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Chapter 12

Viral DNA and cDNA Array in the Diagnosis of Respiratory Tract Infections

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Respiratory Tract Infections (RTIs)

Respiratory tract infections (RTIs) are caused directly or indirectly by any infectious agent that implants and replicates in the respiratory tract, in the pulmonary parenchyma or onto pleural sierose and causes clinical syndromes with prevalent respiratory symptoms [1]. When the respiratory tract is only the first site of infection, patients show respiratory syndromes, but then symptoms involve the specific anatomic district target of the infectious agent, as we can see in some infectious disease: Measles, Scarlet fever, Mononucleosis, Meningococcal disease and Varicella. Even with these limitations RTIs are the most common infections in men and are a significant cause of morbidity and mortality in both developing and developed countries among infants, youngsters and elderly people, and are the first cause of temporary invalidation (absence from work or school), visiting emergency service and family doctor consultancies during the winter season, independently from the age [2]. so they are a great socioeconomic and medical burden. In 2010, the World Health Organization (WHO) estimated that RTIs caused about 3 million deaths worldwide, including developed countries, being the first cause of child mortality [3]. Respiratory infections are common in both hospital and community settings. The third national prevalence survey conducted in 2006 found that infections of the lower respiratory tract (LRTI, not pneumonia) and pneumonia together accounted for 19.9 percent of the Healthcare Associated Infections (HCAIs) in acute hospitals. It I important also to consider the hospital-acquired infections affecting the respiratory tract that cause considerable morbidity and mortality. This type of respiratory infections generally affects those who are affected from serious diseases [4]. RTIs can be classified in, infections of the upper respiratory tract (URIs), which affect the nose, sinuses and throat (common cold, tonsillitis, sinusitis, laryngitis, influenza) and infections of the lower respiratory tract (LRTIs), which

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affect the airways and lungs (influenza, bronchitis, pneumonia, bronchiolitis, tuberculosis, that is a persistent bacterial infection of the lungs). LRTIs include two serious conditions – acute bronchitis and pneumonia:

Acute bronchitis (inflammation of the bronchi) is an acute respiratory infection in which the dominant symptom is coughing without localized infection. It must not not be confused with chronic bronchitis, which is a chronic obstructive pulmonary disease (COPD). Acute bronchitis is usually an infection that is community-acquired and typically it arises as a complication of URI caused by a virus, when bacterial infection supervenes. Children that seem prone to bronchitis generally have poor living conditions (overcrowding,poor hygiene and poor nutrition) and the respiratory disease may be exacerbated by maternal smoking, especially during pregnancy. It I reported that individuals who have experienced childhood bronchitis are at risk of developing further symptoms during their teenage years if they then smoke [5]. Pneumonia (inflammation of the lung) is a serious condition, that caused may death after RTI, especially in older adults and infants. It may be acquired in hospital or the community. The alveoli become filled with pus, air is excluded, and the lung is said to be 'consolidated'.In bronchopneumonia, consolidation is widely distributed; in lobar pneumonia,it is localized [6]. In the community, bacterial pneumonia is most frequently caused by *Streptococcus pneumonia* that infects most commonly people with pre-existinghealth problems, frequently developing as a complication of some other RTI (for example influenza or measles). The establishment of a pharmacological treatment is complicated because some strains of *Streptococcus pneumoniae* are now resistant to penicillin, so vaccination has been recommended in the UK since 2003. The pneumococcal vaccine is part of the childhood immunization program and it is also recommended to people over 65 years of age. It is also recommended for people following splenectomy and those with dysfunction of the spleen, sickle cell disease, coeliac disease, chronic renal disease, chronic respiratory disease, chronic heart conditions, liver disease, diabetes mellitus, immunosuppression and HIV. After vaccination, about 80 per cent of healthy adults develop a good antibody response within three weeks. Other bacteria responsible for community-acquired pneumonia include *Mycoplasma pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila* and *Staphylococcus aureus*, including the strain that produces the Panton–Valentine leukocidin toxin [7]. It I mandatory also to consider hospital acquired pneumonia. It mainly affects critically ill and postoperative patients. Risk factors include obesity, impaired consciousness, a history of smoking and underlying respiratory disease. In hospital, bacteria, viruses or fungi can cause pneumonia, but most hospital-acquired pneumonia is caused by *S. aureus* and Gram-negative opportunists [8]. URIs that involve the nasal passages, pharynx, tonsils and epiglottis are minor infections acquired in the community and are caused by viruses. URIs can, however, have serious consequences for the very young and older adults. They also account for a high proportion of days lost from work and school, so their impact on the health of individuals and their social and economic consequences should not be dismissed. The nasal discharge associated with colds contains viral particles, dead cells from the nasal mucosa and bacteria, but these are of the same type

