

# Investigating the aetiology of respiratory tract infections in children admitted to Tygerberg Children's Hospital using molecular methods and viral culture



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## Abstract

### Introduction

Acute respiratory tract infections cause significant morbidity and mortality worldwide, and are the main reason for the utilisation of health care services. Identifying the aetiological cause of lower respiratory tract infections (LRTIs) is difficult at the best of times, and more than 20 viruses and bacteria have been associated with LRTIs, which cannot be distinguished with clinical examination alone. Viruses can be detected in respiratory samples by a variety of methods, and without exception molecular methods have proven to be more sensitive than non-molecular-based tests. The increased sensitivity of molecular methods may assist in expanding our knowledge of the pathogenesis of severe respiratory tract infections, and could have a positive influence on patient management, infection control, vaccination strategies and public health.

### Aims and objectives

1. Determine the viral causes of lower respiratory tract infections requiring admission in using shell vial culture with immunofluorescent staining and two multiplex PCR assays, the Seeplex<sup>®</sup> RV15 ACE Detection system (Seeplex<sup>®</sup> RV15 ACE) and the Respiratory Multiplex Real-Time RT-PCR LightMix<sup>®</sup> Customised Kit (Resp Multiplex RT-PCR).
2. Compare the Seeplex<sup>®</sup> RV15 ACE and the Resp Multiplex RT-PCR with shell vial culture for the detection of respiratory viruses in routine diagnostic respiratory samples.
3. Examine the demographic and clinical characteristics associated with each respiratory viral pathogen.

### Materials and Methods

One hundred and thirty-eight paediatric patients, admitted to Tygerberg Children's Hospital from May 2010 to August 2010 with a presumptive diagnosis of an acute respiratory tract infection were included in the study. Nasopharyngeal or tracheal aspirates were collected, and all samples were tested by all three diagnostic methods. Clinical, demographic and laboratory data were collected through a systematic review of medical and laboratory records and subsequently anonymised.

## Results

Thirty-seven viruses were detected in 36 samples (26.1%) by shell vial culture with immunofluorescent staining; 169 viruses in 102 samples (73.9%) with the Seeplex® RV15 ACE; and 90 viruses in 73 samples (52.9%) with the Resp Multiplex RT-PCR. Shell vial culture had excellent specificity, but low sensitivity for all of the respiratory viruses. Conversely, the Seeplex® RV15 ACE had excellent sensitivity for all viruses, but slightly lower specificity. This was due to the detection of additional viruses, which may have been true positives due to the increased sensitivity of this assay. The Resp Multiplex RT-PCR had excellent sensitivity and specificity.

At least one respiratory pathogen could be identified in 80% of the patients. At least one virus was detected in 57% of patients, bacterial micro-organisms in 6%, and both viral and bacterial pathogens in 17%. Viral-bacterial co-infections were associated with increased severity compared to other infections, as these children were more likely to receive steroids and a blood transfusion ( $p = 0.002$ ), and more likely to require mechanical ventilation ( $p < 0.001$ ) and admission to the intensive care unit ( $p = 0.04$ ).

## Conclusions

We confirmed that molecular techniques are significantly more sensitive than shell vial culture for the detection of respiratory viruses in children. Due to their highly specific nature and the genetic variability observed in viruses, an excellent, continuous quality control programme is essential to ensure the continued superiority of these assays. Viral-bacterial co-infection is associated with increased severity of LRTIs in children. Further research is needed to elucidate the precise pathogenic and immunologic mechanism of this interaction.

# Opsomming

## Inleiding

Akute lugweg infeksies is verantwoordelik vir beduidende morbiditeit en mortaliteit wêreldwyd en is die hoofrede vir die benutting van gesondheidsdienste. Identifisering van die oorsaak van laer lugweg infeksies is baie moeilik en meer as 20 virusse en bakterieë word hiermee geassosieer. Ongelukkig kan kliniese ondersoek alleen nie onderskei tussen die verskillende organismes nie. Respiratoriese virusse kan deur 'n wye verskeidenheid van toets metodes aangetoon word. Molekulêre metodes is sonder uitsondering meer sensitief as nie-molekulêre metodes. Hul verhoogde sensitiwiteit mag help om ons kennis oor die patogenese van erge lugweg infeksies te verbreed en kan 'n positiewe invloed op pasiëntbehandeling, infeksiebeheer, immunisasie strategieë en publieke gesondheidsorg hê.

## Doel van die Onderzoek

1. Bevestig die virale oorsake van laer lugweg infeksies deur gebruik te maak van "shell vial" kultuur met immunofluoressensie en twee veelvoudige molekulêre toetse, die Seeplex® RV15 ACE en die Resp Multiplex RT-PCR.
2. Vergelyk die Seeplex® RV15 ACE en die Resp Multiplex RT-PCR met "shell vial" kultuur vir die aantoning van respiratoriese virusse in roetine diagnostiese monsters.
3. Onderzoek die demografiese en kliniese eienskappe wat met elke respiratoriese patogeen geassosieer word.

## Metodiek en Materiaal

Een honderd agt-en-dertig kinders wat toegelaat is tot Tygerberg Kinderhospitaal vanaf Mei 2010 tot Augustus 2010 met 'n voorlopige diagnose van 'n akute lugweg infeksie is in die studie ingesluit. Nasofaringeale of trageale aspirate is van elke pasiënt gekollekteer en met al drie diagnostiese metodes ondersoek. Kliniese, demografiese en laboratorium data is gekollekteer deur 'n sistematiese ondersoek van mediese en laboratorium rekords en daarna anoniem gemaak.

## Resultate

Sewe-en-dertig virusse is in 36 monsters (26.1%) aangetoon deur “shell vial” kultuur met immunofluoressensie; 169 virusse in 102 monsters (73.9%) deur die Seeplex® RV15 ACE; en 90 virusse in 73 monsters (52.9%) deur die Resp Multiplex RT-PCR. “Shell vial” kultuur het uitstekende spesifisiteit gehad, maar sensitiwiteit was laag vir al die virusse. Teenoorgesteld hiermee het die Seeplex® RV15 ACE hoë sensitiwiteit vir al die virusse gehad, maar eeffe laer spesifisiteit. Dit was as gevolg van die aantoning van addisionele virusse, wat moontlik ware positiewe resultate kon wees as gevolg van die verhoogde sensitiwiteit van hierdie toets metode. Die Resp Multiplex RT-PCR het uitstekende sensitiwiteit en spesifisiteit gehad.

Ten minste een respiratoriese patogeen is in 80% van die pasiënte geïdentifiseer. Een of meer virusse was in 57% van die pasiënte aangetoon, bakterieë in 6% en beide virale en bakteriële patogene in 17%. Virale-bakteriële ko-infeksies, in vergelyking met ander infeksies, was geassosieer met meer ernstige lugweg infeksies aangesien hierdie kinders meer geneig was om steroïede en ’n bloedtransfusie te ontvang ( $p = 0.002$ ). Hulle het ook meer waarskynlik meganiese ventilasie ( $p < 0.001$ ) en toegang tot die intensiewe sorg eenheid benodig ( $p = 0.04$ ).

## Gevolgtrekkings

Ons het bevestig dat molekulêre tegnieke aansienlik meer sensitief is as “shell vial” kultuur vir die aantoning van respiratoriese virusse in kinders. As gevolg van hul hoogs spesifieke aard en die genetiese variasie waargeneem in virusse, is ’n uitstekende deurlopende kwaliteitsbeheer program noodsaaklik vir die voortgesette uitneemendheid van hierdie metodes. Virale-bakteriële ko-infeksies word geassosieer met meer ernstige laer lugweg infeksies in kinders. Verdere navorsing is nodig om die presiese patogenetiese en immunologiese meganismes van hierdie interaksie toe te lig.

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## Abbreviations

<b>8-MOP</b>	– 8-methoxypsoralen
<b>ARTI</b>	– Acute respiratory tract infection
<b>bp</b>	– Base pairs
<b>cDNA</b>	– Complimentary DNA
<b>CesA3</b>	– Cellulose synthase 3
<b>CHERG</b>	– Child Health Epidemiology Reference Group
<b>CMV</b>	– Cytomegalovirus
<b>CRP</b>	– C-reactive protein
<b>Ct-value</b>	– Cycle threshold value
<b>DFA</b>	– Direct fluorescent antibody assay
<b>DNA</b>	– Deoxyribonucleic acid
<b>DPO</b>	– Dual priming oligonucleotide
<b>dsDNA</b>	– Double-stranded DNA
<b>dNTP</b>	– Deoxyribonucleotide triphosphate
<b>FITC</b>	– Fluorescein-5-isothiocyanate
<b>HBoV</b>	– Human bocavirus
<b>HCoV</b>	– Human coronavirus
<b>HEp-2</b>	– Human laryngeal carcinoma
<b>HEV</b>	– Human enterovirus
<b>HF</b>	– Human fibroblast
<b>HIV-1</b>	– Human immunodeficiency virus type 1
<b>HMPV</b>	– Human metapneumovirus
<b>HPeV</b>	– Human parechovirus
<b>HRV</b>	– Human rhinovirus
<b>ICU</b>	– Intensive care unit
<b>IFA</b>	– Indirect fluorescent antibody assay
<b>IQR</b>	– Interquartile range
<b>LAIV</b>	– Live-attenuated influenza vaccine
<b>LRTI</b>	– Lower respiratory tract infection
<b>MA</b>	– Marker for Set A
<b>MB</b>	– Marker for Set B

<b>MC</b>	– Marker for Set C
<b>MDCK</b>	– Madin-Darby Canine Kidney
<b>NAATs</b>	– Nucleic acid amplification techniques
<b>NC</b>	– Negative control
<b>NNMDs</b>	–Neurologic and neuromuscular disorders
<b>ND</b>	–Not determined
<b>PBS</b>	– Phosphate buffered saline
<b>PC</b>	– Positive control
<b>PCR</b>	– Polymerase chain reaction
<b>PIV1</b>	– Human parainfluenza virus type 1
<b>PIV2</b>	– Human parainfluenza virus type 2
<b>PIV3</b>	– Human parainfluenza virus type 3
<b>PIV4</b>	– Human parainfluenza virus type 4
<b>Resp Multiplex RT-PCR</b>	– Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit
<b>RNA</b>	– Ribonucleic acid
<b>rpm</b>	– Revolutions per minute
<b>RSV</b>	– Respiratory syncytial virus
<b>RT-PCR</b>	– Reverse transcription PCR
<b>SARS</b>	– Severe acute respiratory syndrome
<b>Seeplex® RV15 ACE</b>	– Seeplex® RV15 ACE Detection System
<b>Sens</b>	– Sensitivity
<b>Spec</b>	– Specificity
<b>ssDNA</b>	– Single-stranded DNA
<b>ssRNA</b>	– Single-stranded RNA
<b>SVC</b>	– Shell vial culture
<b>TIV</b>	– Trivalent inactivated influenza vaccine
<b>URTI</b>	– Upper respiratory tract infection
<b>USA</b>	– United States of America
<b>UV</b>	– Ultraviolet
<b>V</b>	– Volt
<b>VTM</b>	– Viral transport medium
<b>w/v</b>	– Weight per volume

## Chapter 1

# Literature review

### 1.1 Introduction

Acute respiratory tract infections (ARTIs) cause significant morbidity and mortality worldwide, and are the main reason for the utilisation of health care services. Upper respiratory tract infections (URTIs) such as rhinitis, pharyngitis and laryngitis occur frequently, with approximately 6 to 9 infections per year in children and 2 to 4 infections per year in adults (Templeton, 2007). Complications of URTIs include acute otitis media, asthma exacerbations, and lower respiratory tract infections (LRTIs) such as pneumonia, bronchitis, and bronchiolitis. According to the Child Health Epidemiology Reference Group (CHERG) an estimated 156 million episodes of childhood pneumonia occurred worldwide in 2000, of which more than 95% were in developing countries and 7% to 13% require hospitalisation (Rudan et al., 2008). Worldwide approximately 1.4 million children under the age of 5 years died due to pneumonia in 2010, with more than three quarters occurring in Africa and South-East Asia (Liu et al., 2012). Almost half of the global deaths due to pneumonia in children less than 5 years of age occur in Africa, whilst less than one fifth of the world's population in this age group live on the continent (Rudan et al., 2008). The number of pneumonia diagnoses made in children decrease with increasing age; from 36 per 1 000 children between 1 and 5 years, to 16 per 1 000 children between 5 and 14 years of age (Jokinen et al., 1993).

### 1.2 Aetiology of lower respiratory tract infections in children

Identifying the aetiological cause of lower respiratory tract infections in children is difficult at the best of times as suitable specimens can seldom be obtained from the lower respiratory tract, and the difficulties in differentiating colonisation or latent infection from active infection. Even more so in developing countries where the necessary diagnostic tools, including invasive methods such as lung biopsies and bronchoalveolar lavage, not to mention diagnostic tests, are not readily available.

Relatively few studies have investigated the aetiology of community-acquired pneumonia in children comprehensively. This is mainly due to the fact that ARTIs may be caused by such a wide spectrum of bacteria and viruses that it is difficult to detect all in a single study, and that many of them require specialised diagnostic tests that are only available at great expense in research laboratories.

Evidence of a possible causative agent has been identified in 42% to 90% of cases, depending on the nature and number of tests utilised in the study. Bacteria are responsible for up to 47%, viruses for up to 39% and mixed viral-bacterial infections for up to 45% of paediatric pneumonia cases (Sinaniotis, 2004; Table 1.1). The main bacterial causes of childhood pneumonia in developing countries are *Streptococcus pneumoniae*, *Haemophilus influenzae type B*, *Staphylococcus aureus* and *Klebsiella pneumoniae* (Rudan et al., 2008).

<b>Table 1.1 Mixed aetiology of paediatric community-acquired pneumonia</b>						
<b>Study</b>	<b>Country</b>	<b>n</b>	<b>Virus only n (%)</b>	<b>Bacteria only n (%)</b>	<b>Mixed viral-bacterial n (%)</b>	<b>Aetiology detected (Total) n (%)</b>
Juvé n et al., 2000	Finland	254	82 (32)	56 (22)	77 (30)	215 (85)
Michelow et al., 2004	USA	154	29 (19)	57 (37)	36 (23)	122 (79)
Cevey-Macherel et al., 2009	Switzerland	99	33 (33)	19 (19)	33 (33)	85 (86)
Hamano-Hasegawa et al., 2008	Japan	1 700	473 (28)	585 (34)	258 (15)	1316 (77)
Tsolia et al., 2004	Greece	75	28 (37)	9 (12)	21 (28)	58 (77)
Okada et al., 2012	Japan	903	311 (34)	253 (28)	173 (19)	737 (82)
Lahti et al., 2009	Finland	76	8 (11)	26 (34)	34 (45)	68 (90)

The leading viral cause of childhood pneumonia is respiratory syncytial virus (RSV), as it accounts for up to 40% of hospitalisations for LRTIs. It is followed by influenza A and B, parainfluenza virus types 1 to 3, and adenovirus (Rudan et al., 2008). However, in addition to these viruses, other viruses have recently been identified as causative agents of ARTIs in children. Until recently, rhinoviruses and coronaviruses were thought to only be common cold agents, but recent studies have shown that they can also cause significant lower respiratory tract disease (El-Sahly et al., 2000). Human metapneumovirus (HMPV) and human bocavirus (HBoV), both identified approximately 10 years ago, have also been associated with ARTIs (Allander et al., 2005; van den Hoogen et al., 2001). Originally it was thought that viruses are responsible for a decreasing percentage of pneumonia cases as the age of the patient group increases (Sinaniotis, 2004), but recent studies utilising advanced molecular techniques indicate that viruses remain an important aetiological agent and may be responsible for a third of pneumonia cases in adults (Jennings et al., 2008; Johansson et al., 2010).



In the study by Juvén and colleagues (2000) 254 Finnish children, hospitalised for community-acquired pneumonia over three years, were investigated. Evidence of an aetiological agent was found in 85% of the patients using a variety of diagnostic techniques. They found a significant difference in the percentage of viral infections in children less than 2 years of age (80%) compared to children more than 5 years of age (37%), whereas the number of bacterial infections remained fairly stable over age (47% vs. 58%). This observation was confirmed by Michelow and colleagues (2004) and Hamano-Hasegawa and colleagues (2008), but only for *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*. The most common viral-bacterial co-infection was human rhinovirus (HRV) with *Streptococcus pneumoniae*, which appears to elicit a stronger immune response than either single infection (Juvén et al., 2000; Lahti et al., 2009). Viral infections appear to cause both structural and functional changes to the respiratory epithelium, leading to enhanced bacterial attachment, immune dysfunction and subsequent super-infection with diminished response to antibiotic therapy (Bakaletz, 1995).

Infections with multiple viruses have been detected in 6.5% to 27% of children presenting with ARTIs. Viruses commonly involved in dual infections include HBoV, RSV, HRV, influenza virus and HMPV (Bonzel et al., 2008; Canducci et al., 2008; Cilla et al., 2008; Do et al., 2011; Richard et al., 2008). There is conflicting evidence regarding the association between dual infections and an increase in disease severity. A number of studies have found an association between dual viral infection and increased disease severity (Cilla et al., 2008; Richard et al., 2008; Semple et al., 2005), whilst others did not (Canducci et al., 2008; Wolf et al., 2006). Richard and colleagues (2008) found that dual-infected children with bronchiolitis have a three times higher risk of intensive care unit (ICU) admission than those with a single infection, with the risk increasing to ten-fold when it is a RSV and HMPV co-infection (Semple et al., 2005).

### **1.2.1 Viral causes of respiratory tract infections**

Several viruses have been linked to both upper and lower respiratory tract infections in children and adults. The basic virological properties of the most important of these viruses are listed in Table 1.1 and Table 1.2 and these viruses are discussed individually.

Cytomegalovirus (CMV) is a ubiquitous, opportunistic pathogen that can cause severe disease, most commonly pneumonia, in patients with impaired cellular immunity, including haematopoietic stem cell (Paris et al., 2009) and solid organ transplant recipients (Johansson et al., 2010), patients with haematologic malignancies (Chemaly et al., 2005), and HIV-1-infected patients (Zampoli et al., 2011). Amongst solid organ transplant recipients, lung transplant recipients are at particular risk for CMV

disease, and CMV disease increases the risk of developing bronchiolitis obliterans in these patients. It occurs most frequently in the first year after transplantation, and decreases the patient's ten-year survival rate by up to 26% (Johansson et al., 2010). In a study conducted in Cape Town, South Africa amongst children with severe pneumonia, HIV-1-infected children were three times more likely to have CMV pneumonia than HIV-1-uninfected children (Zampoli et al., 2011). More than one third of the patients with CMV pneumonia in this study were co-infected with *Pneumocystis jiroveci*, and co-infection has been associated with increased mortality (Boonsarngsuk et al., 2009). More than half of the patients required intensive care, and 25% of all patients with CMV pneumonia passed away during admission. In HIV-1-infected children with severe pneumonia failing to respond to conventional treatment, CMV is responsible for up to 72% of these cases (Goussard et al., 2010). These findings suggest that HIV-1-infected children with severe pneumonia should be treated empirically with gancyclovir until CMV disease can be ruled out.

Human parechovirus (HPeV), like human rhinovirus and human enterovirus, belongs to the family *Picornaviridae*, but has recently been classified into its own genus, *Parechovirus*. Predominantly it has been associated with gastro-intestinal, central nervous system and respiratory diseases (Ehrnst and Eriksson, 1993). Between 13% and 50% of children diagnosed with HPeV infection report respiratory symptoms, including both the upper and lower respiratory tract (Abed and Boivin, 2006; Ehrnst and Eriksson, 1993). HPeV has been detected in 1.2% of respiratory tract specimens collected over a one year period in Scotland (Harvala et al., 2008), and associated respiratory diseases include acute otitis media, sinusitis, conjunctivitis, croup, bronchiolitis and pneumonia (Abed and Boivin, 2006; Berkovich and Pangan, 1968; Watanabe et al., 2007). Another respiratory virus has been co-detected in 20.8% to 70.3% of cases, and in one study 33% of the patients had an underlying chronic medical condition (Abed and Boivin, 2006; Ehrnst and Eriksson, 1993; Harvala et al., 2008). HPeV has also been associated with respiratory disease outbreaks in paediatric wards (Berkovich and Pangan, 1968).

