CLINICAL EPIDEMIOLOGY OF NEWLY DISCOVERED RESPIRATORY VIRUSES

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DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science (Dissertation) in the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted for any degree or examination at any other Universities.

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Signature of candidate

_____ Day of January 2012

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List of abbreviation

dUTP	Deoxyuridine-triphosphatase nucleotides
cDNA	Complementary Deoxyribonucleic acid
CRP	C-reactive Protein
CXR	Chest X-Radiography
CXR-AC	Alveolar Consolidation on Chest Radiography
HBoV	Human Bocavirus
BPV	Bovine Parvovirus
HCoV-HKU1	Human coronavirus HKU1
HCoV-NL63	Human coronavirus NL63
HCoV-229E	Human coronavirus 229E
HCoV-OC43	Human coronavirus OC43
HIV	Human Immunodeficiency Virus
HMPV	Human Metapneumovirus
HRV	Human Rhinovirus
KIPyV	Polyomavirus KI
LRTI	lower respiratory tract infections
MVC	canine minute virus
NPA	Nasopharyngeal Aspirates
PIV	Para influenza Viruses 1 and 3,
PCT	Procalcitonin
PCR	Polymerase chain reaction
RSV	Respiratory Syncytial Virus
RT-PCR	Reverse-Transcription Polymerase Chain of Reaction
PCV	Pneumococcal Conjugate Vaccine
PFU	Plaque Forming Units
RTI	respiratory tract infections
UNG	Uracil-N-glycosylase
URTI	Upper respiratory tract infections
WUPyV	Polyomavirus WU

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Chapter 1: Abstract

Background: Lower respiratory tract infections (LRTI) are a leading cause of morbidity and mortality in young children. A number of new viruses associated with LRTI in young children have recently been discovered. These include Human Bocavirus (HBoV), Human Polyomavirus strains WU (WUPyV) and KI (KIPyV) and Human Coronavirus strains NL63 (HCoV-NL63) and HKU-1 (HCoV-HKU-1). There is, however, limited data on the epidemiology of these newly discovered respiratory viruses in industrializing country settings, including South Africa.

Objective: To determine the clinical epidemiology of HBoV, HCoV-NL63, HCoV-HKU1, HCoV-OC43, HCoV strain 229E (HCoV-229E), WUPyV, KIPyV and human rhinovirus (HRV) in young children.

Methods: Nasopharyngeal aspirates where taken from children who were hospitalized at Chris Hani Baragwanath Hospital between February 2000 and January 2002 with severe LRTI. These children had been enrolled in a double-blind, randomized, placebo-controlled trial of a 9-valent pneumococcal conjugate vaccine (PCV). Nucleic acid extraction was undertaken from archived nasopharyngeal aspirate samples and the respiratory viruses identified using real time duplex PCR. The study was limited to examining samples from HIV uninfected children with LRTI who were less than 24 months of age.

Results: Overall, samples were available for 895 of 1565 nasopharyngeal aspirates, from children hospitalized with LRTI, collected from February 2000 to January 2002. A comparison between those LRTI episodes for which samples were unavailable compared to those for which samples were available indicated that children in whom samples were unavailable were younger than children with available samples (9.9±6.4 vs. 11.8±6.5

months; p<0.0001). In addition there was a higher frequency of wheezing episodes in children for whom samples were unavailable (60.4 vs. 54.6%; p=0.022).

The overall prevalence of the viruses in children with any LRTI were 33.2% for HRV, 21.2% for HBoV, 16.1% for WUPyV, 10.1% for HCoV-OC43, 7.0% for KIPyV, 3.2% for HCoV-NL63, 2.6% for HCoV-HKU-1, and 0.6% for HCoV-229E. There was a higher probability of detecting a selected virus in LRTI episodes among PCV-compared to placebo-recipients for HBoV (24.2% vs. 18.2%, respectively; p=0.028) and HRV (36.7% vs. 29.5%, respectively; p=0.023). Conversely, viruses identified more frequently in LRTI episodes among children who received placebo compared to PCV-recipients included WUPyV (20.2% vs. 12.1%, respectively; p=0.001), KIPyV (10% vs. 4.2%, respectively; p=0.001), HCoV-OC43 (14.1% vs. 6.2%, respectively; p≤0.0001) and HCoV-HKU1 (4.5% vs. 0.1%, respectively; p≤0.0001).

Overall, the prevalence of the studied-viruses in the subgroup of children categorized as having bronchiolitis was 33.8% for HRV, 33.4% for WUPyV, 22.3% for HBoV, 11.1% for HCoV-OC43, 5.3% for KIPyV, 2.3% for HCoV-NL63, 1.9% for HCoV-HKU1 and 0.4% for HCoV-229E. Viruses more commonly identified in placebo- compared to PCV-recipients among children hospitalized with bronchiolitis included WUPyV (20.0% vs. 12.3%, respectively; p=0.029), HCoV-OC43 (15.9% vs. 7.2%, respectively; p=0.004) and HCoV-HKU1 (3.6% vs. 0.5%, respectively; p=0.015).

The prevalence of the newly studied viruses in the subgroup of children categorized as having clinical pneumonia was 30.8% for HRV, 20.3% for HBoV, 16.4% for WUPyV, 9.1% for HCoV-OC43, 8.6% for KIPyV, 4.1% for HCoV-NL63, 3.2% for HCoV-HKU1 and 0.6% for HCoV-229E. Viruses identified more frequently among placebo- compared to PCV-recipients, in those with clinical pneumonia, included WUPyV (20.4% vs. 11.9%, respectively; p=0.013), KIPyV (12.7% vs. 4.1%, respectively; p=0.001),

HCoV-HKU1 (5.3% vs. 0.9%, respectively; p=0.008). Conversely, HCoV-OC43 was identified more frequently in children with clinical pneumonia among PCV- (5.0%) compared to placebo-recipients (2.7%, p=0.004).

There were seasonal peaks, during autumn-winter months (April to June), in the detection of HRV, WUPyV, HCoV-OC43, HCov-NL63 and HCoV-HKU1, whereas KIPyV, HBoV and HCoV-229E were identified perennially.

Conclusion: Prevalence of respiratory viruses is high in industrializing countries and the presence of these viruses is frequently associated with co-infections of more than one etiological agent. In industrializing countries such as in South Africa, the recently identified respiratory viruses play a role in development of pneumonia.

Chapter 2: Background

Acute respiratory infections (ARI) are the main cause of childhood hospitalization and death worldwide, particularly in industrializing countries. In 2000 global estimates of child death showed that 1.9 million children died from ARI and 70% of those occurred in industrializing countries (Williams et al 2002). In 2008 an estimated 5.97 million children less than 5 years of age died due to infectious diseases, including 1.57 million (15% of all under-5 years childhood deaths) because of pneumonia in Africa, Eastern Mediterranean, Southeast Asia, and the Western Pacific. Whereas in industrialized countries including Europe and America, 10% of childhood deaths were due to pneumonia (Black et al 2010). The study by Black et al. also showed that one third of neonatal deaths were due to various infectious causes, including pneumonia which accounted for 4% of deaths in neonates (Black et al 2010). A systematic review on the burden of pneumonia indicated that the incidence of pneumonia in low-income countries was 0.28 episodes/child-year in children less than 5 years of age (Rudan et al 2004). This incidence results in 150.7 million new cases of clinical pneumonia, with 11-20 million (7-13%) severe cases requiring hospitalization. However, in developed countries the incidence of community acquired pneumonia was 0.026 episodes/child-year, resulting in 2 million new cases in children less than 5 years of age (Rudan et al 2004).

Major presentations of lower respiratory tract infections (LRTI) that account for the global burden of disease among young children are bronchiolitis and pneumonia, as defined by the Child Health Epidemiology Reference Group under World Health Organization (Rudan et al 2004). The high mortality due to LRTI in industrializing countries is also associated with a high incidence of pneumonia in these countries (Jokinen et al 1993; Weigl et al 2003). The heightened susceptibility to pneumonia in children from developing countries may include increased exposure to risk factors such

as indoor pollution, overcrowding, malnutrition, low coverage with measles vaccine and other vaccines targeting respiratory pathogens, low birth weight and non-exclusive breastfeeding (Bruce et al 2007; Mulholland et al 2008; Rudan et al 2008).

Advances in medical science have led to the development of vaccines that target known bacterial pathogens including *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib). Vaccines such as the 9-valent pneumococcal conjugate vaccine (PCV) have been shown to reduce the incidence of radiologically confirmed pneumonia in HIV-uninfected children under 5 years by 85%, and by 65% in HIV-infected children in South Africa (Klugman et al 2003). Pneumococcal conjugate vaccines were developed following the successful use of Hib conjugate vaccine in children. PCV-7 (Prevenar) was developed from the seven serotypes that caused the most invasive pneumococcal disease in children in the United Stated and it consists of seven pneumococcal polysaccharides conjugated to an immunogenic carrier protein (Zangwill et al 1996). It has been shown that PCV-7 reduces overall invasive pneumococcal disease by 89.1% (Black S. 2000), with an overall reduction of 20.5% in chest radiograph infiltrates and a 4.3% overall reduction in clinically diagnosed pneumonia (Black et al 2002). In addition a 9-valent pneumococcal conjugate vaccine (PCV-9) was used in Phase III clinical trials in South Africa and Gambia. The PCV-9 includes two extra serotypes in addition to the 7 serotypes from Prevenar vaccine thus resulting in the 9-valent PCV. Using different definition of pneumonia PCV has been shown to reduce pneumonia by 58.1% (Madhi et al 2005) and 76.5% (Cutts et al 2005). Hence, these studies have shown that PCV is effective at preventing radiologically confirmed pneumonia (Klugman et al 2003; Madhi et al 2005).

PCV has also been shown to reduce superimposed pneumococcal infections in children who had been infected with respiratory viruses (Madhi et al 2004). In South Africa the PCV vaccine trial

showed that children with respiratory virus were 31% less likely to be hospitalized for pneumonia. Specifically, PCV reduced pneumonia associated with influenza A/B by 45%, parainfluenza viruses (types 1-3) by 44% and respiratory syncytial virus by 22% (Madhi et al 2004). This study indicated that there was no association of all cause and virus specific bronchiolitis between placebo and PCV-vaccinated children. Hence, data from Madhi et al., implies that pneumonia associated with viruses is a result of bacterial co-infections (Madhi et al 2004). In agreement other studies have shown that hospitalized children with pneumococcal pneumonia may also have viral respiratory infections (Madhi et al 2004; Peltola et al 2004).

Respiratory infections are classified by their symptoms and anatomical site of presentation: upper respiratory tract infections and lower respiratory tract infections. Upper respiratory tract infections (URTI) are infections that occur in sinus, pharynges, epiglottis and laryngotrachie. These infections are self limiting and transitory. They are caused by various organisms including viruses. The viruses infect the upper respiratory tract by invading the mucosa epithelial cells causing destruction, sloughing and loss of cilliary activity. Hence this leads to the obstruction of the paranasal sinuses resulting in sinusitis. Large amounts of proteins and immunoglobulins are excreted as a result of cytokines response. The destruction of the epithelial lining due to virus invasion also contributes to the development of otitis media, as the destruction increases the chance of normal bacterial flora of the mucosa to multiply and invade the epithelial lining. Symptoms associated with URTI include sneezing, sore throat, nasal discharge, cough, headache and myalgia. The symptoms occur after incubation period of 48-72 hours and vary with age and type of virus; complications are rare in URTI (Baron S 1996).

The lower respiratory tract infections (LRTI) include bronchitis, bronchiolitis and pneumonia. Bronchitis and bronchiolitis are due to the inflammation of the mucosa in the bronchial tree, the mucosa becomes hyperemic and edematous and copious bronchial secretions are produced. This leads to the loss of mucociliary function and destruction of respiratory epithelium. Eventually the bronchial walls thicken and the bronchial lumen is narrowed due to excretion of necrotic material, resulting in the obstruction of airways. The narrowing of the bronchial lumen is common in children. The symptoms of bronchitis and bronchiolitis include: cough, coryza, fever, increased respiratory rate, grunting, wheezing and retraction of chest walls. Pneumonia is the inflammation of the lung parynchema and can be grouped into lobar pneumonia (alveolar processes of the entire lobe) and bronchopneumonia (alveolar processes of parts of the lobe). Aerosols of the pathogen causing pneumonia are inhaled into the lower respiratory tract where they infect and multiply in the alveoli, and cause various symptoms including: fever, chest pain, cough, shortness of breath and production of sputum. Other symptoms depend on the type of infectious organism and age of patient; these include confusion, headache, abdominal pain, nausea, vomiting and diarrhoea.

The common symptoms occurring in both upper and lower respiratory tract infection can be described as common cold like symptoms and studies have shown that the only difference between the viral infections causing common cold like symptoms and pneumonia, is the duration of the incubation period (Tyrrell DAJ 1959; Tyrrell DAJ 1993).

Pneumonia may be caused by a diversity of pathogens, including viruses, bacteria and fungi. Initial studies identified *Streptococcus pneumoniae* and *Haemophilus influenzae* as the main bacterial causes of pneumonia (Del Beccaro et al 1992). Other bacteria causing pneumonia include *Staphylococcus aureus,* non-typhoid Salmonella species and less frequent other gram negative

pathogens such as *Klebsiella pneumoniae* and *Eschericia coli* (Bartlett JG 2008). *S. pneumoniae* is the most common bacterium identified in most age groups of children with pneumonia in the absence PCV vaccination (Mulholland et al 1999; Klugman et al 2003). Recent studies have, however, indicated that a significant proportion of pneumococcal pneumonia may be associated with concurrent respiratory viral infections (Henrickson 1998; Madhi et al 2004; Arnold et al 2006; Fry et al 2007). The focus of this project was to characterize the clinical epidemiology of recently discovered respiratory viruses in South African children hospitalized for LRTI, for which there is limited information from South Africa (Smuts and Hardie 2006; Smuts et al 2008; Venter et al 2009).

Studies have shown that the prevalence of respiratory viruses in children with pneumonia decreases with age, with highest prevalence in infants (Henrickson KJ. Viral pneumonia. In: Wald E 1998). Respiratory viruses have been identified in 50% to 90% of young children hospitalized with LRTI including bronchiolitis and pneumonia (Henrickson KJ. Viral pneumonia. In: Wald E 1998; Arnold et al 2006; Fry et al 2007). Respiratory viruses including respiratory syncytial virus (RSV), parainfluenza viruses (PIV) 1 and 3, adenoviruses (AdV), influenza A, rhinovirus (RV), and human metapneumovirus (HMPV), have been collectively identified in up to 90% of pneumonia cases occurring in the first year of life, whereas this declines to 50% in school-aged children and 12% in adults (Murphy et al 1981; Denny et al 1986; Cilla et al 2008; Smuts et al 2008).

Traditionally, isolation and identification of viral relied mainly on cell culture or antigen detection by immunofluorescence assays. Cell culture was considered the gold standards for the identification of respiratory viruses because of its specificity. However it has technical limitations related to timeliness of sample preparation, sample transportation and cell culture conditions including cell-lines used in culture. Additionally, not all respiratory viruses such as RV are easily cultured due to large number of serotypes (Johnston et al 1995). Hence, further molecular and immunological tests were explored to

determine the role of viruses in pneumonia cases. Immunological assays such as direct and indirect immunofluorescence have been developed to detect viral antigens. Although immunofluorescence assays are as specific as cell culture, they have limitations, including the variability of sensitivity in identifying different viruses which range between 48 to 100% (Wong et al 1982)

The discovery of new respiratory viruses has gained momentum following advances in molecular detection of potential pathogens compared to the past reliance on culture and antigen detection of respiratory viruses. Polymerase chain reaction (PCR) has improved the sensitivity and specificity of detection of viral respiratory pathogen from different sample types (Heikkinen et al 2002; Lambert et al 2008). Real time PCR is rapid, more sensitive and specific in detecting respiratory viruses than antigen detections and cell culture (Jennings et al 2004). Real time PCR results from patients in a clinical setting indicates a true reflection of the occurrence of a respiratory infection (Jartti and Korppi 2011). Another advantage with real time PCR techniques includes the ability to detect multiple viruses simultaneously also including viruses that have multiple serotypes such as RV. Commercial kits are available for multiplex PCR assays for most previously known respiratory viruses. More recently a number of new respiratory viruses have been identified, and they have been variably associated with pneumonia in industrialized country settings. The newly discovered respiratory viruses studied in this project are: human coronavirus NL63 (HCoV-NL63) discovered in 2004 (van der Hoek et al 2004)), human coronavirus HKU-1 (HCoV-HKU1) discovered in 2005 (Woo et al 2005; Sloots et al 2006), human bocavirus (HBoV) discovered in 2005 (Allander et al 2005), human polyomavirus WU (WUPyV) and KI (KIPyV) discovered in 2007 (Allander et al. 2007; Gaynor et al 2007). We also included in this study the human coronavirus-229E (HCoV-229E), human coronavirus-OC43 (HCoV-OC43) and human rhinovirus (HRV) for which there is also only limited information from developing countries.

2.1 Human Bocavirus

2.1.1 Molecular background

Human bocavirus (HBoV) belongs to the Parvoviridae family, subfamily Parvovirinae and genus Bocavirus. The family of Parvoviridae consists of the subfamilies densovirinae and parvovirinae. The Parvovirinae subfamily consists of 5 genera including the genus Bocavirus (Allander et al 2005). The genus Bocavirus consists of bovine parvovirus (BPV), canine minute virus (MVC) and human bocavirus. Bocavirus is the second virus from parvoviridae family that is known to cause disease in humans, after the parvovirus B19 which causes erythema infectiosum in childhood. The name evolved from two members of Bocavirus genus, the bovine ("bo") and canine ("ca") parvoviruses. Bocaviruses' DNA is distinct from the non-human viruses but shares homology with the bovine virus and canine virus. The genomic organization of bocavirus consists of two major open reading frames (ORF), encoding the viral capsid proteins (VP1/VP2) and non-structural genes (NS1 and NP1). Another additional ORF is located in the middle of HBoV genome encoding a nonstructural protein (NP-1) that was found to have 47% amino acid similarity to that of MVC and BPV (Allander et al 2005). Bocavirus surface proteins and non-structural genes are highly conserved (Lau et al 2007). Thus molecular epidemiology of bocavirus reveals very little changes within the virus genome. A study in California has shown that there is little variation in the NP1 and NS1 genes of bocavirus over a period of 21 months (Arnold et al 2006). The two genotypes identified in the later study and were identical to genotype ST1 and genotype ST2 previously described (Allander et al 2005; Kesebir et al 2006). In South Africa, phylogenetic analysis of the viral capsid gene revealed that the strains from South Africa (Smuts and Hardie 2006)

align closely with genotype ST2 previously described (Allander et al 2005). Hence this illustrates that bocaviruses circulating around the world are similar. A single lineage of HBoV has been shown to be responsible for both respiratory tract and enteric infections in humans (Lau et al 2006; Lau et al 2007).

