protein), and the viral polymerase (L protein) [5]. The RNA core is surrounded by M protein and covered by a lipid envelope. This envelope contains the three surface glycoproteins (F, SH, and G), in the form of spikes of approximately 13–17 nm.

The core nucleic acids are associated with the P, N, L, M2-1, and M2-2 proteins and form a nucleocapsid 17 nm in diameter. With the help of the G and F proteins, hMPV attaches and fuses to heparan sulphate receptors on the cell surface.

Comparing all the subgroups (A1, A2, B1, and B2), the N gene is found to be most conserved at both the nucleotide and the amino acid levels (91.2% and 98.4%, respectively), while the G gene is the least conserved (79% and 59.2%, respectively) [79].

hMPV is commonly found in children, with high susceptibility rates in children less than 2 years old. hMPV infection in adults normally shows only mild flu-like symptoms. However, in some adult cases (especially elderly adults), hMPV acts as enhancer of chronic obstructive pulmonary disease (COPD), and patients with COPD are more prone to hMPV infection [80]. hMPV infection has also been reported in several immunocompromised patients, such as lung transplant recipients, patients with haematological malignancies, and hematopoietic stem cell transplant recipients [81, 82]. No significant correlation was found between genotype and disease severity [83]. There are conflicting reports on the association between RSV–hMPV co-infection and disease severity; some studies found that co-infection leads to an increased rate of ICU admission and hospital stay [84, 85], but others found no association between co-infection and disease severity [86, 87].

1.6. Respiratory Syncytial viruses

Respiratory syncytial virus (RSV) is a Pneumovirus belonging to the *Paramyxoviridae* family. It is an enveloped RNA virus that expresses 11 proteins. Its genome is a non-segmented single-stranded negative sense RNA [88].

RSV is divided into two types, A and B [89, 90], based on antigenic differences in their glycoproteins G and F [91, 92]. RSV A and RSV B types are further divided into subtypes or genotypes based on the variable domain of the attachment G protein [93, 94]. The genotypes of subgroup A were 8 (GA1–GA7 and SAA1), as those of subgroup B (BA, GB1–GB4, and SAB1–3) [95]. In 2013 a new RSV A genotype was described, NA1, genetically close to GA2 genotype. GA2 genotype and BA genotype are the most common genotypes of RSV subgroups A and B around the world [96]. The attachment glycoprotein (G) is a major structural protein that may be associated with both infectivity and antigenicity [97]. The differences among the various RSV subtypes are located mainly in the ectodomain of the G protein, which share only 44% amino acid sequence identity between the two subgroups, as compared to 83% identity in the transmembrane and cytoplasmic domains [88, 92, 98].

A number of studies were carried out to test whether RSV A or RSV B infections differ in their clinical outcome [99, 100], but no significant differences were found.

RSV has been frequently identified as the most common virus and is associated with severe infections [101, 102]; it is the dominant cause of respiratory tract infection in children under 5 years [103, 104]. RSV is predominant in winter months and it represents the most common agent of severe airway disease in infants and young children. RSV infection may cause major problems in infants less than 1 year of age and can lead to bronchiolitis and bronchopneumonia [13, 50, 88]. For many years, RSV was a well-known cause of lower respiratory tract infection in young children [50, 88], more recently it is recognized as an increasingly important cause of respiratory infection in adults [102, 104], in particular in older adults and in immunocompromised patients. Although the effects of RSV infection in these patients are underestimated [101, 103].

1.7. Parainfluenza viruses

Human parainfluenza viruses (HPIVs) are enveloped, non-segmented, negative- sense, sigle-stranded RNA viruses that are classified in the genera *Respirovirus* (HPIV-1 and HPIV-3) and *Rubulavirus* (HPIV-2, HPIV-4a, HPIV-4b), *Paramyxovirinae* subfamily, *Paramixviridae* family. Subgroups/genotypes of HPIV-1 and HPIV-3 have been reported. HPIV are the second most common cause of lower respiratory tract infections in young children. At least six common structural proteins in the order of 3'-N-P-C-M-F-HN-L-5' are encoded by HPIV genome. A non-structural protein (C), is found in HPIV-1, HPIV-2 and HPIV-3; an additional non-structural protein (V) is detected in HPIV-2 (and maybe HPIV-3) but not in HPIV-1; and a unique non structural protein (D) may also exist in HPIV-3. The N, P and L proteins together with viral RNA form the nucleocapsid core of HPIV. The HN protein is surface glycoprotein that is present in the lipid envelope of HPIV.

HPIV are a common cause of both upper and lower respiratory tract infections, particularly in children, and they commonly re-infect both children and adults [105-109]. Most epidemiological and clinical research has been focused on parainfluenza serotypes 1–3. This has been primarily due to the poor growth characteristics in cell culture of parainfluenza 4 (PIV4), the lack of commercial diagnostic reagents, and historical exclusion from routine diagnostic testing [107].

Although fewer HPIV strains have been detected compared with other respiratory viruses such as RSV, HRV, and HMPV, previous reports suggest that HPIV1 and 3 are the dominant viruses in children with acute respiratory illness (ARI) [110].

HPIV1 and 3 show high prevalence and are associated with up to 12% of acute lower respiratory tract infections in adults [111, 112].

Several studies have reported that HPIV1 infections demonstrate clear outbreaks in autumn, mostly in September and November, every 2 years and HPIV3 causes yearly outbreaks around the globe, mainly in the spring-summer season [113-116]. A recent study suggested that four different types of HPIV cause similar clinical manifestations in patients, and the clinical presentation of HPIV infection may differ depending on patient's age [117].

1.8. Adenoviruses

The human adenoviruses (HAdvs) belong to the genus *Mastadenovirus* in the family *Adenoviridae* and consist of 52 serotypes divided into seven subgenera, A-G. Each serotype is distinguished by its resistance to neutralization by antisera to other known adenovirus serotypes. Advs have a non-enveloped, 20 faces icosahedral virion that consists of a core containing linear double-stranded DNA enclosed by a capsid. The capsid is composed of 252 capsomers, 240 of which are hexons and 12 are pentons. Advs are a common cause of respiratory and gastrointestinal illness in children and young adults. However, of the 52 recognized human serotype, only one-third are associated with a specific human disease; other infections remain asymptomatic. The human serotype associated with diseases can cause a variety of types of clinical illnesses involving almost every human organ system. Illnesses include upper and lower respiratory tract infections, conjunctivitis, cystitis and gastroenteritis. Several studies have found that the enteric adenoviruses are second only to rotaviruses as the causative agents of acute gastroenteritis in infants and young children [118]. Most illnesses caused by Advs are acute and self-limiting; Advs can remain latent in the body (in tonsils, lymphocytes, and adenoidal tissues) for years and be reactivated under certain conditions, such as a change in immune status. The long-term effect of such a latent infection is unknown. A large proportion of infections caused by subgenera A and D tend to be asymptomatic, whereas the species within subgenera B and E tend to result in higher rate of symptomatic respiratory illnesses. Immunity is specie-specific. Over 5% of respiratory diseases in children younger then 5 years of age are due to Adv infections. The initial transmission of Advs is through the nasopharynx. Secondary transmission in households can be as high as 50% due to fecal-oral transmission from children shedding virus in the feces. Advs can be recovered from the throat or stool of an infected child for up to 3 weeks. Advs respiratory infections are also well documented in adults [119, 120]. In 2007 a new variant of Adv 14 appeared among military recruits and general public which caused severe respiratory illness resulting in high rate of hospitalization and significant mortality [121, 122].

1.9. Bocaviruses

Human Bocavirus (HBoV) was discovered in 2005, in Sweden in respiratory samples from children with suspected acute respiratory tract infection (ARTI) by using a large-scale molecular viral screening technique [10]. In 2009-2010 other three human bocaviruses, HBoV2, HBoV3 and HBoV4, was identified in stool samples and associated with gastrointestinal diseases [123, 124]. HBoV1 was detected in individuals of all ages, with a predilection for young children with respiratory symptoms. However, with molecular diagnostics of respiratory tract secretions, HBoV1 may often be seen also in asymptomatic children [125-132].

HBoVs belong to the *Parvoviridae* family, *Parvovirinae* subfamily. They are minute DNA viruses that for replication are highly dependent on cellular functions including the DNA polymerase. Their linear single-stranded genome is only ~5 kb in length with still unknown terminal sequences [133]. By electron microscopy, the structure of HBoV is typical of Parvoviridae, that is, non-enveloped capsid of icosahedral symmetry and diameter of ~25nm [134-137]. The genome putatively encodes two forms of the nonstructural protein NS1 and for HBoVs unique, nuclear phosphoprotein NP1, as well as two major structural proteins, VP1 and VP2 [139-141]. VP1 and VP2 are capsid proteins and have in common the C-terminal part and they only differ in extension of the N-terminus of VP1, the VP1 unique region (VP1u).

Since initial observations, several studies have reported the prevalence of HBoV infection all over the world ranging from 2 to 21.5%, mainly in children younger than 3 years of age where it has been associated with upper and with lower RTIs [141-146]. In a study from Norway, HBoV was detected in 12% of children with RTI and it was the fourth most common virus after RSV, HRV and hMPV [147]. HBoV infections tend to be associated with high rates of coinfections with other viral pathogens such as HRV, AdV, RSV, as well as with bacteria such as *Streptococcus* spp and *Mycoplasma pneumoniae* [145, 147].

In many studies a positive correlation was seen between respiratory illness and high copy numbers of HBoV1 DNA or the presence of HBoV1 monoinfection [145,148-152].

So far, HBoV infection in adults has only been recorded in a few papers [153-155].

In addition to the infections by the previously mentioned respiratory viruses, there may also be different members of the *Herpesviridae* family which may lead serious respiratory diseases in immunocompromised patients.

1.10. Herpesviridae

Members of the *Herpesviridae* family are enveloped double-strand DNA viruses. A typical herpesvirion consists of a core containing a linear double-stranded DNA (the precise arrangement of the DNA within the core is not known); an icosahedral capsid approximately 125 nm in diameter containing 162 capsomers (150 hexons and 12 pentons); an amorphous protein coat called the tegument; and an envelope containing viral glycoprotein spikes on its surface. These glycoproteins confer distinctive properties to each virus and provide unique antigens to which the host is capable of responding.

The eight human-specific herpesviruses are denominated as human herpesviruses 1 to 8 (HHV 1-8), classified into three subfamilies (α-herpesviruses, β-herpesviruses and γ-herpesviruses).

The α-herpesviruses, herpes simplex virus types 1 and 2, and varicella-zoster virus, have a short replicative cycle, induce cytopathology in monolayer cell cultures, and have a broad host range; β-herpesviruses, cytomegalovirus, and human herpesviruses 6 and 7, with a long replicative cycle and restricted host range; and γ-herpesviruses, Epstein-Barr virus and human herpesvirus 8, with a very restricted host range. All herpesviruses code for unique enzymes involved in the biosynthesis of viral nucleic acids. These enzymes are structurally diverse and parenthetically provide unique sites for inhibition by antiviral agents. The synthesis and assembly of viral DNA is initiated in the nucleus. Assembly of the capsid is also initiated in the nucleus; release of progeny virus from the infected cell is accompanied by cell death. Furthermore all herpesviruses establish latent infection within tissues that are characteristic for each virus, reflecting the unique tissue trophism of each member of this family.

Most herpesvirus genes contain a promoter/regulatory sequence spanning 50 to 200 bp upstream of TATA box, a transcription initiation site 20 to 25 bp

downstream of the TATA box, 5'nontranslated leader sequence of 30 to 300 bp, a single major open reading frame with a translation initiation codon that meets the host requirements for efficient initiation, 10 to 30 bp of 3' nontraslated sequence, and a canonical polyadenylation signal with standard flanking sequences, but exist some exceptions. For example genes without a TATA box and initation from a second in frame methionine have been reported. Herpesviruses encode between 70 and 200 genes. After primary infection, all of them remain latent in the organism and cause a lifelong persisting infection [156]. Their reactivation occurs more or less frequently, depending from the viruses and may be more severe in patients immunosuppressed [157-159].

DNA of herpesviruses such as Epstein-Barr virus (HHV 4, EBV), Herpes simplex virus (HHV 1/2, HSV-1/2) and Cytomegalovirus (HHV 5, CMV) is detectable frequently in respiratory tract samples of immunocompromised patients [160-163].

1.10.a. Herpes simplex virus type 1

Herpes simplex virus (HSV) infections are very common in the human population [164]. After primary infection, which usually occurs during childhood (HSV type 1 or HSV-1) or adolescence (HSV type 2 or HSV-2), the virus remains latent for life in the ganglia of sensory neurons, resulting in a large carrier population among adults.

HSV-1 primarily affects the oropharyngeal mucosa, whereas HSV-2 is mainly involved in genital infection. They belong to the α-herpesvirus subfamily.

The HSV virion consists of 4 elements: a core containing the viral DNA, an icosahedral capsid surrounding the core, a largely unstructured proteinaceous layer called the tegument that surrounds the capsid, and an outer lipid bilayer envelope exhibiting spikes on its surface. The HSV-1 genome is a single, linear molecule of double stranded DNA about 152 base pairs in length, but a small fraction of the virion DNA may be circular. It is divided into two segments called long (L) and short (S). Short regions of repeated sequence occur at the

genome ends and between the L and S segments. Each segment is composed of a unique sequence, UL and US, bounded by inverted repeats referred to as *b* and *c*, respectively. They are both flanked by the *a* inverted repeats, so that the final canonical structure is: *ab*-UL-*b'a'c'*-US-*ca* also designated as *a*-TRL-UL-IRL-*a'*-IRS-US-TR*S*-*a* with TR and IR standing for terminal repeats and internal repeats, respectively [165, 166].

A total of 75 genes for known proteins are encoded with 69 of these present in a single copy and three in two copies each. Among the genes encoded are 43 ancestral or core genes present in all α-, β- and γ-herpesviruses. All 43 are located in UL and most are conserved genes involved in vital virus functions such as entry of the virus into a host cell, DNA replication, capsid assembly, packaging DNA into the capsid and exit of the capsid for the host cell nucleus. The non-core HSV-1 genes include all US genes and highly divergent genes found at the segment ends. Non-core genes encode proteins involved in lineage- or species-specific functions such as transcriptional transactivation, immune evasion and host cell recognition.

HSV-1 contains more than 30 distinct proteins that are designed as virion polypeptides (VP) and given serial numbers.

The tegument is largely unstructured except for some apparent icosahedral structure around the pentons and it is composed of at least 20 viral proteins. The most significant proteins associated with the tegument are the VP16 virion transactivator proteins, encoded by the UL48 ORF. The capsid is composed of 162 capsomers. The outside of the capsid is composed of 4 proteins: VP5 (UL19), VP26 (UL35), VP23 (UL18) and VP19C (UL38). The major capsid protein is VP5, it is present in 5 copies in each penton capsomere and six copies in each hexon capsomere in icosahedral envelope. VP26 is present in six copies as a ring on top of the VP5 subunits on each hexon. The envelope consists of a lipid bilayer with about 11 different viral glycoprotein fixed in it.

HSV has been associated with pulmonary disease, mostly in immunocompromised hosts. Some reports showed that HSV is frequently present in the respiratory tract of intensive-care unit (ICU) patients [167-169]. The mucosal damage associated with intubation and mechanical ventilation is a potential trigger for HSV reactivation, resulting in active secretion in the throat [160]. Aspiration from the URT is said to be the main source of LRT HSV

infections [170, 171], but there are also arguments in favour of the reactivation of the virus in the lungs or trachea [172]. The detection of HSV in the URT of ICU patients has recently been associated with a significantly longer ICU stay and increased mortality as compared with HSV DNA-negative patients [160].

1.10.b. Epstein-Barr virus

Epstein-Barr virus (EBV) belongs to the γ-herpesvirus subfamily. Its genome is linear, double-stranded. EBV, the first isolated human tumour virus, was identified in 1964 by Epstein's group in a cell line derived from Burkitt lymphoma [173]. Two major EBV types have been detected in humans: EBV-1 and EBV-2 (also known as types A and B). EBV-1 and EBV-2 differ in geographic distributions. EBV-1 is observed more frequently in most populations. However, EBV-2 is nearly as prevalent as EBV-1 in New Guinea, as well as in equatorial Africa [174].

EBV-1 and EBV-2 differ in the sequence of the genes that code for the EBV nuclear antigens (EBNA-2, EBNA-3A/3, EBNA-3B/4, and EBNA-3C/6) [175]. These differences cause alterations in some biological properties, including transforming potential. Like other herpesviruses, EBV is a DNA virus with a toroid-shaped protein core that is wrapped with DNA, a nucleocapsid with 162 capsomers, a protein tegument between the nucleocapsid and the envelope, and an outer envelope with external virus-encoded glycoprotein spikes [176]. Many EBV ORFs are divided into latent and lytic genes (further divided into immediate early genes, early genes and late genes). Most of these genes are translated into proteins. Several lytic genes encode for human homologues. In addition, some latent genes are non-translated; this is the case for *EBV-encoded RNA* (*EBER)-1* and *-2* [177, 178]. Furthermore, EBV encodes at least 17 micro-RNAs, arranged in two clusters: ten are located in the introns of the viral *BART* gene, and three adjacent to *BHRF1* [179]. The viral genome also contains a series of 0.5-kb terminal direct repeats at either end and internal repeat sequences that serve to divide into

short and long unique sequence domains that have most of the coding capacity [180].

EBV establishes latent infection in lymphocytes and can induce proliferation of the latently infected cells [181]. EBV infection of B cells is mediated through the interaction of the viral envelope glycoprotein gp350/220 with the cellular receptor for the C3d complement component CR2 (CD21) [182-184].

Humans are the only natural host for EBV. It is known that EBV infects > 90% of the world's adult population. Upon infection, the individual remains a lifelong carrier of the virus. EBV is transmitted from host to host via saliva. Primary infection begins at the oropharyngeal epithelium.

EBV is associated with a variety of tumors derived from B cells, T cells, natural killer (NK) cells, and epithelial cells. At present, tumors related to EBV infection include Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's and non-Hodgkin's lymphomas, gastric cancer, breast cancer, leomyosarcomas arising in immunocompromised individuals, central nervous system lymphomas associated with HIV, post-transplant lymphoproliferative disorders.

1.10.c. Human Cytomegalovirus

Human cytomegalovirus (HCMV) is a ubiquitous virus infection with worldwide distribution. HCMV is associated with opportunistic disease that has been recognized in more highly developed areas of the world. HCMV has the largest genome of any known human virus, at 236 kbp in size [185]. The genome is a linear, double-stranded DNA molecule consisting of two unique regions (U_L and U_S), that are flanked by direct repeats (TR_L and IR_L; TR_S and IR_S) [186, 187].

A copy of the terminal direct repeat of approximately 300–600 bp (the *a* sequence) is present in inverted orientation at the junction between IR_L and IR_S [188, 189].