Two novel polyomaviruses, KI polyomavirus and WU polyomavirus, were first identified in 2007 in respiratory samples from patients, mostly children less than 5 years of age, with respiratory tract infections in Sweden and Australia (Allander et al., 2007a; Gaynor et al., 2007). WU polyomavirus has been detected in between 1% and 7% of patients with ARTI, with co-detection of another respiratory virus in 30.8% to 100% of the cases (Abed et al., 2007; Bialasiewicz et al., 2008; Foulongne et al., 2008; Gaynor et al., 2007; Han et al., 2007; Mourez et al., 2009; Norja et al., 2007). Only three studies included a control group, and WU polyomavirus was detected in 4.2% to 6.4% of asymptomatic patients (Abed et al., 2007; Han et al., 2007; Norja et al., 2007). KI polyomavirus has been detected in between 0.6% and

8% of patients with ARTI, with the highest detection rate observed in immunocompromised adults (Allander et al., 2007a; Bialasiewicz et al., 2008; Foulongne et al., 2008; Han et al., 2007; Mourez et al., 2009; Norja et al., 2007). Another respiratory virus was co-detected together with KI polyomavirus in 33% to 83.3% of cases. Only two studies included an asymptomatic control group, and KI polyomavirus was detected in none and 5.4% of the control patients (Han et al., 2007; Norja et al., 2007). Nonetheless, in some symptomatic cases, KI or WU polyomavirus was the only respiratory virus detected despite extensive screening (Gaynor et al., 2007; Han et al., 2007). Further studies are needed to define the precise role of these polyomaviruses in respiratory tract infections.

#### 1.2.1.1 Respiratory syncytial virus

Human respiratory syncytial virus (RSV) was first isolated not from a human, but from a laboratory-confined chimpanzee which displayed symptoms of an upper respiratory tract infection (Morris et al., 1956). Within a year the same virus was detected in infants exhibiting symptoms of respiratory tract infection (Chanock et al., 1957). Since then RSV has been recognised as the leading cause of acute lower respiratory tract infection in children less than 5 years of age worldwide (Hall et al., 2009; Nair et al., 2010; Weber et al., 1998). RSV is responsible for an estimated 33.8 million episodes of acute LRTI, 3.4 million hospitalisations and 66 000 – 190 000 deaths annually in children less than 5 years of age, with 99% of these deaths occurring in developing countries (Nair et al., 2010).

RSV is usually detected during autumn and winter in countries with temperate climates (Hall et al., 2009; Madhi et al., 2006; Manoha et al., 2007; Rodríguez-Auad et al., 2012) or during the rainy season in countries with tropical climates (Do et al., 2011; Weber et al., 1998). RSV can be divided into 2 subtypes (A and B), which can co-circulate during the same season, or one subtype can dominate over the other (Hall et al., 1990). There is conflicting evidence regarding the influence of subtype on disease severity, with either subtype A (Papadopoulos et al., 2004; Walsh et al., 1997) or B (Hornsleth et al., 1998) or neither (Kneyber et al., 1996) associated with more severe disease. Almost all children are infected with RSV within the first two years of life, with more than two-thirds of primary infections occurring within the first year. Primary infection after 12 months of age appears to be less severe than primary infection before this age with fewer cases of LRTI (5.9% vs. 21.6%) and hospitalisation (0% vs. 1.6%). Re-infection with RSV is common in the first five years of life, with more than 75% of children who had been infected in the first year of life being re-infected during the following year. The risk of re-infection and disease severity decreased with increasing number of prior infections and neutralising antibody titre (Glezen et al., 1986).

Table 1.2 Taxonomy of the main viral causes of acute respiratory tract infections					
Order	Family	Subfamily	Genus	Species	
Mononegavirales	Paramyxoviridae	Paramyxovirinae	Respirovirus	Human parainfluenza virus type 1	
				Human parainfluenza virus type 3	
			Rubulavirus	Human parainfluenza virus type 2	
				Human parainfluenza virus type 4	
Nidovirales	Orthomyxoviridae	Pneumovirinae	Pneumovirus	Human respiratory syncytial virus	
			Metapneumovirus	Human metapneumovirus	
		Influenzavirus A	Influenza A virus		
			Influenzavirus B	Influenza B virus	
		Coronaviridae	Coronavirinae	Alphacoronavirus	Human coronavirus 229E
					Human coronavirus NL63
Picornavirales	Picornaviridae		Betacoronavirus	Human coronavirus HKU1	
				Human coronavirus OC43	
				Human enterovirus A – D	
Picornavirales	Parvoviridae	Parvovirinae	Bocavirus	Human bocavirus	
				Mastadenovirus	Human adenovirus A – E

**Table 1.3: Basic characteristics of the main respiratory viruses**

Virus	Virion properties				Genome properties			
	Diameter (nm)	Enveloped vs. non-enveloped	Symmetry	Nucleic acid	Polarity	Length (kilobase pairs)	Segmented vs. non-segmented	
RSV	100 – 350	Enveloped	Spherical or filamentous	ssRNA	Negative	15	Non-segmented	
Influenza A	100 – 300	Enveloped	Spherical or filamentous	ssRNA	Negative	13.5	Segmented	
Influenza B	100 – 300	Enveloped	Spherical or filamentous	ssRNA	Negative	13.5	Segmented	
Adenovirus	90 - 120	Non-enveloped	Icosahedral	dsDNA		36	Non-segmented	
PIV1 - 4	150 – 200	Enveloped	Spherical	ssRNA	Negative	15	Non-segmented	
HCoVs	100 – 160	Enveloped	Spherical	ssRNA	Positive	27 – 32	Non-segmented	
HRV	30	Non-enveloped	Spherical	ssRNA	Positive	7.2 – 8.5	Non-segmented	
HEV	30	Non-enveloped	Spherical	ssRNA	Positive	7.2 – 8.5	Non-segmented	
HBoV	21 – 22	Non-enveloped	Icosahedral	ssDNA		5.5	Non-segmented	
HMPV	150 – 600	Enveloped	Spherical or filamentous	ssRNA	Negative	13	Non-segmented	

RSV has been detected in between 9.5% and 28.7% of children presenting with acute respiratory tract infections (Do et al., 2011; Hall et al., 2009; Juvén et al., 2000; Manoha et al., 2007; Noyola et al., 2007; Rodríguez-Auad et al., 2012), and in up to 89% of children with bronchiolitis (Semple et al., 2005). Coughing, fever, rhinorrhoea, wheezing and respiratory distress are the most frequent clinical findings detected in children with RSV infection. RSV has been associated with both upper and lower respiratory tract infections in children, including pharyngitis, otitis media, asthma, bronchiolitis and pneumonia (Hall et al., 2009; Manoha et al., 2007; Rodríguez-Auad et al., 2012). Severe bronchiolitis requiring hospitalisation in the first year of life appears to be an important risk factor for the subsequent development of recurrent wheezing and asthma throughout childhood and into early adulthood (Rooney and Williams, 1971; Sigurs et al., 2010). RSV has also been responsible for lower respiratory tract infections in both immunocompromised and immunocompetent adults (Dowell et al., 1996; Englund et al., 1988), and nosocomial outbreaks amongst both adults and children in high-risk units (Anak et al., 2010; Kassis et al., 2010).

RSV is responsible for up to 20% of all hospitalisations for ARTI in children with most of these occurring in children less than 6 months of age (Hall et al., 2009). Risk factors for increased disease severity and hospitalisation include prematurity (Hall et al., 2009; Madhi et al., 2006), chronic lung disease of prematurity (Boyce et al., 2000; Navas et al., 1992), congenital heart disease (Boyce et al., 2000; Navas et al., 1992), compromised immunity (Hall et al., 1986; Madhi et al., 2006), Down syndrome (Zachariah et al., 2012), younger age (El Saleeby et al., 2011; Hall et al., 2009), birth close to the start of RSV season (Boyce et al., 2000), lower admission weight, lack of breastfeeding and higher RSV viral load (El Saleeby et al., 2011). However, most cases of RSV infection, including those that require hospitalisation, occur in healthy children without any predisposing risk factors to predict disease severity (Boyce et al., 2000; Hall et al., 2009; Noyola et al., 2007). The mortality rate due to RSV infection has been estimated at between 0% and 4.3% with most of these deaths occurring in children with an underlying chronic disease (Hall et al., 2009; Madhi et al., 2006; Navas et al., 1992; Rodríguez-Auad et al., 2012).

Hospitalisation due to RSV can be prevented in certain high-risk groups with the intramuscular administration of palivizumab, a humanised murine monoclonal antibody against the fusion glycoprotein of RSV. It can decrease the rate of hospitalisation due to RSV by 39% to 78% in these groups (Feltes et al., 2003; The IMPact-RSV Study Group, 1998). Patients eligible to receive palivizumab during the RSV season, according to the American Academy of Pediatrics, include children less than 2 years of age with chronic lung disease of prematurity or haemodynamically significant congenital heart disease requiring

medical therapy; premature infants born less than 32 weeks gestation and premature infants with a gestational age between 32 and 35 weeks with at least one additional risk factor (Committee on Infectious Diseases, 2009). Unfortunately palivizumab is exceedingly expensive and thus inaccessible to most children in developing countries.

More recently motavizumab, a second humanised murine monoclonal antibody against RSV, was derived from palivizumab through the process of affinity maturation (Wu et al., 2007). In a phase 3 clinical trial conducted in several countries worldwide, motavizumab led to a 26% relative reduction in hospitalisation due to RSV compared to palivizumab (Carbonell-Estrany et al., 2010). Unfortunately, during this trial, children who received motavizumab had significantly more skin reactions than those who received palivizumab, and it was not developed further. A fully human monoclonal antibody against the attachment protein of RSV has recently been isolated from patients with laboratory-confirmed RSV using novel single-cell phenotyping technology. In a mouse model this monoclonal antibody was significantly more effective than palivizumab for both prevention and treatment of RSV (Collarini et al., 2009).

#### 1.2.1.2 Influenza virus

Although the first influenza virus was isolated in 1930 from swine (Shope, 1931) the first human influenza virus (influenza A virus) was isolated only three years later with the successful infection of ferrets with nasal washings from patients with influenza symptoms (Smith et al., 1933). It took another three years before the isolated virus could be transmitted successfully from a ferret back to humans during a laboratory incident (Smith and Stuart-Harris, 1936). Influenza B virus was first isolated in 1940 from a child during an outbreak of influenza-like illness in a convalescent home for children with rheumatic heart disease (Francis, 1940). The first influenza C virus was isolated in 1947 from a man with mild respiratory and constitutional symptoms (Taylor, 1949). Influenza A and B are responsible for annual seasonal influenza epidemics, while influenza pandemics have only been associated with influenza A virus (Glezen, 1996). Influenza C virus is primarily associated with mild, self-limiting upper respiratory tract infections, but a few cases of lower respiratory tract infection have been described (Moriuchi et al., 1991).

Annual influenza epidemics occur during the winter months in temperate climates, but influenza virus infections can be detected year round in tropical climates, with a peak in activity detected during the rainy season in some countries (Brooks et al., 2010; Do et al., 2011; Gessner et al., 2011; Neuzil et al., 2002; Peltola et al., 2003). The annual influenza attack rate in children varies from year to year, but can

be as high as 223 influenza infections per 1 000 children (Heikkinen et al., 2004; Neuzil et al., 2002). Influenza A virus has been detected in between 5.4% and 13.4% of children presenting with symptoms of any acute respiratory tract illness, with influenza B virus responsible for 1.4% to 7.8% of cases (Do et al., 2011; Heikkinen et al., 2004; Neuzil et al., 2002; Silvennoinen et al., 2009; Wishaupt et al., 2011). Infection with influenza A virus and influenza B virus present with the same general symptoms including fever, cough, rhinorrhoea, pharyngitis, headache, fatigue and myalgia (Brooks et al., 2010; Peltola et al., 2003; Silvennoinen et al., 2009). Several studies suggest that influenza B virus tends to infect older children and cause myalgia and gastrointestinal disease more often than influenza A virus (Chi et al., 2008; Peltola et al., 2003; Silvennoinen et al., 2009; Silvennoinen et al., 2011).

Influenza virus infection has been associated with a variety of ARTIs, including acute otitis media, croup, bronchiolitis, pneumonia and asthma exacerbations. Influenza viruses were responsible for only 30.4% of influenza-like illness cases in Greek children in a study by Pogka and colleagues (2011), but was the predominant agent in children older than 5 years. Influenza viruses are responsible for between 4% and 16% of all cases of community-acquired pneumonia in children, and most of these occur in children less than 2 years of age (Brooks et al., 2010; Juvén et al., 2000; Laundry et al., 2003). The role of influenza A virus in acute exacerbations of asthma may be underestimated as a recent study demonstrated that it was three times more common in non-hospitalised than hospitalised patients (Mandelcwaig et al., 2010). Influenza viruses are detected in only a small percentage of children with croup, and usually in children older than 5 years of age, but croup due to influenza virus tends to be more severe than croup due to parainfluenza virus (Denny et al., 1983; Peltola et al., 2002).

Influenza virus infection leads to between 36 and 135 hospitalisations per 100 000 children per year. The highest hospitalisation rate (276 per 100 000) is in children less than 6 months of age and more than 50% of all cases occur in children less than 2 years of age (Coffin et al., 2007; Heikkinen et al., 2004; Schrag et al., 2006; Silvennoinen et al., 2011). The risk for hospitalisation due to influenza in healthy children less than 2 years of age is similar to that of previously recognised high-risk adults and children (Rennels et al., 2002). Children spend an average of 3 days in the hospital and those with underlying cardiac or neurologic and neuromuscular disorders (NNMDs) are at increased risk of prolonged hospitalisation (Coffin et al., 2007; Dawood et al., 2010; Peltola et al., 2002; Peltola et al., 2003; Schrag et al., 2006; Silvennoinen et al., 2011). Between 25% and 49% of hospitalised children have at least one underlying high-risk medical condition, with asthma most often reported (Coffin et al., 2007; Dawood et al., 2002; Peltola et al., 2003; Schrag et al., 2006; Silvennoinen et al., 2011). Approximately 12% of children are admitted to the ICU with up to 5.9% requiring mechanical ventilation. Deaths in children due



to influenza are infrequent, less than 0.01%, but almost half of these occur in children without an underlying condition recognised to increase the risk of influenza-associated complications (Bender et al., 2010; Bhat et al., 2005; Coffin et al., 2007; Dawood et al., 2010; Schrag et al., 2006; Silvennoinen et al., 2011). Up to a quarter of children develop influenza-associated complications during their hospitalisation, with a higher incidence among children with a previously recognised high-risk condition than otherwise healthy children. Influenza-associated complications include secondary bacterial pneumonia, respiratory failure, seizures and other NNMDs, myositis, Reye's syndrome, myocarditis and pericarditis. Approximately one in five healthy children develop an influenza-associated complication, with the lowest complication rate in children less than 6 months of age (Coffin et al., 2007; Peltola et al., 2003). During influenza pandemics morbidity and mortality may be markedly increased as was seen during the recent pandemic in 2009 – 2010 (Committee of Infectious Diseases, 2010).

There are two classes of antiviral drugs currently available for the treatment and chemoprophylaxis of influenza virus infection, neuraminidase inhibitors and adamantanes. The adamantanes are currently not recommended for the treatment or prevention of influenza because recent influenza A virus isolates are resistant to these drugs, and they have no activity against influenza B virus (World Health Organization, 2010a). Oseltamivir, an orally administered neuraminidase inhibitor, has been approved for the treatment and prevention of influenza in adults and children older than 1 year of age (Fiore et al., 2011). Oseltamivir decreases the median duration of symptoms in influenza-infected children by 1.5 days if started within 48 hours after symptom onset (Whitley et al., 2001). The second neuraminidase inhibitor, zanamivir, is an inhaled powder and has been approved for the treatment of influenza in adults and children older than 7 years, and for chemoprophylaxis in adults and children older than 5 years of age (Fiore et al., 2011). Treatment with zanamivir decreases the median time to resolution of symptoms of influenza in children by 1.25 days if started within 36 hours of symptom onset (Hedrick et al., 2000). The use of neuraminidase inhibitors for chemoprophylaxis is currently not recommended as a consequence of recently described failures of post-exposure chemoprophylaxis due to the development of oseltamivir resistance, the widespread availability of vaccines for prevention and the preferential use of these drugs for treatment rather than prevention. Recommendations regarding the use of antivirals are influenced by both local and international influenza virus surveillance and resistance data, and clinicians should be aware of any relevant changes (World Health Organization, 2010a). Recently the effectiveness of the neuraminidase inhibitors in the treatment of influenza in healthy adults and children has been brought into question. During systematic review and meta-analysis of available data it was noted that most studies were manufacturer funded with limited results published and available for public scrutiny. The neuraminidase inhibitors were found to have only

a modest effect on the clinical course of influenza illness, and their ability to prevent influenza complications require further study (Jefferson et al., 2009; Shun-Shin et al., 2009; Wang et al., 2012).

Annual vaccination is the most effective way to prevent influenza infection and its consequences. Vaccination can reduce laboratory-confirmed influenza A virus illness in children by up to 95% (Neuzil et al., 2001). There are two vaccines, trivalent inactivated influenza vaccine (TIV) and live-attenuated influenza vaccine (LAIV), available worldwide for the prevention of influenza, but only TIV is available in South Africa currently (Fiore et al., 2010; Schoub, 2012). TIV is approved for use in adults and children older than 6 months of age, while LAIV is only approved for people between 2 and 49 years of age without underlying high-risk medical conditions (Committee on Infectious Diseases, 2010). Children younger than 9 years of age should receive two doses of vaccine one month apart if they have not been vaccinated previously, as children without pre-existing antibodies have lower antibody response rates after single vaccination (Neuzil et al., 2006). Although the World Health Organization recommends annual influenza vaccination for all children between 6 and 23 months of age due to the substantial health care burden of influenza in young children, the South African guidelines only recommend vaccination for children with an underlying medical condition that increases the risk of influenza and its complications (Schoub, 2010; World Health Organization, 2005). Several studies have shown that HIV-1-infected patients have a diminished immune response to influenza vaccination, especially those with high HIV-1 RNA viral loads and low CD4+ counts (Iorio et al., 1997; Kosalaraksa et al., 2011; Yamanaka et al., 2005). Therefore it is recommended in the United States of America that HIV-1-infected children, regardless of their degree of immunosuppression, follow the same recommendations as HIV-1-uninfected children with regards to annual influenza vaccination, with the exception that only the trivalent inactivated vaccine may be used in this group (Fiore et al., 2010). However, all HIV-1-infected children, and not only those less than 9 years of age, may benefit from two doses of TIV (Kosalaraksa et al., 2011).

### 1.2.1.3 Adenovirus

The first adenovirus was isolated in 1953 when Rowe and colleagues (1953) attempted to establish a tissue culture cell line from adenoidal and tonsillar tissue fragments. Adenoviruses were first associated with acute respiratory tract infection in 1954 when it was isolated from a military recruit with atypical pneumonia (Hilleman and Werner, 1954). Since then adenoviruses have been associated with a variety of infections, including both upper and lower respiratory tract infections, acute otitis media, pharyngoconjunctival fever, cystitis and acute gastroenteritis (Carrigan, 1997; Chen et al., 2004; Edwards et al., 1985; Faden et al., 2005; Larrañaga et al., 2000; Ruuskanen et al., 1985). Adenovirus infections

appear to occur more commonly in children, immunocompromised patients, and people living in close quarters, such as chronic care facilities and military barracks.

Adenoviruses have been detected in 4.2% to 21% of patients presenting with acute respiratory tract infections, depending on the population under evaluation, and can be detected year round, with epidemics frequently reported in the spring, early summer and early winter (Brandt et al., 1969; Brandt et al., 1972; Carrigan 1997; Chen et al., 2004; Do et al., 2011; Hong et al., 2001; Juvén et al., 2000; Larrañaga et al., 2000; Rocholl et al., 2004; Ruuskanen et al., 1985). Commonly reported symptoms include fever, cough, nasal congestion, rhinorrhoea, and abnormal breath sounds on auscultation (Chen et al., 2004; Edwards et al., 1985; Hong et al., 2001).

Adenoviruses have been detected in up to 12.6% of children presenting with LRTI, with just less than a third of these co-detected with another respiratory virus. In this particular study adenovirus was second only to RSV as a cause of viral pneumonia (Larrañaga et al., 2000). Pneumonia due to adenovirus infection can cause extensive necrosis, leading to death and severe sequelae such as bronchiectasis and bronchiolitis obliterans (Becroft, 1971). During an outbreak of adenovirus pneumonia in Chile in 1998, 18.4% of the children died and almost half of the survivors developed bronchiolitis obliterans (Castro-Rodriguez et al., 2006). Risk factors for the development of bronchiolitis obliterans included increased length of hospitalisation, intensive care admission with mechanical ventilation, and systemic  $\beta$ -agonist and corticosteroid use. The mortality rate due to adenovirus pneumonia can be as high as 85% in neonates (Abzug and Levin, 1991).

Community-acquired and nosocomial adenovirus respiratory tract infection outbreaks have been reported in paediatric care units, and chronic care facilities (Alpert et al., 1986; Castro-Rodriguez et al., 2006; Faden et al., 2005; Hatherill et al., 2004). Serotypes 3 and 7 are responsible for most of these, and the associated mortality rate is calculated to be between 12% and 29%. Risk factors associated with increased mortality include the presence of an underlying chronic condition, the need for mechanical ventilation and systemic corticosteroid use, and the diagnosis of adenovirus pneumonia or disseminated disease.