2.1.2 Clinical Significance

A summary of selected studies undertaken in children on HBoV is described in Table 2.1. HBoV has been isolated from pooled nasopharyngeal aspirate specimens from children with pneumonia, bronchiolitis and asthma (Allander et al 2005; Kesebir et al 2006; Fry et al 2007). Studies have reported prevalence of HBoV to be 1 to 8% in children less than 5 years with pneumonia (Allander et al 2005; Smuts H and Hardie D 2006; Fry et al 2007) and as high as 19% in children with acute wheezing (Allander et al 2007). Clinical symptoms associated with bocavirus include hypoxia, fever, acute wheezing and abnormal findings on chest radiographs (Kesebir et al 2006). Most studies indicate that HBoV as an autumn/winter seasonality (Allander et al 2005; Smuts H and Hardie D 2006). HBoV has been identified globally in children with LRTI, including in a previous study from South Africa, Table 2.1 (Allander et al 2005; Lu et al 2006; Smuts H and Hardie D 2006; Lau et al 2007). Co-infections of HBoV with other respiratory viruses are common (37% - 83%) in many studies (Allander et al 2005; Kesebir et al 2006; Smuts H and Hardie D 2006; Fry et al 2007). High viral loads of HBoV are associated with pneumonia and acute wheezing in children (Allander et al 2007; Fry et al 2007). An another study it was recommended that HBoV has a similar epidemiological profile as RSV in children (Manning et al 2006).

The role of HBoV as a potential pathogen causing pneumonia is, however, controversial. Kesebir et al., reported the presence of HBoV in 5% of specimens that tested negative for common respiratory viruses and in 0% of specimens from asymptomatic participants (Kesebir et al 2006). In contrast, HBoV was identified in 3.9% of children with LRTI and 1% of asymptomatic children in Thailand (Fry et al 2007). A study in Netherlands also identified HBoV in 5.1% of children with LRTI and 4.8% in children less than 5 years of age without LRTI (van de Pol et al 2009). In addition, HBoV has been identified in stool specimens in children with diarrhoea (Arnold et al 2006; Lau et al 2007). There have only been limited studies in industrializing countries which have examined the prevalence of HBoV in either asymptomatic or symptomatic individuals. In South Africa, HBoV was identified in 11% (38 out of 341) of children less than 2 years old diagnosed with pneumonia (Smuts H and Hardie D 2006).

Table 2.1: Summary of clinical studies on Human Bocavirus

Reference	Study site	Study Year	n	Illness description	HBoV Prevalence	Age in years	Co-infections
(Allander et al 2005)	Sweden	05	540	Asthma, wheezing & bronchiolitis	3.1%	266 paediatrics & 112 adults	Out of 14: 2 HBoV & RSV and 1 HBoV & Adenovirus
(Kesebir et al 2006)	USA	04	425	LRTI	5.2%	<2	Not given
(Fry et al 2007)	Thailand	07	1680	Pneumonia	4.9%	0-65	Out of 22 positives: 1 HCoV-NL63 in control patient, 4 Infl A, 3 HRV, 2 Adenovirus, 1 HPIV, 1 HPIV 3/Infl B, 1 HPIV 3/Adenovirus, 1 HMPV/HRV/HCoV-NL63
(Smuts and Hardie 2006)	South Africa	06	35	LRTI including pneumonia	11%	2-12	Co-infections with RSV 35%, HRV 10%, Flue 13%, HCoV 8% & AdV3%.
(Manning et al 2006)	United Kingdom	05-06	924	URTI	8.2%	<5	7 AdV & 6 RSV, AdV & 1 HPIV 1-3, 4 Influ B & Influ A 1. 2 RSV-Adenovirus
(Lau et al 2007)	China	04-05	400	Hospitalized patient	6.5%	<5	14/93 HBoV-HRV and 2/93 HBoV-HMPV

LRTI: lower respiratory tract infection
URTI: upper respiratory tract infection
n study population size

2.2 Human Polyomaviruses: WUPyV and KIPyV

2.2.1 Background

Human polyomavirus belong to family *Polyomaviridae*; are small non-enveloped double-stranded DNA viruses that infect a variety of avian, rodent and primate species. In immunocompromised patients, viruses from the family *Polyomarividae* induce disease and can be oncogenic (Gardner et al 1971; Padgett et al 1971; Allander et al 2007; Gaynor et al 2007; Feng et al 2008). There are five polyomaviruses that are known to date and these are: BKV (Gardner et al 1971), JCV (Padgett et al 1971), KIPyV (Allander et al. 2007), WUPyV (Gaynor et al 2007) and MCV(Feng et al 2008). BKV causes nephropathy and is associated with renal and urinary tract disorders such as tubular nephritis that can lead to hemorrhagic cystitis. JCV causes primary multifocal leukoencephalopathy (Arthur et al 1986). Molecular genetics of the WUPyV and KIPyV indicate that the two viruses are similar. Both viruses define a novel branch within the *Polyomaviridae* family, and the WUPyV/KIPyV branch is closely related to primate polyomaviruses (Gaynor et al 2007)**.**

2.2.2 Clinical significance

The WUPyV and KIPyV are the only polyomaviruses from the *Polyomaviridae* family that have been isolated from nasopharyngeal aspirates of children with LRTI. A sero-prevalence study indicated seroprevalence in children under 5 years of age of 54% for WUPyV and 56% for KIPyV, similar results were obtained in adults older than 21 years for KIPyV (55%) for and WUPyV (69%) (Kean et al 2009). This study indicated that infection with these viruses occur

early in childhood. Another study identified WUPyV in 2.07% and KIPyV in 1.07% of patients under 15 years of age with upper or lower respiratory infections and >45 years with upper respiratory infections (Abedi Kiasari et al 2008). It has been suggested that polyomaviruses may establish persistent infections in the respiratory tract and become reactivated during respiratory disease of any cause (Abedi Kiasari et al 2008).

A few selected recent published studies on polyomavirus, summarized in Table 2.2, indicate that the prevalence of KIPyV ranges from 1.5% to 2.5% in children less than 5 years of age with LRTI (Bialasiewicz et al 2007; Norja et al 2007) and the prevalence of WUPyV ranged from 0.4% to 7% (Han et al 2007; Lin et al 2008). The identification of KIPyV and WUPyV and its association in the pathogenesis of LRTI in children is also controversial because of a similar prevalence found in ill and asymptomatic children. KIPyV and WUPyV were identified in 0% and 2.6%, respectively, in children with LRTI; and 4.8% and 2.4% respectively in asymptomatic children (van de Pol et al 2009). Another study identified WUPyV in 4.2% of asymptomatic children compared to 7% in children with LRTI (Han et al 2007). A comparison of asymptomatic and symptomatic patient was performed in another study, and it was found that the frequency of KIPyV/WUPyV in children without LRTI was higher (11%) compared to in children with LRTI (7%), although asymptomatic children were older and immunosuppressed compared to the LRTI cases (Norja et al 2007).

Polyomaviridae is known to induce disease in immunocompromised individuals. A cross-sectional study conducted in 300 South African children with LRTI identified WUPyV in 7% and KIPyV in 1% in children less than 2 years old, among whom the prevalence of HIV was 57% and 33% in WUPyV and KIPyV cases, respectively (Venter et al 2009). The study also

identified 5 strains of WUPyV that were unique to South Africa, which clustered with 4 genotypes of existing strains. Most of the WUPyV strains from the study clustered into genotype 1. The KIPyV strains from South Africa were identical to that in Sweden and Australia (Venter et al 2009).

Table 2.2 Summary of clinical studies on Human Polyomaviruses

Reference	Study site	Study Year	n	Illness description	Prevalence of Polyomaviruses	Age in years	Co-infections
(Allander et al 2007)	Sweden	04-05	1300	RTI	0.5%	0-90	3 RSV, 1 HMPV, 1 Influ A
(Gaynor et al 2007)	Australia and USA	03 Australia, 03-06 (USA)	245 (Australia) 410 USA	ARTI	3.6% (Australia) 1.2% (USA)	4-53 (Australia) 4-51 (USA)	HBoV and HRV
(Bialasiewicz et al 2007)	Australia	02-03	951	RTI	2.5% KI	0-95	4 RSV, 1 Influ A, 1 HMPV
(Norja et al 2007)	United Kingdom	07	893	Symptomatic & asymptomatic group	1.11 WUPyV/ 1.56 % KIPyV	< 5	120 RSV, 4 Influ A, 46 Influ B, 35 HPIV, 80 AdV
(Venter et al 2009)	South Africa	06-07	300	LRTI	WUPyV 7% & KIPyV 1%	0-72	[a]6 HRV, 3 HBoV, 2 RSV, 3HBoV-AdV, 2 PIV 1-3, 2 Influ A
(Lin et al 2008)	China	05-06	278	Acute LRTIs	0.4% WUPyV & 0% KIPyV	< 5	Not given
(Abedi Kiasari et al 2008)	United Kingdom,	06-07	371	RTI	KIPyV 2.7% & WUPyV 1.08%	0-79	[b]4 HMPV, 1 HBoV, 1 PIV1, 1 RSV-B

RTI: respiratory tract infection

ARTI: acute respiratory tract infection

LRTI : lower respiratory tract infection

[a] Co-infections were determined in 15/21 (71%) and 2/3 of WUV- and KIV-positive specimens, respectively

[b] HMPV and HBoV were co-infection with WUPyV, HPIV and RSV-B were co-infection with KIPyV

2.3 Human Coronaviruses: HCoV-NL63, HCoV-OC43, HCoV-229E and HCoV-HKU1

2.3.1 Background

Human Coronaviruses are spherical enveloped viruses. Their envelope consists of studded glycoproteins that enclose a matrix which contains a single stranded positive sense RNA associated with nucleoproteins. The glycoproteins have antigenic epitopes that are recognized by the host immune system and account for the diversification of Coronaviruses. The human Coronaviruses belong to the family of *Coronaviridae* as a result of the glycoprotein appearance on the electron microscope (Baron S 1996). The genomic structure of Coronaviruses comprises regions 1a and 1b (encodes non-structural proteins), S (encodes Spike protein), ORF3, E (encodes envelope protein), M (encodes membrane proteins) and N (encodes nucleocapsid protein) genes (van der Hoek et al 2004). Human coronaviruses can be divided in three distinct groups: Group I coronaviruses are HCoV-229E and HCoV-NL63, group II coronaviruses are HCoV-OC43 and HCoV-HKU-1, group III consist of severe acute respiratory syndrome (SARS) virus, which represents an early split from group II (Pyrc et al 2007). In group I all genes for HCoV-NL63 share sequence similarity with HCoV-229E with the exception of the M gene (van der Hoek et al 2004).The GC content of Coronaviruses genome range from 32% (group II) to 34% (group I) (van der Hoek et al 2004; Woo et al 2005).

SARS was the first of the Coronaviruses to have crossed the species barrier from infected civet cats to humans, and to cause a severe lung disease (Li et al 2005). In 2003, SARS caused

800 deaths and 8000 illnesses and has been shown to be absent from human populations (van der Hoek et al 2007). Coronaviruses HCoV-OC43 and HCoV-229E have been previously associated with mild URTI. The later viruses have been circulating in human populations for several years, these viruses are easily cultured and have been shown to cause disease in humans (Tyrell DAJ and Bynoe ML 1965), hence following Koch's postulate stating that: a microorganism must be present in organisms suffering from disease, the microorganism must be isolated from a diseased organism and grown in pure culture, the microorganism should cause disease when introduced into a healthy organism, and lastly the microorganism must be re-isolated from the inoculate of diseased host and shown to be identical to the original causative agent. HCoV-NL63 was initially isolated from a 7 month old girl with coryza, conjunctivitis, fever and bronchiolitis (van der Hoek et al 2004) and HCoV-HKU1 was discovered in respiratory secretions from a 71 year old man with chronic respiratory tract disease, hospitalized with fever, cough and infiltrate chest radiograph (Woo et al 2005). HCoV-NL63 has been shown to grow in monkey kidney culture (van der Hoek et al 2004). HCoV-HKU1 does not fulfil Koch's postulate due to the unavailability of cell culture methods to grow the viruses and there is no animal model available. Hence it is important to establish significant association between the virus and the disease using a control group. Most of the studies of the later viruses do not have a control group involved in the investigation; hence only indicate the occurrence of the virus in ill patients.

The human coronaviruses have a worldwide distribution. These respiratory viruses generally cause mild symptoms, but have a potential to cause severe disease in young children, the elderly and immunocompromised individuals (van Elden et al 2004). Coronaviruses cause infection by invading the respiratory epithelial cells which cause cilia damage. This triggers an

inflammatory response, resulting in nasal secretion, inflammation and swelling. Common symptoms of coronavirus infection are common cold symptoms including sneezing and obstruction of the airways. Immunity against coronavirus infection does not persist; individuals infected with coronaviruses can be infected again with the virus later in the season (Baron S 1996). Coronaviruses infections occur primarily in the autumn and winter seasons.

2.3.2 Clinical Significance

Infections with Coronaviruses have been documented in various studies from both children and adults, including symptomatic and nonsymptomatic individuals. Prevalence of HCoV-229E, HCoV-NL63 and HCoV-OC43 was 0%, 5% and 5% respectively in children less than 2 years of age with LRTI and 0%, 1.4% and 2.8%, respectively in children with URTI (Talbot et al 2009). The latter study suggested that HCoV-OC43 and HCoV-NL63 may be associated with LRTI in children. In support of the association another similar study indicated that the later viruses occur in high frequencies in children with LRTIs and URTIs and that these viruses are prone to cause severe illness in children (van Elden et al 2004). HCoV-NL63 was frequently associated with croup together with influenza virus or PIV-1, in children less than 5 years of age with acute respiratory disease (Choi et al 2006). In another study the prevalence of HCoV-NL63 in children under 16 years of age with acute respiratory disease was 1.7%, in which 31% of the cases presented with croup and 21.4% had bronchiolitis (Han et al 2007). The prevalence of HCoV-HKU1 was 19.2% in patients with acute respiratory disease (Gerna et al 2007). Although a high prevalence of HCoV-HKU1 was reported some studies have indicated lower detection rates of HCoV-HKU1 (Woo et al 2005). However it is suggested that prolonged study periods for investigation of respiratory tract infection should be

implemented in order to estimate prevalence of respiratory infection (Lau et al 2007). HCoV-NL63 and HCoV-HKU infections have been shown to occur in 18% and 30% respectively of febrile seizure cases in children with acute respiratory infection (Chiu et al 2005; Lau et al 2006; van der Hoek et al 2006). HCoV-NL63 has been implicated in kawasaki disease, but no confirmatory study can link the virus to the disease (van der Hoek et al 2006). In group I infections HCoV-229E antibodies have been shown to occur in lower levels as compared to the HCoV-NL63, whereas in group II infections HCoV-OC43 induces high levels of antibodies compared to HCoV-HKU1 (Lau et al 2006).

There are few data for HCoV-NL63 and -HKU1 from South Africa. A study by describing the prevalence of HCoV-NL63 found 2.4% HCoV-NL63 positive children with acute wheezing (Smuts et al 2008). The study also indicates co-circulation of two genotypes HCoV-NL63 A and B lineage; similar findings were observed in Hong Kong (Woo et al 2005).

Table 2.3: Clinical studies on Human Coronaviruses

Reference	Study site	Study Year	n	Illness description	Prevalence of Coronaviruses	Age in years	Co-infections
(van der Hoek et al 2004)	Netherlands	03	614	URTIs or LRTIs	1.14% HCoV-NL63	<1	1 RSV and 1 *Pneumocystis carinii*
(van Elden et al 2004)	Netherlands	03	545	RTI	11% of	Not given	Not given
(Choi et al 2006)	Korea	00-05	515	ARTI	1.6% HCoV-NL63	≤5	#Not given for Human coronaviruses
(Han et al 2007)	United Kingdom	04-06	827	ARTI	1.7% HCoV-NL63	<5	Not given
(Gerna et al 2007)	Italy	03-05	823	RTI	50.9% HCoV-OC43, 26.4% HCoV- 229E, 17% HCoV-NL63, 5.7% nontypeable	1-21	Not given

RTI: respiratory tract infection

ALRTI: acute lower respiratory tract infection

LRTI: lower respiratory tract infection

URTI: upper respiratory tract infection

(Gerna et al 2007) identified HCoV co-infections was identified in 29% of children in first year of life and co-infections were associated with severe respiratory syndromes.

(Han et al 2007) showed that HCoV-NL63 positive patients were diagnosed as croup in 9 (64.2%), bronchiolitis in 3 (21.4%), bronchial asthma exacerbation in 1, and pneumonia in 1. Only single infections were observed from the study

Co-infections were investigated for HBoV with other viruses

2.4 Human Rhinovirus

2.4.1 Molecular background

Human rhinovirus (HRV) is a small non-enveloped single stranded positive sense RNA virus belonging to the *Picornaviridae* family and Enterovirus genus. previously There were 100 immunologically distinct serotypes of rhinoviruses of which 74 belong to HRV-A species, 25 to HRV-B species and a single serotype belonging to the recently identified HRV-C species are associated with more severe respiratory illness (Xiang et al 2008). Currently there are additional strains that have been added to the previous four subgroup namely subgroup A, B, C, uncharacterised clade D which is found to be part of the HRV-A serotype with over 150 strains of human rhinovirus (Lee et al 2007; Palmenberg et al 2009). The newly discovered HRV-C species was implicated as an important cause of febrile illness, wheezing episodes and asthmatic exacerbations in hospitalized children (Lau et al 2007). Using multiplex PCR assays, rhinovirus was identified in 59.7% of 181 nasopharyngeal aspirate samples in children less than 2 years of age with respiratory illness (Lee et al 2007).

2.4.2 Clinical significance

Rhinovirus was initially isolated from individuals with common colds in 1956. Over the past 50 years advances in molecular techniques, have improved the characterization of the clinical spectrum of rhinovirus illness. HRV has been associated with medically significant illness' including acute otitis media, sinusitis and bronchiolitis (Mäkelä et al 1998; Choi et al 2006; Revai et al 2007). Lower respiratory tract infection due to HRV has also been associated with a triggering of asthmatic exacerbation in school-aged children (Johnston et al 1995; Lau et al

2007), acute exacerbation of cystic fibrosis (Rakes et al 1999) and otitis media (Moore et al 2010). Rhinovirus has been shown to be present in asymptomatic patients (Moore et al., 2010) and it has been implicated as an underlying cause of illness in both symptomatic and asymptomatic individuals (van Gageldonk-Lafeber et al 2005). In clinical studies, HRV infections have been associated with an impaired interferon response, which can enhance viral replication in patients with asthma (Jartti and Korppi 2011).

The prevalence of HRV in children with respiratory illness in developing countries is comparable with that of respiratory syncytial virus (RSV) in children less than 5 years. HRV was identified in 28% and RSV in 23% of Vietnamese children less than 5 years old with acute respiratory tract infections (Yoshida et al 2010). Similarly, Nascimento-Carvalho identified HRV in 21% and RSV in 15% of children hospitalized for community acquired pneumonia in Brazil (Nascimento-Carvalho et al 2008). In Kenya the prevalence of HRV in children less than 5 years of age hospitalized for respiratory symptoms and fever was 84% of which 26% belonged to the recently identified group HRVC, 55% group HRV-A and 3% group HRV-B (Miller et al 2009). The latter study suggested that infections with HRV-C resulted in more severe illness with increased supplementary oxygen requirement in children infected with HRV-C compared to children infected with HRV-A alone. These studies suggest an important role for HRV as a possible cause of respiratory illness in children from developing countries. Similarly, in the USA the prevalence was of HRV in children under 5 years of age hospitalized with acute respiratory illness or fever was 16% (167 of 1052 episodes), of which subgroup characterization showed 43.5% to be HRV-A, 4.1% HRV-B and 52.4% HRV-C. Children with HRV-C were more likely to be diagnosed with asthma (Miller et al 2009). HRV has been detected in asymptomatic patients, these patients are regarded as those who are in

incubation period before symptoms are revealed, those that have viral particles and in which genetic material which is still detectable after infection has passed (Peltola et al 2004).