The structure is represented as ab-U_L-b9a9c9-U_S-ca, where, as already mentioned, U_L and U_S denote the long and short unique regions and ba/b9a9

and ca/c9a9 indicate the inverted repeats. It belongs to β-herpesviruses subfamily. As all β-herpesviruses, also HCMV, replicate slowly in cell culture and remain cell-associated. So far, 4 genera of β-herpesviruses have been recognized; their genome has a capacity to encode about 166 gene products. Virions contain a 125 nm icosahedral nucleocapsid composed of 5 herpesvirus core proteins: major capsid protein (MCP, the UL86 gene product) composed of hexons and most pontons; triplexes composed of 2 subunits; the minor capsid protein (TRI1, the UL46 gene product) together with the minor capsid protein binding protein (TRI2, the UL86 gene product); the smallest capsid protein (SCP, the UL48A gene product) that decorates MCP tips; and a portal protein (PORT, the UL104 gene product) that constitutes one specialized penton used for encapsidation of viral DNA.

CMV infection is a leading cause of morbidity and mortality in severely immunosuppressed patients, such as those undergoing allogeneic hematopoietic stem cell or solid organ transplantation [190]. Nevertheless, recent data suggest that CMV may also be a relevant cause of morbidity in patients lacking canonical immunosuppression and displaying various inflammatory processes, including cardiovascular, autoimmune, and chronic bowel diseases [191-193]. In these patients, active CMV infection is detected frequently in either the inflamed tissues or even the blood compartment. Furthermore, active CMV infection, either restricted to the lower respiratory tract or involving both the lower respiratory airways and the systemic compartment, has been shown to occur frequently during critical illness in adult CMV-seropositive patients [194], and has been associated with prolonged ICU hospitalization, extended periods of mechanical ventilation, higher rates of nosocomial infection, and overall mortality [195].

1.11. Viral respiratory infections in intensive care unit adult patients

As previously reported, for adult patients in ICUs viral respiratory infections account for about 30% of pneumonia cases [73]. Influenza A (H1N1) 2009 was an important cause of pneumonia and ARDS in these clinical settings and it was the most frequently virus detected in these patients [18]. However some cases of influenza A (H3N2) have been reported in ICU patients [196] and less frequently also influenza B virus [197]. Other respiratory viruses, like EV and RV, have been involved in severe cases of infections requiring hospitalization in ICU [65, 66, 75, 76]. Some studies found that RSV-hMPV co-infection leads to an increased rate of ICU admission [84, 85].

The significance of detection of herpes viruses in respiratory secretions of critically ill patients is controversial. Detection of HSV1, EBV and CMV is common in ICU patients. In patients requiring mechanical ventilation, herpes viruses are commonly detected from the respiratory tract. However, viral detection does not necessarily mean viral disease. HSV and CMV are the most frequent viruses detected among non-immunosuppressed ICU patients [171, 172]. Patients infected with these viruses show increased morbidity and, especially for CMV, mortality.

HSV has been isolated previously in cell culture from bronchoalveolar lavages from 47 (39%) of 121 critical care patients [167]. There are some data suggesting that HSV and CMV do not carry the same pathogenicity [198]. Lung is considered as the main site of CMV latency and reactivation [198], but not for HSV. HSV-1 can be isolated from the saliva of 1–5% of the general population. In the ICU, the frequency of viral reactivation is higher. Some studies found that high percentage of ICU patients had HSV in the throat [167, 168]. Other studies demonstrated clearly that detection of HSV DNA in respiratory secretions is common and showed that duration of tracheal intubation is correlated with HSV detection [167, 169].

HSV can be detected in the lower respiratory tract of 5–64% of ICU patients, depending on the population and the diagnostic method used [167, 168, 199]. As already mentioned, HSV detection in the lower respiratory tract does not necessarily mean herpetic pulmonary disease [199]. It is not clear if HSV

recovery from lower respiratory tract samples of non-immunocompromised ventilated patients corresponds to viral contamination of the lower respiratory tract from mouth or throat or both, a local tracheobronchial excretion of the virus due to its reactivation without parenchymal involvement, or real HSV bronchopneumonia. More studies showed that there was a significant association between an HSV1 viral load >100,000 copies/ml of BAL and admission to the ICU ($p < 0.0001$), mechanical ventilation ($p < 0.001$) and death ($p < 0.01$) [161, 200, 201]. Cytomegalovirus is known to be a cause of pneumonia or systemic disease in immunocompromised patients [202], but the exact significance of CMV recovery in respiratory samples from non immunocompromised patients is not clear. Many studies showed that reactivation of CMV could lead to an increased duration of ventilation or ICU stay in non-immunosuppressed patients [161, 162, 203, 204]. Regarding CMV associated with pneumonia, the average values of viral load reported in literature vary [205-207]; however a viral load in BAL samples, between $4{,}6x10^4$ and $5x10^5$, was proposed as a threshold for the diagnosis of pneumonia [205, 206].

Regarding EBV, published reports reflect a high degree of variability concerning the prevalence of EBV in BAL samples from patients admitted in ICU [163, 208-210]. Lung et al. [211] detected EBV DNA in exfoliated cells in bronchial washing samples and concluded that the lower respiratory tract is a major reservoir for EBV. Friedrichs et al. [163], thus, concluded that the detection of EBV DNA in BAL may be more a marker of viral persistence than a marker of active infection.

Rapid diagnosis is essential for prompt patient management. Diagnoses of viral respiratory tract infections have been made for many years, generally, by non-molecular approaches such as direct immunofluorescence and viral culture. Although these methods are effective and often complementary, they are time-consuming, labor-intensive, and often lack sensitivity or specificity [212-214]. However, now various nucleic acid amplification tests have been developed as an important tool for the accurate identification of pathogens causing respiratory syndromes [214-217].

1.12. Molecular assays to detect respiratory viruses

Both commercial kits or in "house" methods, in some cases able to detect many respiratory viruses simultaneously, have been described [218-220].

The preference of one test over the other depends on its specificity, sensitivity and turnaround time as well as cost in resource limited settings. Molecular methods have significantly improved the diagnosis of acute respiratory tract infections. These techniques offer high sensitivity and provide specific results within a shorter period of time and for a larger number of pathogens compared to classical methods such as virus isolation or direct fluorescent antibody tests.

Various kinds of commercial molecular systems have been developed for fast and more accurate detection of respiratory viruses.

For example, the EraGen MultiCode-PLx Respiratory Virus Panel (Luminex, Austin, TX, USA) is one assay that couples multiplex PCR chemistry with high-throughput microsphere flow cytometry for simultaneous detection of 17 viruses: influenza A and B; respiratory syncytial virus A and B; parainfluenza 1, 2, 3, 4(a) and 4(b); metapneumovirus; adenovirus B, C, and E; coronovirus NL63, 229E, and OC43; and rhinovirus.

Seegene developed many kits to detect simultaneously different types of respiratory viruses; Anyplex II RV16 Detection kit is one of these, it is a multiplex real-time RT-PCR assay that can detect simultaneously 16 different types of viruses.

Moreover, the FilmArray Respiratory Panel (RP) (Idaho Technology, Inc., Salt Lake City, UT, USA), which consists of a pouch system with a multiplex PCR test, provides the detection of 18 viruses and 3 bacterial respiratory pathogens in about 1 hour.

Recently, AdvanSure™ kit based on multiplex real-time PCR has been developed for simultaneous detection of 14 respiratory viruses. In addition to these, many other kits are available.

1.13. Next-generation sequencing (NGS) to detect respiratory viruses

Next-generation sequencing (NGS), provides high speed and throughput that can produce an enormous volume of sequences. The most important advantage provided by these platforms is the determination of the sequence data from single DNA fragments of a library that are segregated in chips, avoiding the need for cloning in vectors prior to sequence acquisition.

The first next-generation high-throughput sequencing technology was developed in 2005 by Roche, the platform was the 454 FLX pyrosequencing.

In 2007, Illumina released the Genome Analyzer developed by Solexa GA, and subsequently, SOLiD was released by Applied Biosystems. These platforms represent the "second generation" systems, able to sequence populations of amplified template-DNA molecules with a typical "wash-and-scan" technique.

More recently, Life Technologies has developed new sequencing platform named Ion Torrent; this platform, together Heliscope by Helicos and a real-time sequencing platform by Pacific Biosciences, represents the "third-generation". These platforms allow us to sequence single large DNA molecules without the need to halt between read steps [221].

NGS methods have different underlying biochemistries and differ in sequencing protocol (sequencing by synthesis for 454 pyrosequencing, Illumina GA, Ion Torrent PGM and Heliscope, sequencing by ligation for SOLiD), throughput, and for sequence length.

Typical applications of NGS methods in microbiology and virology are discovery of new microorganisms and viruses by using metagenomic

approaches, investigation of microbial communities in the environment and in human body niches in healthy and disease conditions, analysis of viral genome variability within the host (*i.e.*, quasispecies), detection of low-abundance antiviral drug-resistance mutations in patients with human immunodeficiency virus (HIV) infection or viral hepatitis [222, 223].

NGS also led to the detection of viral pathogens in nasopharyngeal aspirate samples from patients with acute lower respiratory tract infections [224], such as a new HEVs [225].

More recently, two influenza-like viruses genome (H17N10 and H18N11) have been demonstrated in bats by NGS [24, 27].

Analysis of full-length viral genome and quasispecies was applied, for example, on total RNAs extracted from the lung of a patient who died for viral pneumonia due to pandemic 2009 influenza A virus (A/H1N1/2009). This analysis revealed nucleotide heterogeneity on hemagglutinin as quasispecies, leading to amino acid changes on antigenic sites which could be relevant for antigenic drift [226]. Furthermore, mutations of HRV genome were explored in a lung transplant recipient infected with the same HRV strain for more than two years [227].

NGS high throughput sequencing technologies have become available in the last few years and are in continuous development and improvement.

2. Aim of the study

Respiratory viruses cause many diseases, from mild to severe illnesses, and contribute significantly to morbidity and mortality worldwide [1]. Different viruses can establish respiratory tract infections, they belong to the *Orthomyxoviridae, Coronaviridae, Picornaviridae, Paramyxoviridae, Adenoviridae, Parvoviridae* and in immunocompromised patients *Herpesviridae* families. Altogether, the etiologic diagnosis of respiratory viral infections has been underestimated so far. In patients with predisposing conditions the outcome of these infections can be more severe, sometimes requiring hospitalization, even in intensive care units (ICU), because of the development of pneumonia and acute respiratory distress syndrome (ARDS). For these patients, in particular, rapid diagnosis is essential. In addition, in ICU patients the significance of the detection of some members of *Herpesviridae* family, like HSV1, CMV and EBV, is controversial.

The aim of this study was to clarify the prevalence of respiratory viruses and herpesviruses, and their role in ICU patients.

Viral detection was performed in both upper and lower respiratory samples in order to compare the viral populations in these two compartments and, possibly, to draw informations concerning the role of the infection in severe cases. As in these patients in particular there is the need of a rapid diagnostics response, large part of this study was devoted to the development of diagnostic assays able to accurately characterise respiratory viruses quickly and at the lowest costs.

These assays could be particularly useful to screen a large number of patients for epidemiological studies and to asses the prevalence in the lower and upper respiratory tract of ICU patients with, regarding CMV, EBV and HSV1, the ultimate goal to understand the clinical significance of this phenomenon.

In addition, for this study a protocol for NGS was developed, able to detect and to characterize one or more viruses in the same clinical samples.

3. Materials and Methods

3.1. Detection of target viruses and primers design

The targets for this study were the more common viruses involved in respiratory disease, in particular in ICU patients. The viruses included in this study were: influenza viruses type A and type B, rhinoviruses, enteroviruses, respiratory syncytial viruses, human metapneumoviruses, coronaviruses group I and II, parainfluenza viruses type 1, 2 and 3, human adenoviruses and some viruses belonging to *Herpesviridae* family (cytomegalovirus, Epstein-Barr virus and herpes simplex type 1 virus).

For each virus about 100 sequences have been downloaded from GenBank (NCBI), in order to identify a conserved and specific region in the genome of each virus able to identify and in case to typify the viruses directly from the clinical sample. These sequences were aligned using ClustalW v1.4 included in BioEdit v7.0.0. For each virus two sets of primers were designed; for influenza A viruses 6 sets of primers were designed. All the primers were designed using Primer3.

The sequence of primers chosen, the gene targets, the lenght of fragments and annealing temperatures are reported in table 1, table 2 and table 3.

Table 1. Primers used to perform RT duplex real time PCRs.

Virus	Gene	Primers	Size	Annealing
PIV 1,3	L	5'gAgACTCTgAgCTgTTTTTTAAC'3 5'gCTgTACTTTCAAATCTCCA'3	71 bp	55°C
PIV 2	L	5'TgCATgTTTTATAACTACTgATCTTgCTAA'3 5'gTTCgAgCAAAATggATTATggT'3	77 bp	55°C
RSV	L	5' AATACAgCCAAATCTAACCAACTTTA'3 5'gCCAAggAAgCATgCAATAAA'3	94 bp	55°C
hMPV	F	5'gAgAgCTgAAAgAATTTgTgAgC'3 5'ggTCCAATgATATTgCTggTgTTA'3	174 bp	55°C
CoV I	polymerase	5'CAACgTATgTgTgCTATAggC'3 5'gTATTAACTATTTCAgCAggAC'3	74 bp	55°C
RV/EV	5'UTR	5'AgTCCTCCggCCCCTgAA'3 5'gAAACACggCACCCCAAAgT'3	120 bp	55°C

Flu A	M	5’TCAggCCCCCTCAAAgCC’3	158 bp	55°C
		5’ gggCACggTgAgCgTRAA’3		
Flu B	M	5’ TagCAgAAggCCATgAAAgCTC’3	94 bp	55°C
		5’ CgTTCCTAgTTTTACTTgCATTgAAT’3		

Table 2. Outer and inner primers for herpesviruses.

Virus	Gene	Primers	Size	Annealing
EBV	polymerase	5’TgCAACAATggACACgCAA’3	505 bp	55°C
		5’CTCCAACgCCATACCCAAgT’3		
		5’TCCgTCAATgCAACggAAgA’3	158 bp	55°C
		5’AgCCAgACATCCATTCggTg’3		
CMV	polymerase	5’ AATCggCgAgTATCTgCTgg’3	480 bp	55°C
	UL54	5’ TCgTAAACgTCCACgTCTgg’3		
		5’CCCgTgTACgAggTCCgTgTg’3	154 bp	55°C
		5’ggTCggAgACATCgCAgTCg’3		
HSV1	polymerase	5’gCgTCATCTACgggggTAAg’3	394 bp	55°C
	UL30	5’TACgggATCCggTCCTTgAT’3		
		5’gggTAAgATgCTCATCAAgggC’3	101 bp	55°C
		5’CgTCgTAAAACAgCAggTCg’3		

Table 3. Primers used to perform PCRs and RT-PCRs for Nextera XT protocol.

Virus	Gene	Primers	Size	Annealing
Flu A	M	5’gAgTCTTCTAACMgAggTCgAAACgTA’3	597 bp	55°C
		5’gCTgCCTgTCACTCgATCC’3		
Flu A	H1	5’AACAAAggTgTAACggCAgC’3	383 bp	55°C
		5’TgCgAATgCATATCTCggTA’3		
Flu A	N1	5’TCCCCCTTggAATgCAgAAC’3	544 bp	55°C
		5’AAgACACCCACgGTCgATTC’3		
Flu A	H3	5’AATgACAACAgCACggCAAC’3	588 bp	55°C
		5’TTggTCCTTgTCCgTAACCg’3		
Flu A	N2	5’ATTggTCAAAgCCgCAATgT’3	725 bp	55°C
		5’TCTgggTgTgTCTCCAACAAg’3		
Flu B	M	5’CACTgTTggTTCggTgggAA’3	367 bp	55°C

		5’ACAAAgCACAgAgCgTTCCT’3		
RV/EV	5’UTR	5’gCACTTCTgTTTCCCCg’3	389 bp	55°C
		5’gAAACACggACACCCAAAgT’3		
RSV	L	5’TAAgRATTgCWAATTCTgAATTAgA’3	501 bp	55°C
		5’TMCCWgCTCCTTCACCTATgA’3		
HMPV	F	5’AACCATMCgRCTTgAgAgTg’3	418 bp	55°C
		5’gCTYCCgTAgACCCCTATCAg’3		
CoVI	polymerase	5’gCYCAYgCTgCTgTTgATTC’3	534 bp	55°C
		5’ACTRgARCCATTgTCWACCTg’3		
CoVII	polymerase	5’gAggAACAgRATgAAATTTAYg’3	502 bp	55°C
		5’AAgCAgTAgTTgCATCACCAC’3		
PIV 1,3	L	5’CAAgARggTMgACTCTTTgC’3	633 bp	55°C
		5’AgCTgCWAgATgKATTgCAC’3		
PIV 2	L	5’TCTCgCAAATCATgCAggTACT’3	496 bp	55°C
		5’gCCTTCAATACCTCCCTTggA’3		
AdV	exon 6	5’CAACACCTAYgASTACATgAA’3	474 bp	55°C
		5’KATggggTARAgCATgTT’3		

3.2. Clinical samples

Pediatric patients: A total of 72 clinical samples from upper respiratory tract of children with respiratory infections collected during the years 2012 and 2013 were analyzed for the detection of respiratory viruses.

Intensive Care Unit patients: A total of 156 clinical samples from upper and lower respiratory tract from 58 patients, hospitalized in ICU, were analyzed to detect the respiratory viruses and herpesviruses. The clinical samples were collected between September 2011 and May 2014.

3.3. Viral nucleic acids extraction

Extraction of viral RNAs and DNAs from clinical samples was carried out using a commercially available kit (QIAamp MinElute Virus, Valencia, CA,

USA) for the simultaneous purification of viral RNA and DNA from body fluids.
This protocol is for purification of viral nucleic acids from 200 µl of body fluids. The elution was carried out in a final volume of 150 µl.

3.4. RT duplex real time PCRs

After RT with random examers (Qiagen, Valencia, CA, USA), four duplex real-time PCR, using EVAGreen fluorescent dye, were developed. A duplex was performed to detect influenza A and influenza B viruses; a second duplex was performed to detect PIVs belonging to *Respirovirus* genera (PIV1 and PIV3) and RSV; the viruses target for the third duplex PCR were PIV type 2 and hMPV; and a fourth duplex was performed to detect CoV I and EV/RV. In particular, after retrotranscription of 10 µl of RNA with random examers, 2X HRM PCR master mix (Qiagen, Valencia, CA, USA) was used. The reaction volume was 25 µl (12,5 µl of master mix, 1,75 µl of each primer [10µM], 5 µl of cDNA and H_2O to reach the final volume). After initial activation step, 30 cycles of amplification (95°C for 10 sec, 55°C for 30 sec, 72°C for 10 sec (acquiring Green)) were performed. For Melting analysis, ramp from 65°C to 95°C was used, rising by 0.1°C each step. The reaction was performed on Rotor Gene 6000 (Qiagen, Valencia, CA, USA). The primers specific for each virus were listed in table 1.