Immunocompromised children appear to be more susceptible to adenovirus infection than their adult counterparts. In a retrospective review of bone marrow transplant recipients at a single transplant centre, almost a third of the children were infected with adenovirus, while only 14% of adults were infected, making children 3.5 times more likely to be infected with adenovirus post-transplantation

(Carrigan, 1997). It was also noted that children tended to develop adenovirus-associated disease within the first 30 days post-transplantation, whereas most adults developed disease more than 90 days post-transplantation. Adenovirus pneumonia has also been associated with graft loss, development of bronchiolitis obliterans, and death in paediatric lung transplant recipients (Bridges et al., 1998).

#### 1.2.1.4 Human parainfluenza viruses

Human parainfluenza virus type 2 (PIV2) was first isolated in 1956 from two infants presenting with acute laryngotracheobronchitis (croup) (Chanock, 1956). Human parainfluenza virus type 1 (PIV1) was also initially isolated from three infants presenting with croup; while human parainfluenza virus type 3 (PIV3) was first isolated from 35 children presenting with acute respiratory illness (Chanock et al., 1958). Human parainfluenza virus type 4 (PIV4) was first isolated from a male college student with symptoms of acute upper respiratory tract infection in 1960 (Johnson et al., 1960). All four human parainfluenza viruses were proven to cause common cold symptoms in healthy volunteers, but with a longer incubation period than what is normally observed with human rhinovirus infections (Tyrrell and Bynoe, 1969; Tyrrell et al., 1959).

The human parainfluenza viruses can cause a wide variety of diseases, ranging from mild upper respiratory tract infections to severe lower respiratory tract infections requiring hospitalisation. They have been detected in up to 74.2% of children presenting with croup (Denny et al., 1983), with PIV1 detected most frequently. Several studies have noted an association between infection with the human parainfluenza viruses, especially PIV1 and PIV2, and croup, with a peak in hospitalisation due to croup coinciding with the peak of parainfluenza virus activity (Counihan et al., 2001; Downham et al., 1974; Hendrickson et al., 1994; Laurichesse et al., 1999; Marx et al., 1997; Reed et al., 1997). PIV1 and PIV2 are detected more frequently in children aged one to two years, while PIV3 and PIV4 are detected more often in children less than one year of age (Laurichesse et al., 1999; Reed et al., 1997). PIV3 is often associated with lower respiratory tract infections such as pneumonia and bronchiolitis, and has been detected in up to 73.6% of children presenting with pneumonia (Downham et al., 1974; Laurichesse et al., 1999). Children infected with PIV3 develop acute otitis media more often than those with PIV1 or PIV2 (Reed et al., 1997), and it has been responsible for a meningitis outbreak in a neonatal unit (Laurichesse et al., 1999). Both upper and lower respiratory tract infections due to PIV3 have been detected in immunocompromised children and adults, including haematopoietic stem cell and solid organ transplant recipients (Dignan et al., 2006; Luján-Zilbermann et al., 2001; Park et al., 2009; Wright and O'Driscoll, 2005). There is no difference in the clinical presentation of infection with the human parainfluenza viruses in HIV-1-infected versus HIV-1-uninfected children, but overall duration of

hospitalisation and mortality rate is higher (Madhi et al., 2002). Less data is available regarding the epidemiology and clinical features of PIV4 infection due to technical difficulties in virus isolation (Canchola et al., 1964), and the lack of rapid immunofluorescence and molecular detection methods until recently (Aguilar et al., 2000; Rubin et al., 1993). PIV4 has been detected mainly in children with both upper and lower respiratory tract infections, but a few cases in adults have been described (Billaud et al., 2005; Downham et al., 1974; Lau et al., 2009; Lindquist et al., 1997; Rubin et al., 1993; Vachon et al., 2006). It has also been detected in a number of children presenting with febrile convulsions or aseptic meningitis (Downham et al., 1974; Lau et al., 2009; Lindquist et al., 1997; Rubin et al., 1993). PIV4 was responsible for an outbreak of acute respiratory infection amongst institutionalised children in Hong Kong (Lau et al., 2005).

Only a relatively small number of studies have investigated the epidemiology of all four of the human parainfluenza viruses simultaneously (Aguilar et al., 2000; Billaud et al., 2005; Downham et al., 1974; Fry et al., 2006; Lau et al., 2009; Laurichesse et al., 1999; Rubin et al., 1993). PIV1 has been detected in 9.5% to 36.4% of all cases of human parainfluenza virus respiratory infection with an autumn to early winter biennial pattern. PIV2 has a similar seasonal pattern with smaller peaks in the late autumn and winter, and has been responsible for 3.1% to 12% of cases. PIV3 is the most commonly detected human parainfluenza virus (37.5% – 74.4%) with peak detection in summer and spring each year. Similar to PIV1 and PIV2, PIV4 can also be detected more frequently in the autumn and winter months, and is at least as prevalent as PIV2 with detection rates of 1.1% to 15.6%.

#### 1.2.1.5 Human coronaviruses

The first two human coronaviruses (HCoVs), HCoV-229E and HCoV-OC43, were isolated from people suffering from the common cold in the mid-1960s (Hamre and Procknow, 1966; McIntosh et al., 1967). Both strains were proven to cause the common cold in studies involving healthy adult volunteers (Bradburne and Somerset, 1972; Bradburne et al., 1967). The volunteers developed typical cold symptoms such as malaise and rhinorrhoea after inoculation, and symptoms generally lasted less than one week. Several subsequent studies have suggested that these HCoVs may be associated with more severe upper and lower respiratory tract infections in the elderly, infants and immunocompromised individuals (Falsey et al., 2002; Folz and Elkordy, 1999; Gagneur et al., 2008; McIntosh et al., 1974; Pene et al., 2003).

With the detection of a novel coronavirus, subsequently named severe acute respiratory syndrome (SARS)-related coronavirus, as the cause of an outbreak of severe acute respiratory syndrome

in 2003, interest in the human coronaviruses was renewed (Drosten et al., 2003). In 2004 another novel coronavirus, HCoV-NL63, was isolated in the Netherlands from a seven month-old infant with fever, coryza, conjunctivitis and bronchiolitis (van der Hoek et al., 2004). In January 2005 a third new coronavirus, HCoV-HKU1, was isolated in Hong Kong from an elderly gentleman with chronic obstructive pulmonary disease presenting with a two-day history of fever and productive cough (Woo et al., 2005). Since then both of the new human coronaviruses have been detected in both adults and children suffering from upper and lower respiratory tract infections worldwide (Arden et al., 2005; Dare et al., 2007; Dominguez et al., 2009; Gaunt et al., 2010; Gerna et al., 2007; Leung et al., 2009; Smuts et al., 2008; Talbot et al., 2009). A clear association between HCoV-NL63 and croup has been demonstrated in a population-based study in children less than three years of age in Germany (van der Hoek et al., 2005). Forty-five percent of the patients who tested positive for HCoV-NL63 alone had croup, in contrast to only 6% of the HCoV-NL63-negative group. Regression analysis indicated that the chance of croup was 6.6 times higher in HCoV-NL63-positive children than in HCoV-NL63-negative children. This association has been confirmed in several studies from the Far East (Han et al., 2007; Leung et al., 2009; Sung et al., 2010; Wu et al., 2008). Esper and colleagues (2005) suggested that HCoV-NL63 may also be associated with Kawasaki disease, but several subsequent studies failed to confirm this association (Baker et al., 2006; Dominguez et al., 2006; Lehman et al., 2009; Shimizu et al., 2005). In a large prospective study conducted in Hong Kong, febrile seizures were more likely to occur in children infected HCoV-HKU1 than those infected with HCoV-OC43, RSV, PIV1 and adenovirus, but for a significantly shorter time period (Lau et al., 2006). Human coronaviruses have also been associated with gastro-intestinal disease (Esper et al., 2010; Gerna et al., 1984; Risku et al., 2010) and multiple sclerosis (Burks et al., 1980; Stewart et al., 1992), but a causal relationship with either disease has not been established.

Peak circulation of human coronaviruses appear to be in the winter in temperate climates, with HCoV-OC43 and HCoV-HKU1 detected more frequently in the late autumn and early winter, and HCoV-NL63 in the late winter to early spring (Dominguez et al., 2009; Gaunt et al., 2010; Gerna et al., 2007). Seasonality appears to be very similar in subtropical Hong Kong (Lau et al., 2006), with the exception of HCoV-NL63, which was detected more frequently in the summer and fall. In tropical climates the human coronaviruses have been detected year-round (Dare et al., 2007). There also appears to be significant year-to-year variation in the circulation of human coronaviruses (Dare et al., 2007; Gaunt et al., 2010). Up to 10.3% of patients with ARTIs have tested positive for human coronaviruses worldwide, with between 30.5% and 50% of these patients co-infected with other respiratory viruses (Dare et al., 2007; Dominguez et al., 2009; Gagneur et al., 2008; Gaunt et al., 2010; Gerna et al., 2007; Lau et al., 2006; Talbot et al., 2009; van Elden et al., 2004). Unfortunately matched controls were included in only two

studies (Dare et al., 2007; van Elden et al., 2004), with HCoV detected in 2.1% and 0.4% of control patients, respectively. HCoV-229E has been associated with nosocomial viral respiratory infection outbreaks in paediatric and neonatal intensive care units (Gagneur et al., 2008).

#### 1.2.1.6 Human rhinovirus

The first human rhinovirus (HRV) was successfully cultured in embryonic human fibroblasts in 1953 (Andrewes et al., 1953), and by the late 1980s one hundred serotypes, divided into two groups (A and B), had been identified (Hamparian et al., 1987). Since then at least 50 more HRV strains have been identified by molecular methods. The majority of these were found to belong to a new group, group C, by analysis of full genome sequences (Palmenberg et al., 2009).

HRVs can be detected throughout the year with peaks in autumn and spring (Chung et al., 2007; Manoha et al., 2007; Monto et al., 1987; Smuts et al., 2011; Winther et al., 2006). HRVs cause common cold symptoms in healthy adult volunteers (Cate et al., 1964) and have been detected in up to 80% of patients presenting with common cold symptoms (Arruda et al., 1997). Symptoms last between five and seven days, and typically include sore throat, coughing, rhinorrhoea, nasal congestion, sneezing, malaise and headache. Human rhinoviruses have also been associated with acute otitis media (Vesa et al., 2001) and sinusitis (Pitkäranta et al., 1997). HRVs have been detected as the sole pathogen in middle ear fluid from children with acute otitis media (Arola et al., 1988) and have also been associated with poor response to antibiotics in bacterially co-infected patients (Sung et al., 1993).

In the last two decades evidence has emerged that HRVs are also involved in lower respiratory tract infections. Infection of bronchial epithelial cells by HRVs has been confirmed *in vitro* (Lopez-Souza et al., 2009) and *in vivo* (Gern et al., 1997; Papadopoulos et al., 2000) by experimental infection of healthy volunteers. Several studies, using molecular techniques, have linked HRV infection both epidemiologically and clinically with pneumonia, bronchiolitis, and acute exacerbations of chronic lung diseases such as asthma and chronic obstructive pulmonary disease. HRVs have been detected in 10% – 45% of children and in 4% – 8% of immunocompromised adults with acute LRTIs (Gern 2010). HRVs have been detected in up to 58% of children presenting with acute wheezing (Jartti et al., 2004; Smuts et al., 2011), and up to 40% of infants subsequently develop recurrent wheezing illness (Martinez et al., 1995). Detection of HRV, and not RSV, seems to be the most important predictor of this progression (Lemanske et al., 2005). Moreover, wheezing illness due to HRV infection at any time in the first three years of life is associated with an almost ten-fold increase in the risk of developing asthma by the age of six years, while wheezing illness due to RSV infection is associated with only a 2.6-fold increased risk (Jackson et al.,



2008). HRV infection also appears to be the leading cause of asthma exacerbations, as HRVs have been detected in 48% – 82% of children and 10% – 48% of adults presenting with this complaint (Gern 2010; Kling et al., 2005). This link is further strengthened by the observation that the incidence of asthma exacerbations appears to follow the same seasonal pattern, with peaks in autumn and spring, as HRV infections (Johnston et al., 1996). An association between increased severity of asthma exacerbation and HRV persistence for more than 6 weeks has also been noted (Kling et al., 2005).

#### 1.2.1.7 Human enterovirus

Although the majority of human enterovirus (HEV) infections are asymptomatic, they have been associated with a wide range of symptoms ranging from mild febrile illness to aseptic meningitis and severe neonatal sepsis. Infection with HEVs have also been associated with upper and lower respiratory tract infections, including pneumonia and bronchiolitis, in both children and adults (González et al., 1999; Jacques et al., 2008; Mizuta et al., 2003). A wide variety of non-polio enteroviruses have been detected in respiratory secretions, including echovirus 6, 11, 13, 16 and 30, coxsackie virus A2, A4, A16, B2 and B3, and enterovirus 68 (Jacques et al., 2008; Mizuta et al., 2003; Oberste et al., 2004), but only enterovirus 68 has been exclusively identified in patients with respiratory tract infections (Oberste et al., 2004).

In a multi-year study amongst French children by Jacques and colleagues (2008) they found a significant spring to fall seasonality with 47% of HEV infections occurring in these months, which was confirmed by Chung and colleagues (2007). Respiratory infection (31%) was the second most common HEV-induced disease entity after aseptic meningitis (47%), with bronchiolitis as the most frequently identified respiratory illness. HEV infection has been associated with 14% – 22% of patients presenting with URTI, and in 17% – 19% of patients presenting with LRTI (Billaud et al., 2003; Chung et al., 2007; Jacques et al., 2008). HEVs have also been detected in 25% of children presenting with acute wheezing (Jartti et al., 2004), and in 12% – 21% of children presenting with bronchiolitis (Andréoletti et al., 2000; Chung et al., 2007; Nascimento et al., 2010).

#### 1.2.1.8 Human bocavirus

Human bocavirus (HBoV) was first identified in 2005 by Allander and colleagues (2005) in respiratory tract samples by means of a novel detection method involving filtration and DNase treatment of samples, random polymerase chain reaction (PCR) amplification and cloning, followed by large-scale sequencing and bioinformatics. Since then HBoV has been detected worldwide, mainly in children under the age of two years with both upper and lower respiratory tract infections (Arnold et al.,



2006; Calvo et al., 2008b; Christensen et al., 2008; Longtin et al., 2008; Moriyama et al., 2010; Nascimento et al., 2010; Smuts and Hardie, 2006; von Linstow et al., 2008).

Human bocaviruses can be detected year-round, with peak detection rates reported in either autumn and winter (Lau et al., 2007; Smuts and Hardie, 2006; Smuts et al., 2008; von Linstow et al., 2008) or spring and early summer (Arnold et al., 2006; Moriyama et al., 2010). The prevalence of HBoV in children presenting with acute respiratory tract illness ranges from 2.5% to 19%, but has only been detected in 0.8% to 1.2% of adults (Allander et al., 2005; Allander et al., 2007b; Arnold et al., 2006; Bastien et al., 2006; Calvo et al., 2008b; Christensen et al., 2008; Do et al., 2011; Esposito et al., 2008; Lau et al., 2007; Longtin et al., 2008; Maggi et al., 2007; Moriyama et al., 2010; Nascimento et al., 2010; Smuts and Hardie, 2006; Smuts et al., 2008; von Linstow et al., 2008). Between 20% and 86% of the patients had symptoms suggestive of a lower respiratory tract infection, and between 17% and 37% of the patients had to be admitted to an intensive care unit. The most common symptoms reported were cough, rhinorrhoea, and fever. Other viruses were co-detected in 33% to 90% of cases where HBoV was detected. The most common co-infecting viruses were HRV, RSV, HEV, adenovirus, and HMPV. Only five of the studies mentioned above screened control patients for the presence of HBoV DNA. Three of the studies failed to detect HBoV in asymptomatic control patients (Allander et al., 2007b; Maggi et al., 2007; Moriyama et al., 2010), while the third study detected HBoV in 8.6% of the control patients (von Linstow et al., 2008). Surprisingly, the fifth study (Longtin et al., 2008) detected HBoV in 43% of the asymptomatic control patients, but in only 13.8% of the symptomatic patients. Two cases of nosocomial HBoV infection in a neonatal ICU have also been described (Calvo et al., 2008a). HBoV has also been detected in up to 19% of children presenting with acute wheezing, making it the fourth most common virus detected in these children after HRV, RSV, and HEV (Allander et al., 2007b; Smuts et al., 2008).

In a study by Arnold and colleagues (2006) it was noted that 16% of patients who tested positive for HBoV had diarrhoea, and not always in conjunction with respiratory tract symptoms. This observation warranted further investigation, as the animal bocaviruses are known to cause severe gastro-intestinal disease in dogs (Thomson and Gagnon, 1978) and calves (Durham et al., 1985). Vincente and colleagues (2007) examined 527 stool samples from children with acute gastroenteritis, irrespective of the presence of respiratory symptoms. HBoV was found in 9.1% of the samples, with other intestinal pathogens co-detected in more than half of them. HBoV was detected in only 2.1% and 0.8% of patients presenting with acute gastroenteritis in two other studies (Lau et al., 2007; Lee et al., 2007). In both of these studies other intestinal pathogens were also co-detected in more than half of the cases. Thus the precise role of HBoV in gastro-intestinal disease still needs to be determined.

### 1.2.1.9 Human metapneumovirus

Human metapneumovirus (HMPV) was first described in 2001 by van den Hoogen and colleagues (2001) after isolating the virus from 28 children with respiratory tract infections in the Netherlands. Since then it has been detected in both children and adults with upper and lower respiratory tract infections worldwide (Bastien et al., 2003; Ebihara et al., 2004; Ijpmma et al., 2004; Manoha et al., 2007; Nissen et al., 2002; Williams et al., 2004).

HMPV can be detected year-round with peak detection rates in winter and early spring, usually after the seasonal peak of RSV (Bastien et al., 2003; Døllner et al., 2004; Esper et al., 2004; Maggi et al., 2003; Manoha et al., 2007; Mullins et al., 2004; Williams et al., 2004). HMPV prevalence in children with acute respiratory tract infection ranges from 2.2% to 25.5% (Bastien et al., 2003; Boivin et al., 2003; Do et al., 2011; Døllner et al., 2004; Ebihara et al., 2004; Esper et al., 2004; Ijpmma et al., 2004; Maggi et al., 2003; Manoha et al., 2007; Mullins et al., 2004; Nascimento et al., 2010; Smuts et al., 2004; Williams et al., 2004). Studies conducted over several years have also noted year-to-year variations in prevalence (Maggi et al., 2003; Manoha et al., 2007; Smuts et al., 2004; Williams et al., 2004). Co-infection rates vary between 4.5% and 39%, and the viruses most often detected with HMPV are RSV, influenza virus and HRV. Only three studies included asymptomatic controls in their study design. Two studies did not detect HMPV in any of the asymptomatic children (Boivin et al., 2003; van den Hoogen et al., 2001), while the third study found HMPV in 1.1% of control patients (Williams et al., 2004).

Upper respiratory tract infections were diagnosed in 8.7% to 44% and lower respiratory tract infections in 35% to 91% of children included in the above-mentioned studies. Up to 37.5% of the children had to be admitted to the intensive care unit. HMPV caused a similar clinical picture to RSV, with fever, cough, rhinorrhoea, and wheezing as the most frequently reported signs and symptoms. However, most studies noted that the mean age of children presenting with RSV infection was less than six months, where the mean age of presentation of HMPV infection was between 6 and 24 months. A study by Semple and colleagues (2005) found that 72% of children admitted to ICU with severe bronchiolitis were dually infected with HMPV and RSV, while only 10% of children admitted to the general wards with moderate disease were co-infected, conferring a ten times increased relative risk of ICU admission. One study investigated HMPV infection in both HIV-1-infected and HIV-1-uninfected children (Madhi et al., 2007). They found that although HMPV was identified in a smaller percentage of HIV-1-infected children with LRTI than HIV-1-uninfected children (5.4% versus 11.1%), the burden of hospitalisation was 5.4 times greater in the HIV-1-infected group.

### 1.3 Diagnosis of viral respiratory tract infections in children

It is widely accepted that infections of the upper respiratory tract are in all probability viral in origin, but more than 20 bacteria and viruses may be the cause of infection in the lower respiratory tract (Templeton, 2007). Clinical examination cannot discriminate between these causative agents, and initial therapy is therefore empirical while the physician waits for the results of the diagnostic tests.

#### 1.3.1 Virus isolation in cell culture

Virus isolation in cell culture has long been considered the “gold standard” for virus detection against which all other methods are measured. Prior to the 1950s and early 1960s laboratories relied mainly on experimental animals for the diagnosis of viral infections. These techniques were only available in selected laboratories usually associated with university research units, prohibitively expensive and time-consuming (Hsiung, 1984). The main advantages of cell culture methods are their ability to isolate and identify a large variety of viruses, even those that were not necessarily expected, and that detection of a virus in cell culture indicates that the virus is viable and replication competent, a finding which is unattainable using most other detection methods. Virus isolation in cell culture also has the potential for high sensitivity as a single infectious virion can be detected. The main disadvantages of this technique are that it is time-consuming, slow and requires technical expertise. The time needed to obtain a positive result can take up to eight days in the case of influenza, and even longer with other respiratory viruses (Leland and Ginocchio, 2007). A variety of different cell lines also need to be inoculated for each specimen as the susceptibility of different cell lines, and even different strains of the same line, may differ for the isolation of rhinoviruses and enteroviruses (Arruda et al., 1996; Chonmaitree et al., 1988). Furthermore, the detection of viruses that do not cause a cytopathic effect in cell culture as well as those that are unable to grow in cell culture require alternative diagnostic methods.