Table 2.4: Clinical studies on Human Rhinovirus in children

Reference	Study site	Study Year	n	Illness description	Prevalence of HRV	Age in years	Co-infection
(Lee et al 2007)	USA	99-01	26	RTIs	59.6%	<1	28 co-infections with at least one other virus
(Lau et al 2007)	China	04-05	1200	LRTIs & URTIs	1.16%	2 months-3 yrs	2 HMPV
(Xiang et al 2008)	China	07	258	LRTIs	5.5%	≤15	1 RSV-HCoV-NL63, 7 RSV and 3HPIV
(Yoshida et al 2010)	Vietnam	07-08	958	Acute RTIs	28%	≤5	Not given
(Miller et al 2009)	Kenya	07	1468	RTIs &/or fever	33%	<5	110 RSV, 17 HMPV, 1 Influ A
(Miller et al 2009)	USA, New York,	01-03	1052	Acute RTIs &/or fever	16%	<5	2 RSV, 0.94 HMPV, 0.62 Influ, 0.62 HPIV

RTI : respiratory tract infection

ARTI: acute respiratory tract infection

LRTI : lower respiratory tract infection

URTI: upper respiratory tract infection

Respiratory viruses have been implicated in the etiology of pneumonia and lower respiratory tract infections. Previous studies have shown that respiratory viruses contribute to bacterial infections by viral destruction of epithelium, viral upregulation of bacterial adhesion molecules such as (PAF) platelet-activating factor receptor and the effect of viral neuraminidase on bacterial adhesion (Madhi et al 2004; Peltola et al 2004). Respiratory viruses including rhinovirus have been implicated in increasing pneumococcal adherence to the epithelial cells (Ishizuka et al 2003). Pneumococcal conjugate vaccines have been shown to induce opsonophagocytic antibodies thus preventing acquisition of new pneumococcal serotype during upper respiratory tract infection (Madhi et al 2004). However, viral susceptibility associated with pneumococcal pneumonia occurs during seven days before the pneumococcal capsule-induced antibody response and maybe a result of depletion of pneumococcal-capsular-antibody specific B cells by the vaccine before opsonophagocytic antibodies can develop (Klugman et al 2003). Vaccines provide useful tools to elucidate the roles of bacteria and respiratory viruses. PCV-9 prevented 31% of pneumonias associated with any of seven respiratory viruses in children in hospital, by preventing superimposed bacterial infections (Madhi et al 2004).

The aim of the study was to reveal prevalence of newly discovered respiratory viruses in children whom had been hospitalized for lower respiratory tract infection.

2.5 Study objectives

1. To establish an in-house multiplex PCR for the identification of select respiratory viruses.
2. To identify, and determine the prevalence of the studied viruses in HIV-uninfected children hospitalized for lower respiratory tract infection.
3. To describe the clinical symptoms during the course of LRTI in the children in whom the viruses were identified.

Chapter 3: Methods

3.1 Study population

This study involved a retrospective analysis of nasopharyngeal aspirate (NPA) samples obtained in children hospitalized for lower respiratory tract infections. The children had been enrolled as part of a phase III double blind, placebo controlled trial of a 9-valent pneumococcal conjugate vaccine (PCV-9) in Soweto, South Africa (Klugman et al 2003). The PCV formulation included serotypes 1, 5, 4, 6B, 9V, 18C, 19F and 23F which were independently conjugated to the non-toxic diphtheria toxoid molecule (also known as cross reactive molecule-197; CRM_{197}). In this trial 39,836 children were enrolled from March 1998 to October 2000 and randomized to receive either three doses of PCV-9 or placebo. Overall 18, 245 received all three doses of study-vaccine and 18,268 received all three doses of placebo as per randomization.

The study-cohort was monitored for hospitalization for LRTI through daily surveillance conducted at Chris Hani Baragwanath hospital for the duration of the trial from March 1998 to October 2005. The first dose of study-vaccine was administered at a mean age (±SD) of 6.6 (±1.2) weeks, second dose at 11.2 (±2.5) weeks and the third dose at 15.9 (±3.8) weeks of age. No booster dose of PCV was given. All hospitalized children were evaluated clinically by one of the study doctors who used a standardized form for documenting clinical signs and symptoms. Children with a LRTI had nasopharyngeal aspirates collected which were analysed for selected respiratory viruses by immunofluorescence assays or by nested PCR SA (Madhi

et al 2004). Testing was initially undertaken at the National Institute of Virology, South Africa and subsequently as from February 2000 at the Respiratory Meningeal Pathogens Unit (RMPRU). The method of NPA collection and archiving of samples in viral transport medium has been previously described (Madhi et al 2004). Initial testing of the samples included testing for RSV, Influenza A and B virus, Parainfluenza viruses type 1-3 and Adenovirus using immunofluorescence assays (Madhi et al 2004). Aliquots of NPA samples tested at the RMPRU were archived at -70°C. These archived samples were subsequently tested for human metapneumovirus by nested RT-PCR as previously detailed by (Madhi et al 2005).

The present study involved further evaluation of available archived NPA samples from study-participants who were hospitalized for LRTI from February 2000 onward. For the purposes of this exploratory study testing of samples was limited to samples obtained from HIV-uninfected children less than two years of age for the presence of emerging respiratory viruses in children hospitalized for LRTI. The study focused on HIV-uninfected children based on an earlier study which indicated that established respiratory viruses such as RSV, influenza viruses, PIV and adenovirus were identified less commonly in HIV-infected (15.7%) than HIV-uninfected children (34.8%, $p<0.0001$) when hospitalized for LRTI (Madhi et al 2000).

3.2 Study-specific definitions

The clinical definition for LRTI, pneumonia and bronchiolitis used in this study are the same as previously described by Madhi et al (2005). Briefly, LRTI was defined as any episode with clinical diagnosis of pneumonia or bronchiolitis done by a study physician, irrespective of symptoms found by clinical examination or chest radiography (CXR) features. Children with LRTI were categorized as having clinical pneumonia if they had evidence of alveolar

consolidation on CXR (CXR-AC) or if they fulfilled the clinical diagnosis of LRTI without wheeze on chest auscultation but had rales and/or bronchial breathing. Children were categorized as having bronchiolitis in the presence of wheezing on chest auscultation performed by one of the study doctors and in the absence of documented alveolar consolidation on chest radiograph or bronchial breathing on chest wall auscultation. CXR-AC was evaluated on the basis of criteria agreed on by the Vaccine Trialist Working Group, coordinated by the WHO (Cherian T 2005).

3.3 Sample extractions and PCR optimization and screening

Internal Control and sample extraction procedure

Archived NPA samples were thawed overnight at 4°C. Samples were briefly vortexed for 15 seconds and centrifuged at 2000 g for 5 minutes. A sample volume of 200µL was spiked with internal controls prior to extraction. The internal controls were used to monitor the efficiency of the nucleic acid extraction, reverse transcriptase reaction, and amplification of target nucleic acids. The selected internal controls in our study were the Newcastle Disease Virus (NDV) a RNA virus and the Lambda phage, a DNA virus internal control. Lambda phage was spiked at a concentration of 10^3 plaque forming units (pfu) per sample, which yielded an average cycle threshold (CT) of 20-35 cycles. NDV spike concentration was 10^4 copies of viral particle/sample, which produced average CT of 20-25 cycles (Table 4.1 in Results section). Controls with a high cycle threshold value (37-40) were considered negative result, therefore if either DNA control was had a low threshold value RNA control was used to validate the extraction, reverse transcription reaction and amplification reaction. Nucleic acids of spiked samples were extracted with the Easymag nucleic acid extraction robot (Biomerieux, France)

and the off-board extraction protocol was selected with the following variable parameters: volumes of sample was 1000µl (200µl of sample and 800µl of lysis extraction buffer), after lysis each sample was transferred to a vessel and 125µl of magnetic silica was added, finally 55 µl were eluted in the extraction kit elution buffer and stored at -70°C for further analysis.

Control DNA

Positive control DNA was obtained as a gift from Dr. Rossen for the viruses HBoV, WUPyV, KIPyV, HRV, HCoV-229E, HCoV-OC43 and HCoV-NL63. The controls were quantified to have 100,000 copies per reaction and a 10-fold dilution of this stock was used routinely as positive controls for all the PCR reaction. For the PCR reactions to detect HCoV-HKU1 viruses, and for which no positive control DNA was available, the control was obtained from clinical samples. Briefly, clinical samples were screened for a positive reaction with a generic amplification protocol and published primer sets followed by confirmation of the fragment length by gel electrophoresis. The positive sample was used as target material to further optimize the PCR, and as positive controls for subsequent reactions. The range of CT for positive control samples for HRV was 25-29 cycles, 18-25 cycles for HBoV, 30-35 cycles for KIPyV, 30-35 cycles for WUPyV, 20-29 cycles for HCoV-NL63, for HCoV-HKU1 was 20-29, HCoV-OC43 was 20-29 and HCoV-229E was 20-29 cycles.

PCR procedure

Complementary DNA was synthesized with the Multiscribe kit without RNAse inhibitor (Applied Biosystems, SA). Reverse transcription-PCR reactions were performed with 10µl of sample eluted from the Easymag nucleic acid extraction machine and 10µL of master mix

according to the manufacturer's recommendations (Appendix: Figure 8.2). The cycling conditions were as follows: 25°C for 10min, 37°C for 2hrs and 85°C for 5sec (Appendix: Figure 8.3). Real time reactions were performed in a total of 25µl with 12.5µl of 2 x Taqman universal polymerase chain reaction master mix with AmpErase-Uracil-N-glycosylase (UNG) (Applied Biosystems, SA). Contamination and false positives from high copy number DNA was eliminated by the AmpErase-Uracil-N-glycosylase (UNG) enzyme present in the reaction mix, which is active during the first incubation step of 50°C for 2min. During PCR dUTP substitute's dTTP in the PCR reaction, the enzyme UNG which is present in the master mix degrades Uracil-containing DNA from previous reactions that may be present in the PCR reaction. In this way products from previous PCR or non-specific or misprimed products produced prior to amplification of target product are eliminated. Hence the initial incubation step of the real-time PCR is the UNG activation which is followed by deactivation at 95°C for 10min. Reactions were performed with 5µL of cDNA for detecting RNA viruses and 5µL of total nucleic acid for detecting DNA viruses. Real time cycling conditions were as follows: 1cycle of 50°C for 2min, 1 cycle of 95°C for 10min, 40 cycles of 95°C for 15s and 60°C for 1min. The primer concentrations for each duplex reaction are shown in Table 3.4.

The optimized cycle thresholds for each respiratory virus control are used as reference for a sample with high DNA concentration, hence a virus with a lower CT indicates that they are detected early in PCR and have a high DNA concentration. Hence the optimal cycles observed above were used as a guideline to show qualitative results for samples with high DNA concentration. Samples with a low DNA concentration are detected later in the PCR and these samples have a high CT. Hence controls with a low DNA concentration are used as a

reference for qualitative results for samples with low DNA concentration. Negative results were deduced by the software as Not Detected.

Primer and probe optimizations and detection limits of the assay

Table 3.1 shows the primer and probe sequences, target gene and published reference for each targeted virus. Virus specific primer and probe sequence combinations were based on previously published sequences and the target regions represented highly conserved regions for each virus. Primer and probe optimizations were performed with the ABI 7500 Real Time PCR machine and Taqman Universal PCR Master Mix. Primer and probe for the internal controls were optimized using internal control DNA and cDNA. Positive controls were used to optimize the primer and probe concentrations for the newly discovered respiratory viruses. The primer dilutions were prepare in the reaction plates, as per the dilution matrix as indicated in Table 3.2. The optimal primer concentration of forward and reverse primers was selected as being the lowest concentration at which there was no effect on the sensitivity of the reaction as determined by the CT and delta reaction using the linear phase of the amplification curve at the threshold. These primer limiting reaction conditions resulted in a primer set that would amplify the target with maximum sensitivity, but would not reach saturation, and thus allow a second target to amplify as a multiplex within the same reaction. PCR optimizations were done and the level of duplex was achieved with pairing of two viruses at a time, the 5 pairs of viruses were detected simultaneously on one plate. The results of the primer matrixes and optimizations are shown in the Appendix, (Tables 8.3-8.6). Duplex reactions were paired in accordance with the type of nucleic acid present in the virus, that is RNA viruses were co-paired separately from DNA viruses. The following multiplex pairs

reactions were performed: HCOV-NL63 and HCoV-OC43; HCoV-HKU and HRV; WUPyV and KIPyV; NDV RNA control and HCoV-229E and HBoV and Lambda DNA control.

These pairings were validated to determine whether there was any loss of sensitivity in the presence of a second target, by performing a template dilution matrix of the two target DNA's, as shown in Table 3.3. Duplexed primer pairs were selected if the paired combination met the following criteria: there was no loss of sensitivity or significant change in CT when both targets are present in the same reaction at any concentration, compared to a single target, and there was no cross reactivity caused by the multiplex. The results of these matrixes are shown in the Appendix, (Tables 8.7-8.11).

Table 3.1: Primer and probes sequences used to detect respiratory viruses of interest

Primer	Gene	Reference	Sequence
HCoV-OC forward	N-gene	(van Elden et al 2004)	CGA TGA GGC TAT TCC GAC TAG GT
HCoV-OC reverse	N-gene	(van Elden et al 2004)	CCT TCC TGA GCC TTC AAT ATA GTA ACC
HCoV-OC Probe	N-gene	(van Elden et al 2004)	5' FAM-TCC GCC TGG CAC GGT ACT CCC T-TAMRA 3'
HCoV-229E forward	N-gene	(van Elden et al 2004)	CAG TCA AAT GGG CTG ATG CA
HCoV-229E reverse	N-gene	(van Elden et al 2004)	AAA GGG CTA TAA AGA GAA TAA GGT ATT CT
HCoV-229E Probe	N-gene	(van Elden et al 2004)	5'VIC-CCC TGA CGA CCA CGT TGT GGT TCA-TAMRA 3'
HCoV-NL forward	N-gene	(Fouchier et al 2004)	GCG TGT TCC TAC CAG AGA GGA
HCoV-NL reverse	N-gene	(Fouchier et al 2004)	GCT GTG GAA AAC CTT TGG CA
HCoV-NL Probe	N-gene	(Fouchier et al 2004)	5' VIC-ATG TTA TTC AGT GCT TTG GTC CTC GTG AT-TAMRA 3'
HKU1 forward	N-gene	In house	CGC CTG GTA CGA TTT TGC C
HKU1 reverse	N-gene	In house	GAA CGA TTA TTG GGT CCA CGT G
HKU1 Probe	N-gene	In house	5' VIC- TGTTGA AGG CTC AGG AAG GTC TGC TTC TAA-TAMRA 3'
HBoV forward	NP1 gene	Dr. J. Rossen	GGA AGA GAC ACT GGC AGA CAA
HBoV reverse	NP1 gene	Dr. J. Rossen	GGG TGT TCC TGA TGA TAT GAG C
HBoV Probe	NP1 gene	Dr. J. Rossen	5' FAM- CTG CGG CTC CTG CTC CTG TGA T -TAMRA 3'
HRV forward	5' UTR	Dr. J. Rossen,	GCC TGC GTG GCT GCC
HRV reverse	5' UTR	Dr. J. Rossen,	CCT GCG TGG CGG CC
HRV forward	5' UTR	Dr. J. Rossen	ACG GAC ACC CAA AGT AGT TGG T
HRV reverse	5' UTR	Dr. J. Rossen	ACG GAC ACC CAA AGT AGT CGG T
HRV Probe	5' UTR	Dr. J. Rossen	5' FAM- TCC GGC CCC TGA ATG TGG CTA A -TAMRA 3'
HRV Probe	5' UTR	Dr. J. Rossen	5' FAM- TCC GGC CCC TGA ATG CGG CTA A -TAMRA 3'
HRV forward	5' UTR	Dr. J. Rossen,	GCC TGC GTG GCT GCC
WU forward	Gp5	Dr. J. Rossen	GGC ACG GCG CCA ACT
WU reverse	Gp5	Dr. J. Rossen	CCT GTT GTA GGC CTT ACT TAC CTG TA
WU Probe	Gp5	Dr. J. Rossen	5' FAM-TGC CAT ACC AAC ACA GCT GCT GAG C 3'
KI forward	NTR	Dr. J. Rossen	ACC TGA TAC CGG CGG AAC T
KI reverse	NTR	Dr. J. Rossen	CGC AGG AAG CTG GCT CAC
KI Probe	NTR	Dr. J. Rossen	5' VIC-CCA CAC AAT AGC TTT CAC TCT TGG CGT GA 3'

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Table 3.2: Primer matrix for all viruses

Primers (uM [c])	1st dilution 1.3uM	2nd dilution 0.6uM	3rd dilution 0.33uM	4th dilution 0.16uM	5th dilution 0.08uM	6th dilution 0.04uM
1st dilution 1.3Um	1.3F[a]/1.3R[b]	1.3F/ 0.6R	1.3F/0.33R	1.3F/0.16R	1.3F/ 0.08R	1.3F/0.04R
2nd dilution 0.6uM	0.6F/1.3R	0.6F/0.6R	0.6F/0.33R	0.6F/0.16R	0.6F/0.08R	0.6F/0.04R
3rd dilution 0.33uM	0.33F/1.3R	0.33F/0.6R	0.33F/0.33R	0.33F/0.16R	0.33F/0.08R	0.33F/0.04R
4th dilution 0.16uM	0.16F/1.3R	0.16F/0.6R	0.16F/0.33R	0.16F/0.16R	0.16F/0.08R	0.16F/0.04R
5th dilution 0.08uM	0.08F/1.3R	0.08F/0.6R	0.08F/0.33R	0.08F/0.16R	0.08F/0.08R	0.08F/0.04R
6th dilution 0.04uM	0.04F/1.3R	0.04F/0.6R	0.04F/0.33R	0.04F/0.16R	0.04F/0.08R	0.04F/0.04R

[a] forward primer

[b] Reverse primer

[c] Molar concentration

Table 3.3: DNA matrixes for all viruses

	1st dilution	2nd dilution	3rd dilution	4th dilution	5th dilution
1st dilution	1st /1st	2nd /1st	3rd /1st	4th /1st	5th /1st
2nd dilution	1st/2nd	2nd /2nd	3rd /2nd	4th /2nd	5th /2nd
3rd dilution	1st/ 3rd	2nd /3rd	3rd /3rd	4th /3rd	5th /3rd
4th dilution	1st/4th	2nd /4th	3rd /4th	4th /4th	5th /4th
5th dilution	1st/ 5th	2nd /5th	3rd /5th	4th /5th	5th /5th

1st dilution is the highest dilution from original stock diluted 10X, followed by 2nd dilution which is diluted 100X, 3rd is diluted 1000X, 4th is diluted 10000X and 5th is diluted 1000000X

Table 3.4: Optimized primer-pairs concentration used in detected target respiratory viruses

Primers-pairs	Concentrations (uM)	Forward	Reverse	Probe
DNA Viruses	HBoV	0.2	0.2	0.3
	Lambda	0.32	0.08	0.3
	Polyomavirus-WU	0.8	0.4	0.3
	Polyomavirus-KI	0.8	0.2	0.3
RNA Viruses	HCoV-229E	0.2	0.2	0.3
	NDV	0.16	0.08	0.3
	HCoV-OC43	0.2	0.2	0.3
	HCoV-NL63	0.2	0.2	0.3
	HCoV-HKU1	0.3	0.3	0.3
	Rhinovirus	0.2	0.2	0.3

3.4 Statistical Analysis

Data were analyzed using STATA (version 8.0; Stata Corp). Proportions were compared using the $\chi 2$ test and the Fisher's exact test if the expected cell value was < 5 observations, an alpha ≤ 0.05 was considered significant. Due to a low frequency of positive coronavirus-229E, the virus was excluded from some of the analysis

Chapter 4: Results

4.1 Sensitivity and Specificity

Using a serial dilution series the limits of detection were determined for each virus. Detection limits are shown in Table 4.1, the detection limits for all the viruses were less than 100 copies/ reaction with exceptions for HRV , WUPyV and KIPyV that were less than 1000 copies/ reaction. In addition controls were paired with each other to show specificity (Appendix Tables 8.7 to 8.12).

