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Development of quantitative multiplex real-time RT-PCR assays for detection of 13 conventional and newly discovered viruses associated with lower respiratory tract infections in children in South Africa

By

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Declaration

The results presented here, follow from research carried out at the Department of Medical Virology, University of Pretoria, under the supervision of Dr. Marietjie Venter. These results are original and have not been submitted in any form to any other university.

Publications and Presentations

***Maiden surname**

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3. James A Berkley, Patrick Munywoki, Mwamajuma Ngama, Sidi Kazungu, John Abwao, Ann Bett, **Ria Lassaunière**, Tina Kresfelder, Pat Cane, Marietjie Venter, J Anthony G Scott and D James Nokes. *Viral etiology of severe pneumonia among Kenyan young infants and children.* Journal of the American Medical Association. In Press. (Impact Factor 23.5).
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Selected Abbreviations Used in Text

Adv	adenovirus
AIDS	acquired immunodeficiency syndrome
ALRI	acute lower respiratory tract infection
BAL	bronchoalveolar lavage
BLAST	basic local alignment search tool
℃	temperature in degrees Celsius
cDNA	complementary DNA
CMV	cytomegalovirus
CPE	cytopathic effect
DNA	deoxyribonucleic acid
E	envelope protein
F	surface fusion glycoprotein
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
G	surface attachment glycoprotein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIT	gastrointestinal
hBoV	human bocavirus
hCoV	human coronavirus
HE	haemagglutinin-esterase glycoprotein
HEF	haemagglutinin-esterase-fusion protein
HIV	human immunodeficiency virus
HN	haemagglutinin-neuraminidase protein
hMPV	human metapneumovirus
HSCT	hematopoietic stem cell transplant
ICU	intensive care unit
IFA	immunofluorescence assay
Ig	immunoglobulin
kb	kilobase
L	RNA-dependent RNA polymerase or large protein
LRTI	lower respiratory tract infection
LRTI NOS	lower respiratory tract infection not otherwise specified
M	matrix protein
M2	second matrix protein

µl	microlitre/s
N	nucleocapsid protein
NA	neuraminidase protein
NPA	nasopharyngeal aspirate
NS	non-structural protein
ORF	open reading frame
P	phosphoprotein
PA	polymerase acid protein
PB1	polymerase basic 1 protein
PB2	polymerase basic 2 protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIV	parainfluenza virus
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-PCR	reverse transcription PCR
S	spike protein
SCID	severe combined immunodeficiency disorder
SH	small hydrophobic
Tm	melting temperature
tMK	tertiary monkey kidney cells
URTI	upper respiratory tract infection
WHO	World Health Organization

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Abstract

Development of quantitative multiplex real-time RT-PCR assays for detection of 13 conventional and newly discovered viruses associated with lower respiratory tract infections in children in South Africa

By

MM Lassauniere

Supervisor: Dr. Marietjie Venter

Department: Medical Virology

Degree: MSc. Medical Virology

Acute lower respiratory tract infection (ALRI) is a major cause of paediatric morbidity and mortality, annually accounting for approximately 2 million childhood deaths worldwide of which up to 90% resides in the developing world. In 12-39% of ALRI cases no aetiological agent is identified, despite comprehensive investigations, thus suggesting that additional unknown agents may be involved. Since 2001 a number of new viruses have been identified that may account for some of these cases including human metapneumovirus (hMPV), human bocavirus (hBoV), and two new coronaviruses (hCoV) NL63 and HKU1. The contribution of the recently identified respiratory viruses to annual seasonal lower respiratory tract disease in Sub-Saharan Africa where human immunodeficiency virus infections may exacerbate respiratory infections is not fully understood. In addition, the role and disease association of many of these viruses as primary or co-infecting pathogens, as well as the underlying factors that may determine the pathogenesis of these viruses, is not yet well defined.

Quantitative multiplex real-time RT-PCR assays were developed and validated for the detection of 13 well recognized and newly identified viral causes of ALRI, including respiratory syncytial virus (RSV), influenza viruses A and B, parainfluenza virus (PIV) types 1, 2, and 3, adenovirus, hMPV, hBoV and hCoV-NL63, -HKU1, -229E, and -OC43. The newly designed assays were subsequently used to facilitate the investigation of the contribution of respiratory viruses in patients requiring hospitalisation or attending outpatient visits in public sector hospitals serving the Pretoria area, South Africa. During 2006, the prevalence of the aforementioned respiratory viruses was determined by investigating the well recognized viruses previously diagnosed by routine immunofluorescence assays (IFA) in 737 respiratory specimens as well as viruses retrospectively detected by multiplex real-time RT-PCR in a sample group of 319 specimens. The epidemiology and disease association of these respiratory viruses in children who were predominantly less than 5 years of age with acute respiratory tract infections was investigated. Specimens were received from 2 public sector hospitals in Pretoria, South Africa. In addition, the disease association of each virus as a single or co-infection in human immunodeficiency virus (HIV) infected/exposed and HIV-uninfected children as well as the role of viral load was investigated.

The multiplex assays could detect 2.5-25 recombinant plasmid DNA/RNA (*in vitro* transcribed) copies/µl, with a co-efficient of variation of less than 3.1%. Validation on 91 known positive respiratory specimens indicated similar specificity to IFA or single-round

PCRs used in the initial identification of the viruses. Application of the multiplex assays to IFA negative specimens improved the detection of respiratory viruses by up to 44%. In children less than 5 years of age RSV was identified in 35.1%, followed by PIV 3 (8.3%); adenovirus (5.6%); influenza A (4.2%); hMPV (4.2%); hBoV (3.8%); hCoV-NL63 (1.6%); influenza B (1.0%); and PIV1, PIV2, hCoV-OC43, hCoV-229E, hCoV-HKU1 in less than 1% of cases. Co-infections were more common for the new viruses ranging from 58% of hMPV cases to 84% for hCoV-NL63 relative to 27% of RSV cases. Viruses were most frequently identified in children <1-year. RSV activity peaked in autumn and winter, PIV 3 in spring, while influenza A and B were mostly detected in winter. The observed seasonal distribution of hBoV and hMPV was less defined compared to traditional viruses, with both viruses showing variability over the two years. Comparable hospitalisation rates were observed for RSV, hMPV, PIV 3 and adenovirus, where approximately 60% of infected children were hospitalised. In addition, a high frequency of hospitalisations was observed in patients for both hMPV and hBoV in HIV-infected/exposed children. Co-infections occurred at higher frequencies with the new viruses, were more frequently associated with severe disease and were frequent in HIV-infected/exposed patients. Viral load was associated with severe RSV disease (p=0.014) however no significant association was observed for the new viruses as single infections. However, where hMPV occurred as a co-infection, higher viral loads of either hMPV or co-infecting agents occurred in severe cases. This association was also observed for hBoV. Most cases of hCoV-NL63 and hCoV-OC43 were co-infections in hospitalised patients.

The newly developed multiplex assays demonstrates an improved sensitivity and scope of detecting respiratory viruses relative to routine antigen detection assays while the quantitative utility may facilitate investigation of the role of co-infections and viral load in respiratory virus pathogenesis. RSV remains the most significant viral cause of paediatric ALRI in South Africa. Viruses not currently included in routine diagnostic assays collectively contributed to 11% of ALRI cases in children <2-years in South African hospitals.

Chapter 1

Literature Survey

1.1 Introduction

Acute lower respiratory tract infection (ALRI) is a major cause of paediatric morbidity and mortality, annually accounting for approximately 2 million childhood deaths worldwide of which up to 90% resides in the developing world (Black *et al.*, 2003; Williams *et al.*, 2002). This may be attributable to the high prevalence of risk factors, such as human immunodeficiency virus (HIV) infection and malnutrition that impair the immune system and thereby predispose individuals to more severe respiratory disease (Caulfield *et al.*, 2004; Clerici *et al.*, 2000; McNally *et al.*, 2007). The burden of respiratory tract infections is however not limited to the paediatric population. Pneumonia, influenza, and influenza-like illnesses are the sixth leading cause of death for all age groups in the USA, accounting for 45 000 deaths annually (Bartlett *et al.*, 2000).

Even though the clinical features of respiratory tract infections are easily recognized, the aetiology of a large percentage of disease remains undetermined. Common respiratory pathogens include *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, *Chlamydophilia pneumoniae*, *Legionella* spp. and a number of viruses belonging to the families Paramyxoviridae, Orthomyxoviridae, Picornaviridae, Adenoviridae, and Coronaviridae. Viral infections and concomitant viral-bacterial infections play a major role in childhood acute respiratory tract infection (figure 1.1), with a limited number of viruses accounting for a large proportion of viral respiratory tract infections (Mackie, 2003; Michelow *et al.*, 2004). While these viruses produce overlapping clinical syndromes, they are virologically distinct. Respiratory syncytial virus (RSV), influenza viruses A and B, parainfluenza viruses (PIV) types 1, 2, and 3, and adenoviruses (Adv) are well recognized respiratory viruses and were traditionally viewed to be the leading causes of viral lower respiratory tract infections. However, in 12-39% of ALRI cases no aetiological agent is identified, suggesting that additional unknown agents may be involved (Jartti *et al.*, 2004). Since 2001 a number of new viruses have been described that may account for these cases including human metapneumovirus (hMPV) (van den Hoogen *et al.*, 2001), human coronaviruses (hCoV) NL63 and hCoV-HKU1 (van der Hoek *et al.*, 2004; Woo *et al.*, 2005), human bocavirus (hBoV) (Allander *et al.*, 2005) and most recently two new polyoma viruses, WU virus (WUV) and KI virus (KIV) (Allander *et al.*, 2007a; Bialasiewicz *et al.*, 2007; Gaynor *et al.*, 2007). Accumulative evidence indicate that the former four are probable causes of ALRI although

frequent co-infections are reported for especially hBoV (Allander *et al.*, 2007b; Anderson, 2007; Arden *et al.*, 2006; Arden *et al.*, 2005; Bosis *et al.*, 2007; Chiu *et al.*, 2005; Kupfer *et al.*, 2007). The role of the polyomaviruses is still in question (Norja *et al.*, 2007; Ren *et al.*, 2008; Venter *et al.*, 2009). In addition, improved sensitivity of diagnostic tests has lead to the increased detection of viruses traditionally associated with mild upper respiratory tract infections in cases of ALRI, including hCoV-OC43, hCoV-229E and rhinoviruses as well as the identification of a new rhinovirus genotype, rhinovirus C (Hayden, 2004; Pene *et al.*, 2003; Vabret *et al.*, 2003; Wisdom *et al.*, 2009).

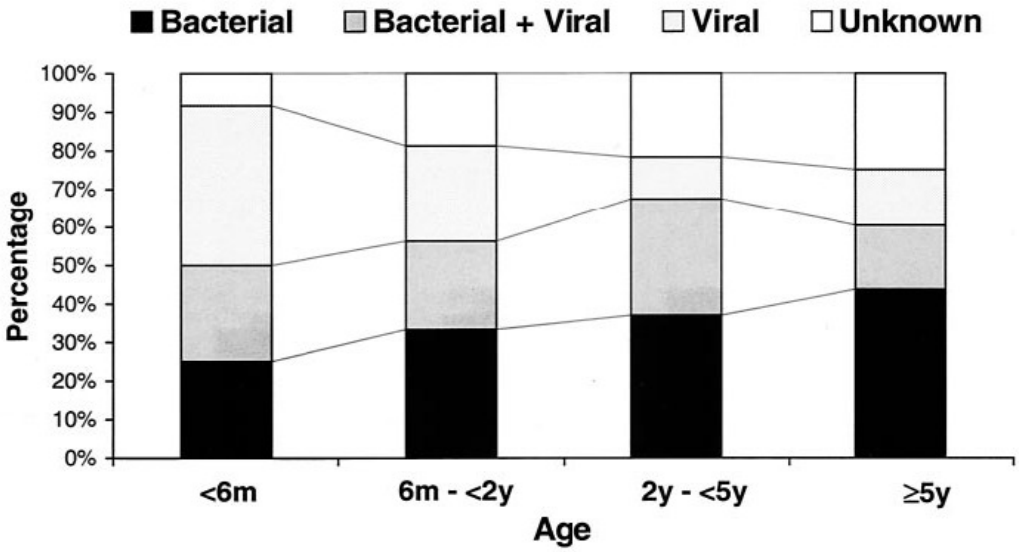

Figure 1.1. Distribution of pathogens associated with lower respiratory infections in children, stratified by age (Michelow *et al.*, 2004).

The newly identified respiratory viruses remained undiscovered up until now due to a number of possible reasons, including: i) multiple virology laboratories use continuous cell lines for virus isolation in which these viruses replicate inefficiently or not at all (e.g. hBoV); ii) certain viruses display very slow replication kinetics in vitro and may therefore have remained undetected; iii) replication of certain respiratory viruses in vitro requires special conditions (e.g. hMPV that requires the addition of trypsin) that have not been used in previous virus discovery studies; and iv) the lack of cross-reactivity in the serologic assays and low sequence homology to known human viral pathogens make the detection of new viruses unlikely with standard serological or molecular techniques (e.g. hCoV-NL63).

At present the gold standard for laboratory diagnosis of respiratory tract viruses rely on viral culture in combination with immunofluorescence assays (IFA). However, IFA has several significant disadvantages including the requirement for highly skilled individuals, it is highly dependent on the quality of the specimen, the throughput is low, and not all respiratory viruses can be detected by IFA (Madeley and Peiris, 2002). Some of these limitations can be surmounted by commercially available rapid RSV and influenza A/B antigen detection assays (adapted enzyme immunoassay, chromatographic immunoassay and direct fluorescent antibody assays) with turnaround times of less than 1 hour (Ohm-Smith *et al.*, 2004; Rahman *et al.*, 2007). However, comparative studies have indicated that these assays lack sensitivity and specificity (Aslanzadeh *et al.*, 2008; Ohm-Smith *et al.*, 2004; Rahman *et al.*, 2007). Overcoming these shortcomings, real-time PCR is a technique that is conceptually simple, highly specific, sensitive, and with a turn around time from sample receipt to result in less than 6 hours (Templeton *et al.*, 2004). In diagnostic laboratories the use of real-time PCR is, however, limited by cost and sometimes by the availability of adequate test sample volume. To overcome these shortcomings and also to increase the diagnostic capacity of real-time PCR, multiplex real-time PCR has been described (Gunson *et al.*, 2006). In multiplex real-time PCR more than one target can be detected in a single reaction, and therefore has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility. The development of multiplex real-time PCR tests with increased sensitivity will permit the detection of both the well recognized and newly discovered viral causes of acute respiratory tract infections allowing for a greater percentage of specimens to be diagnosed.

Even though qualitative real-time PCR in the diagnostic setting has numerous advantages over more traditional detection methods, one of the limitations is that it does not provide insight into the significance of the detected pathogen. In some cases patients may still be shedding low concentration virus from previous infections or have co-infections with less important pathogens. In contrast, quantitative real-time PCR provides qualitative as well as quantitative information. Quantitative tests may aid in the study of virus pathogenesis as well as potential vaccine and antiviral strategies. This may facilitate investigations of the importance of the new viruses when detected by themselves or as co-infections in patients. The advantages of quantitative real-time PCR is that it permits the assessment of viral load at a given time point, facilitates the monitoring of response to

treatment, and offers the possibility to determine the dynamics of virus proliferation (Watzinger *et al.*, 2006). This may provide valuable information in the clinical setting but also answer research questions regarding the association and importance of different respiratory viruses with lower respiratory tract disease.

1.2 Well Recognized Respiratory Viruses

Viruses traditionally associated with acute respiratory tract infections include RSV, PIV, Adv, influenza viruses, hCoV-OC43, hCoV-229E, and rhinoviruses. Of these, the coronaviruses (OC43 and 229E) and rhinoviruses were primarily associated with upper respiratory tract infections (URTI) (Myint, 1995; Turner and Couch, 2007). The principal viruses responsible for lower respiratory tract infections (LRTI) are RSV, PIV and influenza viruses which result in significant hospitalisation rates in young children (Forster *et al.*, 2004; Iwane *et al.*, 2004).

1.2.1 Respiratory syncytial virus

Respiratory syncytial virus, first identified in 1956 (Morris Jr *et al.*, 1956), is recognized as the most important viral agent of severe lower respiratory tract infections in children worldwide. It is a highly contagious respiratory pathogen that has the capacity to infect approximately one-half of susceptible infants during a single RSV epidemic (Parrott *et al.*, 1973). The virus causes annual epidemics and contributes significantly to physician visits, hospitalisation and nosocomial outbreaks (Forster *et al.*, 2004; Iwane *et al.*, 2004).

RSV belongs to the *Pneumovirus* genus within the *Paramyxoviridae* family (sub-family *Pneumovirinae*) and is an enveloped, pleiomorphic virus. The nonsegmented, negative-sense RNA genome encodes 10 proteins namely the non-structural (NS1, NS2), nucleocapsid (N), phosphoprotein (P), matrix (M), small hydrophobic (SH), surface attachment glycoprotein (G), surface fusion glycoprotein (F), second matrix (M2), and RNA-dependent RNA polymerase (L) in the order 3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5' (Collins and Crowe, 2007). Conserved genes include the NS1-, NS2-, SH-, and N protein genes, with the highest nucleotide sequence conservation found within the N protein gene (Chen

et al., 2000; Johnson and Collins, 1989). Thus, making the N protein a favourable target for molecular diagnostic assays although conserved regions within the F, M and L protein have also been targeted (Beck *et al.*, 2010; Fodha *et al.*, 2007; Kuypers *et al.*, 2004; Nauwelaers *et al.*, 2009; Templeton *et al.*, 2004). The G protein is the most variable RSV gene product and is responsible for much of the antigenic diversity of RSV (Collins and Crowe, 2007). The nucleotide and amino acid sequence variability of the G protein progressively accumulates over time, suggesting that it may be under immune pressure (Cane and Pringle, 1995). Of the three glycoproteins expressed on the surface of the virion (F, G and SH), only the F and G glycoproteins have been studied in detail for vaccine development (Singh *et al.*, 2007a). Thus far, recombinant vaccines containing the F glycoprotein seems promising since it induces high titers of neutralizing antibodies and RSV-specific cytotoxic and T-helper lymphocytes (Singh *et al.*, 2007b). In addition, the F protein is conserved among RSV strains, and antibodies induced against an F protein vaccine will provide protection against all RSV strains (Collins and Crowe, 2007).

RSV has a single serotype that is classified into two antigenic subtypes, A and B, which usually co-circulate within geographically confined epidemics, though may vary in relative prevalence from year to year (Hall *et al.*, 1990; Mufson *et al.*, 1988). Sequence analysis of the G protein gene revealed multiple genotypes within both subtype A and B (Peret *et al.*, 1998). Similar to the observed subtype variation, genotype predominance within each subgroup may also shift from year to year and may be as a consequence of selection pressure (Peret *et al.*, 1998; Wertz and Moudy, 2004). While antigenic variation is not necessarily essential for re-infection, growing evidence suggest that by potentially allowing immune evasion, antigenic variability within the attachment protein may contribute to re-infection (Melero *et al.*, 1997).

Infection and re-infection with RSV are frequent during the first years of life (Nokes *et al.*, 2009). One prospective study demonstrated an infection rate of 68.8 per 100 children less than 12 months of age and 82.6 per 100 during the second year of life. All children were infected by age 24 months, of which half experienced re-infections (Glezen *et al.*, 1986). Even though RSV is known primarily as a paediatric pathogen, symptomatic re-infection can occur in individuals of all ages, with elderly persons and individuals with underlying conditions being most at risk of severe morbidity and mortality (Falsey and

Walsh, 2005; Whimbey *et al.*, 1996). Outbreaks of RSV occur annually and predictably within specific climatic regions. In temperate climates, including South Africa, the RSV epidemic occurs from late autumn through to early spring (Gilchrist *et al.*, 1994; Kim *et al.*, 1973; Light, 2007; Madhi *et al.*, 2006; Mufson *et al.*, 1973). In tropical and subtropical regions, despite the absence of a defined winter season, consistent seasons of infection have also been observed. With few exceptions (Chew *et al.*, 1998), RSV infections have been observed mainly during the rainy seasons in tropical regions of China, India, The Gambia, Philippines, and Columbia (Bedoya *et al.*, 1996; Chan *et al.*, 1999; Cherian *et al.*, 1990; Hazlett *et al.*, 1988; Weber *et al.*, 1998).

Primary RSV infection may present with varying degrees of disease severity. In previously healthy infants, primary infection at age 6 weeks to 9 months usually causes upper respiratory tract symptoms (Collins and Crowe, 2007). However, in 25% to 40% of such infections, patients may develop lower respiratory tract infections of which bronchiolitis and pneumonia are the primary manifestations (Choi *et al.*, 2006; Forster *et al.*, 2004; Iwane *et al.*, 2004). In these patients initial symptoms include rhinorrhea that may be accompanied by a loss in appetite, followed by coughing, sneezing and a low grade fever. Shortly after the cough has developed, the patient may begin to wheeze. If the disease is mild, the symptoms may not progress beyond this stage (Collins and Crowe, 2007). Investigations of factors that may predispose individuals to more severe RSV disease in the United States of America indicated younger age (specifically <1 year), black and Hispanic race/ethnicity, male gender, and the presence of chronic underlying illness to be important (Iwane *et al.*, 2004). Infants hospitalised with RSV associated lower respiratory tract infections often report subsequent childhood wheeze, which led to the belief that RSV infection may predispose individuals to asthma later in life. However, it is unclear as to whether RSV infection confers a long-term change in the host that subsequently increases the risk of asthma, or whether the association between RSV and asthma is due to shared genetic predisposition (Kuehni *et al.*, 2009). Although evidence supporting both hypotheses has been reported (Thomsen *et al.*, 2009; Wu *et al.*, 2008), the causative link between RSV and asthma remains inconclusive.

In previously healthy adults RSV infection may be symptomatic in more than 80% of cases. Infections are usually characterized by symptoms including sore throat, cough,

nasal congestion, dyspnoea, wheeze and fever (O'Shea *et al.*, 2005). In elderly persons, especially those with underlying disease, severe pneumonia may occur, which may lead to hospitalisation and the development of adult respiratory distress syndrome, requiring intubation and ventilation (Falsey and Walsh, 2005). RSV infection in severely immunocompromised individuals usually starts with an upper respiratory tract infection, clinically presenting similar to an infection in a normal host, however, may evolve into lower respiratory tract disease in up to 50% of cases (Collins and Crowe, 2007).

1.2.2 Parainfluenza viruses

Human parainfluenza viruses are members of the *Paramyxoviridae* family (sub-family *Paramyxovirinae*) and are pleomorphic, enveloped viruses (Karron and Collins, 2007). These viruses were first isolated and characterized in the late 1950s and are genetically and antigenically divided into types 1 to 4, with type 4 occurring in two antigenic subtypes (A and B). PIVs belong to two different genera: PIV 1 and PIV 3 belong to the *Respirovirus* genus, and PIV 2 and PIV 4 belong to the *Rubulavirus* genus. The negative-sense, single-stranded RNA genomes are organized to encode at least six common structural proteins in the invariant order of 3'-N-P-M-F-HN-L-5' (HN – haemagglutinin neuraminidase) (Karron and Collins, 2007). Each gene encodes a single major protein accept the P gene, which expresses a variety of accessory proteins using two or all three of the reading frames (depending on the virus). The major proteins encoded by the P gene are P, V, and C. All PIVs encode a P protein, whereas most, but not all, encode a V protein and only members of the *Respirovirus* genus encode a C protein (Karron and Collins, 2007). While the N protein and M protein are the most conserved, highly conserved regions within the L protein and HN protein are also present. Of these, the N and HN protein are mostly targeted for molecular diagnostic assays (Hu *et al.*, 2005; Templeton *et al.*, 2004; van de Pol *et al.*, 2007; Watzinger *et al.*, 2004). Conversely, sequence variability on the carboxyl and amino terminus of the HN protein of PIVs (Echevarria *et al.*, 2000; van Wyke Coelingh *et al.*, 1988), and the 5' noncoding region and proximal coding region of the F protein of PIV 3 facilitates investigations into the molecular epidemiology of PIV outbreaks (Karron *et al.*, 1993; Zambon *et al.*, 1998). Antibodies are produced against all PIV proteins. However, the two surface proteins, HN and F, are the only antigens to induce antibodies that neutralize infectivity (Karron and Collins, 2007). Current approaches to PIV vaccines include subunit

HN and F proteins vaccines, intranasal administration of live attenuated strains, recombinant bovine/human viruses, and strains engineered using reverse genetics (Henrickson, 2003). Clinical trails on an intranasally administered live attenuated PIV 3 vaccine (*cp*-45) has shown to be well tolerated, appropriately attenuated, infectious, and immunogenic in children as young as 1 month old (Karron *et al.*, 2003).

PIV occur worldwide and are endemic in the general population, causing infection and illness in infants, young children, the immunocompromised, the chronically ill, and the elderly (Falsey *et al.*, 1995; Glezen *et al.*, 1984; Glezen *et al.*, 2000). Each of the different types of PIV has a distinct epidemiological pattern of outbreaks (Hall, 2001). PIV 1 causes large, defined outbreaks that are marked by sharp biennial rises in croup cases during autumn in both hemispheres (Carballal *et al.*, 2001; Marx *et al.*, 1997). Infections with PIV 1 are rarely observed in infants younger than 1 month and primarily occur in children aged 7 to 36 months, with a peak incidence in the second and third years of life (Henrickson, 2003). Outbreaks of infection with PIV 2 usually follow PIV 1 outbreaks, alternate years with PIV 1, or cause yearly outbreaks (Belshe *et al.*, 1983; Downham *et al.*, 1974; Murphy *et al.*, 1980). The majority of PIV 2 infections occur in children younger than 5 years of age, and even though the peak incidence is between 1 and 2 years of age, significant numbers of infants younger than 1 year are hospitalised each year (Henrickson, 2003). PIV 3 outbreaks occur yearly, mainly during spring and summer, and last longer than outbreaks of PIV 1 and PIV 2. Infants younger than 6 months are particularly vulnerable to infection with PIV 3. Unlike the other PIVs, PIV 3 infections are frequently observed during the first year of life (Henrickson, 2003). PIV 4 is rarely isolated and, hence, relatively unknown and uncharacterized (Henrickson, 2003).

Together with RSV and recently hMPV, PIV form part of the most important family (*Paramyxoviridae*) of respiratory pathogens among children, accounting for 30-40% of all ALRI in infants and children (Denny and Clyde, 1986; Murphy *et al.*, 1980). They are the major cause of laryngotracheobronchitis (croup), but are known to cause a wide spectrum of both upper and lower respiratory disease ranging from a mild, febrile common cold to severe, potentially life-threatening croup, bronchiolitis, and pneumonia. PIV 1 and PIV 2 are the most common pathogens associated with croup (Denny *et al.*, 1983), whereas PIV 3 is more frequently associated with bronchiolitis and pneumonia in infants and young children

(Counihan *et al.*, 2001). In addition, PIV 3 has increasingly become recognized as an important cause of morbidity and mortality in immunocompromised patients, both adults (Glezen *et al.*, 2000; Nichols *et al.*, 2001) and children (Apalsch *et al.*, 1995; Butnor and Sporn, 2003), and in the institutionalized elderly (Todd Faulks *et al.*, 2000). Features of respiratory virus infections in immunocompromised patients include persistent infection and prolonged viral shedding, increased occurrence of progression to pneumonia, and a high mortality rate compared with the general population (Lujan-Zilbermann *et al.*, 2001). In contrast, PIV 4 predominantly causes mild upper respiratory illness in children and adults, though severe symptoms can occur in infants (Lau *et al.*, 2005). Re-infections occur in the presence of pre-existing immunity, but usually cause a mild illness limited to the upper respiratory tract, though elderly and immunocompromised individuals are at risk of serious complications of infection.

