MOLECULAR EPIDEMIOLOGY AND CLINICAL CHARACTERISTICS OF THE HUMAN METAPNEUMOVIRUS IN SOUTH AFRICA

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctorate of Philosophy in the branch of Medicine

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I. DECLARATION

I, Herbert Patrick Ludewick, hereby declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University

Herbert P Ludewick

October 2007

II DEDICATION

This thesis is dedicated to my family, my wife Kerry Ann and sons Kerwin, Kyle and Klyne. Kerry, I thank you for the support that you have given me, I know believe this quote: "*Behind very successful man is a woman*". Thank you for the encouragement to continue when I thought it was not meant to be. I am grateful for the time that you guy's allowed me to be away from home when I needed to complete my laboratory work. To my boys I especially dedicate this work to you guys as I draw my inspiration from you three and encourage you to dream as I am proof that no matter were you come from you can achieve anything.

I would also like to dedicate this work to my mother, Shirley, thank for a solid upbringing it has certainly made me what I am today. To my brother, Arnold and sister, Verna, thank you for the love, respect and encouragement, may GOD bless you all.

III. PUBLICATIONS AND PRESENTATIONS ARISING FROM THE THESIS

A. Publications

1. Madhi SA, Ludewick H, Abed Y, Klugman KP, Boivin G. Human metapneumovirus associated lower respiratory tract infections among hospitalized HIV-1 infected and HIV-1 uninfected African infants. *Clin Infect Dis* 2003 37: 1705 – 1710

2. Boivin G, Mackay I, Sloots TP, Madhi S, Freymuth F, Wolf D, Shemer-Avni Y, Ludewick H, Gray G, LeBlanc E. Global genetic diversity of the human Metapneumovirus fusion gene. *Emerg Infect Dis J* 2004; 10: 1154-1157. (I.F. 5.34)

3. Ludewick HP, Abed Y, van Niekerk N, Klugman KP, Boivin G and Madhi SA, Genetic variability of the human metapneumovirus in South Africa. *Emerg Infect Dis J.* 2005; 11: 1074-8.

4. Madhi SA, Ludewick H, Kuwanda L, van Niekerk N, Cutland C, Little T, Klugman KP. Pneumococcal co-infection with human metapneumovirus. *Journal of Infectious Diseases.* 2006; 193:1236-43.

5 Madhi SA, Ludewick H, Kuwanda L, van Niekerk N, Cutland C, Klugman KP. Seasonality, incidence and repeat human metapneumovirus lower respiratory infections in an area with a high prevalence of human immunodeficiency virus type-1 infection. *The Journal of Pediatric Infectious Disease*. 2007: 26(8):693-699.

B. Presentations

1. *44th ICACC- Interscience Conference on Antimicrobial Agents and Chemotherapy,* Washington DC, USA, 30 October-2 November, 2004. Ludewick H, van Niekerk N, Klugman KP, Madhi SA. (Poster V-1263).

2. *23rd Annual Meeting of the European Society for Paediatric Infectious Diseases; Valencia, Spain, May 1820, 2005.* H. Ludewick, Cutland CL, Van Niekerk N, Kuwanda L, Madhi SA. The importance of human metapneumovirus in African children. (Poster 239).

3. *5th International Symposium on Pneumococci and Pneumococcal Diseases*. Alice Springs, Australia; 2-6 April 2006. Madhi SA, Ludewick H, Kuwanda L, van Niekerk N, Cutland C, Little T, Klugman KP. Pneumococcal co-infection with human metapneumovirus (Poster- P08.03)

4. *12th International Congress on Infectious Diseases. Lisbon, Portugal June 15-18.* Madhi SA, Ludewick H, Kuwanda L, van Niekerk N, Cutland C, Klugman KP. Seasonality, incidence and repeat human metapneumovirus lower respiratory tract infections in HIV-1 infected and HIV-1 uninfected children. (Poster 30.045)

IV. ABSTRACT

The human metapneumovirus is a novel paramyxovirus associated with acute respiratory infections in children, adults, elderly and immunocompromised individuals. It has a worldwide distribution and the prevalence range between 1.5% to 25% in individuals with respiratory infections. Based on phylogenetic analysis 2 distinct genetic groups (A and B) that are sub-divided into four subgroups (A1, A2, B1 and B2) have been shown to circulate. Until recently, there was no information on the molecular epidemiology and the clinical characteristics of the hMPV in Africa, including South Africa, a region with a high prevalence of paediatric human immunodeficiency virus type-1 (HIV) infection.

The molecular epidemiology and clinical characteristics of the hMPV in South Africa was investigated over a three period (2000-2002) in children hospitalized with lower respiratory tract infection. The children were part of a cohort participating in a phase 3 clinical trial investigating the efficacy of a 9-valent-pneumococcal protein-polysaccharide conjugate vaccine (PCV).

The objectives of the study were: i. to investigate the molecular epidemiology of hMPV in South Africa; ii. characterize the burden of hMPV disease and determine the clinical features of hMPV-LRTI in children infected and not infected by HIV; iii. probe the role of *Streptococcus pneumoniae* in the pathogenesis of hMPV-LRTI.

The overall prevalence of hMPV in children hospitalized with lower respiratory tract infections (LRTI) was 7.4%. The mean age of children with hMPV associated LRTI (hMPV-LRTI) in South Africa was 13.3 months (range 1.4-49.2 months), with HIV infected children being older than children not infected with HIV (mean [range] 17.6 [4.5-44.3] vs. 12.3 [1.4-49.2] months; P=0.007). The incidence of hMPV-LRTI was 5.0 (95%C.I.3.3-7.5) fold greater in HIV infected children (incidence rate: 2 504 [95%C.I. 1 683-3 577] per 100 000) than in HIV uninfected children (incidence rate: 505 [95%C.I. 409-618] per 100 000, P<0.0001). Human metapneumovirus was identified less frequently than RSV but more commonly than other studied respiratory viruses.

The double-blind PCV-9 vs. placebo controlled trial was used to probe the role of pneumococcal co-infections contributing to the pathogenesis of severe hMPV-LRTI. The incidence of hospitalization for hMPV-LRTI was reduced by 46% (95%, CI, 25-63; P=0.0002) in PCV-9 vaccinees compared to placebo recipients. This inferred that coinfection with *Streptococcus pneumoniae* was integral to the pathogenesis of hMPV-LRTI requiring hospitalization.

Both groups of the hMPV circulated during the three year period including concurrent circulation of multiple subtypes of the virus. There was a transition from group B to group A subtype virus as the dominant circulating virus over sequential years.

Sequence analysis of the two attachment glycoproteins (F and G), showed the F gene protein to be highly conserved, in contrast the attachment protein gene

(G protein) was highly variable particularly in the extracellular domain between lineages. Repeat hMPV-LRTI by either homologous or heterologous strains within 3 months of each other suggested that natural infection did not confer complete immunity to hMPV.

The present study demonstrated that hMPV is a leading pathogen associated with LRTI among children in Africa and indicated that occult pneumococcal co-infections' were integral in the pathogenesis of hMPV-LRTI requiring hospitalization. Additionally, this is the first study to have characterized the molecular epidemiology of hMPV in Africa and provides insight as to issues that may exist regarding the design of an hMPV vaccine.

V. ACKNOWLEDGEMENTS

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ABBREVIATIONS USED IN THE TEXT

aa	amino acid
ARTI	acute respiratory tract infection
bp	base pairs
CHD	chronic heart disease
CLD	chronic lung disease
CXC-AR	radiologically confirmed alveolar consolidation
hMPV	human metapneumovirus
ITT	intent-to-treat
HIV	human immunodeficiency virus type-1
LRTI	lower respiratory tract infection
NPA	nasopharyngeal aspirate
nt	nucleotide
PCP	*Pneumocystis jiroveci* pneumonia
PCV	pneumococcal conjugate vaccine
PIV1-3	parainfluenza virus type 1-3
PP	per protocol
RSV	respiratory syncytial virus
RTI	respiratory tract infection
RT-PCR	reverse transcriptase polymerase chain reaction
URT	upper respiratory tract infection
VE	vaccine efficacy
WHO	World health Organisation

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The Global Burden of Disease (GBD) study reported that acute respiratory infections together with measles, diarrhea, malaria and HIV/AIDS accounted for more the 50% of deaths in children in 2001 (Lopez et al., 2006). Lower respiratory tract infections are among the leading causes of death in the low-middle income and high-income countries and rank third and fourth respectively (Lopez et al., 2006). Compared to the 1990 statistics there was less of a decline in death rates due to acute respiratory infections in South Asia and sub-Saharan Africa than other areas (Lopez et al., 2006).

Viruses are the most common pathogens identified in infants and young children with a lower respiratory tract infection (LRTI). It is estimated that approximately 3% of children under 1 year of age are hospitalized with severe viral lower respiratory tract infections in the USA (van Woensel et al., 2003). In South Africa viruses were identified from 30% of children hospitalized with severe lower respiratory tract infections (Madhi et al., 2000) .

Several viruses have been shown to cause LRTI in children of which respiratory syncytial virus (RSV) is the most common cause (van Woensel et al., 2003).

In England RSV is responsible for 75% of hospital admission in children under 5 years with bronchiolitis (van Woensel et al., 2003) and in South Africa RSV was the dominant (49.7%) respiratory viral pathogen among children not infected by HIV that were hospitalized for LRTI (Madhi et al., 2000).

Recently a novel respiratory virus, named human metapneumovirus (hMPV), was isolated in the Netherlands (van den Hoogen et al., 2001). Based on the genome constellation and morphological features it has been classified into the family, *Paramyxoviridae* (van den Hoogen et al., 2001), subfamily *Pneumovirinae* and genus, *Metapneumovirus*. It is the only known species from this genus that infects humans and prior to its identification the only other known species of the *Metapneumovirus* genus was the avian pneumovirus (APV).

Human metapneumovirus was initially isolated from 28 children with respiratory tract infections from samples collected over a 20 year period (van den Hoogen et al., 2001) and was identified in 10% of samples from children with respiratory tract infections in which no other pathogen was detected (van den Hoogen et al., 2001).

Serological data provided evidence of infection by hMPV to be present since 1958 among individuals across different age-groups, (van den Hoogen et al., 2001) with almost all children having been infected by 5-10 years of age in the Netherlands, Japan and Ethiopian children (Ebihara et al., 2003; van den

Hoogen et al., 2001; Wolf et al., 2003). In South Africa a study reported an overall seroprevalence rate of 63.5% among children aged from 0 to 36 months, with the highest seroprevalence rate of 92% observed among children age 24-36 months (IJpma et al., 2004).

Following the initial identification of hMPV, it has been associated worldwide as an etiological agent of upper respiratory tract infections (URTI), LTRI, acute wheezing, asthma exacerbation as well as otitis media (Hamelin et al., 2004; van den Hoogen et al., 2004b). Human metapneumovirus has been identified as a pathogen associated with respiratory tract infections across all age-groups as well as among immunocompromised individuals. It is however most prevalent in children younger than 2 years of age with respiratory illness (Maggi et al., 2003; Viazov et al., 2003) and has been detected in children as young as 3-4 months of age (Peiris et al., 2003; van den Hoogen et al., 2003).

1.2. The human metapneumovirus structure

The hMPV is an enveloped, negative sense RNA virus, with pleomorphic spherical or filamentous virions (Peret et al., 2002; van den Hoogen et al., 2001) with characteristics which are similar to other *Pneumoviruses* (Easton et al., 2004).

1.2.1. Genome structure

The genome of the hMPV is approximately 13kb with 93.8% of the genome involved in open reading frames (ORFs) that are separated by short non-coding regions. Most of the ORFs are initiated at nucleotide (nt) 14 of its mRNA and terminate within a gene-end signal (termination sequence). This stop-restart process that guides transcription is characteristic of viruses that belong to the order *Mononegavirus* (Biacchesi et al., 2003).

The genome encodes 8 mRNAs and is organized as follows: 3'-N-P-M-F-M2-SH-G-L-5' (Biacchesi et al., 2003; van den Hoogen et al., 2002) flanked at the 3' end by a leader sequence and the 5' by a trailer sequence. These proteins are common to members of the subfamily, *Pneumovirinae*, however, the genomic organization is similar to avian pneumovirus (APV) (Biacchesi et al., 2003; van den Hoogen et al., 2002; van den Hoogen et al., 2001).

Based on comparisons to the ORFs of APV and RSV, the hMPV proteins are as follows: N= nucleocapsid RNA binding protein, P (phosphoprotein), M (non glycosylated matrix protein), F (fusion glycoprotein), M2-1 (transcription elongation factor), M2-2 (RNA synthesis regulatory factor), SH (small hydrophobic surface protein), G (attachment protein) and L (polymerase subunit).

1.2.1.1 The nucleoprotein (N gene)

The nucleoprotein forms an integral part of the nucleocapsid of the virion and is tightly associated to the RNA genome and gives the RNA genome its helical structure (Easton et al, 2004). The N gene of the hMPV encodes a 394 amino acid protein and shares a 88% amino acid sequence identity with APV-C (Biacchesi et al., 2003; van den Hoogen et al., 2002) and 41% amino acid identity with RSV. Three conserved regions (between amino acids 160-172; 251-263 and 278-327) in the N protein present among members of the family *Paramyxoviridae* were also present in hMPV (van den Hoogen et al., 2002).

1.2.1.2 The phosphoprotein (P gene)

Phosphoproteins of pneumoviruses are associated with the RNA genome and form part of the nucleocapsid and are believed to be involved in both replication and transcription (Easton et al., 2004). hMPV putative phosphoprotein contains one ORF of 294 amino acid that lacks cysteine residues and has a glutamate rich C terminus (van den Hoogen et al., 2002). Its proteome shares 68% identity with the APV-C proteome and similar to other pneumoviruses also has the highly conserved region between amino acid 185-241, thought to be involved in RNA synthesis or maintaining the structure of the nucleocapsid (van den Hoogen et al., 2002).

1.2.1.3 The matrix (M) protein

Matrix proteins lines the inner surface of the lipid membrane that surrounds the core and forms a link between the nucleocapsid and the envelope inactivating the nucleocapsid of pneumoviruses prior to packaging (Easton et al., 2004). Human metapneumovirus M ORF codes a 254 amino acid protein with high amino acid identity to the M protein of APV. Similar to other members (RSV, APV and PVM) of the subfamily *Pneumovirinae*, two secondary ORF, one (54 amino acids) within and the other (33 amino acids) overlapping the major ORF was identified (van den Hoogen et al., 2002), however there have been no reports on proteins synthesized by these secondary ORF (van den Hoogen et al., 2002). The conserved hexapeptide (YTAAVQ) at residues 14-19 present in other pneumoviruses was also present in the putative hMPV M protein.

1.2.1.4 The 22 K (M2) proteins

The genes encoding the M2 proteins of metapneumoviruses contain two overlapping ORFs M2-1 and M2-2 (Easton et al., 2004). The M2-1 is involved in virus RNA synthesis, functioning as a transcription elongation factor (Easton et al., 2004). The M2-1 of hMPV encodes a 187 aa protein and reveals a 84% identity with M2-1 of APV (van den Hoogen et al., 2002). The putative M2-2 ORF is 77 aa in length and starts at nt 512 in the M2-1 (van den Hoogen et al., 2002)

1.2.1.5 The fusion (F) protein

Fusion (F) proteins of the family *Paramyxoviridae* show limited identity to each other but are structurally similar. They are synthesized as inactive precursors (F0) and are cleaved by the host cell proteases generating two subunits, an N-terminal F2 and a larger C-terminal F1 subunit. The activated fusion protein is responsible for the fusion of the viral membrane and the host cell plasma membrane in combination with the attachment protein (Easton et al., 2004; Morrison, 2003). In a study using recombinant hMPV that lack SH and G proteins showed that the F protein may be used as sole surface protein for replication in vivo (Biacchesi et al., 2004b).

The F gene of hMPV encodes a 539 amino acid protein that has 81% identity to the F protein of APV (van den Hoogen et al., 2002). Structurally it is similar to other members of the family *Paramyxoviridae*. The F protein of hMPV is glycosylated containing three potential N glycosylation sites. Fourteen cysteine residues, 12 of which are located in the F1 subunit and 2 in the F2 subunit are present in the ORF and 8 are conserved among all paramyxoviruses.

Unlike RSV that contains two cleavage sites, the hMPV F protein contains only one cleavage site, containing the residues RQSR. The primary sequence of the hMPV F protein contains 3 hydrophobic domains: i. the signal peptide located at the amino terminus of the F2 subunit; ii. a fusion and membrane anchor domains located in the F1 subunit at N; and iii. C termini respectively

(van den Hoogen et al., 2002). The fusion domain is thought to initiate fusion in paramyxoviruses, by inserting into the target membrane (Morrison, 2003). Also present in F protein of hMPV are two heptad repeat domains (HRA and HRB) that are believed to be essential for viral fusion. Both of these domains are located in the F1 subunit, with HRA located adjacent to the fusion domain and HRB adjacent to the membrane anchor domain at the carboxy terminal.

The F protein gene of the hMPV is believed to be the major antigenic determinant that mediates extensive cross-lineage neutralization and protection (MacPhail et al., 2004; Skiadopoulos et al., 2006; Skiadopoulos et al., 2004). Sequencing of the F gene protein have shown it to be highly conserved between strains (Bastien et al., 2003a; Bastien et al., 2003b; Biacchesi et al., 2003; Boivin et al., 2004; Ishiguro et al., 2004; van den Hoogen et al., 2002; van den Hoogen et al., 2004a). Amino acid alignments of the predicted F ORF has revealed the presence of several conserved cysteine residues between groups as well as lineages and the presence of specific substitutions that act as signature sequences to differentiate hMPV groups (Boivin et al., 2004; van den Hoogen et al., 2004a). Some of these substitutions were present in functional domains such as at codon 122 in the fusion domain and codons 135 and 143 in the heptad repeat A region (Boivin et al., 2004).