Table 4.1: Limits of detection of respiratory viruses

Dilution series	1:10 (10^5)	1:100 (10^5)	1:1000(10^5)	1:10000(10^5)	1:100000 (10^5)
HBoV	18[#]	20	24	29	ND*
HRV	25	27	30	ND	ND
WUPyV	30	34	36	ND	ND
KIPyV	30	33	37	ND	ND
HCoV-NL63	20	24	26	31	ND
HCoV-HKU1	20 [a]	24	26	29	ND
HCoV-OC43	20	23	26	29	ND
HCoV-229E	21	25	28	31	ND
Lambda	18	20	22	24	ND
NDV	20	24	29	33	ND

*ND Not detected= negative
[#]Cycle threshold value=positive
($10E^5$) concentration given with DNA extracts from Dr. Rossen with exception to HCoV-HKU 1
a: cycle threshold obtained from patient sample HCoV-HKU 1 from our laboratory

4.2 Demographic features of available and unavailable samples in LRTI episodes

To determine whether there was any sampling bias between the NPA samples that were available for testing from the original study and the samples we used, a selection of demographic and clinical features of the LRTI episodes from the tested samples were compared to those that were not tested. Overall, 895 (57.2%) of 1565 nasopharyngeal aspirates from children under two years of age hospitalized for LRTI, from February 2000 onward, were available for testing (Table 4.2). Samples unavailable for testing were collected from younger children compared to the samples that were available for testing (9.9 months ±6.41 (±SD) vs. 11.8 months ±6.50, respectively; p<0.0001), were associated with a lower mean temperature on admission (37.2°C vs. 37.4°C, respectively; p=0.035) and were more likely to be associated with wheezing (60.4% vs. 54.6%; p=0.022).

Table 4.2: Selected features of LRTI episodes for which samples were tested compared to those in which samples were unavailable for testing in HIV-uninfected children less than two years of age

Demographic features	Samples tested N=895	Samples not tested N=670	P value
% of total	57.19	42.81	
Age in months ± SD	11.83±6.50	9.91±6.41	<0.001
Female (%)	42.5	42.8	0.88
Mean temperature in °C ±SD	37.43±2.07	37.18±0.77	0.035
Bronchial breathing n (%)	30 (3.4)	25 (3.8)	0.65
Wheezing n (%)	488 (54.6	401 (60.4)	0.022
C repitations/ rales n (%)	541 (60.5)	372 (56.0)	0.08
Mean Respiration Rate per minute ±SD	49.94±19.82	51.69±19.74	0.08
Mean weight in Kg ±SD	9.27±3.44	9.14±5.15	0.73
Pneumonia n (%)	464 (51.8)	332 (49.6)	0.11
Bronchiolitis n (%)	431 (48.2)	338 (50.4)	0.11
ICU Admission (%)	12 (1.3)	9 (1.3)	0.96
Deaths due to LRTI (%)	8 (0.89)	13 (1.9)	0.08

4.3 The prevalence of identifying the studied viruses in children hospitalized for LRTIs

The overall prevalence of the newly studied respiratory viruses, stratified by PCV vaccination status, and prevalence thereof in clinical syndromes of bronchiolitis and pneumonia are shown in Table 4.3 to Table 4.5. For comparison purposes, Tables 4.3, 4.4 and 4.5 also illustrates the prevalence of other previously studied respiratory viruses including RSV, PIV 1-3, influenza A/B virus, hMPV and adenovirus in the group of children for whom samples were available for evaluation in this study. The total prevalence of the viruses in children with LRTI were 33.2% for HRV, 21.2% for HBoV, 16.1% for WUPyV, 10.1% for HCoV-OC43, 7.0% for KIPyV, 3.2% for HCoV-NL63, 2.6% for HCoV-HKU-1, and 0.6% for HCoV-229E.

Differences by PCV-vaccination status in the probability of identification of a newly studied viruses included a higher frequency of HBoV (24.2% vs. 18.2%; p=0.028) ,and HRV (36.7% vs. 29.5%; p=0.023) in children vaccinated with PCV compared to placebo recipients (Table 4.3). Conversely, viruses identified more frequently in children who received placebo compared to PCV-recipients included WUPyV (20.2% vs. 12.1%, p<0.0001), HCoV-OC43 (14.1% vs. 10.1%; p<0.0001) and HCoV-HKU1 (14.1% vs. 2.6%; p<0.0001) and KIPyV (10.0% vs. 4.2%; p<0.0001). At least one virus was identified in 82.2% LRTI episodes, which occurred with a higher frequency in placebo- (85.2%) than PCV-recipients (79.3%; p=0.021). In addition, concurrent infection by two viruses were identified in 59.3% overall; and were also more common in the placebo (68.0%) than in the PCV (51.0%; p<0.0001) group. Multiple co-infections with three or more viruses were identified in 16.7% of all LRTI episodes, and likewise also more commonly identified in placebo (21.1%) compared to PCV-recipients (12.3%; p<0.0001)

Table 4.4 indicates the prevalence of studied viruses in children with bronchiolitis. In these children at least one virus was identified in 85.2% of episodes. The overall prevalence of identifying the studied viruses were 33.8% for HRV, 33.4% for WUPyV, 22.3% for HBoV, 11.1% for HCoV-OC435.3% for KIPyV, , 2.3% for HCoV-NL63, 1.9% for HCoV-HKU1 and 0.4% for HCoV-229E. Viruses more commonly identifying in placebo compared to PCV recipients were WUPyV (20.0% vs. 12.3%, respectively; p=0.029) and HCoV-OC43 (15.9% vs. 7.2%, respectively; p=0.004). Viruses more commonly identifying in PCV recipients compared to placebo was HCoV-HKU1 (2.5% vs. 2.1; p=0.015) There was a higher probability of identifying three or more concurrent viruses among placebo (21.1%) compared to PCV recipients (13.6%; p=0.04).

The prevalence of the newly studied viruses in children with clinical pneumonia was 30.8% for HRV, 20.3% for HBoV, 16.4% for WUPyV, 9.1% for HCoV-OC43, 8.6% for KIPyV, 4.1% for HCoV-NL63, 3.2% for HCoV-HKU1 and 0.6% for HCoV-229E; Table 4.5. A total of 79.5% clinical pneumonia episodes were identified to be associated with at least one virus, including those viruses which had been previously studied in these samples. The prevalence of individual viruses was greater in placebo than PCV recipients for WUPyV (20.4% vs. 11.9%, respectively; p=0.013), KIPyV (12.7% vs. 4.1%, p=0.001) andHCoV-HKU1 (5.3% vs. 0.9%, p=0.008). Conversely, HCoV-OC43 was identified more frequently in children who received PCV (5.0%) compared to placebo recipients (2.7%; p=0.004). There were no differences in prevalence of HBoV, HCoV-NL63 and HRV between placebo and PCV recipients hospitalized for clinical pneumonia. There was, however, a higher frequency of identifying at least one virus in the placebo (88.5%) than PCV recipients (74.0%; p=0.005). Overall, two concurrent viruses were identified in 60.3% of clinical pneumonia cases, co-infections were also more common in

placebo (71.8%) compared to PCV recipients (47.5%; p<0.0001). Similarly, 16.4% of all clinical pneumonia episodes were associated with identification of at least three viruses, which also occurred more frequently in placebo (21.2%) compared to PCV recipients (11.0%; p=0.003).

Table 4.3: Prevalence of newly emerging viruses and previously studied viruses in children hospitalized for LRTI

Virus detected	Total N=895 N, [%; 95% CI]	Placebo N=440 N, [%; 95% CI]	Vaccine N=455 N, [%; 95% CI]	p-value[1]
HBoV	190 [21.2; 18.6, 24.1]	80 [18.2; 14.7, 22.1]	110 [24.2; 20.3, 28.4]	0.028
WUPyV	144 [16.1; 13.7, 18.7]	89 [20.2; 16.6, 24.3]	55 [12.1; 9.2, 15.4]	0.001
KIPyV	63 [7.0; 5.5, 8.9]	44 [10; 7.4, 13.2]	19 [4.2; 2.5, 6.4]	0.001
HCoV-NL63	29 [3.2; 2.2, 4.6]	13 [3; 1.6, 5]	16 [3.5; 25.6]	0.86
HCoV-HKU1	23 [2.6; 1.6, 3.8]	20 [4.5; 2.8, 6.9]	3 [0.1; 0.01, 1.2]	<0.0001
HCoV-OC43	90 [10.1; 8.2, 12.2]	62 [14.1; 11, 17.7]	28 [6.2; 4.1, 8.8]	<0.0001
HCoV-229E	5 [0.6; 0.2, 1.3]	4 [0.9; 0.2, 2.3]	1 [0.2; 0.01, 1.2]	0.18
HRV	297 [33.2; 30.1, 36.4]	130 [29.5; 25.3, 34.1]	167 [36.7; 32.3, 41.3]	0.023
***RSV*[2]**	*209* *[23.4; 20.6, 26.3]*	*100* *[23; 19, 27]*	*109* *[24; 20.1, 28.2]*	*0.66*
***Influenza A/B*[2]**	*48* *[5.4; 3.98, 7.1]*	*23* *[5.2;3.3, 7.7]*	*25* *[5.5; 3.6, 8.0]*	*0.86*
***Para influenza 1-3*[2]**	*36* *[4.0; 2.8, 5.5]*	*15* *[3.4; 1.9, 5.6]*	*21* *[4.6; 2.9, 6.9]*	*0.36*
***Adenovirus*[2]**	*22* *[2.5; 1.5, 3.7]*	*13* *[2.9; 1.6, 4.9]*	*9* *[1.97; 0.9, 3.7]*	*0.35*
***hMPV*[2]**	*82* *[9.2; 7.4, 11.2]*	*49* *[11.1; 8.4, 14.5]*	*33* *[7.3; 5.0, 10.0]*	*0.044*
At least 1 virus identified	736 [82.2; 79.6, 84.7]	375 [85.2; 81.6, 88.4]	361 [79.3; 75.3, 82.9]	0.021
2 viruses identified	531 [59.3; 56.0, 62.6]	299 [68; 63.4, 72.3]	232 [51; 46.3, 55.7]	<0.0001
≥3viruses identified	149 [16.7;14.3, 19.3]	93 [21.1;17.4, 25.3]	56 [12.3; 9.4, 15.7]	<0.0001

[1] p-values show a comparison between placebo and vaccine group

[2]Indicate previously detected respiratory viruses from the samples tested in this study

Table 4.4: Prevalence of newly emerging viruses and previously studied viruses in children hospitalized for bronchiolitis

Virus detected	Total N=431 N, [%; 95% CI]	Placebo N=195 N, [%; 95% CI]	Vaccine N=236 N, [%; 95% CI]	p-value[1]
HBoV	96 [22.3; 18.4, 26.5]	36 [18.5; 13.3, 24.6]	60 [25.4; 20, 31.5]	0.08
WUPyV	68 [33.4; 29, 38.1]	39 20.0]14.6, 26.3]	29 [12.3; 8.4, 17.2]	0.029
KIPyV	23 [5.3; 3.4, 7.9]	13 [6.7; 3.6, 11.1]	10 [4.2; 2.1, 7.7]	0.26
HCoV-NL63	10 [2.3; 1.1, 4.2]	4 [2.1; 0.6, 5.2]	6 [2.5; 0.9, 5.5]	1.00
HCoV-HKU1	8 [1.9; 0.8, 3.6]	7 [3.6; 1.5, 7.3]	1 [0.4; 0.01, 2.3]	0.015
HCoV-OC43	48 [11.1; 8.3, 14.5]	31 [15.9; 11.1, 21.8]	17 [7.2; 4.3, 11.3]	0.004
HCoV-229E	2 [0.5; 0.1, 1.7]	1 [0.5; 0.01, 2.8]	1 [0.4; 0.01, 2.3]	0.89
HRV	154 [35.7; 31.2, 40.5]	62 [31.8; 25.3, 38.8]	92 [39; 32.7, 45.5]	0.12
RSV[2]	*117* *[27.2; 23.0, 31.6]*	*48* *[24.6; 18.7, 31.3]*	*69* *[29.2; 23.5, 35.5]*	*0.28*
Influenza A/B[2]	*19* *[4.4;2.7, 6.8]*	*6* *[3.1; 1.1, 6.6]*	*13* *[5.5; 2.9, 9.2]*	*0.22*
Para influenza 1-3[2]	*18* *[4.2; 2.5, 6.5]*	*6* *[3.1; 1.1, 6.6]*	*12* *[5.1; 2.7, 8.7]*	*0.30*
Adenovirus[2]	*9* *[2.1; 0.1, 3.9]*	*6* *[3.1; 1.1, 6.6]*	*3* *[1.3; 0.3, 3.7]*	*0.19*
hMPV[2]	*43* *[9.9; 7.3, 13.2]*	*25* *[12.8; 8.5, 18.3]*	*18* *[7.6; 4.6, 11.8]*	*0.07*
At least 1 virus identified	367 [85.2; 88.4]	168 [86.2;80.5, 90.7]	199 [84,3; 79.0, 88.7]	0.59
2 viruses identified	251 [58.2; 53.4, 62.9]	123 [63.1; 55.9, 69.9]	128 [54.2; 47.7, 60.7]	0.06
≥3viruses identified	73 [17; 13.5, 20.8]	41 [21.0; 15.5, 27.4]	32 [13.6; 9.5, 18.6]	0.04

[1] p-values show a comparison between placebo and vaccine group
[2]Indicate previously detected respiratory viruses from the samples tested in this study

Table 4.5: Prevalence of newly described viruses and previously studied viruses in children hospitalized for pneumonia

Virus detected	Total N=464 N, [%; 95% CI]	Placebo N=245 N, [%; 95% CI]	Vaccine N=219 N, [%; 95% CI]	p-value[1]
HBoV	94 [20.3; 16.7, 24.2]	44 [18; 13.4, 23.3]	50 [22.8; 17.4, 29]	0.19
WUPyV	76 [16.4; 13.1, 20.1]	50 [20.4; 15.5, 26]	26 [11.9; 7.9, 16.9]	0.013
KIPyV	40 [8.6; 6.2, 11.6]	31 [12.7; 8.8, 17.5]	9 [4.1; 1.9, 7.7]	0.001
HCoV-NL63	19 [4.1; 2.5, 6.3]	9 [3.7; 1.7, 6.9]	10 [4.6; 2.2, 8.2]	0.63
HCoV-HKU1	15 [3.2; 1.8, 5.3]	13 [5.3; 2.9, 8.9]	2 [0.9; 0.1, 3.3]	0.008
HCoV-OC43	42 [9.1; 6.7, 12.0]	31 [2.7; 8.8, 17.5]	11 [5; 2.5, 8.8]	0.004
HCoV-229E	3 [0.6; 0.1, 1.9]	3 [0 ; 0.0, 1.7]	0	0.25
HRV	143 [30.8; 26.6, 35.2]	68 [27.8; 22.2, 33.8]	75 [32.2; 28.0, 41.0]	0.13
RSV[2]	*92 [19.8; 16.3, 23.8]*	*52 [21.2; 16.3, 26.9]*	*40 [18.3; 13.4, 24.0]*	*0.43*
Influenza A/B[2]	*29 [6.3; 4.2, 8.8]*	*17 [6.9; 4.1, 10.9]*	*12 [5.5; 2.9, 9.4]*	*0.52*
Para influenza 1-3[2]	*18 [3.9; 2.3, 6.1]*	*9 [3.7; 1.7, 6.9]*	*9 [4.1; 1.9, 7.7]*	*0.81*
Adenovirus[2]	*13 [2.8; 1.5, 4.5]*	*7 [2.9; 1.2, 5.8]*	*6 [2.7; 1.0, 5.9]*	*0.94*
hMPV[2]	*39 [8.4; 6.0, 11.3]*	*24 [9.8; 6.4, 14.2]*	*15 [6.8; 3.9, 1.0]*	*0.25*
At least 1 virus identified	369 [79.5; 75.6, 83.1]	207 [88.5; 79.3, 88.8]	162 [74; 67.6, 79.7]	0.005
2 viruses identified	280 [60.3; 55.7, 64.8]	176 [71.8; 65.8, 77.4]	104 [47.5; 40.7, 54.3]	<0.0001
≥3viruses identified	76 [16.4; 13.1, 20.1]	52 [21.2; 16.3, 26.9]	24 [11; 7.2, 15.8]	0.003

[1] p-values show a comparison between placebo and vaccine group

[2]Indicate previously detected respiratory viruses from the samples tested in this study

4.4 Respiratory viral co-infections in LRTI episodes

Co-infections are defined as an infection of more than 2 viruses detected in one specimen. The viral co-infections of new respiratory viruses and older respiratory viruses were compared. in this context our results revealed a co-infection of more than 2 viruses detected from one specimen, hence the results in Tables 4.6, 4.7 and 4.8 show a higher number of co-infections found in samples positive for each respiratory virus. For instance in WUPyV the number of children positive for WUPyV is a hundred and forty four (144) whereas the co-infections observed is a hundred and sixty, thus indicating that children with WUPyV had more than one virus infection. Overall higher viral co-infections in all three syndromes was observed for the HRV, WUPyV, HBoV and RSV.

Table 4.6 details the prevalence of co-infection occurring in children with LRTI. The table describes the probability of co-infection to occur for each respiratory virus. From table 4.6 a number of significant differences in the detection of respiratory viruses was observed when comparing the co-infections to other viruses detected in a group of children with a respiratory virus. Comparing the different episodes of LRTI where HBoV was detected with another virus, co-infections with HRV were significantly more prevalent than the other co-infections (p=0.027). Comparing detection of respiratory virus with another respiratory virus, the significant viral co-infection was WUPyV with HMPV (n=12; 8.3%; p=0.020), HRV (n= 42; 29.2%; p value=0.045) and HCoV-OC43 (n=20, 13.9%, p=0.006). Eighty two percent of the 190 LRTI episodes in which HBoV was identified were associated with co-infection with one of the other viruses. The top three most common viral co-infection with HBoV were HRV (n=50; 26.3%), WUPyV (n=30; 15.8%) and RSV (n=17; 8.9%).

90% of 144 LRTI episodes in which WUPyV was identified were associated with co-infections with one or two of the other viruses. The top three most common viral co-infections with WUPyV were HRV (29.1%), HBoV (20.8%), RSV (15.9%), HCoV-OC43 (13.9). And 58.5% of 82 LRTI episodes in which HMPV were identified were associated with one of the other viruses. The most common viral co-infections with HMPV was HCoV-OC43 (n=8, 9.8%, p=0.035).