1.2.3 Influenza viruses

Influenza viruses (family *Orthomyxoviridae*) are highly contagious respiratory pathogens that have the capacity to cause local epidemics and worldwide pandemics with significant infection rates. Influenza viruses are enveloped viruses with negative-sense, single stranded, segmented RNA genomes. There are three different genera (types) of influenza viruses – A, B, and C – which may be associated with illness in humans. All A- and B-type influenza viruses possess eight RNA segments, whereas influenza C viruses only possess seven RNA segments (Palese and Shaw, 2007). The genome structures for influenza A- and B-type viruses are similar: the three largest RNA segments (segment 1 to 3) encode the polymerase proteins (PB1 [polymerase basic 1], PB2 [polymerase basic 2], and PA [polymerase acid]), the fourth RNA for haemagglutinin (HA) protein, the fifth RNA for the nucleocapsid protein (N), the sixth RNA the neuraminidase (NA) protein, the seventh and eight RNA for matrix (M) proteins and the non-structural proteins (NS1 and NS2), respectively (Palese and Shaw, 2007). Influenza C viruses follow a similar genome structure. However, the major glycoprotein of influenza C, HEF (haemagglutinin-esterase-fusion), combines the functions of the HA and NA proteins, and thus influenza C viruses contain one less RNA segment than influenza A and B viruses (Palese and Shaw, 2007). The HA genes have a much higher evolutionary rate compared to PB1, PB2, PA, NP, and M (McCullers *et al.*, 2004; Webster *et al.*, 1992; Wright *et al.*, 2007), and is thus used to

define the molecular epidemiology of influenza viruses. Conversely, the slower evolving protein genes, in particular the NP, are targeted for molecular diagnostic assays (Coiras *et al.*, 2004; Smith *et al.*, 2003).

The segmented nature of their RNA genomes allows for the exchange of genetic material (reassortment) between two different influenza viruses co-infecting a single host (Wright *et al.*, 2007). Reassortment can occur for influenza A, B, and C viruses, but has not been observed between the different genera. Influenza pandemics are usually due to a novel influenza A virus subtype created by reassortment (antigenic shift), as influenza A viruses are the most variable with 16 HA subtypes and 9 NA subtypes (Kawaoka *et al.*, 1989; Michaelis *et al.*, 2009a; Scholtissek *et al.*, 1978). Pandemic outbreaks are the most devastating manifestation of influenza, having high attack rates (20% to 40% of the world population) and causing significant mortality. Influenza pandemics have occurred with 10- to 40-year intervals, although reliable records only date back to the 1918 pandemic (Wright *et al.*, 2007). The three previous influenza pandemics are the A/H1N1 pandemic from 1918 through 1919 ("Spanish influenza"), A/H2N2 pandemic from 1957 through 1963 ("Asian influenza"), and A/H3N2 from 1968 through 1970 ("Hong Kong influenza") (Wright *et al.*, 2007). After approximately 40 years since the last influenza pandemic in 1968 the World Health Organization (WHO) declared the most recent influenza pandemic on June 11, 2009 (www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html, 2009). The first information on the emergence of the novel H1N1 influenza A virus dates from 24 April 2009 and was first identified in Mexico (Michaelis *et al.*, 2009b). Due to rapid and frequent international travel, the virus rapidly spread to more than 209 countries and overseas territories with over 14,711 deaths reported by 24 January 2010 (WHO's weekly pandemic update 85 (www.who.int/csr/don/2010_01_29/en/index.html, 2010)). The virus originated from the triple-reassortant swine influenza (H1) virus circulating in North American pigs (Michaelis *et al.*, 2009a). Initial diagnosis of this virus was difficult since routine diagnostic assays that are highly sensitive and specific for the detection of seasonal influenza, including direct immunofluorescence, rapid antigen tests, as well as some PCR assays demonstrated poor sensitivity for the detection of the novel reassortant influenza virus (Ginocchio *et al.*, 2009; Vasoo *et al.*, 2009). In contrast, comparative studies reported a superior sensitivity of certain PCR assays for the detection of all influenza strains including the pandemic H1N1 2009 virus (Ginocchio *et al.*, 2009).

Additionally, annual epidemics occur as a result of the evolution of the surface antigens (HA protein) of influenza A and influenza B (antigenic drift) that allows these viruses to evade the immune response and cause infection despite pre-existing immunity (Wilson and Cox, 1990). The evolution rates of influenza B, and especially influenza C, are considerably slower than for influenza A viruses. The rapid evolution of influenza A and the devastating impact of the resulting epidemics and pandemics accentuate the importance of active surveillance for the identification of novel circulating strains (Wright *et al.*, 2007). In addition, the use of diagnostic assays with the capacity to detect all influenza A subtypes, irrespective of origin, should be encouraged as it will be essential for the diagnosis of reassortant viruses during new pandemics.

Since 1977, two influenza A virus subtypes (H3N2 and H1N1) have been co-circulating with influenza B viruses (Zambon, 1999). The prevalence of these viruses differs temporally and geographically, making influenza virus epidemiology complex. Though overall, influenza viruses are responsible for up to 12-19% of acute respiratory tract infections, with influenza A having significantly higher attack rates than influenza B (www.cdc.gov/flu/weekly/fluactivity.htm). Even though individuals of all ages are at risk of infection, the highest mortality rate is observed in elderly individuals (Simonsen, 1999).

The severity of disease associated with the three different influenza genera differs, with influenza A causing the most severe illness in humans. The severity of influenza A virus infection ranges from asymptomatic infection to primary viral pneumonia that rapidly progresses to a fatal outcome (Zambon, 1999). The principal anatomical site of disease is the trachea and bronchi. Infection generally results in tracheobronchitis, though it may clinically manifest as a common cold, pharyngitis, upper respiratory illness with both nasal and throat symptoms, laryngitis, croup, and bronchiolitis (Cough and Kasel, 1995). Gastrointestinal (GIT) symptoms such as nausea, vomiting, and diarrhoea may also occur, particularly in young children. The most common complications of influenza virus infection are acute sinusitis, otitis media, and pneumonia. Severe primary viral pneumonia occurs in both adults and children, with patients with underlying heart or lung disease being most at risk (Rothberg *et al.*, 2008). In children the clinical manifestations are generally similar to those observed in adults, though there are some distinct differences. Children have higher fever that may be accompanied by febrile convulsions (Price *et al.*, 1976; Toovey, 2008). In

addition, otitis media, croup, pneumonia, and myositis are more frequent in children (Wright *et al.*, 2007). Influenza B viruses cause a similar spectrum of disease to that of influenza A viruses. However, the frequency of severe influenza B virus infections that require hospitalisation is about 4-fold less than influenza A virus (Wright *et al.*, 2007). In addition, extrapulmonary manifestations such as myositis, Reye syndrome and gastrointestinal symptoms are more common with influenza B than A virus infection. In contrast to influenza A and B viruses, influenza C virus infections result in less severe illness, as they predominantly cause upper respiratory tract infections and are rarely associated with severe lower respiratory tract infection (Moriuchi *et al.*, 1991).

1.2.4 Adenovirus

Adenoviruses were first isolated and characterized in 1953 by Rowe *et al*. (Rowe *et al.*, 1953). Human adenoviruses are members of the *Mastadenovirus* genus within the *Adenoviridae* family and are nonenveloped, lytic viruses with a characteristic icosahedral morphology (Berk, 2007). To date, 52 distinct adenovirus serotypes have been identified, which are classified into 7 subgroups or species (A to G) based on distinctive characteristics, including tissue tropism, electrophoretic mobility of structural proteins, sequence homology of their double-stranded DNA genomes, genomic G+C content, and other biological properties (Jones *et al.*, 2007; Kajon *et al.*, 2007). Adenoviruses express at least 10 structural proteins numbered by convention II (hexon), III (penton base), IV (fiber), V (core), VI (minor capsid), VII (core), VIII (minor capsid), IX (minor capsid), μ (core), terminal protein, and the p23 viral protease (Berk, 2007). The hexon gene contains both conserved regions and hypervariable regions. To date, the only PCR-based diagnostic assays that were able to effectively cover all adenovirus subgroups have targeted conserved regions within the hexon protein gene (Ebner *et al.*, 2005; Heim *et al.*, 2003). Whereas, the hypervariable regions within the hexon protein gene are used to determine the molecular epidemiology of adenoviruses (Crawford-Miksza and Schnurr, 1996).

Adv cause a wide variety of common and sporadic infections without a strict one-to-one relationship between serotype and disease (Wold and Horwitz, 2007). However, certain adenoviral subgroups including B, C, D, and E, have been well characterized and different clinical syndromes have been associated with the different subgroups. For example,

subgroup B1 (Adv3, Adv7, and Adv21), subgroup C (Adv1, Adv2, Adv5, and Adv6), and subgroup E (Adv4) are well recognized causes of acute respiratory tract infections, whereas keratoconjunctivitis is associated with serotypes from subgroup D, gastroenteritis with serotypes from subgroup F, and cystitis with serotypes from subgroup B2 (Lenaerts *et al.*, 2008).

Adv infections are endemic and frequently occur in the absence of symptoms (Lenaerts *et al.*, 2008). They are, however, responsible for a small portion of respiratory morbidity in the general population and for 5% to 10% of respiratory illness in children (Berk, 2007; Brandt *et al.*, 1969). Clinical manifestations are generally limited to URTI or pharyngitis, though about 10% of childhood pneumonias are attributable to adenoviruses (Wold and Horwitz, 2007). Respiratory symptoms associated with Adv infections include nasal congestion, coryza and cough, while often being accompanied by systemic manifestations (e.g. generalized malaise, fever, chills, myalgia, and headache) (Wold and Horwitz, 2007). In immunocompetent individuals, Adv infections are usually mild and self-limiting, mostly resolving without sequelae. In these patients, the efficient clearance of Adv infections appear to be primarily T-cell mediated, and therefore patients with impaired T-cell immunity are at greater risk of severe disease (Ljungman, 2004). In neonates and premature infants, who have an immature T-cell immunity, Adv have been shown to cause severe and often fatal infections (Henquell *et al.*, 2009; Rieger-Fackeldey *et al.*, 2000). Similarly, disseminated and potentially life-threatening adenovirus infections are frequently observed in immunocompromised individuals, which includes patients with acquired immunodeficiency syndrome (AIDS), transplant recipients receiving immunosuppressive therapy and individuals with hereditary immunodeficiencies (Adeyemi *et al.*, 2008; Koopmann *et al.*, 2000).

1.3 Newly Described Respiratory Viruses

1.3.1 Human Metapneumovirus

Discovery. In 2001, van den Hoogen *et al.* reported the discovery of a previously unknown paramyxovirus from children suffering from respiratory tract infections in the

Netherlands (van den Hoogen *et al.*, 2001). The virus was isolated from the respiratory secretions of 28 epidemiologically unrelated children during the previous 20 years. Electron microscopy analysis of infected trypsin supplemented tertiary monkey kidney (tMK) cell supernatants revealed the presence of paramyxovirus-like pleiomorphic particles with short envelope projections (figure 1.2). In order to characterize the unknown virological agent genomic sequence information was sought by randomly primed PCR amplification. Subsequent phylogenetic analysis on nucleotide sequences identified the unknown agent as a new member of the *Metapneumovirus* genus (sub-family *Pneumovirinae*, family *Paramyxoviridae*) and was thereafter named human metapneumovirus.

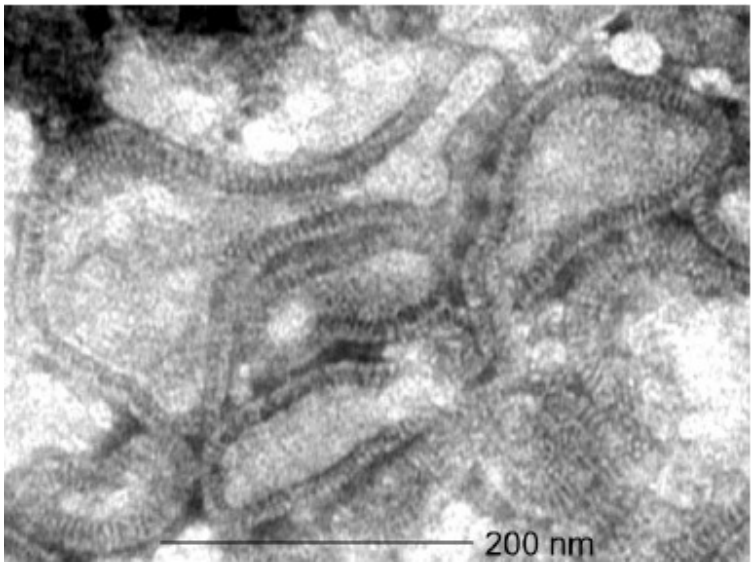

Figure 1.2 Electron micrograph of hMPV particles (van den Hoogen *et al.*, 2001)

HMPV differs from the pneumoviruses (such as RSV), which also belongs to the *Pneumovirinae* sub-family, in that it has a slightly different gene constellation and lacks non-structural proteins NS1 and NS2. The order of the genome for hMPV appears as: 3'-N-P-M-F-M2-SH-G-L-5' (van den Hoogen *et al.*, 2002). Similar to RSV, the complete G protein coding region has high nucleotide and amino acid sequence diversity (as low as 30-37% amino acid identity) (van den Hoogen *et al.*, 2004). Conversely, the F-, N-, M-, and L proteins are highly conserved (Bastien *et al.*, 2003; van den Hoogen *et al.*, 2004), serving as target regions for hMPV diagnostic PCR assays (Boivin *et al.*, 2002; Maggi *et al.*, 2003; van den Hoogen *et al.*, 2003). Van den Hoogen *et al.* suggested several lines of evidence demonstrating that hMPV is an established pathogen in humans. Firstly, nasopharyngeal

aspirate (NPA) samples from patients that tested negative for known respiratory viruses showed that approximately 10% of the unexplained RTI may be associated with hMPV. Secondly, serological analysis revealed that hMPV commonly infects children, since towards the age of 5 years nearly all children were seropositive. Lastly, sera taken from humans in 1958 revealed a 100% seroprevalence, indicating that hMPV has been circulating in the human population for more than half a century (van den Hoogen *et al.*, 2001).

Epidemiology. Soon after its discovery in the Netherlands, various studies that retrospectively screened stored respiratory samples indicated hMPV to account for a significant portion of respiratory tract infections with previously unknown aetiology. HMPV has been identified in 2.3% to 25% of respiratory tract infections worldwide (Boivin *et al.*, 2003; Camps *et al.*, 2008; Choi *et al.*, 2006; Cuevas *et al.*, 2003; Kaida *et al.*, 2006; Smuts *et al.*, 2008), with prevalence rates of hMPV varying from year to year (Aberle *et al.*, 2008; Choi *et al.*, 2006; Maggi *et al.*, 2003; Williams *et al.*, 2006). HMPV is primarily detected in children less than 5 years of age, though immunocompromised or elderly adult populations are also at risk of infection (Agapov *et al.*, 2006; Boivin *et al.*, 2002; Falsey, 2008). The seasonal activity of hMPV has been shown to vary from year to year. In particular, one study that investigated the epidemiology of hMPV over a 7-year period indicated that hMPV seasonal activity was characterized by a biennial rhythm, with spring activity occurring every second year (Aberle *et al.*, 2008). In general, hMPV circulates primarily during late winter and spring where the peak activity often coincides with or follows the peak of RSV activity (Boivin *et al.*, 2003; Chano *et al.*, 2005; Maggi *et al.*, 2003; Williams *et al.*, 2006). Though in Hong Kong, which is situated in a subtropical region, peak hMPV activity has been observed during the spring-summer period (Peiris *et al.*, 2003).

Phylogenetic analysis of fusion (F) and attachment (G) genes have shown that hMPV is represented by 2 broad genetic virus lineages, lineage A and B, with each group divided into genetic sublineages 1 and 2 (Bastien *et al.*, 2003; van den Hoogen *et al.*, 2004). Although significant antigenic and genetic diversity has been noted, there is little evidence that suggest antigenic drift over time (van den Hoogen *et al.*, 2004). Similar to RSV (Peret *et al.*, 2000; Peret *et al.*, 1998), both hMPV lineages can circulate concurrently with varying degrees of prevalence where the ratio of detectable hMPV lineage A to B may shift from

year to year (Agapov *et al.*, 2006; Ludewick *et al.*, 2005; Peret *et al.*, 2002). Re-infection with both homologous and heterologous strains occur, which poses a challenge for vaccine development as infections may occur in the presence of pre-existing immunity (Pelletier *et al.*, 2002; Williams *et al.*, 2006).

Clinical characteristics. Infection with hMPV cause disease similar to that of RSV, though in general, hMPV associated disease appears to be less severe than that observed for RSV (Boivin *et al.*, 2003; Viazov *et al.*, 2003). HMPV associated illness ranges from mild upper respiratory tract infection to severe bronchiolitis or pneumonia, with both healthy children and adults with underlying disease being susceptible to hMPV infection. Prominent clinical features of hMPV infection includes tachypnea, cough, hypoxia, fever, rales, wheezing and changes on chest radiographs such as hyperinflation, infiltrates, and peribronchial cuffing (Agapov *et al.*, 2006; Williams *et al.*, 2004). LRTI in hMPV infected children are frequently diagnosed as bronchiolitis, croup, pneumonia, or acute exacerbation of asthma (Caracciolo *et al.*, 2008; Williams *et al.*, 2004). In addition to LRTI, hMPV contributes to ~5% of URTI, which is comparable to upper respiratory tract episodes caused by RSV, influenza virus, and HPIV (Williams *et al.*, 2006). However, the role that hMPV plays in URTI in otherwise healthy children appears to be less prominent than the role it plays in LRTI in most paediatric populations studied.

In adults, hMPV infection usually presents as a mild, self-limiting upper respiratory tract infection (Falsey, 2008), though fatal hMPV infections have been reported in immunocompromised patients, including hematopoietic stem cell transplant (HSCT) recipients (Cane *et al.*, 2003) and leukemia patients (Pelletier *et al.*, 2002). In adult HSCT recipients, fatal pneumonia (Englund *et al.*, 2006) as well as persistent hMPV infection in the absence of respiratory symptoms have been reported (Debiaggi *et al.*, 2006), while viral shedding and mild respiratory symptoms were associated with hMPV infection in a child with severe combined immunodeficiency disorder. In HIV-infected adults receiving antiretroviral therapy, hMPV has been found in association with acute febrile respiratory illness and bronchospasm (Klein *et al.*, 2010), though appear to be rare in this patient group (Garbino *et al.*, 2008; Muller *et al.*, 2009). In children, however, a comparative study investigating the epidemiology of severe hMPV-associated LRTI in HIV-infected and HIV-uninfected children indicated that the burden of hospitalisation for hMPV-LRTI was 5.4-fold

greater in HIV-infected compared to HIV-uninfected children (Madhi *et al.*, 2007). In addition, it was reported that HIV-infected children with hMPV-LRTI had a higher rate of bacterial co-infection as well as a higher mortality rate compared to HIV-uninfected children. Mild or asymptomatic persistent hMPV infection in these two immunocompromised individuals may suggest that the host's immune response may play a key role in disease pathogenesis. Since both fatal and mild or asymptomatic hMPV infections have been reported in immunocompromised individuals, further studies are required to determine whether such differing outcomes are due to viral, host, or environmental factors.

Strategies focusing on future prevention of hMPV infection include investigations into and establishment of appropriate animal models and reverse genetics techniques for vaccination studies (Biacchesi *et al.*, 2004; MacPhail *et al.*, 2004). Gene-deletion mutant viruses lacking the G or M2 genes have been shown to be immunogenic and provide protection against wild-type hMPV in hamsters (Biacchesi *et al.*, 2005). Subunit F protein vaccines have also been shown to be immunogenic and protective against homologous and heterologous hMPV (Cseke *et al.*, 2007; Herfst *et al.*, 2007). In future, one or more of these approaches may produce the first licensed hMPV vaccine.

1.3.2 Human Bocavirus

Discovery. In 2005 Allander *et al.* described the identification of a novel parvovirus in patients with lower respiratory tract infections (Allander *et al.*, 2005). The finding resulted from pooled cell-free fractions of NPA specimens obtained from mostly paediatric patients with LRTI. The main experimental components applied to the discovery of this novel virus were concentration of viruses by ultracentrifugation, depletion of contaminating nucleic acids by filtration and DNase digestion (Allander *et al.*, 2001), nucleic acids extraction, random PCR amplification (Froussard, 1992), cloning and large-scale sequencing. Subsequent database searches of the sequencing results (BLAST searches) revealed the presence of parvovirus-like sequences in the respiratory samples. Based on genomic structure and amino acid similarity, hBoV was classified into the *Bocavirus* genus (*Parvoviridae* family, sub-family *Parvovirinae*) together with the namesake members of the

genus, *bo*vine parvovirus and *ca*nine minute virus (Allander *et al.*, 2005). Like all members of the *Parvovirinae* subfamily, hBoV has two major open reading frames coding for a non-structural protein (NS1) and two viral capsid proteins (VP1 and VP2), respectively. Between these two genes a third, smaller open reading frame encodes another non-structural protein (NP-1) (Allander *et al.*, 2005). There is relatively little heterogeneity within the NS1 and NP-1 genes, with the NS1 gene being the most conserved of all (Lau *et al.*, 2007a). The greatest nucleotide and amino acid diversity appears to be in the VP1/VP2 genes (Kesebir *et al.*, 2006; Lau *et al.*, 2007a).

To determine the prevalence of hBoV in respiratory samples, Allander *et al.* developed a diagnostic PCR assay targeting the nucleoprotein gene (Allander *et al.*, 2005). HBoV was identified in 3.1% of hospitalised patients with lower respiratory tract infections, though co-infections with other respiratory viruses occurred in 17.6% of patients. Patients with single hBoV infections presented with varying degrees of respiratory distress with 50% of patients suffering from wheezing bronchitis or asthma and two patients had chronic lung disease. From these findings the authors concluded that hBoV was a likely aetiological agent of LRTI in patients as hBoV was primarily found in samples negative for other viruses. However, the co-detection rate determined in the original study may have been underestimated since other viruses were only detected by means of immunofluorescence and cell culture which have been shown to be less sensitive than nucleic acid detection (Oosterheert *et al.*, 2005; Templeton *et al.*, 2005), and other respiratory pathogens such as coronaviruses were not sought by any means.

Epidemiology. Since the first report, hBoV has been shown to be a ubiquitous virus with a worldwide distribution occurring at frequencies ranging from 1.5% to 19.3% in patients with acute respiratory tract infections (Allander *et al.*, 2007b; Bastien *et al.*, 2006). The observed discrepancies in prevalence may be attributable to differences in the sensitivity of detection assays, duration of study periods, technique of nasopharyngeal sampling, and differences in patient selection rather than actual differences in hBoV prevalence. The majority of prevalence studies have also investigated the occurrence of co-infecting viruses in hBoV infected patients and have reported co-infection rates ranging from 17.6% to 83%, with co-infection rates above ~40% being frequently observed (Allander *et al.*, 2005; Fry *et al.*, 2007). The co-infection rate differs between studies due to

differences in the intensity by which other respiratory viruses were sought, sensitivities of the diagnostic assays and sampling size.

Variable observations have been reported regarding the seasonal distribution of hBoV infections. Some studies report year round detection of hBoV with some indicating increased prevalence during autumn, winter and spring (Brieu *et al.*, 2008; Fry *et al.*, 2007; Kesebir *et al.*, 2006; Sloots *et al.*, 2006; Weissbrich *et al.*, 2006), while other studies did not observe a seasonal occurrence at all (Bastien *et al.*, 2006; Maggi *et al.*, 2007). HBoV infection predominantly occurs in patients younger than 4 years, however is seldom observed in children less than 6 months of age which may suggest some degree of protection from maternal antibodies (Fry *et al.*, 2007; Ma *et al.*, 2006; Manning *et al.*, 2006). Seroepidemiological studies have shown that ≥85% of individuals are hBoV-seropositive by the age of 5 years (Endo *et al.*, 2007; Kahn *et al.*, 2008). Together with the infrequent detection of hBoV in adults (Fry *et al.*, 2007; Lau *et al.*, 2007a; Longtin *et al.*, 2008), it may suggest that the majority of the population is infected during childhood followed by some form of immunity.

HBoV and respiratory disease. Since the discovery of hBoV its pathogenic role in respiratory disease has been the subject of much debate. To establish the causative role of hBoV in respiratory disease has been difficult due to the frequent co-detection of hBoV with other respiratory viruses with known pathogenic potential. In addition, Koch's revised postulates cannot be applied to hBoV for reason that neither *in vitro* propagation systems nor an animal model of infection have been available (Allander *et al.*, 2005). The difficulty in addressing causality of putative microbial pathogens identified by sequence-based detection methods has been a subject of debate since the advent of molecular diagnostic techniques. To address this, Fredricks and Relman further revised Koch's revised postulates and proposed molecular guidelines for establishing microbial disease causation (Fredericks and Relman, 1996). To establish a causal role for hBoV, Allander *et al.* proposed the application of the alternative causality criteria developed for molecular diagnostics (Allander, 2008). Various studies have provided data relevant for these criteria, while some criteria have not yet been investigated e.g. presence of hBoV in the lower respiratory epithelium. In support of hBoV causality, some studies have shown that hBoV is more frequently detected in patients with respiratory disease compared to asymptomatic

children (Allander *et al.*, 2007b; Brieu *et al.*, 2008; Garcia-Garcia *et al.*, 2008; Kesebir *et al.*, 2006). In particular, Fry *et al.* indicated that the risk of hBoV infection was four times higher among patients with pneumonia compared to asymptomatic control patients, but found that in the absence of a co-infecting virus, the detection of hBoV in patients with pneumonia was not significantly greater compared to control patients (Fry *et al.*, 2007). In contrast to the above mentioned studies, a recent study reported a significantly higher detection rate in asymptomatic children compared to symptomatic children (43% vs. 13.8%) (Longtin *et al.*, 2008). Results from asymptomatic controls should be interpreted with caution since some studies did not match the symptomatic and asymptomatic groups in age and method of sampling. In particular, respiratory samples obtained from symptomatic patients may vary from asymptomatic individuals in respect to cell count and volume since patients with acute infection will have cell-rich mucoid respiratory secretions produced by an inflammatory process while asymptomatic individuals have very little nasopharyngeal secretions (Schildgen *et al.*, 2008).

There is, however, additional support for an association of hBoV with respiratory disease that is not dependent on asymptomatic controls. Allander *et al.* showed a significant association between high nasopharyngeal hBoV viral load and wheezing (Allander *et al.*, 2007b), whereas another study only observed high viral loads in patients with pneumonia and not in asymptomatic individuals (Fry *et al.*, 2007). In addition, two other studies detected higher viral loads in patients with single hBoV infections compared to patients with co-infections (Brieu *et al.*, 2008; Jacques *et al.*, 2008). These findings may suggest that at a high viral load hBoV could have a pathogenic potential and that detection at low viral loads may reflect long-term virus shedding after clinical recovery, as has been reported for parvovirus B19 (Lefrere *et al.*, 2005; Lindblom *et al.*, 2005). Virus persistence was also suggested by the detection of low levels hBoV in the sera of patients that tested negative for hBoV in respiratory secretions (Allander *et al.*, 2007b). However, overall it does appear as though hBoV viremia may be associated with acute hBoV infection as Allander *et al.* noted that hBoV DNA was more frequently detected in the patients' blood during acute hBoV infection than after recovery (Allander *et al.*, 2007b). In addition, hBoV is seldom detected in the blood of patients with moderate to low nasopharyngeal loads (Allander *et al.*, 2007b; Fry *et al.*, 2007), which once again brings into question the relevance of low hBoV viral loads in the nasopharynx. In order to address this, Kantola *et*

al. collected paired serum samples from hBoV infected patients to investigate serological markers of acute infection (presence of immunoglobulin (Ig) M antibodies and/or rising IgG antibody levels) in patients with high and low nasopharyngeal hBoV viral loads (Kantola *et al.*, 2008). It was observed that, respectively, 86% and 24% of patients with high and low viral loads exhibited serological markers of acute infection. They also observed that 9 patients who fulfilled 3 non-serological criteria for acute hBoV infection (viremia, high viral loads in NPA, lack of other viruses) all had serological evidence of acute hBoV infection (Kantola *et al.*, 2008). Altogether, these findings suggest that high viral load and viremic hBoV infection is associated with acute respiratory disease, while the detection of a low viral load in nasopharynx alone has uncertain relevance. Further studies are needed to strengthen this association.