In the mid 1980s traditional tube culture was succeeded by shell vial culture (SVC), which could provide a diagnosis within 24 to 48 hours. The SVC technique combines centrifugation-enhanced inoculation of clinical specimens onto preformed monolayers of cells and incubation for a predetermined period of time. Virus infection is detected by a method that does not depend on recognition of virus-induced cytopathic effect, but on specific, labelled monoclonal antibodies directed against early viral proteins produced shortly after infection (Leland and Ginocchio, 2007). It was initially thought that centrifugation enhanced infectivity by forcing the viral particles against the cell monolayer, but it was later found that centrifugation mechanically stresses the cells, leading to enhanced cell proliferation and longevity (Hughes, 1993). Advantages of SVC over traditional tube culture include shorter time to

definite diagnosis, including for those viruses that require subsequent blind passages due to poor or slow replication, it is relatively easy to perform and requires less technical expertise to read and interpret. The main disadvantage of this technique is that only those viruses that are specifically sought will be detected, and unforeseen and novel viruses will thus be missed. The processing and reading of shell vial cultures are also subjective and relatively labour-intensive and time-consuming. The success of both traditional tube and shell vial culture depend on the integrity of the sample when it reaches the laboratory. Specimens should be collected, transported and stored under optimal conditions to ensure the viability of any viruses contained therein (Leland and Ginocchio, 2007).

### **1.3.2 Fluorescent antibody methods**

Direct detection of viral antigens in respiratory samples without the need for virus isolation in cell culture has been available for more than 30 years and has become the foundation of numerous laboratories' respiratory diagnostic repertoire. Indirect fluorescent antibody assays (IFAs) has a two-step sandwich format and can provide a result within three hours, whilst direct fluorescent antibody assays (DFAs) incorporates a single monoclonal antibody which is labelled with a fluorescent dye and can provide a result in less than one hour (Leland and Ginocchio, 2007). Immunofluorescent methods appear to be more sensitive for the detection of RSV than culture methods (85% to 99% versus 29% to 74%) (Dunn et al., 2004; Landry and Ferguson, 2000; Matthey et al., 1992). This is mainly due to the fact that RSV is very labile and quickly inactivated if the sample is not refrigerated during transportation and inoculated immediately upon receipt in the laboratory. In one study (Hijazi et al., 1996) where the samples were inoculated within 2 hours of being taken, the sensitivity of cell culture improved to 94%. The sensitivity for adenovirus detection by immunofluorescent methods when compared to viral culture is much lower at 0% to 58%. The corresponding sensitivities for influenza A and B and parainfluenza virus types 1 – 3 are between these extremes (Dunn et al., 2004; Landry and Ferguson, 2000; Matthey et al., 1992). Disadvantages of immunofluorescent methods include the need for an adequate number of respiratory epithelial cells in the specimen, the potentially subjective reading of the slides and its inability to differentiate between viable and non-viable viruses. The overall sensitivity of these methods when compared to cell culture can decrease from 86% to 77% if uninterpretable slides due to non-specific fluorescence and inadequate number of cells are taken into account (Matthey et al., 1992). Due to the decreased sensitivity of this method, especially with regards to adenovirus, subsequent culture of all initial negative and uninterpretable results is recommended (Leland and Ginocchio, 2007).

### 1.3.3 Rapid antigen detection methods

Since the late 1980s an extensive assortment of non-fluorescent, technically less demanding and visually-read assays have been developed for the rapid detection of viral antigens in respiratory samples (Leland and Ginocchio, 2007). Different formats have been developed, including immunochromatographic assays, optical immunoassays, lateral-flow assays, and membrane-bound enzyme immunoassays. These rapid antigen detection methods generally require less than 30 minutes to produce a result, and frequently contain an internal control to monitor the performance of the assay. Rapid antigen assays are commercially available for the detection of RSV and influenza A and B, and are generally less sensitive than virus isolation in cell culture or molecular methods. In contrast with shell vial culture with immunofluorescent staining, which require as little as 10 viable viral particles per shell vial to produce a positive result, these assays may require as many as 1 million viral particles per test to yield a positive result (St George et al., 2002). As with immunofluorescent methods, rapid antigen detection methods appear to be more sensitive for the detection of RSV than for influenza A or B (Leland and Ginocchio, 2007). Thus a negative rapid antigen test result should be confirmed with either culture or molecular methods. These assays are reported to have a high specificity (Leland and Ginocchio, 2007), but it is important to bear in mind that the positive predictive value of the result will be influenced by the level of circulation of that particular virus in the community at the time of testing (World Health Organization, 2010b). Thus, for example, during the summer months when influenza A virus is not circulating in the community, a positive rapid antigen assay result should be confirmed with laboratory-based culture or molecular methods. Conversely, a positive result for influenza A virus, during winter when it is known that the virus is actively circulating in the community, is considered to be a true positive. A positive rapid antigen test also does not rule out co-infection with another respiratory virus, therefore in cases where multi-viral infection is suspected further tests may be warranted. In summary, the performance characteristics of rapid antigen detection methods depend on a wide variety of factors, including the level of technical expertise of the person performing the assay, the age and immune status of the patient, the type of specimen tested, the specific virus tested for, the time elapsed between onset of symptoms and specimen collection, and the time of the year (Leland and Ginocchio, 2007; World Health Organization, 2010b).

### 1.3.4 Molecular methods

Viruses, including non-viable and non-culturable viruses, can also be detected directly in respiratory tract samples using highly sensitive and specific nucleic acid amplification techniques (NAATs), without the need for a lengthy incubation period. With conventional techniques, such as polymerase chain reaction (PCR), amplification and detection using gel electrophoresis or colorimetric

methods, are performed separately. Contemporary techniques, such as real-time PCR, measure the accumulation of amplicons throughout amplification using fluorescent dyes. These assays do not require further sample manipulation post-amplification leading to a decreased turn-around time and lessens the possibility of sample contamination. The performance of molecular assays can vary considerably as a result of different extraction, amplification and detection techniques and instrumentation, primer and probe design, and level of technical expertise. Therefore NAATs require thorough verification and validation before they can be implemented in diagnostic laboratories, and regular quality control monitoring after implementation (Leland and Ginocchio, 2007).

Several studies have compared the virus detection rate in respiratory samples between molecular and non-molecular methods, and all concluded that molecular methods are more sensitive (Freymuth et al., 2006; Kim et al., 2009; Kuypers et al., 2006; Lassaunière et al., 2010; Sanghavi et al., 2012; Templeton et al., 2004; Tiveljung-Lindell et al., 2009; Yoo et al., 2007). Detection rates increased by 5.3% to 37.4%, depending on how many viruses were detected by the molecular methods. Depending on the type and variety of conventional methods employed and the number of different viruses detected by each diagnostic method, the majority of additional viruses detected could be part of those that could not be detected by the conventional methods (Sanghavi et al., 2012; Tiveljung-Lindell et al., 2010), or those that could (Freymuth et al., 2006; Kuypers et al., 2006). Two studies found that molecular methods were not more sensitive than cell culture with regards to detection of influenza A virus (Freymuth et al., 2006; Tiveljung-Lindell et al., 2009). Molecular methods also detected more multi-viral infections (7.2% – 19.8%) compared to non-molecular methods (0% – 1.9%) (Freymuth et al., 2006; Kim et al., 2009; Kuypers et al., 2006; Tiveljung-Lindell et al., 2009).

Advantages of NAATs include its high sensitivity and specificity, relatively short turn-around-time, and multiplexing capabilities. Thus patients will be diagnosed more accurately more often, resulting in swift administration of suitable antiviral medication and implementation of appropriate infection control measures. Its elevated specificity can also be a disadvantage as unexpected viruses will be missed, and the assay's ability to detect continually evolving viruses must be carefully monitored with modification of the primer pairs as needed. Other disadvantages of NAATs include the high costs of instrumentation and reagents, the potential for cross-contamination, and its inability to distinguish between viable and non-viable viruses, and active and latent infection (Leland and Ginocchio, 2007; Mahony, 2008). Reagent costs and hands-on time can be decreased by means of multiplexing, i.e. detecting more than one virus in a single reaction vessel. Disadvantages of using multiplex PCR include the risk of false positive or false negative results due to competition for reagents, the formation of primer dimers, preferential

amplification of one target sequence over others and differing annealing temperatures. Careful optimisation during development of a multiplex PCR assay is therefore required to minimise these disadvantages (Elnifro et al., 2000). In order to prevent false positivity, a novel dual priming oligonucleotide (DPO) system was developed by Seegene Inc. (Seoul, South Korea). In contrast to conventional primers, which is based on a single priming event and mispriming can lead to extension of non-specific primers, the DPO system has two separate primer segments, joined by a deoxyinosine linker (Chun et al., 2007). The longer 5'-segment initiates stable annealing, while the shorter 3'-segment of the primer is designed to block non-specific annealing. Extension will only occur upon successful annealing of both primer segments. In evaluation studies this system proved to be highly specific (100%) with no non-specific bands or false positive results detected (Chun et al., 2007; Kim et al., 2009).

The biggest problem physicians face with regards to the molecular diagnosis of viral respiratory tract infections is the clinical significance of a positive result. It is unclear whether it signifies active infection or shedding due to latent or previous infection. The clinical significance for multi-viral infections with regards to disease severity and clinical outcome also still needs to be determined (Mahony, 2008).

#### **1.4 Motivation for diagnosis of viral respiratory tract infections**

Although many physicians do not feel that the specific diagnosis of a viral respiratory tract infection has a major impact on patient management or outcome, there are a number of reasons why it is important to make a definite diagnosis.

Identifying the aetiological agent will help the clinician to better understand the clinical course and prognosis of the respiratory tract infection, and adjust the patient's management plan accordingly. For example, secondary bacterial infection is an important cause of influenza-associated death, and antibiotic efficacy does not appear to affect the overall probability of death due to this complication. McCullers (2004) demonstrated in a study on mice that treatment of the predisposing influenza infection with a neuraminidase inhibitor improved survival, even when treatment was delayed for up to 5 days. Treatment of the secondary bacterial pneumonia with appropriate antibiotics in the absence of a neuraminidase inhibitor cleared the infection, but did not improve survival. Studies suggest that viral co-infection in children with bronchiolitis leads to more severe disease and an increased risk of admission to ICU (Richard et al., 2008; Semple et al., 2005). Awareness of viral co-infection may thus aid the physician in decisions regarding admission to hospital and level of care.



Timely identification of the causative organism may also have an impact on patient management. Neuraminidase inhibitors, used for the specific treatment of influenza virus infections, need to be administered within 36 to 48 hours of symptom onset for maximal efficacy (Hedrick et al., 2000; Whitley et al., 2001). Ideally influenza virus infection should be confirmed with laboratory testing before treatment is commenced, but treatment should not be delayed whilst waiting for laboratory confirmation. Excessive and inappropriate antibiotic use appears to be the main driving force behind the worldwide increase in antibiotic resistance (van de Sande-Bruinsma et al., 2008). As an estimated 55% of all antibiotic prescriptions for ARTIs in the United States of America (USA) are for infections unlikely to be bacterial in origin (Gonzales et al., 2001), identification of a viral cause should have a substantial impact on antimicrobial resistance patterns and rising health costs. Viral respiratory tract infections can spread effectively in closed environments such as hospital wards, especially in paediatric wards as children often produce copious amounts of respiratory secretions. Knowledge of the viral infections in a ward may guide infection prevention and control practices and the use of vaccination or prophylactic antivirals such as palivizumab in the case of RSV (Templeton, 2007).

Molecular techniques for the diagnosis of viral respiratory tract infections may prove invaluable in outbreak situations, with for example SARS-related coronavirus, avian influenza or pandemic influenza A virus. The rapid detection of these viruses would enable timely implementation of appropriate infection prevention and control practices. As the clinical picture caused by these pandemic viruses and routine seasonal respiratory viruses are virtually indistinguishable, the rapid detection of routine viruses allows the clinician to make an alternative diagnosis, alleviating the need for strict isolation precautions and contact tracing. Louie and colleagues (2004) used molecular techniques to distinguish between SARS-related coronavirus and more common causes of pneumonia in suspected SARS cases in California, USA. Ordinary seasonal viruses were identified in 42% of the suspected SARS cases by molecular methods.

Few studies have investigated the clinical and financial benefits of rapid diagnosis of viral respiratory tract infections, and conflicting results have been found. Two historical cohort studies found that rapid viral diagnosis by fluorescent antibody methods decreased the length of hospital stay by 1.3 – 5.3 days and the duration of antibiotic use, compared to cell culture diagnosis in both children and adults (Barenfanger et al., 2000; Woo et al., 1997). However, rapid viral diagnosis by DFA did not make a difference in length of stay, antibiotic prescription or performance of additional diagnostic tests in a randomised controlled trial conducted in a Canadian children's hospital emergency department (Doan et al., 2009). Rapid viral diagnosis by real-time PCR also does not appear to have an effect on the length of



hospitalisation, antibiotic use or utilisation of additional diagnostic tests in adults or children with ARTIs (Oosterheert et al., 2005; van de Pol et al., 2011; Wishaupt et al., 2011). Although physicians working in a paediatric ICU stated that they would change a patient's antibiotic prescription as a result of a positive respiratory virus PCR result, in practice no changes were made. The main reasons for this discrepancy were clinical suspicion of bacterial co-infection, and waiting for the bacterial culture result before making a final decision (van de Pol et al., 2011). Rapid viral diagnosis decreased total patient costs in the trials where it had a favourable impact on length of antibiotic use and hospital stay (Barenfanger et al., 2000; Woo et al., 1997), whilst it added to patient costs when no impact was seen (Oosterheert et al., 2005). In a cost analysis study by Mahony and colleagues (2009), they found that multiplex PCR was the least expensive method for the diagnosis of viral respiratory tract infections in children, if the prevalence of viral respiratory tract infections was at least 11% and the multiplex PCR was used in place of, and not in conjunction with, other diagnostic methods. The majority of the savings was associated with a decrease in the length of hospital admission.

Using a multiplex PCR instead of the traditional shell vial culture technique for the diagnosis of respiratory tract infections will deliver sensitive and timely results. The overall viral detection rate in respiratory samples will be increased as nucleic acid amplification techniques can detect both newly discovered and older, easily culturable viral pathogens. This may assist in expanding our knowledge of the pathogenesis of severe respiratory tract infections, especially with regards to the role of well-known pathogens thought to exclusively cause mild upper respiratory tract infections, newly discovered viruses, and viral co-infections. These improvements could have a positive influence on patient management, infection control, vaccination strategies and public health.

The Division of Medical Virology receives approximately 700 respiratory tract samples for the detection of respiratory viruses from Tygerberg Hospital per year, of which more than one third are from the paediatric and neonatal intensive care units. Improvement in the overall detection rate in respiratory samples will be particularly beneficial in these units in terms of potential decreased length of stay in ICU and reduction in antibiotic use.

## **1.5 Hypothesis**

Multiplex PCR is a superior method to currently available methods for the diagnosis of viral respiratory tract infections in children.

## 1.6 Aim of study

The aim of the study is to determine the viral causes of lower respiratory tract infections in children requiring admission to Tygerberg Children's Hospital.

## 1.7 Objectives

The objective of this study is to:

1. Determine the viral causes of lower respiratory tract infections requiring admission in using shell vial culture with immunofluorescent staining and two multiplex PCR assays, the Seeplex® RV15 ACE Detection system (Seegene, Korea) and the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit (TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany).
2. Compare the Seeplex® RV15 ACE Detection System and the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit with shell vial culture for the detection of respiratory viruses in routine diagnostic respiratory samples.
3. Examine the demographic and clinical characteristics associated with each respiratory viral pathogen.

## Chapter 2

# Materials and Methods

## 2.1 Materials

### 2.1.1 Patient selection

One hundred and thirty-eight paediatric patients under the age of six years, admitted to Tygerberg Children's Hospital from May 2010 to August 2010 with a presumptive diagnosis of an acute respiratory tract infection, as determined by the attending clinician, were included in the study. The required study size was calculated to be at least 100 patients, based on an 18.2% detection rate for conventional methods in our laboratory and an estimated 19% difference in detection rate between conventional and molecular methods, as determined by previous studies, with 85% power and a significance level of 95% (estimated sample size for the comparison of two proportions). Clinical, demographic and laboratory data were collected through a systematic review of medical and laboratory records and subsequently anonymised.

### 2.1.2 Study definitions

#### 2.1.2.1 Acute respiratory tract infection

In order to calculate the sensitivity and specificity of each diagnostic method for the viruses that could be detected by at least two of the three methods (i.e. RSV, influenza virus A and B, parainfluenza virus types 1 – 3, adenovirus, HMPV, HBoV and the HCoVs), true positive viral infection was defined as detection of a specific respiratory virus by more than one detection method. Samples that tested negative by all detection methods were determined to be true negative. Any sample that tested positive for a particular virus with a single diagnostic method was considered to be a false positive result for that method. Any sample that tested negative for a particular virus with one method, but positive with the two remaining methods was considered to be a false negative result for that method. Sensitivity and specificity could not be calculated for the remaining viruses (i.e. HRV, HEV, PIV4 and CMV) that could only be detected by a single diagnostic method. Viral co-infection was defined as detection of more than one respiratory virus in the same sample. Viral-bacterial co-infection was defined as detection of a virus in the respiratory sample and isolation of a clinically significant bacterial organism from a respiratory or blood sample taken on the same day.

### 2.1.2.2 Patient demographics and clinical data

Children less than 12 months of age were defined as premature if they were born at an estimated gestational age of 36 weeks or less. To determine whether a patient had an underlying chronic medical condition that might predispose the child to severe influenza or RSV infection, a detailed record review was performed. These high-risk medical conditions included chronic pulmonary (including asthma), haemodynamically significant cardiovascular, renal, hepatic, neurologic, haematologic, or metabolic disorders (including diabetes mellitus); immunosuppression (including immunosuppression caused by medications or by HIV-1 infection), long-term aspirin therapy, Down syndrome and birth before an estimated 35 weeks of gestation (Committee on Infectious Diseases, 2009; Fiore et al., 2010; Zachariah et al., 2012). The term mechanical ventilation included any form of artificial ventilation, i.e. both invasive and non-invasive ventilation (e.g. nasal continuous positive airway pressure ventilation).

### **2.1.3 Clinical samples**

Nasopharyngeal or tracheal aspirates were collected from the patients and sent to the Virology laboratory in viral transport medium (VTM) (Highveld Biological, Lyndhurst, South Africa) as part of the routine diagnostic work-up. No additional samples were collected from the patients. During initial specimen processing for shell vial culture, part of the original sample was stored in an additional Eppendorf tube at  $-80^{\circ}\text{C}$  until further processing for molecular methods.

### **2.1.4 Ethical issues**

The study was approved by the Health Research Ethics Committee of the University of Stellenbosch with reference number N09/02/066.

## **2.2 Methods**

### **2.2.1 Shell vial culture with immunofluorescent staining**

Samples were processed according to the standard operating protocol of the Division of Virology by qualified and appropriately trained personnel under Biosafety Level 2 conditions.

The following respiratory viruses were tested for: influenza virus A and B, parainfluenza virus types 1 – 3, respiratory syncytial virus (RSV), adenovirus, cytomegalovirus (CMV), and human metapneumovirus (HMPV).

### 2.2.1.1 Shell vial culture inoculation

The standard operating protocol for virus isolation from nasopharyngeal and tracheal aspirates is as follows:

- The content of the specimen container was made up to 6 ml with VTM if necessary, and mixed briefly with a RX3 vortex mixer (Velp Scientifica, Usmate, Italy).
- 0.2 ml antibiotic cocktail (penicillin, streptomycin and amphotericin B) was added to the container and left standing for 5 minutes.
- The specimen was centrifuged at 2 000 revolutions per minute (rpm) for 10 minutes at 4 °C in a Beckman GPR benchtop centrifuge (Beckman Coulter, California, USA).
- One human fibroblast (HF cells) shell vial and three combination (Madin-Darby Canine Kidney (MDCK) and human laryngeal carcinoma (HEp-2) cells) shell vials were marked with the appropriate specimen number.
- The cell culture medium was removed from each of the shell vials by decanting the liquid into a waste receptacle.
- 0.2 ml of the specimen was inoculated into each shell vial using a sterile glass pipette. The specimens were filtered through a sterile 0.22 or 0.45 µm filter using a syringe if necessary.
- The remaining specimen was stored in two labelled Eppendorf tubes at – 80 °C.
- The inoculated shell vials were centrifuged at 3 000 rpm at room temperature for 45 minutes in a Thermo Scientific Heraeus Megafuge 40 centrifuge (Thermo Fisher Scientific, Maryland, USA).
- 1 ml of maintenance medium (Eagle's Minimum Essential Medium (Lonza, Maryland, USA) with added penicillin and streptomycin for the combination shell vials and RPMI-1640 (Lonza, Maryland, USA) with added foetal calf serum, penicillin and streptomycin for the HF shell vials) were added to each of the shell vials.
- The HF shell vials were incubated at 37 °C and the combination shell vials at 33 °C for a minimum of 40 hours.