1.2.1.6 The small hydrophobic (SH) protein

The precise function of the SH protein is not known and RSV mutants lacking small hydrophobic protein have shown that it is not necessary for attachment, infectivity or virion assembly, but has been shown to impair a host response (Easton et al., 2004). Recombinant hMPV lacking SH have been shown to replicate efficiently in culture suggesting that the SH is not essential for growth in cell culture and when administered to hamsters replicated in upper and lower respiratory tracts (Biacchesi et al., 2004b). Recently it was shown in hamsters that SH may not be a protective antigen (Skiadopoulos et al., 2006).

Human metapneumovirus SH ORF is the largest and encodes a 183 amino acid protein with a low sequence identity to other pneumoviruses. Despite this low amino acid identity, its amino acid composition is relatively similar to APV and RSV with high percentages of serine and threonine residues (Biacchesi et al., 2003; van den Hoogen et al., 2002). The SH protein of hMPV is a type II glycoprotein with a cytoplasmic amino terminus and extracellular carboxy-terminus. The protein is anchored to the plasma membrane by a hydrophobic signal-anchor sequence at the amino terminus. The extracellular domain has 2-4 N linked and 2-3 potential O glycosylation sites, and 9-10 cysteine residues (Biacchesi et al., 2003; Biacchesi et al., 2004a; van den Hoogen et al., 2002).

1.2.1.7 The attachment glycoprotein (G)

Members of the *Paramyxoviridae* family encode two surface glycoproteins, a fusion protein as well as an attachment protein that participates in viral attachment. Depending on the genus of the virus the attachment protein may be hemagglutinin-neuraminidase (HN), hemagglutinin (H) or glycoprotein (G protein) (Morrison, 2001). Members of the subfamily *Pneumovirinae* encode the G protein, a type II mucin-like glycoprotein.

The putative hMPV G ORF is located adjacent to the SH gene and depending on the strain encode a 217-236 amino acid protein (Biacchesi et al., 2003; Peret et al., 2004; van den Hoogen et al., 2002). The predicted hMPV G protein has features consistent with an anchored type II mucin like transmembrane protein as seen for other pneumoviruses (RSV and APV). Consistent with mucin like glycoproteins, the G protein is highly glycosylated as predicted by the high serine-threonine content and in addition has a high proline content (Biacchesi et al., 2003; Peret et al., 2004; van den Hoogen et al., 2002). Hydrophilic profiles revealed an anchored type II transmembrane protein pattern with an intracellular amino terminus that is hydrophilic followed by a short hydrophobic region of approximately 20 amino acids and a mostly hydrophilic extracellular carboxy terminal (Peret et al., 2004; van den Hoogen et al., 2002)

Despite the similar features the predicted hMPV protein has with other pneumoviruses it shows no identities with RSV or APV (van den Hoogen et al., 2002) and lacks some prominent features of RSV. Compared to the RSV the hMPV G protein lacks the second methionyl translational start codon that is necessary for the translation of the soluble secreted form of RSV G protein. The hMPV G protein contains between 1-2 cysteines residues of which one is conserved in the extracellular domain, unlike RSV that contains 4 conserved closely spaced cysteines that overlap with 13 conserved amino acids to form a cystine noose and include an CX3C chemokine motif (Biacchesi et al., 2003).

1.2.1.8 The polymerase protein (L)

The L protein is the major component of the viral RNA-dependent RNA polymerase complex (Easton et al., 2004; van den Hoogen et al., 2002). It is a large protein an the hMPV L ORF encodes a 2005 aa protein (van den Hoogen et al., 2002). It shares the highest homology with APV (64%) and the 4 core polymerase motifs that may be essential for polymerase function are well conserved in hMPV L protein (van den Hoogen et al., 2002).

1.3 Molecular epidemiology of the hMPV

The molecular epidemiology of hMPV is starting to unfold as studies have shown substantial genetic variability between strains particularly in the surface

attachment glycoprotein. It is this variability that may be responsible for the ability of hMPV to cause seasonal epidemics and reinfections.

van den Hoogen et al. performed phylogenetic analysis on partial nucleotide sequences of the N, M, F and L ORFs and showed the existence of 2 distinct genetic groups, A and B; (van den Hoogen et al., 2001). The existence of these genetic groups have been confirmed by other phylogenetic studies and within these groups, two subgroups have been identified (type 1 and 2) (Bastien et al., 2003a; Bastien et al., 2003b; Biacchesi et al., 2003; Boivin et al., 2002; Ebihara et al., 2004b; Ishiguro et al., 2004; Maggi et al., 2003; Peret et al., 2002; Viazov et al., 2003).

Genetic distances have also provided support for the existence of the two genetic groups. Sequence analysis of the genomes of strains from genetic groups A and B from the Netherlands (van den Hoogen et al., 2002) Canada (Biacchesi et al., 2003) and Japan (Ishiguro et al., 2004) showed an overall 80% nucleotide identity between groups A and B (Biacchesi et al., 2003; Ishiguro et al., 2004). There was a high degree of identity within subgroups for all ORFs compared by Biacchesi et al. and Ishiguro et al. (Biacchesi et al., 2003; Ishiguro et al., 2004)

1.3.1 Genetic variation in the surface glycoproteins

Reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequencing has been used to examine the variability of hMPV for molecular

epidemiology studies. Several studies have examined the degree of variation between clinical strains for the surface glycoproteins (F and G gene proteins).

Nucleotide and amino acid identities between genetic groups based on the partial and full length sequences of the F gene showed a low degree of variability between groups, ranging from 81-85% at the nucleotide level and 95% at the amino acid level (Bastien et al., 2003a; Bastien et al., 2003b; Biacchesi et al., 2003; Boivin et al., 2004; Ishiguro et al., 2004; van den Hoogen et al., 2002; van den Hoogen et al., 2004a).

In contrast, there was a high degree of variability at the nucleotide and amino acid level for the attachment glycoprotein. Sequence diversity between group A and B clinical isolates of hMPV were between 50-57% at nucleotide level and 30-37% amino acid level in the Netherlands (van den Hoogen et al., 2004a), 52-58% at nt level and 31-38% at amino acid level in Canada (Peret et al., 2004) and 56-60% at nt level and 31-35% at amino acid level in Japan (Ishiguro et al., 2004).

Within groups higher percentage difference in identities were observed at the nt level (74-100%) compared to the amino acid level (61-100%) (Ishiguro et al., 2004; Peret et al., 2004; van den Hoogen et al., 2004a).
Different lengths of ORFs were also reported between groups. Group A ORFs ranged between 217-235 polypeptides and groups B ORF between 220-236 polypeptides (Ishiguro et al., 2004; Peret et al., 2004; van den Hoogen et al.,

2004a) and are due to either nucleotide substitutions, use of alternative transcription codons or insertions (Ishiguro et al., 2004; Peret et al., 2004; van den Hoogen et al., 2004a). Most of the variation observed in the G protein was in the extracellular domain with the intracellular and transmembrane domains being conserved.

From these studies conducted on hMPV strains isolated from countries in the northern hemisphere the variation may be due to host immunity and extensive glycosylation may reduce its antigenicity by shielding the virus protein with host specific sugars as has been suggested for RSV (Easton et al., 2004).

1.4 Diagnosis of the human metapneumovirus

The use of rapid sensitive diagnostic methods for detection of respiratory viruses is essential. hMPV was originally cultured on tertiary monkey kidney (tMK) cells in the unusual presence of trypsin and induced a cytopathic effect postinfection (van den Hoogen et al., 2001). Subsequently it has been shown to induce cytopathic effects on LLC-tMK2 cell and Vero cells (Hamelin et al., 2004; van den Hoogen et al., 2004a) and can grow efficiently on Hep-2 cells without inducing a cytopathic effect (Chan et al., 2003). The advantage of viral culture is that it will allow for the detection of unknown hMPV lineages, however, the standard culture procedures are difficult and laborious.

The use of monoclonal antibodies (MAbs) for the diagnosis of hMPV has been performed (Percivalle et al., 2005), is rapid and may provide a result within 2 hours after NPA collection (Gerna et al., 2006). The use of MAbs was recently

shown to be less sensitive than molecular approach using RT-PCR, detecting only about 1/3 of the samples positive by RT-PCR (Gerna et al., 2006). Further, as mentioned, by 5-10 years of age all individuals are exposed to hMPV. Therefore serological assays for the diagnosis of current hMPV infections are limited and would require acute and convalescent samples, thus being of limited clinical value.

Reserve transcriptase polymerase chain reaction (RT-PCR) is the current method of choice for the diagnosis of hMPV. Primers sequences from several genes (L, N, F) based on the sequence of the prototype strain from the Netherlands (NL/01/00) have been designed and used for the detection of hMPV, however there is concern that the genetic variability displayed by some genes of hMPV may underestimate its prevalence (Hamelin et al., 2004) and certain primers pairs may not detect both lineages of hMPV (Sarasini et al., 2006; van den Hoogen et al., 2003). Therefore if RT-PCR is used, the primers should be able to detect both genetic groups of the hMPV with good specificity and sensitivity.

Recently the sensitive and rapid real RT-PCR assay which employs the use of fluorescent labeled probes has been used for the detection of hMPV (Boivin et al., 2003; Cote et al., 2003; Kuypers et al., 2005; Mackay et al., 2003; Maertzdorf et al., 2004). This technology may be best suited for diagnosis as carefully designed probes may be used to detect the different lineages of the

virus, it does however, require the use expensive equipment that may not be readily available in all laboratories, particularly in developing countries.

Although PCR may be the goal standard for diagnosing infections, its sensitivity may be a limitation for as it may detect an infection that is currently not causing illness. Further, factors such as degradation of RNA during specimen transport or the stage of patients illness which cannot be controlled for may results in false negatives (Kuypers et al., 2005).

1.5 Clinical epidemiology of hMPV

The hMPV has been identified as the causative pathogen in a substantial proportion of lower respiratory tract infections in young children. Although the mean age of hMPV-associated LRTI is greater than for RSV-LRTI the clinical symptoms of hMPV are indistinguishable from RSV and hMPV is second only to RSV as the cause of bronchiolitis in children (Kahn, 2006).

The prevalence of hMPV has been reported worldwide with a range as low as 1.3% to infection rates as high as 43% in individuals with respiratory tract infections (Table 1.1). Differences in the reported prevalence may be related to differences in the study design and study population between studies. Most of the studies investigated the prevalence of hMPV in hospitalized children; however some studies included all patients enrolled whereas others limited their investigation to samples that were negative for other respiratory viruses. Other factors that may impact on the reported prevalence are i) some studies

were limited to respiratory season, ii) the different ages of children for whom the hMPV was investigated and iii) the diagnostic methodology used (viral culture, RT-PCR, serology) differed between studies.

In addition the frequency of hMPV in the general community has also been reported with differing results. A study conducted in the United Kingdom identified the hMPV in 1.3% of patients with influenza-like illness (ILI) in the (Stockton et al., 2002), in Canada 2.9% of all respiratory tract infections in the community were due to hMPV (Boivin et al., 2003). The differences in prevalence of hMPV between the above two studies may be due to the study in the United Kingdom being restricted to patients with influenza-like illness.
In Germany the prevalence of the hMPV in the general community was 1% in children <3 years of age with LRTI who were negative for other known respiratory pathogens (Konig et al., 2004).

Lung disease, premature birth, underlying heart disease and a compromised immune system are risk factors for severe hMPV disease (Beckham et al., 2005; Dollner et al., 2004; Kahn, 2006; Ulloa-Gutierrez et al., 2004). Human metapneumovirus has been identified in an immunocompromised child with acute lymphoblastic leukemia who presented with bronchiolitis and died due to severe pneumonitis (Pelletier et al., 2002). Human metapneumovirus was also identified in nine percent of adults with haematological malignancies and respiratory tract infections tested, including three of whom died (Williams et al., 2005). In a prospective study conducted on high risk children in Argentina,

hMPV was found to be infrequent (12/567 episodes) but was a severe agent of lung disease (Klein et al., 2006).

The burden of disease of respiratory viruses in South Africa has been shown to be increased in HIV infected children who have a poorer outcome and more severe disease (Madhi et al., 2000), however, there is no information on the impact of HIV on hMPV.

1.5.1 Seasonality and circulation of the hMPV

Data collected in recent reviews show that the hMPV season overlap with RSV and similar to influenza virus A/B and RSV causes seasonal epidemics (Hamelin et al., 2004; Hamelin and Boivin, 2005; Principi et al., 2006; van den Hoogen et al., 2004b) In temperate regions most of the viral activity was reported during the winter-spring month (Hamelin et al., 2004; Hamelin and Boivin, 2005; Principi et al., 2006; van den Hoogen et al., 2004b). In most of these studies, surveillance for hMPV has been limited to the respiratory season which was confirmed in a recent study conducted over a three period (October 2000-June 2003), showing higher hMPV activity in late winter-spring months (Garcia-Garcia et al., 2006). In contrast, viral activity was reported during the late spring-summer months in the subtropics (Peiris et al., 2003).

The ability of hMPV to cause annual epidemics may be due to its genetic variation in the G protein and the existence of two major genetic groups and minor subgroups.

Table 1.1 The Prevalence of human metapneumovirus

Study period[1]	Country	Prevalence (%)[2]	Study population/Comments	Type of study	References
2000 (winter)	Netherlands	7/68 (10)	Children with ARTI	Retrospective	(van den Hoogen 2001)
2000-2001 (winter)	Canada	20/862 (2.3)	hMPV recovered from culture 2 bacterial coinfections	Retrospective	(Boivin et al., 2002)
2001	Australia	23/00 (1.5)	Children with ARTI	Retrospective	(Nissen et al., 2002)
09/00-05/01	Finland	10/132 (8)	Hospitalized children with acute wheezing	Prospective	(Jartti et al., 2002)
10/00-03/01	UK	9/405 (2.2)	All ages with ILI Samples negative for other viruses. HMPV detected in ages <1->65y Community surveillance	Prospective	(Stockton et al., 2002)
11/01-02/02	France	19/337 (6.6)	Hospitalized children with ARTI Negative for other respiratory viruses	Retrospective	(Freymouth et al., 2003)
12/01-05/02	Canada	12/208 (5.8)	Hospitalized children <3y with ARTI 0/51 control hMPV positive 2/12 co-infected with other viruses Real time PCR	Prospective Case control	(Boivin et al., 2003)
10/01-02/02	USA	19/296 (6.4)	Children <5y 1 nosocomial infection 2/19 co-infected	Laboratory based	(Esper et al., 2003)
05/01-08/02	Hong Kong	32/587 (5.5)	Children ≤18y with ARTI Samples collected 1 or 2 a week. 2/32 co infected 1 case of nosocomial transmission	Prospective	(Peiris et al., 2003)

Study period[1]	Country	Prevalence (%)[2]	Study population/Comments	Type of study	References
01/02-05/02	Germany	11/63 (17.5)	Hospitalized children <2 y with ARTI hMPV detection by nested RT-PCR 3 children co-infected	Retrospective	(Viazov et al., 2003)
01/00-05/02	Italy	23/90 (25)	Children with ARTI Incidence varies over 3 years 37% in 2000, 7% in 2001 and 43% in 2002 9/23 co-infected hMPV also detected in 7 plasma samples	Retrospective	(Maggi et al., 2003)
10/01-04/02	Canada	66/445 (14.4)	All ages with ARTI Specimens collected from 4 laboratories	Laboratory based	(Bastien et al., 2003b)
09/00-02/02	Netherlands	48/685 (7.0)	Persons all ages with ARTI Most of hMPV<2y 6/48 co-infected	Laboratory based	(van den Hoogen 2003)
1999-2001 winter season	USA	984 (4.5)	Young and elderly adults 9/217 asymptomatic were hMPV infected	Prospective	(Falsey et al., 2003)
04/02-05/02	Brazil	19/111 (17)	Children <3 years ALRTI Study period correspondence to rainy season 8 coinfected with RSV	Laboratory based cross sectional study	(Cuevas et al., 2003)
1976-2001	USA	49/248 (20)	Children LRTI Negative for other viruses Estimated that 12% of LRTI due to hMPV 15% of URTI due to hMPV 3 episodes of recurrent infection	Retrospective	(Williams et al., 2004)

Study period[1]	Country	Prevalence (%)[2]	Study population/Comments	Type of study	References
06/00-05/03	Japan	57/637 (8.9)	Hospitalized and outpatients children with ARTI Samples collected from 3 different geographical areas	Laboratory based	(Ebihara et al., 2004b)
06/02-08/02	South Africa	8/137 (5.8)	Hospitalized children between ages 15d-13.9y 13 tested HIV positive 1/8 hMPV infected was HIV positive	Prospective	(IJpma et al., 2004)
08/00-09/01	USA	26/668 (3.9)	Hospitalized children <5y with ARI/ARI-related from 2 US cities Nose and throat swabs and not NPA 8/26 >1 mo premature	Population based-prospective	(Mullins et al., 2004)
11/02-04/03	Norway	50/236 (21)	Hospitalized children ARTI 41/50 had LRTI 10/50 co-infected with other viruses 17/50 had CRP 66mg/l on admission (pneumonia)	Prospective	(Dollner et al., 2004)
11/01-10/02	USA	54/668 (8.1)	Children 5 years Specimens previously negative for other viruses 3 children had 2 specimens test positive 7-54 days apart	Retrospective	(Esper et al., 2004)
1998-2002	Argentina	11/100 (11)	Children <5 years with ARTI Samples were negative for Respiratory virus	Retrospective	(Galiano et al., 2004)

Study period[1]	Country	Prevalence (%)[2]	Study population/Comments	Type of study	References
10/00-4/01	Germany	2/620 (<1)	Hospitalized and outpatient children <3 years Negative for other viruses Community acquired hMPV infections	Prospective multicenter	(Konig et al., 2004)
10/00-08/02	USA	54/868 (6.2)	Children ≤18 years	Retrospective study	(McAdam et al., 2004)
12/02-05/03	USA	52/719 (7.2)	Patients 1d-20 years old Real time RT-PCR	Evaluation study	(Kuypers et al., 2005)
11/02-03/03	Italy	42/1505 (2.8)	Children <15 years 7/42 coinfected	Prospective	(Bosis et al., 2005)
08/97-03/00	Korea	26/166 (15.7)	Children < 5years with LRTI 5/26 coinfected with other viruses	Retrospective	(Kim and Lee, 2005)
10/00-06/03	Spain	69/494 (14)	Hospitalized children <2y with ARTI 18/69 co-infected 1 child bacteremia by *S pneumoniae*	Prospective	(Garcia-Garcia , 2006)
03/02-06/04	USA	202/3740 (5.45)	Children Real time PCR	Retrospective	(Agapov et al., 2006)
01/02-11/03	Peru	12/420 (2.9)	All ages (1-<89y) ILI 6/12 grew in culture	Retrospective	(Gray et al., 2006b)

Study period[1]	Country	Prevalence (%)[2]	Study population/Comments	Type of study	References
1982-2001	USA	118/2384 (5)	Infants and children with URTI prospectively followed from average 2.4y old Real time PCR Reinfection with homologous and heterologous strains	Retrospective	(Williams et al., 2006)
11/03-10/04	France	50/589 (8.5)	Hospitalized children <5y with RTI 16/50 dual infections with other viruses 15 co infected with RSV	Prospective	(Foulongne et al., 2006)
06/03-05/05	Argentina	12/567 (2)	Premature infants and children with CHD and CLD	Prospective	(Klein et al., 2006)
11/01-10/02	Israel	68/517 (13)	Hospitalized children <5y with LRTI 209/517 community acquired pneumonia 16/68 co infected with other viruses	Prospective	(Wolf et al., 2006)
12/03-05/04	Italy	40/306 (13.1)	Infants and young children 10/40 coinfected with other viruses	Prospective	(Sarasini et al., 2006)
06/04-05/05	Japan	29/144 (20)	Children <17 y with RTI 103/141 children <3y Nested RT-PCR	Prospective	(Kaida et al., 2006)
10/01-05/04	USA	24/1294 (2.60	All ages 21/24 grew in culture	Retrospective	(Gray et al., 2006a)

Footnote. 1 Study period refers to the month/year, eg.05/00 refers to May 2000. 2. The prevalence is based on the positive hMPV detected/ by either the total number of episodes or number of individuals and the value in parenthesis is the percentage.