Viral co-infections in children categorized as having bronchiolitis was 251 (58.2%) of 451 episodes (Table 4.7). among the cases of bronchiolitis, HBoV was associated with another viral co-infection in 80.2% of 96 cases. Comparing detection of respiratory virus with another respiratory virus the significant viral co-infection was WUPyV were HRV (n=22; 32.3%; p=0.026), and HMPV (n=4; 5.9%; p=0.041), in addition HRV was significantly detected in 27.1% HCoV-OC43 patients (p=0.004)

The prevalence of at least two viral co-infections in children categorized as having clinical pneumonia was 280 (60.2%) of 464 cases (Table 4.8). Among the cases of clinical pneumonia, HBoV was associated with another viral co-infection in 84% of 94 cases. The most common viral co-infections being HRV (n=23, 24.5%), WUPyV (n=15, 19.7%) and RSV (n=8, 8.5%).

Table 4.6: Concurrent infection of newly emerging viruses and previously studied viruses in children that were hospitalized for lower respiratory tract infection

Virus	HBoV N=190	WUPyV N=144	KIPyV N=63	HCoV-NL63 N=29	HCoV-HKU1 N=23	HCoV-OC43 N=90	HCoV-229E N=4	HRV N=297	RSV N=209	Influ A/B N=48	PIV 1-3 N=36	AdenoV N=22	hMPV N=82	Total (%)
HBoV	-	30	12	4	1	9	0	50	17	7	10	6	10	156 (82.1)
WUPyV	30	-	10	6	4	20	1	42	23	7	2	3	12	160 (90)
KIPyV	12	10	-	1	1	9	2	23	9	1	5	1	5	79 (79.7)
HCoV-NL63	4	6	1	-	1	1	0	8	6	2	0	0	3	32 (91)
HCoV-HKU1	1	4	1	1	-	5	1	4	4	0	0	0	4	25 (92)
HCoV-OC43	9	20	9	1	5	-	1	27	11	5	3	1	8	100 (90)
HCoV-229E	0	1	2	0	1	1	-	2	0	0	0	0	1	8 (50)
HRV	50	42	23	8	4	27	2	-	46	7	11	1	21	242 (81.5)
RSV	17	23	9	6	4	11	0	46	-	0	0	0	3	119 (41.0)
Influ A/B	7	7	1	2	0	5	0	7	0	-	0	0	1	30 (62.5)
PIV 1-3	10	2	5	0	0	3	0	11	0	0	-	0	0	31 (86.1)
AdenoV	6	3	1	0	0	1	0	1	0	0	0	-	0	12 (54.5)
Hmpv	10	12	5	3	4	8	1	21	3	1	0	0	-	68 (82.9)

Note: Viruses shown in italics were previously tested for by immunofluorescence assay or RT-PCR for hMPV.
N: represent samples positive for respiratory virus with LRTI

Table 4.7: Concurrent infection of newly emerging viruses and previously studied viruses in children that were hospitalized for bronchiolitis

Virus	HBoV *N= 96	WUPyV N=68	KIPyV N=23	HCoV-NL63 N=10	HCoV-HKU1 N=8	HCoV-OC43 N=48	HCoV -229E N=2	HRV N= 154	RSV N= 117	Influ A/B N=19	PIV 1-3 N=18	AdenoV N=9	hMPV N=43	Totals
HBoV	-	15	5	3	5	1	0	27	9	2	5	1	4	77 (80.2)
WUPyV	15	-	3	1	11	3	1	22	12	3	1	2	4	78 (87.2)
KIPyV	5	3	-	1	4	0	2	8	4	0	3	0	3	33 (69.7)
HCoV-NL63	3	1	1	-	0	0	0	4	3	0	0	0	0	12 (83.3)
HCoV-OC43	5	11	4	0	-	2	1	13	5	2	0	1	5	49 (97.9)
HCoV-HKU1	1	3	0	0	2	-	2	1	2	0	0	0	1	12 (66.7)
HCoV-229E	-	1	2	0	1	0	-	1	0	0	0	0	1	6 (33.3)
HRV	27	22	8	4	13	13	1	-	20	4	3	0	14	117 (75.9)
RSV	9	12	4	3	5	2	0	20	-	0	0	0	3	58 (49.6)
Influ A/B	2	3	0	0	2	0	0	4	0	-	0	0	0	11 (57.9)
PIV 1-3	5	1	3	0	0	0	0	3	0	0	-	0	0	12 (66.7)
AdenoV	1	2	0	0	1	0	0	0	0	0	0	-	0	4 (44.4)
hMPV	4	4	3	0	5	1	1	14	3	0	0	0	-	35 (81.4)

Note: Viruses shown in italics were previously tested for by immunofluroesence assay or RT-PCR for hMPV.
*N: represent samples positive for respiratory virus with bronchiolitis syndrome

Table 4.8: Concurrent infection of newly emerging viruses and previously studied viruses in children that were hospitalized for clinical pneumonia

	HBoV N= 94	WUPyV N=76	KIPyV N=40	HCoV-NL63 N=19	HCoV-HKU1 N=15	HCoV-OC43 N=42	HCoV -229E N=3	HRV N= 143	RSV N= 92	Influ A/B N=29	PIV 1-3 N= 18	AdenoV N=13	hMPV N=39	Totals
HBoV	-	15	7	1	0	4	0	23	8	5	5	5	6	79 (84)
WUPyV	15	-	7	5	1	9	0	20	11	4	1	1	8	82 (92.7)
KIPyV	7	7	-	0	1	5	0	15	5	1	2	1	2	46 (86.9)
HCoV-NL63	1	5	0	-	1	1	0	4	3	2	0	0	3	17 (89.5)
HCoV-HKU1	0	1	1	1	-	3	1	3	2	0	0	0	3	14 (93.3)
HCoV-OC43	4	9	5	1	3	-	0	14	6	3	3	0	3	51 (82.4)
HCoV-229E	0	0	0	0	1	0	-	1	0	0	0	0	0	2 (66.7)
HRV	23	20	15	4	3	14		-	26	3	8	1	7	124 ()86.7
RSV	8	11	5	3	2	6	0	26	-	0	0	0	0	61 (66.3)
Influ A/B	5	4	1	2	0	3	0	3	0	-	0	0	1	19 (65.5)
PIV 1-3	5	1	2	0	0	3	0	8	0	0	-	0	0	19 (94.7)
AdenoV	5	1	1	0	0	0	0	1	0	0	0	-	0	8 (61.5)
hMPV	6	8	2	3	3	3	0	7	0	1	0	0	-	33 (84.6)

Note: Viruses shown in italics were previously tested for by immunofluroesence assay or RT-PCR for hMPV.

N: represent samples positive for respiratory virus with clinical pneumonia diagnosis

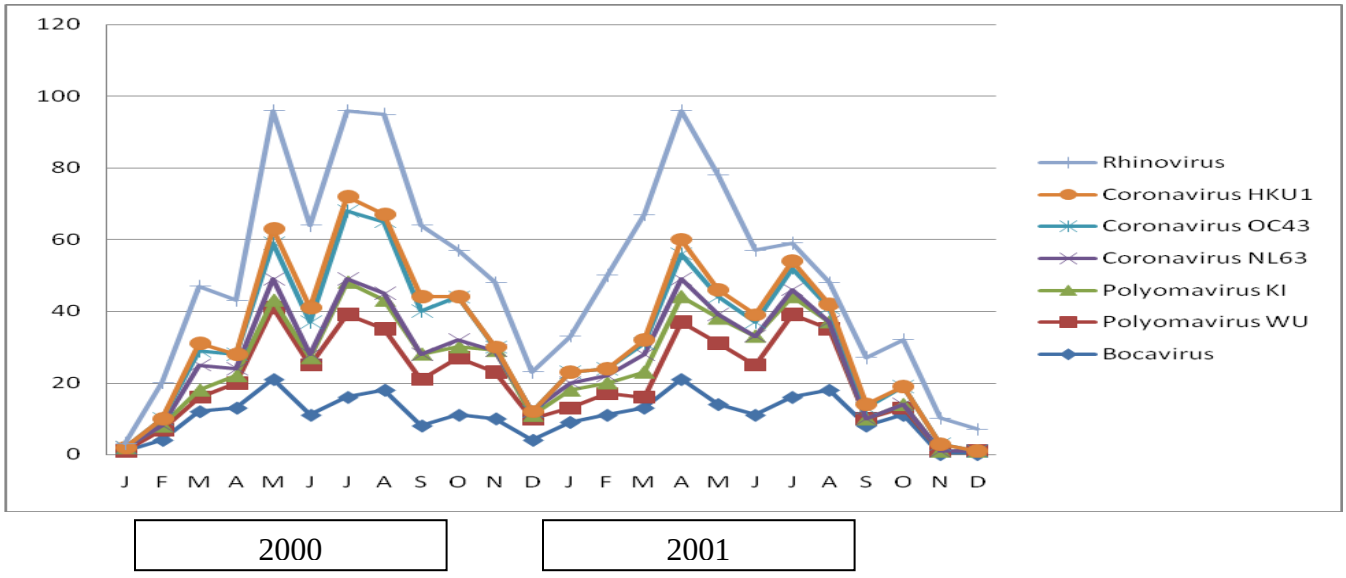


Figure 4.1: Seasonal variation in identifying new respiratory viruses in children hospitalized for LRTI between January 2000 and December 2001

4.5 Seasonality of respiratory viruses

Seasonality was observed for the period of January 2000 to December 2001. Figure 4.1 describes the seasonality of respiratory viruses in children with LRTI less than 2 years of age. The Y-axis shows the number of children with respiratory virus and the X-axis shows the months of the year for sampling. The peaks during winter months (April to August) were found for HRV, HBoV, WUPyV and KIPyV, HCoV-OC43 and HCoV-HKU1 virus over the period that the samples were collected. These months of the year represent Autumn-Winter seasons in South Africa. HCoV-229E was not included in the analysis as it did not have enough samples spread throughout.

4.6 Demographics and clinical presentations associated with newly discovered respiratory viruses

RSV, being the most common virus among the previously studied viruses was used as a referent group with which comparisons with other viruses were made in relation to demographic data, clinical data and laboratory information. A description of the demographic and clinical presentations of the LRTI episodes associated with RSV and the other viruses are shown in Tables 4.9-4.10.

Children with LRTI who tested positive for RSV had a median age of 9.73 and in comparison with children who had LRTI episodes associated with HBoV, WUPyV, KIPyV and HRV were older; i.e. 10.8 months, 13.2 months, 12.2 months and 11.3 months, respectively (Table 4.9). There were, however, no differences in the percentages of babies who were born premature between the children who tested positive for RSV compared to whom tested positive for the other viruses.

Table 4.9: Selected demographic features of children with specific respiratory viruses

Demographic feature	HBoV N=190	WUPyV N=144	KIPyV N=63	HCoV-NL63 N=29	HCoV-OC43 N=90	HCoV-HKU1 N=23	HRV N=297	RSV 209
Age in moths [range]	10.8 [1.4-23.3]	13.2 [1.6-23.9]	12.3 [1.7-23.8]	10.9 [2.2-23.3]	10.6 [1.4-24.0]	12.4 [1.8-22.0]	11.3 [1.6-23.8]	9.7 [1.5-23.8]
	p=0.03	p<0.01	p<0.01	p=0.14	p=0.26	p=0.19	p=0.02	
Female, %	43.7	41.0	47.6	55.2	42.2	52.2	37.4	43.1
	p=0.90	p=69	p=0.52	p=0.22	p=0.89	p=0.40	p=0.19	
% of babies <37 wks GA at birth	20.0	15.3	22.2	10.3	12.2	17.4	13.8	19.6
	p=0,92	p=0.29	p=0.65	p=0.31	p=0.12	p=0.53	p=0.08	

GA: gestational age
p-values compare to RSV

4.7 Clinical Signs and symptoms of identified respiratory viruses

Table 4.10 described the clinical signs and outcome of children in whom the newly identified respiratory viruses were identified and in comparison to LRTI episodes in which RSV had been previously identified. Generally, the signs and outcome of LRTI episodes associated with the newly studied viruses were similar to that observed for RSV-associated LRTI. Exceptions to this (i.e. $p<0.05$) as shown in Table 4.10 included a higher prevalence of identifying bronchial breathing in LRTI episodes associated with KIPyV (60.3%; p=0.027) and HRV (4.04%; p=0.05) compared to RSV associated LRTI (0.95%), Conversely, wheezing on chest auscultation was observed more frequently in RSV associated LRTI episodes (63.2%) compared to LRTI episodes associated with KIPyV (45.2%; p=0.011), HCoV-NL63 (41.4%; p=0.025) and HCoV-HKU1 (39.1%; p=0.025). There was no difference in oxygen saturation at admission, duration of hospital stay or case fatality rate among any of the newly studied viral LRTI episodes compared to RSV associated LRTI. There was a low overall case fatality rate including four associated with HRV, three associated with HBoV and one each associated with WUPyV, KIPyV and HCoV-OC43.

Table 4.10: Selected clinical signs and symptoms and outcome in children with LRTI

Clinical feature	HBoV N=190	WUPyV N=144	KIPyV N=63	HCoV-NL63 N=29	HCoV-OC43 N=90	HCoV-HKU1 N=23	HRV N=297	RSV 209
Diarrhoea, n (%)	49 (25.8) p=0.32	30 (20.8) p=0.88	12 (19.1) p=0.67	5 (17.2) p=0.59	14 (15.6) p=0.23	6 26.1 p=0.62	63 (21.4) p=0.96	45 (21.5)
Seizures, n (%)	5 (2.7) p=0.86	11 (7.7) p=0.02	2 (3.2) p=0.66	1 (3.6) p=0.53	4 (4.5) p=0.46	1 (4.4) p=0.47	15 (5.1) p=0.13	5 (2.4)
Vomiting, n (%)	78 (41.1) p=0.22	54 (37.5) p=0.07	30 (48.4) p=0.86	10 (34.5) p=0.20	37 (41.1) p=0.34	11 (47.8) p=0.95	114 (38.5) p=0.05]	98 (47.1)
Bronchial breathing, n (%)	6 (3.2) p=0.16	5 (3.5) p=0.09	4 (6.4) p=0.03	na	2 (2.22) p=0.59	1 (4.4) p=0.27	12 (4.0) p=0.05	2 (0.96)
Oxygen supplementation, n (%)	110 (57.9) p=0.69	93 (64.6) p=0.36	38 (60.3) p=0.94	17 (58.6) p=0.90	57 (63.3) p=0.57	13 (56.5) p=0.76	177 (59.6) p=0.96	125 (59.81)
Wheezing, n (%)	110 (57.9) p=0.28	79 (54.9) p=0.12	28 (45.2) p=0.01	12 (41.4) p=0.03	56 (62.2) p=0.88	9 (39.1) p=0.03	177 (59.6) p=0.42	132 (63.2)
Oxygen saturation at admission (±SD)	92.41 (4.04) p=0.89	91.76 (5.05) p=0.22	92.00 (5.13) p=0.53	91.28 (3.46) p=0.12	92.98 (3.14) p=0.12	92.96 (2.77) p=0.38	91.65 (8.24) p=0.21	92.35 (3.38)
Axillary temp (±SD)	37.68 (4.03) p=0.26	37.28 (0.87) p=0.66	37.55 (1.03) p=0.11	37.14 (0.85) p=0.46	37.44 (0.89) p=0.28	37.77 (0.93) p=0.01	37.23 (0.78) p=0.11	37.25 (0.74)
Mean cough duration, days (±SD)	4.30 (3.87) p=0.11	4.46 (4.72) p=0.09	4.32 (4.66) p=0.25	4.77 (4.23) p=0.14	4.28 (4.07) p=0.21	3.64 (2.90) p=0.94	4.09 (4.30) p=0.28	3.70 (3.43)
Mean weight, Kg	8.04 p=0.74	9.06 p=0.06	9.26 p=0.08	8.41 p=0.73	8.42 p=0.54	8.64 p=0.54	8.60 p=0.27	8.15
Mean hospital stay, days (±SD)	2.54 (3.35) p=0.22	2.92 (4.62) p=0.91	2.33 (2.77) p=0.20	3.41 (3.01) p=0.54	2.48 (3.70) p=0.29	1.87 (1.55) p=0.15	2.59 (3.78) p=0.26	2.97 (3.65)
Case fatality rate, n (%)	3 (1.6) p=0.10	1(0.69) p=0.41	1 (1.59) p=0.23	0	1 (1.1) p=0.30	0	4 (1.4) p=0.15	0

p-values compare to RSV
na: not available

4.8 Clinical and laboratory findings of newly identified respiratory viruses

Results of laboratory investigations undertaken in the children in whom the newly studied viruses were identified are shown in Table 4.11 and these episodes were compared to RSV associated LRTI episodes as a referent. A C reactive protein measurements of ≥40 mg/l, as a proxy measure of bacterial co-infection, was identified in a higher proportion of LRTI episodes associated with WUPyV (30.0%; p<0.0001), KIPyV (27.1%; p=0.017) and HCoV-NL63 (35%; p=0.03) than RSV associated LRTI episodes (17.0%). Similarly, indirect evidence of a greater proportion of bacterial coinfection as indicated by Procalcitonin of ≥0.2 ug/ml was evident for KIPyV (60%; p=0.023) and HCoV-OC43 (57.4%; p=0.0035) compared to RSV (40.3%). Other bacterial infections was found not significant when compared to RSV.