as are present in health. Bacterial invasion of the damaged epithelium is rare, and antibiotics are seldom required. Acute ear infection occurs as a complication with up to 30 percent of URIs. Because most URIs are self-limiting, their complications are more important than the infections, mastoiditis and other complications of URIs account for nearly 5 percent of all URI worldwide leading to hearing impairment or deafness most of the times in developing countries where there is limited access to adequate medical treatments. RTIs are extraordinary frequent because of a great number of antigenically distinct aetiological agents, their great diffusion and the short period of immunization. Moreover the anatomic structure of the respiratory tract shows high variable physicochemical parameters such as temperature, pH, and humidity that assurance a great number of habitat for various microorganisms. Moreover the respiratory tract is directly connected to the external environment and it is continuously crossed by the airflow that frequently contains irritant agents (atmospheric contaminants, cigarette smoke, cold air, fines) that may injure the local mucosa end predispose to the implantation of microorganisms. Fortunately there is a huge amount of mechanisms of defense that preserve the integrity of this anatomic site, the nasopharyngeal lymphatic system, ciliary cells, salivary lisozima, antibodies, interferons and pulmonary macrophafges. The infectious agents of RTIs can be viruses, bacteria, fungi and protozoa, but they are usually caused by a virus [1, 2]. Bacterial agents causing RTIs can implant directly onto the mucosa of the respiratory tract, but more frequently they superinfect a tract of mucosa previously injured by a virus. *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* are the most common organisms that cause the bacterial superinfection of viral acute sinusitis [8]. The sequence viral-bacterial infection is very frequent and must be considered to establish an appropriate pharmacological treatment. Parasitic infections of the respiratory tract occur worldwide among both immunocompetent and immunocompromised patients and may affect the respiratory system in a variety of ways. The most common parasites involved are, *Ascariasis*, *Schistosoma* and *Toxoplasma gondii*. Since the clinical presentations and radiographic findings of several of these diseases may mimic tuberculosis and malignancy it is important to consider parasitic infections in the differential diagnosis of such respiratory syndromes. If identified early, most parasitic respiratory diseases are curable with medical or surgical treatments [9]. RTIs are transmitted by airflow, pathogens enter the organism trough the upper respiratory tract and sometimes trough the conjunctivae, that have been infected by direct contact especially for viruses, or trough vectors, whereas the elimination of the infecting particles takes place trough cough. Rarely the respiratory tract can be reached by circulating microorganisms in blood. The reservoir for the majority of RTIss are infected humans that guarantee an optimal environment for the survival of the infecting agent, but some microorganisms that occasionally infect humans have animal reservoir; for example in 2002, a new Coronavirus (CoV) emerged in the People's Republic of China, associated with a severe acute respiratory syndrome (SARS) and mortality in humans. The epidemic rapidly spread throughout the world before being contained in 2003, although sporadic cases occurred thereafter in Asia. The virus was thought to be of zoonotic origin from a wild animal reservoir (Himalayan palm civets, *Paguma larvata*), but the definitive host is still unknown [10]. RTIs are generally epidemic because they are very contagious, generally they

are more frequent during autumn and winter when atmospheric pollution is higher and permanence indoor increases. Acquired immunity after RTIs is generally short because the majority of the most common aetiological agents are ineffective immunogens; the immunological response to RTIs generally produces initially IgM and after IgG, so that the presence of IgG has a diagnostic value only if it increases for times or is very high. During RTIs many viral and bacterial immunogens induce the production of secretory IgA that are released in to the muco where they compete for tissue receptors and link to microorganisms. The periodic genetic mutations of RTIs aetiological agents vanish both natural and acquired immunity; each time that genetic mutations that involve major antigens take place, especially for viruses, the diffusion of the infecting agent of RTIs becomes pandemic [11]. An example of immune evasion due to genetic variability is given by INFVs that are dynamic and are continuously evolving. INFVs can change in two different ways: antigenic drift and antigenic shift. Antigenic drift takes place continuously while antigenic shift happens only occasionally. INF A viruses undergo both kinds of changes; INF B viruses change only by the more gradual process of antigenic drift. Antigenic drift refers to small, gradual changes that occur through point mutations in the two genes that contain the genetic material to produce the main surface proteins, hemagglutinin (HA), and neuraminidase (NA). These point mutations occur unpredictably and result in minor changes to these surface proteins. Antigenic drift produces new virus strains that may not be recognized by antibodies to earlier INFVs strains. This is one of the main reasons why people can become infected with INFVs more than one time and why global surveillance is critical in order to monitor the evolution of human INFVs for selection of which strains should be included in the annual production of INF vaccine. In most years, one or two of the three virus strains in the INF vaccine are updated to keep up with the changes in the circulating INFVs. For this reason, the immunization against INF needs to be vaccinated every year. Antigenic shift refers to an abrupt, major change to produce a novel INFV A subtype in humans that was not currently circulating among people. Antigenic shift can occur either through direct animal (poultry)-to-human transmission or through mixing of human influenza A and animal influenza A virus genes to create a new human influenza A subtype virus through genetic reassortment. Antigenic shift results in a new human INFV A subtype [12, 13].