Although all subgroups have been observed circulation in single communities in a single epidemic (Boivin et al., 2002; Esper et al., 2004; Gerna et al., 2005; Mackay et al., 2006; Mackay et al., 2004; Peret et al., 2004; Sarasini et al., 2006; Williams et al., 2006) a single subgroup has been shown to predominate (Mackay et al., 2004; Peret et al., 2004). In Canada, subgroup B2 viruses predominated during the 1998 epidemic and in 2001 –2002 epidemics subgroup A1 predominated (Peret et al., 2004). In Australia the co-circulation of all four subgroups over 4 consecutive years (2001-2004) was observed with one subgroup predominating in each year (2001 subgroup A1; 2002 subgroup A2; 2003 subgroup A2 and B1 and 2004 subgroup B1) (Mackay et al., 2006). Recently a study conducted over a 20 year period in the USA by Williams and co-workers report that all four subgroups have circulated in the community over 20 years with a single lineage dominating (Williams et al., 2006). Similarly a switch in predominant genotype from group A in 2003 to group B in 2004 was reported in another USA study (Agapov et al., 2006) This evidence suggests that like RSV (Sullender, 2000) strains from both groups of the hMPV can co-circulate in a single epidemic with a switch in predominant strain providing evidence that suggests that the hMPV may evade pre-existing community immunity through a switch in viral genotype

1.5.2 Dual infection of human metapneumovirus with other respiratory pathogens

Because of overlapping seasons of respiratory viruses, co-infection with multiple viruses may be common (Hamelin et al., 2004; Hamelin and Boivin, 2005; Principi et al., 2006; van den Hoogen et al., 2004b).

Similarly hMPV has been identified in the presence of other respiratory viruses (Table 1.1). There is however conflicting data regarding the clinical significance of dual viral infection involving hMPV compared to illness that involves hMPV alone (Bosis et al., 2005; Maggi et al., 2003; Williams et al., 2004; Xepapadaki et al., 2004).

In the United Kingdom 70% of children with RSV bronchiolitis who required admission to the intensive care unit were co-infected with hMPV (Greensill et al., 2003). This increased severity due to co-infection between RSV and hMPV was replicated by Semple et al. who found that co-infected children required admission to ICU for mechanical ventilation (Semple et al., 2005). This increased severity was not observed in two other studies which did not detect hMPV in severe RSV-LRTI (Lazar et al., 2004; van Woensel et al., 2006).

In a prospective study involving children younger than three years of age, co-infection with RSV and hMPV was associated with more severe disease, including 60% of co-infected infants requiring ICU admission (Konig et al., 2004). Additionally, co-infection of hMPV with coronavirus in individuals with severe acute respiratory syndrome (SARS) was reported in Hong Kong and Canada (Chan et al., 2003; Chan et al., 2004; Poutanen et al., 2003).

Studies in macaques with the SARS-associated coronavirus did not however result in more severe disease following subsequent infection by hMPV (Fouchier et al., 2003). In contrast, other studies did not find an increase in hMPV associated disease severity being linked with other concurrent viral

infections (Bosis et al., 2005; Maggi et al., 2003; Williams et al., 2004; Xepapadaki et al., 2004) and recently a co-infection rate of 26% was also not associated with more severe disease (Garcia-Garcia et al., 2006).

1.5.3.Bacterial coinfections in viral- associated pneumonia

Epidemiological studies as well as in vitro and animal studies have reported that viral infections predispose to bacterial disease probably by bacterial adherence induced by viruses (Beadling and Slifka, 2004; Hament et al., 1999; Peltola and McCullers, 2004).

Mechanisms through which superinfection might occur are poorly understood but may include physical damage to respiratory tract epithelium by viruses, virus induced immunosupression as well as the up regulation of bacterial host cell receptors through inflammatory response to viral infections (Beadling and Slifka, 2004; Hament et al., 1999; Peltola and McCullers, 2004). Recently, Navarini et al used a mouse model to examine the enhanced susceptibility to bacterial superinfection. They showed that the production of IFN I due to viral infection caused apoptosis of granulocytes, resulting in the inability to control bacterial superinfection (Navarini et al., 2006).

Pneumonia is a major cause of childhood morbidity and mortality, causing over 2 million deaths annually worldwide (Obaro and Madhi, 2006). Bacterial and viral vaccines are effective in limiting disease severity (Beadling and Slifka, 2004). A major obstacle to understanding the efficacy of vaccines against pneumonia and defining the role of bacterial coinfection is the lack of

sensitive tools to diagnose bacterial pneumonia. The WHO recommended tachypnoea with chest –wall indrawing as a clinical screening tool for the management of lower respiratory tract infections (WHO, 1990), however, laboratory confirmation of the bacterial etiology of pneumonia is limited. Although blood cultures may be specific, they lack sensitivity (10-15%) for confirming the bacterial etiology of pneumonia. The usefulness of chest radiographs in pneumonia is also controversial. Recently Madhi (2006) reported that in South Africa the sensitivity of chest radiograph-confirmed alveolar consolidation (CXR-AC) underestimated the burden of pneumococcal pneumonia prevented by pneumococcal conjugate vaccine (PCV) by up to 63% (Madhi, 2006).

Although controversial, biochemical markers such as C-reactive protein (CRP) and procalcitonin have been shown to be useful for diagnosis of pneumococcal pneumonia (Madhi et al., 2005a; Simon et al., 2004). Examining the efficacy of a nine-valent pneumococcal conjugate vaccine, Madhi et al. showed that the efficacy of 9-PCV associated with CXR-AR among HIV uninfected children compared to controls was 21% (P=0.04) and increased to 38% (P=0.05) when associated with CXR-AR and CRP (level ≥ 120mg/l).

The efficacy of the vaccine increased considerably to 64% (P=0.006) when procalcitonin (level 5.0ng/ml) was added to CXR-AR and CRP. More recently, it has been also been shown that a CRP level of ≥ 40mg/l may provide a

better assessment of the effect of PCV in preventing pneumonia compared to chest radiographs and procalcitonin (Madhi et al., 2006).

The use of vaccine as a probe to define the burden of disease may be a powerful tool due the lack of sensitive laboratory diagnostic tools (Obaro and Madhi, 2006). This method also has its shortcoming and may also underestimate the burden of disease as the proportion of disease preventable by the vaccine will only be equal to disease burden when the vaccine is 100 % effective (Obaro and Madhi, 2006). Using the 9-valent PCV to examine the role of *Streptococcoccus pneumoniae* in the etiology of viral pneumonia, Madhi and Klugman showed a 31% overall reduction in the incidence of hospitalization for viral associated pneumonias in all children (HIV infected and HIV uninfected) among the vaccinees (Madhi and Klugman, 2004). This suggested that receipt of PCV-9 reduced the risk of superimposed pneumococcal infection from occurring among PCV-9 vaccinees in children that may have been infected with the respiratory viruses in the community. Overall there was a 45% (p=0.01) reduction in Influenza A, 22% (p=0.08) reduction in RSV and 44% (p=0.02) reduction in PIV types 1-3 associated pneumonia in (Madhi and Klugman, 2004).

There is little evidence of the importance of bacterial co-infections in children with hMPV-associated LRTI. Boivin et al. reported on two cases in whom *Streptococcus pneumoniae* and *Staphylococcus aureus* was present in respiratory secretions from 12 samples concurrent with hMPV infection (Boivin et al., 2002). Dollner et al. reported that children with hMPV-associated

pneumonia had high CRP levels (median, 105.5mg/l [ranged <5-281 mg/l]) most likely reflecting undiagnosed bacterial coinfection in those children. Further in that study children with hMPV-associated LRTI had high temperatures (mean, 39.9^{o}C) (Dollner et al., 2004), a known predictor of bacterial infection (Banya et al., 1996).

1.5.4 Antigenic characteristics

The observed genetic variability may lead to antigenic variability and the two hMPV genetic clusters may represent two distinct antigenic groups. There is conflicting evidence as to whether the two genetic clusters are in fact two antigenic groups.

Using antisera raised in ferrets against the two genetic groups for virus neutralizing assays, van den Hoogen and coworkers showed that the two genetic groups (A and B) were antigenically different based on the definition that a homologous to heterologous neutralization titer of >16 defines a serotype (van den Hoogen et al., 2004a). The antigenic diversity was also addressed by Bastein et al. using western blot analysis and immunoprecipitation of the G protein with polyclonal antibodies raised against strains specific to each group isolated in Canada (Bastien et al., 2004).

This study showed that the detection of the G protein was group specific and also demonstrated that the G protein is N and O linked glycosylated (Bastien et al., 2004). MacPhail et al. studying the replication of hMPV in different animals reported that in Syrian golden hamsters there was cross protection between hMPV subgroups A or B, suggesting that there was no antigenic

difference between the groups and a vaccine based on either group may be effective (MacPhail et al., 2004). A study designed to address the antigenic relatedness of the two groups found them to be highly related antigenically (48% in hamsters and 64%-99% in non-human primates) demonstrating a high level of cross protection (Skiadopoulos et al., 2004). This study also showed that the hMPV F gene is the major neutralization antigen and conferring substantial neutralization and protection across lineages, a finding that was subsequently confirmed (Skiadopoulos et al., 2006), and also showed that the G protein was not a major neutralizing or protective antigen. The antigenic relatedness of the two groups needs to be addressed further as this conflicting evidence may have implications for the development of an effective vaccine.

1.5.5 Reinfection with hMPV

Serological data has shown that the hMPV antibody titer was higher in older individuals (>2 years) compared to younger individuals (Ebihara et al., 2003), suggesting a booster effect probably due to re-infection. Re-infection with the hMPV was reported in a 7 month old immunocompromised child who had severe LRTI and was infected with two genetically distinct hMPV strains (Pelletier et al., 2002).

The symptoms of the first episode was a cold that progressed to bronchiolitis, the second episode which occurred about 10 months later also started with a common cold but deteriorated to bilateral pneumonitis and death (Pelletier et al., 2002). Another study reported a 9 month old girl re-infected with a

heterologous strain of hMPV in a space of 19 days (Ebihara et al., 2004a). Subgroup A1 hMPV was identified as the cause of the initial illness and the second illness was caused by a subgroup B2 virus. The re-infection with subgroup B2 strain was associated with a more severe illness, resulting in the onset of wheezy bronchitis and pneumonia (Ebihara et al., 2004a). Studies by William et al reported that among children with hMPV-LRTI associated illness a recurrent infection was associated with upper respiratory tract (URT) several months/years of age later. This indicated primary immunity induced by infection of the LRTI (lower respiratory tract infection) reduced the severity of subsequent infections and limited the replication of hMPV to the URT (Williams et al., 2004; Williams et al., 2006).

1.5.6 Virulence difference

It is possible that there may be an association between disease severity and hMPV subgroup; however, this remains to be addressed fully. It has been suggested that subgroup A2 may be linked to more severe disease as it has been detected more often than other subgroups (Mackay et al., 2004) Recently a study designed to examine the difference found that group A viruses were more virulent than group B and the difference in severity was not due to underlying medical conditions (Vicente et al., 2006).

In contrast a study in Japan did not find any significant difference for rates of hospitalization between the groups to suggest a difference in severity (Ebihara et al., 2004b) and a study from St Louis, USA found similar rates of illness for either group (Agapov et al., 2006). Such conflicting evidence has also been

reported for RSV (Sullender, 2000) and it has been suggested that difference may be due to the inclusion of only hospitalized patients that were severely ill (Cane, 2001) or that host genetic factors may influence the susceptibility (Stark et al., 2002). Alternatively if studies are not done over an extended time period, the introduction of a new subgroup of virus into the community may be temporally related to more severe disease being observed for that subgroup of virus.

1.5. Objectives

The objectives of this study were:

i. to investigate the molecular epidemiology of hMPV in South Africa;

ii. characterize the burden of severe hMPV associated LRTI; and determine the clinical features of hMPV-LRTI in children infected and not infected by HIV;

iii. probe the role of *Streptococcus pneumoniae* in the pathogenesis of hMPV-LRTI.

CHAPTER 2

STUDY MATERIALS AND METHODS

2.1. Study population

The nasopharyngeal aspirates (NPAs) used in this study were collected from children participating in a phase 3 study of which the primary objective was to determine the efficacy of a PCV-9 in preventing invasive pneumococcal disease and radiographically confirmed pneumonia in South Africa. Details of the study have been previously published (Klugman et al., 2003; Madhi and Klugman, 2004). Briefly stated, recruitment of 39836 children was started on the 1 March 1998 and enrolment of all the subjects was completed by October 2000, with the last child being immunized in December 2000. The first dose of study vaccine was administered at a mean age of 6.6 (standard deviation [S.D.] 1.2) weeks and a further two doses of study-vaccine were administered at 11.2 (S.D. 2.5) and 15.9 (S.D.3.8) weeks respectively, with no booster dose of PCV given. Surveillance for study-outcome cases was hospital-based and continued until 15th November 2001, at which stage the data were analyzed for the primary objectives of the study. Thereafter, investigators and laboratory staff remained blinded to the randomization arm of the individual subjects and surveillance continued until October 2005. All children that were hospitalized were clinically evaluated by one of the study-doctors who used a standardized form for documenting signs and symptoms. The decision to hospitalize children was done as part of routine patient management by the attending physicians and independent of the study.

Children would either have been referred from a primary health-care clinic or self-referred directly to the hospital by the parent. There is no known difference in clinical criteria for hospitalization of HIV infected and HIV uninfected children. The present study is limited to surveillance that occurred from 1st January 2000 until 31st December 2002 as prior to this period samples were not routinely archived.

2.2 Collection of nasopharyngeal aspirates

Nasopharyngeal aspirates were collected within 24 hours of admission to hospital and surveillance was conducted by a study staff doctor at the Chris Hani-Baragwanath hospital. Nasal secretion samples were obtained using a nasogastric tube (FG 8 x 10 cm in length, Ven Medical Products, South Africa), attached to a 5 ml syringe containing 3ml of normal saline. The saline was injected into the nasopharynx and aspirated immediately. An aspirate of 1-2 ml was obtained from the children. On arrival at the laboratory the samples were aliquoted and tested for other respiratory viruses. An aliquot was stored at –70^{o}C was subsequently used for the testing of hMPV in this study.

2.3 Respiratory virus identification

The NPA samples were used to test for common respiratory viruses. The specimens were centrifuged and the pellet was spread onto a microscope slide and fixed. Initially screening was performed by a direct pooled immunofluorescent test for respiratory viruses.

Positive samples were tested for RSV by means of mouse anti-RSV monoclonal fluorescent antibody (Chemicon International Inc; Temecula, California, USA). Specimens that tested negative for RSV were subsequently examined for each of seven respiratory viruses (RSV, influenza A and B, PIV 1,2,3 and adenovirus) using specific monoclonal fluorescein conjugated antibodies (Chemicon International; Temecula, California, USA).

2.4. Identification of hMPV

2.4.1 RNA extractions

Viral RNA was extracted from the stored frozen nasopharyngeal aspirates using the QIAamp viral RNA kit (Qiagen). Viscous NPAs that were difficult to pipette were homogenized with QIAshredder homogenizer columns (Qiagen, Hilden, Germany) and the flow-through was used for viral RNA extraction. The extraction was performed according to the manufacturer's instructions. A volume of the thawed NPA was treated for RNases and lysed with a buffer provided by the manufacturer. This was followed by the addition of ethanol for precipitation of the viral RNA. Following centrifugation the viral RNA becomes attached to a membrane with the help of the carrier RNA. The attached viral RNA was washed twice and eluted with RNase-free water provided by the manufacturer and stored at –20^{o}C.

2.4.2. RT-PCR and nested PCR: F gene

A nested RT-PCR with primers designed for the fusion (F) gene was used for the identification of hMPV from NPAs. The F gene is highly conserved and a

nested RT-PCR assay was necessary to increase the sensitivity for detection and increase the yield of PCR product for sequencing.