All duplex real time PCRs were developed using standards prepared with reference strains obtained from the National Institute for Biological Standards and Controls (NIBSC) or already available in the laboratory. The standards were prepared by cloning according to the standard protocol of pGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA). The plasmid DNA was purified by QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). To obtain the RNA standard for each virus, each product of the cloning was transcribed with T7 RNA polymerase (Promega, Madison, Wisconsin, USA).

3.5. PCRs and real time PCRs to detect herpes viruses

To detect herpes viruses (CMV, EBV and HSV1) three nested PCRs were developed, using the primers listed in table 2. Applied Biosystems Master Mix was used for each nested PCRs; in particular, after denaturation, 35 cycles of amplification were performed (95°C for 30 sec, 55°C for 30, 72°C for 1 min), followed by a final extention at 72°C for 7 min. The reaction volume was 20 µl (2 µl of 10X master mix, 0,6 µl of 200 µM dNTPs, 1,6 µl of MgCl2 [25mM], 1 µl of each primer [10µM], 0,2 µl of Taq 100U, 5 µl of extracted DNA and H_2O to reach the final volume).
The second step was performed similarly (30 cycles of amplification and 2 µl of the products of the first step).

The positive clinical samples obtained with these PCRs were analyzed by quantitative real-time PCRs.
To perform the calibration curves, serial dilutions of DNA calibrator for each virus were used.
These calibrators consisted of DNA sequences obtained by the cloning of the template of the product of the PCR of each virus (using the inner primers, table 2), cloned in the pGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA). The plasmid DNA was purified by QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA).
Quantitative real-time PCRs were performed using 2X HRM PCR master mix (Qiagen, Valencia, CA, USA) . The reaction volume for each amplification was 25 µl (12,5 µl of master mix, 1,75 µl of each primer [10µM], 5 µl of DNA and H_2O to reach the final volume).
After initial activation step, 40 cycles of amplification (95°C for 10 sec, 55°C for 30 sec, 72°C for 10 sec (acquiring Green)) were performed. For Melting analysis, ramp from 80°C to 90°C was used, rising by 0.1°C each step. The reaction was performed on Rotor Gene 6000 (Qiagen, Valencia, CA, USA).

3.6. PCRs and RT-PCRs for Nextera XT protocol (Illumina)

To perform the PCRs to be used with Nextera XT protocol, Illumina recommends to amplify more than 300 bp to ensure even coverage across the length of the DNA fragment. This is because the tagmentation reaction cannot add an adapter right at the distal end of a fragment. It is important to design the PCR primers so that these amplify about 100 bp longer than the desired insert to be sequenced.

Nextera XT use an enzymatic DNA fragmentation step and thus it can be more sensitive to DNA input compared to mechanical fragmentation methods.

The sets of primers used to perform the PCRs and RT PCRs were reported in table 2 (outer primers for herpesviruses) and in table 3.

Overall 17 sets of primers were used in order to detect and amplify in the same clinical samples one or more viruses. Applied Biosystems Master Mix was used to perform PCRs for adenovirus, CMV, EBV and HSV1. In particular, after denaturation, 35 cycles of amplification, for each amplification, were performed (95°C for 30 sec, 55°C for 30, 72°C for 1 min), followed by a final extention at 72°C for 7 min. The reaction volume was 20 µl (2 µl of 10X master mix, 0,6 µl of 200 µM dNTPs, 1,6 µl of MgCl2 [25mM], 1 µl of each primer [10µM], 0,2 µl of Taq 100U, 5 µl of extracted DNA and H_2O to reach the final volume).

After RT with random examers (Qiagen, Valencia, CA, USA), the same protocol was applied to detect all RNA viruses.

After each amplification, the PCR products were quantified by Qubit dsDNA BR Assay system (Invitrogen), Illumina recommends to use a fluorimetric method specific for duplex DNA.

Five microliter of all positive and negative templates (17 overall) for each sample were pooled.

Each pool was purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), in order to remove the contaminants.

Finally, the purified pools were quantified using Qubit dsDNA BR Assay system (Invitrogen) and adjusted in order to add 1 ng of each pool to the next step, because the Nextera XT DNA Sample Preparation Kit protocol is optimized for 1 ng of input DNA total.

For sample quantification 2 µl of each DNA sample with 198 µl of the Qubit working solution were used.

3.7. Nextera XT protocol to NGS (Illumina)

The Nextera XT DNA Sample Preparation Kit uses an engineered transposome to simultaneously fragment and tag ("tagment") input DNA, adding unique adapter sequences in the process. A limited-cycle PCR reaction uses these adapter sequences to amplify the insert DNA. The PCR reaction also adds index sequences on both ends of the DNA.

The Nextera XT protocol consists of 5 steps:

Tagmentation of Input DNA: During this step input DNA is tagmented (tagged and fragmented) by the Nextera XT transposome. The Nextera XT transposome, as already mentioned, simultaneously fragments the input DNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent steps.

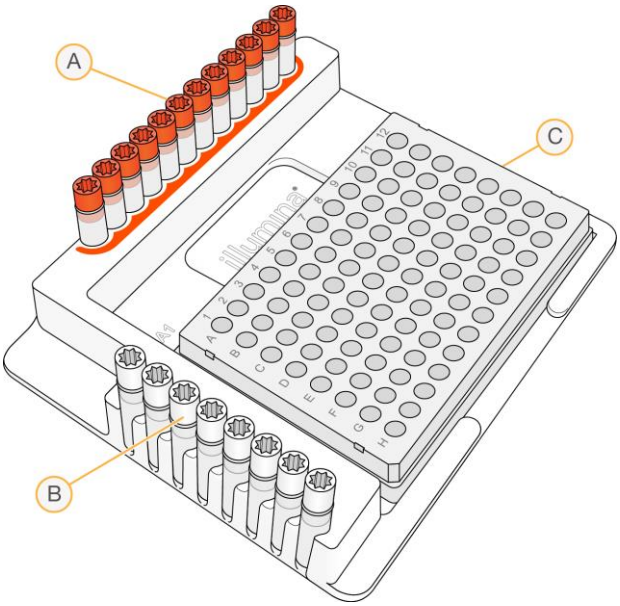
The protocol for this step is as follows:
Add 10 µl of TD Buffer (Tagment DNA Buffer) to each well of a plate and subsequently add 5 µl of input DNA at 0.2 ng/µl (1 ng total) to each sample well. Add 5 µl of ATM (Amplicon Tagment Mix) to the wells containing input DNA and TD Buffer. Now, cover the plate with Microseal 'B' and centrifuge at 280 xg at 20°C for 1 minute. After, place the plate in a thermocycler and run the following program:

- ✓ 55°C for 5 minutes
- ✓ Hold at 10°C

Carefully remove the Microseal “B” seal and add 5 µl of NT Buffer (Neutralize Tagment Buffer) to each well of the plate; gently pipette up and down 5 times to mix. Cover the plate with Microseal 'B', centrifuge at 280 xg at 20°C for 1 minute. Place the plate at room temperature for 5 minutes.

PCR amplification: In this step, the tagmented DNA is amplified via a limited-cycle PCR program. The PCR step also adds index 1 (i7) and index 2 (i5) (figure 1) and sequences required for cluster formation.

Figure 1. TruSeq Index Plate Fixture (96 libraries).



A Index primer 1 (i7)

B Index primer 2 (i5)

C plate

In this step it is critical to use the full amount of recommended input DNA and to use the right combination of the index.

The protocol for this step is as follows:

Add 15 µl of NPM (Nextera PCR Master Mix) to each well of the plate, previously incubated at room temperature for 5 minutes; add 5 µl of index 2 primers to each column of the plate and add 5 µl of index 1 primers to each row of the plate. Now, gently pipette up and down 3 to 5 times to mix, cover the plate with Microseal 'A' and centrifuge at 280 xg at 20°C for 1 minute.

After centrifugation it is possible to perform the PCR, using the following program:

72°C for 3 minutes

95°C for 30 seconds

12 cycles of:

95°C for 10 seconds

55°C for 30 seconds

72°C for 30 seconds

72°C for 5 minutes

Hold at 10°C

PCR Clean-Up: This step uses AMPure XP beads to purify the library DNA, and provides a size selection step that removes very short library fragments from the population.

After amplification, the plate has to be centrifuged at 280 xg for 1 min (20˚C) to collect condensation. A new plate is required to transfer 50 µl of the PCR product and to add 30 µl of AMPure XP beads to each well. Gently pipette mix up and down 10 times and incubate at room temperature without shaking for 5 minutes. Subsequently, place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared. With the plate on the magnetic stand remove and discard the supernatant. With the plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows: add 200 µl of freshly prepared 80% ethanol to each sample well; incubate the plate on the magnetic stand for 30 seconds; carefully remove and discard the supernatant. Repeat this step two times. With the plate still on the magnetic stand, allow the beads to air-dry for 15 minutes. After, remove the plate from the magnetic stand and add 52.5 µl of RSB (Resuspension Buffer) to each well of the plate. Gently pipette mix up and down 10 times and incubate at room temperature for 2 minutes.

Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared. Now, transfer 50 µl of the supernatant to the new plate.

Library normalization: This process normalizes the quantity of each library to ensure more equal library representation in the pooled sample.

For this step a new plate is required to transfer 20 µl of the supernatant. Add 45 µl of the combined LNA1 (Library Normalization Additives 1)/LNB1 (Library

Normalization Beads 1) to each well of the plate. Seal the plate with Microseal 'B' and shake the plate on a microplate shaker at 1,800 rpm for 30 minutes. Place the plate on a magnetic stand for 2 minutes and confirm that the supernatant has cleared. With the plate on the magnetic stand remove and discard the supernatant.
Remove the plate from the magnetic stand and wash the beads with 45 µl of LNW1 (Library Normalization Wash 1), seal the plate with Microseal 'B' and shake the plate on a microplate shaker at 1,800 rpm for 5 minutes. Place the plate on a magnetic stand for 2 minutes and confirm that the supernatant has cleared, remove and discard the supernatant. Remove the plate from the magnetic stand and repeat the same wash with LNW1.
Remove the plate from the magnetic stand and add 30 µl of 0.1 N NaOH to each well. Seal the plate with Microseal 'B' and shake the plate on a microplate shaker at 1,800 rpm for 5 minutes.

During the 5 minute elution, apply the SGP (StoraGe Plate) barcode plate sticker to a new 96-well PCR plate and add 30 µl of LNS1 (Library Normalization Storage Buffer 1) to each well of this plate.

After the 5 minute elution, place the plate on the magnetic stand for 2 minutes or until the supernatant appears clear. Now, transfer the supernatant from this plate to the SGP plate. Seal the SGP plate with Microseal 'B' and then centrifuge at 1,000 xg for 1 minute.

Library pooling:

Centrifuge the SGP plate at 1,000 xg for 1 minute at 20°C to collect condensation. Transfer 5 µl of each library to be sequenced from the SGP plate to an Eppendorf tube.
Add 576 µl of HT1 (Hybridization buffer) to the new Eppendorf tube and transfer in this tube 24 µl of the library to be sequenced. Mix this tube by vortexing and incubate at 96°C for 2 minutes.

After the incubation, invert the tube 1-2 times to mix and immediately place in the ice-water bath for 5 minutes. In the end, load the tube into a thawed MiSeq reagent cartridge into the Load Samples reservoir.

3.8. MiSeq sequencing (Illumina)

Before starting the run in MiSeq it is fundamental to create the sample sheet. The sample sheet is used to record information about our samples for later use in data analysis. This step also identifies any incorrect index combinations, allowing re-design before the library prep starts. To create the sample sheet the Illumina Experiment Manager (IEM) was used.

The run on the MiSeq was performed in paired-end, 2x250 cycles.

During cluster generation, single DNA molecules are bound to the surface of the flow cell, and then bridge-amplified to form clusters.

Following cluster generation, clusters are imaged using LED and filter combinations specific to each of the four fluorescently labeled dideoxynucleotides. After imaging of one tile of the flow cell is complete, the flow cell is moved into place to expose the next tile. The process is repeated for each cycle of sequencing. Following image analysis, the software performs primary analysis, which includes base calling, filtering, and quality scoring.

3.9. Data analysis

The quality of the sequence run was monitored by Sequencing Analysis Viewer (SAV) (Illumina).

When the run is complete, the MiSeq Reporter analysis software launches automatically to perform secondary analysis, which includes alignment and variant calling. Subsequently, the reads were aligned with the reference genome previously create as FASTA format file.

4. Results

Altogether, for this study 228 clinical samples from 130 patients, collected between September 2011 and May 2014, were analyzed in order to understand what are the most common viral populations in the two compartments, and which are potentially associated with severe respiratory disease.
Out of the 130 patients, 58 were hospitalized adults in ICU and 72 were children both inpatients and outpatients. From 58 ICU patients a total of 156 samples have been analyzed, from upper and lower respiratory tract.

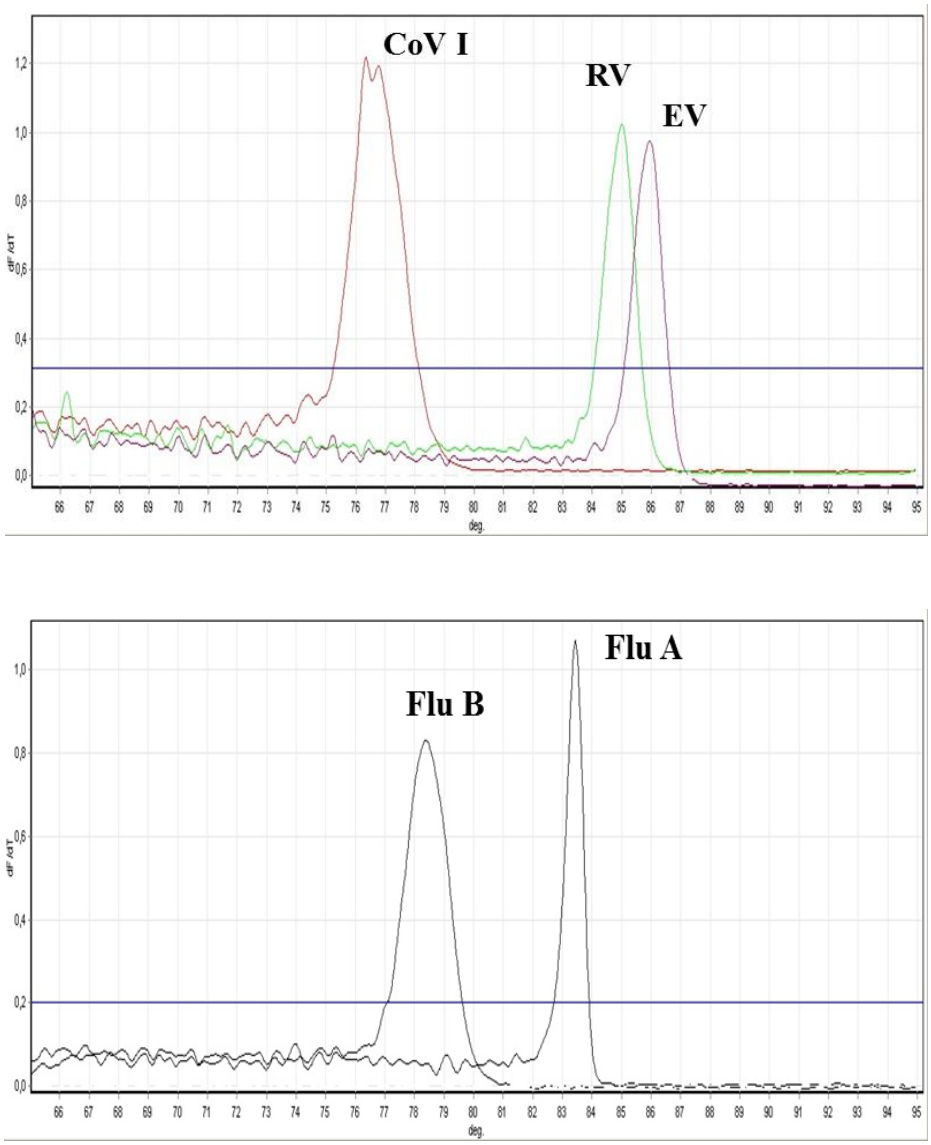
4.1. Detection of RNA viruses by RT real-time duplex PCR

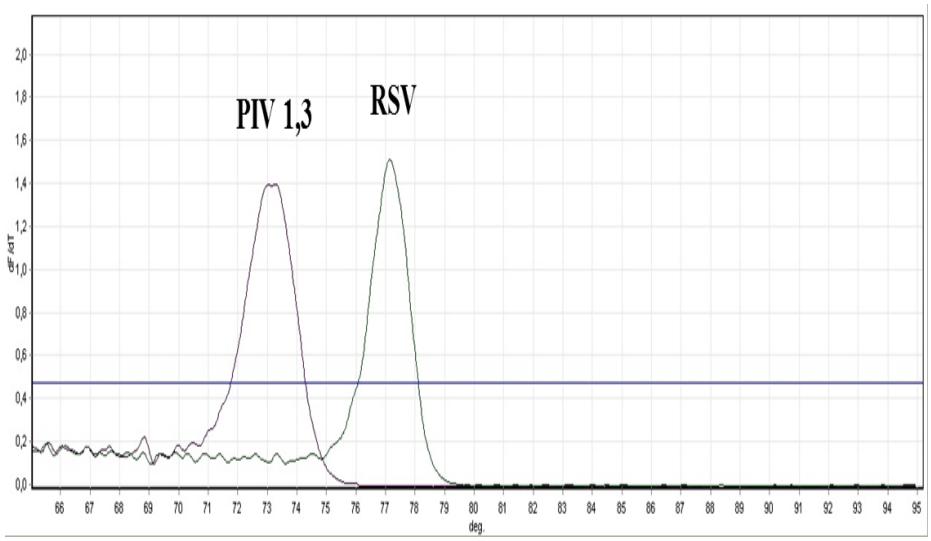
RT real-time duplex PCRs, using EvaGreen fluorescent dye, were used to identify and characterize the main respiratory RNA viruses directly from clinical samples. Each viral RNA sequence shows a characteristic Melting profile. (Figure 2)
All duplex real-time PCRs were developed using standards prepared with reference strains obtained from the National Institute for Biological Standards and Controls (NIBSC) or already available in the laboratory. In order to assess the efficiency and sensitivity of each duplex real-time PCR seventy-two clinical samples from children were analysed in this study. Six children were positive for influenza A and 4 were positive for influenza B viruses; 10, 6 and 2 children were positive for EV/RV, RSV and CoV I respectively.
One hundred fifty-six clinical samples from upper and lower respiratory tract from ICU patients were analysed. Twenty patients were positive for influenza A viruses and 2 were positive for influenza B viruses. The other respiratory viruses detected were: RV/EV (2 adult patients) and hMPV (1 adult patient).
The figure 3 shows the rate of respiratory viruses detected in all patients (adults and children).
In particular for all adult patients positive for influenza A and B viruses, both upper and lower respiratory tract samples were positive. Also in one adult patient positive for EV/RV, the virus was detected in both samples; while for

another one only the upper respiratory tract sample was positive for EV/RV. The hMPV was present only in the upper respiratory samples. The results obtained for clinical samples from ICU patients were shown in figure 4 and in table 4.