2. Viral RTIs

At least two hundred different viruses can establish RTIs and they belong to the *Adenoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Picornaviridae*, *Coronaviridae*, *Herpesviridae* and *Parvoviridae* familes. Viruses the most frequently associated with RTIs are, Adenovirus (ADV), BoV, CoV, Enterovirus (ENTV), INFV A, B, C, Metapneumovirus (hMetV), Parainfluenza (IPV) viruses 1, 2, 3, 4, Rhinoviruses (RV), Respiratory Syncitial Viruses (RSV). While it is true that respiratory viruses place a greater burden on people in developing countries, these viruses are still a big health threat in the developed world, where over 100 million people have been killed INFV in the last century alone (Piralla et al. 2011). In children, viruses are responsible for the majority of RTIs, with bacteria thought to be responsible for fewer than 15% of cases; acute pharyngitis is caused by viruses in more

than 70% percent of cases in young children, mild pharyngeal redness and swelling and tonsil enlargement are typical [2, 15]. The most pathogenetic viruses for humans are, INFV and RSV with high mortality rate in elderly people and infants respectively. INFVs have the highest evolution rate, being the INF A viruses those causing the most severe and expansive outbreaks. Genetic variations in INF A viruses usually lead to global epidemics or pandemics; the latest FluA (H1N1) outbreak in 2009 was originated by a variant INF A H1N1 of swine origin, classified by the World Health Organization (WHO) as level 6 alert, pandemic [16]. Viruses cause most URIs, with RVs accounting for the 25-30% of cases, IPV, ADV, RSV, INFV 25-35%, CoV 10% and ENTV (Coxsackievirus, CoxV) less than 5% [17]. Acute viral infections predispose to bacterial infections of the sinuses and middle ear, and aspiration of infected secretions and cells can result in LRIs. To date the most common causes of viral LRIs are RSVs. They tend to be highly seasonal, unlike PINFV, the next most common cause of viral LRIs. The epidemiology of influenza viruses in children in developing countries deserves urgent investigation because safe and effective vaccines are available. Before the effective use of measles vaccine, the Measles virus was the most important viral cause of respiratory tract–related morbidity and mortality in children in developing countries. ADVs are medium-sized (90-100 nm), non-enveloped icosohedral viruses with double-stranded DNA. There are over 50 types that are immunologically distinct that can cause infections in humans. ADVS are relatively resistant to chemical and physical agents and to adverse pH conditions and can live for a long time outside the body and most commonly cause respiratory illness. The symptoms of ADV infection can range from the common cold to pneumonia, croup, and bronchitis. Some ADVs types can cause other illnesses such as gastroenteritis, conjunctivitis, cystitis, and less commonly, neurological disease. Infants and people with weakened immune systems are at high risk for severe complications of ADV infection and some people infected with ADV can have ongoing infections in their tonsils, adenoids, and intestines that do not cause symptoms. They can shed the virus for months or years [18].Human bocavirus (hBoV), the second parvovirus potentially pathogenic to humans after Parvovirus B19 (Pb19), was discovered by PCR in respiratory samples collected from young children with respiratory diseases in Sweden in 2005. Since the first description of hBoV as a possible human pathogen of lower respiratory tract infections in children, it has been detected in at least 19 countries in the five continents. HBoV infections shows a variety of clinical symptoms; the most common symptoms in hBoV-infected children without coinfections are cough (85%), followed by rinorrhea (67%), fever (59%), difficulty in breathing (48%), diarrhea (16%), conjunctivitis (9%) and rash (9%), body temperature ranging from 37.5 to 40.2^{o}C, wheezing; nausea, sore throat, headache and myalgia were also recorded in hBoV-infected children of older age and adults. The age distribution of hBoV-infected humans ranges from 10 days to 60 years, but hBoV was primarily detected in young children aged 6 months to 3 years. That a peak detection of hBoV is among children of 6 to 24 months of age [19]. CoVs (order Nidovirales, family Coronaviridae, genus Coronavirus) are a diverse group of large, enveloped, positive-stranded RNA viruses that cause respiratory and enteric diseases in humans and other

animals. There are three groups of CoV; groups 1 and 2 contain mammalian viruses, while group 3 contains only avian viruses. Within each group, CoVs are classified into distinct species by host range, antigenic relationships, and genomic organization. The viruses can cause severe disease in many animals, and several viruses, including infectious bronchitis virus, feline infectious peritonitis virus, and transmissible gastroenteritis virus, are significant veterinary pathogens. Human coronaviruses (hCoVs) are found in both group 1 (HCoV-229E) and group 2 (HCoV-OC43) and are responsiblefor ~30% of mild upper respiratory tract illnesses [19]. In March 2003, a novel CoV, SARS-CoV was discovered in association with cases of severe acute respiratory syndrome (SARS). The sequence of the complete genome of SARS-CoV has been now determined; it is 29,727 nucleotides in length, has 11 open reading frames, and the genome organization is similar to that of other CoVs. Phylogenetic analyses and sequence comparisons showed that SARS-CoV is not closely related to any of the previously characterized CoV. By late April 2003, over 4300 SARS cases and 250 SARS-related deaths were reported to WHO from over 25 countries around the world. Most of these cases occurred after exposure to SARS patients in household or healthcare settings. The incubation period for the disease is usually from 2 to 7 days. Infection is usually characterized by fever, which is followed a few days later by a dry, non-productive cough, and shortness of breath. Death from progressive respiratory failure occurs in about 3% to nearly 10% of cases. Evidence of SARS-CoV infection has been documented in SARS patients throughout the world. SARSCoV RNA has frequently been detected in respiratory specimens, and convalescent-phase serum specimens from SARS patients contain antibodies that react with SARS-CoV [19]. Enteroviruses (ENTV) genus belongs to the *Picornaviridae* family positive single stand ssRNA viruses. Current taxonomy divides non-polio human ENTVs into four species (human ENTVs A to D), including a total of 108 serotypes. Individual serotypes have different temporal patterns of circulation and can be associated with different clinical manifestations. Although the majority of human ENTV infections remain asymptomatic, these viruses are associated with various clinical syndromes, ranging from minor febrile illness to severe and potentially fatal pathologies, including aseptic meningitis, encephalitis, myopericarditis, acute flaccid paralysis, and severe neonatal sepsis-like disease. ENTVs are responsible for a wide range URIs and LRTIs occurring in adults and infants. These viruses are considered as the third etiological cause of bronchiolitis in young infants aged 1-12 months. Moreover, several clinical case studies reported the etiological role of the Cox A16, the ENTV 71 and of a newly discovered genotype ENTV-104 in the development of acute or fatal pneumonia indicating that ENTVs belonging to species A to C can be responsible for severe LRTIs in immunocompetent infants or adults. Taking into account the recent epidemiological and clinical data and because of frequent mutations and intra-species enteroviral RNA genomic recombination events, the respiratory strains of ENTV are considered also as potential agents of emerging infectious diseases in human populations [21]. There are three types of INFV: A, B, and C. Only INF A viruses are further classified by subtype on the basis of the two main surface glycoproteins HA and NA. INF type A viruses can infect people, birds, pigs, horses, and