2.4.2.1 RT-PCR: F gene

Single step RT-PCR assays have an advantage over a two step RT-PCR as it is fast and decreases the risk of contamination. To prevent the possibility of contamination, a single step RT-PCR assay was considered because of its advantages over a two-step assay. hMPV viral RNA was amplified using the SUPERSCRIPT One-Step RT-PCR kit (Invitrogen). The viral RNA was initially amplified by RT-PCR in a 50µl reaction mix as recommended by the manufacturer and was subsequently optimized for a 25µl reaction, considerably reducing the cost for each reaction. The primers used for the primary RT-PCR reaction span the F gene and correspond to nucleotide sequences position 3052-3069 (primer Fatg) and 3844-3862 (primer Frev) in the NL/1/00 genome (accession number AF371337) and are shown in Table 2.1 were used for the diagnosis of hMPV.

Each 25µl reaction contained 12.5µl of a 2X reaction mix that was provided by the manufacturer of the kit, 0.6µM of each primer (final concentration, Fatg/Frev Table 2.1), 3mM $MgSO_4$ (final concentration), 0.4mM dNTPs (dTTP, dATP, dGTP, dCTP at final concentrations), 0.25µl of RT/*Taq* enzyme mix and 5µl of viral RNA all made up to 25µl with sterile water.

The RT-PCR reaction was performed in an Eppendorf Mastercycler (Eppendorf) as follows: reverse transcription at 50^{o}C for 30 min, an initial denaturation step at 94^{o}C for 2 min, followed by 35 cycles at 94^{o}C for 30 sec,

45°C for 45 sec and 68°C for 1min. No template controls (NTCs) and a negative control (specimens that were negative for hMPV) was included.

2.4.2.2 Nested PCR: F gene

To increase the sensitivity and the yield for sequencing, a second round of PCR was performed. Two microliters of the RT-PCR product was used as the starting template for the nested PCR. A 50µl reaction containing at final concentrations the following reagents: 1X reaction buffer, 3mM $MgCl_2$, 400µM dNTP, 0.6µM each primer (Ffor/Fnest Table 2.1, corresponding to nucleotide positions 3130-3149 and 3794-3810, respectively in the NL/1/00 genome, accession number: AF371337) and 1.25U *Taq* made up to volume with sterile water, was prepared for each reaction.

The cycling parameters were as follows: initial denaturation for 2 min at 94°C, followed by 30 cycles at 94°C, 48°C and 72°C for 1 min each and a final extension at 72°C for 7 min.

2.4.3 RT-PCR:G protein gene

The G gene was also analyzed using a nested RT-PCR approach. The primers for the detection of the G gene are shown in Table 2.1 and correspond to nucleotide position 6262-6285 (HMPVGunivF) and 7181-7204 (HMPVGunivR) in the NL/1/00 genome accession number, AF371337, this primer amplified an 800-1000bp region of the hMPV G protein gene.

The viral RNA was reverse transcribed at 50^{o}C for 30 min, followed by an initial denaturation at 95^{o}C for 3 min, 38 cycles of PCR as follows: 94^{o}C for 1 min, 59^{o}C for 1 min and 72^{o}C for 2 min and a final extension at 72^{o}C for 7 min. To increase the yield for sequencing a nested PCR was performed using the same primers set.

2.5 Analysis of the PCR product

The nested PCR products for both the F and G genes were visualized on a 2% ethidium bromide stained agarose gel on a UV transilluminator and photographs of the gels were taken with a Kodak polariod camera. In addition to the positive control a molecular weight marker (DNA molecular marker VI, Roche diagnostics, Mannheim, Germany) was included to verify a positive result.

2.6 Nucleotide sequencing

The F and the G gene were sequenced in this study. The PCR products were purified using the QIAquick gel extraction kit (QIAGEN, GmbH) and sequenced in both directions. Cycle sequencing for the F gene was performed with the nested primers (Ffor and Frev) and for the G gene the primers used for detecting the G gene (HMPVGunivF and R) in Table 2.1. Sequencing of the PCR product was carried out using the BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems, Foster City, Calif) on the ABI 310 Genetic Analyzer (Applied Biosystems). The nucleotide sequences were

edited using Chromas version 1.43 software and saved as text files in FASTA format.

2.6.1 Nucleotide sequence accession numbers

The hMPV nucleotide sequences from this study have been deposited in Genbank under the following accession numbers AY694693 to AY694784 for the F gene and AY848859 to AY848919 for the G gene.

2.7 Phylogenetic analysis and genetic identities

Nucleotide sequences from F and G genes were aligned with CLUSTAL X 1.64b (Thompson et al., 1997) using the multiple alignment option. Phylogenetic trees were constructed on the aligned nucleotide sequences with the neighbour-joining (NJ) method using the Nucleotide: Kumira 2-parameters in MEGA version 2.1 (Kumar et al., 2001a). Statistical evaluations of the NJ trees were carried out by bootstrap evaluation (500 bootstrap replicates).

Genetic identities for both nucleotide and amino acid were computed on the aligned sequences with Bioedit software. The identities were determined as the proportion of differences, i.e. the number of pair-wise nucleotide or amino acid differences divided by the total number of nucleotides or amino acids in the sequenced region.

The potential structure of the attachment protein (G protein) was predicted using the hydropathy plot of Kyte and Doolittle (Kyte and Doolittle, 1982) in the Bioedit software package. The hydrophobicity score for the amino acids were between -4.5 and 4.5.

2.8 HIV testing

The HIV infection status of individual subjects who were hospitalized was determined using two HIV ELISA test **(**Axsym® and Murex* HIV 1+2, Murex Diagnostic Limited, Dartford, England). A HIV PCR (Roche Amplicor version 1.5, Nutley, NY) test was used to confirm the infections status of children <18 month of age if the ELISA test was reactive or if any child had a non-reactive HIV ELISA test despite the presence of stigmata of acquired immunodeficiency virus syndrome (AIDS).

2.9 C-reactive protein (CRP)

C-reactive protein (CRP) tests were performed using immunoturbidometry (717 Automated Analyzer, Boehringer Mannheim/Hitachi, Mannheim, Germany) at the National Health Laboratory Service, South Africa.

Samples were sent for testing either by the attending physician at the time of admission of the child to hospital or serum that was obtained within 12 hours of admission that was stored at -70^{0}C was retrospectively analyzed for CRP when available.

Table 2.1 Sequences of primers used for the detection and sequencing of human metapneumovirus

Primers	Gene	NL/1/00 corresponding nucleotide positions	Sequence (5'-3')
Fatg	Fusion protein (F gene)	3052-3069	ATGTCTTGGAAAGTGGTG
Frev	Fusion protein (F gene)	3844-3862	CCATGTAAATTACGGAGC
Ffor	Fusion protein (F gene)	3130-3149	TCATGTAGCACTATAACT
Fnest	Fusion protein (F gene)	3794-3810	TCTTCTTACCATTGCAC
HMPVGunivF	Attachment protein (G gene)	6262-6285	GAGAACATTCGRRCRATAYATG
HMPVGunivR	Attachment protein (G gene)	7181-7204	AGATAGACATTRACAGTGGATTCA

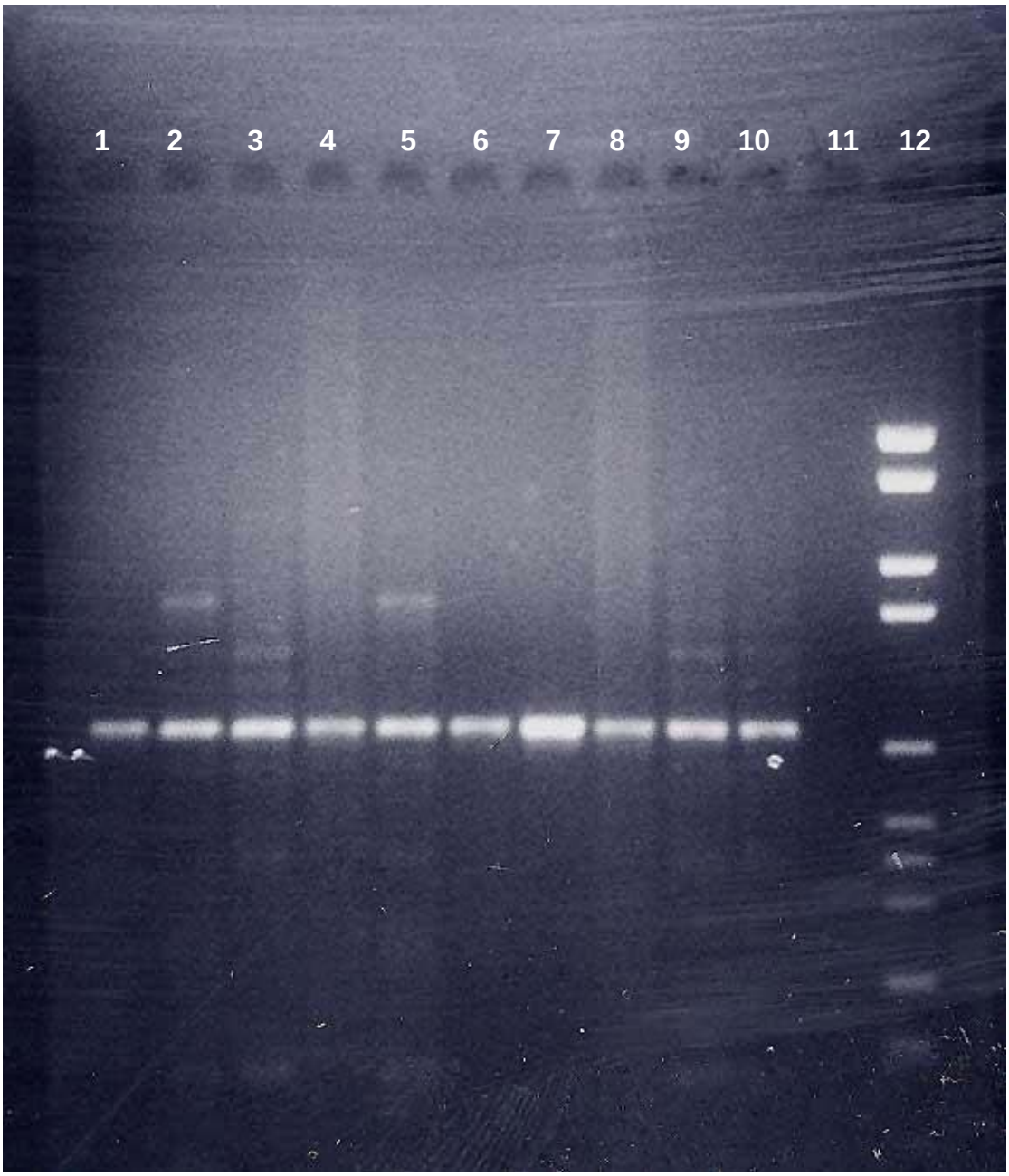


Figure 2.1 A 2% ethidium bromide stained agarose gel showing the nested PCR product as amplified using primers spanning the F gene. Lanes 1-9 are different patient samples that are positives for hMPV. Lane 10 is a positive control sample provided by Dr Guy Boivin, lane 11 is a NTC and lane 12 is a 100 bp molecular weight marker.

2.10 Statistical Analysis

Statistical analysis was performed using Epi Info version 6.04c (Atlanta, Georgia, USA), SAS (SAS Institute, Cary, North Carolina, USA) and STAT version 8.0 (StataCorp LP, College Station, TX, USA). Continuous and categorical variables were analyzed using unpaired Student t-test and Mantel-Haenszel chi-square test, respectively. Fisher's exact test was used when a cell had an expected value of fewer than five observations. Median (25^{th}-75^{th} centiles) were used for all age-related analyses and the mean and standard deviation (S.D) were calculated for continuous variables. Relative risks (R.R) and 95% confidence intervals ($CI_{95\%}$) were used in describing risk differences between HIV infected and HIV uninfected children.

As receipt of PCV may have impacted on the epidemiology of hMPV associated hospitalization as has been described for other respiratory viruses (Madhi and Klugman, 2004), the calculation of the incidence rate of hospitalization for hMPV-LRTI was limited to placebo recipients.

2.10.1 Vaccine Efficacy (VE)

Vaccine efficacy was calculated using the vaccine efficacy calculation in Epi Info version 6.04d for cohort studies. This is based on the formula: V.E. (%) = (incidence rate in the unvaccinated – incidence rate in the vaccinated)/ incidence rate in unvaccinated} x 100. All children that were randomized were included in the intent-to-treat (ITT) analysis from the day of receiving their first dose of study vaccine.

Children were considered to be fully vaccinated and included in the per protocol (PP) analysis if the event occurred more than 14 days following the third dose of study vaccine and the child received all the study- vaccines as per planned schedule.

Only the first episode of any clinical syndrome was included in the respective "vaccine efficacy" calculation. Trends in hMPV-LRTI between vaccinees and placebo recipients among children <6.0 months, 6.1-12.0 months, 12.1-24 months and >24.0 months of age at the time of hospitalization were calculated using Mantel-Haenszel chi-square. An alpha of ≤ 0.05 was considered as significant.

2.11 Definitions

The clinical definitions of the spectrum of LRTI listed below have been previously defined by Madhi et al. and have been adopted for this study as well (Madhi, 2003).

2.11.1 Severe lower respiratory tract infection (LRTI)

The diagnosis of LRTI was based on WHO clinical criteria, which was complemented by pulse oximetry. Children were enrolled if they had a history of cough of less than two weeks duration and fulfilled at least one of the following criteria: 1) tachypnea – defined as a respiratory rate >50/breath/min between 2-12 months of age and 40 breaths/min in older children; and the presence of lower chest wall in drawing (and/or intercostals recession in

malnourished children); 2) An arterial oxygen saturation of <90% room air, as measured by pulse oximetry.

2.11.2 Respiratory viral associated LRTI

Children with LRTI in whom respiratory virus antigen was detected by immunofluorescence from NPAs were categorized as having viral associated LRTI.

2.11.3 Bronchiolitis

A clinical diagnosis of bronchiolitis was made if the child was less than two years of age and had bilateral diffuse wheezing with or without the presence of bilateral crackles, or in the presence of clinical and/or radiographic signs of hyperinflation the absence of any adventitious sounds and other evidence of airspace infiltration on chest radiograph.

2.11.4 Pneumonia

Children who have evidence of crackles in the absence of wheezing, or no adventitious sounds on chest auscultation in the presence of any airspace consolidation on chest radiograph, or alveolar consolidation on chest radiograph were categorized as having pneumonia.

CHAPTER 3

PREVALENCE AND BURDEN OF DISEASES OF THE HMPV IN HIV UNINFECTED AND HIV INFECTED CHILDREN HOSPITALIZED WITH LRTI OVER A 3 YEAR PERIOD IN SOUTH AFRICA

3.1 Study sample

Figure 3.1 gives a summary of the children that were hospitalized with LRTI (bronchiolitis and pneumonia) during the study period January 2000 to December 2002. During this period there were 3176 episodes of LRTI requiring hospitalization of which NPA were performed on 3069 (96.6%) samples. Of the 3069 episodes, 2715 (88.5%) samples were available for hMPV testing. The HIV status was available for only 2678 (98.6%). There was no significant difference (P > 0.99) between the samples for which NPA were done and the samples that were available for hMPV testing, both overall and between HIV infected and HIV uninfected.

Children in whom NPA samples were unavailable for further hMPV testing (354 [11.5%] of 3069) were younger median [range] age-months: 9.3 [1.4-54.7] *vs* 13.8 [1.2-56.1], P <0.001) and were 1.9 fold (95% C.I 1.5-2.5) more likely to have one of the other respiratory viruses identified (133 [37.65] of 354 *vs* 645 [23.8%] of 2715; P<0.0001) than in whom NPA were available for hMPV testing. These differences were evident in HIV infected as well as in

HIV uninfected children. There was however no clinical or demographic differences regarding LRTI episodes for which NPA were unavailable.

3.2 hMPV in hospitalized children

Overall a total of 3302 NPA samples were tested for hMPV in children with respiratory illness of which 2715 samples were from children with LRTI. This study is limited to children with LRTI as the samples from children with other respiratory illness were not done in a systematic manner.

Human metapneumovirus was identified from 230 NPA samples tested in children with a respiratory tract illness. A clinical diagnosis of LRTI was made in 202 (87.8%) of the 230 hospitalizations associated with hMPV and the remaining 28 (12.2%) episodes were associated with respiratory symptoms in children with a non-LRTI illness. Except for a single HIV infected vaccine recipient with lymphocytic interstitial pneumonitis, the remaining 27 non-LRTI cases of hMPV-associated hospitalization occurred in HIV uninfected children. The spectrum of illness' (including multiple diagnosis) observed in these HIV uninfected children included: nine children with acute exacerbation of hyper-reactive airway disease/asthma, eight episodes of upper respiratory tract infection, eight episodes of febrile convulsions, two episodes each of break-through epileptic seizures or gastroenteritis and one case each of pneumococcal septicemia and tuberculosis meningitis.