The results of the duplex PCRs were confirmed by sequencing positive samples and by comparison with other assays, including commercial, validated, assays, (r-gene for respiratory viruses detection, Biomérieux) which gave similar results.

Figure 2. Characteristic Melting profile of each viral RNA sequence.




PIV 1,3
RSV
dF/dT
deg.

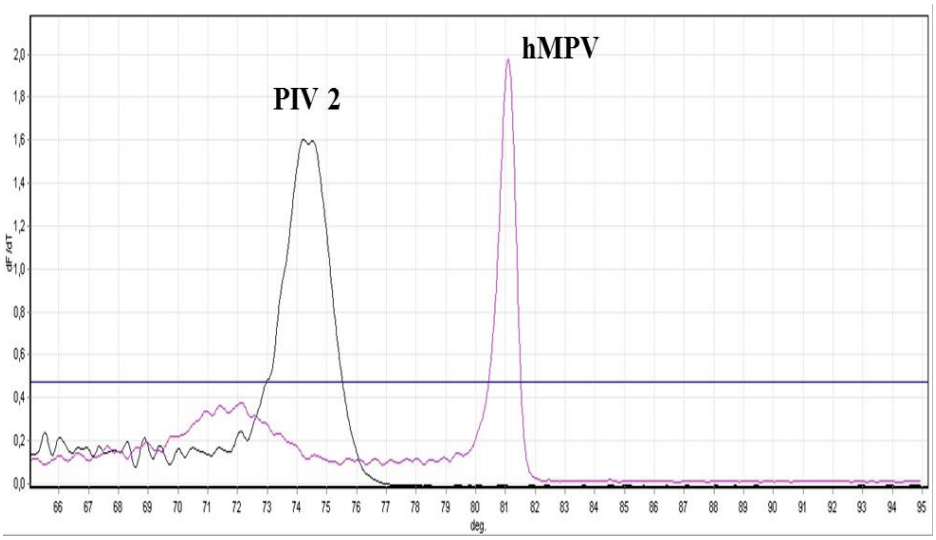

hMPV
PIV 2
dF/dT
deg.

Figure 3. Rate of respiratory viruses detected in all patients.

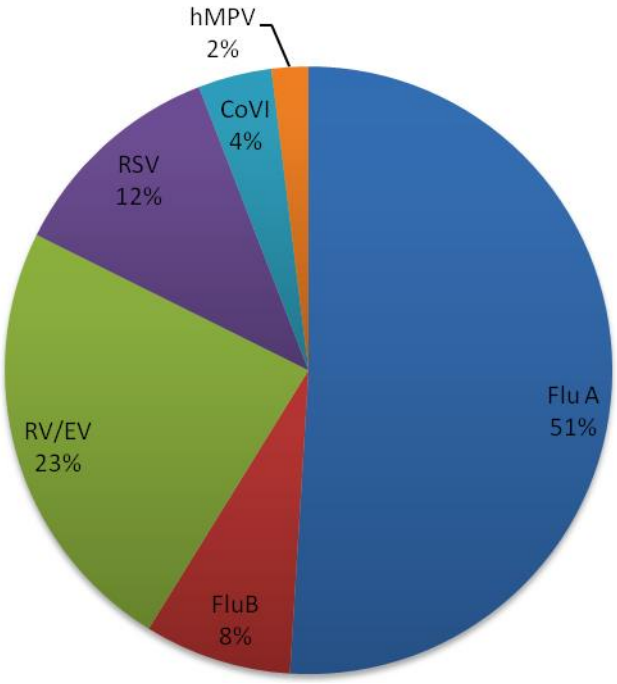


Figure 4. Respiratory viruses detected in positive ICU adult patients.

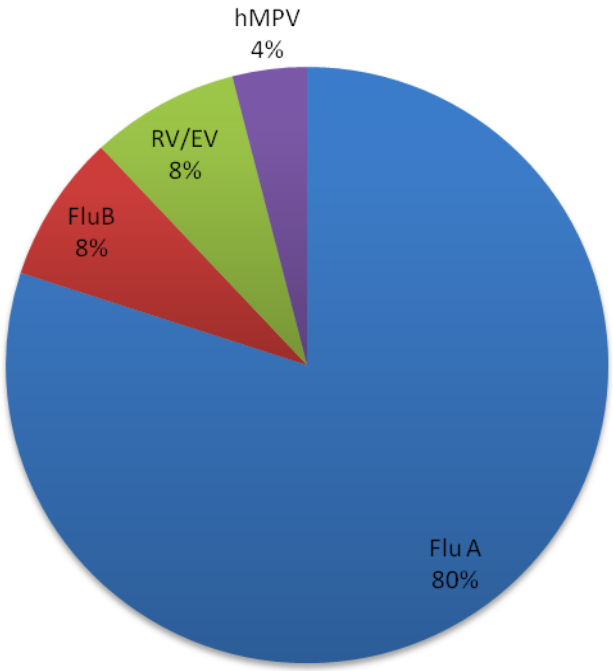


Table 4. Results obtained for clinical samples from ICU adult patients.

Virus	TS	BAL	TS/BAL
Flu A	-	-	20
Flu B	-	-	2
RV/EV	1	-	1
HMPV	1	-	-

TS: throat swab

BAL: broncho-alveolar lavage

4.2. Detection of herpesviruses by nested-PCRs

One hundred fifty-six clinical samples from upper and lower respiratory tract from 58 ICU patients were analysed to detect herpesviruses. For thirty-eight patients only one specimen (from upper and lower respiratory tract) collected the first day of hospitalization was available. For twenty patients, instead, 2 specimens, collected at different time periods, were available.
Altogether, 37 of 58 (about 64%) patients were positive for one or more herpesviruses in at least one sample.

EBV was detected in 25 out of 58 patients, either as a single infection or as mixed infection. Ten patients with the specimen collected the first day of hospitalization were positive for EBV DNA only; EBV was present in upper respiratory tract samples from 7 of these patient, while it was present in lower respiratory tract samples from 1 patients and in both upper and lower respiratory tract samples from 2 patients. Moreover, 6 patients showed EBV in mixed infections. Regarding patients with 2 different specimens collected at different times, 3 were positive for EBV DNA. In particular, one patient showed EBV DNA in upper respiratory tract sample collected on the first day of hospitalization only, another one showed the EBV infection in lower respiratory tract sample collected the first day of hospitalization and also in the same clinical sample collected after 9 days of hospitalization. The third patient showed EBV infection in upper respiratory tract sample collected on the first

day of hospitalization and both in upper and lower respiratory tract sample collected after 8 days. Six patients, with the samples collected at different times, showed EBV DNA together with DNA from other herpesviruses. The results are summarized in the table 5.

Table 5. EBV results.

Patients	First sample			Second sample		
	TS	BAL	Mix	TS	BAL	Mix
1	+	-	-	n.a.	n.a	n.a
2	+	-	-	n.a.	n.a	n.a
3	+	-	-	n.a.	n.a	n.a
4	+	-	-	n.a.	n.a	n.a
5	+	-	-	n.a.	n.a	n.a
6	+	-	-	n.a.	n.a	n.a
7	+	-	-	n.a.	n.a	n.a
8	-	+	-	n.a.	n.a	n.a
9	+	+	-	n.a.	n.a	n.a
10	+	+	-	n.a.	n.a	n.a
11	-	-	+	n.a.	n.a	n.a
12	-	-	+	n.a.	n.a	n.a
13	-	-	+	n.a.	n.a	n.a
14	-	-	+	n.a.	n.a	n.a
15	-	-	+	n.a.	n.a	n.a
16	-	-	+	n.a.	n.a	n.a
17	+	-	-	-	-	-
18	-	+	-	-	+	-
19	+	-	-	+	+	-
20	-	-	-	-	-	+
21	-	-	+	-	-	+
22	-	-	+	-	-	-
23	-	-	-	-	-	+
24	-	-	+	-	-	-
25	-	-	+	-	-	-

In this study CMV was detected in 15 patients, either as single or mixed infection. Only 2 patients showed CMV single infection. In one of these cases, CMV was detected in upper and lower respiratory tract sample collected on the first day of hospitalization and was not present any more in the clinical sample collected after 5 days. The second patient showed CMV infection in the first

lower respiratory tract sample and in the upper and lower respiratory tract sample collected after 5 days. CMV results are reported in the table 6.

Table 6. CMV results.

Patients	First sample			Second sample		
	TS	BAL	Mix	TS	BAL	Mix
1	-	-	+	n.a.	n.a	n.a
2	-	-	+	n.a.	n.a	n.a
3	-	-	+	n.a.	n.a	n.a
4	-	-	+	n.a.	n.a	n.a
5	-	-	+	n.a.	n.a	n.a
6	-	-	+	n.a.	n.a	n.a
7	+	+	-	-	-	-
8	+	-	-	+	+	-
9	-	-	-	-	-	+
10	-	-	-	-	-	+
11	-	-	+	-	-	-
12	-	-	+	-	-	+
13	-	-	+	-	-	-
14	-	-	+	-	-	-
15	-	-	+	-	-	-

Also HSV1 was detected in 15 patients out of 58. Three patients with the specimen collected on the first day of hospitalization showed single HSV1 infection; HSV1 was present in upper respiratory tract sample from one of these patients, as well as in lower respiratory tract sample from one of these patients and in both upper and lower respiratory tract sample from one of these patients. Moreover, 3 patients showed HSV1 in mixed infections. Regarding patients with 2 specimens collected in different time periods, 5 showed HSV1 single infection and 4 showed HSV1 mixed infection. The results for HSV1 are showed in the table 7. The 7 patient showed HSV1 in the second lower respiratory tract sample, collected after 7 days of hospitalization. The 9, 10 and 11 patients showed HSV1 in the first and in the second upper respiratory tract samples (collected after 5 days), both in the upper and lower respiratory tract sample collected after 20 days of hospitalization, in the first upper respiratory tract sample and in the second upper and lower respiratory tract sample collected after 6 days, respectively.

Table 7. HSV1 results.

Patients	First sample TS	BAL	Mix	Second sample TS	BAL	Mix
1	-	+	-	n.a.	n.a	n.a
2	+	-	-	n.a.	n.a	n.a
3	+	+	-	n.a.	n.a	n.a
4	-	-	+	n.a.	n.a	n.a
5	-	-	+	n.a.	n.a	n.a
6	-	-	+	n.a.	n.a	n.a
7	-	-	-	-	+	-
8	-	+	-	-	-	-
9	+	-	-	+	-	-
10	-	-	-	+	+	-
11	+	-	-	+	+	-
12	-	-	-	-	-	+
13	-	-	+	-	-	-
14	-	-	+	-	-	+
15	-	-	+	-	-	-

Mixed herpesviruses infections were not rare, 14 of 58 patients showed mixed herpesviruses infections. The results are summarized in table 8.

Table 8. Mixed herpesvirus infections detected in this study.

Patients	**Mixed infection**	**Clinical samples**
1	EBV/CMV	TS/BAL
2	EBV/CMV	TS
3	EBV/CMV/HSV1	TS
4	EBV/CMV	TS/BAL
5	EBV/CMV	TS/BAL
6	CMV/HSV1	TS/BAL
7	EBV/CMV	TS
8	EBV/ HSV1	TS
9	EBV/CMV/HSV1	BAL
10	EBV/CMV/HSV1	BAL

11	EBV/CMV/HSV1	TS/BAL
12	CMV/HSV1	BAL
13	EBV/CMV	TS/BAL
14	EBV/CMV	BAL

4.3. Quantification of herpesviruses in upper and lower respiratory samples

The exact significance of herpeviruses presence in respiratory samples from non immunocompromised ICU patients is not clear; to understand better their role all herpesviruses positive clinical samples obtained by the nested-PCRs were analyzed by quantitative real-time PCRs.

EBV DNA viral load in TS samples was higher than in BAL samples; the range was between $1x10^3$ copies number/ml and $3x10^9$ copies number/ml.

The range of CMV DNA load was between $1x10^3$ copies number/ml and $9x10^5$ copies number/ml.

In general, all patients HSV1 positive had high viral load in TS and in BAL samples (between 10^3 copies number/ml and 10^9 copies number/ml).

In the tables 9, 10 and 11 there are summarized the quantitative results expressed in copies number/ml for EBV, CMV and HSV1 virus respectively.

Table 9. Quantitative results expressed in copies number/ml for EBV DNA.

Patients	First sample		Second sample	
	TS	BAL	TS	BAL
1	$1x10^6$	9x10	n.a.	n.a.
2	$2x10^5$	neg	n.a.	n.a.
4	$5x10^6$	neg	n.a.	n.a.
6	$3x10^9$	$1x10^6$	n.a.	n.a.
7	$1x10^3$	n.q.	n.a.	n.a.
8	neg	$2x10^3$	n.a.	n.a.
9	$1x10^6$	neg	n.a.	n.a.

11	$3x10^5$	$3x10^3$	n.a.	n.a.
12	$1x10^5$	$3x10^3$	n.a.	n.a.
13	$3x10^3$	neg	n.a.	n.a.
15	$1x10^3$	neg	n.a.	n.a.
16	$4x10^3$	neg	n.a.	n.a.
17	$1x10^5$	neg	n.a.	n.a.
18	n.q.	neg	n.a.	n.a.
19	$3x10^3$	neg	n.a.	n.a.
20	$7x10^3$	neg	n.a.	n.a.
21	$2x10^3$	neg	$2x10^2$	neg
23	$1x10^2$	neg	$6x10^3$	$5x10^3$
24	neg	$1x10^3$	neg	$4x10^3$
26	$3x10^3$	neg	neg	neg
30	$1x10^5$	neg	neg	neg
32	neg	neg	$1x10^5$	$3x10^2$
33	neg	n.q.	neg	$1x10^3$
36	$1x10^3$	neg	$6x10^2$	neg
37	neg	$2x10^3$	neg	neg

n.a. not available

n.q. not quantifiable

In 14 patients, EBV DNA was demonstrated in the first sample only in TS, in 4 it was present in TS in higher amount than in BAL, whereas in 4 patients it was present in BAL only. In patients 21 and 36 the EBV DNA load in the TS in the second sample decreased of about 1 log in comparison with the first TS sample. In patients 24 the EBV DNA was present only in the BAL in both the first and second sample at the same level.

Table 10. Quantitative results expressed in copies number/ml for CMV DNA.

Patients	First sample		Second sample	
	TS	BAL	TS	BAL
1	$4x10^5$	$9x10^5$	n.a.	n.a.
2	$2x10^3$	$5x10^3$	n.a.	n.a.
4	$3x10^3$	$2x10^3$	n.a.	n.a.
6	$7x10^5$	$2x10^5$	n.a.	n.a.
10	neg	$8x10^3$	n.a.	n.a.
11	$9x10^5$	neg	n.a.	n.a.
21	neg	neg	$1x10^3$	neg
24	neg	neg	neg	$2x10^3$
26	$4x10^2$	neg	neg	neg
27	$6x10^3$	$2x10^4$	neg	neg
31	neg	$2x10^3$	$9x10^3$	8x10

32	$2x10^2$	neg	neg	n.q.
35	neg	$5x10^3$	neg	neg
36	neg	$4x10^3$	neg	neg
37	n.q.	neg	neg	$6x10^3$

As regards CMV DNA, in 4 cases it was shown in the TS only, in 4 cases it was present at the same level in both TS and BAL, whereas in 5 cases it was detectable in the BAL only. In patients 27 the CMV DNA load was slightly higher in BAL than in TS.

Table 11. Quantitative results expressed in copies number/ml for HSV1 DNA.

Patients	First sample		Second sample	
	TS	BAL	TS	BAL
3	$2x10^5$	neg	n.a.	n.a.
4	$4x10^7$	neg	n.a.	n.a.
5	neg	$1x10^3$	n.a.	n.a.
10	$3x10^6$	neg	n.a.	n.a.
14	$6x10^5$	$3x10^6$	n.a.	n.a.
20	$2x10^6$	neg	n.a.	n.a.
21	neg	neg	$4x10^5$	neg
22	neg	neg	neg	$4x10^6$
24	neg	$3x10^5$	neg	neg
25	neg	$2x10^6$	neg	neg
28	$8x10^5$	neg	$4x10^4$	neg
29	neg	neg	$4x10^6$	$5x10^6$
32	$2x10^8$	$1x10^4$	$1x10^7$	$1x10^8$
34	$2x10^3$	neg	$6x10^5$	$1x10^9$
35	$1x10^9$	$7x10^5$	$1x10^9$	$1x10^9$

As regards HSV1 DNA, in 6 cases it was shown in TS only, in 4 cases it was shown in BAL only.

In patients 14, 29, 32, 34 and 35 HSV1 DNA was detected both in TS and in BAL. In patient 29 only in the second sample HSV1 DNA was detected.

4.4. Development of a protocol to detect respiratory viruses by NGS (MiSeq, Illumina Platform)

This protocol was developed with the aim to analyse a total of 60 clinical samples from the upper and lower samples (from 30 ICU patients) previously analyzed with RT duplex real-time PCRs and with nested PCRs to detect herpesviruses. To develop the protocol 30 standards have been prepared with reference strains obtained from the National Institute for Biological Standards and Controls (NIBSC) or already available in the laboratory from upper and lower respiratory tract.

For influenza A virus, the following standards were prepared: for subtypes H1N1, H3N2, H5N1 and H7N7. Furthermore, one pool of DNA standards (Adv, CMV, HSV1) and one pool of RNA standards (Flu B, Flu A (H1N1), HMPV) were prepared.

4.5. Preliminary results using Nextera-XT protocol to MiSeq, Illumina Platform

Altogether Nextera-XT protocol to Miseq was applied to 24 clinical samples from 12 ICU patients, and to 6 reference strains obtained from the National Institute for Biological Standards and Controls (NIBSC) or already available in the laboratory.

Before to applied Nextera-XT protocol all clinical samples and all standards were amplified using 17 sets of primers and subsequently all amplicons of each sample were pooled.