Table 4.11: Clinical and laboratory findings in children with LRTI

Clinical feature	HBoV N=190	WUPyV N=144	KIPyV N=63	HCoV-NL63 N=29	HCoV-OC43 N=90	HCoV-HKU1 N=23	HRV N=297	RSV 209
Median CRP-mg/dl [range]	13 [1-446.2]	18 [1-446.2]	14.6 [1-331.2]	18 [1-269.4]	15 [1-315.2]	24.95 [0-251]	13 [1-344]	10 [1-290]
CRP ≥40 mg/dl; n (%)	26 (20.5) p=0.47	30 (30.0) p<0.01	13 (27.1) p=0.02	7 (35.) p=0.03	13 (22.4) p=0.37	5 (31.3) p=0.17	44 (21.9) p=0.27	24 (17.0)
Median PCT-ug/ml [range]	0.10 [0.01-44.2] p=0.79	0.15 [0.08-69.7] p=0.01	0.34 [0.09-30.9] p=0.02	0.14 [0.09-5.1] p=0.52	0.24 [0.01-13.9] p=0.09	0.10 [0.10-11.3] p=0.33	0.18 [0.01-105.6] p=0.02	0.12 [0.08-47.5]
PCT ≥0.2 ug/ml; n (%)	43 (37.7) p=0.68	43 (47.8) p=0.28	27 (60.0) p=0.02	8 (44.4) p=0.74	31 (57.4) p=0.04	6 (42.9) p=0.85	92 (49.5) p=0.11	50 (40.3)
CXR-AC; n (%)	29 (15.3) p=0.78	28 (19.4) p=0.44]	15 (23.8) p=0.17	4 (13.8) p=1.00	19 (21.1) p=0.31	5 (21.7) p=0.51	55 (18.5) p=0.51	34 (16.3)
[1]Bacteria in BC; n (%)	21 (1.1)	28 (19.4)	10 (15.9)	2 (6.9)	9 (10.0)	8 (34.8)	43 (14.5)	21 (10)

p-values compare to RSV
na: not available
CRP: C-reactive Protein
PCT: Procalcitonin
CXR-AC: Alveolar Consolidation on Chest Radiography
[1] Bacteria grown from patient's blood culture

Table 4.12: Bacteria found in blood cultures in children with LRTI and newly discovered respiratory viruses

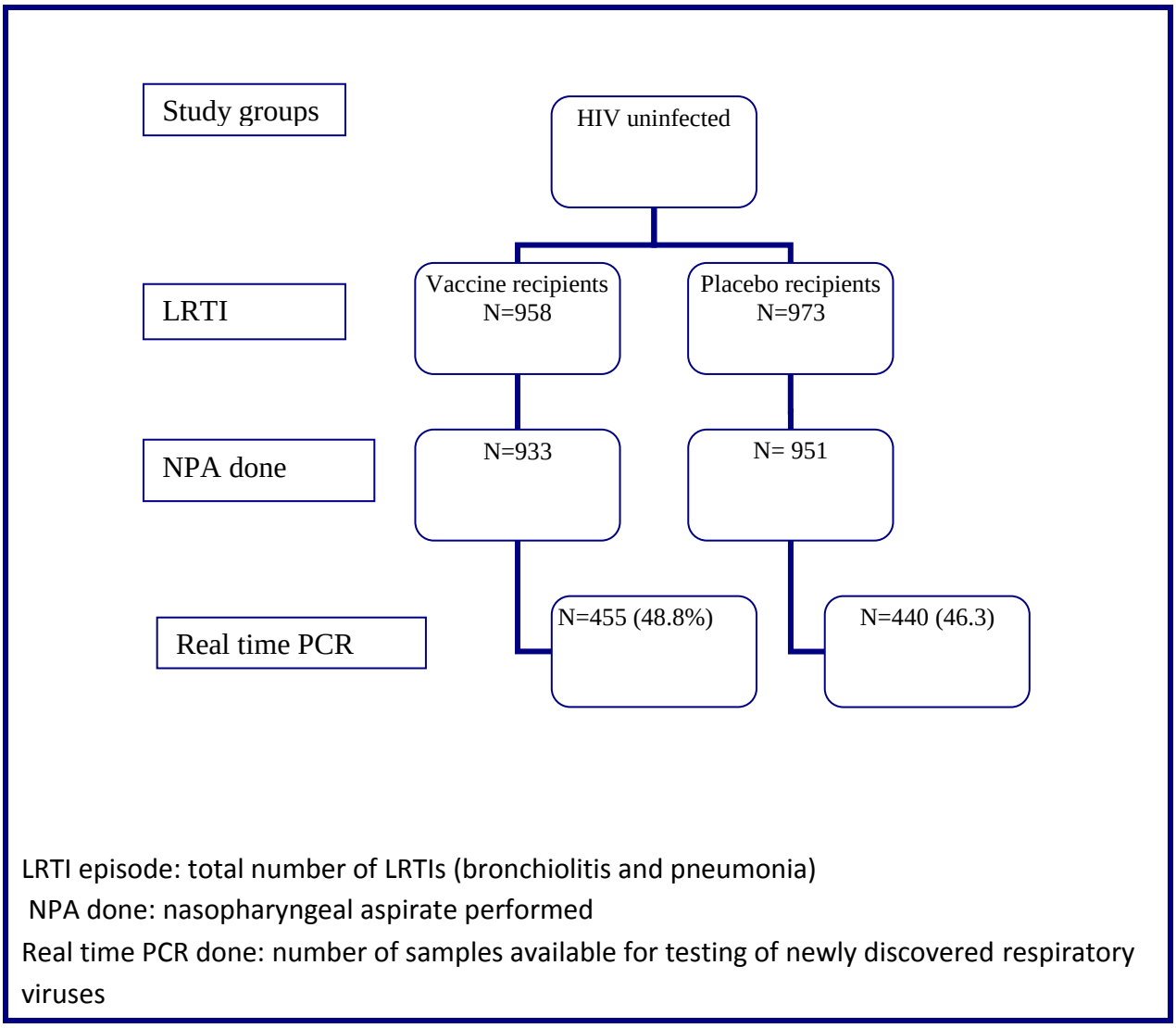
Clinical feature	HBoV N=190	WUPyV N=144	KIPyV N=63	HCoV-NL63 N=29	HCoV-OC43 N=90	HCoV-HKU1 N=23	HRV N=297	RSV 209
S.pneumoniae, n	1 p=0.73	2 p=0.37	0 p=0.77	0 p=0.87	1 p=0.52	0 p=0.90	3 p=0.45	1
H. influenzae, n	0 p=0.52	0 p=0.59	0 p=0.77	0 p=0.87	0 p=0.69	0 p=0.90	2 p=0.63	1
[1] Other bacteria, n	20 p=0.40	26 p=0.01	10 p=0.11	2 p=0.47	8 p=0.91	8 p=0.001	38 p=0.19	19
No bacteria n	159	107	50	27	77	14	234	174

p-values compare to RSV

[1] Other bacteria: *Staphylococcus epidermidis, corynebacterium, Salmonella, nocardia species, Escherichia coli, Streptococcus agalactiae, Bacillus bacterium, Pseudomonas aeruginosa, Campylobacter, Staphylococcus aureus, Propionibacterium, Bimorrela catarrhalis, Viridans Streptococcus, Enterococcus faecalis, Alkaligines spp, Morganella morganii, Lactobacillus genus, Klebsiella genus, Acinetobacter lwoffii, Peptostreptococcus species, Candida albicans, Streptococcus milleri group, Micrococcus genus, Shigella flexneri, Neisseria meningitides, Haemophilus parainfluenzae, Citrobacter freundii, Mycobacterium tuberculosis, Neisseria subclavia, Escherichia vulneris.*

4.9 Bacterial co-infection

Table 4.12 describes bacterial co-infections found in samples positive for respiratory viruses. Bacterial analysis on the blood in children with LRTI revealed that HCoV-HKU1 (n=8, 34.8%, p=0.000) and WUPyV (n=26, 18.1%, p=0.011) was found to be significantly different compared with RSV in diagnosis for other bacteria, there was no further significance observed. However possibility of contamination is observed for Corynebacteria.



LRTI episode: total number of LRTIs (bronchiolitis and pneumonia)
NPA done: nasopharyngeal aspirate performed
Real time PCR done: number of samples available for testing of newly discovered respiratory viruses

Figure 4.2: Summary of children who participated in the PCV trial under two years hospitalized for LRTI since 1 February 2000.

Chapter 5: Discussion

Respiratory viruses have been associated with pneumonia (Henrickson 1998; Madhi et al 2004; Arnold et al 2006; Fry et al 2007). Various studies have detected respiratory viruses in symptomatic and asymptomatic patients with respiratory illness (van Elden et al 2004; Allander et al 2005; Kesebir et al 2006; Fry et al 2007; Han et al 2007; van de Pol et al 2009). Recently PCR has been used in laboratory and clinical settings as a diagnosis tool as it is an effective technique to detect genetic material of pathogens (van der Hoek et al 2004; Allander et al 2005; Woo et al 2005; Sloots et al 2006; Gaynor et al 2007). PCR is currently used to detect respiratory viruses as it has been recognized to be as sensitive and specific as other more conventional methods (virus isolation, cell culture and serology) (Heikkinen et al 2002; Lambert et al 2008). Hence PCR has increased the ability to detect viral pathogens including new and known respiratory viruses in clinical samples. In this study we have used PCR to identify and enumerate respiratory viruses, in HIV negative children less than 2 years old with lower respiratory tract infection.

Prevalence of newly discovered respiratory viruses in children hospitalized for LRTIs

In the current study the prevalence of newly described respiratory viruses was reported overall and it was stratified by PCV vaccination status; we also report on the prevalence thereof by clinical syndromes of bronchiolitis and clinical pneumonia. The prevalence's reported are in general higher than previous published data on newly discovered respiratory viruses; nevertheless, the published data from various studies also report similar high prevalence of respiratory viruses associated when stratified by clinical syndromes LRTI (Choi et al 2006), pneumonia (Fry et al 2007; Franz et al 2010; Khadadah et al 2010), wheeze (Allander et al 2007; Smuts et al 2008) and bronchiolitis (Khadadah et al

2010). However bronchitis, bronchiolitis and pneumonia have been collectively associated with respiratory viruses (Gerna et al 2007).

Prevalence, clinical presentation and co-infections with other viruses and bacteria for HBoV

The overall prevalence of HBoV was high, 21.2% for LRTI, 22.3% for bronchiolitis and 20.3% for clinical pneumonia. In general, HBoV was more prevalent in South African children with LRTI in comparison to other studies, where the prevalence of HBoV ranged between 1% and 8% (Allander et al 2005; Smuts and Hardie 2006; Fry et al 2007). However, our data were similar to data describing the prevalence of HBoV associated with different clinical presentations, such as 19% in children with wheeze (Allander et al 2007) and 19.3% in children with acute respiratory illness (Bonzel et al., 2008). In the earlier studies (Allander et al 2005; Smuts H and Hardie D 2006), a conventional PCR method was used to enumerate HBoV, whereas the more recent studies (Allander et al 2007; Bonzel et al 2008) have used either quantitative or qualitative real time PCR. This difference in techniques can be a possible explanation for the higher prevalence of HBoV found in our study, as real time PCR is more specific and sensitive to detect genetic material in even the least concentrated samples.

When stratified by PCV status, HBoV was most prevalent in the PCV-recipients compared to placebo-recipients for all clinical syndromes. This is in contrast with data for most of the viruses described in this study whose prevalence was either unaffected, or even decreased with PCV vaccination. This unexpected result suggests that not only is HBoV unable to create the conditions which favour the outgrowth of vaccine serotypes of pneumococci, but also that LRTI associated with HBoV appears to be enhanced by PCV vaccination. This conclusion needs to be approached with caution due to bias in sample selection from the original sample set collected during the vaccine trial. The present data

suggests that continued surveillance for HBoV infections is needed in light of the rollout of PCV vaccination in South Africa.

In 82% of LRTI episodes where HBoV was detected real-time PCR was also positive for at least one other respiratory virus. In other studies that have also reported on the prevalence of co-infections with HBoV in children with respiratory tract disease a 16% HBoV co-infection rate was observed (Bonzel et al 2008) and 43% (Manning et al 2006), 18% (Choi et al 2006), 39.1% (Weissbrich et al 2006) and 83% rates were detected in children with pneumonia (Fry et al 2007). This wide range is most likely due to differences in the number of pathogens reported as co-infections and the limitations in the methods used for detection. In the latter publications HBoV co-infections were more frequently associated with RSV (24%), HPIV (43%), HRV (42%) and Influenza (9%) (Fry et al 2007). Other studies showed co-infections of HBoV with RSV (14.3%) and adenovirus (7.1%) (Allander et al 2005) or with HRV (50%) and RSV (62%) (Fry et al 2007). In a South African study co-infection with a range of viral and bacterial pathogens was found in 37% of HBoV-positive children (Smuts and Hardie 2006). In our study we detected HBoV co-infections in: 26% with HRV, 8.9% with RSV, 3.2% with adenovirus and 7.4% with HCoV-strains. The co-infections rates of HBoV with other viruses in our study are consistent with other studies that have reported on HBoV co-infections with other respiratory viruses in children younger than 24 months (Manning et al 2006; Smuts and Hardie 2006; Weissbrich et al 2006; Allander et al 2007; Fry et al 2007).

Using data from a previous study in our unit, 1.1% of samples that were positive for HBoV were identified to have a correspondent positive bacterial blood culture, indicative of a bacteria-viral co-infection. Of the HBoV positive samples 15.3% had a CXR-AC, 20.5% had an elevated CRP greater than 40mg/dl and 37.7% had elevated PCT greater than 0.2 ug/ml. Three children who tested positive for

HBoV died. These findings are indicative that children infected with HBoV had severe (bacterial and viral) infections. Children who tested positive for HBoV experienced vomiting (41.1%), diarrhea (25.8%), seizures (2.7%), bronchial breathing (6.0%), wheezing (57.9%) and were in need of oxygen supplementation (57.9%) throughout treatment. Similarly, studies have found clinical symptoms including wheezing (Allander et al 2007) and oxygen supplementation (Fry et al 2007) in children with HBoV infection. Our findings show that HBoV prevalence is similar to RSV in LRTI clinical syndrome.

One of the 3 children who died who were positive for HBoV was co-infected with HRV and the other two children had either bacterial infection from *E. coli* or from *S. pneumoniae*. C reactive protein values for the children with bacterial infections were 278 and 10 respectively.

Prevalence, clinical presentation and co-infections with other viruses and bacteria for HRV

The prevalence of HRV was 33.2% for LRTI, 33.8% for bronchiolitis and 30.8% for clinical pneumonia. Other studies have detected HRV in 32% (Talbot et al 2009; Franz et al 2010), 34% (Khadadah et al 2010) and 56% (Choi et al 2006) of children with lower respiratory tract infection. The prevalence of HRV infection noted in studies in children with clinical pneumonia ranged from 22% (Franz et al 2010) to 42% (Khadadah et al 2010). In a study from Kuwait where 28% of the total specimens collected from LRTI episodes were positive for at least one of the 10 respiratory viruses tested showed that 37% and 42% of those presenting with bronchiolitis and pneumonia had HRV in their samples, respectively. (Khadadah et al 2010). In this stud 58% of the patients with LRTI were less than 1 year old (Khadadah et al 2010). These studies mentioned above used both conventional (Choi et al 2006; Khadadah et al 2010) and real-time PCR (Talbot et al 2009; Franz et al 2010). Hence factors that play a role in estimating the prevalence of HRV in different populations, the age of the subjects sampled and method used to detect the respiratory viruses is essential in the analysis.

The HRV is notoriously known for its diversity, hence has a slight advantage over other viruses for spreading faster and higher pathogenetic potential (Arden et al 2010). Human rhinovirus ability to escape host immunity, attributed to the error prone viral RNA polymerase, enhances its ability to survive longer in patients by generating variants. Hot spots regions have been identified in the viral capsid genes VP1, VP2 and VP3 during acute infections (Cordey et al 2010). Therefore most previewed prevalence studies have utilized viral capsid genes to detect HRV. Our study also used these regions, as it increased the potential to detect more than one variant

Despite a higher prevalence of HRV, co-infections of HRV with RSV (15.5%), HBoV (16.8%), WUPyV (14.1%), HMPV (7%), KIPyV (7.7%) and 3.7% with PIV 1-3 was found in our study. Other studies have shown co-infections of HRV with PIV 3 (7.1%) and RSV (50%) in children hospitalized for lower acute respiratory tract infections (Xiang et al 2008). Co-infections results from other studies have also shown that the most prevalent co-infections were due to RSV, HMPV, Influenza and PIV in children with asthma (Miller et al 2009). HRV-A and HRV-C strains of HRV show the higher degree of co-infections (Miller et al 2009).

Patients' clinical information from which respiratory viruses were detected is essential in linking the presence of a particular respiratory virus and to disease severity. Children positive for HRV were found to have the following symptoms: seizures (5.1%), diarrhoea (21.4%), vomiting (38.5%), bronchial breathing (4.0%) and 59.6% required oxygen supplementation and were also found to have wheezing. Other studies have shown a significant finding of fever and cough as clinical symptoms associated with HRV-A and HRV-C infection in children, respectively (Miller et al 2009). There were four case fatalities with HRV, of these deaths 3 patients had no bacterial or other viral infections and one patient was co-infected with WUPyV. We have also shown that HRV prevalence is similar to that of RSV, suggesting

that HRV plays a role in the development and severity of LRTI in children less than 24 months. When stratified by PCV vaccination status, HRV had a similar prevalence to HBoV where it was more prevalent in the PCV vaccinated recipients for all clinical syndromes.

Occurrence of HRV in the lower respiratory tract of children in our study and other studies supports the findings that HRV replicates and causes disease in the lower respiratory tract. In particular, a higher prevalence of clinical pneumonia indicates that infections with HRV of epithelial cells increases adherence of pneumococcus and that HRV impairs immune response of macrophages to bacterial lipopolysaccharide and lipoteichoic acid (Oliver et al 2008).

Prevalence, clinical presentation and co-infections with other viruses and bacteria for HCoV-strains

The human coronaviruses are viruses commonly known to cause mild respiratory symptoms including mild cough, running nose and malaise (van der Hoek et al 2007). However in immunocompetent individuals including children, HCoV are less prone to cause severe illness than in immunocompromised individuals due to their vulnerable immune system (van der Hoek et al 2007). In our study the overall prevalence of HCoV strains was 16.5% of LRTI, 15.9% for bronchiolitis and 17% for clinical pneumonia. Of the 4 strains tested the most prevalent strain was HCoV-OC43 in all clinical syndromes. Children who received placebo were more likely to have HCoV-OC43 infection than other strains of HCoV. Published data have shown that the majority (74.5%) of HCoV infections was associated with lower respiratory tract infections including bronchitis, bronchiolitis and pneumonia, compared to only 25.5% of upper respiratory tract infections which include rhinitis, pharyngitis and laryngitis (Gerna et al 2007). Other published reports have associated HCoV-NL63 and –OC43 with a substantial burden of lower respiratory illness and medically attended upper respiratory infections in

previously healthy children (Talbot et al 2009). Comparing human coronaviruses HCoV-OC43 and HCoV-NL63 had the highest infections in children diagnosed for LRTIs, bronchiolitis and clinical pneumonia in our study. Other studies have also found a higher prevalence of HCoV-OC43 and HCoV-NL63 in children (Hofmann et al 2005). In our study the least detected HCoV strain was the HCoV-229E strain. From other studies evidence of low prevalence of HCoV-229E was found using both molecular (i.e. PCR) and serological methods (virus neutralization) in individuals suffering from URTIs and LRTIs and their findings are similar to our study (Hofmann et al 2005; Talbot et al 2009). Both HCoV-OC43 and HCoV-229E share similar genomic sequences and form the group I of HCoV, in addition these two strains cause severe respiratory diseases in children under the age of 2 years and immunocompromised adults (van der Hoek et al 2004; van der Hoek et al 2007). Our study also showed that HCoV-strains occur in children less than 2 years of age, with a median age ranging from 9.73 months to 12.43 months. Nevertheless the difference in human coronaviruses prevalence is also noticeable in the populations around the world.

Due to their differences in prevalence we should speculate whether these viruses exhibit similar clinical symptoms. Wheezing is often one of the clinical symptoms associated with the presence of respiratory viruses in children, followed by fever and coughing. In our study we report that compared to RSV both HCoV-NL63 and HCoV-HKU1 infections cause significant more wheezing 41.4%, p=0.03 and 39.1%, p=0.03, respectively. Whereas other studies have indicated an association of URTIs and HCoV-NL63 (van der Hoek et al 2004), URTIs includes symptoms ranging from sneezing, sore throat, nasal discharge, cough, headache and myalgia (Baron S 1996). In addition a South African study also implicated the presence of HCoV-NL63 in children with acute wheezing (Smuts et al 2008). On the other hand, HCoV-NL63 has been associated with croup among infants with LRTIs (Choi et al 2006; Gerna et al 2007). Additional clinical data found in our study include elevated C reactive

measurements of more than ≥40 mg/dl that were observed in 35% of children with HCoV-NL63, indicative of an active inflammation. A highly bacterial infection was observed in 8% of children with HCoV-HKU1. In our study HCoV-NL63 positive children were found to have an elevated CRP measurement, indicative of a severe infection in these patients.