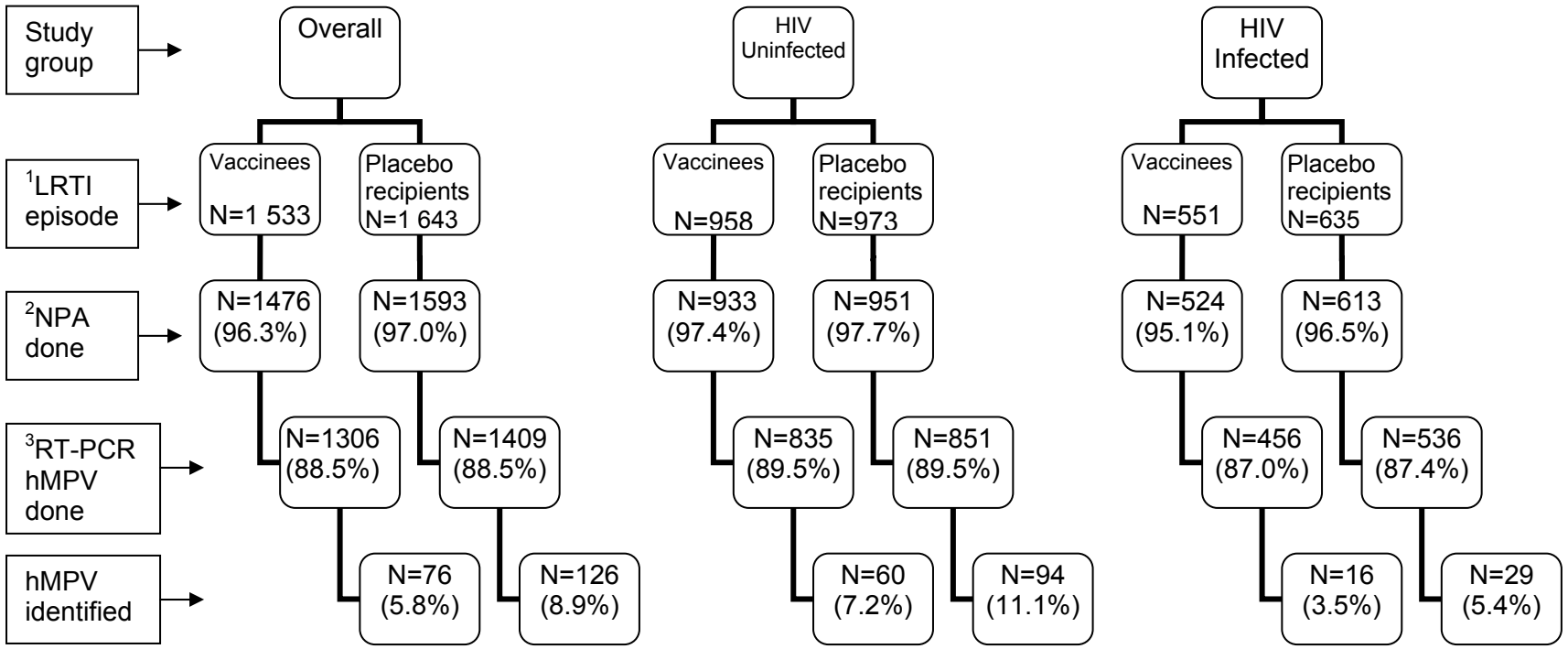


Figure 3.1: Summary of children hospitalized for lower respiratory tract infection that were investigated for human metapneumovirus infection in South Africa[1]LRTI episodes= total number of lower respiratory tract infections (bronchiolitis or pneumonia). [2]NPA done= nasopharyngeal aspirate performed to test for respiratory viruses other than hMPV. [3]RT-PCR done= Number of NPA samples that were available for reverse-transcriptase polymerase chain reaction (RT-PCR) assay to detect hMPV.

3.3 Prevalence of hMPV in episodes of LRTI

Among the 2715 episodes of LRTI, 202 (7.4%) samples were positive for hMPV (Figure 3.1). hMPV was identified in 154 (9.1%) of 1686 samples in HIV uninfected children compared to 45 (4.5%) of the 992 samples in HIV infected children (P=0.001). Overall hMPV was identified in 76 children that were given the PCV and 126 that were given placebo (P=0.01). Of the 76 PCV recipients positive for the hMPV, 60 (78.9%) were HIV uninfected and 16 (21.1%) were HIV infected compared to placebo recipients where 94 (76.4%) were HIV uninfected and 29 (23.6%) were HIV infected. The HIV infection status was unknown for three of the children with hMPV that had received placebo.

3.4 Burden of hMPV-LRTI hospitalization in HIV-infected and – uninfected children not vaccinated with PCV

The data from children that were given the placebo were used to investigate the burden of hMPV. Samples were available for hMPV testing in 2715 (85.5%) of the 3176 episodes of LRTI hospitalizations. Among the placebo recipients that were available for hMPV testing, 1409 (85.8%) of the 1643 episodes of LRTI required hospitalization. There was no difference in the proportion of LRTI episodes for which samples were available for hMPV testing between HIV uninfected children (851 [87.5%]) of 973) and HIV infected children (536 [84.4%] of 635, P=0.08). Human metapneumovirus was identified in a lower proportion of HIV infected children (29 [5.4%] of 536) than HIV uninfected children (94 [11.1%] of 973, P=0.004**;** Table 3.1).

Table 3.1: Prevalence of identifying human metapneumovirus (hMPV) compared to other studied respiratory viruses in children not vaccinated with a pneumococcal conjugate vaccine.

Virus identified	Overall[1]		HIV uninfected children[2]		HIV infected children[3]	
	N (%)[4]	O.R. (95% C.I.)[5]	N (%)[4]	O.R. (95%C.I.)[5]	N (%)[4]	O.R. (95%C.I.); P=[5]
RSV[6]	240 (15.1)	0.55 (0.44-0.70)[8]	201 (21.1)	0.46 (0.35-0.61)[8]	31 (5.1)	1.07 (0.62-1.86); P=0.79
Influenza A virus	73 (4.6)	2.04 (1.50-2.78)[8]	46 (4.8)	2.44 (1.67-3.58)[8]	22 (3.6)	1.59 (0.87-2.91); P=0.10
PIV 1-3[7]	52 (3.3)	2.56 (1.82-3.62)[8]	33 (3.5)	3.45 (2.26-5.31)[8]	18 (2.9)	1.89 (1.0-3.59); P=0.04
Adenovirus	32 (2.0)	4.79 (3.18-7.26)[8]	27 (2.8)	4.25 (2.69-6.75)[8]	5 (0.8)	6.96 (2.54-20.6); P<0.0001
HMPV	126 (8.9)	Not applicable	94 (11.1)	Not applicable	29 (5.4)	Not applicable

[1]Total 1 643 LRTI episodes of which 1 593 tested for viruses other than hMPV and 1409 samples available for hMPV testing. [2] Total 973 LRTI episodes of which 951 tested for viruses other than hMPV and 851 samples available for hMPV testing. [3] Total 635 episodes of LRTI of which 613 episodes tested for viruses other than hMPV and 536 samples available for hMPV testing. [4]Number in column refers to number of isolates and value in parenthesis is a percentage of the number of samples tested for that virus. [5]Refers to comparing prevalence of identifying the specified virus relative to that of identifying hMPV. [Odds ratio (O.R.) 95% confidence interval (95%C.I.); P value (P=)]. [6]RSV: respiratory syncytial virus. [7] PIV: parainfluenza virus type 1-3. [8]P value <0.0001

The overall incidence of hMPV-LRTI was however 5.0 (95%C.I.3.3-7.5) fold greater in HIV infected children (incidence rate: 2 504 [95%C.I. 1 683-3 577] per 100 000) than in HIV uninfected children (incidence rate: 505 [95%C.I. 409-618] per 100 000, P<0.0001). The estimated incidence of hMPV-LRTI in these children, after adjusting for those episodes of LRTI for which specimens were unavailable for hMPV testing, was 2 936 [95%C.I.2 042-4 079] per100 000 in HIV infected children and 575 [95%C.I. 472-695] per 100 000 in HIV uninfected children, R.R.5.4; 95%C.I. 3.5-7.5, P<0.0001.

3.5 Seasonality off hMPV in relation to other studied respiratory viruses

Figure 3.2 shows the seasonality of hMPV in relation to the other viruses, including all episodes of LRTI that were investigated for the other viruses. Human metapneumovirus was identified throughout each of the three years; however identification of hMPV peaked during the autumn-winter months (April-August). The season appears to follow the RSV season and precede the influenza and parainfluenza seasons.

3.6 Prevalence of hMPV in relation to other studied respiratory viruses

Among all the children not vaccinated with PCV, hMPV was identified (126 [8.9%] of 1 409, Figure 3.1) less frequently than RSV (15.1%; P<0.0001), but more commonly than influenza A/B virus (4.6%; P<0.0001), PIV 1-3 virus (3.3%; P<0.0001) and adenovirus (2.0%; P<0.0001; Table 3.1).

Similarly, identification of hMPV (11.1%) was less common than RSV (21.1%; P<0.000) but more frequent than any of the other studied viruses (P<0.0001, Table 3.1) in HIV uninfected children. Among HIV infected children hMPV (5.4%; Table 3.1) was identified more commonly than parainfluenza type 1-3 (2.9%; P=0.04) and adenovirus (0.8%; P=0.001). These comparisons did not differ significantly when restricting the analyses to only those episodes of LRTI for which samples were available for hMPV testing (data not shown)

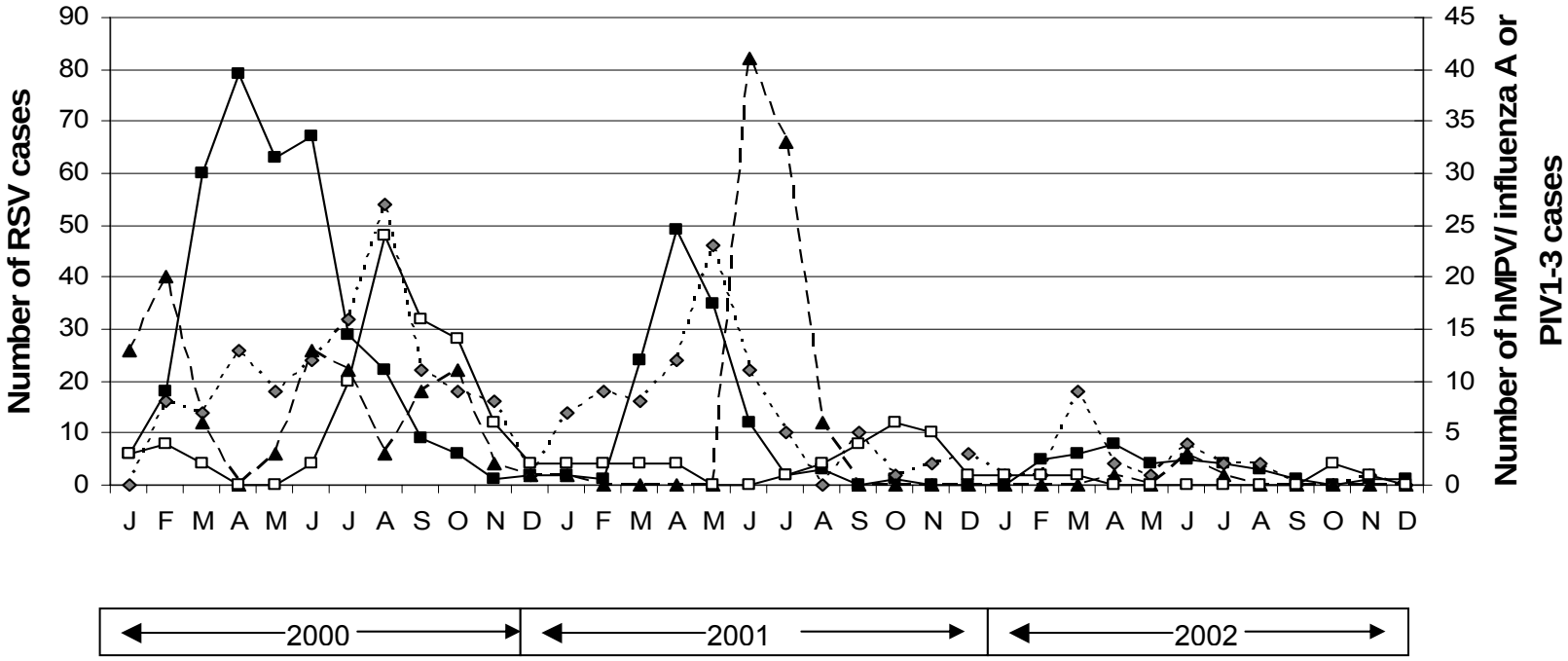

Number of RSV cases
Number of hMPV/ influenza A or PIV1-3 cases
90
80
70
60
50
40
30
20
10
0
45
40
35
30
25
20
15
10
5
0
J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
2000
2001
2002

Figure 3.2: Seasonal variation in identifying different respiratory viruses in children hospitalized for lower respiratory tract infection between January 2000 until December 2002.

Legend: ——■——:respiratory syncytial virus (RSV); - - -▲- - - :influenza A virus; - - -♦ - - -: human metapneumovirus (hMPV); ——□——:parainfluenza virus type 1-3.

Note: Includes all the respiratory viruses that were identified during this study period in the cohort of children participating in the phase 3 pneumococcal conjugate vaccine efficacy study. Samples were only available on a subgroup of children (N=2 715 [88.5%] of 3 069 children on whom nasopharyngeal swabs were performed) for human metapneumovirus testing.

3.7 Clinical features of human metapneumovirus associated lower respiratory tract infections

Among children in whom hMPV was identified, the only statistically significant difference in the clinical features listed in Table 3.2 between PCV and placebo recipients was that HIV infected PCV recipients were less likely (2 [12.5%] of 16 vs. 13 [44.8%] of 29, P=0.05) to have alveolar consolidation on chest radiographs. The data from PCV and placebo recipients were therefore combined when analyzing the demographic and clinical features of children with hMPV-LRTI (Table 3.2). The overall mean age of children with hMPV-LRTI was 13.3 months and HIV infected children with hMPV-LRTI were older (mean 17.6 months) than HIV uninfected children (12.3 months; P=0.007). Furthermore, HIV infected children had a longer duration of hospital stay (5.8 vs. 4.1 days, P=0.003) as well as a higher mortality rate (4.4 vs. 0%, P=0.05) compared to HIV uninfected children (Table 3.2). Both of the children who died were HIV infected and were males aged 4.5 and 21.7 months. The younger of these children had concurrent *Pneumocystis jiroveci* pneumonia (PCP). The older HIV infected child who died was not investigated for PCP. The only child that required mechanical ventilatory support was a 4 month old HIV uninfected child from whom RSV was concurrently identified from the nasal aspirate.

Additional differences observed between HIV infected children and HIV-uninfected children are shown in Table 3.2. These included the observations that HIV infected children were more likely to have concurrent bacteremia (16.3% vs. 0%, P<0.0001), had higher median CRP levels (43mg/l; P=0.04)

nd were more likely to present with pneumonia than bronchiolitis (P=0.0001). *Pneumocystis jiroveci* pneumonia was diagnosed in two (25%) of eight HIV infected children with hMPV-LRTI in whom an immunofluorescence assay was performed to identify *Pneumocystis jiroveci* cysts. Concurrent respiratory viral infections occurred in one HIV infected child and 12 (7.8%) of 154 HIV uninfected children. This mainly involved the co-presence of respiratory syncytial virus (8 [61.5%] of 13], Table 3.2.

3.8 Discussion

Serological diagnostic testing requires paired sera (acute and convalescent sample) and hMPV replicates slowly in culture and may also yield false positives (Ebihara et al., 2004b) making both of these methods unsuitable for clinically investigating for hMPV-associated disease. RT-PCR is sensitive, less labour intensive and does not require sophisticated equipment. It has been used by most studies that have investigated the prevalence of hMPV (Hamelin et al., 2004; Hamelin and Boivin, 2005). The present study used a nested RT-PCR assay to detect for hMPV RNA isolated from stored nasopharyngeal aspirates. The primary RT-PCR was done using a one step approach to avoid contamination that may have arisen due to the high throughput (2715 samples) of samples. The nested PCR was used to increase the sensitivity of PCR as the yield of RNA from stored NPA may be suboptimal.

Table 3.2: Demographic and clinical characteristics of human immunodeficiency virus type-1 (HIV) infected and -uninfected children hospitalized for human metapneumovirus (hMPV) associated lower respiratory tract infections

Demographic/ clinical feature	Overall N=202	HIV infected N=45	HIV uninfected N=154	P value[1]
Median age (range)- months	13.3 (1.4-49.2)	17.6(4.5-44.3)	12.3 (1.4-49.2)	0.007
Male: female	1.3:1	1.4:1	1.3:1	0.84
Gestational age <37 weeks at birth (%)	42 (20.8)	11 (24.4)	31 (20.1)	0.53
Mean (S.D.) oxygen saturation[2]	92.0 (4.2)	90.8 (6.0)	92.4 (3.4)	0.03
Mean (S.D.) axillary temperature ^{o}C	37.4 (0.8)	37.6 (0.9)	37.4 (0.8)	0.18
Bronchiolitis (%)	80 (39.6)	7 (15.6)	73 (47.4)	0.0001
Clinical pneumonia (%)	119 (58.9)	38 (84.4)	81 (52.6)	0.0001
Median (range) CRP-mg/l[3]	23 (1-435)	43 (2-435)	18 (1-405)	0.04
Median (range) WCC x10^{9} cells/ml[4]	10 (3-30)	10.2 (3-21.3)	10 (4.3-30)	0.81

Median (range) procalcitonin –ug/ml[5]	0.2 (0.1-85.1)	1.3 (0.1-85.1)	0.2 (0.1-43.0)	0.62
Alveolar consolidation on CXR (%) [6]	49 (24.3)	15 (33.3)	34 (22.1)	0.12
Bacteria from blood culture[7]	7 (3.7)	7 (16.3)	0 (0)	<0.0001
Other viruses cultured[8]	13 (6.4)	1 (2.2)	12 (7.8)	0.30
Mean (range) hospital stay- days	3.5 (1-35)	5.8 (1-21)	4.1 (1-35)	0.003
Case fatality rate (%)	2 (0.01)	2 (4.4)	0 (0)	0.05

[1]Comparing HIV infected to HIV uninfected children. [2] Measured by a pulse oximeter in room-air upon admission to hospital. [3]C-reactive protein (CRP) test performed in 27 HIV infected and 91 HIV uninfected children. [4]Involves 10 observations in HIV infected and 19 observations in HIV uninfected children. WCC=white cell count.[5]Involved 9 observations in HIV infected and 83 observations in HIV uninfected children. [6]Alveolar consolidation on chest radiograph using study-specific interpretation and definition criteria.(Cherian et al., 2005)[7] Involved 189 observations overall, 43 in HIV infected children and 146 in HIV uninfected children in whom blood cultures were performed. Bacteria cultured in HIV infected children were *Streptococcus pneumoniae* (n=4), *Salmonellla* sp. (n=2) and *Streptococcus viridans* (N=1). Further 20 contaminants cultured in HIV infected and uninfected children each.[8] Other viruses identified were respiratory syncytial virus (1 in HIV infected and 8 in HIV uninfected children), and three influenza A virus and one adenovirus in HIV uninfected children.