other animals, but wild birds are the natural hosts for these viruses. INF viruses type A are divided into subtypes and named on the basis of two proteins on the surface of the virus: HA and NA. There are 16 known HA subtypes and 9 known NA subtypes and many different combinations of HA and NA proteins are possible. Only some INF A subtypes (i.e., H1N1, H1N2, and H3N2) are currently in general circulation among people, other subtypes are found most commonly in other animal species. For example, H7N7 and H3N8 viruses cause illness in horses, and H3N8 also has recently been shown to cause illness in dogs. Only INF A viruses infect birds, and all known subtypes of INF A viruses can infect birds. Typically, wild birds do not become sick when they are infected with avian INF A viruses, however, domestic poultry, such as turkeys and chickens, can become very sick and die from avian flu, and some avian INF A viruses also can cause serious disease and death in wild birds. Highly pathogenic avian INF A virus strains (HPAI) can cause severe illness and high mortality in poultry. More recently, some HPAI viruses (e.g., H5N1) have been found to cause no illness in some poultry, such as ducks. Avian INF A viruses of the subtypes H5 and H7,including H5N1, H7N7, and H7N3 viruses, have been associated with HPAI, and human infection with these viruses have ranged from mild (H7N3, H7N7) to severe and fatal disease (H7N7, H5N1). In general, direct human infection with avian INFVs occurs very infrequently, and has been associated with direct contact (e.g., touching) infected sick or dead infected birds (domestic poultry). INF B viruses are usually found only in humans. Unlike INF A viruses, these viruses are not classified according to subtype. INF B viruses can cause morbidity and mortality among humans, but in general are associated with less severe epidemics than INF A viruses. Although INF type B viruses can cause human epidemics, they have not caused pandemics. INF C viruses cause mild illness in humans and do not cause epidemics or pandemics. These viruses are not classified according to subtype [22].

HMPV is a respiratory viral pathogen that causes a spectrum of illnesses that range from asymptomatic infection to severe bronchiolitis. In 2001, van den Hoogen et al described the identification of this new human viral pathogen from respiratory samples submitted for viral culture during the winter season. Half of the initial 28 hMPV isolates were cultured from patients younger than 1 year, and 96% were isolated from children younger than 6 years. Seroprevalence studies revealed that 25% of all children aged 6-12 months who were tested in the Netherlands had detectable antibodies to hMPV; by age 5 years, 100% of patients showed evidence of past infection. Separate reports from all areas of the world support the early contention that this newly discovered virus is ubiquitous, and, like human respiratory RSV infection, is seasonal in nature. Although the description of this viral pathogen was first described in children, subsequent reports have highlighted the importance of hMPV as a cause of respiratory illness in adults of all ages in patients with cancer, in the elderly population (as a cause of serious LRTI), and in adults with underlying chronic medical conditions [23]. Human parainfluenza viruses (hPIV) belong to the *Paramyxoviridae* family and are negative-sense, single-stranded RNA viruses that show fusion and hemagglutinin-neuraminidase glycoprotein "spikes" on their surface. There are