Viruses with low prevalence may be misrepresented in terms of their associations with symptoms observed in the different studies, and this is true for HCoV-229E as it was the least detected virus amongst the coronaviruses in our study. In addition most studies are biased in the selection of samples, as specimens from sick patients are normally used in viral studies due to the need to diagnose and treat the patients. Hence future studies need to approach the question of asymptomatic viral prevalence in a manner that will educate researchers on the biological pathways respiratory viruses follow in both unhealthy and healthy individuals without or without symptoms.

The highest rates of co-infections of HCoV-OC43 were with HMPV (8.9%), WUPyV (13.9%) and HRV (27.1%). Although the importance of these findings in terms of disease severity remains elusive in our case, however other studies have associated severe respiratory disease with multiple co-infections of HCoV-strains with other viruses as compared to single infection of HCoV strains in children less than one years of age (Gerna et al 2007).

Prevalence, clinical presentation and co-infections with other viruses and bacteria for WUPyV

The prevalence of WUPyV detected in our study was higher compared to most of the published available data, as mentioned above detection methodology and patient age have a heavy role in the detection of respiratory viruses. Human polyomaviruses have been shown to have high seroprevalence particularly in younger children and the elderly compared to young adults (Abedi

Kiasari et al 2008). In addition their abundance during childhood and the presence of other viruses may have some bearings to the development of severe disease (Han et al 2007). Thus, studies have come to a conclusion that human polyomaviruses establish persistent infection in the respiratory tract and become reactivated during respiratory disease of any cause (Han et al 2007; Abedi Kiasari et al 2008). With that in notion, human polyomaviruses have been detected in asymptomatic patients (Han et al 2007; van de Pol et al 2009), in some cases these patient are immunosuppressed (Norja et al 2007). Still the significance of these findings remains elusive, as it is further complicated by co-infection with other respiratory viruses.

We found an overall co-infection with WUPyV associated with LRTI in 90.0% of samples, 54.2% for bronchiolitis and 56.9% for clinical pneumonia. In overall LRTI high number of co-infections of WUPyV was observed with HRV (29.2%), HMPV (8.3%) and HCoV-OC43 (13.9%). In specimens collected from bronchiolitis cases the highest co-infection rates of WUPyV were with HRV (32.3%) and HBoV (22.1%). WUPyV co-infections in clinical pneumonia syndrome was not statistically significant but was the commonest co-infection with HBoV, as it had the highest co-infections than other viruses. Other studies have found co-infections of WUPyV with of HRV (40.5%) and HBoV (27.02%) (Gaynor et al 2007) and with HMPV (50%) and with HBoV (25%) (Abedi Kiasari et al 2008) with various clinical presentations. Hence our data showed a higher prevalence of co-infection of WUPyV with HBoV. With this knowledge, WUPyV can be regarded as a virus that facilitates secondary infections by other viruses or bacteria or can be acquired early in life and reactivated later as a result of infection.

In our study WUPyV infection was associated with seizures (7.7%). Although other studies have associated WUPyV infection with cough, fever and wheezing (Zhuang et al 2010), there was no association of WUPyV with clinical features from our findings when compared to RSV.. Clinical

features of the children in whom WUPyV was detected indicated a raised CRP to 20.5% ,a raised PCT level to 47.8% and 19.4% of CXR-AC. Hence, these clinical findings of WUPyV positives were associated with severe respiratory infection. In addition 19.4% of WUPyV positive specimens were associated with blood culture positive for various bacteria. One case fatality was observed in this scenario. In most studies published to date looking at WUPyV presence clinical data was not presented and only diagnosis of respiratory significance is indicated, hence this impedes to link the presence of the virus with the severity of disease. However, when we used clinical data WUPyV is implicated in playing a role in the severity of illness.

Although we confirmed the presence of WUPyV in children with pneumonia, its role still remains unclear due to the high frequency of co-infection with other respiratory viruses. This lack of distinction is possibly associated with the use of highly sensitive PCR for detection of positive samples, which is able to detect pathogens even in low copy numbers, and is thus unable to discriminate between infection and asymptomatic carriage.

Prevalence, clinical presentation and co-infections with other viruses and bacteria for KIPyV

We found a prevalence of 8.6% of KIPyV in children with clinical pneumonia, 7.0% in children with LRTI and 5.3% in children with bronchiolitis. The prevalence of this virus in our samples was higher than most published data (normally less than 5%) for all syndromes (Bialasiewicz et al 2007; Abedi Kiasari et al 2008; van de Pol et al 2009; Venter et al 2009). Major difference in prevalence from our study compared to other study should be approached with caution, due to differences in methods used in processing the samples and analysis, also including the season of sampling that in our case included the winter months. Age of patient also plays an important role, as previous data supported the

presence of polyomaviruses early in childhood (Abedi Kiasari et al 2008). Bialasiewicz et al. also detected a higher prevalence in younger children (Bialasiewicz et al 2007).

Comparing our findings we found that 14.3% of KIPyV positive specimens were co-infected with RSV, 7.9% with HMPV and 1.6% with Influenza A/B and other studies have found 16.7% KIPyV co-infections with RSV, 4.2% with HMPV and 4.2% with Influenza A. Similar results have been found in other studies (Allander et al 2007; Bialasiewicz et al 2007; Gaynor et al 2007; Abedi Kiasari et al 2008). Different publications have suggested that KIPyV infections do not cause disease, in particular pneumonia and bronchiolitis (Norja et al 2007; Jiang et al 2009). However, our findings show a significant association of KIPyV infection in the development of bronchiolitis and clinical pneumonia. Also children infected with KIPyV had a significantly increased CRP and PCT measurement than normal.

Although there are some evidences that implicate that KIPyV has a role in respiratory infections, it still remains unclear that it causes disease due to a high frequency of co-detecting other respiratory viruses in KIPyV positives samples. In addition, lack of asymptomatic control in our studies and published studies complicates the link between respiratory tract disease and presence of KIPyV.

Seasonality

Although our data are spread throughout four seasons, peaks of respiratory viruses were observed during winter months (April to August) for HRV, HBoV, WUPyV and KIPyV, HCoV-NL63, HCoV-OC43 and HCoV-HKU1. Similar results have been shown before for HBoV to occur in winter months in South Africa and Australia (Sloots et al 2006; Smuts and Hardie 2006). In addition KIPyV has been shown to have similar seasonality in Australia (Bialasiewicz et al 2007). Seasonality for WUPyV has been

demonstrated in the Northern hemisphere in the United Kingdom (Norja et al 2007). Human coronaviruses are also found in winter months particularly the strains HCoV-HKU1, HCoV-OC43 and HCoV-NL63 (Gaunt et al 2010). Temporal association for HCoV-229E season was not determined as we had only four positives for our study, similar sporadic trends have however been identified in the United Kingdom (Edinburgh) (Gaunt et al 2010).

Limitation of the study

The complete set of clinical samples collected during the surveillance period of the original parent study was not available for the present study due to the loss of samples. This loss was a result of either insufficient sample caused by repeat testing for other assays, or poor sample quality following prolonged storage. As a result, this study is limited by the potential bias in samples selection with respect to PCV vaccination status, and within the described clinical syndromes, and hence the true incidence of these viruses cannot be reported, and thus reporting is limited to the prevalence of the viruses within the context of these limitations.

In addition we need to keep in mind that methodology and sampling procedure play a role in detecting true prevalence and incidences, making it difficult perform direct comparisons with other studies. Most studies have also showed that sampling during childhood can be biased in detecting higher rates of respiratory viruses in younger children than in older children. Our study was also biased as it only focused on the detection of respiratory viruses in infants.

In the current study it was not possible to validate our real time PCR viral panel against a known method of detection. It would had been of interesting to use different methods such as conventional PCR vs. real time PCR to detect the viruses and to determine the difference between the two methods

in detection limit, sensitivity and sensitivity. A validated panel of known samples positive for each respiratory virus was not used in this study and this limited our knowledge on the performance of the PCR assay. However we optimized our PCR assay (concentration of reagents in the duplex reaction and determination of the detection limits by limiting serial dilutions of known positive samples) against the different viruses tested.

Lastly our study had no asymptomatic patients to compare the prevalence of the respiratory viruses tested. This limited our knowledge in determining actual pathogens responsible for causing disease. Sero-epidemiological studies were also unfortunately impossible to perform in the cohort of our children. Additional data on HIV-infected children would have added more information with regards to the symptoms experience in both HIV-infected and un-infected, hence showing pathogenicity of the newly respiratory viruses in these children.

Other limitations include the fact that molecular epidemiology was not included in the study, to show differences in strains in South Africa when compared with the rest of the world. Again knowledge of molecular sequences enhances ability to treat patients representing with lower respiratory tract infect and in the diagnostic tools to identify the viruses responsible.

Chapter 6: Conclusions

The role of the newly discovered respiratory tract viruses is clinically significant, as these viruses exist in patients presenting with respiratory diseases in particular lower respiratory tract diseases. Although newly discovered respiratory viruses do not fulfill the totality of the Koch's postulate stating that "microorganism must be isolated in diseased organism and grown in pure culture, microorganism should cause disease when introduced to healthy organism, and lastly the microorganism must be reisolated from the inoculate of diseased host and shown to be identical to the original causative agent", nevertheless they fulfill part of Koch's postulate "a microorganisms must be present in organisms suffering from diseases". Therefore strategies such as vaccine interventions can be used to evaluate the role that these respiratory viruses play in elucidate etiology of pneumonia. Although respiratory viruses roles are indefinite additional studies of pneumonia in clinical settings have shown presence of respiratory viruses in patients with pneumonia (Madhi et al 2000; Madhi et al 2005; Arnold et al 2006; Allander et al 2007; Rudan et al 2008)

The high detection rates of respiratory viruses is a true representation of respiratory tract infection, as molecular techniques such as PCR have been used in most studies to compare the prevalence of respiratory viruses in symptomatic and asymptomatic patients, indicating that PCR positives specimens are not residuals left over from previous infections from earlier infections of the virus of interest hence PCR is a indication of clinical and sub-clinical respiratory infection (Jartti and Korppi 2011).

The project presented in this thesis reveals the persistence of newly described respiratory viruses in children who suffered from pneumonia and their prevalence in children who receive placebo or PCV. Hence we showed that these viruses are also present in South Africa. Although their role is remains elusive, presence of newly respiratory viruses indicates that they assist in establishing lower respiratory infections including pneumonia and bronchiolitis. Further investigations of the study would include genotyping positive samples from this study and constructing phylogenetic lineage of respiratory viruses in South Africa. Additional studies focusing on HIV-infected children, older children particularly of school age and contacts of children with these respiratory viruses identified would aid in further understanding their role in respiratory disease..

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Appendix

8.1.1 Lambda Phage Growth Protocol: Induction of Lambda lysogen

- Grow culture of lambda lysogen overnight at 30°C.
- Inoculate fresh LB and grow culture at 30°C till an OD of 0.2 is reached (540nm).
- Transfer to 44°C waterbath –shake flask for 10min in waterbath.
- Put culture on shaker at 37°C for 1hr 45min.
- Microfuge culture for 1min.
- Discard supernatant.
- Vortex briefly to resuspend pellet in 0.5ml LM medium. (L broth and 10mM $MgCl_2$).
- Add 20 µL of chloroform.
- Vortex for 10s
- Store lysate (Figure 8.1) in cold 4°C.

8.1.2 Titering lambda phage

- Into a test tube add 2ml LB, 40ul 1M $MgCl_2$, 20ul E.coli (stationary phase), vortex.
- Add 2ml sloppy agar, mix by rolling between palms and pour onto LA plate with 10mM $MgCl_2$.
- When sloppy agar hardens, incubate at 42°C for 15 min.
- In the meantime, prepare serial dilutions (10^{-1}-10^{-6}) of lambda in 20ul.
- When the plate has dried spot 10ul of each dilution onto a single plate. Wait till spots are absorbed. Incubate at 37°C until plaques are visible.
- Where single plaques are visible-count plaques in each spot:

  Pfu/ml= # of plaques X dilution factor/ vol spotted.

8.1.3 Elution of Phage

- Add 5ml if CSM to plate with plaques and store at 4°C for several hours (1-2hrs) with intermittent, gently shaking.
- With a Pasteur pipette, harvest as much of the CSM as possible and place in a 15ml polypropylene tube.
- Add 1ml of fresh CSM to the plate and store it for 15min in a tilted position to allow all of the fluid to drain into one area. Again remove the CSM and combine it with the harvest. Add 0.1ml of chloroform to the pooled CSM, vortex briefly and centrifuge at 3000rpm for 10min at 4°C. recover 8.1.4

8.1.4 Cleaning phage from bacterial lysis

- Add DNAse I and RNAse I at 1ug/ml each. Tilt the culture gently to mix in the enzymes without dispensing the chloroform. Incubate at room temperature for about 20min.
- Split the lysate into two halves and centrifuge out the bacterial debris for 10min at 10000rpm.
- Save 10ul of supernatant in eppendorf tube (use in column prep to check for DNA quality). Decant the remaining supernatant into new tubes.
- Add 7ml of 20% Glycerol (w/v) PEG-8000 and 2.5M NaCl solution to each tube. Store ON on ice in 4°C.

- Centrifuge the PEG-precipitate for 15min at 8000 rpm. Decant the supernatant and invert the tubes over paper towel for 5-10min to drain. Don't dry out pellet.
- Resuspend each pellet in 1ml CSM. Rinse the sides of the tubes well since precipitate often is stuck to the sides rather than the bottom of the tube.
- Pool the resuspensions for each phage in one of the tubes and transfer into new tube. Add 2ml chloroform and centrifuge at 10000rpm for 10min.
- Transfer the aqueous phase to two eppendorf tubes and perform two additional chloroform extractions 0.5ml of chloroform
- Combine the aqueous phases and add an equal volume of 10mM Tris-HCL.

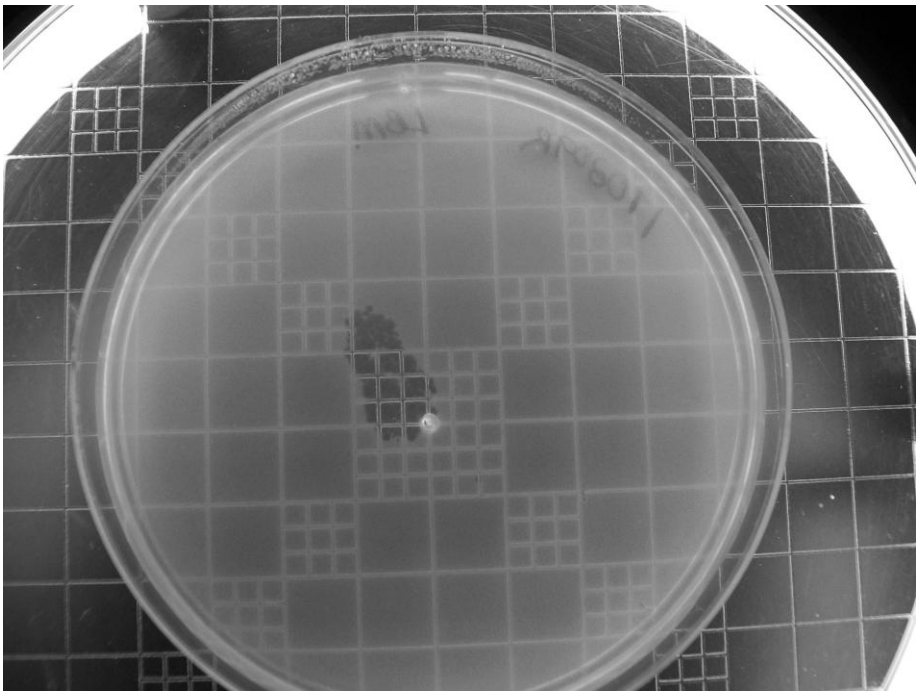


Figure 8.1: Lambda phage plaque lysate

1. Allow the kit components to thaw on ice.

2. Referring to the table below, calculate the volume of components needed to prepare the required number of reactions.

Note: Prepare the RT master mix on ice.

Component	Volume/Reaction (µL)	
	Kit with RNase Inhibitor	Kit without RNase Inhibitor
10× RT Buffer	2.0	2.0
25× dNTP Mix (100 mM)	0.8	0.8
10× RT Random Primers	2.0	2.0
MultiScribe™ Reverse Transcriptase	1.0	1.0
RNase Inhibitor	1.0	—
Nuclease-free H_2O	3.2	4.2
Total per Reaction	10.0	10.0

IMPORTANT! Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

WARNING **CHEMICAL HAZARD.** 10× Reverse Transcription Buffer may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate eyewear, clothing, and gloves.

3. Place the 2× RT master mix on ice and mix gently.

Figure 8.2: Applied Biosystems RT-PCR Protocol

IMPORTANT! These conditions are optimized for use with the High-Capacity cDNA Reverse Transcription Kits.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 sec	∞

Set the reaction volume to 20 µL.

Figure 8.3: Applied Biosystems RT-PCR cycling conditions on light cycler

Table 8.1: Design of primer matrix reactions for all primers

Components	Quantity	SI units
Initial primer concentration	1600	nM
Stock concentration	10000	nM
Volume of primer in reaction	5	µl
Total volume of reaction	25	µl
# of wells for matrix	36	
Total volume 1st tube dilution	66	µl
vol. of primer in 1st tube dilution	44	µl
vol. of water to add in 1st tube dilution	11	µl

Table 8.2: Q-PCR set-up for primer matrix reaction mix

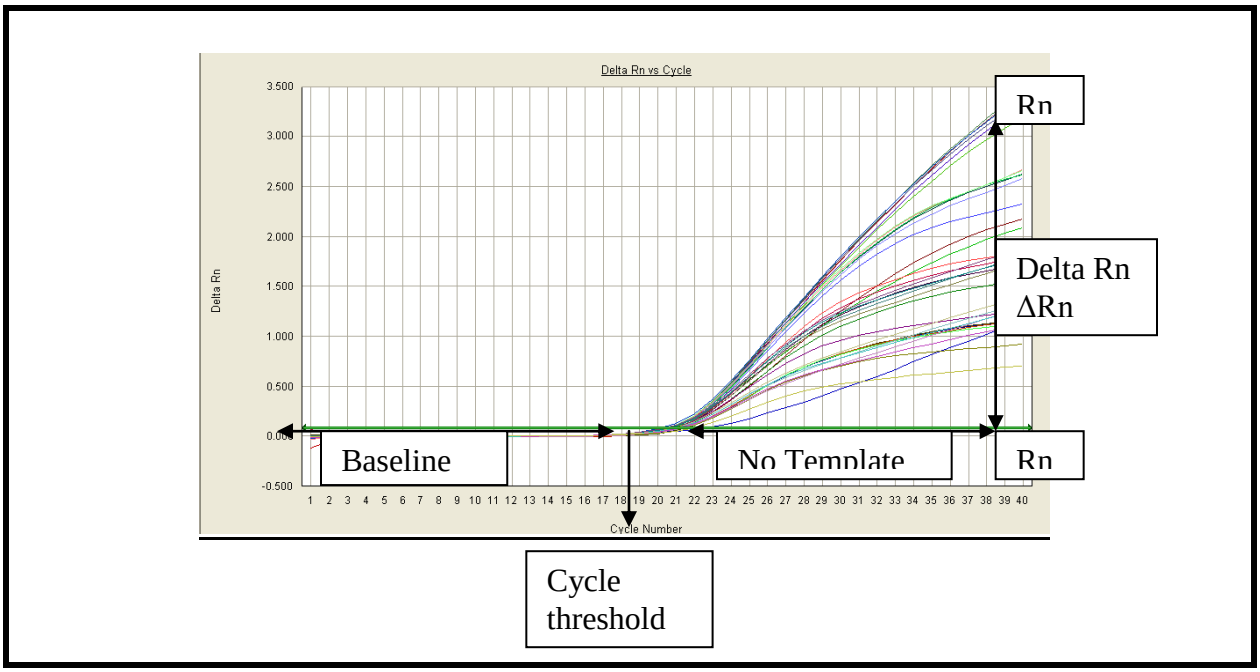
Components	Initial concentration	Final concentration	Volumes (µL)/Reaction	Volume (µL)/37 Reactions
Master mix	2X	1X	12.5	462.5
Forward primer	Various	Various	5	
Reverse primer	Various	Various	5	
Probe	10µM	0.3µM	0.6	22.2
DNA			1	37
Water			0.9	33.3

8.2 Primer optimizations

Optimal primer concentrations were selected at the linear phase of reaction using linear phase graphs. Two criterions apply when selecting primer pairs and these are the Delta Rn and cycle threshold. Tables 8.3-8.4 illustrate the Delta Rn and the cycle thresholds. Control primer sets that were used to detect respiratory virus are limiting primer pairs . In a PCR reaction detecting two targets at the same time it is necessary to limit primers in order for the majority of species to be stopped before it can limit common reactants available for amplification of the minority species.