Although more sensitive quantitative methods may be used to detect hMPV RNA, detecting hMPV in as low as 5 copies of RNA (Cote et al., 2003; Mackay et al., 2003; Maertzdorf et al., 2004), these methods require the used of expensive real time detection systems. Therefore the use of a nested PCR assay may be sufficient to detect for the presence of hMPV RNA used in the present study and reported elsewhere (Kaida et al., 2006). However despite the sensitivity of the methods used it is still possible that factors such as storage and transport of samples may lead to the underestimation of the prevalence of hMPV in the present study.

The overall prevalence of hMPV RNA among the children hospitalized with LRTI over the study period (2000-2002) was 7.4%. Among the placebo vaccinated group, there were more cases of hMPV–LRTI associated hospitalization in HIV uninfected (11.1%) compared to HIV infected children (5.4%), however the overall measured incidence rate was 5.0 fold higher for HIV infected children than HIV uninfected, emphasizing the increase burden of hMPV associated LRTI in HIV-1 infected children.

The overall estimated probable incidence of hMPV in HIV uninfected children in the present study (505-575 per 100 000) was greater than that reported by Peiris et al. in Hong Kong for children <6 years of age (i.e. 422 per 100 000) (Peiris et al., 2003). Furthermore, the present study probably underestimated the incidence of hMPV related hospitalizations since the estimates focused on hMPV-LRTI and excluded children hospitalized for non-LRTI hMPV related respiratory illness, e.g. asthma. Recently, Williams et al. observed that only

2% of children with hMPV associated LRTIs required hospitalization (Williams et al., 2004) suggesting that the overall incidence of hMPV-LRTI in HIV uninfected children may be as high as 28 750 per 100 000 children as the present study was limited to hospitalized children. As children with non-LRTI illness were not systematically investigated for respiratory viruses during the course of this study it is not possible to provide an estimate of the overall burden of hMPV associated hospitalizations.

The peak period for hMPV-LRTI appeared to occur after the peak for RSV LRTI and preceded the peak observed for influenza virus associated LRTI in the years 2000 and 2001, the imbalance in sample availability between episodes of LRTI associated with the presence and absence of the other viruses, makes it difficult to draw any definite conclusions on the exact timing of the hMPV epidemics. Nevertheless, the data indicate that hMPV was identifiable throughout the year and the peak period for hMPV-LRTI hospitalizations occurred within the same window period when the incidence of hospitalization peaked for respiratory syncytial virus, influenza virus and parainfluenza virus associated LRTI. Similar to the findings from a Canadian study,(Boivin et al., 2003) the RSV epidemic was more prolonged than the hMPV epidemic. In addition, perennial identification of hMPV coupled with peak periods as identified in the present study have also been reported in Hong Kong and North America (Peiris et al., 2003; Williams et al., 2004).

In the present study the hMPV was second to RSV as the most frequently identified respiratory virus in HIV uninfected children (11.1% vs. 21.1%); and

as common as RSV (5.1% vs. 5.4% for hMPV) in HIV infected children, thus highlighting it's importance to the pathogenesis of LRTI. It is possible that the importance of the role of hMPV in children with LRTI compared to the other studied viruses may be over-estimated due to differences in the methods used for identifying the various viruses. Although the sensitivity of direct immunofluorescence testing is reported to be greater than 90% (Gardner, 1970) the use of RT-PCR for identifying hMPV may however have resulted in a bias in favor of detecting hMPV.

Despite identifying hMPV less frequently in HIV infected compared to HIV uninfected children with LRTI, the absolute burden of hMPV-LRTI was 5.0-5.4 fold greater in HIV infected than –uninfected children This observation is in keeping with our findings for other respiratory viruses (Madhi et al., 2000). This is due to the heightened susceptibility of HIV infected children to other respiratory pathogens, hence, viruses being proportionately less common among these children compared with HIV uninfected children. The differences in the clinical spectrum of hMPV-LRTI observed between HIV infected and HIV uninfected children were similar to observations of differences in the clinical presentation of other respiratory viruses between HIV infected and HIV uninfected children (Madhi et al., 2002a; Madhi et al., 2002b; Madhi et al., 2001). The complexity of pneumonia in HIV infected children is once again highlighted by the higher prevalence of bacterial co-infections including the broader repertoire of bacteria which cause infections in HIV infected children, as well as that concurrent *Pneumocystis jiroveci* was identified in 25% of the HIV infected children who were investigated for PCP. These factors may

explain the higher median CRP levels, longer duration of hospitalization and the higher mortality rate in HIV infected compared to HIV uninfected children hospitalized for hMPV-LRTI. The older median age of hMPV-LRTI is most likely due to HIV infected children remaining at risk of developing hMPV-LRTI beyond the age-group period when the risk of hMPV-LRTI is reduced in HIV uninfected children. The clinical characteristics described in Table 3.2 did not differ statistically between PCV and placebo recipients except for radiologically confirmed pneumonia. Nevertheless, the inclusion of PCV recipients in this analysis may have inadvertently biased the clinical presentation toward a milder illness as shown later in this thesis PCV vaccination was associated with a 58% reduction in hMPV–LRTI hospitalization (Chapter 5).

In conclusion the present study demonstrates that the human metapneumovirus is a common respiratory pathogen in South Africa that is associated with a higher risk of LRTI associated hospitalizations among HIV infected than HIV uninfected.

CHAPTER 4

MOLECULAR EPIDEMIOLOGY OF HUMAN METAPNEUMOVIRUS IN SOUTH AFRICA

4.1 Human metapneumovirus genotyping

92 (40%) of the 230 samples that tested positive for the hMPV by RT-PCR were randomly selected from each month for which hMPV was isolated and genotyped by sequencing part of the F gene using primers as described in chapter 2. Forty (43.4%) samples were selected from the 2000 epidemic, 34 (37%) samples from 2001 and 18 (19.6%) samples from 2002. Due to the limited amount of RNA only 61 (66%) of the 92 hMPV positives were available for sequencing of the attachment glycoprotein (G) gene.

4.2 Phylogenetics Analysis based on the sequences of hMPV F gene

Phylogenetic analysis based on the partial sequences of the hMPV F gene for the 92 selected strains demonstrated the presence of two major genetic groups (A and B) and 2 subgroups (1 and 2) in South Africa during the study period. The presence of these groups (A and B) and subgroups (1 and 2) were supported by bootstrapping (bootstrap values of 100%) and the clustering with prototype strains from the Netherlands and Canada.

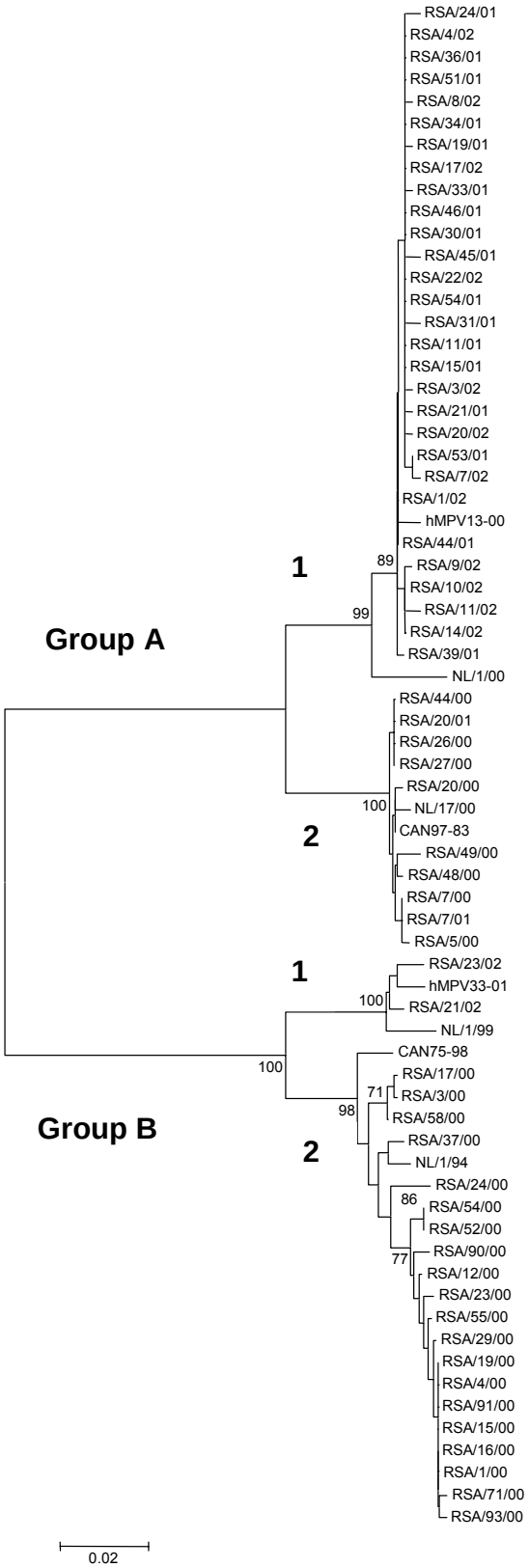
The nomenclature adopted in this study to classify or genotype the South African strains (viz: A1, A2, B1 and B2) has been used by van den Hoogen et al and proposed by Mackay et al as the standard nomenclature to define the groups and subgroups (Mackay et al., 2004; van den Hoogen et al., 2004a).

Most of the strains, 56 (60.9%) clustered together with the prototype A strains (NL/1/00, NL/17/00, hMPV13-00 and CAN97-83) from the Netherlands and Canada. The remaining 36 (39.1%) strains clustered with the group B prototypes (NL/1/99, NL/1/94, CAN95-98 and hMPV33-01) from the Netherlands and Canada. Thirty eight (67.9%) of the 56 group A strains clustered with the group A1 prototypes from the Netherlands (NL/1/00) and Canada (hMPV13-00). The other 18 (32.1%) group A strains clustered with prototypes strains NL/17/00 and CAN97-83, representing subgroup A2. The majority of the group B strains (91.7%) clustered with prototype strains B2 from the Netherlands (NL/1/94) and Canada (CAN75-98) and the other 2 groups B strains, clustered with the subgroup B1 prototypes.

From the topology of the tree (Figure 4.1) subgroup B2 was the most divergent and although the South African hMPV strains clustered with both Canadian and Netherlands prototypes, the South African subgroup A1 virus clustered more closely with the Canadian prototype.

4.3 Multiple genotypes of hMPV based on attachment glycoprotein (G) gene

Phylogenetic analysis based on the sequence of the G gene supported the existence of two distinct genetic lineages and two sub lineages. From the topology of the tree (Figure 4.2) and supported by bootstrap values 70-100%, it appears that multiple genotypes may exist within each group as described for RSV (Sullender, 2000). Applying the method describe for RSV to assign genotypes (sequences that clustered together with bootstrap value of 70-100% (internal nodes at the internal branches) are considered a genotype (Peret et al., 2000; Peret et al., 1998) to the hMPV, multiple lineages may also exist for hMPV. From the topology and supported by bootstrap values, subgroup A1 may be divided into 5 genotypes, subgroup A2 into 2 genotypes, B1 into 2 genotypes and B2 into 6 possible genotypes.


RSA/24/01
RSA/4/02
RSA/36/01
RSA/51/01
RSA/8/02
RSA/34/01
RSA/19/01
RSA/17/02
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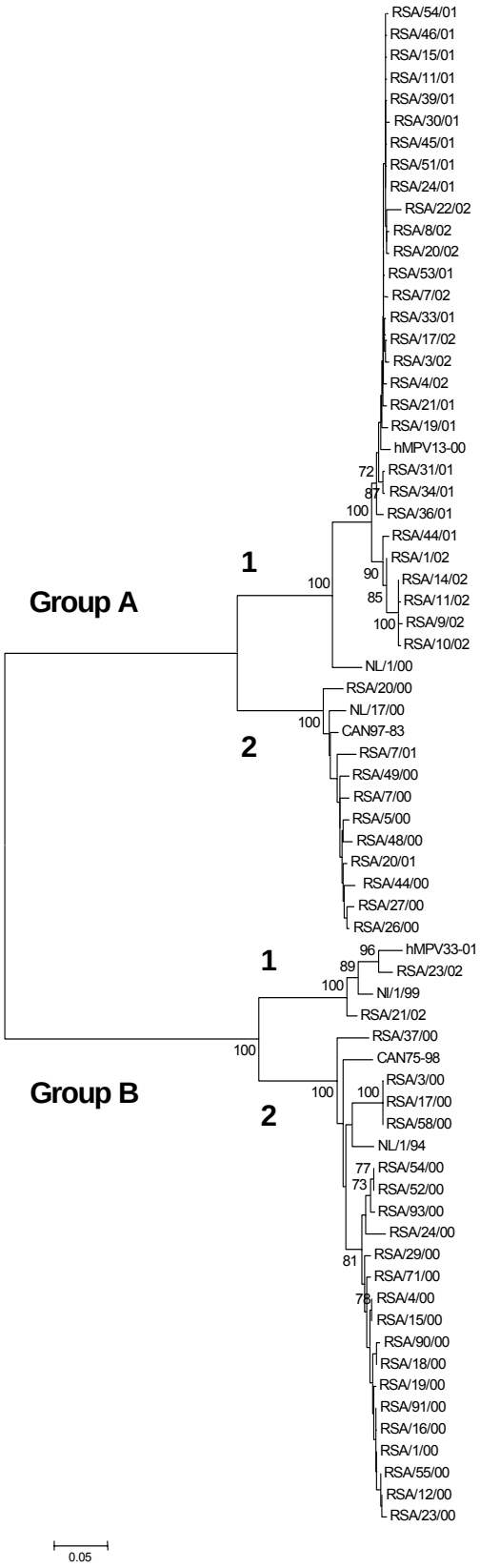
RSA/46/01
RSA/30/01
RSA/45/01
RSA/22/02
RSA/54/01
RSA/31/01
RSA/11/01
RSA/15/01
RSA/3/02
RSA/21/01
RSA/20/02
RSA/53/01
RSA/7/02
RSA/1/02
hMPV13-00
RSA/44/01
RSA/9/02
RSA/10/02
RSA/11/02
RSA/14/02
RSA/39/01
NL/1/00
RSA/44/00
RSA/20/01
RSA/26/00
RSA/27/00
RSA/20/00
NL/17/00
CAN97-83
RSA/49/00
RSA/48/00
RSA/7/00
RSA/7/01
RSA/5/00
RSA/23/02
hMPV33-01
RSA/21/02
NL/1/99
CAN75-98
RSA/17/00
RSA/3/00
RSA/58/00
RSA/37/00
NL/1/94
RSA/24/00
RSA/54/00
RSA/52/00
RSA/90/00
RSA/12/00
RSA/23/00
RSA/55/00
RSA/29/00
RSA/19/00
RSA/4/00
RSA/91/00
RSA/15/00
RSA/16/00
RSA/1/00
RSA/71/00
RSA/93/00
1
2
1
2
89
99
100
100
100
71
98
86
77
Group A
Group B
0.02

Figure 4.1. Neighbour-joining trees based on nucleotide sequences from the partial F gene open reading frame from 61 South African hMPV isolates. The trees were computed with MEGA version 2.1 using the Nucleotide: Kumira 2-parameters (Kumar et al., 2001a). Bootstrap probabilities for 500 replicas are shown at the branch nodes. Only values of 70-100% are indicated. Isolates from South Africa are indicated by RSA followed by the isolate number and year (e.g. RSA/18/02). The viruses from Canada (CAN97-83, hMPV13-00, CAN75-98 and hMPV33-01) and the Netherlands (NL/1/00, NL/17/00, NL/1/99 and NL/1/94) are prototypes from each subgroup.


RSA/54/01
RSA/46/01
RSA/15/01
RSA/11/01
RSA/39/01
RSA/30/01
RSA/45/01
RSA/51/01
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RSA/8/02
RSA/20/02
RSA/53/01
RSA/7/02
RSA/33/01
RSA/17/02
RSA/3/02
RSA/4/02
RSA/21/01
RSA/19/01
hMPV13-00
RSA/31/01
RSA/34/01
RSA/36/01
RSA/44/01
RSA/1/02
RSA/14/02
RSA/11/02
RSA/9/02
RSA/10/02
NL/1/00
RSA/20/00
NL/17/00
CAN97-83
RSA/7/01
RSA/49/00
RSA/7/00
RSA/5/00
RSA/48/00
RSA/20/01
RSA/44/00
RSA/27/00
RSA/26/00
hMPV33-01
RSA/23/02
NI/1/99
RSA/21/02
RSA/37/00
CAN75-98
RSA/3/00
RSA/17/00
RSA/58/00
NL/1/94
RSA/54/00
RSA/52/00
RSA/93/00
RSA/24/00
RSA/29/00
RSA/71/00
RSA/4/00
RSA/15/00
RSA/90/00
RSA/18/00
RSA/19/00
RSA/91/00
RSA/16/00
RSA/1/00
RSA/55/00
RSA/12/00
RSA/23/00
72
87
100
100
90
85
100
1
2
Group A
96
89
100
100
100
77
73
81
78
1
2
Group B
0.05

Figure 4.2. Neighbour-joining trees based on nucleotide sequences from the G gene open reading frame from 61 South African hMPV isolates. The trees were computed with MEGA version 2.1 using the Nucleotide: Kumira 2-parameters (Kumar et al., 2001a). Bootstrap probabilities for 500 replicas are shown at the branch nodes. Only values of 70-100% are indicated. Isolates from South Africa are indicated by RSA followed by the isolate number and year (e.g. RSA/18/02). The viruses from Canada (CAN97-83, hMPV13-00, CAN75-98 and hMPV33-01) and the Netherlands (NL/1/00, NL/17/00, NL/1/99 and NL/1/94) are prototypes from each subgroup

4.3 Genetic diversity in the hMPV

Genetic variability was determined by nucleotide sequencing of a 581 base pair fragment in the F gene and the entire G gene protein. The predicted amino acid sequence was determined using MEGA version 2.1 (Kumar et al., 2001b).