Clinical characteristics. Clinical characteristics associated with hBoV infections are not distinguishable from those ascribed to other respiratory viruses (Allander *et al.*, 2005; Kleines *et al.*, 2007). Frequently reported clinical signs associated with hBoV infection are wheezing, respiratory distress, fever, cough, and rhinorrhea, with chest radiographs showing abnormal findings such as hyperinflation or interstitial infiltrates (Choi *et al.*, 2006; Kesebir *et al.*, 2006). The most common clinical diagnoses given to hBoV-positive patients includes upper respiratory tract infections, bronchiolitis, pneumonia, bronchitis, and acute exacerbation of asthma (Calvo *et al.*, 2008; Choi *et al.*, 2006).

In addition to respiratory symptoms, hBoV-positive patients often present with gastro-intestinal symptoms including vomiting, abdominal pain and/or diarrhoea (Brieu *et al.*, 2008; Lau *et al.*, 2007a). Previous studies have identified hBoV in faecal samples of patients with gastroenteritis suggesting a causative role (Lau *et al.*, 2007a; Vicente *et al.*, 2007). Indeed, the two other members of the *Bocavirus* genus (bovine parvovirus and canine minute virus) do cause enteric disease in their natural host (Durham *et al.*, 1985; Mochizuki *et al.*, 2002). However, the potential role of hBoV in gastroenteritis will necessitate further investigation.

1.3.3 Human Coronaviruses

Coronaviruses are large, enveloped, positive-strand RNA viruses within the order Nidovirales. The viruses are irregularly shaped with distinctive long, petal-shaped spikes on

the surface, giving them a crown-like appearance (hence, the name coronavirus) (Lai *et al.*, 2007). Coronaviruses are known to infect both animals and humans, with human coronaviruses belonging to the *Coronavirus* genus in the *Coronaviridae* family. Viruses within the *Coronavirus* genus are divided into three serologically distinct groups. Group I contains the prototype human hCoV-229E strain, the newly described human hCoV-NL63 strain, and numerous animal coronaviruses (Fouchier *et al.*, 2004; van der Hoek *et al.*, 2004). Group II contains the prototype mouse hepatitis virus, human hCoV-OC43, human hCoV-HKU1, SARS-CoV, and multiple animal coronaviruses (Gorbalenya *et al.*, 2004; Woo *et al.*, 2005), while group III have only been identified in birds (Spaan *et al.*, 2004). The single-stranded RNA genome of coronaviruses is large (27-31 kb) and complex (Stephensen *et al.*, 1999). The most highly conserved genomic sequences are found in the 20 kb polymerase gene, which covers the 5' two-thirds of the coronavirus genome (Snijder and Spaan, 1995). The polymerase gene has two large open reading frames (ORFs), ORF 1a and ORF 1b. Within ORF 1b, there are very highly conserved regions coding for conserved functions e.g. polymerase and helicase activity (Stephensen *et al.*, 1999). Following the polymerase gene are the genes encoding the four structural proteins: spike (S), envelope (E), membrane, and nucleocapsid (N). Even though these genes are homologous between coronaviruses, the level of conservation of the spike and membrane protein genes is so low that only the ORF 1b is considered truly informative for differentiation between the three coronavirus groups through phylogenetic analysis (Drosten *et al.*, 2003; Snijder *et al.*, 2003). However, within a coronavirus group genetic variability among strains can be investigated through phylogenetic analysis of the spike protein gene (Moes *et al.*, 2005). In addition to the polymerase protein gene and the four structural protein genes, coronaviruses contain a variable number of unique characteristic ORFs encoding non-structural proteins and the haemagglutinin-esterase (HE) glycoprotein, which differ markedly among coronaviruses in nucleotide sequence, gene order, number, and method of expression, but are conserved within the same group and are therefore called group-specific proteins (Lai *et al.*, 2007).

Prior to the advent of nucleic acid detection methods, human coronaviruses were difficult to detect and epidemiologic data has subsequently been rare. However, in recent years various research groups have developed in-house nucleic acid detection assays to allow more extensive investigations into the epidemiology and clinical characteristics

associated with these viruses as described below for human coronaviruses OC43, 229E, NL63, and HKU1.

Human Coronavirus OC43

The first human coronavirus, isolated in 1965 from a boy with a typical common cold, was shown to have the capacity to produce colds by inoculation of healthy volunteers with culture fluids (Tyrrell and Bynoe, 1965). The virus was provisionally named B814 virus and was only later referred to as OC43, which relates to its exclusive growth in organ culture. During the 1960's hCoV-OC43 was clearly linked to minor upper respiratory tract infections, however in 1974 it was found to also be associated with lower respiratory disease in particular pneumonia, bronchiolitis and croup (McIntosh *et al.*, 1974). Even though there was then already strong evidence towards more severe hCoV-OC43 associated disease, the majority of studies have only recently launched more elaborate investigations into the epidemiology and clinical characteristics associated with hCoV-OC43 (Chiu *et al.*, 2005; Lau *et al.*, 2006; Vabret *et al.*, 2003).

HCoV-OC43 infects individuals of all ages and is generally the most frequently detected human coronavirus (Dare *et al.*, 2007; Gerna *et al.*, 2006; Lau *et al.*, 2006; Vabret *et al.*, 2003), occurring at a prevalence of between 1% and 2% in patients with acute respiratory tract infections (Arden *et al.*, 2006; Lau *et al.*, 2006). Even though hCoV-OC43 is detected infrequently, it is however not uncommon, as a recent seroprevalence study reported a 90.8% seroprevalence rate in adults (Severance *et al.*, 2008). HCoV-OC43 has been shown to mainly occur during winter (Chiu *et al.*, 2005; Lau *et al.*, 2006; Moes *et al.*, 2005), which may explain why studies that investigated it primarily during winter months reported higher detection frequencies (Sloots *et al.*, 2006; Vabret *et al.*, 2003).

HCoV-OC43 is a widespread virus which, together with hCoV-229E, is responsible for one-third of common colds in children and adults (Myint, 1995). In addition, hCoV-OC43 has also been associated with both mild and severe lower respiratory tract infections in hospitalised patients and outpatients (Papa *et al.*, 2007; Vabret *et al.*, 2003). Although individuals of all ages are at risk of infection, the observed hCoV-OC43 associated disease differ among different age groups, with more severe infection usually observed during the

first year of life or in adult patients with co-morbidities and/or immunocompromised conditions (Garbino *et al.*, 2006; Gerna *et al.*, 2006; Gorse *et al.*, 2009). In concordance, El-Sahly *et al.* indicated that pneumonia and bronchiolitis was primarily associated with children <5 years of age, while acute exacerbation of asthma was observed in older children and adults, and pneumonia and exacerbation of chronic obstructive pulmonary disease was associated with older patients (El-Sahly *et al.*, 2000). In particular, hCoV-OC43 has shown to be an important pathogen of nosocomial viral respiratory infection in neonates (Gagneur *et al.*, 2002), while also being a major cause of morbidity in long-term age-care facilities causing influenza-like illness (Birch *et al.*, 2005).

Human Coronavirus 229E

Human coronavirus 229E was first described by Hamre and Procknow in 1966 (Hamre and Procknow, 1966). The virus was recovered by standard cell culture techniques from six students suffering from colds. The following year, cultured hCoV-229E was administered to healthy adult volunteers at the Common Cold Research Unit in Salisbury, England, where it was shown to be an aetiological agent of the common cold (Bradburne *et al.*, 1967).

HCoV-229E is usually detected less frequently than other coronaviruses, primarily occurring at a prevalence of less than 1% (Chiu *et al.*, 2005; Dare *et al.*, 2007; Lau *et al.*, 2006). Similar to hCoV-OC43, infections with hCoV-229E occur in individuals of all ages with the majority of adults (91.3%) having been previously exposed to the virus (Dare *et al.*, 2007; Severance *et al.*, 2008). A seroprevalence rate of 57.1% in children less than 2 months of age and 65% in children between 2.5 to 3.5 years of age suggests that primary infection is acquired early in life (Dijkman *et al.*, 2008; Shao *et al.*, 2007). The discrepancy observed between the very low detection rates and high seroprevalence rates may suggest that the virus is either not detected due to a very short shedding period or that it only causes symptomatic disease in a small number of individuals. In support of the latter hypothesis, Alper *et al.* (Alper *et al.*, 2008) indicated that hCoV-229E does cause subclinical infections in children.

Where hCoV-229E was observed in association with symptomatic respiratory disease, patients were found to suffer mostly from mild URTI (Myint, 1995). However, in some

instances the frequency of hCoV-229E associated LRTI was comparable to that of URTI (Gerna *et al.*, 2007a). Individuals most at risk of developing more severe hCoV-229E associated disease are the very young as well as older persons with serious co-morbidities and/or immunocompromising conditions (Gagneur *et al.*, 2008; Garbino *et al.*, 2006; Gorse *et al.*, 2009). In paediatric patients, hCoV-229E associated LRTI is often diagnosed as acute bronchiolitis, pneumonia or asthma exacerbation, whereas in older persons pneumonia and exacerbation of chronic obstructive pulmonary disease is frequently observed (Dare *et al.*, 2007; El-Sahly *et al.*, 2000; Theamboonlers *et al.*, 2007). HCoV-229E has also been shown to cause severe pneumonia in hematopoietic stem cell transplant recipients thereby indicating that coronaviruses should be considered as potential aetiological agents of pneumonia in immunocompromised patients (Pene *et al.*, 2003).

Even though hCoV-229E and -OC43 share similarities regarding epidemiology and clinical characteristics, the burden of hCoV-229E associated disease in neonatal intensive care units and long-term age-care facilities is generally greater in comparison to hCoV-OC43 (Falsey *et al.*, 2008; Gagneur *et al.*, 2008). Falsey *et al.* detected hCoV-229E in 8% of senior day-centre attendees (Falsey *et al.*, 1997). None of the patients died nor were hospitalised but lower respiratory tract involvement was present. Similarly, in 2008 the same group reported a detection rate of 10.5% in older patients with LRTI which was higher than RSV, Influenza A and B, and HPIV-3 (Falsey *et al.*, 2008). Altogether these findings indicate that hCoV-229E is a common cause of moderately debilitating acute respiratory illness among older persons in long-term care facilities.

Human Coronavirus NL63

Discovery. The novel human coronavirus NL63 was first described by van der Hoek *et al.* in The Netherlands in 2004 (van der Hoek *et al.*, 2004). The virus was isolated from a 7-month-old child suffering from bronchiolitis and conjunctivitis. Diagnostic tests performed on the respiratory sample yielded negative results for all known respiratory viruses. The clinical sample was subsequently inoculated into tertiary monkey kidney cells (*Cynomolgus* monkey), where cytopathic effect (CPE) was observed. The infectious agent was then passaged onto a monkey kidney cell line (LLC-MK2 cells) and analysed further by the

VIDISCA method, which is a means to identify unknown pathogens using molecular biology tools and is essentially based on the cDNA-AFLP technique (Bachem *et al.*, 1996). The experimental layout of the VIDISCA method is indicated in figure 1.3. The major components of the method are: i) enrichment of viral nucleic acids by centrifugation; ii) removal of interfering chromosomal and mitochondrial DNA by DNase treatment; iii) nucleic acid extraction; iv) cDNA synthesis using random hexamers; v) second strand cDNA synthesis; vi) restriction enzyme digestion of double-strand cDNA; vii) ligation of specific restriction enzyme anchors to digested material; viii) selective 2-round PCR amplification using primers specific to the ligated anchors; ix) cloning and sequencing of amplicons from 2nd round PCR. Sequence analysis revealed similarity to members of the *Coronaviridae* family, though significant sequence divergence with known coronaviruses was apparent. Subsequent phylogenetic analysis indicated that hCoV-NL63 was a member of group I coronaviruses and was most closely related to hCoV-229E. To determine if the occurrence of hCoV-NL63 was an isolated event or whether it was circulating in humans, the founding authors developed an hCoV-NL63 specific diagnostic PCR which identified seven additional individuals carrying the virus. Both children and adults were infected and patients suffered from either URTI or LRTI (van der Hoek *et al.*, 2004).

Shortly after the first publication on hCoV-NL63, a second group in The Netherlands described essentially the same virus, which they named hCoV-NL. Fouchier *et al.* isolated the virus from a nose swab sample collected from an 8-month-old boy suffering from pneumonia in April 1988 (Fouchier *et al.*, 2004). The virus was also initially cultured on tMK cells and was thereafter passaged onto Vero cells. Electron microscopy revealed the presence of coronavirus-like particles and subsequent sequencing of amplicons obtained from coronavirus family specific primers indicated that the virus was more closely related to hCoV-229E than any of the other coronaviruses. HCoV-NL was subsequently detected in four additional children suffering from respiratory tract infections of which three had underlying disease. The similarity between hCoV-NL and the previously described hCoV-NL63 was very high (98% at the nucleotide level) and it can therefore be concluded that these two virus isolates represents the same species (van der Hoek *et al.*, 2006).

In 2005, Esper *et al.* described the identification of a similar group I coronavirus in respiratory specimens obtained from children less than 5 years old and was designated

New Haven coronavirus (hCoV-NH) (Esper *et al.*, 2005). Limited sequence data suggested that the then newly identified hCoV-NH was closely related to NL63 and NL, and that these viruses are strains of the same species. Unlike the discovery methods employed by Van der Hoek *et al.* and Fouchier *et al.*, Esper *et al.* used molecular probes that target regions of the genome conserved among human and animal coronaviruses. The advantage of this approach is that it negates the propagation of the virus in cell culture and may prove useful for future discovery of coronaviruses that are not easily propagated in cell culture.

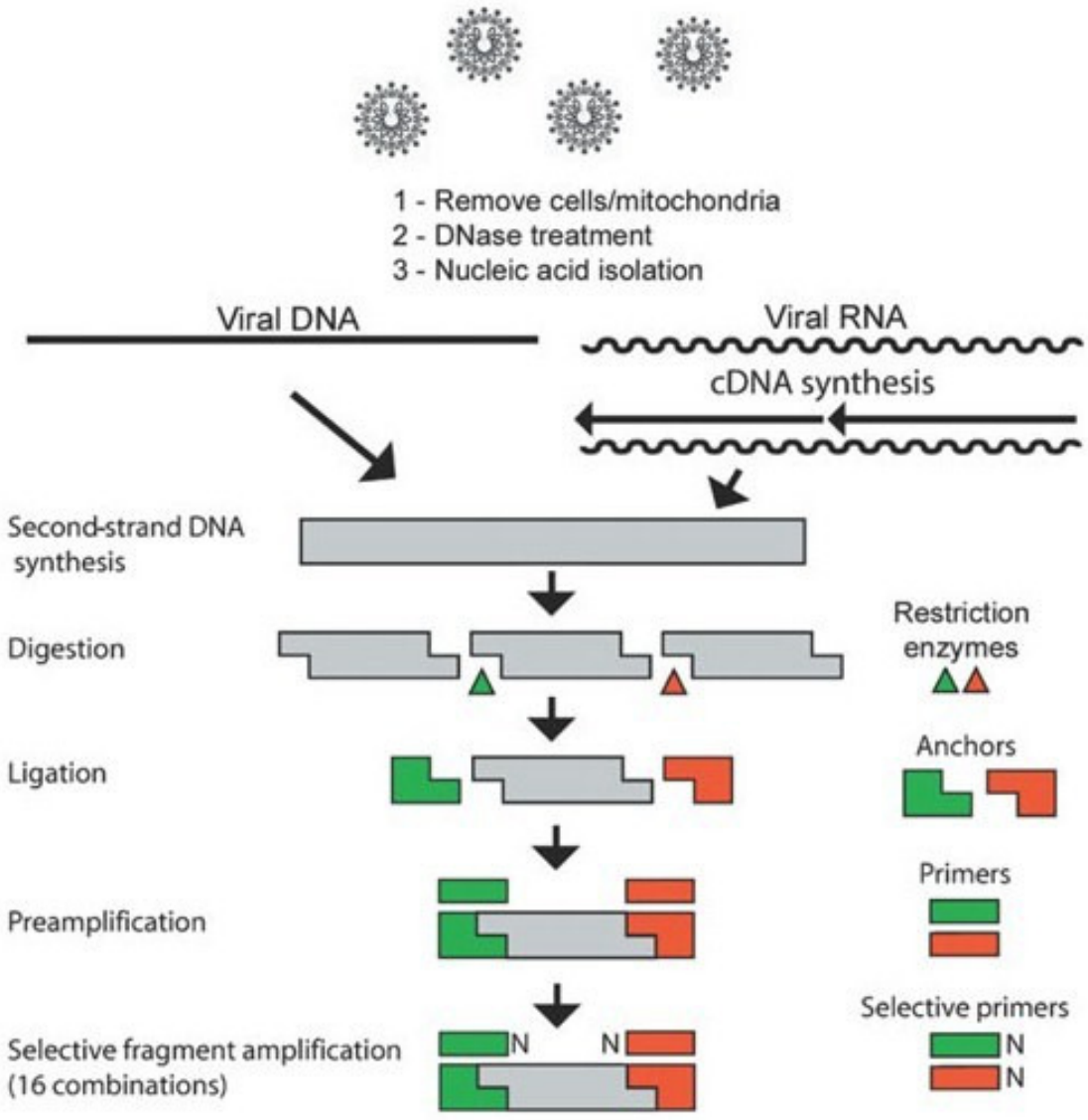

Figure 1.3 The VIDISCA method. Schematic overview of steps incorporated in the performance of the VIDISCA method (from van der Hoek *et al.* (van der Hoek *et al.*, 2004)).

Epidemiology. Since the first description./.n in 2004, various studies have reported the presence of hCoV-NL63 in their various populations indicating a worldwide distribution (Arden *et al.*, 2005; Bastien *et al.*, 2005a; Chiu *et al.*, 2005; Dare *et al.*, 2007; Vabret *et al.*, 2005). These reports showed a prevalence of 0.6% to 9.3% in patients with respiratory tract infections, with some studies indicating annual prevalence variation (Gerna *et al.*, 2006; Han *et al.*, 2007; Koetz *et al.*, 2006). Frequent occurrence of respiratory viral co-infections was commonly reported (Arden *et al.*, 2005; Dare *et al.*, 2007; Gerna *et al.*, 2007a; van der Hoek *et al.*, 2005). The seasonal occurrence of hCoV-NL63 showed different patterns in different geographical regions. In temperate regions, hCoV-NL63 infections appear to peak in winter (Arden *et al.*, 2005; Bastien *et al.*, 2005b; van der Hoek *et al.*, 2005), while a spring-summer predominance is observed in subtropical areas (Choi *et al.*, 2006; Lau *et al.*, 2006). HCoV-NL63 causes infection and illness in individuals of all ages (Dare *et al.*, 2007), though is frequently detected in children under the age of 5 years (Bastien *et al.*, 2005a; Chiu *et al.*, 2005; Han *et al.*, 2007). In particular, Lambert *et al.* showed that hCoV-NL63 is an important pathogen in community-based illness in children, especially in those who attend child care (Lambert *et al.*, 2007). However, elderly individuals and those with underlying disease are also at risk of infection (Bastien *et al.*, 2005a; Garbino *et al.*, 2006). Similar to hCoV-229E, a seroprevalence rate of 45.2% in children less than 2 months of age and 75% in children between 2.5-3.5 years of age suggests that primary infection with hCoV-NL63 occurs early in life (Dijkman *et al.*, 2008; Shao *et al.*, 2007).

Clinical characteristics. HCoV-NL63 infection occurs in both previously healthy individuals and those with underlying disease (Chiu *et al.*, 2005; Fouchier *et al.*, 2004). The clinical symptoms associated with hCoV-NL63 respiratory tract infection are comparable to those observed for influenza, RSV, and other coronaviruses (Bastien *et al.*, 2005b). HCoV-NL63 has been found in association with both upper- and lower respiratory tract infections where patients may present with a wide range of clinical signs including respiratory distress, wheezing, crepitations, apnoea, cough, rhinorrhea, fever, and parahilar infiltrates in the event of a LRTI (Arden *et al.*, 2005; Bastien *et al.*, 2005b; Vabret *et al.*, 2005). HCoV-NL63 is considered to be a major cause of croup (Choi *et al.*, 2006; van der Hoek *et al.*, 2005), but has also been found in association with bronchiolitis, pneumonia, acute asthma exacerbation, and febrile seizures (Arden *et al.*, 2005; Bastien *et al.*, 2005b; Papa *et al.*, 2007). Although hCoV-NL63 associated disease has led to hospitalisation, it has been

reported to be less severe in comparison to RSV and is less common in cases of acute LRTI associated with pneumonia (Arden *et al.*, 2005; Choi *et al.*, 2006; Koetz *et al.*, 2006; van der Hoek *et al.*, 2005).

Human Coronavirus HKU1

Discovery. In 2005, Woo *et al.* (Woo *et al.*, 2005) reported the discovery of a novel group II coronavirus in a 71-year-old man suffering from pneumonia. The patient was hospitalised with a fever and productive cough with a chest radiograph showing patchy infiltrates. Direct antigen detection assays, reverse transcription PCR (RT-PCR), and viral culture could not identify respiratory viruses in the patient's NPA specimens, prompting them to search for alternative causative agents. Similar to the method employed in the identification of hCoV-NL by Fouchier *et al.* (Fouchier *et al.*, 2004), Woo *et al.* made use of primers targeting a region in the polymerase gene that is conserved among all coronaviruses. The nucleotide sequence identified from the resulting amplicon indicated significant sequence divergence with known coronaviruses while genomic analysis revealed close association with group II coronaviruses. This novel coronavirus was designated human coronavirus HKU1 (hCoV-HKU1). The clinical significance of hCoV-HKU1 in the index patient was made evident by the presence of high viral loads which coincided with the patient's acute symptoms. In addition, the decrease in viral load was accompanied by recovery from disease and the development a specific antibody response to the recombinant nucleocapsid protein of the virus. A preliminary study on 400 NPA's collected during the SARS-period identified the presence of hCoV-HKU1 in another adult patient suffering from pneumonia and had viral loads comparable to those of the index patient (Woo *et al.*, 2005).

Epidemiology. Large epidemiological studies (enrolled >1000 patients) performed over one or more years generally report hCoV-HKU1 detection frequencies of between 0.3% and 1% (Dare *et al.*, 2007; Dominguez *et al.*, 2009; Esper *et al.*, 2006; Lau *et al.*, 2006), though higher frequencies have been reported (Kuypers *et al.*, 2007). Esper *et al.* proposed that the infrequent detection of hCoV-HKU1 may suggest that it is an uncommon virus or that it has properties that reduce the likelihood of detection such as a short period of viral shedding, or that hCoV-HKU1 may be a common virus that causes symptomatic disease in

only a relatively small percentage of infected individuals (Esper *et al.*, 2006). The latter appears to be the most likely explanation as a seroprevalence study indicated that 59.2% of adults have had previous exposure to the virus (Severance *et al.*, 2008). HCoV-HKU1 were detected predominantly during the winter months in prevalence studies (Esper *et al.*, 2006; Gerna *et al.*, 2007a; Lau *et al.*, 2006; Sloots *et al.*, 2006; Vabret *et al.*, 2006). Similar to other human coronaviruses, hCoV-HKU1 infects individuals of all ages (Gerna *et al.*, 2007a; Vabret *et al.*, 2006; Woo *et al.*, 2005), with children under the age of 2 years, immunocompromised adults and individuals with co-morbidities, especially those of the respiratory and cardiovascular system, being most at risk (Esper *et al.*, 2006; Gerna *et al.*, 2007a; Sloots *et al.*, 2006; Talbot *et al.*, 2009).

Disease association. HCoV-HKU1 associated disease may vary from mild URTI to severe LRTI (Esper *et al.*, 2006; Lau *et al.*, 2006). Overall, URTI is more frequently observed in hCoV-HKU1 infected patients, though an association with pneumonia, bronchiolitis, acute asthma exacerbation, and febrile seizures have also been reported (Esper *et al.*, 2006; Gerna *et al.*, 2007a; Lau *et al.*, 2006; Vabret *et al.*, 2008). One study looked in particular at patients with hCoV-HKU1 associated pneumonia and observed that most patients had relatively mild disease, were hospitalised only briefly and made a quick recovery (Woo *et al.*, 2005). Clinical symptoms associated with hCoV-HKU1 infection include rhinorrhea, cough, fever, and parahilar infiltrates in the event of a LRTI (Esper *et al.*, 2006; Gerna *et al.*, 2007a).

1.4 Diagnosis of Respiratory Viruses

1.4.1 Cell culture

Cell culture has long been considered the “gold standard” for the diagnosis of viruses and is the procedure that sets diagnostic virology apart from the other areas of diagnostic microbiology. Cell cultures are used in the virology diagnostic laboratories for the isolation and identification of viruses, the production of antigens used in serology, and the propagation of viruses as control materials. It is a highly sensitive method that by nature amplifies the amount of putative virus, facilitating detection and characterization. In

addition, it provides an isolate of viable virus that, if necessary, facilitates further characterization. However, important disadvantages of cell culture include the necessity of specialized facilities and expertise, relative prolonged time to detection, expense, and only a relatively limited range of viruses can be detected by cell culture (e.g. *in vitro* culture of hBoV and hCoV-HKU1 have been unsuccessful to date (Gerna *et al.*, 2007a; Woo *et al.*, 2005)).

Cell culture permits the detection of all the well recognized respiratory viruses with the exception of coronaviruses, with different cell lines being optimal for the primary isolation of the different respiratory viruses (Cough and Kasel, 1995; Henrickson, 1995; Hierholzer, 1995; Tristram and Welliver, 1995). Efficient *in vitro* replication of influenza viruses, some serotypes and strains of parainfluenza viruses, and human metapneumovirus in continuous cell lines requires exogenous protease (trypsin) in the cell culture medium (Henrickson, 1995; van den Hoogen *et al.*, 2001). Although it is recommended for the recovery of these viruses, the addition of trypsin is not standard practice in some laboratories, which is in part reason why human metapneumovirus has remained undiscovered until 2001. One particular limitation of cell culture is that some viruses may produce relatively inconspicuous cytopathic effects (CPE) (Agapov *et al.*, 2006; Henrickson, 1995), making differentiating between the respective viruses difficult without additional aids such as antigen detection techniques or nucleic acid detection assays. This limitation can be addressed by the use of shell vial cultures, which is a modified cell culture technique that combines the elements of cell culture and antigen detection to achieve rapid culture-based detection. The method involves the centrifugation of the specimen onto the cell monolayer, where after an incubation period of a minimum of 16 hours viral growth is detected by antigen detection procedures, whether CPE is visible or not (Storch, 2007).

Even though cell culture is progressively being replaced by immunologic and molecular methods it will remain important regionally to maintain a source for analyzing genetic and antigenic change in virus populations and for the discovery of unknown viruses (van den Hoogen *et al.*, 2001; van der Hoek *et al.*, 2004).

1.4.2 Antigen detection

Antigen detection of respiratory viruses may be achieved by various methods. Enzyme-linked, radio-, and fluoroimmunoassays have all been used for the detection of respiratory viral antigens (Korppi *et al.*, 1986; Scalia *et al.*, 1995; Storch, 2000). However, these assays are not commercially available for all respiratory viruses. At present, the most generally used antigen detection method is immunofluorescence assays (IFA), which uses serotypic immune animal antisera conjugated with fluorescein isothiocyanate (FITC) (Madeley and Peiris, 2002). Immunofluorescence performed directly on the respiratory specimen without prior culture amplification is widely used for the diagnosis of RSV, PIV, influenza, and adenovirus infections. Compared to cell culture, this method has shown highly variable detection rates, ranging from 22-100% and averaging between 50-75% (Henrickson, 2004). The observed variability is attributable to differences in the specimen collection, specimen processing, antibody production, and testing procedure.