Two experiments were performed:

First experiment: only one patient (two clinical samples) and 4 reference strains were analysed in this experiment. The reference strains were AdV, CMV, a pool with DNA viruses (AdV, CMV, HSV1) and a pool with RNA viruses (hMPV, influenza A (H1N1) and influenza B). The clinical samples

previously analysed with nested PCRs were EBV and CMV positive. The standard protocol of Nextera-XT suggests to perform the reaction without purifying the amplicon pools. In this experiment, Nextera-XT protocol was applied to amplicon pools with and without purification and moreover it was applied to amplicon pools frozen at -20°C and to fresh amplicon pools. The preliminary results obtained suggest that the quality of experiment is better for the fresh amplicon pools than amplicon pools frozen. There wasn't any difference in the quality between the fresh amplicon pools with and without purification, while the quality of experiment was improved with the purification of amplicon pools frozen. In the table 12 the results of reference strains AdV and CMV obtained with MiSeq sequencing after Nextera-XT protocol are reported. The fourth column reports the number of reads mapped in paired-end, this parameter shows the quality of experiments. The 1, 2 and 3 samples in the table indicate AdV frozen reference strain with purification, fresh AdV without purification and fresh AdV with purification respectively. The 4, 5 and 6 samples indicate CMV frozen reference strain with purification, fresh CMV without purification and fresh CMV with purification respectively.

Table 12. Results for reference strains AdV and CMV obtained with MiSeq sequencing.

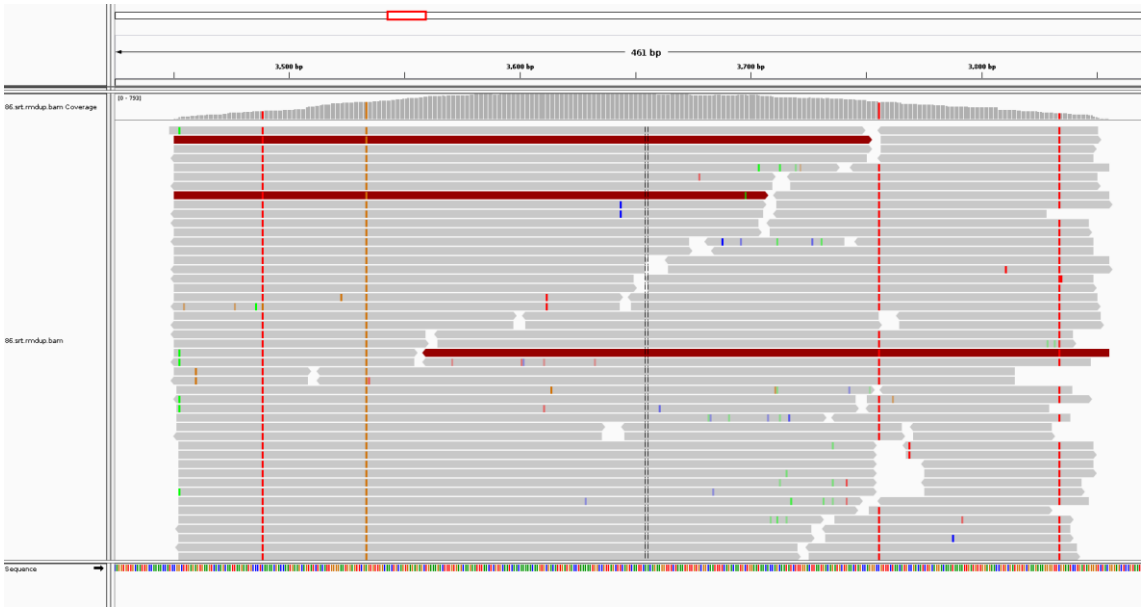
Sample	Total reads	Reads mapped	Reads mapped in paired-end
1	476376	313193	4358
2	318933	176536	15067
3	473754	243106	19336
4	67043	30009	29415
5	105344	92705	89617
6	108757	94406	92691

Second experiment: a total of 24 clinical samples from 12 patients were analysed. The analysed clinical samples included frozen amplicon pools with purification. The results obtained with MiSeq platform are in agreement with

results obtained with RT duplex real-time or nested PCRs. Nineteen clinical samples from 20 patients were negative for all viruses detected, only 5 clinical samples from 4 patients were positive for one or more viruses. The TS from 2 patients was positive for EBV and CMV DNA. The BAL of one of this patients was positive for CMV DNA. The BAL of another patient was positive for CMV DNA and the TS of another one was positive for EBV DNA.

To visualize the results of the date analysis, IGV (Integrative Genomic Viewer) software was used. The figure 5 shows an example of data generated in this study using IGV software. This software uses color and transparency to highlight interesting events in the data; positions with evident mismatches with respect to the reference are highlighted with color bars; doubtful base mismatches are displayed with transparency. An error in the reading is displayed with a colored line.

Figure 5. Visualization of generated data for hMPV using IGV software (reference strain AY145296.1).



5. Discussion

Respiratory viruses may cause many severe disease and contribute significantly to morbidity and mortality worldwide [1]. The aim of this study was a better knowledge of viral agents involved in respiratory infections in ICU patients.

In patients with predisposing conditions the outcome of these infections can be more severe, sometimes requiring hospitalization, even in intensive care units (ICUs), because of the development of pneumonia and acute respiratory distress syndrome (ARDS). For adult patients in the ICUs, respiratory viruses account for about 30% of pneumonia cases, with mortality rates comparable to those of bacterial pneumonia [14] and thus rapid diagnosis is essential for the management of these patients to this aim.

To achieve this goal, diagnostic assays able to accurately characterise respiratory viruses quickly and at the lowest costs were developed.

Four duplex RT real-time PCRs were developed. These assays were previously evaluate on 72 upper respiratory samples from children. The results of the duplex real-time PCRs were confirmed by sequencing positive samples and by comparison with other assays, including commercial, validated, assays, which gave similar results. The sensitivity of these duplex real-time PCRs is about 1 copy number/µl.

Then, a total of 156 clinical samples from upper and lower respiratory tract of 58 adult patients hospitalized in ICU were analysed. In 80% of positive adult patients influenza A viruses were detected, in 8% influenza B viruses and rhinovirus/enterovirus and in 4% metapneumoviruses. In particular in all ICU adult patients positive for Influenza A or B viruses, the virus was demonstrated in both upper or lower respiratory tract samples. For an adult patient positive for RV/EV, the virus was detected in both samples; while for another only the upper respiratory tract sample was positive. Only one upper respiratory tract sample from one patient was positive for hMPV.

The results obtained in this study were in agreement with other published studies that showed influenza virus as the most common virus detected in ICU patients, followed by rhinoviruses [18-20].

In ICU patients the detection of HSV1, EBV and CMV is common, but the significance of the detection of these viruses in respiratory secretions of critically ill patients is controversial. HSV and CMV are the most frequent herpesviruses detected in non-immunosuppressed ICU patients, requiring mechanical ventilation [171, 198].

In this study, three nested PCRs were developed to detect herpesviruses (CMV, EBV and HSV1). To understand better their role in ICU patients all herpesviruses positive clinical samples obtained by nested-PCRs were further analyzed by quantitative real-time PCRs.

Altogether, 37 of 58 (about 64%) patients were positive for one or more herpesviruses.

EBV was detected in 25 patients of 58, either as single or mixed infection; CMV and HSV1 were detected in 15 patients either as single or mixed infection. Mixed infections were not rare.

In general, for EBV viral load in TS samples was higher than in BAL samples. These results could suggest a possible viral contamination of the lower respiratory tract from mouth or throat or both.

The data published for this purpose reflect a high degree of variability concerning the prevalence of EBV in BAL samples from patients admitted in ICU [163, 208-210]. Lung et al. [211] detected EBV DNA in exfoliate cells in bronchial washing samples and concluded that the lower respiratory tract is a major reservoir for EBV. Friedrichs et al. [163] concluded that the detection of EBV DNA in BAL may be a marker of viral persistence more than a marker of active infection.

In this study CMV was detected as single infection only in two patients. In one of these, CMV was detected in upper ($6x10^3$ copies number/ml) and lower respiratory tract sample ($2x10^4$ copies number/ml) collected on the first day of hospitalization and not in the clinical samples collected after 5 days. The second patient showed CMV DNA in the first lower respiratory tract sample only ($2x10^3$ copies number/ml) whereas after 5 days it was detectable in both the upper ($9x10^3$ copies number/ml) and lower respiratory tract sample (8x10 copies number/ml).

Regarding CMV association with pneumonia, the average values of viral load reported in literature vary [205-207]; however a viral load in BAL samples,

between $4{,}6x10^4$ and $5x10^5$ copies number/ml, has been proposed as a threshold for the diagnosis of pneumonia [205, 206]. None of the patients analysed in this study had a viral load within this range only in BAL sample.

HSV1 was detected in 15 patients of 58 as single or mixed infection. Eight patients showed single HSV1 infection and 7 showed HSV1 in mixed infection. In general, all patients HSV1 positive had high viral load in TS and in BAL samples (avarage value 10^6).

It is not clear if HSV1 presence in lower respiratory tract samples of non-immunocompromised ventilated patients corresponds to viral contamination of the lower respiratory tract from mouth or throat or both, a local tracheobronchial excretion of the virus due to its reactivation without parenchymal involvement, or real HSV bronchopneumonitis. Several studies showed that there was a significant association between an HSV1 viral load >100,000 copies/ml of BAL and admission to the ICU ($p<0.0001$), mechanical ventilation ($p<0.001$) and death ($p<0.01$) [160, 168, 200, 201]. In our study 3 patients had an HSV1 DNA viral load in BAL > 100,000 copies/ml.

Altogether, it can be hypothesized that the presence of the viral sequences in the upper respiratory samples only is not indicative of a lower respiratory tract infection. The contemporary detection of viral genome sequences in both upper and lower respiratory samples is not always indicative of a lower respiratory tract infection. It could be indicative of a lower respiratory tract infection if the viral load is higher in the lower respiratory tract in comparison with the upper respiratory tract. The detection of viral genome sequences in lower respiratory samples only seems to suggest a lower respiratory tract infection. Analysing our results, 4 patients were EBV DNA positive in BAL only; the patient number 8 (table 9) had a viral load of $2x10^3$ in BAL sample collected the first day of hospitalization, a second sample was not available. The patient 24 (table 9) had a viral load of 10^3 in BAL sample collected the first day of hospitalization as well as in the second sample collected after 9 days of hospitalization; also the patient 37 (table 9) showed a viral load in BAL of $2x10^3$ collected the first day of hospitalization and not in the sample collected after few days. On these basis, EBV infection of the lower respiratory tract could be suspected mainly in patient 24.

As regards CMV DNA it was shown only in BAL in 5 cases concerning the patients 10, 24, 35, 36 and 37 (table 10) who showed a viral load of approximately 10^3. Moreover in 3 cases the positivity showed to be transient. Altogether in these cases the role of CMV infetction in the respiratory disease could not be demonstrated.

HSV1 DNA only in BAL was detected in patients 5, 22, 24 and 25 (table 11). The first sample collected from the patient 5 showed a viral load of $1x10^3$, a second sample was not available. The patients 24 and 25 showed a viral load of $3x10^5$ and $2x10^6$ in the samples collected the first day of hospitalization and not in the samples collected after few days, respectively. This transient positivity is not suggestive of an infection of the lower respiratory tract by HSV1.

It is important analyse the clinical samples collected at different times, in particular when the viral genome is detected in both upper and lower respiratory samples. In these cases, the analysis of the successive sample could help to understand better the role of viral infections in the lower respiratory tract diseases.

The assays described could be particularly useful to screen a large number of patients for epidemiological studies and to assess the prevalence in the lower and upper respiratory tract of ICU patients with, regarding CMV, EBV and HSV1, the ultimate goal to understand the clinical significance of this phenomenon.

The possible contribute of the use of the NGS to the knowledges of the viruses involved in upper and lower respiratory tract infections in ICU patients was also studied.

First the protocol was developed using standard controls at different concentration.

The results obtained with MiSeq platform were in agreement with those given by the traditional assays developed in this study. However it is not possible to do any conclusions because of the low number of samples examined.

Nextera-XT protocol to MiSeq platform has been used. To perform this experiment and data analysis at least about one week is required.

NGSs may have some hurdles, as cost-effectiveness, high-throughput formats for clinical settings, turnaround time, the requirement for investments in bioinformatics tools, databases, and data management, training of personnel, and the reporting and interpretation of guidelines upon the identification of viruses of which the clinical relevance is not clear. Furthermore, this approach is difficult to apply for diagnostic routine, nevertheless it might be applied to samples that remain negative with routine diagnostics and it could be useful for epidemiological studies.

To complete this study it might be useful to analyse a larger number of samples and design new sets of primers in regions less conserved of each virus in order to identify new possible viral variant or better characterize the virus present in the clinical samples.

In fact, an advantage of using a NGS approach to detect viruses in clinical specimens is that it can also be used to obtain information regarding the virus species and/or type of virus that was identified.

6. References

1. Williams B.G., Gouws E., Boschi-Pinto C., Bryce J., Dye C., Estimates of world wide distribution of child deaths from acute respiratory infections. Lancet Infect 431-432 Dis 2, 25–32 2002.

2. Mäkelä M.J., Puhakka T., Ruuskanen O., Leinonen M., Saikku P., Kimpimäki M., Blomqvist S., Hyypiä T., Arstila P., Viruses and bacteria in the etiology of the common cold. J Clin Microbiol 36:539-42 1998.

3. Dong J., Olano J.P., McBride J.W., Walker D.H., Emerging pathogens: challenges and successes of molecular diagnostics. Journal of Molecular Diagnostics, vol. 10,no. 3, pp. 185–197, 2008.

4. Carstens E.B., Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2009). Archives of Virology, vol. 155, no. 1, pp. 133–146, 2010.

5. van den Hoogen B.G., de Jong J.C., Groen J., Kuiken T., de G.R., Fouchier R.A., Osterhaus A.D., A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat. Med. **7**, 719-724, 2001.

6. Ksiazek T.G., Erdman D., Goldsmith C.S., Zaki S.R., Peret T., Emery S., Tong S., Urbani C., Comer J.A., Lim W., Rollin P.E., Dowell S.F., Ling A.E., Humphrey C.D., Shieh W.J., Guarner J., Paddock C.D., Rota P., Fields B., DeRisi J., Yang J.Y., Cox N., Hughes J.M., LeDuc J.W., Bellini W.J., Anderson L.J., SARS Working Group, A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. 348, 1953-1966, 2003.

7. Woo P.C., Lau S.K., Chu C.M., Chan K.H., Tsoi H.W., Huang Y., Wong B.H., Poon R.W., Cai J.J., Luk W.K., Poon L.L., Wong S.S., Guan Y., Peiris J.S., Yuen K.Y., Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J. Virol. 79, 884-895, 2005.

8. van der Hoek L., Sure K., Ihorst G., Stang A., Pyrc K., Jebbink M.F., Petersen G., Forster J., Berkhout B., Uberla K., Human coronavirus NL63 infection is associated with croup. Adv. Exp. Med. Biol. 581, 485-491, 2006.

9. Zaki A.M., van Boheemen S., Bestebroer T.M., Osterhaus A.D.M.E., Fouchier R.A.M., Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N. Engl. J. Med. 367, 1814-1820, 2012.

10. Allander T., Tammi M.T., Eriksson M., Bjerkner A., Tiveljung-Lindell A., Andersson B., Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc. Natl. Acad. Sci. USA. 102, 12891-12896, 2005.

11. Raoult D., La Scola B., Birtles R., The discovery and characterization of Mimivirus, the largest known virus and putative pneumonia agent. Clin Infect Dis 45: 95–102, 2007.

12. Sinaniotis C.A., Viral pneumonia in children: incidence and aetiology. Paediatric Respiratory Reviews, vol. 5, supplement 1, pp. S197–S200, 2004.

13. Perez C.M.B., Prevalence of viral pathogens among pediatric patients admitted for pneumonia in a local tertiary hospital. PIDSP Journal, vol. 13, no. 1, 2012.

14. Choi S.H., Hong S.B., Ko G.B., Lee Y., Park H.J., Park S.Y., Moon S.M., Cho O.H., Park K.H., Chong Y.P., Kim S.H., Huh J.W., Sung H., Do K.H., Lee S.O., Kim M.N., Jeong J.Y., Lim C.M., Kim Y.S., Woo J.H., Koh Y., Viral infection in patients with severe pneumonia requiring intensive care unit admission. The American Journal of Respiratory and Critical Care Medicine, vol. 186, no. 4, pp. 325–332, 2012.

15. Østby A.C., Gubbels S., Baake G., Nielsen L.P., Riedel C., Arpi M., Respiratory virology and microbiology in intensive care units: a prospective cohort study. APMIS 121: 1097–1108 2014.

16. Woodhead M., Welch C.A., Harrison D.A., Bellingan G., Ayres J.G., Community-acquired pneumonia on the intensive care unit: secondary analysis of 17 869 cases in the ICNARC Case Mix Programme Database. Crit Care. 10(Suppl 2): S1, 2006.

17. Wilson P.A., Ferguson J., Severe community acquired pneumonia: an Australian perspective. Inter Med J 35:699–705, 2005.

18. Cilloniz C., Ewig S., Ferrer M., Polverino E., Gabarrus A., de la Bellacasa J.P., Mensa J., Torres A., Community acquired polymicrobial pneumonia in the intensive care unit: aetiology and prognosis. Crit Care 15:R209, 2011

19. Cameron R.J., de Wit D., Welsh T.N., Ferguson J., Grissell T.V., Rye P.J., Virus infection in exacerbations of chronic obstructive pulmonary disease requiring ventilation. Intensive Care Med 32:1022–9, 2006.

20. Garbino J., Gerbase M.W., Wunderli W., Kolarova L., Nicod L.P., Rochat T., Kaiser L., Respiratory viruses and severe lower respiratory tract complications in hospitalized patients. Chest 125:1033–9, 2004.

21. Kowalinski E., Zubieta C., Wolkerstorfer A., Szolar O.H., Ruigrok R.W., Cusack S., Structural analysis of specific metal chelating inhibitor binding to the endonuclease domain of influenza pH1N1 (2009) polymerase. PLoS Pathog. 8, e1002831, 2012.

22. Zhu X., Yang H., Guo Z., Yu W., Carney P.J., Li Y., Chen L.M., Paulson J.C., Donis R.O., Tong S., Stevens J., Wilson I.A., Crystal structures of two subtype N10 neuraminidase-like proteins from bat influenza A viruses reveal a diverged putative active site. Proc Natl Acad Sci U.S.A. 109, 18903–18908, 2012.

23. Sun X., Shi Y., Lu X., He J., Gao F., Yan J., Qi J., Gao G.F., Bat-derived influenza hemagglutinin H17 does not bind canonical avian or human receptors and most likely uses a unique entry mechanism. Cell Rep 3, 769–778, 2013.