### 2.2.1.2 Immunofluorescent staining

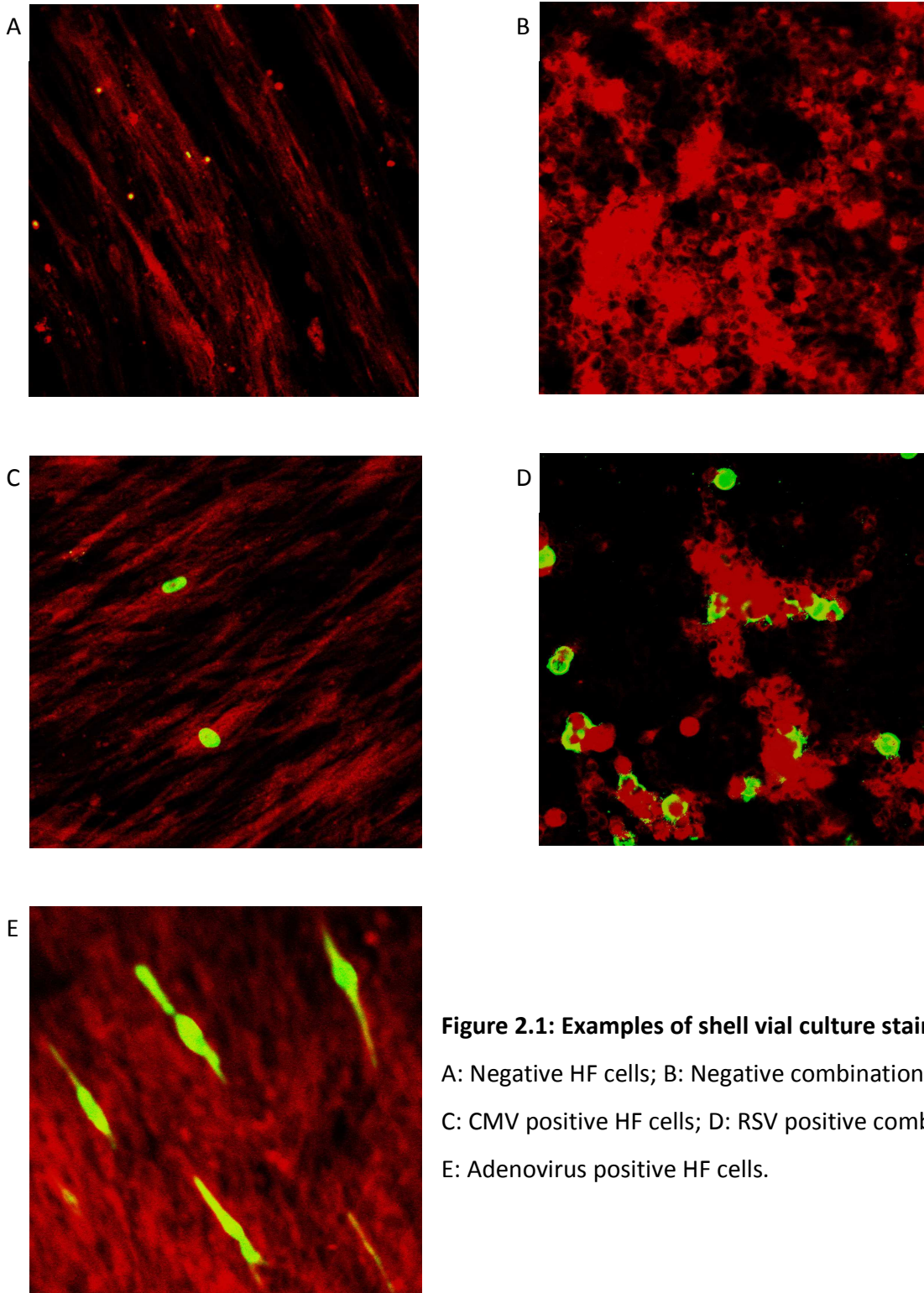
- Immunofluorescent staining was performed using the Millipore Respiratory Virus Screen kit (Chemicon International, California, USA), Millipore Mouse Anti-Cytomegalovirus Monoclonal Antibody (Chemicon International, California, USA), and Millipore Human Metapneumovirus IFA Reagent (Chemicon International, California, USA).
- The maintenance medium was removed from two of the combination shell vials and the HF shell vial by decanting the liquid into a waste receptacle.

- The remaining combination shell vial was kept in the incubator at 37 °C.
- Each shell vial was rinsed once with phosphate buffered saline (PBS).
- 2 ml cold acetone was added to each of the shell vials and allowed to stand at – 20 °C for 20 minutes to fix the cells to the coverslip.
- The coverslips were removed from the shell vials, taking care to keep the cell-side up, and mounted on labelled microscope slides with Entellan® Rapid Embedding Agent for Microscopy (Electron Microscopy Sciences, Pennsylvania, USA).
- The HF coverslip was divided in half with Entellan® Rapid Embedding Agent for Microscopy.
- A sufficient amount of Millipore Respiratory Virus Screen (a mixture of monoclonal antibodies against influenza virus A and B, parainfluenza virus types 1 – 3, adenovirus and RSV) was added to one of the combination coverslips to cover the cells.
- A sufficient amount of Millipore Human Metapneumovirus IFA Reagent was added to the second combination coverslip.
- A sufficient amount of Millipore Mouse Anti-Cytomegalovirus Monoclonal Antibody was added to one half of the HF coverslip, and Millipore Adenovirus Monoclonal Antibody (Chemicon International, California, USA) was added to the other half.
- The slides were incubated in a humid chamber at 37 °C for 30 minutes.
- The slides were rinsed twice in PBS with 1% Tween® 20 Detergent (Merck KGaA, Darmstadt, Germany) and once in PBS to remove excess monoclonal antibody solution.
- A sufficient amount of fluorescein-5-isothiocyanate (FITC)-labelled Anti-Mouse IgG Conjugate was added to each of the coverslips to cover the cells and the slides were incubated in a humid chamber at 37 °C for 30 minutes.
- The slides were rinsed twice in PBS with 1% Tween® 20 Detergent and once in PBS to remove excess conjugate solution.
- The slides were mounted under a cover glass using a drop of glycerol as a mounting fluid.
- The slides were examined using an Olympus BX60 fluorescence microscope (Olympus, Pennsylvania, USA) at 100 – 200 x magnification for cells exhibiting characteristic apple-green fluorescence. Detailed examination was carried out at 400 x magnification when necessary.
- When the combination coverslip stained with the Millipore Respiratory Virus Screen was positive, the remaining combination coverslip was used for confirmation and identification of the specific virus.
- The maintenance medium was removed from the combination shell vial and rinsed once with PBS.

- The cells were scraped off the inside of the shell vial with a 1ml plastic bulb pipette and transferred to a labelled Eppendorf tube.
- The tube was centrifuged in a Beckman Microfuge® 16 centrifuge (Beckman Coulter, California, USA) at 14 800 rpm for 30 seconds.
- The excess PBS was removed and the cell pellet was resuspended in 500 µl PBS.
- The tube was centrifuged in a Beckman Microfuge® 16 centrifuge at 14 800 rpm for 30 seconds.
- The excess PBS was removed and the cell pellet was resuspended in 150 µl PBS.
- 10 µl of the cell suspension was pipetted onto each of 9 wells on a labelled Teflon®-coated multi-well slide.
- The slide was allowed to air dry at room temperature for 30 minutes and fixed with cold acetone at – 20 °C for 20 minutes.
- A specific monoclonal antibody against a specific virus was added to each well in the following order:
  - Well 1 – Parainfluenza virus type 1
  - Well 2 – Parainfluenza virus type 2
  - Well 3 – Parainfluenza virus type 3
  - Well 4 – Influenza virus A
  - Well 5 – Influenza virus B
  - Well 6 – RSV
  - Well 7 – Adenovirus
  - Well 8 – Respiratory Virus Screen
  - Well 9 – Normal mouse antiserum (to detect non-specific fluorescence)
- The remainder of the immunofluorescent staining procedure was followed as described above and the slide was examined using an Olympus BX60 fluorescence microscope at 100 – 200 x magnification for cells exhibiting characteristic apple-green fluorescence.
- The type of staining pattern depends on the infecting virus and its growth pattern and examples can be seen in Figure 2.1.
  - *Parainfluenza virus types 1 – 3*: Fluorescent staining is confined to the cytoplasm and punctate with irregular inclusions.

- *Influenza virus A and B*: Fluorescence can be either nuclear, cytoplasmic or both. Nuclear fluorescent staining is uniformly bright, but cytoplasmic staining is frequently punctate with large inclusions.
- *RSV*: Fluorescent staining is confined to the cytoplasm, punctate with small inclusions and may be associated with syncytia.
- *Adenovirus*: Fluorescence can be either nuclear, cytoplasmic or both. Nuclear fluorescent staining is uniformly bright, but cytoplasmic staining is frequently punctate.
- *CMV*: Fluorescent staining is confined to the nucleus.
- *HMPV*: Fluorescence can be either nuclear, cytoplasmic or both.
- *Negative cells*: Negative cells stain a dull red colour due to the Evans blue counterstain.





**Figure 2.1: Examples of shell vial culture staining patterns.**

A: Negative HF cells; B: Negative combination cells;

C: CMV positive HF cells; D: RSV positive combination cells;

E: Adenovirus positive HF cells.

### 2.2.2 Seeplex® RV15 ACE Detection System

The Seeplex® RV15 ACE Detection System (Seegene, Inc., Seoul, Korea) is a multiplex RT-PCR which tests for the viruses listed in Table 2.1. The reported lower limit of detection of this assay is 100 copies per reaction.

Set A	Set B	Set C
Adenovirus serogroups A – E	Coronavirus OC43/HKU1	HBoV
Coronavirus 229E/NL63	Rhinovirus	Influenza B
Parainfluenza type 2	RSV type A	HMPV
Parainfluenza type 3	Influenza A	Parainfluenza type 4
Parainfluenza type 1	RSV type B	Enterovirus

#### 2.2.2.1 Nucleic acid extraction

Viral DNA and RNA were extracted from respiratory samples using the automated NucliSENS® easyMAG® nucleic acid extraction system (bioMérieux, Boxtel, the Netherlands), according to manufacturer's instructions with a few modifications. Briefly, 1 000 µl of the stored sample (or 500 µl if there was insufficient volume available) was added to each plastic vessel well. Lysis buffer was automatically added by the instrument and the mixture was incubated at room temperature for 10 minutes on-board the instrument. 100 µl of silica was then added to each of the wells, followed by automatic magnetic separation. The nucleic acid was eluted to a final volume of 20 µl and stored at – 20 °C until further processing.

#### 2.2.2.2 Reverse transcription

For reverse transcription of purified viral RNA the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Maryland, USA) was used according to the protocol optimised for use with the Seeplex® RV15 ACE Detection System (Seeplex® RV15 ACE).

- 8 µl of the extracted nucleic acid, 0.2 µg random hexamers, and 3 µl nuclease-free water were added to a sterile, nuclease-free tube on ice.
- The tube was incubated at 80 °C for 3 minutes in a GeneAmp® PCR System 9700 (Applied Biosystems, California, USA), chilled on ice for 2 minutes and spun down briefly in a Spectrafuge Mini Centrifuge (Labnet International, New Jersey, USA).
- 4 µl 5X reaction buffer, 1 µl Ribolock™ RNase inhibitor (20 u/µl), 2 µl 10mM dNTP mix, and 1 µl RevertAid™ M-MuLV Reverse Transcriptase (200 u/µl) were added to the tube for a final reaction volume of 20 µl.

- The tube was incubated at 37 °C for 90 minutes and then at 94 °C for 2 minutes in a GeneAmp® PCR System 9700.
- The tube was chilled on ice for 2 minutes and spun down briefly in a Spectrafuge Mini Centrifuge before storage at – 20 °C until amplification.

### 2.2.2.3 Amplification

Amplification was carried out according to the manufacturer's instructions. Each sample was simultaneously amplified in three separate reactions (Set A, B, and C). The content of each reaction is listed in Table 2.2.

Reagent	Content	Volume (µl)
5X RV15 PM	<ul style="list-style-type: none"> <li>— Primer pairs for pathogens in each set (A, B or C)</li> <li>— Primer pair for internal control</li> <li>— Template for internal control</li> </ul>	4
8-MOP solution	8-methoxypsoralen (8-MOP) to prevent carry-over contamination	3
2X Multiplex Master Mix	<ul style="list-style-type: none"> <li>— DNA polymerase</li> <li>— Buffer containing dNTPs</li> <li>— MgCl<sub>2</sub> and stabilisers</li> </ul>	10
Nucleic acid sample		3
<b>Total volume</b>		<b>20</b>

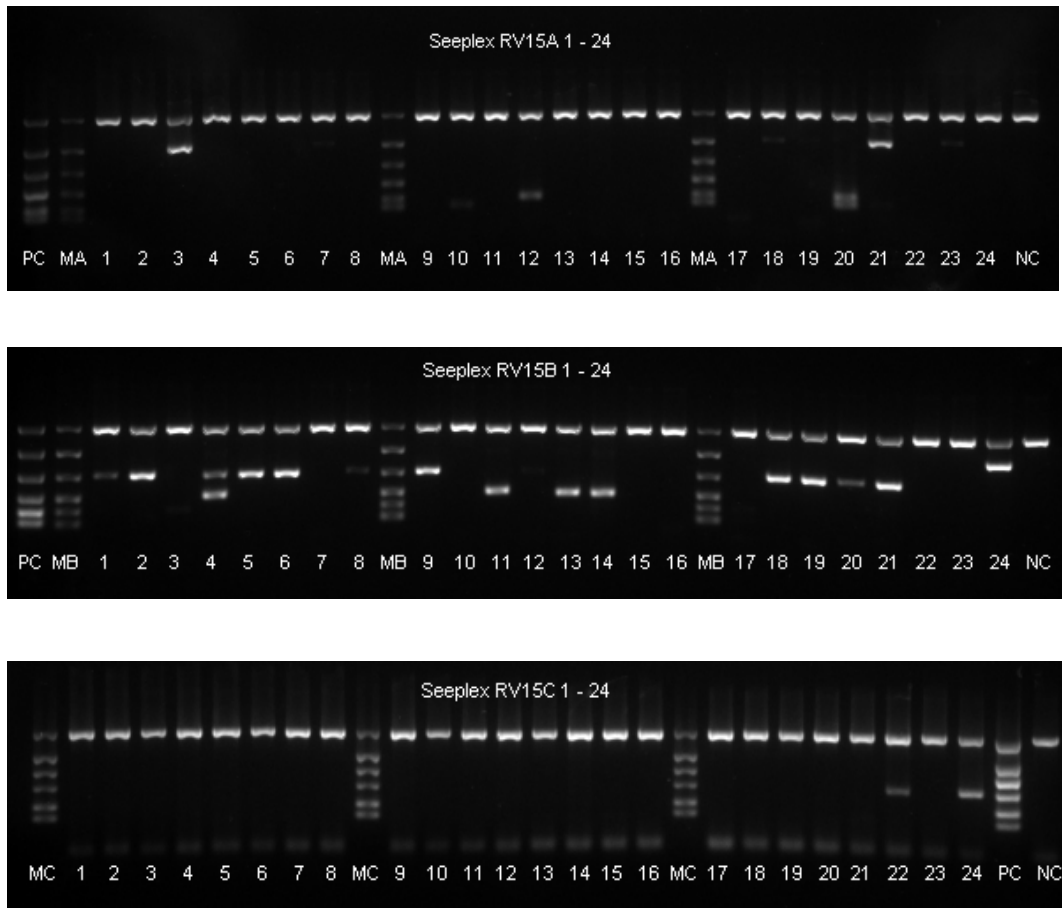
All reactions were performed on a GeneAmp® PCR System 9700 using the PCR protocol listed in Table 2.3. A positive and negative control, provided by the manufacturer, was included in each run. After completion of the amplification reaction the tubes were irradiated with UV-light at 365 nm for 20 minutes to prevent carry-over contamination.

Segment	Temperature	Duration
Initiation	94 °C	15 minutes
40 cycles	Denaturation	94 °C
	Annealing	60 °C
	Elongation	72 °C
Final elongation	72 °C	10 minutes

#### 2.2.2.4 Detection

The PCR products were visualised by electrophoresis at 60V on a 2% (w/v) agarose gel stained with ethidium bromide for 45 minutes. All of the gels were visualised using the UVIprochemi II D-77 LS-26M gel documentation system (UVItec, Cambridge, United Kingdom). The specific respiratory virus was identified by comparison of the amplicon size (Table 2.4) with the reference band size as indicated by the Seeplex® RV15 ACE marker for each set, provided by the manufacturer (Figure 2.2).

<b>Table 2.4: Seeplex® RV15 ACE amplicon size and target gene</b>		
<b>RV15 ACE Detection Set A</b>	<b>Amplicon size (bp)</b>	<b>Target gene</b>
Internal control	850	CesA3
Adenovirus	534	Major core protein
Coronavirus 229E/NL63	375	Spike
Parainfluenza virus type 2	264	HN
Parainfluenza virus type 3	189	F
Parainfluenza virus type 1	153	HN
<b>RV15 ACE Detection Set B</b>	<b>Amplicon size (bp)</b>	<b>Target gene</b>
Internal control	850	CesA3
Coronavirus OC43/HKU1	578	ORF1b
Rhinovirus A/B/C	394	5'UTR
RSV A	269	F
Influenza A	206	M1
RSV B	155	F
<b>RV15 ACE Detection Set C</b>	<b>Amplicon size (bp)</b>	<b>Target gene</b>
Internal control	850	CesA3
Human bocavirus 1/2/3/4	579	NS1
Influenza B	455	NA
Human metapneumovirus	351	F
Parainfluenza virus type 4	249	HN
Enterovirus	194	5'UTR



**Figure 2.2: Example of a Seeplex® RV15 ACE detection via gel electrophoresis.** More than one respiratory virus was successfully co-detected in several samples. PC = Positive Control; NC = Negative Control; MA = Marker for Set A; MB = Marker for Set B; MC = Marker for Set C.

#### 2.2.2.5 Controls

A positive and negative control, provided by the manufacturer, was included in each run. An internal control was added to each sample after extraction and co-amplified and co-detected with the target DNA from the clinical specimen. The internal control is a DNA plasmid incorporating the cellulose synthase 3 (*CesA3*) gene of *Arabidopsis thaliana*, commonly known as the mouse-ear cress, a small flowering plant native to Asia, Europe and northwest Africa. It has no homology to either human or viral genomic DNA and helped to identify samples containing substances which may interfere with PCR amplification.

In addition, the 8-MOP system was used to prevent possible carry-over contamination as it is known to intercalate into and covalently crosslink double-stranded nucleic acid after activation with light at between 320 and 400 nm.

### 2.2.3 Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit

The Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit (TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany) is a multiplex real-time RT-PCR which tests for the viruses listed in Table 2.5. The reported lower limit of detection of this assay is  $2.5 \times 10^4$  copies/ml (Lassaunière et al., 2010).

	Probe Label LightCycler® Red 610	Target gene	Probe Label LightCycler® Red 640	Target gene	Probe Label LightCycler® Red 670	Target gene
Panel 1	RSV	NP	HMPV	F	HBoV	NS1
Panel 2	PIV1 & PIV2	HN	PIV3	NP	HCoV-NL63	ORF1a
Panel 3	Influenza A	NS1	Influenza B	NP	Adenovirus	Hexon
Panel 4	HCoV-OC43	ORF1b	HCoV-HKU1	ORF1b	HCoV-229E	NP

#### 2.2.3.1 Nucleic acid extraction

Viral DNA and RNA were extracted once from respiratory samples using the automated NucliSENS® easyMAG® nucleic acid extraction system as described above. The nucleic acid was eluted to a final volume of 20 µl and stored at – 20 °C until further processing.

#### 2.2.3.2 Reverse transcription

For reverse transcription of purified viral RNA the RevertAid™ First Strand cDNA Synthesis Kit was used as described above. The reverse transcription reaction product was stored at – 20 °C until amplification.

#### 2.2.3.3 Amplification and detection

Amplification and detection was performed with the LightCycler® FastStart DNA Master<sup>PLUS</sup> HybProbe Kit (Roche, Basel, Switzerland) on a LightCycler® 480 Instrument (Roche, Basel, Switzerland) according to the instructions of the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit (Resp Multiplex RT-PCR).