IFA is directed primarily at the detection of viral antigens expressed in respiratory epithelial cells, therefore specimens must provide adequate cells for examination. The ideal respiratory specimens include NPA specimens, nasal washes, or nasopharyngeal swabs. Tracheal aspirates and bronchoalveolar lavage (BAL) fluid can also be used. The process of IFA include clarification of the specimen and pelleting of cells by centrifugation, spotting of cells onto microscope slides, drying and fixation, staining of slides with monoclonal antibodies specific for the viruses being sought, and examination of the slides using a fluorescent microscope with ultraviolet epi-illumination (Storch, 2000).

The major advantages of IFA over cell culture are increased turnaround time and lack of requirements for viable virus in the specimen. However, IFA has several important disadvantages. Firstly, IFA requires individual skill at all points including the collection of the specimen, extracting the cells, making and staining the slide preparations, and reading the results under the microscope (Madeley and Peiris, 2002). Secondly, the throughput by IFA is low as each specimen is handled and read individually (Madeley and Peiris, 2002). Thirdly, IFA is highly dependent on the quality of the specimen (amount of cells) and with specimen deterioration occurring during transit, it is imperative that a “cold chain” for transporting specimens is maintained and that lines of communication with the laboratory

should be as short as possible (Madeley and Peiris, 2002). Fourthly, not all respiratory viruses can be detected by IFA (e.g. rhinoviruses due to the multiplicity of antigenic types) (Madeley and Peiris, 2002). Lastly, IFA are not suitable for the discovery of new viruses and for those that have been recently identified (with the exception of hMPV) there are no commercial IFA available.

1.4.3 Nucleic acid detection methods

At present, the most commonly used techniques for the diagnosis of viral respiratory tract infections is cell culture usually in combination with immunofluorescence assays (Fischbach, 2009). However, in recent years, nucleic acid detection of pathogenic DNA and RNA viruses by means of PCR have gained increased popularity due to a combination of improved sensitivity and specificity, a potential for automatization, and increased turnaround time (Ieven, 2007; Watzinger *et al.*, 2006).

Nucleic acid detection by means of PCR involves three steps: i) extraction of nucleic acids from a sample; ii) nucleic acid amplification; and iii) detection of amplified products. Originally, detection of PCR amplicons relied on gel electrophoresis in the presence of ethidium bromide that allows subsequent visualization of the amplicons during UV irradiation. As an alternative, PCR amplicons may be captured onto a solid phase and detected by an enzyme immunoassay. These methods are, however, time consuming and necessitates multiple PCR product handling steps that increases the risk of carryover contamination and false positive results in subsequent assays. In comparison, real-time PCR is significantly faster with amplification and detection performed simultaneously in sealed tubes, thus obviating the need for post-amplification processing and subsequently significantly reduces the risk of carryover contamination (Ieven, 2007; Watzinger *et al.*, 2006).

Numerous chemistries are available to perform real-time PCR including dual labelled probes (TaqMan™ probes), molecular beacons, minor groove binding probes (MGB), fluorescence resonance energy transfer (FRET) hybridization probes, intercalating dyes (e.g. SYBR green) and fluorescent labelled primers such as Sunrise™, Lux™ or Scorpion primers™. The principle of the different probe chemistries with associated advantages and

disadvantages are listed in table 1.1 (Gunson *et al.*, 2006; Lee *et al.*, 2004).

Comparative studies have shown that for the detection of respiratory viruses real-time RT-PCR is significantly more sensitive than conventional detection methods (i.e. cell culture and IFA) (Gharabaghi *et al.*, 2008; Kuypers *et al.*, 2006; Smith *et al.*, 2003; van de Pol *et al.*, 2007). Various single target real-time PCR assays have been described for the viruses various respiratory viruses (as reviewed by M. Ieven (Ieven, 2007)), though individual detection of the respective viruses is costly. This limitation can, however, be overcome with multiplex PCR, which entails simultaneous independent amplification of multiple pathogens in a single PCR reaction. In addition to reducing overall testing cost, multiplex PCR can significantly increases throughput and reduces turnaround time (Gunson *et al.*, 2006). Detection of viral respiratory pathogens by multiplex real-time PCR has been described previously (Brittain-Long *et al.*, 2008; Gunson *et al.*, 2005; Templeton *et al.*, 2004). These studies employ the use of hydrolysis probes (Brittain-Long *et al.*, 2008; Gunson *et al.*, 2005) and molecular beacons (Templeton *et al.*, 2004) as probe chemistry which are highly sensitive to hybridization mismatches. Due to the high mutation frequencies of respiratory viral genomes this may result in false negative results (Gunson *et al.*, 2006). In contrast, fluorescence resonance energy transfer (FRET) hybridization probes can tolerate up to 6 mismatches (Zaayman *et al.*, 2009). In addition, the probes are not hydrolyzed during the amplification reaction, thereby facilitating generation of amplicon-specific melting curves for further discrimination and incorporation of multiple targets per reaction. Therefore, FRET hybridization probes may be best suited for multiplex detection of respiratory viruses.

Even though PCR has significant advantages over conventional diagnostic assays, it has some important limitations. Firstly, designing oligonucleotide primers is dependent on previous knowledge of a microorganism's genome as well as the ability of publicly available sequence databases to suitably represent all variants of that organism. Secondly, carryover contamination that produces false positive results may cause considerable problems in the routine implementation of PCR in the diagnostic laboratory. Lastly, in some instances PCR may be too sensitive, detecting a microbe that is present at non-pathogenic levels, though this may be addressed by quantitative analysis of microorganisms (Ieven, 2007; Watzinger *et al.*, 2006).

Table 1.1 Comparison of the different chemistries available for real-time PCR (adapted from Gunson, Collins and Carman, 2006 (Gunson *et al.*, 2006))

Chemistry	Advantages	Disadvantages	Principle (Lee *et al.*, 2004)
Molecular beacons	Specific	Susceptible to probe mis-match, expensive, reduced fluorescence, less available	The 5' (labelled with fluorescent dye) and 3' (labelled with a quencher dye) sequence of the probe is complementary, forming a hairpin structure that brings the two fluorescent moieties together, quenching fluorescence. Sequence dependent binding of the probe opens the hairpin 'loop' sequence, quenching of the fluorescent dye is abolished and amplification is detected.
Dual labelled probes	Specific, many publications available,increased fluorescence, less susceptible to probe mismatch. Many manufacturers	Probe mismatch can lead to false negatives	The probes are labelled with a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end. Unbound the dyes are in close proximity and the quencher suppresses fluorescence emission of the reporter dye. Bound to a target sequence, the *Taq* DNA polymerase hydrolyses the probe, releasing the reporter dye into solution. Quenching of the fluorescent reporter dye is abolished and amplification is measured at each extension step.
Minor groove binders	Specific, increased fluorescence produced,can be used in small conserved areas,designed for SNP detection	Susceptible to mismatches, few suppliers	The principle is the same as for dual labelled probes. The difference lies with the addition of minor groove binders e.g. dihydrocyclopyrroloindole at the 3' end. This chemical modification increases the Tm of the probe.
FRET probes	Can readily detect single nucleotide differences, exact match to DNA for signal to be released, can be used in non-PCR amplification assays	Requires strict optimization of probe design, requires accurate thermal denaturation profiles to prevent interference with hybridisation	This chemistry relies on two probes binding in close proximity. One probe is labelled with a donor dye at the 3' end (e.g. Fluorescein) and the other probe labelled with an acceptor dye at the 5' end (e.g. LCRed640). During the annealing step the probes bind to the same strand in a head-to-tail arrangement (1-5 nucleotides apart), bringing the two dyes into close proximity. An appropriate light source excites the donor dye. Fluorescence energy is transferred from the donor dye to the acceptor dye which then emits fluorescence. Amplification is detected during the annealing step.
Labelled primers (e.g. sunrise, scorpion, lux)	Cheap (no probe needed), sensitive as probe based technology, less homology needed (no probe region)	Primer–dimer formation, strict design criteria	The principle is similar to that of molecular beacons. The difference is that the probe is incorporated into PCR product, which results in the breaking of fluorescence emission suppression by the quencher dye. Amplification is detected after each extension step.
Intercalating dyes	Cheap, use on large regions	Primer-dimer, less specific than other methods, variable melting peaks	The dyes intercalate in a non-specific manner into double-stranded DNA molecules. In solution the dyes do not emit fluorescence. Though, when bound to DNA fluorescence is emitted when excited by an appropriate light source.

1.5 Quantification of Viral Loads

Quantitative detection of viruses has numerous advantages and applications in both the research and diagnostic setting. It may aid in the study of virus pathogenesis, response to treatment, development of vaccine and antiviral strategies, as well as serve as a prognostic marker (Watzinger *et al.*, 2006). Examples thereof included the routine monitoring of human immunodeficiency virus (HIV) viral load that serves as a prognostic marker in HIV infected patients (Mellors *et al.*, 1996), whereas quantification of hepatitis B virus DNA is used to monitor antiviral therapy as well as to determine infectivity (Berger *et al.*, 2001; Wiseman *et al.*, 2009). Similarly, it has been shown that the monitoring of Epstein-Barr virus, adenovirus and human papillomavirus type 16 viral loads may allow assessment of the risk of developing virus associated disease and may be predictive for the development of symptoms and disease outcome (Gustafson *et al.*, 2008; Sato *et al.*, 2008; Ylitalo *et al.*, 2000). In these instances the quantitative detection of viruses may be of clinical significance to prevent severe clinical virological complications and may present a basis for appropriate initiation of antiviral therapy and clinical management of disease. Quantification of viruses may be preformed by various methods and can be collectively grouped into three major types of assays: biological, physical and molecular.

1.5.1 Biological quantitative assays

Biological quantitative assays include the plaque assay and various endpoint methods, all of which require infectivity of cell cultures (as reviewed by Condit (Condit, 2007)). These assays only measure the presence of infectivity and may not count all particles present in a preparation, even many that, in fact, are infectious.

The plaque assay is based on the ability of a single infectious particle to produce macroscopic cytopathology (plaque) on an otherwise normal monolayer of cells cultured in a semisolid medium. Following inoculation of monolayers, the virus spreads from cell to cell, resulting in a localized plaque. After a suitable incubation period, the infected monolayers are stained and the plaques counted. The concentration of infectious virus in the original sample can then be calculated (Boeckh and Boivin, 1998). Viruses that can not

be adapted to a plaque assay (e.g. coronaviruses), but still cause some detectable pathology in cell culture, can be quantified using an endpoint method.

End point methods entail the inoculation of tissue cultures with replicates of serially diluted virus preparations with low concentrations showing infection in all replicate inoculations, while high dilutions show infection in none of the inoculations and some dilutions show infection in only certain replicate inoculations. Statistical methods allow one to calculate the dilution of virus that results in infection in 50% of replicate inoculations. Virus titres are subsequently expressed as the tissue culture infectious dose 50 ($TCID_{50}$). Endpoint quantification methods may be performed in any appropriate assay system e.g. embryonic eggs (virus titre expressed as egg infectious dose or EID_{50}) or experimental laboratory animals (yielding lethal dose or LD_{50}) (Condit, 2007).

A disadvantage of biological quantitative assays is that it is time consuming as well as difficult to perform and has numerous inherent limitations regarding the assay's ability to accurately and reliably quantify viral concentrations in patient samples (Condit, 2007).

1.5.2 Physical quantitative assays

Physical quantitative assays include direct virus particle count by means of electron microscopy and haemagglutination (as reviewed by Condit (Condit, 2007)). Unlike biological quantitative assays, these assays do not give any measures of viral infectivity as viral replication is not required.

Virus particle count may be performed directly using an electron microscope by mixing a sample of purified virus with microscopic marker particles of known concentration. In the resulting solution, virus particles are subsequently counted relative to the amount of beads, resulting in an accurate determination of the virus particle concentration in the original solution (Condit, 2007). In the event that viruses produce attachment proteins that bind to substituants (e.g. sialic acid) that are abundant on a variety of cell types, including erythrocytes, haemagglutination can be used for the quantification of viruses. Quantification of viruses by means of haemagglutination is carried out by adding serially diluted virus particles to a fixed concentration of erythrocytes and transferred to wells with rounded

bottoms. Erythrocytes that did not agglutinate will be free to roll to the bottom of the well, forming a dense, easily recognizable cluster of cells, or button, while erythrocytes that are agglutinated will not be free to roll to the bottom of the well, but instead evenly coat the bottom surface of the well. One haemagglutination unit is defined as the minimum concentration of virus required to cause agglutination, where the titre of the virus solution, expressed as haemagglutination units per millilitre (HA units/ml), can be calculated taking into account the serial dilution (Condit, 2007).

1.5.3 Molecular quantitative assays

In comparison to biological and physical quantitative methods, quantitative PCR offers several theoretical advantages, including i) the potential stability of the assay after specimen freezing and thawing; ii) a lower threshold of detection; iii) a less subjective readout; and iv) an assay that is unaffected by therapeutic passive neutralizing antibodies or experimental antiviral agents.

Real-time PCR, in particular, is rapidly becoming a standard for the detection and quantification of pathogenic DNA and RNA viruses and is firmly established as a mainstream research technology (Bustin and Mueller, 2005). Quantification with real-time PCR technology is based on its ability to detect the number of amplicons generated during each amplification cycle in real-time. Quantification of the target is performed during the exponential phase of PCR, when the product first becomes detectable and is therefore, neither affected by limiting reagents, nor cycling conditions, which affects endpoint analysis-based PCR assays (i.e. quantitative PCR approaches based on data analysis at the end of PCR) (Bustin and Mueller, 2005). The potential for high-throughput, together with frequent introduction of improved or novel chemistries, its reliability and reproducibility, has also seen to the introduction of quantitative real-time PCR-based assays in the diagnostic setting (Boeckh and Boivin, 1998). However, commercial quantitative real-time PCR assays are available for only a limited number of viral pathogens including hepatitis B and C, HIV, cytomegalovirus, human papilloma virus, and SARS-associated coronavirus (Watzinger *et al.*, 2006). In different clinical settings the quantitative monitoring of virus infections has become indispensible to patient and disease management and has led to the

development of in-house real-time quantitative assays for several other viral targets (as reviewed by Watzinger, 2006 (Watzinger *et al.*, 2006)).

Quantitative real-time PCR assays have been described for adenovirus (Ebner *et al.*, 2005; Heim *et al.*, 2003), RSV (Fodha *et al.*, 2007; Gueudin *et al.*, 2003; van Elden *et al.*, 2003), hMPV (Boivin *et al.*, 2003; Deffrasnes *et al.*, 2005; Kuypers *et al.*, 2005), hBoV (Allander *et al.*, 2007b; Lin *et al.*, 2007; Sloots *et al.*, 2006), influenza A and B (Smith *et al.*, 2003; van Elden *et al.*, 2001; Watzinger *et al.*, 2004), parainfluenza virus 1-4 (Hu *et al.*, 2005; Watzinger *et al.*, 2004), hCoV-NL63 (van der Hoek *et al.*, 2005), hCoV-HKU1 (Woo *et al.*, 2005), hCoV-OC43 (Vijgen *et al.*, 2005), and hCoV-229E (Vijgen *et al.*, 2005). Since the influencing factors that drive the divergent disease severities observed for respiratory tract infections have not yet been fully elucidated, some of these assays have been employed to determine the association between respiratory viral load and disease severity. Previous studies have proposed an association between disease severity and RSV viral load, as it was found that patients with more severe respiratory disease often had higher viral loads (DeVincenzo *et al.*, 2005; Gueudin *et al.*, 2003; Martin *et al.*, 2008). However, contrasting observations has also been described (Wright *et al.*, 2002). Previous investigations have found that increased hMPV viral load was frequently associated with hospitalisation, patients between 7 and 12 months and lower respiratory tract involvement (Bosis *et al.*, 2008; Gerna *et al.*, 2007c; Kuypers *et al.*, 2005), whereas high hBoV viral loads were associated with both upper and lower respiratory tract infections (Choi *et al.*, 2008). Patients with single hBoV infections have also been found to present with higher viral loads than patients with co-infections (Brieu *et al.*, 2008; Christensen *et al.*, 2008; Jacques *et al.*, 2008), though contrasting findings have been reported (Neske *et al.*, 2007). Contrary to hMPV and hBoV, quantitative studies on human coronaviruses are limited. Quantification assays for hCoV-OC43 and 229E have been described (Vijgen *et al.*, 2005), but to date have not been applied to clinical investigations. While quantitation of hCoV-HKU1 and hCoV-NL63 has only been described once in each case with no observations made about the association between viral load and disease severity (van der Hoek *et al.*, 2005; Woo *et al.*, 2005). Overall, the clinical relevance of viral load in patients with acute respiratory tract infections remains poorly understood and requires further investigation.

Unlike blood borne viruses such as HIV and hepatitis B and C, quantification of viral loads in respiratory specimens is challenging due to the non-homogenous nature of the specimens, thus, requiring methods for standardization of specimens to ensure that specimens have similar concentrations. To standardize respiratory specimens, relative quantification to a house keeping gene as internal control (GAPDH) have been employed (Gueudin *et al.*, 2003), although its relevance in respiratory specimens have been questioned. Most investigations of respiratory viral loads did not apply any method of standardization (Gerna *et al.*, 2008; Kuypers *et al.*, 2004; Martin *et al.*, 2008; Perkins *et al.*, 2005).

1.6 Study Objectives

To develop a novel rapid assay that can detect all major viral causes of respiratory infections as well as newly identified viruses recognized as important causes of ALRI in developed countries and to investigate the contribution of respiratory viruses in patients requiring hospitalisation or attending outpatient visits in public sector hospitals serving the Pretoria area, South Africa

Specific Objectives

- To develop and optimize quantitative multiplex real-time RT-PCR assays utilizing FRET hybridization probes for differential diagnosis of RSV A and B, Influenza A&B, Parainfluenza 1,2,3, Adenovirus, human Metapneumovirus, human Bocavirus, and human Coronaviruses OC43, 229E, HKU1 and NL63.

- To identify hospitalised paediatric patients infected with well recognized viruses (RSV, PIV1-3, influenza A/B, adenovirus), newly discovered viruses (hMPV, hBoV, hCoV-NL63, hCoV-HKU1) and viruses traditionally associated with upper respiratory tract infections (hCoV-OC43, hCoV-229E) in cases of paediatric lower respiratory tract infections to determine the incidence and importance of the new viruses in lower respiratory tract disease in South Africa.

- To quantify the viral load of viruses that occurs as co-infections and single infections. To determine if co-infections have an affect on disease severity as well as to determine if any specific virus plays a dominant role and to determine if viral load is associated with disease severity.

Chapter 2

A Novel Multiplex Real-Time RT-PCR Assay with FRET Hybridization Probes for the Detection and Quantification of 13 Well Recognized and Newly Identified Respiratory Viruses

2.1 Introduction

Acute lower respiratory tract infection (ALRI) is a leading cause of paediatric morbidity and mortality (Murray, 2001). The most common viral agents associated with respiratory tract infections are respiratory syncytial virus (RSV), influenza viruses A and B, parainfluenza viruses (PIV) 1 to 3, and adenoviruses (Adv). In recent years several new viruses associated with ARI have been identified including. human metapneumovirus (van den Hoogen *et al.*, 2001); human coronaviruses (hCoV) – hCoV-HKU1 (Woo *et al.*, 2005) and hCoV-NL63 (van der Hoek *et al.*, 2004); and human Bocavirus (hBoV) (Allander *et al.*, 2005) and most recently two new polyoma viruses, WU virus (WUV) and KI virus (KIV) (Allander *et al.*, 2007a; Bialasiewicz *et al.*, 2007; Gaynor *et al.*, 2007). Accumulative evidence indicate that the former four are probable causes of ALRI although frequent co-infections are reported for especially hBoV (Allander *et al.*, 2007b; Anderson, 2007; Arden *et al.*, 2006; Arden *et al.*, 2005; Bosis *et al.*, 2007; Chiu *et al.*, 2005; Kupfer *et al.*, 2007). The role of the polyomaviruses is still in question (Abed and Boivin, 2008; Barzon *et al.*, 2009; Norja *et al.*, 2007; Ren *et al.*, 2008; Venter *et al.*, 2009). In addition, improved sensitivity of diagnostic tests has lead to the increased detection of viruses traditionally associated with mild upper respiratory tract infections in cases of ALRI, including hCoV-OC43, hCoV-229E and rhinoviruses as well as the identification of a new rhinovirus genotype, rhinovirus C (Arden *et al.*, 2010; Hayden, 2006; Jartti *et al.*, 2004; Pene *et al.*, 2003; Vabret *et al.*, 2003; Wisdom *et al.*, 2009).

At present, the most commonly used techniques for the diagnosis of viral respiratory tract infections is cell culture usually in combination with direct immunofluorescence assays (IFA) (Fischbach, 2009). Recently nucleic acid detection methods have become more readily available for the diagnosis of virus infections (Watzinger *et al.*, 2006). Comparative studies have shown that for the detection of respiratory viruses real-time RT-PCR is significantly more sensitive than conventional detection methods (Gharabaghi *et al.*, 2008; Kuypers *et al.*, 2006; Smith *et al.*, 2003; van de Pol *et al.*, 2007). The majority of real-time RT-PCR detection assays for respiratory viruses are only qualitative of nature. Even though qualitative real-time PCR in the diagnostic setting has numerous advantages over more traditional detection methods, one of the limitations is that it does not discriminate between current infections or shedding of low concentration of virus from previous infections. The

significance of co-infections and less common viruses are also not portrayed. In contrast, quantitative real-time PCR provides qualitative as well as quantitative information. The advantages of quantitative real-time PCR is that it permits the assessment of viral load at a given time point, facilitates the monitoring of response to treatment, and offers the possibility to determine the dynamics of virus proliferation (Watzinger *et al.*, 2006). Quantitative tests may aid in the study of virus pathogenesis, development of vaccine and antiviral strategies, and will facilitate investigations of the importance of the recently identified respiratory viruses when detected by themselves or as co-infections in patients. Quantification of viral loads in respiratory specimens is challenging due to the non-homogenous nature of the specimens, thus, requiring methods for standardization of specimens ensuring specimens have similar concentrations. To standardize respiratory specimens, relative quantification to an house keeping gene as internal control (GAPDH) have been employed (Gueudin *et al.*, 2003), although its relevance in respiratory specimens have been questioned. However, most investigations of respiratory viral loads did not apply any method of standardization (Gerna *et al.*, 2008; Kuypers *et al.*, 2004; Martin *et al.*, 2008; Perkins *et al.*, 2005).

Qualitative detection of viral respiratory pathogens by multiplex real-time PCR has been described previously (Brittain-Long *et al.*, 2008; Gunson *et al.*, 2005; Templeton *et al.*, 2004). These studies employ the use of hydrolysis probes (Brittain-Long *et al.*, 2008; Gunson *et al.*, 2005) and molecular beacons (Templeton *et al.*, 2004) as probe chemistry which are highly sensitive to hybridization mismatches. Due to the high mutation frequencies of respiratory viral genomes this may result in false negative results (Gunson *et al.*, 2006). In contrast, fluorescence resonance energy transfer (FRET) hybridization probes can tolerate up to 6 mismatches (Zaayman *et al.*, 2009). In addition, the probes are not hydrolyzed during the amplification reaction, thereby facilitating generation of amplicon-specific melting curves for further discrimination and incorporation of multiple targets per reaction.

In this study, the development and application of a novel quantitative real-time RT-PCR assay utilizing FRET probes for the differential diagnosis of well recognized and recently identified respiratory viruses are described. This consisted of 4 multiplex reactions covering 13 different viruses that may be run individually or concurrently.

2.2 Materials and Methods

2.2.1 Viruses and clinical specimens

Tissue culture positive controls were available for RSV subtype A and B, PIV 1- 3, Influenza A, and B. Clinical specimens that tested positive by PCR for hMPV, hCoV-NL63 and hCoV-OC43 were obtained from Dr. Jan Kimpen, University of Utrecht, The Netherlands, and hBoV from Dr. Heidi Smuts, University of Cape Town, South Africa, while RNA from positive clinical specimens for hCoV-HKU1 and hCoV-229E were obtained from Dr Astrid Vabret, University Hospital of Caen, France. For the validation of the multiplex assays, 91 specimens that previously tested positive for each virus were also collected. In addition, respiratory specimens (n=270) that tested negative for conventional respiratory viruses by direct immunofluorescence with the Light Diagnostics™ Respiratory Panel 1 Viral Screening and Identification Kit for RSV A & B, PIV 1,2, and 3, Influenza A and B, and Adenovirus (Millipore, USA) or the rapid BD Directigen™ RSV test (BD, Franklin Lakes, NJ, USA) were collected over 1 year by the Department of Medical Virology, University of Pretoria/National Health Laboratory Services Tshwane Academic Division. Specimens included (nasopharyngeal aspirates (NPA), bronchoalveolar lavage (BAL) specimens, endotracheal aspirates, and sputum specimens.

2.2.2 Processing of specimens

Upon receipt phosphate buffered saline (PBS) was added to NPAs to a final volume of 1ml before vortexing the specimen and clarifying it of cells and mucus at 3000 × g for 5min. Supernatants were removed and stored at -70℃ for PCR analysis.

2.2.3 RNA extraction

Nucleic acids from cultured virus controls were extracted from 200µl cell culture fluid using the High Pure PCR Template kit (Roche, Mannheim, Germany), according to manufacturer's instructions. Total nucleic acids from clinical specimens were extracted from 200µl clarified NPA with the automated MagNA Pure nucleic acid isolation system (Roche, Mannheim, Germany), using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche,

Mannheim, Germany), according to the manufacturer's instructions. Negative water controls were included in every run.

2.2.4 Primer and probe design

For each virus, multiple sequence alignments of all known strains identified by BLAST search analysis were performed in ClustalX (version 1.81) (Thompson *et al.*, 1997), and conserved regions identified for primer and probe design. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) to have melting temperatures of ~60ºC. FRET probes were designed with the support of TIBMOL Biological (Berlin, Germany) and were evaluated using the LightCycler Probe Design Software 2.0 (version 1.0) on site. The layout of the multiplex assays including primer and probe sequences are indicated in table 2.1.

2.2.5 Real-time RT-PCR

cDNA was synthesized using Expand Reverse Transcriptase (Roche, Mannheim, Germany). Real-time PCR reactions were performed using the LightCycler FastStart DNA Master^{PLUS} HybProbe kit (Roche, Mannheim, Germany) and contained 4µl of 5× Master Mix, 100nM of each probe, variable concentrations of primers, and 5µl of cDNA reaction mixture as template in a final volume of 20µl. Real-time PCR cycling was performed on a Light Cycler 2.0 (Roche, Mannheim, Germany) as follows: 95℃ for 10 min followed by 45 cycles of 95℃ for 10 s, 58℃ for 8 s, 54℃ for 8 s, 50℃ for 2 s (ramp rate 5℃/s), and 72℃ for 11 s, followed by melting curve analysis at 95℃ for 10 s, 40℃ for 30 s and 80℃ for 0 s (ramp rate 0.1℃/s).