4.3.1 Variability of the fusion gene protein

Only part of the F gene was sequenced, therefore the identities presented here are estimates of the entire gene. From the partial sequence an amino acid alignment presenting amino acids (44-236 of the prototype Netherlands strain (NL/1/00)) was compared with the prototypes as shown in Appendix 1 (only strains that differed are shown).

The estimated nucleotide and amino acid identities showed a high percentage identity for the F gene (Table 4.1). Between the major groups (A and B), the estimated identities ranged between 83-85% at the nucleotide level and between 93.2-95.8% at the amino acid level. Within groups (Table 4.1) identities ranged between 93-95% between A1-A2 and 98-100% between B1-B2 at the nucleotide level. At the amino acid level the identities were 93.3-97.9% between A1-A2 and 98.4% between B1-B2.

Of the 92 South African hMPV that were sequenced, the predicted amino acid alignments are only shown for strains that differed from the Netherlands prototypes (Appendix 1). Within this region, between amino acid 44-236 (corresponding to NL/1/00), cysteine residues were conserved in all South

African strains at position 60 and 182. Two potential conserved N linked glycosylation sites in the predicted F protein were observed in all the South Africa strains. Group specific amino acid residues were present at positions 122, 135, 139, 167, 175 and 233 differentiating between groups A and B. Amino acid substitutions at various positions were exclusive to subgroups A1 (amino acids [aa] 61, 82, 143), A2 (aa 61, 143, 185) and subgroups B1 (aa 46, 143, 179) and B2 (aa 143).

4.3.2 Variability of the G gene protein

Sequence data showed the G gene to be high variable (Table 4.1). The G gene identities were 45.1-53.1% between groups at the nucleotide level and 22.4-27.6% at the amino acid level. There was also variability within groups, however not as high as between groups (Table 4.1).

The predicted G ORF amino acid alignments of unique South African strains with prototypes from Netherlands and Canada are shown in Appendix 2. Sequence variation due to nucleotide substitutions and insertions led to variable lengths in polypeptides ranging from 228 amino acid residues (subgroup A2) to 240 amino acid residues (subgroup B2). The hMPV G ORF's of subgroups A2 and B1 terminated using the TAA codon whereas the subgroup B2 isolates terminated by TAG codon. For both genetic groups (A and B) a conserved cysteine residue was present in the intracellular domain. A second cysteine residue was present in all but two group B viruses (RSA/71/00 and RSA/90/00) were observed.

Table 4.1 The human metapneumovirus (hMPV) F and G gene nucleotide and amino acid identities of the South African strains over three consecutive years (2000-2002)

		% nucleotide (amino acid) identities			
	Subgroups	A1	A2	B1	B2
Gene					
F	A1	99-100 (98.4-100)	93-95 (96.3-97.9)	83.8-84.1 (93.2-94.3)	82.7-84.5 (94.3-95.8)
	A2		99-100 (99.4-100)	83-83.8 (94.3)	83.1-85 (95.3-95.8)
	B1			98-100 (100)	93-95 (98.4)
	B2				96-100 (99.4)
G	A1	95.4-100 (87.5-100)	72.8-74.7 (55-63.6)	45.9-47.8 (24.3-26.2)	47.4-48.7 (22.4-26.7)
	A2		95-100 (88.6-98.1)	50.3-51.8 (25.4-27.7)	51-53.1 (23.6-27.6)
	B1			93.2 (87.9)	77.4-80.5 (58.2-62.8)
	B2				93.2-100 (82.8-100)

The predicted G ORF revealed a high serine and threonine content ranging from 30.7-34.9% for group A and from 30.6-36.6% for group B isolates. The proline content varied among the subgroups with subgroups A2 ranging from 7.6-9.0%, A1 ranging from 9.0-9.9%, B1 from 7.8-8.7% with B2 containing the lowest proline content ranging from 3.7-5.2%. There was only one conserved potential N-linked glycosylation site that was located at the junction of the intracellular and transmembrane domain.

The potential structure of the G protein was predicted using hydrophobicity plots using the procedure of Kyte and Doolittle (Kyte and Doolittle, 1982) was determined using Bioedit version 5.09 (Hall, 1999) for all subgroups A1, A2, B1 and B2 (Figure 4.3-4.6). Based on these plots the G protein has a transmembrane domain. Present in all subgroups was a hydrophilic N terminus followed by a short hydrophobic region of 20 amino acid and a C terminus that was predominantly hydrophilic.

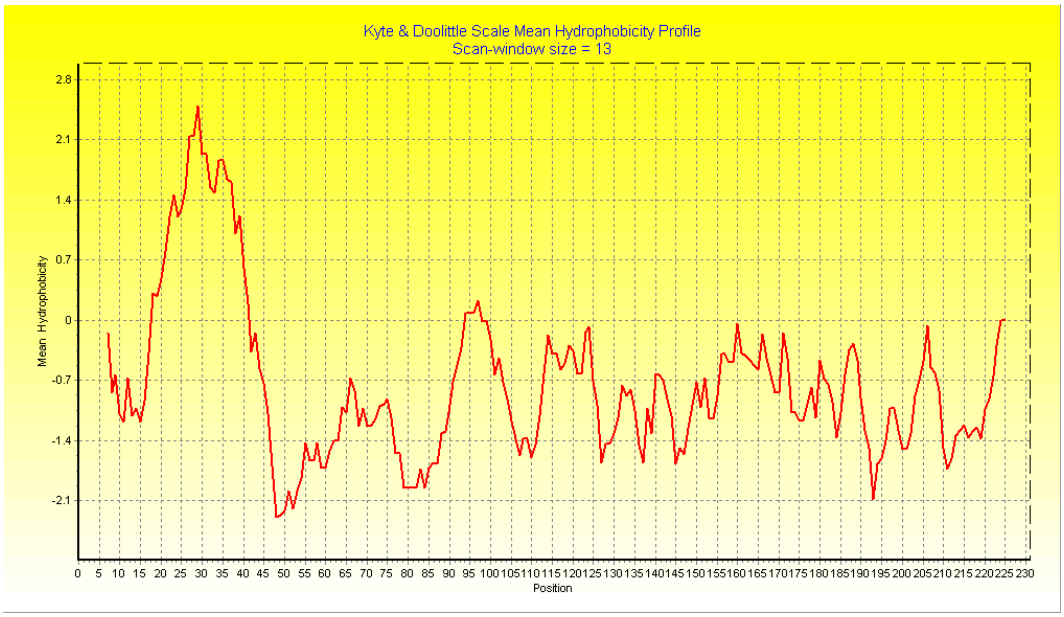

Figure 4.3 A Hydrophobicity plot from the hMPV subgroup A1 (RSA54/01) G protein. The hydrophobic region is above the zero value on the y axis and hydrophilic below the zero value. The scale on the x axis indicates the amino acid residues beginning with the N-terminal.

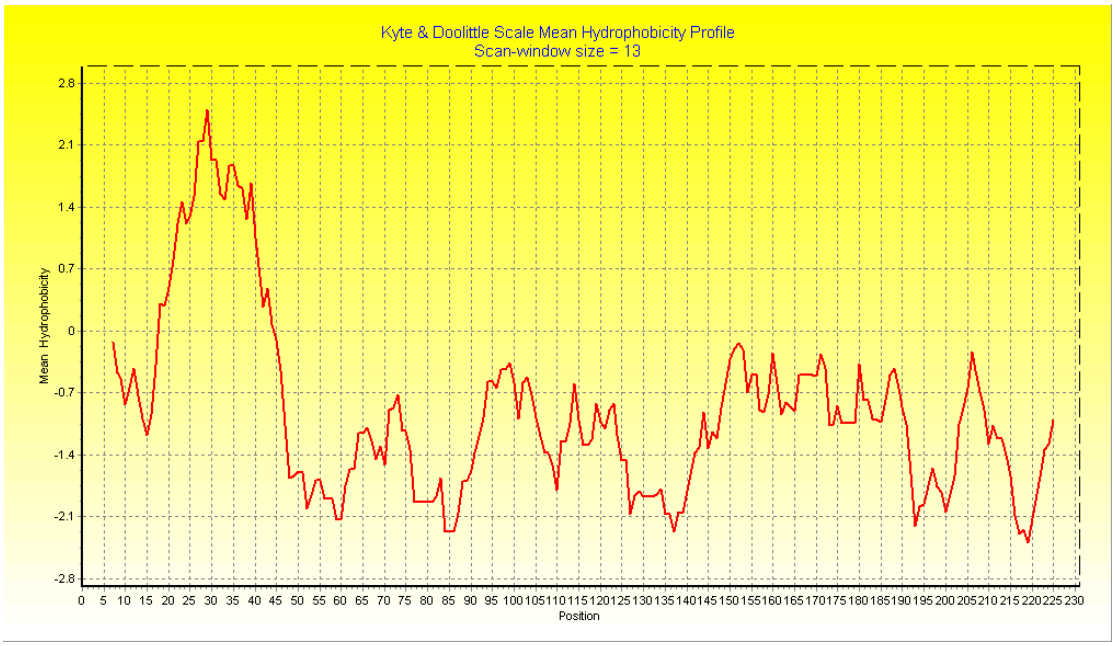

Figure 4.4 A Hydrophobicity plot from the hMPV subgroup A2 (RSA7/00) G protein. The hydrophobic region is above the zero value on the y axis and hydrophilic below the zero value. The scale on the x axis indicates the amino acid residues beginning with the N-terminal.

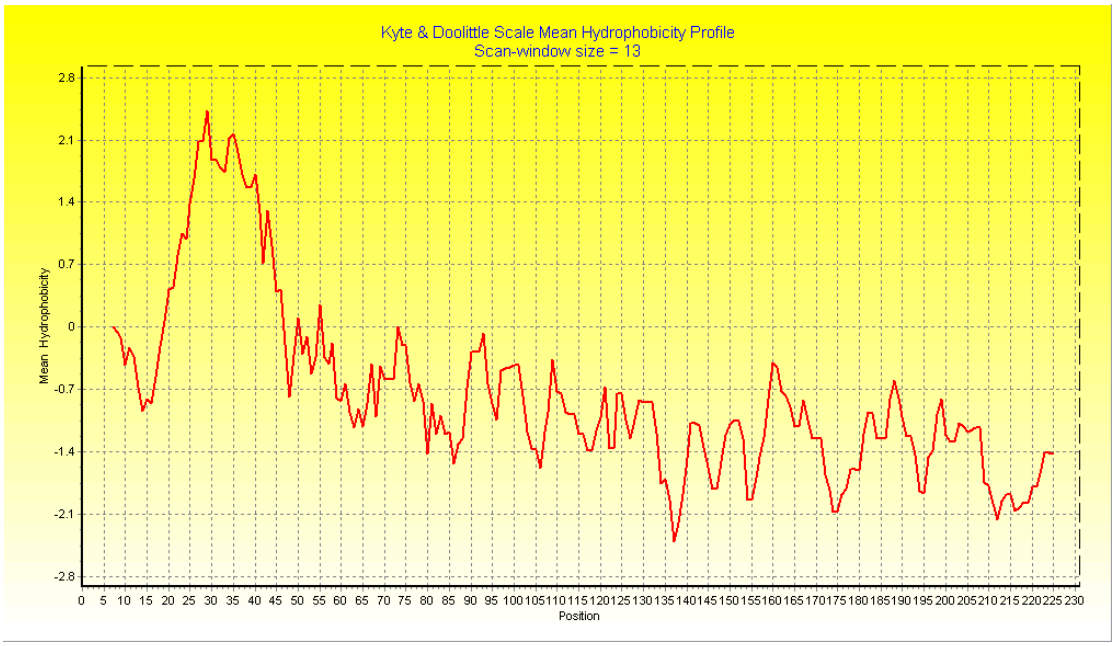

Figure 4.5 A Hydrophobicity plot from the hMPV subgroup B2 (RSA4/00) G protein. The hydrophobic region is above the zero value on the y axis and hydrophilic below the zero value. The scale on the x axis indicates the amino acid residues beginning with the N-terminal.

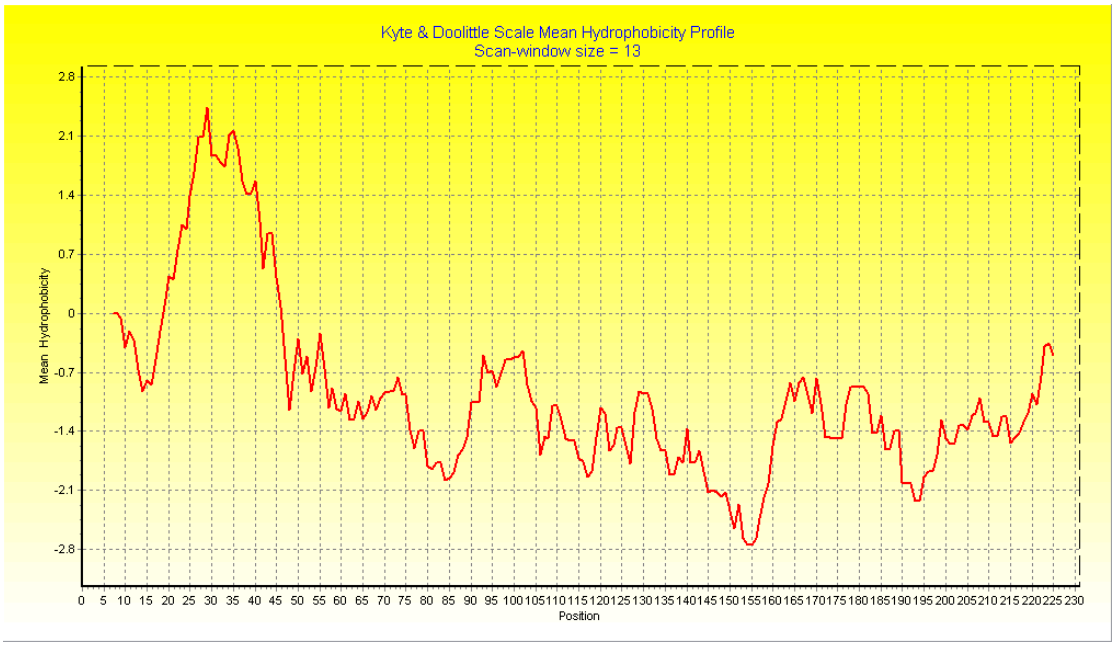

Figure 4.6 A Hydrophobicity plot from the hMPV subgroup B1 (RSA23/02) G protein. The hydrophobic region is above the zero value on the y axis and hydrophilic below the zero value. The scale on the x axis indicates the amino acid residues beginning with the N-terminal.B1 RSA23/02

4.4 Circulation pattern of genotypes over 3 years in a single South African community

All subgroups circulated over the study period, and at least two of the 4 subgroups co-circulated in each year, with one subgroup dominating (Table 4.2). Subgroup B2 and A2 co-circulated during the 2000 epidemic, with 72.5% of the circulating viruses belonging to subgroup B2. In 2001 three subgroups of hMPV co-circulated, A1, A2 and B2 with the majority of infections caused by the A1 (67.7%). Subgroup B2 viruses significantly declined (4 [11.8%] of 34) in 2001 compared to 2000 (29 [72.5%] of 40; $P<0.0001$). The number of infections in 2002 was down and subgroup A1 and B1 co-circulated, with subgroup A1 responsible for 83.3% of all infections. Subgroups A2 and B2 were not detected in 2002

4.5 Discussion

Genetic variability is a strong indicator of positive selection and affects the ability of a virus to continue circulating in a population. Such variability poses a challenge for the future development of vaccines against hMPV as molecular epidemiology studies, including in South Africa, indicate there to be a broad diversity of genetic groups in sequential epidemics.

The hMPV is a novel respiratory pathogen that resembles the RSV causing seasonal epidemics with symptoms that are indistinguishable from RSV. Molecular genetic studies have shown that like RSV, there are two distinct genetic groups of hMPV that cause the seasonal epidemic. These studies

have been limited to the developed world and no studies have investigated the molecular epidemiology of hMPV in countries that have high prevalence of paediatric HIV infection.

The present study reports on the largest community-based phylogenetic study of hMPV in Africa that examines both surface glycoproteins and provides evidence for the circulation of hMPV in a single African community over 3 consecutive years. Based on phylogenetic analysis and genetic distances two distinct genetic groups (A and B) and subgroups (1 and 2) were found. This is consistent with reports from elsewhere (Bastien et al., 2004; Bastien et al., 2003a; Boivin et al., 2002; Esper et al., 2004; Gerna et al., 2005; Mackay et al., 2004; Peret et al., 2004; Peret et al., 2002; van den Hoogen et al., 2004a). The clustering of the South African strains with strains from the Netherlands and Canada suggests a temporal distribution of hMPV variants. Furthermore, in addition to the 4 lineages (A1, A2, B1 and B2) multiple sub lineages (Huck et al., 2006) may exist for hMPV that needs to be characterized at the antigenic level and the clinical impact characterized.

The existence of these two major genetic groups (A and B) and the presence of multiple lineages observed here and reported by others (Bastien et al., 2004; Huck et al., 2006; Schildgen et al., 2004) impacted on the ability of hMPV to cause seasonal epidemics in South Africa.

Table 4.2 Distribution of human metapneumovirus (hMPV) genotype subgroups over three consecutive years (2000-2002) in a single community

		Subgroups			
Year	Total	A1	A2	B1	B2
2000	40	0	11(27.5)[1]	0	29(72.5)
2001	34	23(67.7)	7(20.5)	0	4(11.8)
2002	18	15(83.3)	0	3(16.7)	0
2000-2002	92	38	18	3	33

[1]Value in parenthesis is a percentage of individual subgroups of virus in relation to the rest of the viruses identified in a particular year.