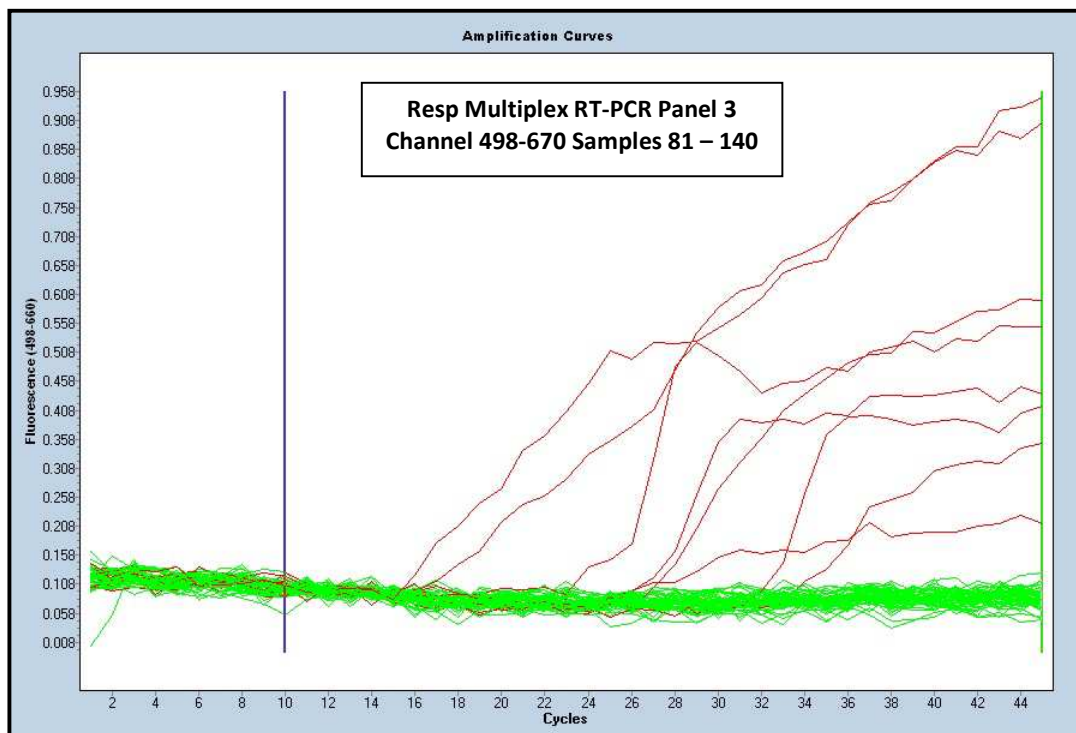
Each lyophilized Panel Primer and Probe Master Mix was reconstituted with 400 µl nuclease-free water. Each sample was amplified in four separate reactions (Panel 1 – 4). The components of each reaction were pipetted into a LightCycler® 480 Multiwell Plate 96 (Roche, Basel, Switzerland) using a QIAgility automated pipettor (Qiagen GmbH, Hilden, Germany). The content of each reaction is listed in



Table 2.6. Each LightCycler® 480 Multiwell Plate 96 was sealed manually with a LightCycler® 480 Sealing Foil (Roche, Basel, Switzerland) before it was transferred to the LightCycler® 480 Instrument.

<b>Table 2.6: Resp Multiplex RT-PCR reagent volumes</b>	
<b>Reagent</b>	<b>Volume (µl)</b>
Primer and Probe Master Mix (Panel 1 – 4)	4
Lightcycler® FastStart DNA Master <sup>PLUS</sup> HybProbe Master Mix	4
Nucleic acid sample	4
Nuclease-free water	8
<b>Total volume</b>	<b>20</b>

All reactions were performed on a LightCycler® 480 Instrument using the PCR protocol listed in Table 2.7. A positive control provided by the manufacturer, and a negative control (nuclease-free water) were included in each run. Result analysis was performed using the LightCycler® 480 Software, Version 1.5 (Roche, Basel, Switzerland), as described in the LightCycler® 480 Instrument Operator's Manual, with the colour compensation mode enabled to compensate for overlap between fluorescence channels (Figure 2.3).



**Figure 2.3: Example of amplification curves generated by the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit.** Each red line represents a positive amplification curve while the green lines indicate negative results.

<b>Table 2.7: Resp Multiplex RT-PCR protocol</b>				
<b>Denaturation</b>				
<b>Cycles</b>	1	<b>Analysis Mode</b>	None	
<b>Temperature</b>	<b>Duration</b>	<b>Ramp Rate (°C/s)</b>	<b>Acquisition Mode</b>	<b>Acquisitions (per °C)</b>
95 °C	10 min	4.40	None	
<b>Cycling</b>				
<b>Cycles</b>	45	<b>Analysis Mode</b>	Quantification	
<b>Temperature</b>	<b>Duration</b>	<b>Ramp Rate (°C/s)</b>	<b>Acquisition Mode</b>	<b>Acquisitions (per °C)</b>
95 °C	10 s	4.40	None	
58 °C	20 s	2.20	None	
54 °C	20 s	2.20	None	
48 °C	20 s	2.20	Single	
72 °C	15 s	4.40	None	
<b>Melting</b>				
<b>Cycles</b>	1	<b>Analysis Mode</b>	Melting Curves	
<b>Temperature</b>	<b>Duration</b>	<b>Ramp Rate (°C/s)</b>	<b>Acquisition Mode</b>	<b>Acquisitions (per °C)</b>
95 °C	60 s	4.40	None	
40 °C	60 s	2.20	None	
75 °C		0.03	Continuous	5
<b>Cooling</b>				
<b>Cycles</b>	1	<b>Analysis Mode</b>	None	
<b>Temperature</b>	<b>Duration</b>	<b>Ramp Rate (°C/s)</b>	<b>Acquisition Mode</b>	<b>Acquisitions (per °C)</b>
40 °C	30 s	2.20	None	

### 2.3 Statistical analysis

Statistical analyses were performed with STATISTICA 10.0 (StatSoft, Inc., Tulsa, USA). Sensitivity and specificity of each multiplex PCR was calculated using 2 x 2 tables. Categorical variables were compared by using two-way tables and the Pearson  $\chi^2$  test and continuous variables were compared with the Kruskal-Wallis test. A two-tailed p-value of  $\leq 0.05$  was considered significant for all statistical tests.



## Chapter 3

# Results

### 3.1 Study population

One hundred and thirty-eight children below the age of six years, with a clinical picture suggestive of acute respiratory tract infection, were included in the study from May 2010 to August 2010. Clinical information from only 125 children could be obtained for further analysis. The median age of the patients was 3 months (interquartile range (IQR) 1.2 – 7 months), and two-thirds of the patients were less than 6 months of age. Eighty-three (60%) patients were male, and 51 children had been born prematurely with a median gestational age of 29 weeks.

The median duration of hospitalisation was 14 days (IQR 8 – 33.5 days) and more than a quarter of patients (35/125) were admitted for more than 30 days. More than half of the patients (64/125) required mechanical ventilation for a median duration of 7 days (IQR 3 – 13.25 days), including ten patients who received nasal continuous positive airway pressure ventilation and did not require intensive care admission. Fifty-four children were admitted to the intensive care unit during their hospitalisation for a median duration of 7 days (IQR 4 – 14 days). Twelve children died during the study period, including five infants born prematurely, two children with measles, one child with congenital rubella infection, and two children with immunosuppression due to HIV-1-infection and chronic granulomatous disease respectively.

At least one underlying medical condition that predisposes to severe respiratory tract infection was detected in 64% (80/125) of the patients. Prematurity, cardiac lesions and immunosuppression were the most frequently reported underlying high-risk medical conditions. Ten children had more than one underlying high-risk medical condition. Eighty-five children were tested for HIV-1 because of exposure to HIV-1, and 13 (15.3%) were found to be HIV-1-infected. The HIV-1-infected children had a median absolute CD4+ count of  $700 \times 10^6$  cells/L, a median CD4+ percentage of lymphocytes of 14.8%, and a median log HIV-1 viral load of 5.88. All of the HIV-1-infected children were started on antiretroviral therapy.

## 3.2 Diagnostic methods

### 3.2.1 Shell vial culture with immunofluorescent staining

Thirty-seven viruses were detected in 36 samples (26.1%) by shell vial culture with immunofluorescent staining (Figure 3.1), including one sample positive for both PIV1 and PIV3 (Table 3.1). RSV was detected most often ( $n = 23$ ), followed by adenovirus ( $n = 6$ ). Two samples were positive for CMV, which was not tested for by any of the other methods. The highest percentage of positive specimens by shell vial culture was found in children aged 12 – 23 months (Figure 3.2). Only one sample was received from a child in the 18 – 23 months old category, and all three diagnostic methods were able to detect a respiratory virus in this sample.

Virus	Consensus result*		Shell vial culture		Seeplex® RV15 ACE		Resp Multiplex RT-PCR	
	Total	Of which multiple	Total	Of which multiple	Total	Of which multiple	Total	Of which multiple
RSV	38	6	23	0	46	22	43	10
Influenza A	ND	ND	0	0	14	14	0	0
Influenza B	ND	ND	0	0	0	0	0	0
Adenovirus	12	2	13	0	18	11	13	4
PIV1/PIV2	8	3	3	1	17	14	8	3
PIV3	7	2	3	1	10	9	7	2
PIV4	ND	ND	ND	ND	2	0	ND	ND
HCoVs	6	1	ND	ND	7	5	7	2
HRV	ND	ND	ND	ND	40	19	ND	ND
HEV	ND	ND	ND	ND	2	1	ND	ND
HBoV	4	3	ND	ND	5	4	4	4
HMPV	8	5	1	0	8	6	8	5
CMV	ND	ND	2	0	ND	ND	ND	ND

\* Consensus (true positive) result is defined as the detection of the specific virus by at least two out of the three diagnostic methods. ND = not determined.

PIV1 and PIV2 were grouped together as the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit (Resp Multiplex RT-PCR) cannot distinguish between the two. HCoVs were grouped together as so few were detected, and the Seeplex® RV15 ACE Detection System (Seeplex® RV15 ACE) cannot distinguish between HCoV-OC43 and HCoV-HKU1 or between HCoV-NL63 and HCoV-229E.

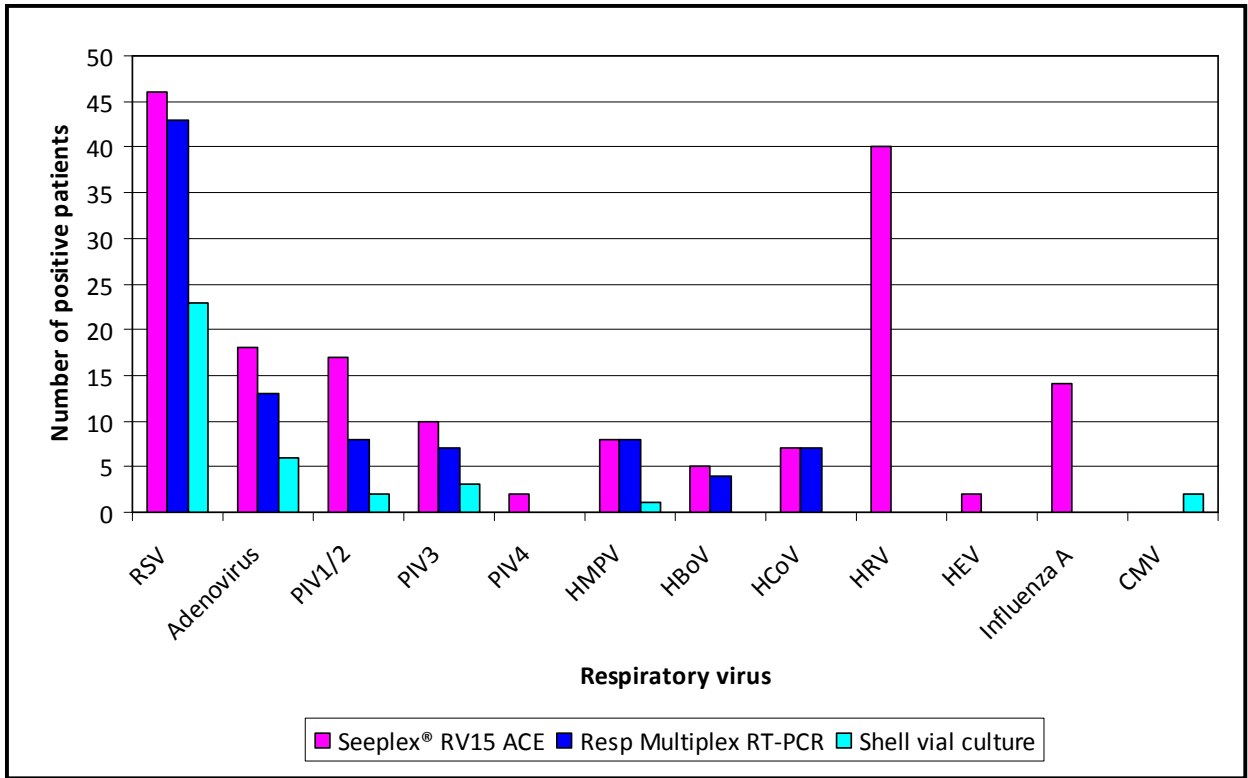


Figure 3.1: Distribution of viruses detected by all three diagnostic methods.

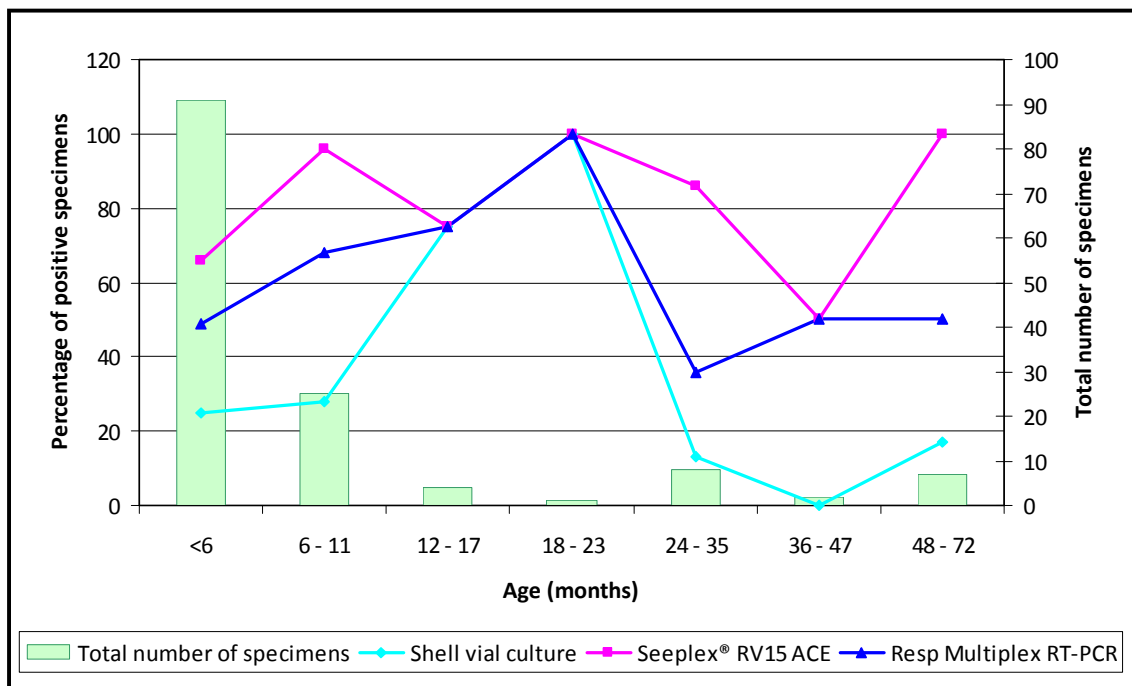
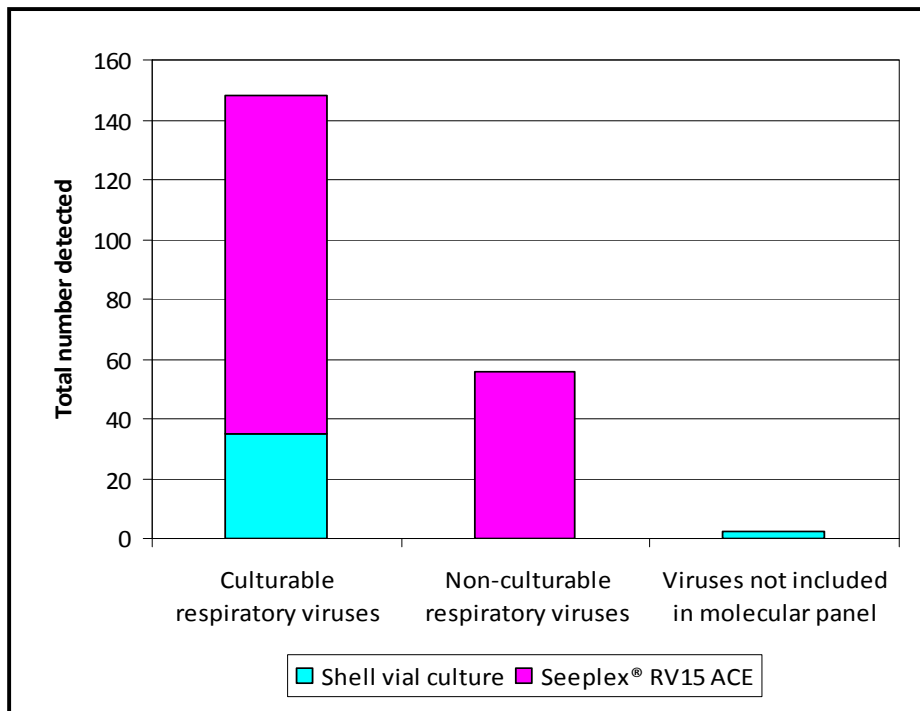


Figure 3.2: Proportion of specimens found to be positive for respiratory viruses by all three diagnostic methods, by age of the patient.

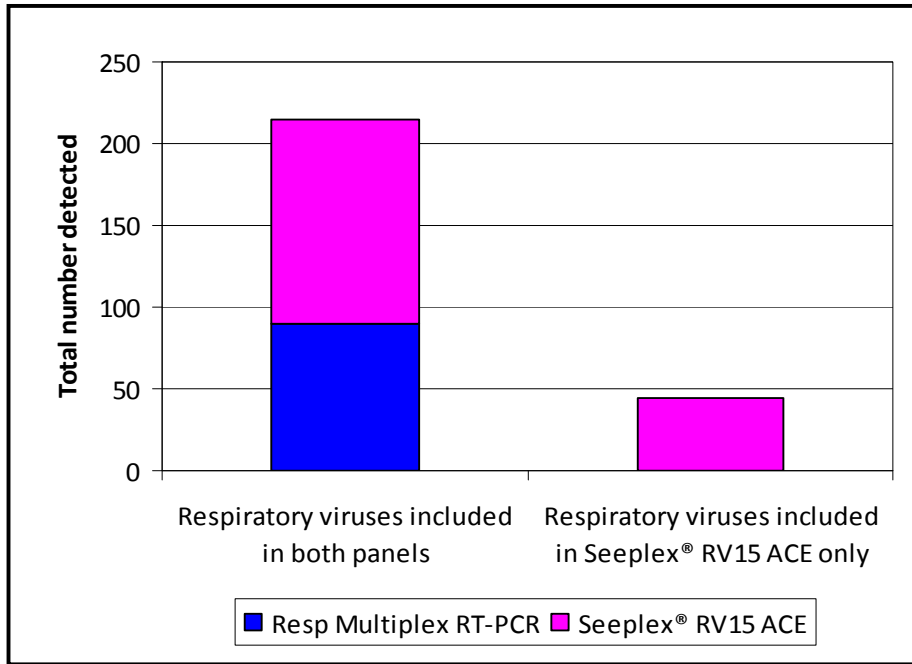
### 3.2.2 Seeplex® RV15 ACE Detection System

One hundred and sixty-nine viruses were detected in 102 samples (73.9%) with the Seeplex® RV15 ACE Detection System (Table 3.1). A single virus was detected in 61 samples, two viruses were detected in 22 samples, and three or more viruses were detected in sixteen samples. More than 60% of samples from all age groups, except for children between 36 and 47 months of age, tested positive with this method (Figure 3.2). RSV was detected most often ( $n = 46$ ), including three patients who tested positive for both RSV subtype A and subtype B, followed by HRV ( $n = 40$ ) and adenovirus ( $n = 18$ ) (Figure 3.1). RSV subtype A ( $n = 51$ ) was detected far more frequently than subtype B ( $n = 5$ ). Almost equal numbers of RSV and HRV were detected in single and multi-viral infections, whereas PIV3, HBoV and HMPV were detected more often together with other respiratory viruses. Influenza A virus was found exclusively in multi-viral infections, while PIV4 was only detected as a sole pathogen. Influenza B virus was not detected in any of the patients. The most commonly detected dual virus combinations were RSV and HRV, adenovirus and HRV, and PIV3 and HRV (all  $n = 3$ ).