four serotypes (1 through 4) and two subtypes (4a and 4b) that show different clinical and epidemiologic features. The virion varies in size (average diameter between 150 and 200 nm) and shape, is unstable in the environment (surviving a few hours on environmental surfaces), and is readily inactivated with soap and water. HPIV are common causes of RTIs in infants and young children; the most distinctive clinical feature of HPIV-1 and HPIV-2 is croup (i.e., laryngotracheobronchitis or swelling around the vocal chords and other parts of the upper and middle airway); HPIV-1 is the leading cause of croup in children, whereas HPIV-2 is less frequently detected. HPIV-3 is more often associated with bronchiolitis (swelling of the small airways leading to the lungs) and pneumonia. HPIV-4 is detected infrequently, and is less likely to cause severe disease; but it may be more common than once thought. HPIVs can cause repeated infections with all serotypes throughout life. Reinfections usually manifested by an upper respiratory tract illness (e.g., a cold, sore throat). HPIVs can also cause serious LRTIs with repeat infection (e.g., pneumonia, bronchitis, and bronchiolitis), especially among older adults and patients with compromised immune systems. The incubation period (time from exposure to the virus to onset of symptoms) for HPIVs generally ranges from 2 to 7 days [24]. RVs are small (30 nm), nonenveloped viruses that contain a single-strand RNA genome within an icosahedral (20-sided) capsid, that RV can be transmitted by aerosol or direct contact. The nasal mucosa is the primary site of onculation, although the conjunctiva may also be involved, though to a lesser extent. RVs attache their selves to the respiratory epithelium and spreads locally. The optimum temperature for RVs repolication is 33-35°C and so does not replicate efficiently at body temperature. This could be the major reason why RVs replicate well in the nasal passages and upper trachebronchial tree but not so well in the lower respiratory tract. RVs are well known for causing the common cold, although they have also been implicated in causing bronchitis and asthma attacks. There is little or no cross-protection between serotypes, making it very difficult to make vaccines. These viruses seems to affect children first, and then there are many modes of transmission that range from aerosol to direct hand-to-hand contact [25]. RSV, is a respiratory virus negative-sense, single-stranded RNA of the family *Paramyxoviridae* that infects the lungs and breathing passages. Most otherwise healthy people recover from RSV infection in 1 to 2 peeks. However, infection can be severe in some people, such as certain infants, young children, and older adults. In fact, RSV is the most common cause of bronchiolitis (inflammation of the small airways in the lung) and pneumonia in children under 1 year of age in the United States. In addition, RSV is more often being recognized as an important cause of respiratory illness in older adults. Initially isolated RSV from chimpanzees with URI as the causative agent of most epidemic bronchiolitis cases. Subsequently, RSV has been associated LRTIs infection in infants and multiple epidemiologic studies have confirmed the role of this virus as the leading cause of LRT infection in infants and young children. Peak of incidence of occurrence of severe RSV disease is observed at age 2-8 months. Overall, 4-5 million children younger than 4 years acquire an RSV infection, and more than 125,000 children are hospitalized annually in the United States because of this infection. This translates to 3-9 per 1000 children younger than 1 year who are hospitalized annually for this condition. Virtually all children have had at least one RSV infection by

their third birthday. The WHO has targeted RSV for vaccine development, which is not surprising, given the prevalence and potential severity of this condition [26]. Other less common cause of viral URI is Herpes simplex virus (HSV).

Table 1 resumes viral cusative agents of Respiratory Tract Infection (RTIs).

Virus group	**Antigenic types**	**RTIs**
Rhinoviruses	100 types and 1 subtypes	Common Cold, Pharyngitis, Acute Laryngitis, Sinusitis, Acute Bronchitis, Bronchiolitis, Acute Pneumonia
Coronavirus	3 or more types	Common Cold, Pharyngitis, Acute Laryngitis, Acute Bronchitis
Parainfluenza viruses	4 types	Common Cold, Pharyngitis, Acute Laryngitis, Acute Laryngotracheobronchitis (Croup), Sinusitis, Acute Bronchitis, Bronchiolitis, Acute Pneumonia
Respiratory Syncytial virus	2 types	Common Cold, Acute Laryngotracheobronchitis (Croup), Acute Bronchitis, Bronchiolitis, Acute Pneumonia
Influenza viruses	3 types	Common Cold, Pharyngitis, Acute Laryngitis, Acute Laryngotracheobronchitis (Croup), Sinusitis, Acute Bronchitis, Bronchiolitis, Acute Pneumonia
Adenoviruses	47 types	Common Cold, Pharyngitis, Acute Laryngitis, Sinusitis, , Acute Bronchitis, Bronchiolitis, Acute Pneumonia
Human Metapneumovirus		Common Cold, , Acute Laryngitis, Bronchiolitis, Acute Pneumonia
Rubeola virus		Common Cold, Acute Bronchitis, Acute Pneumonia
Enteroviruses	5 types	Common cold, Pharyngitis, Acute Bronchitis, Bronchiolitis, Acute Pneumonia
Rubella virus		Common cold, , Acute Bronchitis
Varicella Zooster virus		Common Cold, Oral cavity Infections, Acute Pneumonia
Herpes Simplex viruses	2 types	Pharyngitis, Epiglottitis, Oral cavity Infections, Acute Pneumonia
Cytomegalovirus		Pharyngitis, Oral cavity Infections, Acute Pneumonia
Human Immunodeficency virus	1 type	Pharyngitis
Epstein Barr virus		Epiglottitis
Human herpes virus 6		Acute Pneumonia

Table 1. Viral cusative agents of Respiratory Tract Infection (RTIs)

The relative importance of individual viral agents in early life is open to debate. Certainly, RSV, RV, PIV, and INFV are predominant in the published data. However there are several factors limiting the ability to draw a definitive conclusion about which virus is the most common or important: differences in the way that data are collected (PCR versus immunoassay) between and within studies and the impact of assay sensitivity; differences in study design affecting age, recruitment criteria, and which viruses are studied. About viral aetiology and infant hospitalization due to respiratory infection, INFVs, ADV, hMPV, PIV, RVs, and RSV can all cause bronchiolitis, necessitating hospitalization; RSV has most commonly been reported to be the main cause of hospitalization due to bronchiolitis and increased disease severity, followed by RV and then by influenza virus and viral coinfection is relatively common, occurring in about 20% of cases. Even there is no consensus on the effect of coinfection on disease severity, coinfection with both hMPV and RSV increased the intensive care unit admission rate. While knowledge of which virus is predominant is relevant for the design of vaccines and specific prophylactic treatments, what can be observed is the similarity of symptoms caused by a wide range of viral agents. It may therefore be more appropriate to focus on ways to target the symptoms and not the agent. This may be especially relevant when an excess immune response causes the disease or when there are multiple serologically distinct subtypes circulating.