2.2.6 DNA sequencing

PCR amplicons for DNA sequencing were gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), according to the manufacturer's instructions. DNA sequencing was performed with specific primers using the ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (version 3.1) on an ABI PRISM 3130 DNA sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Table 2.1 Primers and probes for real-time RT-PCR multiplex assays

Multiplex	Target	Primer	Sequence (5'-3') [concentration in nM]	Gene	Authors (Ref.)
1	Respiratory Syncytial Virus	Sense Primer	ATG GCT CTT AGC AAA GTC AA [800nM]	Nucleoprotein	This study
		Antisense Primer 1	TTC TGC ACA TCA TAA TTA GGA G [400nM]		(Gunson *et al.*, 2005)
		Antisense Primer 2	TTT TGC ACA TCA TAA TTG GGA GT [400nM]		This study
		Donor Probe	**AAT GAT ACA CTG TCA TCC AGC AAA TAC AC-FL**[a]		This study
		Acceptor Probe 1	[b]**LCRed610-TTC AAC GTA GTA CAG GAG ATA ATA TTG-PH**[c]		This study
		Acceptor Probe 2	**LCRed610-TCC AAC GGA GCA CAG GA-PH**		This study
	Human Metapneumovirus	Sense Primer	CAA CTG TTT ACT ACC CAA ATG A [300nM]	Fusion Protein	This study
		Antisense Primer	ATA GGG TGT CTT CCT GTG C [300nM]		This study
		Donor Probe	**TGT TGC ATT CCY TTG AYT GCT CAG CAA C-FL**		This study
		Acceptor Probe	**LCRed640-TTG ATM CCW GCT GCT GTG TCG CA-PH**		This study
	Human Bocavirus	Sense Primer	GGG TGT GTT AAT CAT TTG AAC [400nM]	NS1 Protein	This study
		Antisense Primer	CAC TGT CTC TAT GCT TGA CG [400nM]		This study
		Donor Probe	**CTT TGC AGG TTC CAC CCA ATC C-FL**		This study
		Acceptor Probe	**LCRed670-TGC ATT AAG CAC TCC TCC CAC CAA-PH**		This study
2	Parainfluenza Virus Type 1	Sense Primer	TAT CAA TTG GTG ATG CAA TAT AT [300nM]	Hemagglutinin-Neuraminidase	This study
		Antisense Primer	TCC TGT TGT CGT TGA TGT C [300nM]		This study
		Donor Probe	**TAA CCT AAT TGT AAA ACC TGC CCT ATA TCT-FL**		This study
		Acceptor Probe	**LCRed 610-CAC ATC CTT GAG TGA TTA AGT TTG ATG AAT-PH**		This study
	Parainfluenza Virus Type 2	Sense Primer	CCA TTT ACC TAA GTG ATG GAA T [300nM]	Hemagglutinin-Neuraminidase	This study
		Antisense Primer	ATG AGA CCA CCA TAT ACA GGA [300nM]		This study
		Donor Probe	**GAT CTA GCT GAA CTG AGA CTT GCT TTC TAT T-FL**		This study
		Acceptor Probe	**LCRed 610-TGA TAC CTT TAT TGA AAG AGT CAT ATC TCT TCC-PH**		This study
	Parainfluenza Virus Type 3	Sense Primer	TGT ATT TAT CAA AGG GAC CAC [300nM]	Nucleoprotein	This study
		Antisense Primer	TCC AGA TAT GAT CTT CCC GTC [300nM]		This study
		Donor Probe	**CAT ACA TGG TGA GTT CGC ACC AG-FL**		This study
		Acceptor Probe	**LCRed640-CAA CTA TCC TGC CAT ATG GAG YTA TGC-PH**		This study
	Human Coronavirus NL63	Sense Primer	ATA AAC TTG ATA CTG GTG CAC AA [400nM]	ORF1a	This study
		Antisense Primer	TCT AAT GTT ATA CTT AAA ACT ACG TGT C [400nM]		This study
		Donor Probe	**CTA TTA TGA AGC ATG ATA TTA AAG TTA TTG CC-FL**		This study
		Acceptor Probe	**LCRed670-AGG CTC CTA AAC GTA CAG GTG TTA TTT-PH**		This study
3	Influenza A	Sense Primer	GAC CRA TCC TGT CAC CTC TGA C [1000nM]	M2 Protein	WHO Recommended
		Antisense Primer	AGG GCA TTY TGG ACA AAK CGT CTA [1000nM]		WHO Recommended
		Donor Probe	**GGG ATT TTR GGR TTT GTG TTC ACG CTC ACC-FL**		This study
		Acceptor Probe	**LCRed 610- CCC AGT GAG CGA GGA CTG CAG C–PH**		This study
	Influenza B	Sense Primer	AGA GTT GGA CTT GAY CC [300nM]	Nucleoprotein	(Smith *et al.*, 2003)
		Antisense Primer	CAT AGG CAG TCT TGG CT [300nM]		(Smith *et al.*, 2003)
		Donor Probe	**GGA AGC ACA MTC CCC AGA AGA TCA GGT-FL**		(Smith *et al.*, 2003)
		Acceptor Probe	**LCRed640-AAC TGG TGT TGC GAT CAA AGG AGG TGG-PH**		(Smith *et al.*, 2003)
	Adenovirus	Sense Primer	GCC CCA GTG GKC TTA CAT GCA CAT C [300nM]	Hexon Protein	(Heim *et al.*, 2003)
		Antisense Primer	GCC ACG GTG GGG TTT CTA AAC TT [300nM]		(Heim *et al.*, 2003)
		Donor Probe 1	**AAC TGC ACC AGA CCC GGA C-FL**		This study
		Acceptor Probe 1	**LCRed670-AGG TAC TCC GAA GCA TCC TGT CC-PH**		This study
		Donor Probe 2	**AAC TGC ACC AGC CCG GG-FL**		This study
		Acceptor Probe 2	**LCRed670-AGG TAC TCC GAG GCG TCC TGG-PH**		This study
4	Human Coronavirus OC43	Sense Primer	CAT GCA ATG GCA ATA AGA T [400nM]	ORF1b	This study
		Antisense Primer	TTG AAA GGC ACT TAT ATT AGC A [400nM]		This study
		Donor Probe	**CAA AGG TTG AAT CAA CCT TAT CAC TTC T-FL**		This study
		Acceptor Probe	**LCRed610-CAC ATG TGA GTA TAA GCG CTT CTG AAG-PH**		This study
	Human Coronavirus HKU1	Sense Primer	TTG TTG TTC ACA TGG TGA TAG [500nM]	ORF1b	This study
		Antisense Primer	TTG CGT ATA CTT AAA TCT TCA ATC [500nM]		This study
		Donor Probe	**CTG CTA GTA CCA CCA GGC TTA ACA T-FL**		This study
		Acceptor Probe	**LCRed640-AGC AAC CGC CAC ACA TAA CTA TTT CA-PH**		This study
	Human Coronavirus 229E	Sense Primer	CTC AAT CTC GGA ATC CTT C [300nM]	Nucleoprotein	This study
		Antisense Primer	CTC TGA GAA CGA GCA AGA CT [300nM]		This study
		Donor Probe	**GGG TAC TCC TAA GCC TTC TCG TAA TCA-FL**		This study
		Acceptor Probe	**LCRed670-TCC TGC TTC TTC TCA AAC TTC TGC C-PH**		This study

[a] The acceptor fluorophore. The number following LightCycler (LC) Red is the wavelength at which fluorescence is emitted and amplification is detected
[b] The donor fluorophore, fluorescein (FL)
[c] Phosphate (PH) on 3' end to prevent extension of probe by DNA polymerase

2.2.7 Quantification of viral loads

RNA standards for quantification. Target regions for each respective virus and the GAPDH internal control were amplified with the Expand High Fidelity PCR kit (Roche, Mannheim, Germany) using specific primers. Amplicons were gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and cloned into the pJET1.2/blunt cloning vector (Fermentas, Ontario, Canada) according to the manufacturer's instructions.

In vitro transcription. *In vitro* transcription was performed with the TranscriptAid™ T7 High Yield Transcription Kit (Fermentas, Ontario, Canada), according to the manufacturer's instructions. Plasmid DNA was removed by addition of 2U of DNase I at the end of the reaction and incubated at 22ºC for 8 hours. RNA was purified with the RNeasy Plus Mini Kit (Qiagen, Hamburg, Germany) and the concentration determined with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260nm.

Viral loads were quantified against a standard curve produced by amplifying serial dilutions of RNA transcripts for each respective virus as well as for the internal control. Viral loads are presented as DNA/RNA copies detected in 1ml of processed NPA specimens.

2.2.8 Statistical analysis

The confidence level of data analysed was determined with the Student's t-test with two-tailed distribution.

2.2.9 Analytical sensitivity of multiplex assays

The detection limits of the multiplex assays were determined by testing 10-fold serial dilutions of the quantified RNA transcripts. Detection of each dilution was performed in triplicate. The detection limit was defined as the lowest concentration where all three replicates could be detected. For DNA viruses dilutions series of plasmid DNA was used.

2.3 Results

2.3.1 Multiplex development

Optimization of the multiplex assays was done on culture controls or on specimens that previously tested positive by PCR in independent laboratories. The newly designed real-time RT-PCR assays were initially optimized as monospecific assays. To assess the specificity of the primers and probes, the multiplex assays were tested for cross-reactivity among the 13 viruses using control specimens with input copy numbers of approximately 1×10^4 to 1×10^6 copies per real-time RT-PCR reaction. In all cases each virus was only detected by its specific primer and probe set, no cross-reaction was observed. The monospecific assays were subsequently combined to form four multiplex assays and optimized further to achieve similar amplification efficiencies for all combined PCR assays. The four multiplex assays comprise of the following viruses: ***Multiplex 1*** – RSV A and B, hMPV, hBoV; ***Multiplex 2*** –PIV 1, 2, and 3, hCoV-NL63; ***Multiplex 3*** – Influenza A, Influenza B, Adenovirus; ***Multiplex 4*** – Coronavirus OC43, HKU1, and 229E. Viruses were combined into the various multiplex assays according to shared similarities in clinical manifestations and susceptible age groups. The sensitivity of the assays was determined in triplicate through the amplification of serially diluted *in vitro* transcribed RNA from cloned controls. Detection limits for RNA viruses ranged from 2-25 *in vitro* transcribed RNA copies/µl and 25 plasmid DNA copies/µl for DNA viruses (Table 2.2). To assess the inter-run precision of the respective assays, extraction and real-time RT-PCR detection was performed on triplicate aliquots from viral suspensions. Three independent real-time RT-PCR runs performed showed a coefficient of variation of between 0.03% - 3.05% (Table 2.2), indicating high reproducibility of the real-time RT-PCR assays. All four multiplexes were optimized to be performed under the same cycling conditions for simultaneous use. In the event that a 60 nucleotide conserved region could not be identified, a technique described by Pont-Kingdon *et al.* (17) was used to overcome this. This technique was applied to probes used for the detection of RSV A and B, and PIV-2.

To determine if amplification competition may lead to interference during simultaneous quantification of more than one virus in a single reaction, quantified controls were added together at varying concentrations and quantified concurrently. The results indicated that

simultaneous quantification of two viruses with varying concentrations and with input DNA/RNA of more than 2.5×10^2 copies/µl (i.e. detected before amplification cycle 28), showed a coefficient of variation of <3.5%, indicating that the multiplex assays can accurately quantify two viruses in a single reaction. However, when a second virus is detected after amplification cycle 28 (i.e. input DNA/RNA less than 2.5×10^2 copies/µl) or a third virus was present, all viruses could be detected but not accurately quantified. In these events the viruses were quantified separately to ensure accurate quantitation of viral load. However, for diagnostic purposes simultaneous qualitative detection of more than one virus can be achieved to a detection limit of approximately 2.5×10^2 copies/µl.

Table 2.2 Validation of multiplex real-time RT-PCR assays on 91 known positive clinical specimens

Virus and Method of Control Detection	Number of Known Positive Clinical Samples	Samples Detected with Multiplex Assays	Detection Limit	Inter-run coefficient of variation (%)
Direct Immunofluorescence Assay (IFA)				
RSV	30	30	2.5 RNA copies/µl	0.42
PIV 1	8	8	2.5 RNA copies/µl	0.31
PIV 2	1[a]	1	25 RNA copies/µl	1.46
PIV 3	8	8	25 RNA copies/µl	0.91
Influenza A	8	8	25 RNA copies/µl	0.29
Influenza B	4	4	25 RNA copies/µl	0.44
Adv	5	5	25 DNA copies/µl	0.38
PCR				
hMPV	7	7	2.5 RNA copies/µl	3.05
hCoV-NL63	5	5	2.5 RNA copies/µl	0.21
hCoV-HKU1	1	1	2.5 RNA copies/µl	0.03
hCoV-OC43	4	4	25 RNA copies/µl	2.93
hCoV-229E	1	1	2.5 RNA copies/µl	Not Done[b]
Nested PCR				
hBoV	9	5[c]	25 DNA copies/µl	1.14

[a] Only one clinical specimen was available for validation of this virus. However, in addition, three PIV 2 positive specimens that previously tested negative by routine immunofluorescence were detected in the negative specimen screen.
[b] Due to limited sample volume the inter-assay coefficient of variation for hCoV-229E could not be determined
[c] In 4 specimens, hBoV could only be detected by semi-nested PCR

2.3.2 Evaluation of multiplex assays

Clinical evaluation. The multiplex assays were validated on a total of 91 clinical specimens that previously tested positive for the presence of respiratory viruses by IFA,

PCR or semi-nested PCR. With the exception of bocavirus, that was only detected in 5/9 specimens, viruses were detected in all the known positive specimens evaluated (Table 2.2). Bocavirus was previously identified in the positive control specimens by semi-nested PCR. In 4/9 specimens bocavirus could not be detected by a single round PCR. The detection limit of bocavirus by the real-time PCR is 25 copies/µl, suggesting high sensitivity although this is not as sensitive as the conventional semi-nested PCR by which it was initially detected (Smuts and Hardie, 2006).

Negative specimen screening. Real-time RT-PCR screening of 270 respiratory specimens negative for conventional viruses by routine diagnostic tests identified new viruses in 40/270 (14.8%) cases and 79/270 (29.3%) conventional viruses missed by routine diagnostic assays. Overall, respiratory viral detection was improved by 44%. Human bocavirus (n=14), human metapneumovirus (n=15), and human coronavirus-NL63 (n=5) were detected in the IFA negative group. Although HCoV-HKU1 and hCoV-229E were not detected in the IFA negative specimen group, both were detected in the IFA positive control specimens each as a co-infection with RSV.

Table 2.3 Additional respiratory viruses detected in 270 respiratory specimens that tested negative by routine diagnostic assays

Viruses routinely screened for by IFA panel		
Virus	Number of detections	Number of Co-infections *(%)*
RSV	**35**	**2** *(5.7%)*
PIV 1	**2**	**1** *(50%)*
PIV 2	**3**	**1** *(33%)*
PIV 3	**14**	**2** *(14.3%)*
Influenza A	**4**	**2** *(50%)*
Influenza B	**3**	**0**
Adv	**18**	**7** *(38.9%)*
Total	**79** *(29.3%)*	
Viruses not in routine diagnostic assays		
Virus	Number of detections	Number of Co-infections *(%)*
hMPV	**14**	**5** *(35.7%)*
hBoV	**15**	**8** *(53.3%)*
hCoV-NL63	**5**	**4** *(80%)*
hCoV-HKU1	**0**	**0**
hCoV-229E	**0**	**0**
hCoV-OC43	**6**	**5** *(83.3%)*
Total	**40** *(14.8%)*	

In addition, 79 (29.3%) of the IFA negative specimens tested positive for viruses already included in the IFA panel, suggesting a marked improved sensitivity over the IFA. Amplicons from specimens with positive results were sequenced to ensure that the higher positivity rate observed with real-time RT-PCR detection was not due to low specificity of the assays. Non-specific detection was not observed. Co-infections were detected in 6.7% of specimens (Table 3). Viruses could be detected in all the different types of respiratory specimens (NPA, BAL, endotracheal aspirates, and sputums).

2.3.3 Identification of nucleotide base changes

Melting curve analysis of RSV positive clinical specimens revealed four distinct melting curves with melting temperatures (Tm) of 59ºC, 54ºC, 51ºC, and 47ºC (Figure 2.1) identified in 100/131 (76.3%), 20/131 (15.3%), 4/131 (3.1%), and 7/131 (5.3%) of RSV infected patients, respectively. Sequencing of the RSV target regions identified the presence of single base mutations in the region where the probe hybridized to the template (Figure 2.1) despite complete conservation in specimen sequences on Genbank in this area. This confirms the robustness of the FRET hybridization probe technology to tolerate mismatches and overcoming variability of RNA viruses.

2.3.4 Validation of the multiplex assay for quantification of viral load

To evaluate the quantitative utility of the assays, the viral load of 58 RSV infected infants (age 12 days to 8 months) as well as any co-infected viruses detected by the multiplex PCR was determined. The patients were divided into patients with mild disease (infants that visited outpatients but did not require hospitalisation) (n=32) and severe disease (ICU patients) (n=26). Demographics of the patients included in the study are listed in Table 2.4a. HIV was the only immunocompromising condition present in the patients studied. The majority of patients were immunocompetent (HIV negative) and the difference in age between the two groups was not statistically significant. To compensate for inter-specimen variation, all NPAs were suspended to a final volume of 1ml upon receipt and clarified from all cells and mucus. The minimum volume of unprocessed specimens received were ~200µl, consequently the addition of PBS only resulted in a dilution factor of

1:5 or lower. Therefore, the virus concentration measured in the processed specimen differed less than 1 log factor from the original specimen.

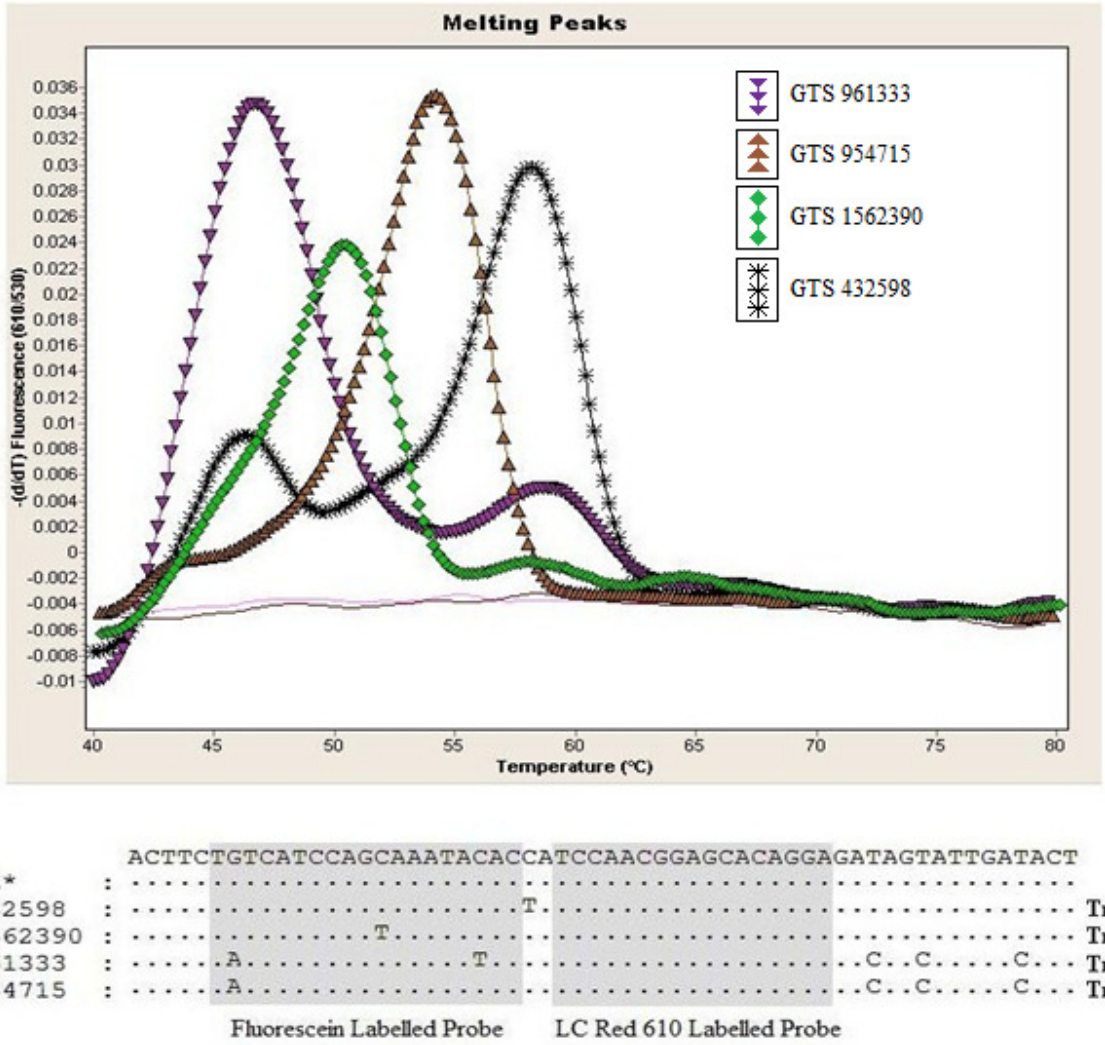

Figure 2.1 Melting curve analysis illustrating the detection of four different RSV A strains identified with the RSV specific fluorescence resonance energy transfer (FRET) hybridization probes. Comparison of the nucleotide sequences of the RSV A strains relative to a reference strain (RSV A*, GenBank accession number X00001) indicating the single nucleotide mismatches corresponding to the four different melting curve profiles. Dots indicate conserved bases, whereas sequence variations are indicated by the nucleotide base.

Two quantification approaches were compared (i) Relative quantification (RSV viral load relative to a stably expressed housekeeping gene (GAPDH) as an internal control and

normalising factor to adjust for the amount of host cells in the original sample) and (ii) Absolute quantification (RSV viral load alone). For diagnostic purposes, the GAPDH real-time RT-PCR assay was optimized as an internal control to confirm effective nucleic acid extraction from specimens. Even though the supernatant fraction of the NPA specimens were used, GAPDH was continuously detected in all supernatant fractions at concentrations ranging between 1.59×10^5 to 1.41×10^9 copies/ml of processed specimens (mean: 2.69×10^8 copies/ml, median: 1.03×10^8 copies/ml), confirming its value as an extraction control. Relative quantification against GAPDH showed no statistical significant differences between the mild and severe disease groups (p=0.65). However, with absolute quantification, a significant difference could be observed between the two groups (Table 2.4b). In the mild disease group, the mean RSV viral load was 3.44×10^9 copies/ml with a median viral load of 7.4×10^8. The RSV viral load in the severe disease group was significantly higher (p = 0.014) with a mean RSV viral load of 3.35×10^{10} copies/ml, and a median viral load of 6.04×10^9 copies/ml. In addition, when compared to the mild disease group, the severe disease group had a higher frequency of viral loads more than 1×10^{10} copies/ml and a lower frequency of viral loads less than 1×10^5 copies/ml.

Co-infections occurred in 11/58 (19%) RSV infected infants. Co-infections with influenza A (4 patients) and hMPV (5 patients) occurred most followed by PIV 3 (2 patients). Co-infections with hBoV, Adv, and hCoV-NL63 were only detected once in each case. Viral loads of the co-infected patients are indicated in table 2.4b. Analysis of specimens revealed that in co-infected patients more severe disease was associated with either a high RSV viral load or a high viral load of a co-infecting virus with known pathogenic potential, e.g. influenza A and PIV 3. However, the numbers of analyzed specimens were too low to determine any statistical significance. The one patient with lower viral loads was co-infected with three viruses (RSV, Adv, and hCoV-NL63) and was 12 days old which may have contributed to the observed disease severity.

Table 2.4 Comparison of RSV infected patients with mild and severe disease. a) Demographics of RSV single infected patients and RSV infected patients with co-infections b) Comparison of viral loads in RSV infected patients with mild and severe disease in the absence or presence of co-infections.

a)

Patient with single RSV infections		
	Mild Disease (n=26)	**Severe Disease (n=21)**
Age		
Mean	2m27d	2m22d
Median	2m25d	1m27d
Sex		
Males	18 Males (69.2%)	14 Males (66.7%)
HIV Status		
HIV PCR Positive	23.08%	4.76%
HIV Serology Positive, PCR Negative	7.69%	9.52%
HIV Serology Negative	19.23%	42.86%
Unknown	50.00%	42.86%
RSV infected patients with co-infections		
	Mild Disease (n=6)	**Severe Disease (n=5)**
Age		
Mean	2m20d	1m7d
Median	2m23d	1m8d
Sex		
Males	4 Males (66.7%)	4 Males (80.0%)
HIV Status		
HIV PCR Positive	0.00%	0.00%
HIV Serology Positive, PCR Negative	0.00%	0.00%
HIV Serology Negative	16.70%	20.00%
Unknown	83.40%	80.00%

b)

Patients with Mild Disease			
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load RSV (copies/ml)**
Single infections			
RSV	**26**	-	mean: 3.44×10^9 (median: 7.40×10^8)
Co-infections with:			
Influenza A	**2**	2.5×10^2 and 1.01×10^4	1.79×10^3 and 2.32×10^9
hMPV	**3**	4.85×10^4 to 1.33×10^6	4.85×10^6 to 2.13×10^9
hBoV + hMPV + Influenza A	**1**	$7.25 \times 10^{4\ (hBoV)}$, $8.25 \times 10^{4\ (hMPV)}$, $6.03 \times 10^{4\ (Influenza\ A)}$	9.83×10^9
Patients with Moderate/Severe Disease			
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load RSV (copies/ml)**
Single infections			
RSV	**21**	-	mean: 3.35×10^{10} (median: 6.04×10^9)
Co-infections with:			
Influenza A	**1**	1.7×10^9	3.00×10^6
hMPV	**1**	1.03×10^5	1.30×10^{11}
PIV 3	**2**	4.73×10^8 and 3.48×10^9	7.33×10^9 and 1.16×10^{11}
Adv + hCoV-NL63	**1**	$3.75 \times 10^{3\ (Adv)}$ and $3.75 \times 10^{3\ (hCoV\text{-}NL63)}$	3.43×10^6

2.4 Discussion

In this study we described the development of four quantitative multiplex real-time RT-PCR assays that can be run concurrently for the detection of 13 well recognized and newly identified viruses. For the detection of conventional respiratory viruses the multiplex assays was shown to be as specific as the routinely used commercial immunofluorescence assay but also to be significantly more sensitive, increasing the detection of conventional viruses by 29.3%. Inclusion of the additional respiratory viruses in the detection assay increased the overall detection frequency by a further 9.7%. The assay demonstrated the ability to detect a high frequency of co-infections with a detection limit ranging between 2.5 copies/µl to 25 copies/µl (corresponding to 2.5×10^3 to 2.5×10^4 copies/ml). These detection limits should be fitting, as previously reported viral loads in respiratory specimens ranged between 1×10^3 to 1×10^{11} copies/ml (Kuypers *et al.*, 2006). Viral loads less than 1×10^3 copies/ml have been described, though the clinical relevance at such low levels have been questioned (Allander *et al.*, 2007b; van der Hoek *et al.*, 2005).

Real-time PCR assays targeting the polyomaviruses WU and KI as well as rhinoviruses were not included in the development of the multiplex assays for various reasons. WUV and KIV were identified after the development of the multiplex assays, though more importantly, their clinical relevance is still in question (Bialasiewicz *et al.*, 2008; Norja *et al.*, 2007). Even though a strong association is observed between these viruses and the respiratory tract (Bialasiewicz *et al.*, 2008), additional studies need to confirm their causative role in respiratory disease. Conversely, the rhinoviruses were excluded since infection with these viruses is typically associated with the common cold and seldom with more severe disease (Turner and Couch, 2007). Evidence towards an increasing association with more severe disease has only recently come to light with the identification of a new and distinct rhinovirus group (rhinovirus C) (Arden *et al.*, 2006; Kistler *et al.*, 2007; Lau *et al.*, 2007b; McErlean *et al.*, 2007; Renwick *et al.*, 2007; Wisdom *et al.*, 2009; Xiang *et al.*, 2008). However, evidence for a high co-infection rate still makes the causative role questionable. In light of these recent findings, future studies will include the revision of the multiplex assays as to allow detection of rhinoviruses.