More than half of the additional viruses (78/132) detected with the Seeplex® RV15 ACE Detection System over shell vial culture were for viruses culturable by shell vial culture (Figure 3.3). This is in keeping with several previous reports (Freythuth et al., 2006; Kim et al., 2009; Kuypers et al., 2006). Conversely, most of the additional viruses (44/79) detected with this assay over the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit were for the viruses not detected by this assay (Figure 3.4).



**Figure 3.3: Additional viruses detected by Seeplex® RV15 ACE compared with viruses cultured by shell vial culture.**



**Figure 3.4: Additional viruses detected by Seeplex® RV15 ACE compared with viruses detected by Resp Multiplex RT-PCR.**

### 3.2.3 Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit

This assay detected ninety viruses in 73 samples (52.9%), with dual infections detected in nine samples and triple infections detected in four samples (Table 3.1). Similar to shell vial culture, the viruses most commonly detected were RSV ( $n = 43$ ) and adenovirus ( $n = 13$ ) (Figure 3.1). HBoV was only detected together with other respiratory pathogens, while PIV3, RSV and adenovirus were detected more often as single viral infections. Unfortunately influenza A virus could not be detected in any samples, due to technical difficulties with the assay, which persisted on repeat testing (Figure 3.5). Even the positive control did not produce an amplification curve. The reason for this failure is unclear, as the same extracted nucleic acid and complementary DNA (cDNA) was used in all assays and the 3<sup>rd</sup> panel of this assay could be read in all other channels, ruling out hardware problems and extraction or reverse transcription errors. Influenza B virus was also not detected in any samples. RSV and adenovirus ( $n = 3$ ) was the dual virus combination most often detected.

Most of the additional viruses (44/53) detected by the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit over shell vial culture were for viruses culturable by shell vial culture (Figure 3.6). Children between 6 and 23 months of age had the highest proportions of positive samples with this assay (Figure 3.2).

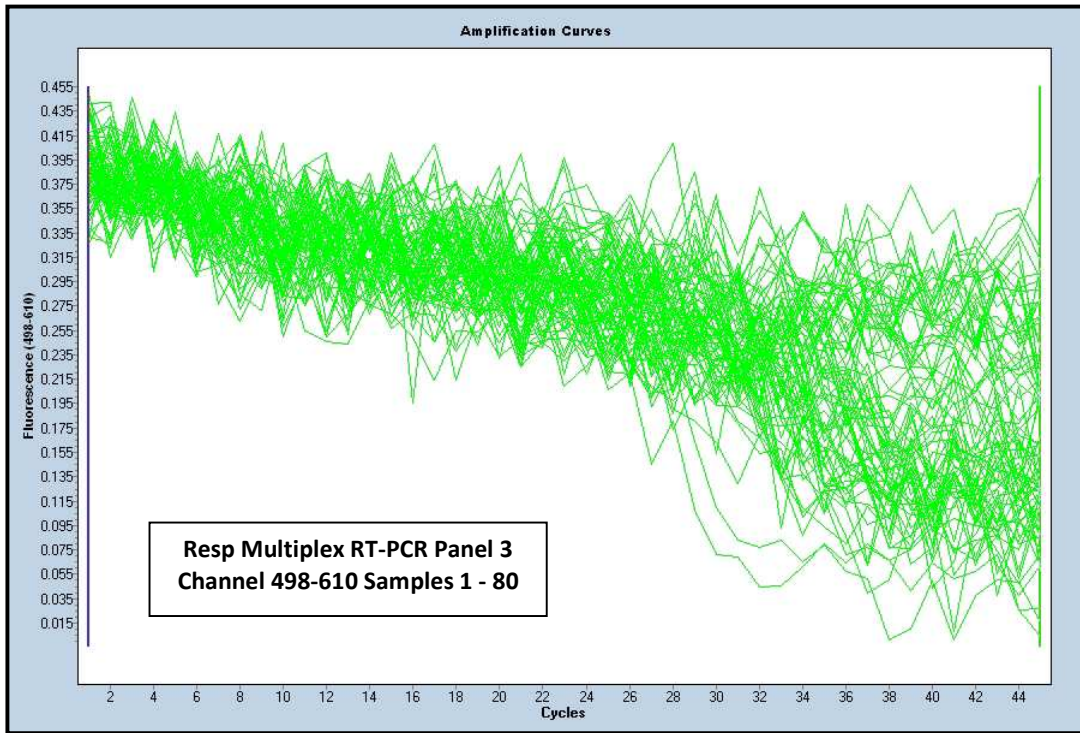


Figure 3.5: Unsuccessful amplification of influenza A virus with the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit.

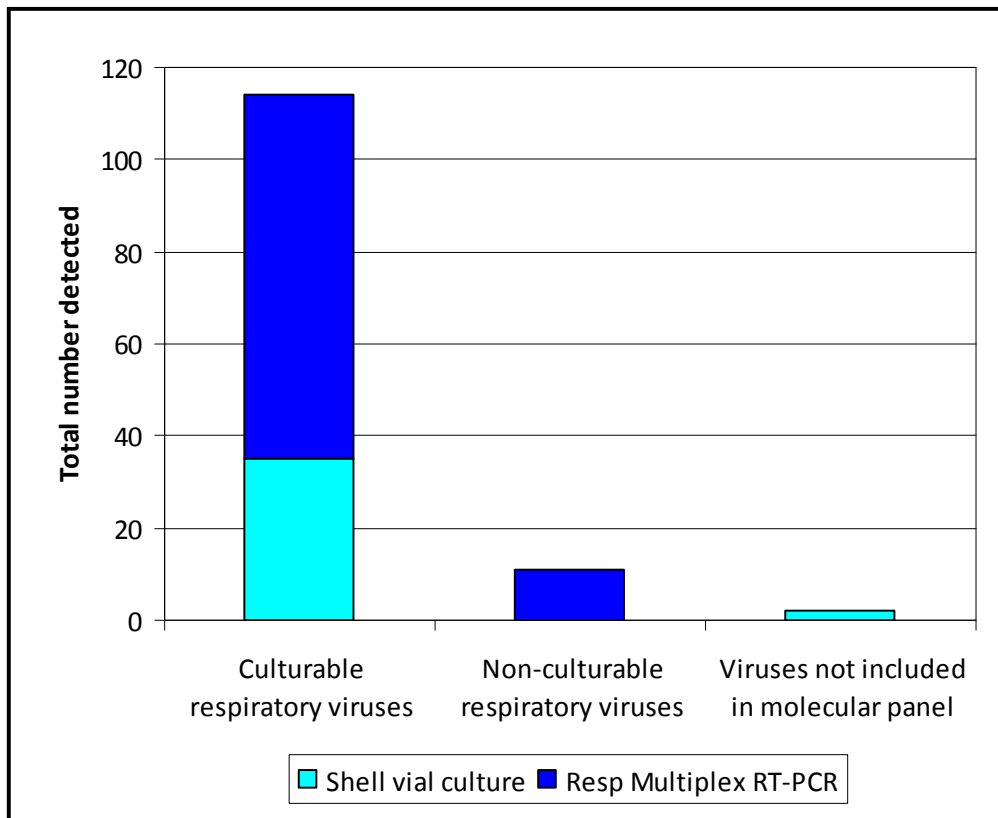


Figure 3.6: Additional viruses detected by Resp Multiplex RT-PCR compared with viruses cultured by shell vial culture.

### 3.2.4 Sensitivity and specificity

The sensitivity and specificity of each assay was calculated using 2 x 2 tables for the viruses that could be detected by at least two of the three methods (i.e. RSV, influenza virus A and B, parainfluenza virus types 1 – 3, adenovirus, human coronaviruses, HBoV and HMPV) (Table 3.2). Unfortunately sensitivity and specificity for each assay with regards to detection of influenza B virus could not be determined as none were detected during the study period. Sensitivity and specificity for influenza A virus could also not be determined, as the virus was only detected by the Seeplex® RV15 ACE Detection

Table 3.2: Sensitivity and specificity of all three diagnostic methods for detection of respiratory viruses in 138 clinical specimens												
Virus	True positive	Shell vial culture			Seeplex® RV15 ACE			Resp Multiplex RT-PCR				
		n	Sens (%)	Spec (%)	n	Sens (%)	Spec (%)	n	Sens (%)	Spec (%)		
RSV	38	23	60.5	100	43	100	95.0	43	97.4	94.1		
Adenovirus	12	6	50	100	18	100	95.2	13	100	99.2		
PIV1/PIV2	8	2	25	100	17	100	93.1	8	100	100		
PIV3	7	3	42.9	100	10	100	97.7	7	100	100		
HMPV	8	1	12.5	100	8	100	100	8	100	100		
HBoV	4	ND	ND	ND	5	100	99.3	4	100	100		
HCoV's	6	ND	ND	ND	7	100	99.2	7	100	99.2		

Sens = Sensitivity; Spec = Specificity; ND = Not determined.

True positive was defined as detection of a specific respiratory virus by more than one detection method

System. PIV1 and PIV2 were grouped together as the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit cannot distinguish between the two. The human coronaviruses were also grouped together as very few were detected, and the Seeplex® RV15 ACE Detection System cannot distinguish between HCoV-OC43 and HCoV-HKU1 or between HCoV-NL63 and HCoV-229E.

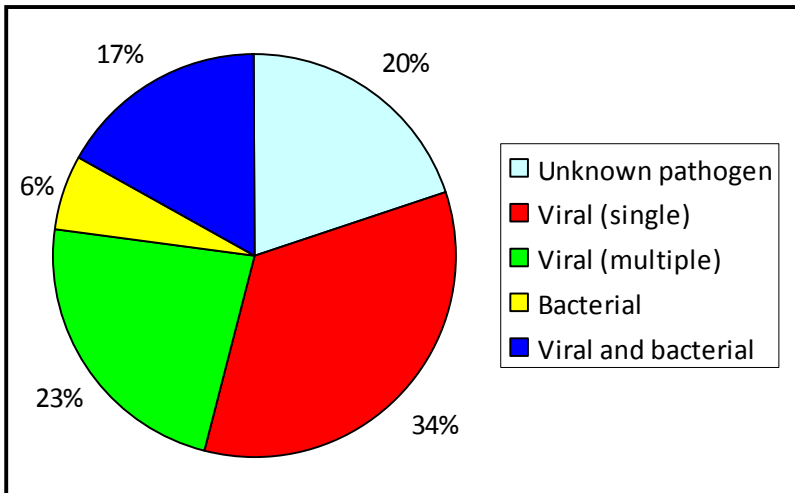
Shell vial culture with immunofluorescent staining had excellent specificity (100%), but low sensitivity for all of the respiratory viruses, ranging from 12.5% for HMPV to 60.5% for RSV. Conversely, the Seeplex® RV15 ACE Detection System had excellent sensitivity (100%) for all viruses, but slightly lower specificity, ranging from 93.1% for PIV1/PIV2 to 100% for HMPV. The additional viruses detected by the Seeplex® RV15 ACE Detection System, which contributed to the lower specificity of this assay, may have been true positives as this assay has an improved lower limit of detection compared to the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit. The Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit had excellent sensitivity, 100% for all viruses except for RSV (97.4%), and specificity, ranging from 94.1% for RSV to 100% for parainfluenza virus types 1 – 3, HBoV and HMPV.

The results obtained with the assay with the highest detection rate (i.e. the Seeplex® RV15 ACE Detection System), together with the two CMV positive results by shell vial culture only, were used for further analysis of the clinical characteristics associated with viral-viral and viral-bacterial co-infections.

### **3.3 Aetiology of respiratory tract infections in children**

At least one respiratory pathogen could be identified in 111 (80%) of the patients (Figure 3.7). A virus was found in three quarters (103/138) of the patients, with a single virus detected in 47 (34%) cases. The proportion of respiratory tract infections due to a single virus infection increased with age, while the proportion of children in whom a respiratory pathogen could not be identified decreased with age (Figure 3.8). However, it should be kept in mind that very few samples were received from children older than 12 months of age (22/138). RSV and HRV were the most commonly detected viruses in single viral ( $n = 18$  and  $n = 14$ ), multi-viral ( $n = 14$  and  $n = 16$ ) and viral-bacterial infections ( $n = 11$  and  $n = 10$ ). The multi-viral combinations most often detected were HRV and PIV3, and HRV and adenovirus (both  $n = 3$ ). The human coronaviruses, HEV, PIV4, CMV and HBoV were detected in less than 5% of all patients. Pneumonia ( $n = 21$ ), bronchopneumonia ( $n = 18$ ) and bronchiolitis ( $n = 10$ ) were the most common discharge diagnoses made in children with viral lower respiratory tract infections, while only two children, one with HRV and one with HCoV-OC43, were diagnosed with an upper respiratory tract infection.

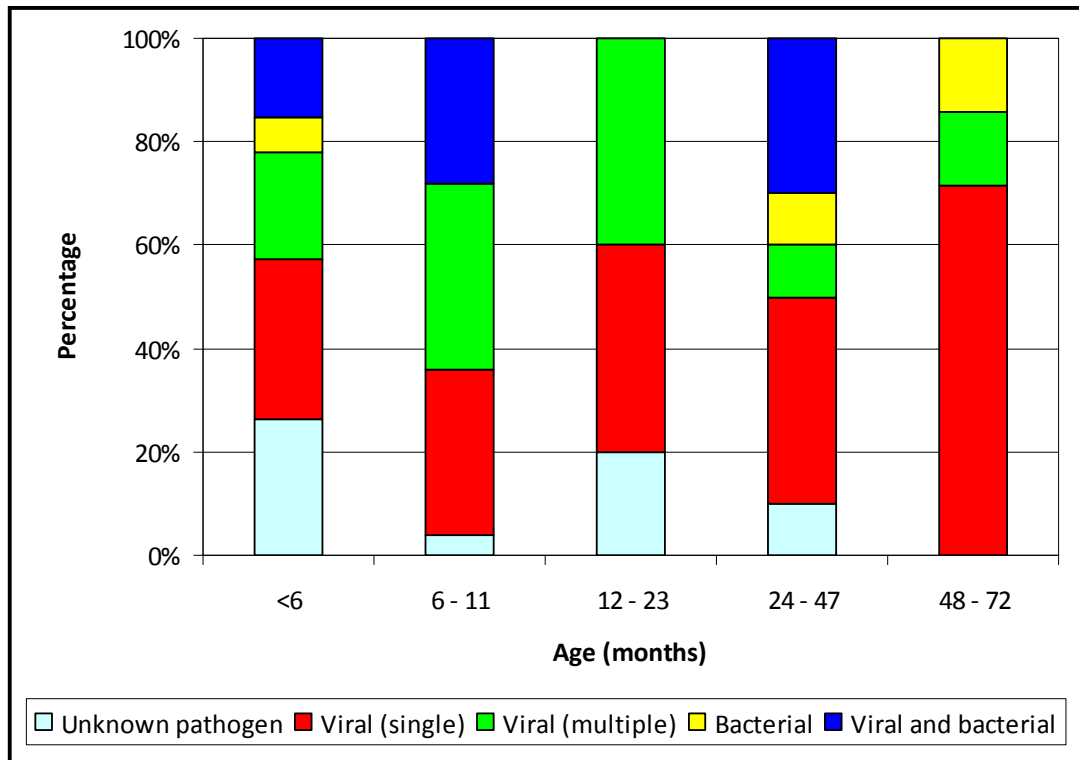




**Figure 3.7: Aetiology of respiratory tract infections in 138 hospitalised children less than 6 years of age.**

Respiratory samples from only 41 patients (30%) were sent for both bacterial and viral culture, while blood culture samples for bacterial diagnosis were collected from 91 patients (66%) on the same day as the respiratory sample. In 41 patients (30%) no samples were sent for bacterial diagnosis. A clinically significant bacterial micro-organism was detected in 32 (23%) patients, with at least one virus co-detected in three quarters of these patients. In 25 patients the organism was detected in the respiratory sample, in 5 patients it was found in the blood sample, and in two patients an organism was found in both sample types. Eight patients had more than one bacterial micro-organism detected in their tracheal aspirate, including one patient with three organisms, and one patient had two organisms in their blood culture sample. The bacterial micro-organisms most often identified were *Klebsiella pneumoniae* (n = 9), of which five isolates were extended spectrum  $\beta$ -lactamase producers, *Acinetobacter baumannii* (n = 7), and *Pseudomonas aeruginosa* (n = 4). Two children each were diagnosed with *Staphylococcus aureus* and *Streptococcus pneumoniae*, and one of these children was co-infected with *Haemophilus influenzae type B*. Four children in the study were diagnosed with pulmonary tuberculosis, of which one child had multi-drug resistant tuberculosis.

The viral-bacterial co-infections most often detected were HRV and *Klebsiella pneumoniae*, RSV and *Klebsiella pneumoniae*, and HRV and *Acinetobacter baumannii* (all n = 2). Pneumonia (n = 9) and bronchopneumonia (n = 4) were the most common discharge diagnoses noted in children with mixed viral-bacterial co-infections.



**Figure 3.8: Distribution of pathogens associated with paediatric respiratory tract infections, stratified by age.**

The demographic and clinical characteristics of the patients by infection type are shown in Table 3.3. The median age of the children where no pathogen could be detected was significantly younger than that of children with single viral, multi-viral and viral-bacterial infections ( $p = 0.004$ ). There were no significant differences in the children's haemoglobin or C-reactive protein (CRP) levels, white blood cell or platelet count, apart from the differences in neutrophil percentage ( $p = 0.038$ ) and CRP level ( $p = 0.009$ ) between the children with viral-bacterial co-infections and those without an identifiable pathogen. Viral-bacterial co-infections were associated with increased severity compared to other infections, as these children were more likely to receive steroids and a blood transfusion ( $p = 0.002$ ), and more likely to require mechanical ventilation ( $p < 0.001$ ) and admission to the intensive care unit ( $p = 0.04$ ). There was also a trend towards increased mortality in this group, but it did not reach significance ( $p = 0.08$ ). Comprehensive analysis of the clinical importance of the different respiratory viruses was not feasible due to the small numbers of each virus observed in the single viral category.

**Table 3.3: Demographic and clinical characteristics of hospitalised children with respiratory tract infections**

Characteristic	Viral (single) (n = 47)	Viral (multiple) (n = 32)	Viral- Bacterial (n = 24)	Bacterial (n = 8)	Unknown pathogen (n = 27)
Median age (months)	3	5	4	2	1
Male gender	29 (62)	17 (53)	14 (58)	6 (75)	17 (63)
Born prematurely	14 (30)	11 (34)	7 (29)	2 (25)	17 (63)
Median gestational age at birth (weeks)	30	29	32	30	28
Median white blood cell count (x 10 <sup>9</sup> /L)	12.1	11.5	14.0	13.4	9.8
Neutrophils percentage	56.0	56.5	67.6	55.4	44.6
Lymphocytes percentage	31.7	31.4	19.1	28.6	32.0
Median haemoglobin (g/dL)	10.5	10.0	10.0	10.9	10.2
Median platelets (x 10 <sup>9</sup> /L)	422	405	348	610	343
Median CRP (mg/L)	14	5.5	47	15.5	4
Received steroids	8 (17)	9 (28)	11 (46)	1 (13)	3 (11)
Received blood transfusion	12 (26)	9 (28)	14 (58)	2 (25)	7 (26)
Mechanical ventilation	19 (40)	9 (28)	16 (67)	5 (63)	15 (56)
Median duration of ventilation (days)	11	5	7.5	7	4.5
Admission to ICU	15 (32)	10 (31)	16 (67)	3 (38)	10 (37)
Median duration of ICU admission (days)	14	4	8	13	4.5
Median duration of hospitalisation (days)	11.5	9	18.5	35.5	21
Died during hospitalisation	5 (11)	2 (6)	5 (21)	0	0
Underlying high-risk medical condition	30 (64)	15 (47)	10 (42)	4 (50)	21 (78)
Chronic pulmonary disease	6	1	2	0	0
Cardiac disease	6	2	0	1	6
Immunosuppression	7	4	1	0	2
Prematurity	12	8	7	2	16
Neurologic disease	0	1	0	1	1
Down syndrome	3	0	0	0	1
≥ 2 underlying high-risk medical condition	3 (6)	2 (6)	0	0	5 (19)

All values in parentheses are percentages.

## Chapter 4

# Discussion

One hundred thirty-eight hospitalised children less than six years of age with suspected lower respiratory tract infections were studied to determine the possible viral aetiology of their infections, using both conventional culture and molecular methods.