3. Diagnosis of viral RTIs

Respiratory viruses, that belong to several taxonomic families, show overlapping clinical signs and symptoms. In clinical practice, a specific virus is often not identified due to the lack of sensitive tests and/or the presence of as-yet-unknown pathogens [27]. Because of the great variety of possible pathogenic agents involved, and because of the high frequency of coinfections, especially among young children, whose immune system is still developing, it is mandatory to use diagnostic methods that allow multiple, sensitive, efficacious and rapid identification of all possible viruses simultaneously possibly present in the clinical sample [27]. Respiratory viruses have become increasingly recognized as serious causes of morbidity and mortality in immunocompromised patients. Rapid and sensitive detection of respiratory viruses is essential for early diagnosis and administration of appropriate antiviral therapy as well as for effective implementation of infection control measures.

Rapid diagnosis of respiratory viruses can enable:

- To establish a direct antiviral therapy, when available, that is crucial considering that antivirals are only effective if administered in the early stages of infection ;
- To abolish unnecessary use of antibiotics, that are often prescribed to patients infected with respiratory viruses, with the result in no relief from symptoms and likelihood that antibiotic resistance will occur in concomitant bacteria ;
- To understand the viral natural history and pathophysiology, which may allow physicians to better understand potential complications[28];
- To implement appropriate personal protective equipment and measures, such as quarantine of infected patients, to minimize spread and prevention of unnecessary isolation (often at great expense) of uninfected individuals; particularly important with

newly emerging or re-emerging pathogens, including severe acute respiratory syndrome caused by CoV, highly pathogenic avian INF and swine-origin INFV H1N1 (S-OIV H1N1) [29];
- To perform accurate epidemiological studies, that allow clinicians to identify populations at risk and determine which populations should consider vaccination (if a suitable vaccine is available);
- finally, to implement rapid viral diagnosis that significantly decreases length of hospital stay and unnecessary laboratory testing [30].

However, even with the best viral detection assays currently available, a specific pathogen cannot be identified in 20% to 50% of RTIss. Existing viral diagnostic methods are limited in sensitivity and scope [31]. Several works suggest that respiratory viruses are underdiagnosed and they might be responsible for a considerable part of the total number of non characterized acquired pneumonia cases [32, 33, 34, 35, 36]. Traditional diagnostics methods besides being too slow, laborious and with a low sensitivity threshold, do not identify common viruses and high incidence viruses, like RV, or new viruses, like CoV. Emerging viruses like MetV or BoV recently discovered and with a very high clinical incidence, especially among children, are not detected neither with the use of traditional techniques nor with more modern methods of molecular diagnostics[37]. The discovery of respiratory viruses occurred initially between 1933 and 1965 when INFV, ENTV, ADV, RSV, RV, PINV and CoV were found by virus culture. In the 1990s, the development of high throughput viral detection and diagnostics instruments increased diagnostic sensitivity and enabled the search and the discover also of new viruses [38]. Since many respiratory viruses can present with similar signs and symptoms, it is impossible to differentiate one virus infection from another clinically. The clinician therefore relies on the laboratory to identify the virus. Many clinicians commonly diagnose patients syndromically with influenza or influenza-like illness without laboratory identification of a virus. The positive predictive value (PPV) of a clinical diagnosis of influenza virus infection in an adult case ranged from 18% to 87% compared with cases of laboratory-confirmed influenza virus infection. During periods of high INF virus activity, a clinical diagnosis based on acute onset of high fever and cough can be highly predictive of influenza (PPV, 79% to 87%; negative predictive value [NPV], 39% to 75%). The consequences of not identifying INF virus in a nursing home or on a hospital ward could be catastrophic. INFVs outbreaks in a hospital can be devastating given the wide range of immunocompromised patients (cancer patients and transplant recipients) that are highly susceptible to life-threatening influenza virus infection. In either setting, specific antiviral agents such as M2 channel inhibitors (amantidine and rimantidine) or NA inhibitors (oseltamivir and zanamavir) can be prescribed; however, these drugs are effective only when given within the first 24 h following infection. The traditional methods by which respiratory viruses are routinely diagnosed: virus culture, serology, immunofluorescence/antigen detection, and nucleic acid/PCR-based tests. The gold standard for the diagnosis of respiratory virus infections, virus isolation by culture, takes days to weeks (shell vial assays are not available for the diagnostics of all respiratory viruses), and many new viruses remain unculturable [39]. Virus culture consists in infecting cell lines with clinical samples; virus isolation is performed using three or four cell lines and, together with embryonated hen eggs for INFV, provides the means for isolating respiratory