In comparison to conventional detection methods, real-time probe based PCR assays have been shown to detect respiratory viruses with increased sensitivity and specificity (Gharabaghi *et al.*, 2008; Kuypers *et al.*, 2006; Smith *et al.*, 2003; van de Pol *et al.*, 2007). A pitfall of real-time PCR is that the more commonly used probe chemistries (hydrolysis probes and molecular beacons) are highly sensitive to mismatches, which may lead to false negative results if point mutations occur (Gunson *et al.*, 2006). In contrast, the assays described in this study utilize two FRET hybridization probes which have the advantage of being able to tolerate a greater number of mismatches than hydrolysis probes or molecular beacons, decreasing the occurrence of false negative results substantially. The benefit of FRET probes was illustrated here; where point mutations was detected in the sites where the probe hybridized to the target template in several clinical specimens despite complete conservation of this area on Genbank. An obstacle when designing FRET hybridization probes is that a conserved area of approximately 60nt is needed for the two probes to bind. In highly variable virus genomes this might pose a problem. However, this can be overcome by using an oligonucleotide probe complementary to conserved regions flanking a variable region as described by Pont-Kingdon *et al.* (Pont-Kingdon and Lyon, 2005). Binding of the probe brings the two conserved regions together, resulting in the variable region to loop out, thereby omitting variable sequences. If a 60 nucleotide conserved region could not be identified, this technique was successfully applied to overcome this complication.

In addition to being highly sensitive and specific, the assay has a turnaround time of <3 hours and covers 13 viruses. The test is currently optimized for the Light Cycler 2 but could be transferred to the Light Cycler 480 for 96 well format. For paediatric patients, rapid diagnosis of respiratory tract infections can lead to improved management and reduce unnecessary antibiotic use and length of hospitalisation (Barenfanger *et al.*, 2000; Woo *et al.*, 1997). Rapid diagnosis is also imperative in managing nosocomial spread of respiratory viruses to high risk patients e.g. bone marrow transplant patients and cancer recipients (Hohenthal *et al.*, 2001; Miall *et al.*, 2002; Whimbey *et al.*, 1995; Whimbey *et al.*, 1997).

The quantitative application of the assay will contribute to addressing questions on viral pathogenesis as demonstrated in a series of single and co-infected RSV positive clinical specimens from patients with mild and severe disease. The quantitative utility of the assays

on clinical specimens was initially validated on RSV infected patients, where RSV and associated co-infecting viruses were quantified. Pathogenesis studies on viruses other than RSV will be described elsewhere. Since respiratory specimens are non-homogenous and may vary in consistency (degree of viscosity due to the presence of mucous), volume, and manner of collection, specimens to be used for quantification studies have to be standardized. To address this, all NPA's included in the present investigation were reconstituted to the same volume and clarified of cells and mucus upon receipt. Two methods of quantification were compared to determine the most appropriate protocol for respiratory specimens: i) quantification of RSV copy number relative to a stably expressed internal control (GAPDH); and ii) absolute quantification of standardized NPA's. Relative quantification of RSV to GAPDH yielded no statistical significant difference between mild and severe disease (p=0.653). These results correspond to the findings of Gueudin *et al.* (Gueudin *et al.*, 2003). A recent study (Semple *et al.*, 2007) has shown that a large percentage of RSV and hMPV virus particles are not cell associated and that there is no significant difference between viral loads in whole or cell-free fractions of the same specimens, thus indicating that the amount of RSV particles are neither related nor proportional to the amount of cells present in a sample. These findings suggest that application of an internal control (housekeeping gene) to normalize for differences in variation between specimens may therefore not be appropriate.

Absolute quantification of RSV viral loads in standardized specimens in the current study indicated a statistical significant (p=0.014) difference between mild and severe disease. These findings correspond to results from similar studies (DeVincenzo *et al.*, 2005; Fodha *et al.*, 2007; Martin *et al.*, 2008). Other variables that may influence outcome of disease (age, immune status, and risk factors) were constant between the two groups studied. In addition to the quantification of single RSV infections, the viral loads in patients with co-infections were also determined. The presence of a co-infection alone does not appear to result in more severe disease, since co-infection occurred at the same frequency in both mild and severe ALRI groups. Other studies observed similar findings in the case of hMPV co-infections (Garcia-Garcia *et al.*, 2006; Wilkesmann *et al.*, 2006). A higher RSV viral load in combination with the co-infection, or a high viral load of PIV 3 or influenza A was associated with more severe ALRI supporting the application of multiplex quantification of viral loads in virus pathogenesis studies.

In conclusion, the findings of our study demonstrate the application of the multiplex real-time PCR assays with FRET hybridization probes to detect respiratory viruses and the associated viral load. The assays may facilitate studies of virus pathogenesis and facilitate investigations of the importance of the recently identified respiratory viruses in ALRI, but also confirm the relevance of a positive PCR finding in acute disease.

Chapter 3

The Role of Well Recognized and Newly Identified Respiratory Viruses in Acute Respiratory Tract Infection

3.1 Introduction

Acute lower respiratory tract infection (ALRI) is a major cause of paediatric morbidity and mortality, annually accounting for approximately 2 million childhood deaths worldwide (Williams *et al.*, 2002). The majority of ALRI associated deaths occur in the developing world (Williams *et al.*, 2002). This may be attributable to the high prevalence of risk factors, such as human immunodeficiency virus (HIV) infection and malnutrition that impairs the immune system and thereby predispose individuals to more severe respiratory disease (Fraker *et al.*, 2000). HIV exposed infants (HIV-uninfected infants born from HIV-infected mothers) are also at risk for severe respiratory disease (McNally *et al.*, 2007). Even though antiretroviral therapy have significantly reduced mother-to-child HIV transmission, infants exposed to HIV are still immunocompromised due to reduced transplacental transfer of protective antibodies from HIV-infected mothers to their children (de Moraes-Pinto *et al.*, 1996), as well as having altered CD4 immunity during the first year of life (Clerici *et al.*, 2000; Nielsen *et al.*, 2001). In addition, the administering of antiretroviral therapy (in particular nevirapine) may further drive the HIV exposed infant's immune system into an anergic/immunodeficient state (Schramm *et al.*, 2006).

Viral infections and concomitant viral-bacterial infections play a major role in childhood ALRI (Juven *et al.*, 2000; Nascimento-Carvalho *et al.*, 2008). Traditionally respiratory syncytial virus (RSV), influenza viruses A and B, parainfluenza viruses (PIV) types 1, 2, and 3, and adenoviruses were viewed to be the leading causes of viral respiratory tract infections. However, since 2001 a number of novel viruses associated with respiratory disease were identified including human metapneumovirus (hMPV) (van den Hoogen *et al.*, 2001), human bocavirus (hBoV) (Allander *et al.*, 2005), and two human coronaviruses (hCoV) HKU1 (Woo *et al.*, 2005) and NL63 (van der Hoek *et al.*, 2004). Since their discovery, it has been shown that all of the newly identified viruses have a worldwide distribution and contribute to lower respiratory tract infections (LRTI). In addition to the newly described respiratory viruses, two coronaviruses, hCoV-OC43 and hCoV-229E, identified in the 1960's has been commonly associated with minor upper respiratory tract infections (URTI) but have also been associated with more severe clinical symptoms in infants, immunocompromised individuals and elderly persons (Vabret *et al.*, 2003; van Elden *et al.*, 2004). At present, the contribution of these viruses to the burden of respiratory

disease in South Africa is poorly described since none of these viruses are currently targeted by routine diagnostic assays.

A comprehensive investigation into the prevalence of well recognized and newly described respiratory viruses and their association with respiratory tract infections in South Africa has not been done previously. In this study we aim to evaluate the relative prevalence and epidemiological characteristics of respiratory viruses occurring in patients with acute respiratory tract infection seeking medical care at regional hospitals in Pretoria, Gauteng province, South Africa. In addition, we aimed to determine the role hBoV, hMPV and coronavirus infections in HIV infected and HIV exposed individuals.

3.2 Materials and Methods

3.2.1 Patients and specimens

From January 2006 to December 2006, 737 respiratory specimens obtained from patients of all ages presenting with acute respiratory tract infections were submitted to the Department of Medical Virology, University of Pretoria/National Health Laboratory Services Tshwane Academic Division for respiratory viral diagnosis. Respiratory specimens were received from three hospitals serving the Pretoria region i.e. Steve Biko Academic Hospital, Kalafong Secondary Hospital, and 1-Military Hospital. Ethical clearance to record demographic and clinical information of patients was obtained from the University of Pretoria Research Ethics Committee (Protocol number S15/2008). From the total specimens received during 2006, 244 specimens that previously tested negative and 75 that previously tested positive by routine diagnostic assays were retrospectively screened by multiplex real-time RT-PCR analysis. Specimens were selected randomly with equal distribution throughout the year.

3.2.2 Processing of specimens

Upon receipt phosphate buffered saline (PBS) was added to NPAs to a final volume of 1ml, vortexed to dislodge the cells from the mucus and clarified at 3000 × g for 5min.

Supernatants were removed and stored at -70℃ for PCR analysis.

3.2.3 Routine diagnostic assays

Direct immunofluorescence (IFA) was performed on the cell pellets of the processed specimens using the Light Diagnostics™ Respiratory Panel 1 Viral Screening and Identification Kit (Millipore, USA) that detects RSV A & B, PIV 1,2, and 3, Influenza A and B, and Adenovirus, and the Light Diagnostics™ Cytomegalovirus (CMV) Immunofluorescence Assay (Millipore, USA) for the detection of CMV. For the detection of RSV alone, a rapid antigen detection ELISA using the rapid BD Directigen™ RSV test (BD, Franklin Lakes, NJ, USA) was performed on request.

3.2.4 RNA extraction

Refer to Chapter 2, Materials and Methods section 2.3.2.

3.2.5 Real-time RT-PCR

Refer to Chapter 2, Materials and Methods section 2.2.5.

3.2.6 Calculation of respiratory virus frequencies

The confidence level of data analysed was determined with the Student's t-test with two-tailed distribution. The prevalence of each virus in the complete specimen group was extrapolated as follows: Conventional respiratory viruses = ((Respective virus percentage identified by RT-PCR in IFA negative sample group × total IFA negative samples) + number of virus present in IFA positive group) ÷ total number of specimens in the study group. New respiratory viruses = ((Respective virus percentage identified by RT-PCR in IFA negative sample group x total IFA negative samples) + (Respective virus percentage identified by RT-PCR in IFA positive sample group x the total positive sample)) ÷ (total number of specimens in group).

3.3 Results

3.3.1 Patient characteristics

Respiratory specimens were obtained from patients of all ages, with 709/737 (96%) of patients being under the age of 5 years. The mean age among patients was 17 months (median: 3 months) and 56% of the population group were male. The demographics of the patients are indicated in table 3.1. From the total specimens received during 2006 a sample group consisting of 244/468 (52%) specimens that previously tested negative and 75/269 (28%) specimens that previously tested positive with routine diagnostic methods were retrospectively collected for multiplex real-time RT-PCR analysis. The monthly proportion of selected specimens was at least 16% of the total specimens received by the virology diagnostic laboratory per month. The patients in the sample group did not differ significantly from the patients in the population group with respect to mean age, sex and HIV status (Table 3.1.).

Table 3.1. Demographic characteristics of patients

Demographics characteristic	All Patients	Patients in Sample Group
Age		
Mean	1 year 5 months	1 year 8 months
Median	3 months	3 months
Range	0 days to 87 years	0 years to 77 years
Percentage < 5 years	96%	97%
Sex		
Males	56%	55%
HIV Status		
HIV negative	94 (12.8%)	45 (14.1%)
HIV exposed, not infected	105 (14.2%)	60 (18.1%)
HIV infected	124 (16.8%)	70 (21.9%)
Unknown	414 (56.2%)	144 (45.1%)
Total	**737**	**319**

3.3.2 Virus detection

For the duration of January to December 2006, the virology diagnostic laboratory received a total of 737 respiratory specimens. Overall, routine diagnostic assays could identify 269 viral aetiological agents in 256/737 (34.7%) respiratory specimens leaving

481/737 (65.3%) cases undiagnosed. A sample group of 244/481 (43.3%) undiagnosed respiratory specimens were randomly selected for virus detection by real-time RT-PCR, which detected one or more viruses in a further 88/244 (36%) specimens. In addition, real-time RT-PCR detected co-infections at a higher frequency than routine diagnostic assays, 6.6% and 1.8% respectively.

To study the presence of viruses in hospitalised patients compared to non-hospitalised patients, the patients in the study group were subdivided into 3 categories according to disease severity: mild – patients seeking medical care at the outpatients department (not hospitalised); moderate – patients hospitalised in general wards; severe – patients requiring intensive care. In patients less than 5 years of age, routine diagnostic assays detected viruses at equal frequencies in patients with mild and moderate disease (37% in each case), with a slightly lower frequency in patients with severe disease (30%), with similar frequencies of co-infections detected between the three groups (~2%). Real-time RT-PCR detected viruses at higher frequencies in comparison to routine diagnostic assays. In patients less than 5 years of age, real-time RT-PCR detected viruses most frequently in outpatients (mild disease, 59%), followed by patients with moderate disease (44%) and patients with severe disease (32%). The number of co-infections detected was markedly higher in the group screened by real-time RT-PCR assays. Co-infections were detected in 4% to 11% of patients, as opposed to 2% detected by routine diagnostic assays. The highest rate of co-infections were observed in the mild disease group with 11%, followed by the moderate disease group with 7% and the severe disease group with 4%. Routine diagnostic assays were able to detect up to two viruses in a single specimen, whereas more than two viruses were only detected by real-time RT-PCR assays in 5 (1.6%) specimens.

Only 28/737 (3.8%) specimens were obtained from patients older than 5 years, of which 11/28 (39%) specimens were selected for real-time RT-PCR analysis. Routine diagnostic assays identified viral aetiological agents in 7/28 (25%) patients with the majority detected in hospitalised patients. Real-time RT-PCR identified respiratory viruses in an additional 4/11 (36%) patients of which only one patient previously tested positive for the presence of respiratory viruses. In contrast to patients under the age of 5 years, co-infections were only detected in hospitalised patients.

3.3.3 Prevalence of respiratory viruses

Extrapolated frequencies of well recognized respiratory viruses were calculated from the addition of viruses detected by routine diagnostic assays and extrapolated numbers from those detected by real-time RT-PCR. Extrapolated frequencies are indicated in table 3.2.

Overall, RSV was detected most, occurring at a frequency of 34.6%, followed by PIV 3 (8.8%), Adv (5.8%), and influenza A (4.6%). PIV 1 and 2, influenza B and CMV were detected at frequencies below 1% (Table 3.3.). A total of 319/737 (43.3%) specimens were screened for the presence of viruses not included in routine diagnostic assays. Of these, hMPV and hBoV occurred most frequently (4.1% each), followed by hCoV-NL63 (1.6%) and hCoV-OC43 (0.6%). HCoV-229E and hCoV-HKU1 were only detected once at a frequency of 0.3% each. During 2006 the polyomaviruses were also screened with conventional PCR (Venter *et al.*, 2009). Polyomavirus WU was detected in 6.5% and polyomavirus KI in 0.8% of cases. Ninety six percent of patients included in the study were under the age of 5 years. Consequently, the frequencies at which the viruses were detected are biased towards children under the age of 5 years, therefore does not reflect prevalence in patients of all ages. This is supported by very little to no change in virus frequencies when only studying patients under the age of 5 years (Table 3.2.).

3.3.4 Age distribution

The age distribution of patients infected with well recognized viruses is indicated in figure 3.1a. The age distribution of patients with ALRI associated with each virus differed. RSV, PIV-3, Influenza A and B, hMPV, PIV-1 and PIV-2 were predominantly identified in children <1 year old (92%, 84%, 75%, 69%, and 93% of infected patients, respectively). The Coronaviruses occurred mostly in children <12 months old. HCoV-NL63 was detected in patients between 12 days and 13 months old, and hCoV-OC43 in patients between 8 months and 6 years. HCoV-HKU1 and hCoV-229E was each detected once in patients 4 months and 2 months old, respectively. Adenovirus and hBoV infections occurred at increased prevalence in patients >6 months old (67% and 65% of infected patients, respectively). In adult patients, only hMPV (30 year old male), RSV (77 and 87 year old females with ALRI) and adenovirus (58 year old male) were detected.

Table 3.2 Viruses identified in respiratory specimens from patients with acute respiratory tract infections

	Routine diagnostic detection in 737 specimens	Real-time RT-PCR detection in 244 IF negative specimens	Extrapolated number of viruses in total IF negative specimens (n=481)	Extrapolated total of viruses in all specimens	Extrapolated Virus Frequency	Extrapolated Virus Frequency in Patients < 5 years of age (n=709)
RSV	**186** (182*)	**36** (35*)	**71** (67*)	**257** (249*)	34.9%	35.1%
PIV 1	**2** (2*)	**1** (0*)	**2** (0*)	**4** (2*)	0.5%	0.3%
PIV 2	**0** (0*)	**3** (3*)	**6** (6*)	**6** (6*)	0.8%	0.8%
PIV 3	**37** (36*)	**13** (12*)	**28** (23*)	**63** (59*)	8.8%	8.3%
Influenza A	**26** (24*)	**4** (3*)	**8** (6*)	**34** (30*)	4.6%	4.2%
Influenza B	**1** (1*)	**3** (3*)	**6** (6*)	**7** (7*)	0.9%	1.0%
Adv	**14** (13*)	**15** (14*)	**30** (27*)	**44** (40*)	5.9%	5.6%
CMV	**3** (3*)	not tested	N/A	**3** (3*)	0.4%	0.4%
Total	**269 (261*)**	**75 (70*)**	**148 (135*)**	**417 (396*)**	**56.8%**	**55.7%**

	Real-time RT-PCR detection in 244 IF negative specimens	Real-time RT-PCR detection in 75 IF positive specimens	Total viruses detected	Extrapolated total number of viruses	Extrapolated Virus Frequency	Extrapolated Frequency of Virus Detected in Patients < 5 years of age
hMPV	10	3	**13** (13*)	**30** (30*)	4.1%	4.2%
hBoV	12	1	**13** (13*)	**27** (27*)	3.7%	3.8%
hCoV-NL63	4	1	**5** (5*)	**11** (11*)	1.5%	1.6%
hCoV-OC43	3	0	**3** (2*)	**6** (4*)	0.8%	0.6%
hCoV-229E	0	1	**1** (1*)	**3** (3*)	0.4%	0.4%
hCoV-HKU1	0	1	**1** (1*)	**3** (3*)	0.4%	0.4%
WU	19	3	**21** (20*)	**48** (45*)	6.5%	6.3%
KI	3	0	**3** (3*)	**6** (6*)	0.8%	0.8%
Total	**51**	**10**	**60 (58*)**	**134 (129*)**	**18.2%**	**18.2%**

(n*) - The number of viruses detected in patients under the age of 5 years.

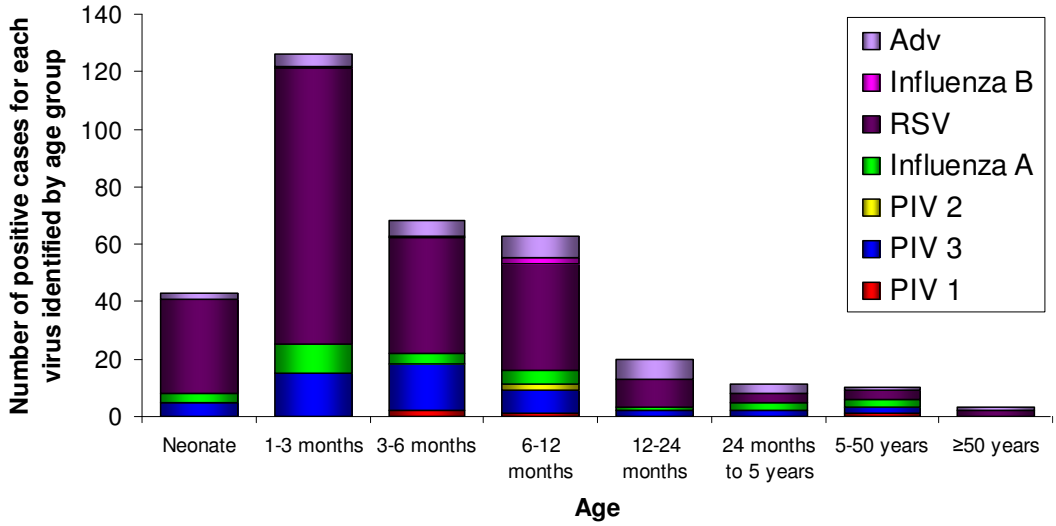

Figure 3.1a Age distribution associated with well recognized viruses (RSV, Influenza A&B, PIV 1-3, and Adv)

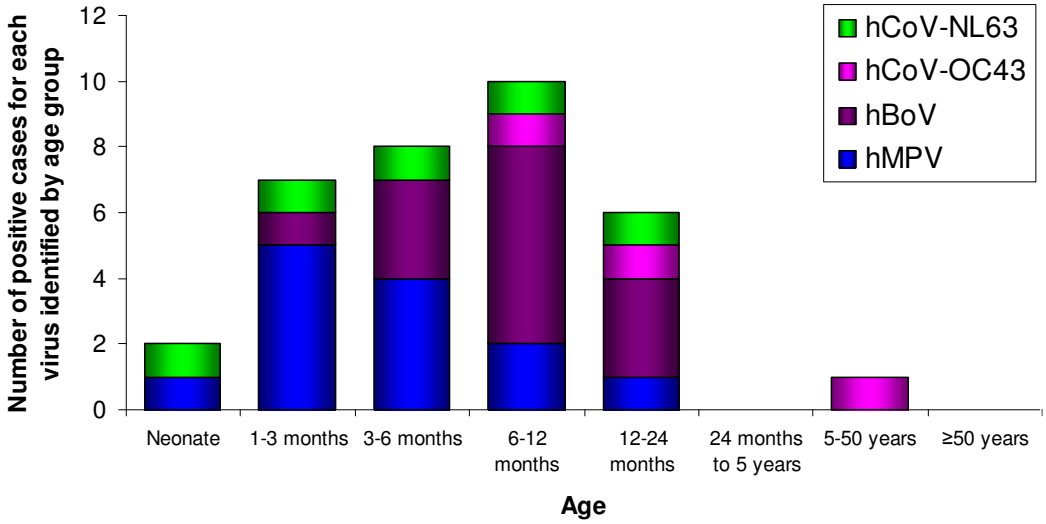

Figure 3.1b Age distribution associated with newly identified viruses (hMPV, hBoV, and coronaviruses)

3.3.5 Seasonal distribution

Two consecutive years (2006 and 2007) were studied to determine the seasonal occurrence of the respective respiratory viruses. Data from 2007 were kindly provided by Dr. Tina Kresfelder in our group. The monthly distributions of respiratory viruses detected during 2006 and 2007 are indicated in figure 3.2.

RSV occurred with increased prevalence during autumn and mid-winter (76% of infections), with a peak occurring in April 2006 and May 2007. The prevalence of PIV 3 increased during late winter and peaked in spring, with the highest number of infections detected in October. Influenza A peaked from April to September in 2006 but had 2 peaks in 2007 (April-May and August-September), however overall 87% of influenza A infections occurred from April to August (mid-autumn to late winter). Influenza B was detected in winter and early-spring. Adenovirus occurred throughout the year. HMPV and hBoV were sporadically detected throughout 2006 with a more defined seasonal occurrence in 2007.

HMPV predominantly occurred between mid-autumn and early spring (70% of infections), while hBoV occurred at a slightly higher frequency during mid-winter and mid-spring (58% of infections). HMPV and hBoV followed similar seasonal patterns during 2006 and 2007, often occurring during the same months which may suggest co-circulation of these viruses. Coronaviruses occurred throughout the year, with hCoV-OC43 predominantly detected during winter and spring, whereas hCoV-NL63 was primarily detected from mid-summer to mid-autumn (91% of infections). HCoV-229E was only detected on two occasions both in autumn. HCoV-HKU1 was only detected once in August.

3.3.6 Disease association

Role of Co-infections. Viral detection by IFA only identified co-infections in 5.3% of RSV cases, 12.2% of Influenza A, 16.7% of PIV 3 cases and 24.4% of Adenovirus cases (Table 3.3a). Co-infections were detected in 27% of RSV cases; 25% of PIV 3, 44.4% of Adenovirus and 55% of Influenza virus A cases when the multiplex PCR that included more viruses and have increase sensitivity was used (Table 3.3a). Overall, the new viruses had a much greater number of co-infections, 58.1% for hMPV; 61% for hBoV; 84% for hCoV-NL63; 75% for hCoV-OC43 (Table 3.3b). Too few of the other coronaviruses were detected for a meaningful conclusion regarding co-infections.

Disease severity in single infections. Of the patients with single RSV, PIV 3, Influenza A, and Adenovirus infections, 59%, 63%, 38%, and 60%, respectively, required hospitalisation or intensive care treatment (Table 3.3a). Patients with single hMPV, hBoV, hCoV-NL63, and hCoV-OC43 infections, 8/14 (57.1%), 9/13 (69.2%), 2/2 (100%), and 3/3

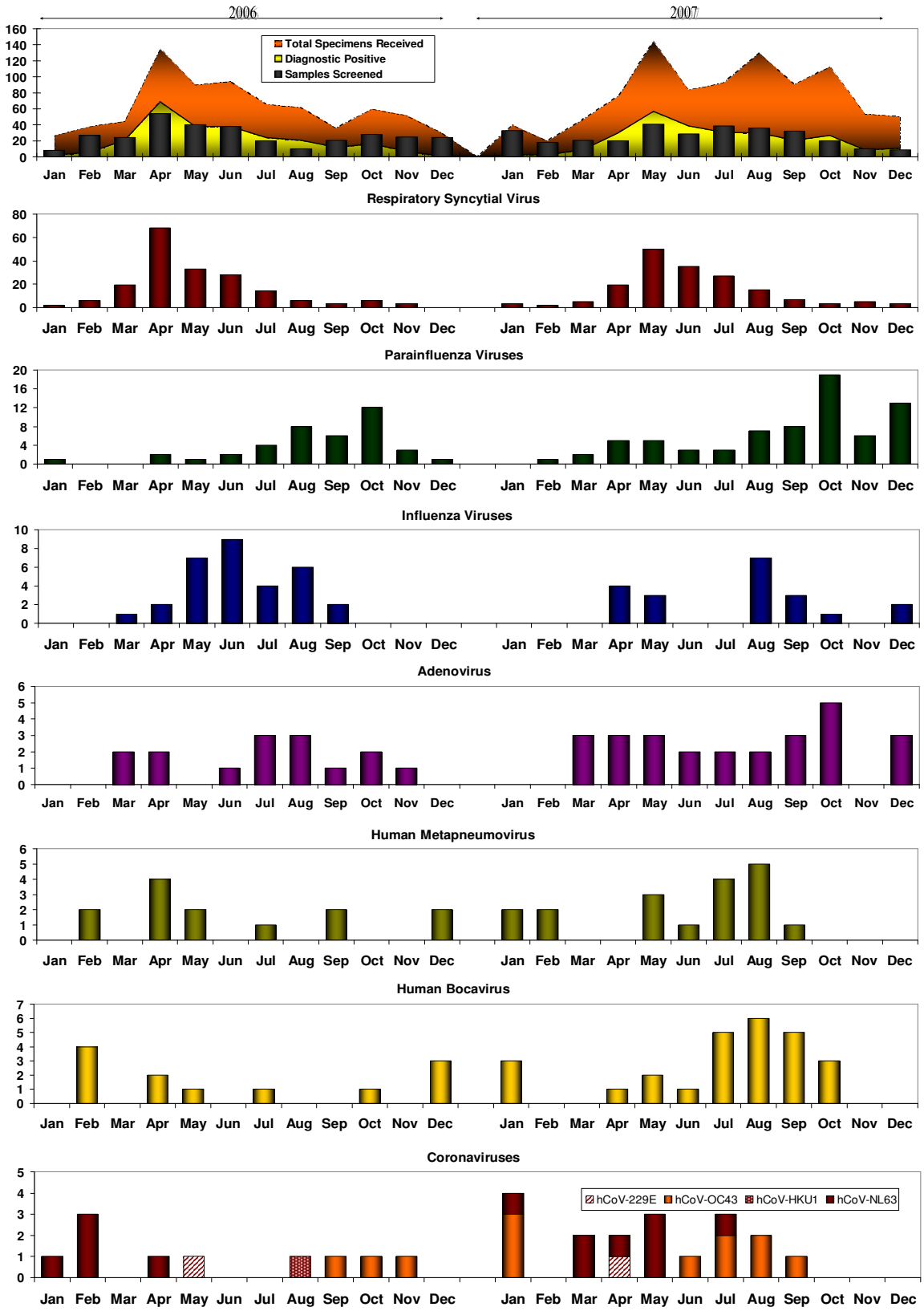

Figure 3.2. Monthly occurrence of acute respiratory tract infections associated with viruses detected in patients from January 2006 to December 2007

(100%), respectively, required hospitalisation or intensive care treatment (Table 3.3b). Inadequate numbers of other viruses were detected to draw a conclusion on severity.