Hence varying the concentrations of primers, the observed trends are attributed to the change in occurring and detection of the target DNA. In this step we were observing how

different concentration of primers affects the detection of target DNA. Thus determining the pair which is most suitable for the detection of respiratory viruses. Table 8.5 illustrates primer matrix with Delta Rn and cycle threshold of 18 for bocavirus. For all respiratory viruses primer concentrations were chosen to ensure that in the PCR reaction they would detected the target virus yielded a maximum Delta Rn value and with a low cycle threshold.



Graph 8.1: Amplification curve of NDV showing log view of primer matrix

Table 8.3: Cycle threshold and fluorescence intensity obtained from primer matrix dilution of NDV and Lambda controls.

Detector: NDV	Reporter: FAM	Threshold: 0.086788	Detector: LAMBDA	Reporter: VIC	Threshold: 0.0329748
Primers	Cycle threshold	Delta Rn	Primers	Cycle threshold	Delta Rn
1.3F/1.3R	20	3.5	1.3F/1.3R	13	1.2
1.3F/0.6R	20	2.1	1.3F/0.6R	12	1.3
1.3F/0.32R	20	1.1	1.3F/0.32R	13	0.8
1.3F/0.16R	20	2.2	1.3F/0.16R	12	1.0
1.3F/0.08R	20	1.6	1.3F/ 0.08R	13	0.8
1.3F/0.04R	21	1.2	1.3F/0.04R	13	0.5
0.6F/1.3R	21	3.5	0.6F/1.3R	12	1.3
0.6F/0.6R	21	3.5	0.6F/0.6R	13	1.3
0.6F/0.3R	21	3.4	0.6F/0.32R	13	1.3
0.6F/0.16R	21	2.6	0.6F/0.16R	13	1.2
0.6F/0.08R	21	1.7	0.6F/0.08R	13	0.8
0.6F/0.04R	21	1.2	0.6F/0.04R	13	0.6
0.32F/1.3R	21	3.4	0.32F/1.3R	13	1.3
0.32F/0.6R	21	3.3	0.32F/0.6R	13	1.3
0.32F/0.32R	21	3.2	0.32F/0.32R	13	1.3
0.32F/0.16R	21	2.6	0.32F/0.16R	13	1.2
0.32F/0.08R	21	1.8	0.32F/0.08R	13	1.0
0.32F/0.04R	21	1.2	0.32F/0.04R	14	0.6
0.16F/1.3R	21	2.6	0.16F/1.3R	14	1.1
0.16F/0.6R	21	2.7	0.16F/0.6R	14	1.2
0.16F/0.32R	21	2.7	0.16F/0.32R	14	1.3
0.16F/0.16R	21	2.3	0.16F/0.16R	14	1.2
0.16F/0.08R	21	1.8	0.16F/0.08R	14	0.9
0.16F/0.04R	21	1.1	0.16F/0.04R	14	0.5
0.08F/1.3R	21	1.8	0.08F/1.3R	14	1.0
0.08F/0.6R	20	1.9	0.08F/0.6R	14	1.0
0.08F/0.32R	21	1.7	0.08F/0.32R	14	1.0
0.08F/0.16R	20	1.8	0.08F/0.16R	14	1.0
0.08F/0.08R	22	1.3	0.08F/0.08R	14	0.8
0.08F/0.04R	22	0.9	0.08F/0.04R	14	0.5
0.04F/1.3R	22	1.3	0.04F/1.3R	14	0.8
0.04F/0.6R	22	1.3	0.04F/0.6R	14	0.8
0.04F/0.32R	22	1.4	0.04F/0.32R	14	0.8
0.04F/0.16R	22	1.3	0.04F/0.16R	14	0.7
0.04F/0.08R	22	1.1	0.04F/0.08R	14	0.6
0.04F/0.04R	22	0.7	0.04F/0.04R	14	0.4

R: Reverse primer and F: forward primer

Table 8.4: Cycle threshold and fluorescence intensity obtained from primer matrix dilution of HCoV-OC43, HcoV-NL63 and HcoV-229E.

Detector: HcoV-OC43	Reporter: FAM	Threshold: 0.231013	Detector: HcoV-NL63	Reporter : VIC	Threshold: 0.231013	Detector: HcoV-229E	Reporter: VIC	Threshold: 0.231013
Primers	Cycle threshold	Delta Rn	Primers	Cycle threshold	Delta Rn	Primers	Cycle threshold	Delta Rn
1.6F/ 1.6R	24	10.0	1.6F/ 1.6R	24	3.0	1.6F/ 1.6R	24	5.4
1.6R / 0.8F	24	10.5	1.6R / 0.8F	24	2.3	1.6R / 0.8F	24	5.3
1.6R /0.4F	24	10.7	1.6R /0.4F	24	2.2	1.6R /0.4F	24	5.0
1.6R/0.2F	24	8.7	1.6R/0.2F	24	2.1	1.6R/0.2F	24	3.9
1.6R/ 0.1F	24	11.5	1.6R/ 0.1F	25	3.1	1.6R/ 0.1F	24	5.6
1.6R/0.05F	24	11.3	1.6R/0.05F	25	3.1	1.6R/0.05F	24	5.2
0.8R/1.6F	24	10.6	0.8R/1.6F	25	3.1	0.8R/1.6F	24	4.9
0.8F/0.8R	24	8.7	0.8F/0.8R	25	2.6	0.8F/0.8R	24	4.1
0.8R/0.4F	24	12.3	0.8R/0.4F	25	2.9	0.8R/0.4F	24	5.1
0.8R/0.2F	24	11.0	0.8R/0.2F	25	3.1	0.8R/0.2F	24	4.6
0.8R/0.1F	24	10.2	0.8R/0.1F	25	3.1	0.8R/0.1F	24	4.4
0.8R/0.05F	24	8.5	0.8R/0.05F	25	2.5	0.8R/0.05F	24	3.7
0.4R/1.6F	24	10.8	0.4R/1.6F	26	2.8	0.4R/1.6F	24	4.5
0.4R/0.8F	24	10.2	0.4R/0.8F	26	2.8	0.4R/0.8F	24	4.2
0.4R/0.4F	24	9.0	0.4R/0.4F	26	2.8	0.4R/0.4F	24	3.8
0.4R/0.2F	24	7.7	0.4R/0.2F	26	2.4	0.4R/0.2F	24	3.4
0.4R/0.1F	25	10.0	0.4R/0.1F	26	3.0	0.4R/0.1F	24	5.4
0.4R/0.05F	24	10.5	0.4R/0.05F	26	2.3	0.4R/0.05F	24	5.3
0.2R/1.6F	24	10.7	0.2R/1.6F	26	2.2	0.2R/1.6F	24	5.0
0.2R/0.8F	24	8.7	0.2R/0.8F	26	2.1	0.2R/0.8F	24	3.9
0.2R/0.4F	24	11.5	0.2R/0.4F	26	3.1	0.2R/0.4F	24	5.6
0.2R/0.2F	23	11.3	0.2R/0.2F	24	3.1	0.2R/0.2F	23	5.2
0.2R/0.1F	25	10.6	0.2R/0.1F	26	3.1	0.2R/0.1F	24	4.9
0.2R/0.05F	25	8.7	0.2R/0.05F	26	2.6	0.2R/0.05F	24	4.1

R: Reverse primer and F: forward primer

Table 8.5: Cycle threshold and fluorescence intensity obtained from primer matrix dilution of HcoV-HKU1, HRV and HboV

Detector: HBoV	Reporter: FAM	Threshold: 0.231013	Detector: HRV	Reporter: FAM	Threshold: 0.231013	Detector: HCoV-HKU 1	Reporter: VIC	Threshold: 0.231013
Primers	Cycle threshold	Delta Rn	Primers	Cycle threshold	Delta Rn	Primers	Cycle threshold	Delta Rn
1.6F/ 1.6R	32	3.4	1.6F/ 1.6R	24	3.3	1.3F/ 1.3R	25	5.4
1.6R / 0.8F	32	3.0	1.6R / 0.8F	24	3.5	1.3F/ 0.6R	25	5.3
1.6R /0.4F	32	3.0	1.6R /0.4F	24	3.5	1.3F/0.32R	25	5.0
1.6R/0.2F	33	3.1	1.6R/0.2F	24	3.3	1.3F/0.16R	25	3.9
1.6R/ 0.1F	33	3.0	1.6R/ 0.1F	24	3.5	1.3F/ 0.08R	25	5.6
1.6R/0.05F	33	2.9	1.6R/0.05F	24	3.4	1.3F/0.04R	25	5.2
0.8R/1.6F	33	2.9	0.8R/1.6F	24	3.2	0.6F/1.3R	25	4.9
0.8F/0.8R	33	2.8	0.8F/0.8R	24	2.8	0.6F/0.6R	25	4.1
0.8R/0.4F	33	2.9	0.8R/0.4F	24	3.1	0.6F/0.32R	25	5.1
0.8R/0.2F	33	2.5	0.8R/0.2F	24	3.2	0.6F/0.16R	25	4.6
0.8R/0.1F	33	2.6	0.8R/0.1F	24	3.0	0.6F/0.08R	25	4.4
0.8R/0.05F	33	2.4	0.8R/0.05F	24	2.6	0.6F/0.04R	25	3.7
0.4R/1.6F	33	2.5	0.4R/1.6F	24	3.4	0.32F/1.3R	25	4.5
0.4R/0.8F	33	2.1	0.4R/0.8F	24	3.1	0.32F/0.6R	25	4.2
0.4R/0.4F	33	2.6	0.4R/0.4F	24	2.9	0.32F/0.32R	24	3.8
0.4R/0.2F	33	1.8	0.4R/0.2F	25	2.3	0.32F/0.16R	25	3.4
0.4R/0.1F	33	3.4	0.4R/0.1F	24	3.3	0.32F/0.08R	25	5.4
0.4R/0.05F	33	3.0	0.4R/0.05F	24	3.5	0.32F/0.04R	25	5.3
0.2R/1.6F	34	3.0	0.2R/1.6F	24	3.5	0.16F/1.3R	25	5.0
0.2R/0.8F	34	3.1	0.2R/0.8F	24	3.3	0.16F/0.6R	25	3.9
0.2R/0.4F	33	3.0	0.2R/0.4F	24	3.5	0.16F/0.32R	25	5.6
0.2R/0.2F	33	2.9	0.2R/0.2F	24	3.4	0.16F/0.16R	25	5.2
0.2R/0.1F	35	2.9	0.2R/0.1F	24	3.2	0.16F/0.08R	25	4.9
0.2R/0.05F	35	2.8	0.2R/0.05F	24	2.8	0.16F/0.04R	25	4.1

R: Reverse primer and F: forward primer

Table 8.6: Cycle threshold and fluorescence intensity obtained from primer matrix dilution of WUPyV and KIPyV

Detector: WUPyV	Reporter: FAM	Threshold: 0.051122	Detector: KIPyV	Reporter: VIC	Threshold: 0.051122
Primers	Cycle threshold	Delta Rn	Primers	Cycle threshold	Delta Rn
1.6F/ 1.6R	34	3.4	1.6F/ 1.6R	34	3.3
1.6R / 0.8F	34	3.0	1.6R / 0.8F	34	3.5
1.6R /0.4F	34	3.0	1.6R /0.4F	34	3.5
1.6R/0.2F	34	3.1	1.6R/0.2F	34	3.3
1.6R/ 0.1F	34	3.0	1.6R/ 0.1F	34	3.5
1.6R/0.05F	34	2.9	1.6R/0.05F	34	3.4
0.8R/1.6F	34	2.9	0.8R/1.6F	34	3.2
0.8F/0.8R	34	2.8	0.8F/0.8R	35	2.8
0.8R/0.4F	34	2.9	0.8R/0.4F	35	3.1
0.8R/0.2F	34	2.5	0.8R/0.2F	34	3.2
0.8R/0.1F	35	2.6	0.8R/0.1F	34	3.0
0.8R/0.05F	35	2.4	0.8R/0.05F	35	2.6
0.4R/1.6F	35	2.5	0.4R/1.6F	34	3.4
0.4R/0.8F	34	2.1	0.4R/0.8F	34	3.1
0.4R/0.4F	36	2.6	0.4R/0.4F	35	2.9
0.4R/0.2F	36	1.8	0.4R/0.2F	35	2.3
0.4R/0.1F	36	3.4	0.4R/0.1F	35	3.3
0.4R/0.05F	36	3.0	0.4R/0.05F	35	3.5
0.2R/1.6F	36	3.0	0.2R/1.6F	35	3.5
0.2R/0.8F	36	3.1	0.2R/0.8F	34	3.3
0.2R/0.4F	36	3.0	0.2R/0.4F	36	3.5
0.2R/0.2F	36	2.9	0.2R/0.2F	36	3.4
0.2R/0.1F	36	2.9	0.2R/0.1F	36	3.2
0.2R/0.05F	36	2.8	0.2R/0.05F	36	2.8

R: Reverse primer and F: forward primer

8.3 Limits of detection: Duplexing results

Duplex PCR is the simultaneous detection of two target sequences. Real time PCR makes it possible to co-detect target sequences as it uses specific probes labeled with unique fluorescent dyes, hence using different wave lengths it is possible to detect different “colors”. Taqman probes are specifically used for this purpose. These probes consist of a quencher dye at the 3’ end of probe and a fluorescent dye at the 5’ end of probe. When the quencher dye is in close proximity to the fluorescent dye there is no emission of fluorescence, however during the annealing/extension period of the PCR reaction the 5’ nuclease activity of DNA polymerase enzyme cleaves the probe, separating the quencher dye from the fluorescent dye resulting in increase of fluorescence. The accumulation of the PCR product is detected and the fluorescence is monitored. The main goal of developing a duplex PCR is to accurately quantitate the amount of each target present without interference from competition or inhibition. In the present study HBoV was duplexed with Lambda DNA control. Table 8.7 shows the 10 fold serial dilutions of both viruses. There was no competition and inhibition for both the Bocavirus and Lambda virus in the duplex PCR, as throughout the PCR Lambda maintains the cycle threshold at which corresponds to a single-plex PCR. Similar results were observed for all the duplex pairs of NDV and HCoV-2289E (Table 8.8), HRV and HCoV-HKU 1 (Table 8.9), HCoV-NL63 and HCoV-OC43 (Table 8.10), WUPyV and KIPyV (Table 8.11), used in this thesis.

Table 8.7: DNA matrix of Bocavirus and Lambda

Bocavirus / Lambda	10E5	10E4	10E3	10E2	10E1
10E5	18 \ 18	18 \ 20	18 \ 26	18 \ 29	ND \ ND
10E4	20 \ 18	20 \ 21	20 \ 25	21 \ 28	ND \ ND
10E3	22 \ 18	22 \ 21	22 \ 25	22 \ 28	ND \ ND
10E2	24 \ 18	24 \ 22	24 \ 25	25 \ 28	ND \ ND
10E1	ND \ ND	ND \ ND	ND \ ND	ND \ ND	ND \ ND

Table 8.8: DNA matrix of NDV and HCoV-229E

NDVHCoV-229E / NDV	10E5	10E4	10E3	10E2	10E1
10E5	22 \ 20	22 \ 24	22 \ 29	22 \ 33	ND \ ND
10E4	25 \ 19	25 \ 23	20 \ 27	21 \ 31	ND \ ND
10E3	29 \ 19	29 \ 23	29 \ 27	30 \ 30	ND \ ND
10E2	36 \ 19	32 \ 23	33 \ 27	32 \ 31	ND \ ND
10E1	ND \ ND	ND \ ND	ND \ ND	ND \ ND	ND \ ND

Table 8.9: DNA matrix of HRV and HCoV- HKU 1

HRV / HCoV-HKU 1	10E5	10E4	10E3	10E2	10E1
10E5	20 \ 25	18 \ 27	18 \ 30	18 \ ND	ND \ ND
10E4	24 \ 26	24 \ 26	24 \ 29	24 \ ND	ND \ ND
10E3	26 \ 25	26 \ 27	27 \ 31	26 \ ND	ND \ ND
10E2	29 \ ND	30 \ ND	29 \ ND	29 \ ND	ND \ ND
10E1	ND \ ND	ND \ ND	ND \ ND	ND \ ND	ND \ ND

Table 8.10: DNA matrix of HCoV-NL63 and HCoV- OC43

HCoV-NL63 / HCoV-OC43	10E5	10E4	10E3	10E2	10E1
10E5	20 \ 21	20 \ 24	21 \ 27	20 \ 31	ND \ ND
10E4	23 \ 20	23 \ 24	23 \ 26	23 \ 32	ND \ ND
10E3	26 \ 20	26 \ 24	26 \ 27	25 \ 31	ND \ ND
10E2	30 \ 20	31 \ 25	31 \ 27	30 \ 31	ND \ ND
10E1	ND \ ND	ND \ ND	ND \ ND	ND \ ND	ND \ ND

Table 8.11: DNA matrix of WUPyV and KIPyV

KIPyV / WUPyV	10E5	10E4	10E3	10E2	10E1
10E5	30 \ 30	20 \ 33	21 \ 37	20 \ 39	ND \ ND
10E4	34 \ 30	33 \ 33	34 \ 36	34 \ ND	ND \ ND
10E3	36 \ 30	36 \ 34	36 \ 37	37 \ ND	ND \ ND
10E2	39 \ 39	ND \ ND	ND \ ND	ND \ ND	ND \ ND
10E1	ND \ ND	ND \ ND	ND \ ND	ND \ ND	ND \ ND

Table 8.12: Validation of PCR reactions

Internal control	Positive control	Sample	Result interpretation
Positive	Positive	Positive	Valid
[1]Negative	Positive	Positive	Valid
Positive	Negative	Positive	Valid

[1]IC negative due to competition for reagents with positive specimens