viruses. Tissue cultures can take up to 15 days, therefore, the infection can often be resolved before the infectious agent is defined. Shell vial culture (SVC), first described in the early 1990s for murine Cytomegalovirus (CMV) is a modification of the conventional cell culture technique for rapid detection of viruses *in vitro* that involves inoculation of the clinical specimen on to cell monolayer grown on a cover slip in a shell vial culture tube, followed by low speed centrifugation and incubation. In this system the low speed centrifugation enhances viral infectivity to the susceptible cells because it produces a minor trauma to the cell surface by the low speed centrifugation mechanical force and enhances the viral entry in to the cells, reducing the total time taken for the virus to produce infection of cells. Shell vials of R-Mix, a combination of mink lung cells and human adenocarcinoma cells (strains Mv1Lu and A549) enable the detection of respiratory viruses from prospective clinical respiratory specimens [40]. The rapidity of the technique without any compromise on sensitivity has made SVC very popular in the field of clinical virology [41]. SVC as traditional cell culture assay requires specific technical and manual skills and is performed only in specialized laboratories. Isolation in cell culture, with the traditional method or the SVC assay is time consuming and have low sensitivity, but it is still considered the gold standard for the diagnosis of viral infections because molecular methods may not necessarily indicate that the virus is causing disease, viral RNA has been detected in asymptomatic children and during viral persistence. For these reasons traditional cell culture and SVC assay are still routinely used diagnostically and have a predominant role in epidemiological studies, are used to follow the course of an infection. A variety of serological tests including the hemagglutination inhibition (HAI) test, complement fixation, and enzyme immunoassay (EIA) are used for testing paired acute- and convalescent-phase sera for diagnosing infections, and in the case of INFV, HAI is able to subtype the virus as being H1 or H3 virus. EIAs was introduced in the 1980s and 1990s, lacks in sensitivity and is usually relegated to point-of-care testing in defined settings. CFT and haeagglutination-inhibition HAI techniques are usually used for serology. Any serological diagnosis is going to be retrospective because the antibody response to a viral infection can take 2 weeks to develop, but serological tests as cell culture assays are used in epidemiological studies. Serology assays that test blood samples for either virus-specific antibodies or viral antigen by a functional assay are labor-intensive and slow to produce results. Direct fluorescent antibody (DFA) staining of cells derived from nasopharyngeal swabs or nasopharyngeal aspirates (NPA) became the mainstay for many laboratories and provide a rapid test result in about 3 h. Direct fluorescent antibody (DFA) but suffers from low sensitivity and is available for only a limited number of respiratory viruses [42]. Molecular tests are more sensitive than other diagnostic approaches, including virus isolation in cell culture, SVC), DFA staining, and EIA, and now form the backbone of clinical virology laboratory testing around the world. The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA or cDNA across several orders of magnitude, generating thousands to millions of copies of a particular sequences. Reverse transcription-PCR (RT-PCR), that produces a cDNA template from an RNA template and amplify the cDNA target is a highly sensitive method for diagnosis of viral infection and has been used successfully in children with RSV. This method was found to be 100-fold more sensitive than single-round PCR and was capable of detecting 0.05 PFU of tissue culture-passaged virus. Multiplex polymerase chain reaction (PCR) assays introduced in the last ten years to avoid separate amplifications

of the viruses under investigation that are resource intensive, time consuming and labor intensive and can detect up to 19 different viruses in a single test using numerous primer couples that have the same annealig temperature. Several multiplex PCR tests are now commercially available and tests are working their way into clinical laboratories, but the majority of the multiplex PCR assays have not included recently discovered respiratory pathogens and require validation of results by post PCR hybridization or semi/nested PCR which make the assay cost ineffective and increases chances of cross contamination. The appearance of eight new respiratory viruses, including the SARS CoV in 2003 and swine-origin INF A/H1N1 in 2009, in the human population in the past nine years has tested the ability of virology laboratories to develop diagnostic tests to identify these viruses. Nucleic acid amplification procedures including PCR, nucleic acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP) were developed for most respiratory viruses by the end of the decade, and today, these highly sensitive NAATs are used in the routine clinical laboratory for detecting respiratory viruses. The profile of viruses detected in RTIs is changing due to the increasing use of nucleic acid-based diagnostic screens and the discovery of newly isolated viruses. Knowledge of the infecting agent does not routinely alter treatment except insofar as a positive viral identification will reduce the inappropriate use of antibiotics and may allow the cohorting of patients to reduce nosocomial infection. Several "new" viruses have been characterized, in part triggered by especially RT-PCR. Recently isolated respiratory viral agents include human hMPV, found in samples from children with RSV-like bronchiolitis who were RSV negative; hBoV, discovered by a random PCR screen of respiratory tract samples; and two new polyomaviruses, WU and KI. The discovery of new agents of infection is important because they may play a role as coinfecting agents, altering disease severity. Newly discovered viruses may also be important in future outbreaks; for example, the severe acute respiratory syndrome (SARS) caused by a CoV [43]. Polymerase chain reaction (PCR) testing is rapid and highly sensitive. At times it seems that it has supplanted culture isolation as a new gold standard for the detection of respiratory viruses in a research setting. However, most PCR tests target only 1 virus at a time, making these assays cumbersome when screening a clinical specimen for all viruses that have a PCR test available. Moreover, reliable PCR assay need to be developed to detect or identify novel viruses. Molecular technology has better sensitivity and the development of multiplex amplifications makes it possible to detect a broader panel of viruses. ADV, PIV, hCoV, BoV can now be detected by multiplex assays. These assays are based on different types of technology, such as ligation-dependent probe amplification (MLPA1), dual priming oligonucleotide (DPO) technology, target specific primer extension (TSPE), or target-specific extension (TSE). For the simultaneous detection of up to 20 viruses, a number of multiplex PCR assays have been proposed [44, 45, 46, 47, 48]. It is believed that PCR has replaced tissue culture and serology as the gold standard for the detection of respiratory viruses owing to its speed, availability and versatility. Even if molecular detection has many proven advantages over standard virological methods, tissue culture remains an important method for detecting novel viral mutations within a virus population, for the detection of novel viruses and for phenotypic characterization of viral isolates [48]. Recently, DNA microarray testing has emerged as a promising new technology for broad-spectrum virus detection [49, 50, 51]. Panviral DNA microarrays represent the most robust approach for massively parallel viral surveillance and detection. The Virochip (Virochip; University of California San Francisco