Role of HIV co-infection in disease severity. In children, HIV status were analyzed according to HIV exposure, i.e. exposed uninfected (seropositive but DNA PCR negative); HIV-infected (DNA PCR positive), HIV-negative (sero-negative) and unknown in cases where patients refused HIV testing. The frequency of HIV-infection/exposure in hospitalised children with single infections for RSV was 38/59 (65%), for PIV 3 10/19 (53%), for adenovirus 5/9 (55%), for hMPV 5/8 (62.5%), for hBoV 7/9 (77.7%), for hCoV-NL63 2/2 (100%), and for hCoV-OC43 3/3 (100%) (Table 3.3a and 3.3b). The frequency of co-infections was significantly higher for all viruses, except hCoV-NL63, in hospitalised HIV-infected/exposed individuals namely for RSV 12/20 (60%); PIV 3 5/7 (72%); adenovirus 5/8 (62%); hMPV 8/10 (80%); hBoV 12/15 (80%); hCoV-OC43 5/8 (62.5%) (Table 3.3a and 3.3b). Co-infections with hCoV-NL63 in hospitalised patients were predominantly detected in HIV-uninfected patients (5/8 (62.5%) patients).

3.3.7 Clinical characteristics of patients with single infections

In order to define the clinical features of the newly described respiratory viruses in South African patients, clinical data were collected from the patient's medical files for all positive specimens. To analyze the clinical presentation of the new viruses, symptoms of patients with single infections with each of the new viruses identified during both 2006 and 2007 were analyzed relative to a RSV-, PIV 3-, and Influenza A infected patients (Table 3.4). For this analysis, viruses detected during both 2006 and 2007 were studied.

In RSV infected patients, respiratory distress, crepitations and wheezing were often observed and patients were frequently diagnosed with bronchiolitis, bronchopneumonia or pneumonia, asthma exacerbation and apnoea attacks were also observed. The clinical diagnoses associated with hMPV infections were less diverse in comparison to RSV and hBoV infections. In this cohort, hMPV infections were associated with URTI, bronchiolitis, pneumonia and bronchopneumonia, with clinical presentations of wheezing, crepitations and respiratory distress. HMPV infected patients often required hospitalisation (57.1%) and were frequently hospitalised for more than 7 days (87.5%), however rarely admitted to ICU.

Table 3.3a Demographics of patients with well recognized respiratory virus infections

	RSV (n=418)		PIV 1 (n=13)		PIV 2 (n=14)		PIV 3 (n=108)		Adv (n=63)		Influenza A (n=49)		Influenza B (n=14)	
# of Co-infections detected by IFA	19/360 (5.3%)		2/11 (18.2%)		2/8 (25.0%)		15/90 (16.7%)		10/41 (24.4%)		5/41 (12.2%)		2/6 (33.3%)	
# of Co-infections detected by multiplex assays	37/137 (27%)		4/4 (100%)		1/6 (16.7%)		10/40 (25.0%)		12/27 (44.4%)		10/18 (55.6%)		2/10 (20.0%)	
Age														
Mean	1y1m		1y2m		2y1m		1y6m		2y10m		2y		11m	
Median	3m		7m		5m15d		5m		8m15d		4m15d		9m	
Sex (% males)	53%		54%		71%		51%		59%		63%		50%	
All infected patients identified by multiplex real-time RT-PCR														
	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection
HIV Status	**n=100**	**n=37**	**n=0**	**n=4**	**n=5**	**n=1**	**n=30**	**n=10**	**n=15**	**n=12**	**n=8**	**n=10**	**n=8**	**n=2**
HIV Negative	14 (14%)	11 (30%)	-	-	2 (40%)	-	2 (7%)	3 (30%)	1 (7%)	4 (33%)	5 (63%)	2 (20%)	-	-
HIV Exposed	42 (42%)	15 (41%)	-	3 (75.0%)	-	-	8 (27%)	4 (40%)	4 (27%)	3 (25%)	3 (37%)	5 (50%)	3 (37.5%)	1 (50.0%)
HIV Positive	22 (22%)	4 (11%)	-	1 (25%)	1 (20%)	-	10 (33%)	2 (20%)	5 (33%)	3 (25%)	-	2 (20%)	3 (37.5%)	1 (50.0%)
Unknown	22 (22%)	7 (19%)	-	-	2 (40%)	1 (100%)	10 (33%)	1 (10%)	5 (33%)	2 (17%)	-	1 (10%)	2 (25.0%)	-
Patients with Moderate to Severe Disease														
	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection
Total	**59/100 (59%)**	**20/37 (54%)**	**0/0 (0%)**	**3/4 (75%)**	**2/5 (40.0%)**	**1/1 (100%)**	**19/30 (63%)**	**7/10 (70%)**	**9/15 (60%)**	**8/12 (67%)**	**3/8 (38%)**	**6/10 (60%)**	**3/8 (37.5%)**	**2/2 (100%)**
HIV Status														
HIV Negative	11 (19%)	7 (35%)	-	-	-	-	1 (5%)	2 (29%)	1 (11%)	3 (37%)	1 (33%)	2 (33%)	-	-
HIV Exposed	24 (41%)	9 (45%)	-	2 (66.7%)	-	-	3 (16%)	3 (43%)	2 (22%)	2 (25%)	2 (66%)	1 (17%)	1 (33.3%)	1 (50.0%)
HIV Positive	14 (24%)	3 (15%)	-	1 (33.3%)	-	-	7 (37%)	2 (29%)	3 (33%)	3 (37%)	-	2 (33%)	2 (67.7%)	1 (50.0%)
Unknown	10 (17%)	1 (5%)	-	-	2 (100%)	1 (100%)	8 (42%)	-	3 (33%)	-	-	1 (17%)	-	-

Table 3.3b Demographics of patients infected with hMPV, hBoV and coronaviruses (hCoV) NL63, -HKU1, -OC43, and -229E

	hMPV (n=29)		hBoV (n=35)		NL63 (n=13)		hCoV-HKU1 (n=1)		hCoV-OC43 (n=12)		hCoV-229E (n=2)	
Number of Co-infections	15 (*51.7%*)		22 (*62.9%*)		11 (*84.6%*)		1 (*100%*)		9 (*75%*)		1 (*50%*)	
Age												
Mean ± SD	7m		1y1m		7m		4m		1y3m		2m and 1m17d	
Median	4m		8m		6m		-		6m		-	
Sex (% males)	52%		49%		46%		0%		42%		100%	
All patients	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection
HIV Status	**n=14**	**n=15**	**n=13**	**n=22**	**n=2**	**n=11**	**n=0**	**n=1**	**n=3**	**n=9**	**n=1**	**n=1**
HIV Negative	2 (14.3%)	2 (13.3%)	2 (15.4%)	2 (9.1%)	-	5 (45.4%)	-	-	-	3 (33.3%)	1 (100%)	1 (100%)
HIV Exposed	5 (35.7%)	4 (26.7%)	5 (38.5%)	6 (27.3%)	1 (50.0%)	2 (18.2%)	-	1 (100%)	2 (66.7%)	2 (22.2%)	-	-
HIV Positive	2 (14.3%)	4 (26.7%)	3 (23.1%)	10 (45.5%)	1 (50.0%)	2 (18.2%)	-	-	1 (33.3%)	3 (33.3%)	-	-
Unknown	5 (35.7%)	5 (33.3%)	3 (23.1%)	4 (18.2%)	-	2 (18.2%)	-	-	-	1 (11.1%)	-	-
Patients with Moderate to Severe Disease	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection	Single Infection	Co-Infection
Total	**8/14 (57.1%)**	**10/15 (66.6%)**	**9/13 (69.2%)**	**15/22 (68.2%)**	**2/2 (100%)**	**8/11 (72.7%)**	**0%**	**0%**	**3/3 (100%)**	**8/9 (88.9%)**	**1/1 (100%)**	**-**
HIV Status												
HIV Negative	1 (12.5%)	1 (10.0%)	1 (11.1%)	2 (13.3%)	-	5 (62.5%)	-	-	-	2 (25.0%)	1 (100%)	-
HIV Exposed	3 (37.5%)	4 (40.0%)	4 (44.4%)	3 (20.0%)	1 (50.0%)	2 (25.0%)	-	-	2 (66.7%)	2 (25.0%)	-	-
HIV Positive	2 (25.0%)	4 (40.0%)	3 (33.3%)	9 (60.0%)	1 (50.0%)	1 (12.5%)	-	-	1 (33.3%)	3 (37.5%)	-	-
Unknown	2 (25.0%)	1 (10.0%)	1 (11.1%)	1 (6.7%)	-	-	-	-	-	1 (12.5%)	-	-

Single hBoV infections were more frequently detected in older patients, males and patients with nutritional deficits than any of the other viruses studied. URTI were diagnosed in 2/13 (15.4%) patients whereas LRTI occurred in 11/13 (84.6%) of patients with single infections. HBoV infected patients presented with a wide range of clinical manifestations including bronchiolitis, bronchitis, asthma exacerbation, apnoea attacks and croup, but were most frequently diagnosed with bronchopneumonia and pneumonia. In addition, gastrointestinal (GIT) symptoms were more frequently associated with hBoV than any of the other viruses. None of hBoV infected patients were admitted to ICU, however, 9/13 (69.2%) patients required hospitalisation of which 6/9 (66.7%) were hospitalised for more than 7 days.

Human coronaviruses seldom occurred in the absence of another virus making it difficult to determine their association with clinical disease. HCoV-NL63 was only detected as the sole aetiological agent in two patients, both requiring hospitalisation in the intensive care unit due to severe pneumonia. One patient, which was also diagnosed with croup, was premature and exposed to HIV while the other patient was HIV positive. Similarly, hCoV-OC43 was detected as a single infection in only three patients, all of which required hospitalisation. One patient suffered from apnoea attacks and 2/3 patients had LRTI, of which one was diagnosed with bronchiolitis. HCoV-229E was only detected as the sole aetiological agent in one HIV negative, 1.6 month old male suffering from a LRTI and apnoea attacks. The patient was admitted to ICU where he received oxygen supplementation. HCoV-HKU1 was only detected on one occasion as a co-infection with PIV 3 in a 4 month old, HIV negative patient suffering from bronchopneumonia. It is difficult to establish an association between hCoV-HKU1 and the presenting symptoms due to the co-infection with PIV 3.

In total 7 deaths were associated with viral respiratory tract infection of which five patients were infected with RSV, one with hMPV, and one with hBoV. The demographics and clinical characteristics of these patients are indicated in table 3.5. All five patients infected with RSV suffered from LRTI of which three were diagnosed with pneumonia and four patients were co-infected with a bacterial pathogen of which the relevance varies. RSV was only identified as the sole aetiological agent in one 4 month old patient diagnosed with

Table 3.4 Clinical characteristics of patients solely infected with RSV, PIV 3, Influenza A, hMPV, hBoV, hCoV-NL63 and hCoV-OC43, respectively

	RSV [a] (n=88)	PIV 3 (n=30)	Influenza A (n=8)	hMPV (n=14)	hBoV (n=13)	hCoV-NL63 (n=2)	hCoV-OC43 [b] (n=3)	hCoV-229E (n=1)
Demographics								
Mean Age (months) ± SD	3.9 ± 3.3	5.8 ± 4.2	5.1 ± 2.9	6.1 ± 4.8	10.9 ± 7.3	3 mo & 6 mo	3.1 ± 1.9	1.6 months
Sex (Males %)	59.1%	40%	83.3%	61.50%	76.90%	0%	33.30%	100.00%
ICU admission	26 *(29.5%)*	7 *(23.3%)*	0	1 *(7.1%)*	0	2 *(100.0%)*	0	1 *(100%)*
Premature	16 *(18.9%)*	5 *(16.7%)*	1 *(12.5%)*	0	0	1 *(50.0%)*	1 (33.3%)	0
Died	7 *(8.0%)*	1 *(3.3%)*	0	1 *(7.1%)*	1 *(7.7%)*	0	0	0
O2 supplementation	44 *(50.0%)*	17 *(56.7%)*	2 *(25.0%)*	7 *(50.0%)*	7 *(53.8%)*	1 (50.0%)	2 (66.7%)	*1 (100%)*
Malnourished (wasted)	13 *(14.8%)*	5 *(16.7%)*	1 *(12.5%)*	1 *(7.1%)*	4 *(30.8%)*	0	1 (33.3%)	0
Undernourished (stunted)	5 *(5.7%)*	0	0	1 *(7.1%)*	2 *(15.4%)*	0	0	0
Unknown nutritional status	20 *(22.7%)*	4 *(13.3%)*	1 *(12.5%)*	2 *(14.3%)*	3 *(23.1%)*	2 *(100%)*	1 *(33.3%)*	1 *(100%)*
Clinical characteristics								
Respiratory distress	39 *(44.3%)*	17 *(56.7%)*	0	10 *(71.4%)*	7 *(53.8%)*	0	1 (33.3%)	0
Crepitations	45 *(51.1%)*	18 *(60.0%)*	3 *(37.5%)*	7 *(50.0%)*	4 *(30.8%)*	0	1 *(33.3%)*	1 *(100%)*
Wheezing	24 *(27.3%)*	10 *(33.3%)*	2 *(25.0%)*	5 *(35.7%)*	5 *(38.5%)*	1 *(50.0%)*	1 (33.3%)	0
Lobar consolidation	2 *(2.3%)*	2 *(6.7%)*	0	0	1 *(7.7%)*	0	0	0
GIT symptoms	16 *(18.2%)*	3 *(10.0%)*	2 *(25.0%)*	3 *(21.4%)*	6 *(46.2%)*	0	1 *(33.3%)*	1 *(100%)*
Clinical diagnosis								
URTI	4 *(4.5%)*	0	2 *(25.0%)*	1 *(7.1%)*	2 *(15.4%)*	0	0	0
LRTI	76 *(86.4%)*	30 *(100%)*	6 *(75.0%)*	11 *(78.6%)*	11 *(84.6%)*	2 *(100%)*	2 (66.7%)	1 *(100%)*
Bronchiolitis	23 *(26.1%)*	10 *(33.3%)*	3 *(37.5%)*	4 *(28.6%)*	1 *(7.7%)*	0	1 (33.3%)	0
Bronchopneumonia	19 *(21.6%)*	7 *(23.3%)*	1 *(12.5%)*	1 *(7.1%)*	4 *(30.8%)*	0	0	0
Pneumonia	21 *(23.9%)*	16 *(53.3%)*	3 *(37.5%)*	4 *(28.6%)*	4 *(30.8%)*	2 *(100%)*	0	0
Bronchitis	5 *(5.7%)*	0	0	0	1 *(7.7%)*	0	0	0
LRTI not otherwise specified	8 *(9.1%)*	0	0	2 *(14.3%)*	1 *(7.7%)*	0	1 (33.3%)	1 *(100%)*
Croup	1 *(1.1%)*	2 *(6.7%)*	0	0	1 (7.7%)	1 *(50.0%)*	0	0
Asthma exacerbation	3 *(3.4%)*	0	0	0	1 *(7.7%)*	0	0	0
Apnoea attacks	7 *(8.0%)*	3 *(10.0%)*	0	0	1 *(7.7%)*	0	1 (33.3%)	1 *(100%)*

[a] Patient files were only available for 88/137 (64%) of hospitalised and outpatients with RSV single infections as identified by real-time RT-PCR
[b] Patient files were only available for 2 our of 3 hCoV-OC43 infected patients.

pneumonia that was HIV positive, malnourished and undernourished. HMPV was identified in a 30 year old male 7 days after admission to the medical ICU, approximately two months later he died due to pneumonia and overwhelming sepsis with *Klebsiella pneumoniae*. The death is therefore not associated with hMPV. Over a 16 day period, hBoV was detected on three different occasions in a malnourished 5 month old, HIV positive male, admitted to hospital with bronchopneumonia. The patient had a *Streptococcus pneumoniae* and a CMV co-infection on the day of admission with *Staphylococcus aureus* detected in the blood seven days post-admission. The patient succumbed to disease sixteen days after being admitted to the hospital.

3.4 Discussion

The contribution and disease association of respiratory viruses, especially the recently described viruses, in developing countries with a high burden of HIV-AIDS is currently poorly described. In order to address this, the present study focused on the contribution of 13 respiratory viruses to hospitalisations and outpatient visits in hospitals in South Africa using specimens submitted over a 2-year period to a diagnostic laboratory serving public sector hospitals in the Pretoria region, Gauteng, South Africa. HIV seroprevalence amongst antenatal clinic attendees in Gauteng was 29.3% by 2008 (www.doh.gov.za/docs/nassps-f.html, 2009).

The majority of specimens received were from children less than 5 years of age. Collectively most patients testing positive were less than 1 year old. RSV remained the most frequent detected virus in this group followed by hBoV and PIV3, hMPV, Adenovirus, hCoV-NL63 and influenza A while the other viruses were all detected in <3% of cases. Polyomavirus WU was also detected in 8% of cases in 2006 (Venter *et al.*, 2009). RSV infections peaked in children <6 months while PIV 3 and hMPV had a broader peak up to 12 months. Influenza and adenovirus showed a peak between 6-12 months but also occurred in older children. HBoV was detected infrequently in patients <6 months old suggesting a possible role for maternal antibodies in protection.

Table 3.5 Clinical characteristics and demographics of seven patients that died in association with three respiratory viruses (RSV, hMPV, hBoV)

Patient	Viral infections	Bacterial co-infection	Age	Sex	HIV status	Diagnosis	Clinical findings	Nutritional status	Respiratory Distress	Oxygen received
1	RSV	*Moraxella catarrhalis*	5 mo	F	Unknown	LRTI NOS[a]	Crepitations, GIT symptoms[b]	Unknown	Yes	Yes
2	RSV	Coagulase Negative Staphylococci	11 mo	M	Unknown	LRTI NOS	Crepitations, GIT symptoms	Normal	Yes	Yes
3	RSV	*Escherichia coli*	2 mo	F	HIV Negative	Pneumonia	Crepitations	Normal	Yes	Yes
4	RSV	*Klebsiella pneumoniae, Pseudomonas aeruginosa*	28 mo	M	HIV Positive	Bilateral pneumonia	Crepitations, GIT symptoms	Malnourished	Yes	Yes
5	RSV	None	4 mo	F	HIV Positive	Pneumonia	Crepitations, GIT symptoms	Malnourished, Undernourished	No	Yes
6	hMPV	*Klebsiella pneumoniae*	30 yrs	M	Unknown	Pneumonia	Crepitations	Unknown	Yes	Yes
7	hBoV, CMV	*Streptococcus pneumoniae, Staphylococcus aureus*	5 mo	F	HIV Positive	Bronchopneumonia	GIT symptoms	Malnourished	Yes	Yes

[a] LRTI NOS – Lower respiratory tract infection not otherwise specified
[b] GIT – Gastrointestinal symptoms

The observed seasonal distribution of hBoV and hMPV was less defined compared to the well recognized viruses, with both viruses showing variability over the two years. This confirms findings from developed countries in the northern hemisphere where hBoV and hMPV that had variable seasonal occurrences (Aberle *et al.*, 2008; Bastien *et al.*, 2006; Maggi *et al.*, 2007). In addition, the observed seasonal distribution of hCoV-NL63 appeared to resemble that of tropical regions (Lau *et al.*, 2006) despite the small numbers detected in the present study.

RSV is most frequently associated with hospitalisation in paediatric patients, followed by PIV 3 and influenza A (Forster *et al.*, 2004; Iwane *et al.*, 2004), though it has been shown that hMPV associated hospitalisation rates may exceed those of the latter two viruses (Cilla *et al.*, 2009). In our study population, ~60% of children infected with RSV, hMPV, PIV 3 and adenovirus were hospitalised (moderate-to-severe disease group). As a single infection, hBoV associated cases had the highest number of moderate-to-severe acute respiratory tract infection (69%) in the 35 patients identified with single infections over the 2-year period. Establishing a causal relationship between hBoV and respiratory tract disease has been hampered by unusually high co-infection rates (Allander *et al.*, 2007b; Anderson, 2007), usually with viruses of known pathogenic potential although it has been identified as single infections with patients with respiratory disease in several studies (Brieu *et al.*, 2008; Calvo *et al.*, 2008; Choi *et al.*, 2006). In the present study the identified 13 cases of single hBoV infections were associated with bronchiolitis, bronchitis, pneumonia, bronchopneumonia, asthma exacerbation, and croup.

Hospitalisation rates associated with hMPV have previously been shown to be 5-fold higher in HIV-infected compared to HIV-uninfected patients (Madhi *et al.*, 2007). Our findings demonstrated a high frequency of hospitalisations in patients with both hMPV and hBoV in HIV-infected/exposed children. Hospitalisations due to single infections with hCoV-NL63 and hCoV-OC43 were exclusively in HIV-infected/exposed children suggesting a possible correlation with acute LRTI in immunosuppressed patients.

In addition to increasing the diagnostic yield by ~40%, real-time RT-PCR detected a notably higher co-infection rate compared to IFA, which may be attributable to the increased sensitivity and inclusion of new viruses. Co-infections were frequently detected in HIV-infected/exposed patients, with co-infection frequencies being

particularly high in patients infected with the recently described respiratory viruses (51.7% to 84.6%), though high co-infection rates were also observed for adenovirus and influenza A (44.4% and 55.6%, respectively). No significant difference was visible in disease severity as measured by hospitalisation between single and co-infections for RSV; slightly more hospitalisations for co-infections for PIV 3; Adenovirus and hCoNL63; but significantly more hospitalisations for co-infections than single infections for influenza A, hMPV and hBoV.

In conclusion, recently described respiratory viruses contribute to acute LRTI and hospitalisations in children in South Africa. Prevalence rates of the different respiratory viruses were comparable to those of countries with a significantly lower HIV-AIDS burden, yet respiratory viruses occurred at a high frequency in HIV-infected/exposed children who may experience more severe disease. Using molecular screening techniques RSV was demonstrated to contribute significantly more to moderate-to-severe ALRI than previously appreciated with antigen detection techniques and remains the most important viral cause of acute LRTI in hospitalised children in South Africa. One of the limitations of this study is that not all patients had consented to be tested for HIV. Case control studies in a HIV cohort may determine the impact of these viruses on hospitalisation rates in HIV-exposed/infected patients compared to HIV-uninfected patients.

Chapter 4

The Association of Viral Load and Co-Infection with Clinical Course of Disease in Children with Acute Respiratory Tract Infection

4.1 Introduction

In recent years, real-time PCR detection of pathogenic DNA and RNA viruses has globally revolutionized the research and diagnostic industry. Real-time PCR detection assays have the ability to detect minute amounts of nucleic acids and provide a platform for both qualitative as well as quantitative studies. Quantitative detection of viruses has numerous advantages and applications in both the research and diagnostic setting. For example, routine monitoring of human immunodeficiency virus (HIV) viral load serves as a prognostic marker in HIV infected patients (Mellors *et al.*, 1996), whereas quantification of hepatitis B virus DNA is used to monitor antiviral therapy as well as to determine infectivity (Berger *et al.*, 2001; Wiseman *et al.*, 2009). Similarly, it has been shown that the monitoring of Epstein-Barr virus, adenovirus and human papillomavirus type 16 viral loads may allow assessment of the risk of developing virus associated disease and may be predictive for the development of symptoms and disease outcome (Gustafson *et al.*, 2008; Sato *et al.*, 2008; Ylitalo *et al.*, 2000). In these instances the quantitative detection of viruses may be of clinical significance to prevent severe clinical virological complications and may present a basis for appropriate initiation of antiviral therapy and clinical management of disease.

In contrast to the above mentioned examples, the clinical relevance of viral load in patients with acute respiratory tract infections is less defined. Individuals infected with respiratory viruses, such as respiratory syncytial virus (RSV), often present with varying degrees of disease severity (Ogra, 2004). Previous studies have proposed an association between disease severity and RSV viral load, as it was found that patients with more severe respiratory disease often had higher viral loads (DeVincenzo *et al.*, 2005; Gueudin *et al.*, 2003; Martin *et al.*, 2008). However, contrasting observations where RSV disease severity was not influenced by viral load has also been described (Wright *et al.*, 2002). Similar to RSV, the association of viral load with clinical disease in recently described respiratory viruses (human metapneumovirus (hMPV), human bocavirus (hBoV), human coronaviruses (hCoV) HKU1 and NL63) and viruses not currently included in routine diagnostic assays (hCoV-OC43 and hCoV-229E) are not well described, in particular not in HIV infected or HIV exposed individuals.

Previous investigations have found that increased hMPV viral load was frequently associated with hospitalisation, age (older than 7 months) and lower respiratory tract

involvement (Bosis *et al.*, 2008; Gerna *et al.*, 2007c; Kuypers *et al.*, 2005), whereas high hBoV viral loads were associated with both upper and lower respiratory tract infections (Choi *et al.*, 2008). In addition, studies have shown that patients with single hBoV infections had higher viral loads than patients with co-infections (Brieu *et al.*, 2008; Christensen *et al.*, 2008; Jacques *et al.*, 2008), though contrasting findings have been reported (Neske *et al.*, 2007). In contrast to hMPV and hBoV, quantitative studies on human coronaviruses are limited. Quantification assays for hCoV-OC43 and 229E have been described (Vijgen *et al.*, 2005), but to date have not been applied to clinical investigations. While quantitation of hCoV-HKU1 and hCoV-NL63 has only been described once in each case with no observations made about the association between viral load and disease severity (van der Hoek *et al.*, 2005; Woo *et al.*, 2005).

In this study we aimed to determine the role of viral load in the severity of disease as well as to describe the role of co-infections in acute respiratory tract infection. In addition, we aimed to determine if HIV infection and HIV exposure influence respiratory viral load.

4.2 Material and Methods

4.2.1 Specimens

During 2006 and 2007, specimens that tested positive by real-time RT-PCR for hMPV (n=31), hBoV (n=39), hCoV-NL63 (n=13), hCoV-OC43 (n=12), hCoV-229E (n=2), and hCoV-HKU1 (n=1) were collected for quantitative analysis of viral loads. Results obtained for the RSV viral loads (n=58) as determined in Chapter 2, section 2.3.4 was included for comparison. Specimens were obtained from patients seeking medical care at regional hospitals in Pretoria, Gauteng, South Africa. Where indicated patients were grouped according to disease severity, namely mild disease – patients seeking medical care at the outpatients department (not hospitalised); and moderate to severe disease – patients hospitalised in general wards and/or hospitalised in the intensive care unit (ICU).

4.2.2 Processing of specimens

Specimens were processed as described in Chapter 2, section 2.2.2.

4.2.3 RNA extraction

Refer to Chapter 2, Materials and Methods section 2.3.2.

4.2.4 Quantitative real-time RT-PCR

Refer to Chapter 2, Materials and Methods section 2.2.5.

4.2.5 Quantification of viral loads

RNA standards for quantification were prepared as described in Chapter 2, section 2.2.7. Viral loads were quantified against a standard curve produced by amplifying serial dilutions of RNA transcripts for RNA viruses and recombinant plasmids for DNA viruses. A standard of known concentration was included in each quantitative real-time RT-PCR run. Viral loads are presented as DNA/RNA copies detected in 1ml of processed NPA specimens.

4.2.6 Statistical analysis

The confidence level of data analyzed was determined with the Mann-Whitney test. Statistical analysis was carried out with GraphPad InStat version 3.06.