### 4.1 Viral diagnostic methods

The shell vial culture technique with immunofluorescent staining detected a respiratory virus in 26.1% of all samples, which is in keeping with reported detection rates for this method (Kim et al., 2009; Sanghavi et al., 2012; Templeton et al., 2004; Tiveljung-Lindell et al., 2009). The Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit doubled the detection rate by detecting a virus in 52.9% of the samples, whilst the Seeplex® RV15 ACE Detection System identified a viral pathogen in an additional 44 samples to increase the diagnostic yield to 73.9%. This increase in detection rate between conventional culture and molecular methods is also in agreement with previous observations, with the 47.8% difference between shell vial culture and the Seeplex® RV15 ACE Detection System one of the largest described (Freymuth et al., 2006; Lassaunière et al., 2010; Sanghavi et al., 2012; Tiveljung-Lindell et al., 2009). In keeping with previous studies (Freymuth et al., 2006; Kim et al., 2009; Sanghavi et al., 2012), the majority of additional viruses detected by both molecular methods were viruses that are also detectable by shell vial culture (i.e. culturable viruses). The increased sensitivity of molecular methods over conventional methods is therefore most likely due to a) the detection of lower virus titres (below the sensitivity of culture methods); b) the detection of viral nucleic acid of viruses that have lost their viability, possibly *in vivo* or during transport; and c) the detection of non-culturable viruses and replication incompetent viruses. The role of the increased sensitivity of molecular methods for low titre specimens is substantiated by previous studies where the Ct-value of viruses detected by real-time PCR only, is significantly higher than the Ct-value of viruses detected by both real-time PCR and conventional methods (Kuypers et al., 2006; Templeton et al., 2004). All three diagnostic methods were able to detect viral co-infections in similar proportions as reported previously (Freymuth et al., 2006; Kim et al., 2009; Kuypers et al., 2006; Tiveljung-Lindell et al., 2009). Once again the Seeplex® RV15 ACE Detection System has one of the highest viral co-infection detection rates (27.5%) reported. The lower co-detection rate of shell vial culture with immunofluorescent staining (0.7%) during this study may be due to overgrowth of

the faster growing virus, interfering with the growth of the slower growing virus, or the technique's inability to detect the co-pathogen.

The performances of all three assays with regards to the respiratory viruses detectable by more than one of the assays were compared using a consensus positive result (i.e. detection of a specific respiratory virus by more than one detection method) as the true positive result. The Seeplex® RV15 ACE Detection System and the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit yielded comparable sensitivities for most of the respiratory viruses that were detectable by both methods, and both were vastly superior to shell vial culture with regards to the viruses detectable by all three methods. Unfortunately the assays' ability to detect both influenza A and B could not be evaluated as none of the assays detected influenza B virus in any sample, whilst only the Seeplex® RV15 ACE Detection System was able to detect influenza A virus in 14 samples. It is unlikely that the influenza viruses detected by this assay were false reactive as the samples were collected during the winter season when influenza viruses circulate in South Africa (Gessner et al., 2011) and both influenza A and B were detected in samples collected from other patients in the community during the study period as part of the Viral Watch Influenza Sentinel Surveillance programme (Maree L, personal communication, 31 July 2012). The reason for the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit's inability to detect influenza virus A remains unclear. As stated previously, the same extracted nucleic acid and cDNA were used in all experiments, and both of the other detection channels of Panel 3 could be read, as could the other panels in this particular detection channel. It is thus unlikely to be due to sample inhibition or instrument failure. Perhaps this particular hybridisation probe set was damaged during manufacturing or transport, leading to indiscriminate hybridisation or erroneous light emission.

Shell vial culture had 100% specificity with regards to all relevant viruses as it detected no additional viruses that were not detected by the molecular assays. Both of the molecular assays had slightly lower specificities, with both detecting additional viruses not detected by any of the other assays. As a consensus true positive was used, the additional viruses detected may have been true positives if this assay is more sensitive than the other assays. This may be the case with regards to the Seeplex® RV15 ACE Detection System as it has a reported lower limit of detection of 100 copies per reaction. With a 1ml sample input volume during the initial extraction process, this equates to a lower limit of detection of 100 copies/ml. The lower limit of detection of the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit is reported to be 25 000 copies/ml of specimen. It is important to remember that the sensitivity of any RT-PCR assay to detect RNA viruses can also be influenced by the efficiency of the reverse transcription reaction. The efficiency of the RNA-to-cDNA conversion can be negatively

influenced by a) the enzyme and primer method used; b) the degree of RNA fragmentation; and c) the presence of large quantities of non-specific background RNA together with a relatively smaller quantity of target RNA (Bustin and Nolan, 2004). It would have been more helpful to use another, previously validated, NAAT as a tie-breaker to determine whether a result is a true positive, instead of only using the consensus result between two molecular techniques as the gold standard. Alternatively the discrepant samples could have been sequenced and phylogenetically analysed to confirm or disprove the result.

Despite the increased sensitivity and specificity demonstrated by both NAATs, there are a few drawbacks to both assays that should be kept in mind. The Seeplex® RV15 ACE Detection System is a multiplex PCR assay that requires a separate detection step after amplification. This adds an additional step to the test procedure where carry-over contamination can occur and it also increases the turn-around-time. The separate reverse transcription and amplification steps, instead of a one-step system, can also lead to potential sample contamination. The 8-MOP system with irradiation of the samples after amplification, but before detection, should help in regard with potential contamination. Another drawback of this assay is the lack of an extraction and reverse transcription control. As the internal control is only added to each sample after these steps, and the positive and negative controls also do not go through these steps, there is no mechanism in place to ensure that a negative result is truly negative and not due to problems encountered during these steps. A solution to this problem could be to include a known positive sample containing an RNA virus in each run to serve as an extraction and reverse transcription control.

The Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit is a multiplex real-time PCR that has a shorter turn-around-time than the Seeplex® RV15 ACE Detection System. Unfortunately this assay does not have an internal control in each sample to monitor the extraction, reverse transcription or amplification steps, and any sample inhibition will not be detected. It is unclear why the internal control, which was reported during the initial development (Lassaunière et al., 2010), was removed from the assay upon manufacturing. As with the Seeplex® RV15 ACE Detection System, this assay also requires reverse transcription and amplification to be performed in separate steps, thus allowing for the possibility of carry-over contamination. An advantage of the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit over the Seeplex® RV15 ACE Detection System is that it can provide an estimated semi-quantitative result. Previous studies have linked a higher viral load as detected by real-time PCR to increased disease severity for several respiratory viruses (Bosis et al., 2008; El Saleeby et al., 2011; Li et al., 2010; Scagnolari et al., 2012).

One of the main advantages of molecular methods over conventional culture techniques is their ability to detect viruses that are unculturable or difficult to culture, and thus previously undiagnosed. Now that we are able to detect these viruses they are increasingly identified in severe cases, for example in immunocompromised patients, but their precise role in the disease process still has to be elucidated. This is further complicated by the fact that NAATs are unable to distinguish between replication-competent and -incompetent viruses. A detectable virus may therefore only be remnants from a previous infection, without active contribution to the current infection. It must also be kept in mind that most diagnostic molecular techniques only test for specific agents due to the precise nature of the assays. Thus other pathogens that may be responsible for the patient's illness will remain undetected. This is of importance in our South African setting where a large proportion of the population is immunocompromised due to infection with or exposure to HIV-1. In these cases viruses such as CMV should be included in the differential diagnosis of severe lower respiratory tract infection in a child, but unfortunately both the Respiratory Multiplex Real-Time RT-PCR LightMix® Customised Kit and the Seplex® RV15 ACE Detection System do not test for this virus, and it has to be tested for separately. Due to the genetic variability and near constant drift observed in especially the RNA viruses, all molecular diagnostic tests must be monitored continuously with updating of the necessary primers and probes when indicated.

Molecular techniques such as multiplex PCR and real-time PCR are essential for the future exploration of the epidemiology, clinical presentation and impact of respiratory viruses in both adults and children, and it will become even more important in the management of these patients when additional antiviral medications against the organisms become available.

## **4.2 Aetiology of respiratory tract infections in children**

The aetiology of respiratory tract infections in children under the age of six years could be determined in 80% of the children included in this study using both conventional culture and molecular methods. This is in keeping with previous studies where a pathogen could be detected in up to 90% of paediatric community-acquired pneumonia cases (Cevey-Macherel et al., 2009; Hamano-Hasegawa et al., 2008; Juvén et al., 2000; Lahti et al., 2009; Michelow et al., 2004; Tsolia et al., 2004). Viruses were associated with 57% of the respiratory tract infections in this study, bacteria with 6%, and mixed viral-bacterial infections were detected in 17% of cases. This is the highest percentage of paediatric respiratory tract infections ascribed to viruses, but in the previous studies fewer different viruses were tested for or less sensitive diagnostic tests were used. Conversely, the percentage of cases ascribed to

bacterial micro-organisms is one of the lowest described, but not all possible bacterial causes of ARTIs were tested for in this study. The limited testing for bacterial micro-organisms in this study is most likely also responsible for the smaller proportion of cases attributed to mixed viral-bacterial infections.

In keeping with previous studies, RSV (31%) was the leading viral cause of ARTIs in the children of this study (Hall et al., 2009; Nair et al., 2010; Weber et al., 1998). As in previous studies utilising NAATs, HRV (29%) was detected in a sizable proportion of patients, followed by adenovirus (13%) and influenza virus A (10%) (Do et al., 2011; Gern, 2010; Juvén et al., 2000; Laundry et al., 2003; Okada et al., 2012). The role of HRV in the aetiology of LRTIs remains controversial, but evidence is emerging that it is a significant cause of pneumonia, bronchiolitis, and acute asthma exacerbations (Gern, 2010; Kling et al., 2005). In contrast with previous observations (Laurichesse et al., 1999; Reed et al., 1997), PIV1 and PIV2 were detected most often in children less than one year of age, as were PIV3 and PIV4. However, this observation may have been influenced by the fact that more than three quarters of all samples were collected from children less than 12 months of age. This bias towards infants less than one year of age may also explain why the median age of children infected with HMPV was similar to that of RSV (2.6 months vs. 2.5 months), and not significantly older as had been reported previously (Boivin et al., 2003; Døllner et al., 2004; Manoha et al., 2007; Mullins et al., 2004). Consistent with previous observations, HBoV was mainly detected together with another respiratory virus (Calvo et al., 2008b; Christensen et al., 2008; Moriyama et al., 2010), while a larger proportion of HMPVs were co-detected with another viral pathogen than previously reported (Bastien et al., 2003; Boivin et al., 2003; Williams et al., 2004). Although no influenza B viruses were detected in this study, it remains an important cause of viral pneumonia in both adults and children (Hamano-Hasegawa et al., 2008; Jennings et al., 2008).

As in previous studies, almost one quarter of the children presenting with ARTI in this study was infected with more than one virus, with RSV, HRV and influenza virus A most often involved (Canducci et al., 2008; Cilla et al., 2008; Do et al., 2011; Richard et al., 2008). Viral co-infections were most frequently observed in children younger than 2 years of age, which is most likely due to the greater incidence of viral respiratory tract infections as a whole in this age group. The large number of children with underlying medical conditions (64%), and thus increased susceptibility, may also have contributed to the large percentage of viral co-infections. There are conflicting results regarding the clinical importance of multi-viral respiratory tract infections, with some studies demonstrating increased disease severity and a higher risk of ICU admission with multi-viral infections (Richard et al., 2008; Semple et al., 2005) while others, including this study, failed to show this association (Canducci et al., 2008; Wolf et al., 2006). Differences in detection methods and their respective sensitivities, seasonal variation in circulating



viruses, variation in sampling methods which could underestimate detection rates, the use of diverse clinical parameters to define disease severity, increased host susceptibility and virus-specific host-virus interactions could contribute these conflicting results.

In contrast to previous observations (Juvén et al., 2000; Michelow et al., 2004; Rudan et al., 2008), the bacterial micro-organisms most often identified in this study were *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. The low detection rates for *Streptococcus pneumoniae* and *Haemophilus influenzae* type B were in all probability influenced by the inclusion of vaccines against these organisms in the Expanded Programme on Immunisation in South Africa since 2009 and 1999, respectively (Group for Enteric, Respiratory and Meningeal disease Surveillance in South Africa, 2012). The relatively high detection rate of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* most likely reflects nosocomial acquisition, as these organisms are widely associated with hospital-acquired and ventilator-associated pneumonia (Jones, 2010). Although almost a third of patients were not tested for a bacterial respiratory pathogen, 17% of the patients were co-infected with viral and bacterial pathogens. Only 5% of the bacterial micro-organisms detected in this study were isolated in blood culture samples, which is in keeping with the expected detection rate for this method (British Thoracic Society Standards of Care Committee, 2002). Atypical and difficult to culture bacterial micro-organisms, such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, are responsible for between 3% and 35% of all cases of pneumonia in children (Juvén et al., 2000; Lahti et al., 2009; Michelow et al., 2004; Tsolia et al., 2004), but unfortunately were not included in this study. It may be presumed that the proportion of patients in which a bacterial or mixed viral-bacterial infection could be identified would increase if these organisms were also included in the diagnostic molecular panel.

Often it is very difficult to determine the significance of bacterial micro-organisms isolated from respiratory tract samples as they may only represent colonisation. However, these commensal organisms may also be responsible for the lower respiratory tract infection. For example the Gram-negative bacilli have been identified as a major cause of hospital-acquired and ventilator-associated pneumonia, leading to prolonged hospitalisation and increased costs (Zar and Cotton, 2002). Recently, Hiram and colleagues (2011) proposed a novel way of determining the clinical significance of commensal bacterial micro-organisms in respiratory tract samples utilising RT-PCR. They developed a pneumonia “battlefield hypothesis” whereby the ratio of pathogenic organisms detected (as determined by the RT-PCR cycle threshold value (Ct-value) of the organism) to human surfactant protein C concentration in the sample (as determined by the RT-PCR Ct-value) may be indicative of the pathogenic

role of the organism. During ARTI the numbers of both the causative bacterial micro-organism and inflammatory cells will increase at the site of infection, while commensal organisms will lag behind.

Mixed viral-bacterial respiratory tract infections were detected in 17% of the children in this study. This is slightly lower than the percentage reported by several studies (Cevey-Macherel et al., 2009; Juvén et al., 2000; Lahti et al., 2009; Tsolia et al., 2004), but in keeping with others (Hamano-Hasegawa et al., 2008; Michelow et al., 2004). This lower co-detection rate is probably due to the limited variety of diagnostic tests for bacterial micro-organisms used in this study. Mixed viral-bacterial respiratory tract infections appear to be more common in children less than 2 years of age, probably reflecting the higher incidence of viral respiratory tract infections, especially RSV, in this age group (Korppi, 1999). It is commonly observed that viral respiratory tract infections pave the way for bacterial super-infection, often with commensal organisms found in the patient's own upper respiratory tract. Several mechanisms have been proposed to explain this phenomenon, including a) virus-induced immune dysfunction, which may interfere with antimicrobial penetration at the site of infection; b) enhanced bacterial attachment to the basement membrane exposed after destruction of the respiratory epithelial cells by the preceding virus infection; c) cough reflex impairment; and d) loss of virus-infected ciliated cells with subsequent accumulation of respiratory secretions (Bakaletz, 1995; Korppi, 1999). It has also been shown in an animal model that the neuraminidase activity of influenza virus exposes various cellular structures that can serve as bacterial receptors, leading to enhanced attachment and invasion by *Streptococcus pneumoniae*. This interaction could explain the high mortality due to secondary bacterial pneumonia observed during several influenza virus pandemics, and the reduction in secondary complications observed in several studies where patients were treated with one of the neuraminidase inhibitors, or vaccinated against influenza (Peltola and McCullers, 2004). In a large randomised, double-blind, placebo-controlled study to evaluate the efficacy of a 9-valent pneumococcal conjugate vaccine, it was noted the vaccine also prevented a substantial proportion of hospitalisations due to virus-associated pneumonia, indicating that bacterial co-infection occurs in a large percentage of cases attributed to viruses alone (Madhi et al., 2004). In this study neither *Streptococcus pneumoniae*, *Haemophilus influenzae type B* nor *Staphylococcus aureus* was co-detected with influenza A virus in any patient.

It has previously been shown in several studies that acute phase markers such as CRP and white blood cell count are significantly higher in patients with bacterial respiratory tract infections than in patients with viral respiratory tract infections (Cevey-Macherel et al., 2009; Korppi and Kröger, 1993; Okada et al., 2012). However, other studies, including this study, have indicated that these laboratory

findings are poor indicators of a possible aetiology in respiratory tract infections, especially with regards to the atypical bacteria, and low values do not rule out bacterial infection (British Thoracic Society Standards of Care Committee, 2002; Burman et al., 1991; Hamano-Hasegawa et al., 2008; Michelow et al., 2004; Tsolia et al., 2004). Several studies have suggested that procalcitonin is a better marker than CRP to distinguish between bacterial and viral infections, and bacterial infections and non-infective causes of inflammation (Simon et al., 2004). Unfortunately procalcitonin measurement is not readily performed in Tygerberg Children's hospital as the test must be referred to another laboratory with subsequent delays in results.

The clinical impact of mixed viral-bacterial infections remains unresolved, but evidence is mounting that mixed infections cause more severe disease than sole viral or sole bacterial infections. In a study to evaluate the clinical response to antibiotic therapy in patients with community-acquired pneumonia, it was noted that patients with mixed infections took longer to become afebrile after commencing antibiotic therapy than individual viral or bacterial infections (Juvén et al., 2004). It was also noted that half of the patients who were considered to have treatment failure, defined as fever lasting more than 48 hours after initiating antibiotic therapy, had mixed infections. All of the children with community-acquired pneumonia in another study who failed to respond to antimicrobial therapy were found to have mixed viral-bacterial infections (Honkinen et al., 2012). Co-infection with HRV and *Streptococcus pneumoniae* has been independently associated with increased severity in adults with community-acquired pneumonia (Jennings et al., 2008), while co-infection with influenza virus and *Staphylococcus aureus* causes more severe and often fatal pneumonia in children (Reed et al., 2009). Mixed viral-bacterial infections have been associated with longer hospitalisation and an increased risk of PICU admission than viral infection alone in children with cancer and neutropaenia (Torres et al., 2012), and a trend towards prolonged hospitalisation was noted in children with invasive pneumococcal disease with concomitant viral infection (Techasaensiri et al., 2010). In this study children with viral-bacterial co-infection were significantly more likely to receive a blood transfusion and steroids. They were also more likely to require mechanical ventilation and intensive care, with a trend towards increased mortality. This confirms the findings of another study conducted in Cape Town, South Africa, where paediatric patients admitted to the PICU with mixed viral-bacterial respiratory tract infections were more likely to require mechanical ventilation than children infected with a viral pathogen only (Ghani et al., 2012). The length of ICU admission and total hospital admission was also significantly longer in these patients, but there was no increased risk of mortality.

### 4.3 Limitations

There are a few limitations to this study. Firstly, the study was conducted at a single centre for a limited time period. Tygerberg Children's hospital is a tertiary-level hospital that provides specialised care to a large proportion of the Western Cape and beyond. Thus patients with mild or moderate illness may have been underrepresented in our study as they would have been treated at their local clinic or day hospital where diagnostic testing for respiratory tract infections is rarely, if ever, performed. This is reflected in the large percentage of patients requiring mechanical ventilation (51%) and intensive care admission (43%). The seasonality of the viruses tested for during this study can vary significantly from season to season, and sometimes from year to year. The significance of certain viruses may therefore have been over- or underestimated due to the short study period. Thus the results of our study may not be generalisable to other settings.

Another limitation of the study is that a control group was not included. A control group is especially important with regards to determining the clinical significance of the newly discovered and newly recognised lower respiratory tract viruses, such as HBoV, HMPV, HEV and HRV. It must be kept in mind that detection of viral RNA or DNA in respiratory samples does not necessarily prove involvement of that particular virus in the patient's pathology or symptomatology. But detection of these viruses in a significantly higher percentage of symptomatic patients, or the inability to detect them in asymptomatic controls, will add to the body of evidence strengthening their role in respiratory infections.

As stated previously, molecular diagnostic methods only test for the specific agents they are designed to detect. The assays used in this study are unable to detect several viruses implicated in the aetiology of viral respiratory tract infections, most notably CMV, the respiratory polyomaviruses and the human parechoviruses. These viruses may contribute significantly to the extent and severity of respiratory tract infections in children, both singly and in combination with other viruses or bacteria, and this requires further investigation.

### 4.4 Conclusions

In conclusion we found that molecular techniques are significantly more sensitive and specific than shell vial culture with immunofluorescent staining for the detection of viruses in children with acute respiratory tract infections. However, due to their highly specific nature and the genetic variability observed in viruses, an excellent, continuous quality control programme is essential to ensure the continued superiority of these assays.

RSV remains the most common cause of acute lower respiratory tract infections in children, with HRV also detected in a significant number of cases. The precise role of HRV in the aetiology of lower respiratory tract infections remains unknown, but evidence is mounting to confirm its increasing importance (Gern, 2010). The role of other, more recently described viruses, such as HBoV, HMPV and the respiratory polyomaviruses, also require further study.

In this study we confirmed the increased severity of viral-bacterial co-infection in children with acute respiratory tract infections. Further research is needed to elucidate the precise pathogenic and immunologic mechanism of this interaction, and the relative importance of the specific viral and bacterial pathogens involved. The impact of treatment of either or both of the infecting pathogens, especially when more antiviral treatments and vaccines become available, also requires further study.

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