24. Zhu X., Yu W., McBride R., Li Y., Chen L.M., Donis R.O., Tong S., Paulson J.C., Wilson I.A., Hemagglutinin homologue from H17N10 bat influenza virus exhibits divergent receptor-binding and pH-dependent fusion activities. Proc Natl Acad Sci U.S.A. 110, 1458–1463, 2013.

25. Garcia-Sastre A., The neuraminidase of bat influenza viruses is not a neuraminidase. Proc Natl Acad Sci U.S.A. 109, 18635–18636, 2012.

26. Tong S., Zhu X., Li Y., Shi M., Zhang J., Bourgeois M., Yang H., Chen X., Recuenco S., Gomez J., Chen L.M., Johnson A., Tao Y., Dreyfus C., Yu W., McBride R., Carney P.J., Gilbert A.T., Chang J., Guo Z., Davis C.T., Paulson J.C., Stevens J., Rupprecht C.E., Holmes E.C., Wilson I.A., Donis R.O., New world bats harbor diverse influenza A viruses. PLoS Pathog. 9, e1003657, 2013.

27. Ying W., Yan W., Boris T., Yi S., George F. G., Bat-derived influenza-like viruses H17N10 and H18N11. Trends in Microbiology, Vol. 22, No. 4, 2014.

28. Suzuki Y., Suzuki T., Holland R.E. Jr, Chambers T.M., Kiso M., Ishida H., Kawaoka Y., Sialic acid species as a determinant of the host range of influenza A virus. J Virol 74:11825-11831, 2000.

29. Palese P., Tobita K., Ueda M., Compans R.W., Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology 61, 397–410 46, 1974.

30. Liu C., Eichelberger M.C., Compans R.W., Air G.M., Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J Virol 69, 1099–1106, 1995.

31. Rebecca J., Garten C., Todd Davis Colin A., Shu R., Lindstrom S., Balish A., Sessions W.M., Xu X., Skepner E., Deyde V., Okomo-Adhiambo M., Gubareva L., Barnes J., Smith C.B., Emery S.L., Hillman M.J., Rivailler P., Smagala J., de Graaf M., Burke D.F., Fouchier R.A.M., Pappas C., Alpuche-

Aranda C.M., López-Gatell H., Olivera H., López I., Myers C.A., Faix D., Blair P.J., Yu C., Keene K.M., Dotson P.D. Jr., Boxrud D., Sambol A.R., Abid S.H., St. George K., Bannerman T., Moore A.L., Stringer D.J., Blevins P., Demmler-Harrison G.J., Ginsberg M., Kriner P., Waterman S., Smole S., Guevara H.F., Belongia E.A., Clark P.A., Beatrice S.T., Donis R., Katz J., Finelli L., Bridges C.B., Shaw M., Jernigan D.B., Uyeki T.M., Smith D.J., Klimov A.I., Cox N.J.. Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H1N1) Influenza Viruses Circulating in Humans. Science 325, 197 DOI: 10.1126/science.1176225, 2009.

32. Kanegae Y., Sutiga S., Endo A., Ishide M., Senya S., Osake K., Nerome K., Oya A., Evolutionary pattern of the hemagglutinin gene of influenza B virus isolated in Japan: Co-circulating lineages in the same epidemic season. J Virol 64, 2860–2865, 1990.

33. Rota P.A., Wallis T.R., Harmon M.W., Rota J.S., Kendal A.P., Nerome K., Co-circulation of two distinct evolutionary lineages of influenza type B virus since 1983. Virology 175, 59–68, 1990.

34. Byarugaba D.K., Erima B., Millard M., Kibuuka H., Lukwago L., Bwogi J., Mimbe D., Mworozi E.A., Sharp B., Krauss S., Mworozi E.A., Sharp B., Krauss S., Webby R.J., Webster R.G., Martin S.K., Wabwire-Mangen F., Ducatez M.F., Genetic analysis of influenza B viruses isolated in Uganda during the 2009–2010 seasons. Virol J 10, 11. doi:10.1186/1743-422X-10-11, 2013.

35. World Health Organization. Recommended composition of influenza virus vaccines for use in the 2012–2013 northern hemisphere influenza season. Wkly. Epidemiol. Rec. 2012, 87, 83–94.

36. Al-Tawfiq J., Memish Z., Middle East respiratory syndrome coronavirus: epidemiology and disease control measures. Infection and Drug Resistance 7 281–287, 2014.

37. Reina J., Lopez-Causape C., Rojo-Molinero E., Rubio R., Clinico-epidemiological characteristics of acute respiratory infections caused by coronavirus OC43, NL63 and 229E. Rev Clin Esp Jun 2014.

38. Lau S.K., Woo P.C., Yip C.C., Tse H., Tsoi H.W., Cheng V.C., Lee P., Tang B.S., Cheung C.H., Lee R.A., So L.Y., Lau Y.L., Chan K.H., Yuen K.Y., Coronavirus HKU1 and other coronavirus infections in Hong Kong. J Clin Microbiol 44(6):2063-71, 2006.

39. Drosten C., Gunther S., Preiser W., van der Werf S., Brodt H.R., Becker S., Rabenau H., Panning M., Kolesnikova L., Fouchier R.A., Berger A., Burguière A.M., Cinatl J., Eickmann M., Escriou N., Grywna K., Kramme S., Manuguerra J.C., Müller S., Rickerts V., Stürmer M., Vieth S., Klenk H.D., Osterhaus A.D., Schmitz H., Doerr H.W., Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 15;348(20):1967-76, 2003.

40. Gralinski L., Baric R., Molecular Pathology of Emerging Coronavirus Infections. J Pathol doi: 10.1002/path.4454, 2014.

41. Li F., Structural analysis of major species barriers between humans and palm civets for severe acute respiratory syndrome coronavirus infections. J Virol. 2008 Jul;82(14):6984-91. doi: 10.1128/JVI.00442-08.

42. http://www.who.int/csr/don/2014_07_23_mers/en/).

43. http://www.promedmail.org/direct.php?id=20140912.2770252.

44. Hijawi B., Abdallat M., Sayaydeh A., Alqasrawi S., Haddadin A., Jaarour N., Alsheikh S., Alsanouri T., Novel coronavirus infections in Jordan, April 2012: epidemiological findings from a retrospective investigation. East Mediterr Health J 19 Suppl 1:S12–S18, 2013.

45. Assiri A., McGeer A., Perl T.M., Price C.S., Al Rabeeah A.A., Cummings D.A., Alabdullatif Z.N., Assad M., Almulhim A., Makhdoom H., Madani H., Alhakeem R., Al-Tawfiq J.A., Cotten M., Watson S.J., Kellam P., Zumla A.I., Memish Z.A., KSA MERS-CoV Investigation Team., Hospital outbreak of Middle East respiratory syndrome coronavirus. N Engl J Med 369(5):407–416, 2013.

46. World Health Organization. Middle East Respiratory Syndrome Coronavirus (MERS-CoV). Summary and Literature Update – as of 27 March 2014. Geneva: World Health Organization; 2014. Available from: http://www.who.int/csr/disease/coronavirus_infections/MERS_CoV_Update_27_March_2014.pdf?ua=1. Date accessed September 11, 2014.

47. Assiri A., Al-Tawfiq J.A., Al-Rabeeah A.A., Al-Rabiah F.A., Al-Hajjar S., Al-Barrak A., Flemban H., Al-Nassir W.N., Balkhy H.H., Al-Hakeem R.F., Makhdoom H.Q., Zumla A.I., Memish Z.A., Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. Lancet Infect Dis 13(9):752–761, 2013.

48. World Health Organization. Middle East respiratory syndrome coronavirus (MERS-CoV) – update. Geneva: World Health Organization; 2014. Available from: http://www.who.int/csr/don/2014_06_13_mers/en/. Date accessed July 16, 2014.

49. Bastien N., Anderson K., Hart L., Van Caeseele P., Brandt K., Milley D., Hatchette T., Weiss E.C., Li Y. Human coronavirus NL63 infection in Canada. J Infect Dis 191:503–506, 2005.

50. Debiaggi M., Canducci F., Ceresola E.R., Clementi M., The role of infections and coinfections with newly identified and emerging respiratory viruses in children. Virol J, 27;9:247. doi: 10.1186/1743-422X-9-247, 2012.

51. Jin Y., Zhang R.F., Xie Z.P., Yan K.L., Gao H.C., Song J.R., Yuan X.H., Cheng W.X., Hou Y.D., Duan Z.J., Newly identified respiratory viruses associated with acute lower respiratory tract infections in children in Lanzou, China, from 2006 to 2009. Clin Microbiol Infect 18:74–80, 2011.

52. Kuypers J., Martin E.T., Heugel J., Wright N., Morrow R., Englund J.A., Clinical disease in children associated with newly described coronavirus subtypes. Pediatrics 119:e70–e76, 2007.

53. Gaunt E.R., Hardie A., Claas E.C., Simmonds P., Templeton K.E., Epidemiology and clinical presentations of the four human coronaviruses 229E, HKU1, NL63, and OC43 detected over 3 years using a novel multiplex real-time PCR method. J Clin Microbiol 48:2940–2947, 2010.

54. Oberste M.S., Maher K., Kilpatrick D.R., Flemister M.R., Brown B.A., Pallansch M.A., Typing of human enteroviruses by partial sequencing of VP1. J Clin Microbiol 37: 1288-1293, 1999.

55. Oberste M.S., Maher K., Kilpatrick D.R., Pallansch M.A. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol 73: 1941-1948, 1999.

56. Tapparel C., Junier T., Gerlach D., Van-Belle S., Turin L., Cordey S., Muhlemann K., Regamey N., Aubert J.D., Soccal P.M., Eigenmann P., Zdobnov E., Kaiser L., New respiratory enterovirus and recombinant rhinoviruses among circulating picornaviruses. Emerg Infect Dis 15:719–726, 2009.

57. Piralla A., Rovida F., Baldanti F., Gerna G., Enterovirus genotype EV-104 in humans, Italy, 2008–2009. Emerg Infect Dis 16:1018–1021, 2010.

58. Yozwiak N., Skewes-Cox P., Gordon A., Saborio S., Kuan G., Balmaseda A., Ganem D., Harris E., DeRisi J.L., Human Enterovirus 109: a novel

interspecies recombinant enterovirus isolated from a case of acute pediatric respiratory illness in Nicaragua. J Virol 84: 9047-9058, 2010.

59. Lukashev A.N., Drexler J.F., Kotova V.O., Amjaga E.N., Reznik V.I., Gmyl A.P., Grard G., Taty R., Trotsenko O.E., Leroy E.M., Drosten C., Novel serotypes 105 and 116 are members of distinct subgroups of Human enterovirus C. J Gen Virol 93: 2357-2362, 2012.

60. Tokarz R., Hirschberg D.L., Sameroff S., Haq S., Luna G., Bennett A.J., Silva M., Leguia M., Kasper M., Bausch D.G., Lipkin W.I., Genomic analysis of two novel human enterovirus C genotypes found in respiratory samples from Peru. J Gen Virol 120-7. doi: 10.1099/vir.0.046250-0, 2012.

61. Daleno C., Piralla A., Scala A., Baldanti F., Usonis V., Principi N., Esposito S., Complete genome sequence of a novel human enterovirus C (HEV-C117) identified in a child with community-acquired pneumonia. J Virol 86: 10888-10889, 2012.

62. Daleno C., Piralla A., Usonis V., Scala A., Ivaskevicius R., Baldanti F., Principi N., Esposito S., Novel human Enterovirus C infection in child with community-acquired pneumonia. Emerg Infect Dis 18: 1913-1915, 2012.

63. Daleno C., Greenberg D., Piralla A., Scala A., Baldanti F., Principi N., Esposito S., A novel human enterovirus C (EV-C118) indentified in two children hospitalised because of acute otitis media and community-acquired pneumonia in Israel. J Clin Virol 56: 159-163, 2013.

64. Daleno C., Piralla A., Scala A., Baldanti F., Greenberg D., Principi N., Esposito S., CAP-PRI Study Group, Full genome sequence of a novel human enterovirus C (HEV-C118) isolated from two children with acute otitis media and community-acquired pneumonia in Israel. Genome Announc doi:pii: e00121-12, 2013.

65. Miller E.K., Edwards K.M., Weinberg G.A., Iwane M.K., Griffin M.R., Hall C.B., Zhu Y., Szilagyi P.G., Morin L.L., Heil L.H., Lu X., Williams J.V., New Vaccine Surveillance Network, A novel group of rhinoviruses is associated with asthma hospitalizations. J Allergy Clin Immunol, 123:98–104, 2009.

66. Bochkov Y.A., Gern J.E., Clinical and molecular features of human rhinovirus C. Microbes Infect 14:485–494, 2012.

67. Lau S.K., Yip C.C., Tsoi H.W., Lee R.A., So L.Y., Lau Y.L., Chan K.H., Woo P.C., Yuen K.Y., Clinical features and complete genome characterization of a distinct human rhinovirus (HRV) genetic cluster, probably representing a previously undetected HRV species, HRV-C, associated with acute respiratory illness in children. J. Clin. Microbiol. 45:3655–3664, 2007.

68. Piralla A., Rovida F., Campanini G., Rognoni V., Marchi A., Locatelli F., Gerna G.,Clinical severity and molecular typing of human rhinovirus C strains during a fall outbreak affecting hospitalized patients. J. Clin. Virol. 45:311–317, 2009.

69. Linsuwanon P., Payungporn S., Samransamruajkit R., Posuwan N., Makkoch J., Theanboonlers A., Poovorawan Y., High prevalence of human rhinovirus C infection in Thai children with acute lower respiratory tract disease. J Infect 59, 115–121. doi: 10.1016/j.jinf.2009.05.009, 2009.

70. Wisdom A., Kutkowska A.E., McWilliam Leitch E.C., Gaunt E., Templeton K., Harvala H., Simmonds P., Genetics, recombination and clinical features of human rhinovirus species C (HRV-C) infections; interactions of HRV-C with other respiratory viruses. PLoS ONE 4:e8518. doi: 10.1371/journal.pone.0008518, 2009.

71. Smuts H.E., Workman L.J., Zar H.J., Human rhinovirus infection in young African children with acute wheezing. BMC Infect. Dis. 11:65. doi:10.1186/1471-2334- 11-65, 2011.

72. Mosser A.G., Vrtis R., Burchell L., Lee W.M., Dick C.R., Weisshaar E., Bock D., Swenson C.A., Cornwell R.D., Meyer K.C., Jarjour N.N., Busse W.W., Gern J.E., Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues. Am J Respir Crit Care Med 171, 645–651.doi: 10.1164/rccm.200407-970OC, 2005.

73. Greenberg S.B., Viral respiratory infections in elderly patients and patients with chronic obstructive pulmonary disease. Am J Med, 112(Suppl 6A):28S–32S, 2002 Review.

74. Goka E.A., Vallelj P.J., Mutton K.J., Klapper P.E., Single, dual and multiple respiratory virus infections and risk of hospitalization and mortality. Epidemiol. Infect., doi:10.1017/S0950268814000302, 2014.

75. Linsuwanon P., Payungporn S., Samransamruajkit R., Theamboonlers A., Poovorawan Y., Recurrent human rhinovirus infections in infants with refractory wheezing. Emerg Infect Dis 15(6):978–980, 2009.

76. Franz A., Adams O., Willems R., Bonzel L., Neuhausen N., Schweizer-Krantz S., Ruggeberg J.U., Willers R., Henrich B., Schroten H., Tenenbaum T., Correlation of viral load of respiratory pathogens and co-infections with disease severity in children hospitalized for lower respiratory tract infection. J Clin Virol 48(4):239– 245, 2010.

77. Marguet C., Lubrano M., Gueudin M., Le Roux P., Deschildre A., Forget C., Couderc L., Siret D., Donnou M.D., Bubenheim M., Vabret A., Freymuth F., In very young infants severity of acute bronchiolitis depends on carried viruses. PLoS ONE 4(2):e4596, 2009.

78. Gern JE., The ABCs of rhinoviruses, wheezing, and asthma. J Virol 84(15):7418–7426, 2010.

79. Piyaratna R., Tollefson S.J., Williams J.V., Genomic analysis of four human metapneumovirus prototypes. Virus Res 160:200–5, 2011

80. Boivin G., Abed Y., Pelletier G., Ruel L., Moisan D., Cote S., Peret T.C., Erdman D.D., Anderson L.J., Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-tract infections in all age groups. J Infect Dis 186:1330–4, 2002.

81. Williams J.V., Martino R., Rabella N., Otegui M., Parody R., Heck J.M., Crowe J.E. Jr., A prospective study comparing human metapneumovirus with other respiratory viruses in adults with hematologic malignancies and respiratory tract infections. J Infect Dis 192:1061–5, 2005.

82. Dokos C., Masjosthusmann K., Rellensmann G., Werner C., Schuler L.S., Muller K.M., Schiborr M., Ehlert K., Groll A.H., Fatal human metapneumovirus infection following allogeneic hemato-poietic stem cell transplantation. Transpl Infect Dis 15:97–101, 2013.

83 Papenburg J., Carbonneau J., Isabel S., Bergeron M.G., Williams J.V., De Serres G., Hamelin M.È., Boivin G., Genetic diversity and molecular evolution of the major human metap-neumovirus surface glycoproteins over a decade. J Clin Virol 58:541–7, 2013.

84. Semple M.G., Cowell A., Dove W., Greensill J., McNamara P.S., Halfhide C., Shears P., Smyth R.L., Hart C.A., Dual infection of infants by human metapneumovirus and human respiratory syncytial virus is strongly associated with severe bronchiolitis. J Infect Dis 191:382–6, 2005.

85. Foulongne V., Guyon G., Rodie`re M., Segondy M., Human metapneumovirus infection in young children hospitalized with respiratory tract disease. Pediatr Infect Dis J 25:354–9, 2006.

86. Van Woensel J.B., Bos A.P., Lutter R., Rossen J.W., Schuurman R., Absence of human metapneumovirus co-infection in cases of severe respiratory syncytial virus infection. Pediatr Pulmonol 41:872–4, 2006.

87. Lazar I., Weibel C., Dziura J., Ferguson D., Landry M.L., Kahn J.S., Human metapneumovirus and severity of respiratory syncytial virus disease. Emerg Infect Dis 10:1318–20, 2004.

88. Cane P.A., Molecular epidemiology of respiratory syncytial virus. Rev Med Virol 11: 103–116, 2001.

89. Hendry R.M., Godfrey E., Anderson L.J., Fernie B.F., McIntosh K. Quantification of respiratory syncytial virus polypeptides in nasal secretions by monoclonal antibodies. J Gen Virol 66 (Pt8): 1705–1714, 1985.