[UCSF]) is a panviral DNA microarray capable of detecting all known viruses, as well as novel viruses related to known viral families in a single assay. The Virochip has been used to indentify SARS, Xenotropic murine leukemia virus-related (a novel Retrovirus) from patients with familial prostate cancer, and a novel clade of human RV [52]. The Virochip has also proven to be successful in a clinical veterinary setting by successfully identifying a novel CoV from a beluga whale held in an aquatic containment facility and by identifying foot-and-mouth disease virus (FMDV) in ticks collected from a livestock market in Nairobi, Kenya. However, the usefulness and sensitivity of the Virochip platform have not been tested on clinical veterinary specimens [53]. In-house microarray platforms have been designed to detect all known viruses, as well as novel viruses related to known viral families (Virochip; University of California San Francisco [UCSF]). These Virochip consists of 22 000 oligonucleotide probes representing all 1800 fully or partially sequenced viruses in GenBank as of Fall 2004 [54]. The performance of the Virochip in respiratory virus detection has been tested using virally infected tissue culture cells and in selected patient cohorts, and it demonstrated high sensitivity and specificity [55]. To date, the Virochip has not been compared directly with standard diagnostic tests for viruses in a clinical setting; thus, the Virochip has been compared with conventional clinical DFA- and PCR-based testing in the detection of respiratory viruses associated with RTIs in children [56]. We report In this study the analysis of 10 clinical samples from patients, with respiratory tract infection symptoms that resulted negative for Influenza A(H1N1)v infection, and 8 samples from Quality Control for Molecular Diagnostics (QCMD) with known viral load of types and subtypes of 17 respiratory viruses, by the Clinical Array Technology (CLARTR) PneumoVir kit® (Geomica), a 120 spots array that make possible the specific identification of: INFV A, B and C; IPV 1, 2, 3 and 4 (subtypes A and B); RSV type A (RSV-A) and type B (RSV-B); RV; MPV (subtypes A and B); ENT (Echovirus); ADV; CoV and BoV. An internal control is included, to assure that the amplification step is performed successfully and to avoid false negative results. We used a proprietary image processing software, installed in a reader (SAICLART®), that is able to detect and resolve the genotypes automatically, avoiding the subjectivity that may introduce the user interaction, and provide fast, accurate and reproducible results. All samples have been analyzed three times and all results have been confirmed by single virus RT PCR (Roche) and Light Cycler, Roche, detector. All viruses detected by PneumoVir kit in the analyzed samples have been confirmed by RT PCR (Roche), in some cases at a sensitivity level higher than what was declared from the manufacturer. We detected single infection with: RSVA, MPV A, Coronavirus 229. One coinfection of MPV A, RSV A, RSV B, one coinfection of Corona 229, IPV3, RSV A, and one coinfection of BoV, MPV A. Only in the sample with the coinfection Corona 229, IPV3, RSV A, the Real Time PCR did not confirm the presence of IPV3 genome. Therefore we believe that the CLART can readily detect respiratory viruses in various clinical respiratory samples (pharyngeal and nasal swabs, nasopharyngeal lavage, pharyngeal exudates). The signal intensity increased according to the viral titer. These data directly correlate sample viral titers with the successful detection by the CLART and highlight the importance of sensitivity when utilizing the Virochip platform in a clinical settings. The CLART positively identified respiratory viruses in the all QCMD samples randomly mixed demonstrating high specificity inside the range of the sensitivity of the method. Together, the data in this report demonstrate that the CLART can successfully detect respiratory viruses frequently found in human respiratory swabs. The ability of the CLART

to positively detect viruses with a high degree of genetic variance, as is found in the specimens tested here, is a benefit that may outweigh concerns regarding costs and turnaround time. Furthermore, the advantages in the technical effort, cost, and turnaround time involved in using the CLART as a viral discovery platform far exceed those of next-generation sequencing platforms. In conclusion CLART provides, at very competitive cost, a system capable of detecting and identifying simultaneously several respiratory viruses, in clinical specimens with high sensitivity and specificity.

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