4.3 Results

4.3.1 Human Metapneumovirus

HMPV was previously identified in 31 respiratory specimens obtained from 29 patients with acute respiratory disease. HMPV was identified as the sole aetiological agent in 14/29 (51.7%) patients, of which 8/14 (57.1%) patients had moderate to severe disease. The viral loads of patients with single infections and patients with co-infections

are indicated in table 4.1. Patients solely infected with hMPV had viral loads ranging from 1.19 × 10^5 to 4.68 × 10^7 copies/ml (mean: 8.92 × 10^6; median: 2.86 × 10^6). Higher hMPV viral loads were only detected in one co-infected patient (up to 2.33 × 10^9 copies/ml), however this was only observed on one occasion and the overall difference in viral load in patients with single infections (n=14) versus patients with co-infections (n=15) was not found to be significant (p=0.31). Higher nasopharyngeal viral loads were observed in patients with moderate to severe disease (mean: 1.32 × 10^7 copies/ml; median: 3.06 × 10^6 copies/ml), than in patients with mild disease (mean: 3.20 × 10^6 copies/ml; median: 2.74 × 10^6 copies/ml), although not statistically significant (p=0.75).

HMPV infected patients were frequently co-infected with another respiratory virus (15/29 *(51.7%)* patients). Co-infections with hBoV occurred most (n=8), followed by RSV (n=6), hCoV-NL63 (n=2), and only once with influenza A, influenza B, hCoV-OC43, and polyomavirus WU. Co-infected patients with moderate to severe disease frequently had either a high hMPV viral load (>1×10^6 copies/ml) or the co-infecting virus was present at a high viral load. In comparison, lower viral loads of both hMPV and co-infecting viruses were frequently observed in co-infected patients with mild disease (Table 4.1).

Table 4.1 Comparison of viral loads in human metapneumovirus infected patients with mild and moderate to severe disease in the absence or presence of a co-infecting virus

	Patients with Mild Disease		
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load hMPV (copies/ml)**
Single infections			
hMPV	**6**	-	mean: 3.20 × 10^6 (median: 2.74 × 10^6)
Co-infections with:			
hBoV	**1**	5.98 × 10^6	1.45 × 10^7
RSV	**3**	4.85 × 10^6 to 2.13 × 10^9	4.85 × 10^4 to 1.33 × 10^6
WU	**1**	Not quantified	6.25 × 10^5
RSV + hBoV + Influenza A	**1**	9.83 × $10^{9\ (RSV)}$, 7.25 × $10^{4\ (hBoV)}$, 6.03 × $10^{4\ (Influenza\ A)}$	8.25 × 10^4
	Patients with Moderate/Severe Disease		
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load hMPV (copies/ml)**
Single infections			
hMPV	**8**	-	mean: 1.32 × 10^7 (median: 3.06 × 10^6)
Co-infections with:			
hBoV	**4**	6.15 × 10^6 to 3.35 × 10^9	3.55 × 10^5 to 2.33 × 10^9
RSV	**1**	1.30 × 10^{11}	1.03 × 10^5
hCoV-OC43	**1**	6.93 × 10^4	2.58 × 10^7
RSV + Influenza B	**1**	1.39 × 10^6 + 3.93 × 10^7	4.00 × 10^6
hBoV + hCoV-NL63	**2**	3.23 × $10^{9\ (hBoV)}$ + 8.15 × $10^{6\ (hCoV-NL63)}$ 2.04 × $10^{4\ (hBoV)}$ + 5.70 × $10^{7\ (hCoV-NL63)}$	1.36 × 10^6 2.73 × 10^5

In one patient, hMPV and hBoV were co-detected in serial respiratory specimens taken from a 1 year old female diagnosed with bronchopneumonia and croup. At the time of admission the patient was malnourished, HIV positive and had heart lesions (patent ductus arteriosus). The patient was hospitalised for 20 days during which time three nasopharyngeal specimens were submitted to the diagnostic virology laboratory for routine testing of respiratory viruses on day 3, 6, and 12 post-admission. On all three occasions the patient tested negative for the presence of RSV, PIV 1-3, influenza A and B, and adenovirus with the routine immunofluorescence panel, whereas real-time RT-PCR identified hMPV and hBoV. The nasopharyngeal viral load of both hMPV and hBoV remained high during the period of testing (Table 4.2.), which may be indicative of an active infection and inefficient clearance of the viral infection due to an impaired immune system. In addition, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* was identified in tracheal aspirates from this patient.

Table 4.2 Human metapneumovirus and human bocavirus viral loads in three specimens taken from a single patient hospitalised for 20 days

	hMPV	hBoV
Day 3 post-admission	3.80E+07	1.84E+08
Day 6 post-admission	8.73E+07	3.95E+08
Day 12 post-admission	3.25E+07	6.75E+08

4.3.2 Human Bocavirus

Human bocavirus was previously detected in 39 respiratory specimens obtained from 35 patients suffering from acute respiratory disease. HBoV was identified as single infections in 13/35 (37.1%) patients. Viral loads of patients with single infections as well as co-infections are indicated in table 4.3. To establish if there is an association between nasopharyngeal hBoV viral loads and disease severity, the viral loads of patients with single hBoV infections suffering from mild (n=4) and moderate to severe disease (n=9) were compared. The hBoV viral load in patients with mild disease range from 8.23×10^5 to 4.53×10^7 copies/ml (mean: 1.32×10^7 copies/ml; median: 3.44×10^6 copies/ml), whereas patients with moderate to severe disease had hBoV viral loads ranging from 1.25×10^4 to 5.28×10^9 copies/ml (mean: 7.34×10^8 copies/ml; median: 2.35×10^6 copies/ml) (Table 4.3.). Higher hBoV viral loads ($>1\times10^8$ copies/ml) were only observed in patients with moderate to severe disease, however very low viral loads ($<1\times10^5$ copies/ml) were also observed in 3/9 (33.3%) patients of which 2 patients were

HIV infected as well as malnourished and one patient was HIV exposed. In comparison to patients with mild disease, the mean hBoV viral load in patients with moderate to severe disease was higher by approximately 1 $\log_{10}$ factor but the difference was not found to be statistically significant (p=0.71) because of the sample sizes.

Table 4.3 Comparison of viral loads in human bocavirus infected patients with mild and moderate to severe disease in the absence or presence of a co-infecting virus

	Patients with Mild Disease		
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load hBoV (copies/ml)**
Single infections			
hBoV	**4**	-	mean: 1.32×10^{7} (median: 3.44×10^{6})
Co-infections with:			
PIV 3	**2**	1.97×10^{6} and 1.30×10^{6}	1.12×10^{4} and 7.20×10^{6}
hCoV-NL63	**1**	1.21×10^{6}	5.28×10^{6}
hMPV	**1**	1.45×10^{7}	5.98×10^{6}
Influenza A	**1**	1.56×10^{6}	3.58×10^{6}
Adenovirus	**1**	3.53×10^{5}	3.58×10^{6}
Polyomaviruses WU + KI	**1**	Not quantified	1.12×10^{4}
RSV + hMPV + Influenza A	**1**	9.83×10^{9} (RSV), 8.25×10^{4} (hMPV), 6.03×10^{4} (Influenza A)	7.25×10^{4}
	Patients with Moderate/Severe Disease		
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load hBoV (copies/ml)**
Single infections			
hBoV	**9**	-	mean: 7.43×10^{8} (median: 2.34×10^{6})
Co-infections with:			
hMPV	**4**	3.55×10^{5} to 2.33×10^{9}	6.15×10^{6} to 3.35×10^{9}
PIV 3	**2**	1.64×10^{7} and 8.38×10^{9}	1.25×10^{4} and 2.30×10^{6}
RSV	**2**	1.35×10^{6} and 2.85×10^{6}	2.30×10^{6} and 3.08×10^{6}
hCoV-OC43	**1**	1.58×10^{6}	4.30×10^{9}
Adenovirus	**1**	7.40×10^{3}	6.10×10^{4}
Polyomavirus WU	**1**	Not quantified	4.95×10^{5}
Polyomavirus WU + Rhinovirus	**1**	Not quantified	1.56×10^{10}
hMPV + hCoV-NL63	**2**	1.36×10^{6} (hMPV), 8.15×10^{6} (hCoV-NL63) 2.73×10^{5} (hMPV), 5.70×10^{7} (hCoV-NL63)	3.23×10^{9} 2.04×10^{4}

Moderate to severe disease occurred at similar frequencies in both patients with single hBoV infections (69.2%) and co-infected patients (68.2%). HBoV was frequently co-detected with a wide range of respiratory viruses including hMPV (n=8), parainfluenza virus (PIV) 3 (n=4), RSV (n=3), hCoV-NL63 (n=3), Influenza A (n=2), Adenovirus (n=2), Polyomaviruses WU (n=3) and KI (n=1). In the presence of another virus hBoV viral loads ranged from 1.25×10^{4} to 1.56×10^{10} copies/ml (mean – 1.34×10^{9} copies/ml; median – 3.08×10^{6} copies/ml), which did not differ significantly from patients with single infections (p=0.92). However, similar to patients with single hBoV infections, high hBoV viral loads of $>1\times10^{9}$ copies/ml as well as higher HIV infection and

HIV exposure rates were observed in co-infected patients with moderate to severe disease (Table 4.3.).

In one instance, over a 17 day period, hBoV was repeatedly detected in a malnourished, 6 month old, HIV positive male diagnosed with bronchopneumonia. The patient was hospitalised in a general paediatric ward where he received supplemental oxygen and ultimately succumbed to disease 17 days post-admission. On day 1, 8, and 12 post-admission NPA specimens were submitted for diagnosis of respiratory viruses. Routine diagnostic immunofluorescence could not detect RSV, PIV1-3, Influenza A/B, or Adv in any of the three NPA specimens, whereas real-time RT-PCR detected hBoV on all three occasions. The viral loads (copies/ml) measured on days post-admission were as follow: Day 1 – 2.50 x 10^4; Day 8 – 7.75 x 10^5; and Day 12 – 1.85 x 10^6. Over a 12 day period the hBoV viral load increased by 2 $\log_{10}$ factors, which may be interpreted as an indication of active viral replication. In addition to hBoV, bacterial culture from blood identified a *Streptococcus pneumoniae* infection (on the day of admission) and a nosocomial *Staphylococcus aureus* infection (on day 7 post-admission). He also tested positive for CMV in viral culture and IgM serology. This demonstrates the vulnerability of immunocompromised patients to opportunistic infections.

4.3.3 Human Coronavirus NL63

HCoV-NL63 seldom occurred in the absence of another virus and was only identified as the sole aetiological agent in two immunocompromised paediatric patients. Both patients were hospitalised in the intensive care unit with severe pneumonia and had viral loads of 4.00 × 10^5 copies/ml and 1.56 × 10^6 copies/ml, which did not differ significantly from viral loads observed in patients with co-infections (Table 4.4). Overall, the mean viral load of hCoV-NL63 in 13 patients with respiratory disease was 2.17×10^7 copies/ml (median: 1.21×10^7 copies/ml).

Co-infections were frequently observed in HCoV-NL63 infected patients (11/13 (84.6%)), with RSV occurring most (n=4), followed by Adv (n=3), hBoV (n=3), hMPV (n=2), PIV 1 (n=1), and rhinovirus (n=1). In contrast to the previously mentioned hMPV and hBoV, the majority of hCoV-NL63 co-infected patients were immunocompetent. In comparison to patients with mild disease, hCoV-NL63 co-infected patients with moderate to severe disease had a higher frequency of hCoV-NL63 viral loads above

1×10^6 copies/ml. However, one HIV negative, 12 day old patient admitted to the intensive care unit with a lower respiratory tract infection and apnoea attacks had very low viral loads of hCoV-NL63 (3.75×10^3 copies/ml) and two co-infecting viruses, RSV (9.83×10^5 copies/ml) and Adv (3.75×10^3 copies/ml).

Table 4.4 Comparison of viral loads in human coronavirus NL63 infected patients with mild and moderate to severe disease in the absence or presence of a co-infecting virus

	Patients with Mild Disease		
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load NL63 (copies/ml)**
Single infections			
hCoV-NL63	**0**	-	-
Co-infections with:			
RSV	**1**	4.15×10^8	2.50×10^5
hBoV	**1**	5.28×10^6	1.21×10^6
Rhinovirus	**1**	Not quantified	1.39×10^5
	Patients with Moderate/Severe Disease		
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load NL63 (copies/ml)**
Single infections			
hCoV-NL63	**2**	-	4.00×10^5 and 1.56×10^6
Co-infections with:			
RSV	**2**	3.53×10^5 and 3.58×10^6	9.73×10^5 and 2.43×10^6
Adv	**1**	2.85×10^6	3.05×10^6
RSV + Adv	**2**	$9.53 \times 10^{8\ (RSV)}$, $1.34 \times 10^{4\ (Adv)}$ $9.83 \times 10^{5\ (RSV)}$, $3.75 \times 10^{3\ (Adv)}$	8.00×10^5 3.75×10^3
RSV + PIV 1	**1**	$2.50 \times 10^{7\ (RSV)}$, $5.00 \times 10^{7\ (PIV\ 1)}$	2.06×10^8
hBoV + hMPV	**2**	$3.23 \times 10^{9\ (RSV)}$, $1.36 \times 10^{6\ (PIV\ 1)}$ $2.04 \times 10^{4\ (hBoV)}$, $2.73 \times 10^{5\ (hMPV)}$	8.15×10^6 5.70×10^7

4.3.4 Human Coronavirus OC43

Similar to hCoV-NL63, hCoV-OC43 infected patients had an unusually high co-infection rate (9/12 (75%) patients). HCoV-OC43 was identified as the sole aetiological agent in only three patients with moderate to severe disease, which had viral loads ranging from 2.50×10^5 to 2.50×10^8 copies/ml (mean: 7.39×10^7; median: 7.39×10^5 copies/ml). All three patients were immunocompromised due to either HIV infection or HIV exposure and two of the three patients suffered from lower respiratory tract infections.

HCoV-OC43 was co-detected with RSV (n=3), Adv (n=2), and only once with hBoV, hMPV, PIV 1, and PIV 2. Only one co-infected patient who had very low viral loads of both hCoV-OC43 (2.80×10^4 copies/ml) and Adv (2.78×10^4 copies/ml) had mild

disease. In comparison to this patient, co-infected patients with moderate to severe disease had either a high hCoV-OC43 viral load or the co-infecting virus was present at a high viral load (Table 4.5.).

Table 4.5 Comparison of viral loads in human coronavirus OC43 infected patients with mild and moderate to severe disease in the absence or presence of a co-infecting virus

	Patients with Mild Disease		
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load OC43 (copies/ml)**
Single infections			
hCoV-OC43	**0**	-	-
Co-infections with:			
Adv	**1**	2.78×10^4	2.80×10^4
	Patients with Moderate/Severe Disease		
	Number of infections	**Viral Load of Co-Infecting Virus (copies/ml)**	**Viral Load OC43 (copies/ml)**
Single infections			
hCoV-OC43	**3**	-	mean: 7.39×10^7 (median: 2.78×10^5)
Co-infections with:			
RSV	**3**	6.60×10^5 to 9.78×10^6	1.35×10^5 to 1.20×10^7
Adv	**1**	3.35×10^4	5.48×10^7
hBoV	**1**	4.30×10^9	1.58×10^6
hMPV	**1**	2.58×10^7	6.93×10^4
PIV 1	**1**	2.58×10^4	3.43×10^5
PIV 2	**1**	4.20×10^6	4.35×10^4

4.3.5 Human Coronaviruses 229E and HKU1

HCoV-229E was detected on two occasions, once as a single infection and once in the presence of RSV. HCoV-229E was identified as the sole aetiological agent in a 1.5 month old, HIV negative male suffering from a lower respiratory tract infection and apnoea attacks. The patient was admitted to the intensive care unit where he received supplemental oxygen. The nasopharyngeal hCoV-229E viral load measured on the day of admission was 2.50×10^3 copies/ml. In another patient, hCoV-229E was co-detected with RSV in a 2 month old, HIV negative male diagnosed with bronchiolitis. On the day of admission, the viral load of hCoV-229E was 1.25×10^4 copies/ml, whereas the viral load of RSV was 1.25×10^7 copies/ml.

HCoV-HKU1 was only detected once in the presence of a co-infecting virus (PIV 3) in a hospitalised 4 month old, HIV negative patient with bronchopneumonia. The viral load of hCoV-HKU1 was 1.46×10^6 copies/ml, whereas PIV 3 occurred at a viral load of 3.64×10^8 copies/ml.

4.4 Discussion

Quantitation of viruses has previously supported the understanding of virus pathogenesis, disease management and clinical outcome (Berger *et al.*, 2001; Gustafson *et al.*, 2008; Mellors *et al.*, 1996; Sato *et al.*, 2008; Wiseman *et al.*, 2009; Ylitalo *et al.*, 2000). At present, the role of viral load in respiratory infections is unclear. Previous investigations have indicated an association between high RSV viral loads and severe respiratory disease (DeVincenzo *et al.*, 2005; Gueudin *et al.*, 2003; Martin *et al.*, 2008), though contrasting findings have been reported (Wright *et al.*, 2002). Correspondingly, we identified a positive correlation between RSV viral load and disease severity, with RSV viral load being significantly higher (p=0.014) in single infected patients with severe disease compared to patients with mild disease (Chapter 2, section 2.3.4). A similar association was observed in RSV infected patients with co-infections, where either a high RSV viral load or a high viral load of the co-infecting virus was observed in patients with severe disease.

Contrary to these findings, a significant association between hMPV viral load and disease severity was not observed in the present study. This is in disagreement to previous investigations that have indicated a possible relationship between high nasopharyngeal hMPV viral load and more severe disease (Bosis *et al.*, 2008; Gerna *et al.*, 2007c; Kuypers *et al.*, 2005). A possible reason for this discrepancy may be the small sample size evaluated in this study or factors other than viral load may drive hMPV associated disease severity. However, in hMPV infected patients with co-infections, viral load does appear to play a role as higher hMPV viral loads or higher viral loads of a co-infecting virus was more frequently observed in patients with moderate to severe disease, compared to co-infected patients with mild disease. In one instance it was possible to follow an hMPV and hBoV co-infection in a hospitalised patient diagnosed with bronchopneumonia and croup. The high viral loads of hMPV and increasing viral loads of hBoV in multiple specimens confirm that the patient was actively infected at the time. Over a 9 day period the viral loads did not decrease, which may be due to inefficient viral clearance caused by a compromised immunity as the patient was both HIV positive and malnourished. Similarly, it was found that all immunocompromised hMPV co-infected patients had moderate to severe disease. Fatal hMPV infection and hMPV persistence have previously been described in organ transplant recipients and once in an infant with severe combined immunodeficiency

disorder (SCID) (Abed and Boivin, 2008; Debiaggi *et al.*, 2007; Englund *et al.*, 2006), which may suggest that immunocompromised patients are at risk for more severe disease.

The causal role of hBoV in respiratory disease has been debated due to the unusually high co-infection rate associated with hBoV infections as well as its occurrence in asymptomatic children (Calvo *et al.*, 2008; Fry *et al.*, 2007; Garcia-Garcia *et al.*, 2008; Manning *et al.*, 2006). However, we identified hBoV at a high viral load as the sole virus in several patients with respiratory disease, which may confirm its pathogenic potential. This is in agreement to previous studies (Brieu *et al.*, 2008; Calvo *et al.*, 2008; Choi *et al.*, 2006). In this study, higher hBoV viral loads were more frequently observed in patients with moderate to severe disease than in patients with mild disease, suggesting that hBoV occurring at a high viral load may be associated with more severe disease. However, hBoV was also found at low viral loads in three immunocompromised patients with lower respiratory tract disease, which may suggest that even at a low viral load hBoV may cause severe disease in immunocompromised individuals. This is in contrast to a study that proposed that at low concentrations hBoV appears to be an innocent bystander (Gerna *et al.*, 2007b). It was also found that hBoV viral loads in patients with single infections did not differ from patients with co-infections, which is in agreement with the findings of Neske *et al.* (2007) (Neske *et al.*, 2007). However, observations where hBoV viral loads were lower in co-infected patients than in single infected patients, have been reported (Brieu *et al.*, 2008; Christensen *et al.*, 2008; Jacques *et al.*, 2008). The presence of high hBoV viral loads in co-infected patients may be explained by insufficient viral clearance caused by an impaired immune system, since the majority of hBoV infected patients with co-infections were immunocompromised due to either HIV infection or HIV exposure. In addition, one fatal case associated with active hBoV infection was in an immunocompromised infant, who most probably succumbed to sepsis caused by a nosocomial *Staphylococcus aureas* infection.

A previous study has shown that the risk of immunocompromised patients contracting coronavirus were significantly higher compared to immunocompetent patients (Gerna *et al.*, 2006). In the present study, hCoV-NL63 and hCoV-OC43 single infections were only detected in HIV infected or HIV exposed infants suffering from moderate to severe lower respiratory tract infections, which may suggest that

immunocompromised individuals are at risk for more severe hCoV-NL63 and hCoV-OC43 associated disease. Overall, hCoV-NL63 co-infected patients with moderate to severe disease had high viral loads, with the exception of a 12 day old patient with very low viral loads. In this instance, the very young age of the patient may have predisposed him to more severe disease. Van der Hoek *et al.* observed significantly lower hCoV-NL63 viral loads in co-infected patients compared to single infected patients and suggested that the interference effect may be due to direct competition for the same target cell in respiratory organs (van der Hoek *et al.*, 2005). However, we observed that hCoV-NL63 viral loads were equally high in both patients with single- and co-infections, therefore indicating that the presence of another virus does not necessarily inhibit hCoV-NL63 virus proliferation. The majority of hCoV-NL63 co-infected patients were immunocompetent. Therefore this observation could not be ascribed to insufficient viral clearance. In comparison to hCoV-NL63, low viral loads of hCoV-OC43 were frequently observed in co-infected patients. Mild disease was observed in patients with low viral loads of both hCoV-OC43 and the co-infecting virus, whereas moderate to severe disease was associated with high viral loads of either hCoV-OC43 or the co-infecting virus. In addition, patients with single hCoV-OC43 infections had high viral loads. This may suggest that at low viral loads hCoV-OC43 may be associated with less severe respiratory disease, while higher viral loads could result in more severe disease. HCoV-HKU1 was only detected once in the presence of PIV 3. In this instance, the high viral load of a co-infecting virus with known pathogenic potential makes it difficult to determine the role of hCoV-HKU1 in the respiratory disease.

The clinical relevance of viruses detected at low levels by highly sensitive PCR assays has been debated. In the present study, it was observed that, in the absence of a co-infecting virus, hBoV and hCoV-229E infected patients with lower viral loads ($<1\times10^5$ copies/ml) had clinical characteristics associated with moderate to severe respiratory disease, suggesting that viral loads of $<10^5$ copies/ml may still cause clinical disease and thus making it difficult to determine the 'minimum' viral load associated with clinical disease. Therefore, the detection of respiratory viruses at low levels should not be considered inconsequential. A limitation of this study is that the time of sample collection relative to the onset of disease was not known as respiratory viruses were detected and quantified retrospectively. It has previously been shown that viral load may vary with respect to the time of sampling relative to the time of disease onset, with higher viral loads associated with early sample collection (1 or 2 days after onset of

disease) (van der Hoek *et al.*, 2005). In light of this, certain significant associations with viral load may have been overlooked due to differences in sampling time.

In conclusion, the findings of our study illustrate that quantitative studies may aid in the understanding of viral pathogenesis as higher viral loads of respiratory viruses was found in association with disease severity, but also that viruses present at low levels in immunocompromised and immunocompetent patients can also cause disease. The presence of low level respiratory viruses in patients with more severe disease emphasizes the importance of highly sensitive nucleic acid detection of respiratory viruses. This study also suggests that viruses detected during acute infection by PCR may be interpreted as disease causing if a viral load of $>10^5$ copies/ml were present.

Chapter 5

Concluding Remarks

Respiratory viruses are well recognized aetiological agents of severe respiratory tract infections in particularly in children less than 5 years of age. In addition to well recognized respiratory viruses (RSV, PIV, influenza viruses, and adenovirus) recent studies identified additional viruses associated with respiratory tract infections (i.e. hMPV, hBoV, hCoV-NL63 and hCoV-HKU1). Most of these viruses have been exhaustively studied in developed countries. However, the role and contribution of these viruses in developing countries with a high HIV-AIDS burden remains poorly described.

The primary objective of this study was to develop a novel rapid assay that can detect all major viral causes of respiratory infections as well as newly identified viruses recognized as important causes of ALRI in developed countries and to investigate the contribution of respiratory viruses in patients requiring hospitalisation or attending outpatient visits in public sector hospitals serving the Pretoria area, South Africa.

In this study, quantitative multiplex RT-PCR assays were developed for the detection and differentiation of 13 respiratory viruses, well recognized and newly described. Compared to routine IFA assays, the multiplex assays were shown to be highly reproducible with improved sensitivity, specificity and turnaround time. The probe chemistry included in the assays was shown to tolerate nucleotide mismatches and may therefore decrease the occurrence of false negatives. The assays were subsequently applied to investigate the epidemiological characteristics and disease association of the various respiratory viruses in Pretoria, Gauteng, South Africa.

Application of the newly developed multiplex assays facilitated in the investigation of the importance of the recently identified respiratory viruses as well as viruses traditionally associated with URTI. High co-infection rates were associated with these viruses made it difficult to draw significant conclusions about their disease association. However, where identified as single infections, these viruses were associated with hospitalised patients suffering from bronchiolitis, bronchopneumonia and pneumonia. These findings thus demonstrated that the recently described respiratory viruses contribute to ALRI and hospitalisations in children in South Africa. The seasonal distribution of the recently described viruses was less defined than that observed for the well recognized viruses. The age distribution of the various respiratory viruses differed, though it was shown that the most susceptible age group was less than one year of age. Prevalence rates of the different respiratory viruses were comparable to those of

countries with a significantly lower HIV-AIDS burden, yet respiratory viruses occurred at a high frequency in HIV-infected/exposed children who may experience more severe disease. Using molecular screening techniques RSV was demonstrated to contribute significantly more to moderate-to-severe ALRI than previously appreciated with antigen detection techniques and remains the most important viral cause of acute LRTI in hospitalized children in South Africa. In addition, for HIV-infected/exposed children, a high frequency of hospitalization was observed for patients infected with the recently identified respiratory viruses, suggesting a possible correlation with acute LRTI in immunosuppressed patients. However, a limitation of this study is that not all patients had consented to be tested for HIV. In future case control studies in a HIV cohort may determine the impact of these viruses on hospitalization rates in HIV-exposed/infected patients compared to HIV-uninfected patients.

Assessment of the viral load illustrated a positive correlation between viral load and disease severity for RSV. This association was not shared for hMPV and hBoV as single infections. A higher viral load of the co-infecting agents, however, occurred in severe cases of hMPV and hBoV. Even though viral load appeared to be associated with increased disease severity, low viral loads were also observed in patients with severe ALRI. These findings illustrated that quantitative studies may aid in the understanding of viral pathogenesis as higher viral loads of respiratory viruses was found in association with disease severity, but also that viruses present at low levels in immunocompromised and immunocompetent patients may also cause disease. In future, quantification of serial specimens may aid in further understanding the phase of infection, virus proliferation and pathogenesis in these patients. In addition, the presence of low level respiratory viruses in patients with more severe disease emphasizes the importance of highly sensitive nucleic acid detection of respiratory viruses. This study also suggests that viruses detected during acute infection by PCR may be interpreted as disease causing if a viral load of $>10^5$ copies/ml were present. A limitation of the study is that the time of sample collection relative to disease onset was not known seen as respiratory viruses were quantified retrospectively. As viral load may vary with respect to the time of sampling relative to time of disease onset, certain significant associations with viral load may have been overlooked due to differences in sampling time. Although in this study it could be assumed that the specimens were taken when patients were admitted to hospital.

Overall, the findings of the present study illustrated the application of the multiplex real-time PCR assays with FRET hybridization probes to detect respiratory viruses and the associated viral load. Subsequent application of these assays demonstrated that viruses not currently included in routine diagnostic assays contribute to ALRI and hospitalisation in children in South Africa. The inclusion of these viruses in routine diagnostic procedures should therefore be encouraged.

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