90. Tsutsumi H., Onuma M., Suga K., Honjo T., Chiba Y., Chiba S., Ogra PL., Occurrence of respiratory syncytial virus subgroup A and B strains in Japan, 1980 to 1987. J Clin Microbiol 26: 1171–1174, 1988.

91. Mufson M.A., Orvell C., Rafnar B., Norrby E., Two distinct subtypes of human respiratory syncytial virus. J Gen Virol 66 (Pt10): 2111–2124, 1985.

92. Sullender W.M., Respiratory syncytial virus genetic and antigenic diversity. Clin Microbiol Rev 13: 1–15, table of contents, 2000.

93. Jones L.P., Zheng H.Q., Karron R.A., Peret T.C., Tsou C., Anderson L.J., Multiplex assay for detection of strain-specific antibodies against the two variable regions of the G protein of respiratory syncytial virus. Clin Diagn Lab Immunol 9: 633–638, 2002.

94. Levine D.A., Platt S.L., Dayan P.S., Macias C.G., Zorc J.J., Krief W., Schor J., Bank D., Fefferman N., Shaw K.N., Kuppermann N., Multicenter RSV-SBI Study Group of the Pediatric Emergency Medicine Collaborative Research Committee of the American Academy of Pediatrics, Risk of serious bacterial infection in young febrile infants with respiratory syncytial virus infections. Pediatrics 113: 1728–1734, 2004.

95. Parveen S., Sullender W.M., Fowler K., Lefkowitz E.J., Kapoor S.K., Broor S., Genetic variability in the G protein gene of group A and B respiratory syncytial viruses from India. J. Clin.Microbiol. 44, 3055–3064.doi:10.1128/JCM.00 187-06, 2006.

96. Tran D.N., Pham T.M., Ha M.T., Tran T.T., Dang T.K., Yoshida L. M., Okitsu S., Hayakawa S., Mizuguchi M., Ushijima H., Molecular epidemiology and disease severity of human respiratory syncytial virus in Vietnam. PLoS ONE 8:e45436. doi: 10.1371/journal.pone.0045436, 2013.

97. Rueda P., Delgado T., Portela A., Melero J.A., García-Barreno B., Premature stop codons in the G glycoprotein of human respiratory syncytial viruses resistant to neutralization by monoclonal antibodies. J Virol 65, 3374–3378, 1991.

98. Johnson P.R., Jr., Olmsted R.A., Prince G.A., Murphy B.R., Alling D.W., Walsh E.E., Collins P.L., Antigenic relatedness between glycoproteins of human respiratory syncytial virus subgroups A and B: evaluation of the contributions of F and G glycoproteins to immunity. J Virol 61: 3163–3166, 1987.

99. Brandenburg A.H., van Beek R., Moll H.A., Osterhaus A.D., Claas E.C., G protein variation in respiratory syncytial virus group A does not correlate with clinical severity. J Clin Microbiol 38: 3849–3852, 2000.

100. Kneyber M.C., Brandenburg A.H., Rothbarth P.H., de Groot R., Ott A., de Groot R., Ott A., van Steensel-Moll HA., Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch Dis Child 75: 137–140, 1996.

101. Nokes D.J., Ngama M., Bett A., Abwao J., Munywoki P., English M., Scott J.A., Cane P.A., Medley G.F., Incidence and severity of respiratory syncytial virus pneumonia in rural Kenyan children identified through hospital surveillance. Clin Infect Dis 49(9):1341–1349, 2009.

102. Turner P., Turner C., Watthanaworawit W., Carrara V., Cicelia N., Deglise C., Phares C., Ortega L., Nosten F.. Respiratory virus surveillance in hospitalised pneumonia patients on the Thailand-Myanmar border. BMC Infect Dis 16;13:434. doi: 10.1186/1471-2334-13-434, 2013.

103. Zou L.R., Zhou J., Li H., Mo Y.L., Chen Q.X., Fang L., Wu J., Wu D., Huang P., Ke C.W., Etiology survey on virus of acute respiratory infection in Guangzhou from 2006 to 2009. Chinese Journal of Prevention Medicine 45(9): 825–829, 2011.

104. Zhao B., Shen J., Gao Y., Yu X., Zhang X., Wu F., Detection for respiratory viruses in Shanghai with multiplex PCR from 2009 to 2010. Journal of Hygiene Research 40(5): 635–637, 2011.

105. Frost H.M., Robinson C.C., Dominguez S.R., Epidemiology and Clinical Presentation of Parainfluenza Type 4 in Children: A 3-Year Comparative Study to Parainfluenza Types 1–3, J Infect Dis 209:695–702, 2013.

106. Ren L., Gonzalez R., Xie Z., Xiong Z., Liu C., Xiang Z., Xiao Y., Li Y., Zhou H., Li J., Yang Q., Zhang J., Chen L., Wang W., Vernet G., Paranhos-Baccalà G., Shen K., Wang J., Human parainfluenza virus type 4 infection in Chinese children with lower respiratory tract infections: a comparison study. J Clin Virol 51:209–212, 2011.

107. Aguilar J.C., Pérez-Breña M.P., García M.L., Cruz N., Erdman D.D., Echevarría J.E. Detection and identification of human parainfluenza viruses 1, 2, 3, and 4 in clinical samples of pediatric patients by multiplex reverse transcription-PCR. J Clin Microbiol 38:1191–1195, 2000.

108. Chiu C.Y., Rouskin S., Koshy A., Urisman A., Fischer K., Yagi S., Schnurr D., Eckburg P.B., Tompkins L.S., Blackburn B.G., Merker J.D., Patterson B.K., Ganem D., DeRisi J.L., Microarray detection of human parainfluenzavirus 4 infection associated with respiratory failure in an immunocompetent adult. Clin Infect Dis 43:e71–e76, 2006.

109. Lau S.K.P., To W., Tse P.W.T., Chan A.K.H., Woo P.C.Y., Tsoi H., Leung A.F.Y., Li K.S.M., Chan P.K.S., Lim W.W.L., Yung R.W.H., Chan K., Yuen K., Icrobiol J., Human Parainfluenza Virus 4 Outbreak and the Role of Diagnostic Tests. J Clin Microbiol 43:4515–4521, 2005.

110. Reed G., Jewett P.H., Thompson J., Tollefson S., Wright P.F., Epidemiology and clinical impact of parainfluenza virus infections in otherwise healthy infants and young children <5 years old. J Infect Dis 175, 807–813. doi:10.1086/513975, 1997.

111. Azevedo A.M., Durigon E.L., Okasima V., Queiroz D.A., de Moraes-Vasconcelos D., Duarte A.J., Grumach A.S., Detection of influenza, parainfluenza, adenovirus and respiratory syncytial virus during asthma attacks in children older than 2 years old. Allergol Immunopathol 31, 311–317, 2003.

112. Matsuse H., Kondo Y., Saeki S., Nakata H., Fukushima C., Mizuta Y., Kohno S., Naturally occurring parainfluenza virus 3 infection in adults induces mild exacerbation of asthma associated with increased sputum concentrations of cysteinyl leukotrienes. Int Arch Allergy Immunol 138, 267–272.doi: 10.1159/000088728, 2005.

113. Knott A.M., Long C.E., Hall C.B., Parainfluenza viral infections in pediatric outpatients: seasonal patterns and clinical characteristics. Pediatr Infect Dis J 13, 269–273.doi:10.1097/00006454- 199404000-00005, 1994.

114. Counihan M.E., Shay D.K., Holman R.C., Lowther S.A., Anderson, L.J., Human parainfluenza virus-associated hospitalizations among children less than five years of age in the United States. Pediatr Infect Dis J 20, 646–653.doi: 10.1097/00006454-200107000-00003, 2001.

115. Hall C.B., Respiratory syncytial virus and parainfluenza virus. N Engl J Med 344, 1917–1928.doi: 10.1056/NEJM200106213442507, 2001.

116. Mizuta K., Abiko C., Aoki Y., Ikeda T., Matsuzaki Y., Itagaki T., Katsushima F., Katsushima Y., Noda M., Kimura H., Ahiko T., Seasonal patterns of respiratory syncytial virus, influenza A virus, human metapneumovirus, and parainfluenza virus type 3 infections on the basis of virus isolation data between 2004 and 2011 in Yamagata, Japan. Jpn J Infect Dis 66, 140–145. doi: 10.7883/yoken.66.140, 2013.

117. Liu W.K., Liu Q., Chen D.H., Liang H.X., Chen X.K., Huang W.B., Qin S., Yang Z.F., Zhou R., Epidemiology and clinical presentation of the four human parainfluenza virus types. BMC Infect.Dis. 13:28. doi: 10.1186/1471-2334-13-28, 2013.

118. Arena C., Amoros J.P., Vaillant V., Balay K., Chikhi-Brachet R., Varesi L., Arrighi J., Blanchon T., Carrat F., Hanslik T., Falchi A., Simultaneous investigation of influenza and enteric viruses in the stools of adult patients consulting in general practice for acute diarrhea. Virol J 18;9:116. doi: 10.1186/1743-422X-9-116, 2012.

119. Cesario TC., Viruses associated with pneumonia in adults. Clin Infect Dis 55(1):107-13. doi: 10.1093/cid/cis297. Review, 2012.

120. Gu L., Liu Z., Li X., Qu J., Guan W., Liu Y., Song S., Yu X., Cao B., Severe community-acquired pneumonia caused by adenovirus type 11 in immunocompetent adults in Beijing. J Clin Virol 54(4):295-301. doi: 10.1016/j.jcv.2012.04.018, 2012.

121. Tate J.E., Bunning M.L., Lott L., Lu X., Su J., Metzgar D., Brosch L., Panozzo C.A., Marconi V.C., Faix D.J., Prill M., Johnson B., Erdman D.D., Fonseca V., Anderson L.J., Widdowson M.A., Outbreak of severe respiratory disease associated with emergent human adenovirus serotype 14 at a US air force training facility in 2007. J Infect Dis 15 199(10):1419-26. doi: 10.1086/598520, 2009.

122. Kajon A.E., Lu X., Erdman D.D., Louie J., Schnurr D., George K.S., Koopmans M.P., Allibhai T., Metzgar D., Molecular epidemiology and brief history of emerging adenovirus 14-associated respiratory disease in the United States. J Infect Dis 202(1):93-103. doi: 10.1086/653083, 2010.

123. Arthur J.L., Higgins G.D., Davidson G.P., Givney R.C., Ratcliff R.M., A novel bocavirus associated with acute gastroenteritis in Australian children. PLoS Pathog 5(4):e1000391. doi: 10.1371/journal.ppat.1000391, 2009.

124. Kapoor A., Slikas E., Simmonds P., Chieochansin T., Naeem A., Shaukat S., Alam M.M., Sharif S., Angez M., Zaidi S., Delwart E., A newly identified bocavirus species in human stool. J Infect Dis 15;199(2):196-200. doi: 10.1086/595831, 2009.

125. Regamey N., Frey U., Deffernez C., Latzin P., Kaiser L., Swiss Paediatric Respiratory Research Group, Isolation of human bocavirus from Swiss infants with respiratory infections. Pediatric Infectious Disease Journal 26: 177–179, 2007.

126. Schildgen O., Müller A., Allander T., Mackay I.M., Völz S., Kupfer B., Simon A., Human bocavirus: passenger or pathogen in acute respiratory tract infections? Clinical Microbiology Reviews 21: 291–304, 2008.

127. Yu J.M., Li D.D., Xu Z.Q., Cheng W.X., Zhang Q., Li H.Y., Cui S.X., Miao-Jin, Yang S.H., Fang Z.Y., Duan Z.J., Human bocavirus infection in children hospitalized with acute gastroenteritis in China. Journal of Clinical Virology 42: 280–285, 2008.

128. Cheng W.X., Jin Y., Duan Z.J., Xu Z.Q., Qi H.M., Zhang Q., Yu J.M., Zhu L., Jin M., Liu N., Cui S.X., Li H.Y., Fang Z.Y., Human bocavirus in children hospitalized for acute gastroenteritis: a case-control study. Clinical Infectious Diseases 47: 161–167, 2008.

129. von Linstow M.L., Høgh M., Høgh B., Clinical and epidemiologic characteristics of human bocavirus in Danish infants: results from a prospective birth cohort study. Pediatric Infectious Disease Journal 27: 897–902, 2008.

130. Campe H., Hartberger C., Sing A., Role of human bocavirus infections in outbreaks of gastroenteritis. Journal of Clinical Virology 43: 340–342, 2008.

131. Blessing K., Neske F., Herre U., Kreth H.W., Weissbrich B., Prolonged detection of human bocavirus DNA in nasopharyngeal aspirates of children with respiratory tract disease. Pediatric Infectious Disease Journal 28: 1018–1019, 2009.

132. Martin E.T., Fairchok M.P., Kuypers J., Magaret A., Zerr D.M., Wald A., Englund J.A., Frequent and prolonged shedding of bocavirus in young children attending daycare. Journal of Infectious Diseases 201: 1625–1632, 2010.

133. Lüsebrink J., Schildgen V., Tillmann R.L., Wittleben F., Böhmer A., Müller A., Schildgen O., Detection of head-to-tail DNA sequences of human bocavirus in clinical samples. PLoS One 6: e19457, 2011.

134. Christensen A., Nordbø S.A., Krokstad S., Rognlien A.G., Døllner H., Human bocavirus in children: mono-detection, high viral load and viraemia are associated with respiratory tract infection. Journal of Clinical Virology 49: 158–162, 2010.

135. Söderlund-Venermo M., Lahtinen A., Jartti T., Hedman L., Kemppainen K., Lehtinen P., Allander T., Ruuskanen O., Hedman K., Clinical assessment and improved diagnosis of bocavirus-induced wheezing in children, Finland. Emerging Infectious Diseases 15: 1423–1430, 2009.

136. Brieu N., Gay B., Segondy M., Foulongne V., Electron microscopy observation of human bocavirus (HBoV) in nasopharyngeal samples from HBoV infected children. Journal of Clinical Microbiology 45: 3419– 3420, 2007.

137. Gurda B.L., Parent K.N., Bladek H., Sinkovits R.S., DiMattia M.A., Rence C., Castro A., McKenna R., Olson N., Brown K., Baker T.S., Agbandje-McKenna M., Human bocavirus capsid structure: insights into the structural repertoire of the Parvoviridae. Journal of Virology 84: 5880–5889, 2010.

138. Dijkman R., Koekkoek S.M., Molenkamp R., Schildgen O., van der Hoek L. Human bocavirus can be cultured in differentiated human airway epithelial cells. Journal of Virology 83: 7739–7748, 2009.

139. Cecchini S., Negrete A., Virag T., Graham B.S., Cohen J.I., Kotin R.M., Evidence of prior exposure to human bocavirus as determined by a retrospective serological study of 404 serum samples from adults in the United States. Clinical and Vaccine Immunology 16: 597–604, 2009.

140. Chen A.Y., Cheng F., Lou S., Luo Y., Liu Z., Delwart E., Pintel D., Qiu J., Characterization of the gene expression profile of human bocavirus. Virology 403: 145–154, 2010.

141. Fry A.M., Lu X., Chittaganpitch M., Peret T., Fischer J., Dowell S.F., Anderson L.J., Erdman D., Olsen S.J., Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J Infect Dis 195:1038–1045, 2007.

142. Hustedt J.W., Christie C., Hustedt M.M., Esposito D., Vazquez M., Seroepidemiology of human bocavirus infection in Jamaica. PLoS One7(5):e38206. doi: 10.1371/journal.pone.0038206, 2012.

143. Kesebir D., Vazquez M., Weibel C., Shapiro E.D., Ferguson D., Landry M.L., Kahn J.S., Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis 194:1276–1282, 2006.

144. Maggi F., Andreoli E., Pifferi M., Meschi S., Rocchi J., Bendinelli M., Human bocavirus in Italian patients with respiratory diseases. J Clin Virol 38:321–325, 2007.

145. Allander T., Jartti T., Gupta S., Niesters H.G., Lehtinen P., Osterback R., Vuorinen T., Waris M., Bjerkner A., Tiveljung-Lindell A., van den Hoogen B.G., Hyypiä T., Ruuskanen O., Human bocavirus and acute wheezing in children. Clin Infect Dis 44:904–910, 2007.

146. Ghietto L.M., Camara A., Zhou Y., Pedranti M., Ferreyra S., Frey T., Camara J., Adamo M.P., High prevalence of human bocavirus 1 in infants with lower acute respiratory tract disease in Argentina, 2007–2009. Braz J Infect Dis 16:38–44, 2012.

147. Christensen A., Nordbo S.A., Krokstad S., Rognlien A.G., Dollner H., Human bocavirus commonly involved in multiple viral airway infections. J Clin Virol 41:34–37, 2008.

148. Hedman L., Soderlund-Venermo M., Jartti T., Ruuskanen O., Hedman K. Dating of human bocavirus infection with protein-denaturing IgG-avidity assays-Secondary immune activations are ubiquitous in immunocompetent adults. J Clin Virol 48:44–48, 2010.

149. Wang K., Wang W., Yan H., Ren P., Zhang J., Shen J., Deubel V. Correlation between bocavirus infection and humoral response, and co-infection with other respiratory viruses in children with acute respiratory infection. J Clin Virol 47:148–155, 2010.

150. Bastien N., Brandt K., Dust K., Ward D., Li Y. Human bocavirus infection, Canada. Emerg Infect Dis 12, 848-850, 2006.

151. Gerna G., Piralla A., Campanini G., Marchi A., Stronati M., Rovida F., The human bocavirus role in acute respiratory tract infections of pediatric

patients as defined by viral load quantification. New Microbiol 30:383–392, 2007.

152. Dina J., Nguyen E., Gouarin S., Petitjean J., Parienti J.J., Nimal D., Brouard J., Freymuth F., Vabret A., Development of duplex real-time PCR for detection of two DNA respiratory viruses. J Virol Methods 162:119–125, 2009.

153. Ren L., Gonzalez R., Wang Z., Xiang Z., Wang Y., Zhou H., Li J., Xiao Y., Yang Q., Zhang J., Chen L., Wang W., Li Y., Li T., Meng X., Zhang Y., Vernet G., Paranhos-Baccalà G., Chen J., Jin Q., Wang J., Prevalence of human respiratory viruses in adults with acute respiratory tract infections in Beijing, 2005—2007. Clinical Microbiology and Infection, vol. 15, no. 12, pp. 1146–1153, 2009.

154. Yu H., Cauchemez S., Donnelly C.A., Zhou L., Feng L., Xiang N., Zheng J., Ye M., Huai Y., Liao Q., Peng Z., Feng Y., Jiang H., Yang W., Wang Y., Ferguson N.M., Feng Z., Transmission dynamics, border entry screening, and school holidays during the 2009 influenza A (H1N1) pandemic, China. Emerging Infectious Diseases, vol. 18, no. 5, pp. 758–766, 2012.

155. Manoha C., Espinosa S., Aho S.L., Huet F., Pothier P., Epidemiological and clinical features of hMPV, RSV and RVs infections in young children. Journal of Clinical Virology, vol. 38, no. 3, pp. 221–226, 2007.

156. Preiser W., Doer H.W., Vogel J.U., Virology and epidemiology of oral herpesvirus infections. Med Microbiol Immunol 192:133–136, 2003.

157. Ogata M., Satou T., Kawano R., Yoshikawa T., Ikewaki J., Kohno K., Ando T., Miyazaki Y., Ohtsuka E., Saburi Y., Kikuchi H., Saikawa T., Kadota J., High incidence of cytomegalovirus, human herpesvirus-6, and Epstein-Barr virus reactivation in patients receiving cytotoxic chemotherapy for adult T cell leukemia. J Med Virol 83:702–709. doi:10.1002/jmv. 22013, 2011.

158. Ljungman P., Molecular monitoring of viral infections after hematopoietic stem cell transplantation. Int J Hematol 91:596–601. doi:10.1007/s12185-010-0570-4, 2010.

159. Vogel J.U., Otte J., Koch F., Gümbel H., Doerr H.W,, Cinatl J. Jr., Role of human cytomegalovirus genotype polymorphisms in AIDS patients with cytomegalovirus retinitis. Med Microbiol Immunol 202(1):37–47. doi: 10.1007/s00430-012-0244-3, 2013.

160. De Vos N., Van Hoovels L., Vankeerberghen A., Van Vaerenbergh K., Boel A., Demeyer I., Creemers L., De Beenhouwer H., Monitoring of herpes simplex virus in the lower respiratory tract of critically ill patients using real-time PCR: a prospective study. Clin Microbiol Infect 15: 358–363, 2009.

161. Chiche L., Forel J.M., Roch A., Guervilly C., Pauly V., Allardet-Servent J., Gainnier M., Zandotti C., Papazian L., Active cytomegalovirus infection is common in mechanically ventilated medical intensive care unit patients. Crit Care Med 37: 1850–1857, 2009.

162. Limaye A.P., Boeckh M., CMV in critically ill patients: pathogen or bystander? Rev Med Virol 20: 372–379, 2010.

163. Friedrichs I., Bingold T., Keppler O.T., Pullmann B., Reinheimer C., Berger A., Detection of herpesvirus EBV DNA in the lower respiratory tract of ICU patients: a marker of infection of the lower respiratory tract? Med Microbiol Immunol 202:431–436, 2013.

164. Whitley R.J., Roitzman B., Richman D.D., Whitley R.J., Hayden F.G., Herpes simplex viruses in Clinical virology. In Fields Virology 2nd. Washington, DC: ASM Press 375–401, 2002.

165. Sheldrick P., Berthelot N., Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harb Symp Quant Biol 39:667-78, 1975.

166. Wadsworth S., Jacob R.J., Roizman B., Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitions. J Virol 15:1487-97, 1975.

167. Bruynseels P., Jorens P.G., Demey H.E., Goossens H., Pattyn S.R., Elseviers M.M., Weyler J., Bossaert L.L., Mentens Y., Ieven M., Herpes simplex virus in the respiratory tract of critical care patients: a prospective study. Lancet 362:1536–1541, 2003.

168. Luyt C.E., Combes A., Deback C., Aubriot-Lorton M.H., Nieszkowska A., Trouillet J.L., Capron F., Agut H., Gibert C., Chastre J., Herpes simplex virus lung infection in patients undergoing prolonged mechanical ventilation. Am J Respir Crit Care Med 175:935–942, 2007.

169. Smith C.A., Conroy L.T., Pollock M., Ruddy J., Binning A., Mc Cruden E.A.B., Detection of Herpes Viruses in Respiratory Secretions of Patients Undergoing Artificial Ventilation. Journal of Medical Virology 82:1406–1409, 2010.

170. Ramsey P.G., Fife K.H., Hackman R.C., Meyers J.D., Corey L., Herpes simplex virus pneumonia: clinical, virologic, and pathologic features in 20 patients. Ann Intern Med, 97: 813–820, 1982.

171. Prellner T., Flamholc L., Haidl S., Lindholm K., Widell A., Herpes simplex virus the most frequently isolated pathogen in the lungs of patients with severe respiratory distress. Scand J Infect Dis 24:283–292, 1992.

172. Tuxen D.V., Cade J.F., McDonald M.I., Buchanan M.R.C., Clark R.J., Pain M.C.F., Herpes simplex virus from the lower respiratory tract in adult respiratory distress syndrome. Am Rev Respir Dis 126: 416–419, 1982.

173. Epstein M.A., Achong B.G., Barr Y.M., Virus particles in cultured lymphoblasts from Burkitt's lymphma. Lancet, 283: 702–703. doi:10.1016/S0140-6736(64)91524-7, 1964.

174. Young L.S., Yao Q.Y., Rooney C.M., Sculley T.B., Moss D.J., Rupani H., Laux G., Bornkamm G.W., Rickinson A.B., New type B isolates of Epstein-Barr virus from Burkitt's lymphoma and from normal individuals in endemic areas. J Gen Virol 68 (Pt 11):2853-62, 1987.

175. Sample J., Young L., Martin B., Chatman T., Kieff E., Rickinson A., Kieff E., Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. J Virol 64(9):4084-92, 1990.

176. Liebowitz D., Kieff E., Epstein-Barr virus. In: The Human Herpesvirus. Roizman B, Whitley RJ, Lopez C, editors, New York, pp. 107–172, 1993.

177. Kieff E.,Epstein-Barr virus and its replication. In: Fields Virology. Fields BN, Knipe DM, Howley P et al., editors. Philadelphia, PA: Lippincott–Raven, pp.2343–2396, 1996.

178. Kieff E., Rickinson A.B., Epstein-Barr virus and its replication. In: Fields Virology. Fields BN, Knipe DM, Howley P et al., editors. Philadelphia, PA: Lippincott– Raven, pp. 2511–2575, 2001.

179. Cai X., Schafer A., Lu S., Bilello J.P., Desrosiers R.C., Edwards R., Raab-Traub N., Cullen B.R., Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. PLoS Pathog, 2: e23 doi:10.1371/ journal.ppat.0020023 PMID:16557291, 2006.

180. Cheung A., Kieff E., Long internal direct repeat in Epstein-Barr virus DNA. J Virol, 44: 286–294. PMID:6292491, 1982.

181. Young L.S., Rickinson A.B. Epstein-Barr virus: 40 years on. Nat Rev Cancer, 4: 757–768. doi:10.1038/ nrc1452 PMID:15510157, 2004.

182. Fingeroth J.D., Weis J.J., Tedder T.F., Strominger J.L., Biro P.A., Fearon D.T., Epstein- Barr virus receptor of human B lymphocytes is the C3d receptor

CR2. Proc Natl Acad Sci USA, 81: 4510–4514.doi:10.1073/pnas.81.14.4510 PMID:6087328, 1984.

183. Fingeroth J.D., Clabby M.L., Strominger J.D., Characterization of a T-lymphocyte Epstein-Barr virus/C3d receptor (CD21). J Virol, 62: 1442–1447. PMID:2831405, 1988.

184. Tanner J., Weis J., Fearon D., Whang Y., Kieff E., Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. Cell, 50: 203–213. doi:10.1016/0092-8674(87)90216-9 PMID:3036369, 1987.

185. Dolan A., Cunningham C., Hector R.D., Hassan-Walker A.F., Lee L., Addison C., Dargan D.J., McGeoch D.J., Gatherer D., Emery V.C., Griffiths P.D., Sinzger C., McSharry B.P., Wilkinson G.W., Davison A.J., Genetic content of wild-type human cytomegalovirus. J Gen Virol 85, 1301–1312, 2004.

186. Weststrate M.W., Geelen J.L., van der Noordaa J., Human cytomegalovirus DNA: physical maps for restriction endonucleases BglII, hindIII and XbaI. J Gen Virol 49(1):1-21, 1980.

187. Davison A.J., Dolan A., Akter P., Addison C., Dargan D.J., Alcendor D.J., McGeoch D.J., Hayward G.S., The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. J Gen Virol Apr;84(Pt 4):1053, 2003.

188. Spaete R.R., Mocarski E.S., Regulation of cytomegalovirus gene expression: alpha and beta promoters are trans activated by viral functions in permissive human fibroblasts. J Virol 56(1):135-43, 1985.

189. Tamashiro J.C., Spector D.H., Terminal structure and heterogeneity in human cytomegalovirus strain AD169. J Virol. 59(3):591-604, 1986.

190. Solano C., Navarro D., Clinical virology of cytomegalovirusinfection following hematopoietic transplantation. Future Virol 5:111–124, 2010.

191. So¨derberg-Naucle´r C., HCMV microinfections in inflammatory diseases and cancer. J Clin Virol 41:218–223, 2008.

192. Nu´n˜ ez J., Chilet M., Sanchis J., Bodı´ V., Nu´n˜ez E., Min˜ana G., Tormo N., Clari M.A., Pellicer M., Chorro F.J., Lla`cer A., Navarro D., Prevalence and prognostic implications of active cytomegalovirus infection in patients with acute heart failure. Clin Sci (Lond) 119:443–452, 2010.

193. Nu´n˜ ez J., Chilet M., Blasco M.L., Clari M.A., Sanjuan R., Mun˜ oz-Cobo B., Bodı´ V., Costa E., Bravo D., Sanchis J., Min˜ana G., Navarro D., Low rate of detection of active cytomegalovirus (CMV) infection early following acute myocardial infarction. Atherosclerosis 222:295–297, 2012.

194. Blanquer J., Chilet M., Benet I., Aguilar G., Mun˜ oz-Cobo B., Tellez A., Costa E., Bravo D., Navarro D., Immunological insights into the pathogenesis of active CMV infection in non-immunosuppressed critically ill patients. J Med Virol 83:1966–1971, 2011.

195. Kalil A.C., Florescu D.F., Prevalence and mortality associated with cytomegalovirus infection in non-immunosuppressed patients in the intensive care unit. Crit Care Med 37:2350–2358, 2009.

196. Peris A., Zagli G., Bernardo P., Bonacchi M., Cozzolino M., Perretta L., Azzi A., Cianchi G., H3N2 Virus as Causative Agent of ARDS Requiring Extracorporeal Membrane Oxygenation Support. Case Rep Med 2014:560208. doi: 10.1155/2014/560208, 2014.

197. Gutiérrez-Pizarraya A., Pérez-Romero P., Alvarez R., Aydillo T.A., Osorio-Gómez G., Milara-Ibáñez C., Sánchez M., Pachón J., Cordero E., Unexpected severity of cases of influenza B infection in patients that required

hospitalization during the first post-pandemic wave. J Infect 65(5):423-30. doi: 10.1016/j.jinf.2012.07.004, 2012.

198. Balthesen M., Messerle M., Reddehase M.J., Lungs are a major organ site of cytomegalovirus latency and recurrence. J Virol 67:5360–5366, 1993.

199. Oud L., Comment on: 'nosocomial viral ventilator-associated pneumonia in the intensive care unit' by Daubin et al. Intensive Care Med 32:613, 2006.

200. Frobert E., Billaud G., Casalegno J.S., Eibach D., Goncalves D., Robert J.M., Lina B, Morfin F., The clinical interest of HSV1 semi-quantification in bronchoalveolar lavage. Journal of Clinical Virology 58 265– 268, 2013.

201. Costa C., Sidoti F., Saldan A., Sinesi F., Balloco C., Simeone S., Lorusso M., Mantovani S., Merlino C., Solidoro P., Cavallo R., Clinical impact of HSV-1 detection in the lower respiratory tract from hospitalized adult patients. Clin Microbiol Infect 18(8):E305–7, 2012.

202. Anderson L.J., Major trends in nosocomial viral infections. Am J Med 91:107S–111S, 1991.

203. Ziemann M., Sedemund-Adib B., Reiland P., Schmucker P., Hennig H., Increased mortality in long-term intensive care patients with active cytomegalovirus infection. Crit Care Med 36: 3145–3150, 2008.

204. von Muller L., Klemm A., Weiss M., Schneider M., Suger-Wiedeck H., Durmus N., Hampl W., Mertens T., Active cytomegalovirus infection in patients with septic shock. Emerg Infect Dis 12: 1517–1522, 2006.

205. Henke-Gendo C., Ganzenmueller T., Kluba J., Harste G., Raggub L., Heim A., Improved Quantitative PCR Protocols for Adenovirus and CMV With an Internal Inhibition Control System and Automated Nucleic Acid Isolation. Journal of Medical Virology 84:890–896, 2012.

206. Chemaly R.F., Yen-Lieberman B., Castilla E.A., Reilly A., Arrigain S., Farver C., Avery R.K., Gordon S.M., Procop G.W., Correlation between viral loads of cytomegalovirus in blood and bronchoalveolar lavage specimens from lung transplant recipients determined by histology and immunohistochemistry. J Clin Microbiol 42:2168–2172, 2004.

207. Riise G.C., Andersson R., Bergström T., Lundmark A., Nilsson F.N., Olofsson S., Quantification of cytomegalovirus DNA in BAL fluid: a longitudinal study in lung transplant recipients. Chest 118(6):1653-60, 2000.

208. Gooskens J., Templeton K.E., Claas E.C., van Bussel M.J., Smit V.T., Kroes A.C., Quantitative detection of herpes simplex virus DNA in the lower respiratory tract. J Med Virol 79:597–604, 2007.

209. Costa C., Elia M., Astegiano S., Sidoti F., Terlizzi M.E., Solidoro P., Botto S., Libertucci D., Bergallo M., Cavallo R., Quantitative detection of Epstein-Barr virus in bronchoalveolar lavage from transplant and nontransplant patients. Transplantation 86:1389–1394.doi:10.1097/TP.0b013e3181890415, 2008.

210. Costa C., Delsedime L., Solidoro P., Curtoni A., Bergallo M., Libertucci D., Baldi S., Rinaldi M., Cavallo R., Herpesviruses detection by quantitative real-time polymerase chain reaction in bronchoalveolar lavage and transbronchial biopsy in lung transplant: viral infections and histopathological correlation. Transplant Proc 42:1270–1274. doi: 10.1016/j.transproceed.2010.03. 086, 2010.

211. Lung M.L., Lam W.K., So S.Y., Lam W.P., Chan K.H., Ng M.H., Evidence that respiratory tract is major reservoir for Epstein-Barr virus. Lancet 1(8434):889–892, 1985.

212. Vallieres E., Renaud C., Clinical and economical impact of multiplex respiratory virus assays. Diagn Microbiol Infect Dis 76:255–61, 2013.

213. Zheng X., Todd K.M., Yen-Lieberman B., Kaul K., Mangold K., Shulman S.T., Unique finding of a 2009 H1N1 influenza virus-positive clinical sample suggests matrix gene sequence variation. J Clin Microbiol 48:665–6,2010.

214. Cho C.H., Lee C.K., Nam M.H., Yoon S.Y., Lim C.S., Cho Y., Kim Y.K., Evaluation of the AdvanSure™ real-time RT-PCR compared with culture and Seeplex RV15 for simultaneous detection of respiratory viruses. Diagnostic Microbiology and Infectious Disease 79; 14–18 2014.

215. Bellau-Pujol S., Vabret A., Legrand L., Dina J., Gouarin S.,.Petitjean-Lecherbonnier J., Pozzetto B., Ginevra C., Freymuth F., Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. J Virol Methods 126:53–63, 2005.

216. Mahony JB., Detection of respiratory viruses by molecular methods. Clin Microbiol Rev 21(4):716–47, 2008-

217. Caliendo AM., Multiplex PCR and emerging technologies for the detection of respiratory pathogens. Clin Infect Dis (Suppl 4):S326–30, 2011.

218. Bierbaum S., Forster J., Berner R., Rücker G., Rohde G., Neumann-Haefelin D., Panning M., CAPNETZ study group, Detection of respiratory viruses using a multiplex real-time PCR assay in Germany, 2009/10. Arch Virol 159(4):669-76. doi: 10.1007/s00705-013-1876-3, 2013.

219. Kim H., Hur M., Moon H.W., Yun Y.M., Cho H.C., Comparison of two multiplex PCR assays for the detection of respiratory viral infections. Clin Respir J doi: 10.1111/crj.12083, 2013.

220. Pretorius M.A., Madhi S.A., Cohen C., Naidoo D., Groome M., Moyes J., Buys A., Walaza S., Dawood H., Chhagan M., Haffjee S., Kahn K., Puren A., Venter M., Respiratory Viral Coinfections Identified by a 10-Plex Real-Time Reverse-Transcription Polymerase Chain Reaction Assay in Patients

Hospitalized With Severe Acute Respiratory Illness South Africa, 2009–2010. The Journal of Infectious Diseases 206(S1):S159–65, 2012.

221. Schadt E.E., Turner S. Kasarskis A., A window into third-generation sequencing. Hum Mol Genet19, R227–R240, 2010.

222. MacConaill L., Meyerson, M., Adding pathogens by genomic subtraction. Nat Genet 40, 155.380–382, 2008.

223. Tang P., Chiu C., Metagenomics for the discovery of novel human viruses. Future Microbiol 5, 177–189.195, 2010.

224. Yang J., Yang F., Ren L., Xiong Z., Wu Z., Dong J., Sun L., Zhang T., Hu Y., Du J., Wang J., Jin Q., Unbiased parallel detection of viral pathogens in clinical samples using a metagenomic approach. .J Clin Microbiol 49, 3463–3469, 2011.

225. Yozwiak N.L., Skewes-Cox P., Gordon A., Saborio S., Kuan G., Balmaseda A., Ganem D., Harris E., DeRisi J.L., Human enterovirus 109: A novel interspecies recombinant enterovirus isolated from a case of acute pediatric respiratory illness in Nicaragua. J Virol 84, 9047–9058, 2010.

226. Kuroda M., Katano H., Nakajima N., Tobiume M., Ainai A., Sekizuka T., Hasegawa H., Tashiro M., Sasaki Y., Arakawa Y., Hata S., Watanabe M., Sata T., Characterization of quasispecies of pandemic 2009 influenza A virus (A/H1N1/2009) by de novo sequencing using a next-generation DNA sequencer. PLoS One 5, doi:10.1371/journal.pone.0010256, 2010.

227. Tapparel C., Cordey S., Junier T., Farinelli L., Van Belle S., Soccal P.M., Aubert J.D., Zdobnov E., Kaiser L., Rhinovirus genome variation during chronic upper and lower respiratory tract infections. PLoS One 6, doi:10.1371/journal.pone.0021163, 2011.

Ringraziamenti

Ringrazio la Prof.ssa Alberta Azzi, a cui sento di esprimere una infinita riconoscenza per i consigli, il sostegno e i suggerimenti con i quali ha supportato questo lavoro.