The study shows the co-circulation of both groups A and B in the same epidemic in a single community consistent with other reports (Boivin et al., 2002; Esper et al., 2004; Gerna et al., 2005; Mackay et al., 2006; Mackay et al., 2004; Peret et al., 2004; Sarasini et al., 2006; Williams et al., 2006). Although all four sub-lineages were found to circulated in South Africa during the study they did not all co-circulate in each year as was observed in Australia (Mackay et al., 2006) and in Italy (Gerna et al., 2005). In South Africa three subgroups co-circulated in 2001 and two subgroups in other years. The present study showed that in 2000 subgroup B2 predominated (72.5% of circulating strains) but declined in 2001 (11.8% of circulating stains). In 2001 subgroup A1 emerged and replaced subgroup B2 as the predominant strain, predominating in 2001 and 2002. The emergence of B1 strains in 2002 and its absence in previous years may have been due to pre-existing community immunity and not to diagnostic assay limitations as was suggested by others (Mackay et al., 2004).

The predominance of subgroup A1 during 2001 was not restricted to South Africa as it was also detected as the predominating circulating virus in Australia and Italy during the same year (Gerna et al., 2005; Mackay et al., 2004) suggesting a global distribution in the same year.

The annual change in the circulation pattern and a switch in predominating strains in successive years reported here and by others (Gerna et al., 2005; Mackay et al., 2006; Mackay et al., 2004; Peret et al., 2004) provide evidence that pre-existing immunity may result in a change in dominant hMPV.

Similar findings in changes of the dominant group of virus that emerges, fostered by a high prevalence of pre-existing community immunity to the other viral group, has been documented for RSV (Cane et al., 1994; Coggins et al., 1998; Peret et al., 2000; Peret et al., 1998)

The significance of the higher number of group A strains (60.1%) compared to group B strains (39.1%) detected in the present study period is unclear, but may be due to differences in virulence between the 2 groups or to immunity in the community. Several studies have proposed that group A viruses may be more virulent than group B virus (Esper et al., 2004; Mackay et al., 2004; Vicente et al., 2006), however, this observation has not been noted by others (Agapov et al., 2006; Ebihara et al., 2004b). An association between viral genotype and clinical severity cannot be made from the present study as the patients used in the present study were all hospitalized with LRTI.

Genetic diversity may lead to antigenic variability and surface glycoproteins are believed to be the major neutralizing and protective antigens. To address the genetic variability of strains from South Africa strains the fusion gene and the entire attachment glycoprotein were sequenced. The F protein gene of the hMPV is a surface glycoprotein and believed to the major antigenic determinant that mediates extensive cross-lineage neutralization and protection (MacPhail et al., 2004; Skiadopoulos et al., 2006; Skiadopoulos et al., 2004). The low variability observed here for the F gene is in keeping with other studies that have shown it to be highly conserved (Bastien et al., 2003a; Biacchesi et al., 2003; Boivin et al., 2004; Peret et al., 2004; van den Hoogen

et al., 2004a). Further, the structural features based on the partial sequence of the hMPV F protein were similar to those reported by others within this region (between amino acids 44-236). The two potential N-linked glycosylation sites as well as the conserved cysteine residues at amino acid positions 60 and 182 were also present in strains from other geographical regions (Boivin et al., 2004)

The presence of specific amino acids in the F gene that may distinguish the hMPV between groups appears to be universal as these specific amino acids have also been observed in isolates from other geographical areas (Boivin et al., 2004). In addition to the group and subgroup specific amino acids observed in the partial sequence of the F gene in the present study other amino acids in other regions of the F protein have also been identified as group differentiation markers (van den Hoogen et al., 2004a).

In contrast to the F gene protein, a high degree of variation was observed for the G gene in this study, confirming reports by other (Biacchesi et al., 2003; Peret et al., 2004; van den Hoogen et al., 2004a). Most of the variation was observed in the extracellular domain and was due to nucleotide substitutions, in frame insertions and the use of alternative termination transcription codons (TAA or TAG) producing polypeptides of variable lengths. The high level of variation observed in the present study for the hMPV G gene from clinical samples also confirms that the variation, as observed by another study (Peret et al., 2004), was not due the passage of hMPV in cell culture. This high level

of variation particularly at the amino acid level for the G gene protein may be due to positive selection, which may be the result of immunological pressure.

The structural features of the hMPV G protein such as the high serine-threonine content, high proline content and a variable number of possible N-linked glycosylation sites together with hydrophilic amino and carboxy termini as the observed here for both groups (A and B) are consistent with previous studies (Bastien et al., 2004; Peret et al., 2004; van den Hoogen et al., 2004a) that suggest the G attachment protein to be an anchored type II mucin like transmembrane protein.

In summary, this is the first study to describe the molecular epidemiology of the hMPV over three consecutive seasons in Africa. The study shows that four genetic sub lineages of hMPV circulate in Africa with a switch in predominating group in successive seasons. The high genetic variability in the G gene causing amino acid changes suggests strong selective pressure.

CHAPTER 5

PNEUMOCOCCAL CO-INFECTION WITH HUMAN METAPNEUMOVIRUS

5.1 Study Aim

This aspect of the study aimed at defining the minimal role of pneumococcal co-infection in the pathogenesis of hMPV-associated LRTI, by using PCV-9 as a probe to determine the role of *S. pneumoniae* in hMPV associated LRTI.

Of the 202 samples that were positive for hMPV, 149 were from children that had been fully vaccinated per protocol; i.e. the child received all three study doses of vaccine within protocol defined periods. 195 children were included in the intent-to-treat (ITT) analysis; i.e. following receipt of the first dose of study vaccine. The HIV status was available for 199 (98.5%) of the 202 children in whom hMPV was identified.

5.2 The effect of pneumococcal conjugate vaccine on the incidence of hospitalization for hMPV-associated pneumonia

In fully vaccinated children, the incidence of hospitalization for at least one episode of hMPV-associated LRTI was reduced by 46% (P = 0.0002) overall, 45% (P=0.002) in HIV uninfected children and by 53% (P=0.035) in HIV infected children (Table 5.1). The intent-to-treat (ITT) estimates (Table 5.2) of vaccine efficacy (VE) for most of the outcomes were not significantly different to the estimates in the per-protocol (PP) analysis.

There were no differences observed in the vaccine efficacy intent-to-treat analysis for hMPV LRTI across the various age-groups overall (P=0.58), in HIV uninfected children (P=0.51) or in HIV-infected children (P=0.98). There was a trend to a lesser effect of the vaccine in reducing the incidence of hMPV-associated LRTI in vaccine recipients <6.0 months of age, compared with that in older children (Table 5.3).

Overall there was a significant reduction in clinical pneumonia among vaccinees (58%; P=0.0001), in HIV uninfected children (55%; P=0.003) and in HIV infected children (65%; P=0.02). In addition, using the WHO criteria for severe/very severe pneumonia as an outcome, a 44% (P=0.003) reduction was observed overall, 40% (P=0.02) in HIV uninfected children and 53% (P=0.04) in HIV infected children (Table 5.1).

5.2.1 The impact of PCV on the incidence of hMPV associated "bacterial pneumonia"

The impact of PCV on the incidence of hMPV-associated "bacterial pneumonia" was assessed using outcomes that are more specific for "bacterial pneumonia"; viz radiologically confirmed pneumonia (CXR-AC) and LRTI associated with an elevated CRP of ≥40mg/l.

5.2.1.1 Chest radiograph confirmed pneumonia

Chest radiographs were available for 176 (90.3%) of the 195 children with hMPV LRTI, 134 (89.9%) of 149 HIV uninfected children and 39 (90.7%) of 43 HIV infected. Overall a 56.0% (P=0.02) reduction in hMPV pneumonia

Table 5.1: Percentage efficacy of pneumococcal conjugate vaccine by per protocol analysis in the prevention of human metapneumovirus associated respiratory tract infections.

hMPV associated measured	Overall				HIV uninfected				HIV infected			
	Vaccine	Placebo	Efficacy 95%C.I.	P value	Vaccine	Placebo	Efficacy 95%C.I.	P value	Vaccine	Placebo	Efficacy 95%C.I.	P value
LRTI[1]	52	97	46 25; 62	0.0002	41	74	45 19; 62	0.002	11	23	53 3; 77	0.035
Clinical pneumonia	26	62	58 34; 73	0.0001	19	42	55 22; 74	0.003	7	20	65 19; 85	0.020
CXR-AC[2]	11 [47][5]	25 [88]	56 11; 78	0.02	9 [36]	15 [68]	40 -37; 74	0.31	2 [11]	10 [20]	80 10; 96	0.04
Bronchiolitis	26	35	26 -23; 55	0.25	22	32	31 -18; 60	0.17	4	3	-24 -83;238	0.99
WHO severe pneumonia[3]	41	73	44 18; 62	0.003	31	52	40 7; 62	0.02	10	21	53 0; 78	0.04
CRP ≥40mg/l[4]	9 [31]	26 [59]	65 26; 84	0.007	7 [23]	18 [46]	61 7; 84	0.05	2 [8]	8 [13]	75 -16; 95	0.11

[1]LRTI=lower respiratory tract infection. [2]hMPV LRTI associated with alveolar consolidation on chest radiograph (CXR-AC). [3]World Health Organization clinically diagnosed LRTI. [4]hMPV LRTI with C-reactive protein (CRP) ≥40mg/l. [5]Value in squared parenthesis is total number of the LRTI episodes for which the test was performed.

associated with CXR-AC was observed (Table 5.1). There was also a significant reduction of 80% (P=0.04) in HIV infected children with the CXR-AC outcome. A significant reduction was not observed (40%, P=0.31) for HIV ninfected children for radiologically confirmed pneumonia, but the power of the study to detect a significant difference of this magnitude was only 17%.

5.2.1.2. LRTI associated with elevated CRP

Overall measurements for CRP levels were available for 116 (59.5%) of the 195 hMPV infected, 88 (59.1%) of the 149 HIV-1 uninfected children and 27 (62.8%) of the 43 HIV-1 infected children. There was a 65% (P=0.007) reduction in the incidence of hMPV pneumonia with a CRP level of ≥ 40 mg/l in fully vaccinated recipients (Table 5.1). A significant reduction was only observed for the HIV uninfected (61%, P=0.05). There was no difference in the proportion of children tested for CRP between PCV and placebo recipients (Table 5.1 and 5.2).

5.2.2 Effect of PCV on bronchiolitis

A non-significant reduction in the incidence of hospitalization for hMPV-associated bronchiolitis among vaccinees recipients in the entire study population (VE, 25%, P=0.21) and HIV uninfected children (VE, 25%, P=0.23) was observed (Table 5.2).

5.2.3 Coinfection with other pathogens

Streptococcus pneumoniae was isolated from 4 (2.1%) of the 189 episodes of hMPV-associated LRTI for which bacterial blood cultures were performed on children. All of the episodes of *S. pneumoniae* bacteremia in children with LRTI occurred in HIV infected children, including 1 (6.3%) of 16 in PCV-9 recipients and 3 (11.1%) of 27 in placebo recipients.

Overall, among children investigated for all episodes (first and subsequent) of hMPV-associated LRTI, the prevalence of co-infection with other respiratory viruses was 4.1-fold (95%, CI, 1.1-18.8) greater in PCV-9 recipients (9 [11.8%] of 76) than placebo recipients (4 [3.2%] of 126) (P=0.02). Similarly, HIV uninfected vaccine recipients with hMPV associated LRTI were 3.5 fold (95% CI, 0.9-16.4) more likely to be co-infected with other respiratory viruses (8[13.3%] of 60) than were placebo recipients (4 [4.3%] of 94) (P=0.06).
In addition there was one HIV infected PCV-9 recipient with hMPV-associated LRTI in whom a viral co-infection was identified.

Table 5.2: Percentage efficacy of pneumococcal conjugate vaccine by intent-to-treat analysis in the prevention of human metapneumovirus associated respiratory tract infections.

HMPV associated outcome	Overall				HIV uninfected				HIV infected			
	Vaccine	Placebo	Efficacy 95%C.I.	P value	Vaccine	Placebo	Efficacy 95%C.I.	P value	Vaccine	Placebo	Efficacy 95%C.I.	P value
LRTI[1]	72	123	42 22; 56	0.0002	57	92	38 14; 56	0.004	15	28	47 1; 72	0.04
Clinical pneumonia	38	78	51 28; 67	0.0002	27	52	48 7; 67	0.005	11	25	56 12; 78	0.02
CXR-AC[2]	14 [63][5]	33 [113]	58 21; 77	0.005	12 [49]	21 [85]	43 -16; 72	0.12	2 [14]	12 [25]	84 26, 96	0.015
Bronchiolitis	34	45	25 -18; 52	0.21	30	40	25 -20; 53	0.23	4	3	-24 -83;237	0.99
WHO severe pneumonia[3]	56	93	39 16; 57	0.002	42	65	35 5; 56	0.03	14	25	45 -6; 71	0.07
CRP ≥40mg/l[4]	14 [42]	32 [74]	56 18; 77	0.008	10 [32]	21 [56]	52 -1; 77	0.05	4 [10]	10 [17]	60 -26; 88	0.17

[1]LRTI=lower respiratory tract infection. [2]hMPV LRTI associated with alveolar consolidation on chest radiograph (CXR-AC). [3]World Health Organization clinically diagnosed LRTI. [4]hMPV LRTI with C-reactive protein (CRP) ≥40mg/l. [5]Value squared parenthesis is total number of the LRTI episodes for which the test was performed.

Table 5.3: Percentage efficacy of pneumococcal conjugate vaccine by intent-to-treat analysis in the prevention of human metapneumovirus associated lower respiratory tract infections by age-groups at time of hospitalization.

Age-group	Overall				HIV uninfected children				HIV infected children			
	Vaccine	Placebo	Efficacy 95%C.I.	P value	Vaccine	Placebo	Efficacy 95%C.I.	P value	Vaccine	Placebo	Efficacy 95%C.I.	P value
<6.0 mo.	19	22	14 -59; 53	0.63	17	18	6 -83; 51	0.87	2	3	34 -295;89	0.65
6.1-12.0 mo.	17	35	52 14; 73	0.012	13	27	52 7; 75	0.027	4	7	43 -93; 83	0.36
12.1-24.0 mo.	23	38	40 -1; 64	0.053	20	30	33 -17; 62	0.16	3	7	58 -64; 89	0.20
>24.0 mo.	17	31	45 1; 70	0.04	10	19	47 -13; 76	0.09	7	12	42 -46; 77	0.24

5.3 Discussion

Animal-model and in vitro studies have shown that respiratory viral infections increase the susceptibility to bacterial co-infections (Beadling and Slifka, 2004; Hament et al., 1999). Defining the role of bacterial coinfection in humans is hindered by the absence of sensitive tools to diagnose bacterial pneumonia. Experimental tools aimed at improving the sensitivity of diagnosis of bacterial pneumonia indicate that approximately one-third of children with RSV-associated pneumonia may have pneumococcal co-infections (Juven et al., 2000; Michelow et al., 2004). Validating the sensitivity and specificity these experimental assays is problematic in the absence of a reference standard against which they can be evaluated.

Recently, Madhi and Klugman showed that 3 doses of PCV could reduce the incidence of hospitalization for respiratory viral associated pneumonia by 31% (95% CI=15-43%; P=0.00009) (Madhi and Klugman, 2004) suggesting that prevention of pneumococcal pneumonia by vaccination reduced the severity of viral infections and likelihood thereof progressing to severe pneumonia requiring hospitalization. By inference, superimposed bacterial infection, including *S. pnemoniae,* appear to be important in the pathogenesis of respiratory viral associated pneumonia.

The current study suggests that bacterial co-infection, particularly *S. pneumoniae*, is an integral part in the pathogenesis of hMPV infections progressing to pneumonia. The estimated 58% overall reduction in clinical pneumonia in vaccinees provides only a conservative estimate of the

prevalence of pneumococcal co-infections in children with hMPV associated pneumonia. The true prevalence of pneumococcal co-infections in children with hMPV associated pneumonia may be even higher than that inferred here. Factors that may have resulted in under-estimating the importance of pneumococcal co-infections in children with hMPV associated pneumonia include: (i) the PCV used in our study only includes 9 of 90 different pneumococcal serotypes, albeit those most commonly responsible for invasive disease; (ii) PCV efficacy against non-bacteremic pneumococcal pneumonia may be less than that observed against invasive disease (83-98%) (Madhi and Klugman, 2004; Madhi et al., 2005b) and (iii) there may be an excess of non-bacteremic pneumonias due to non-vaccine pneumococcal serotypes in the vaccinees.

The role of CRP in discriminating between viral and bacterial infections is controversial (Simon et al., 2004) probably due to the lack of sensitive tools for identification of bacterial infections, Madhi et al. reported that CRP is useful and improves the specificity of chest radiographs in diagnosing penumococcal pneumonia (Madhi et al., 2005a). The present study shows that PCV reduced the incidence of hMPV pneumonia when CRP levels were ≥ 40 mg/l, suggesting that the findings by Dollner et al most likely reflected undiagnosed bacterial coinfection in those children (Dollner et al., 2004). In contrast, CRP levels have been found to be lower in children with hMPV-associated bronchiolitis (Dollner et al., 2004; Jartti et al., 2004), providing indirect evidence that bacterial coinfections may be less important in children with bronchiolitis.

In summary, the findings of this study suggest that children hospitalized for hMPV-associated pneumonia should be treated with antibiotics to cover for superimposed pneumococcal infections.

CHAPTER 6

REINFECTION WITH HOMOLOGOUS AND HETEROLOGOUS STRAIN OF HMPV

6.1 Repeat hospitalization for hMPV associated illness

Eight children had repeated episodes of hospitalization for hMPV associated illness which was spaced 30 and 307 days apart. Two of the 8 children with repeated episodes of hMPV hospitalization were HIV infected children and six were HIV uninfected. Fifteen (88.2%) of the 17 episodes of hospitalization for hMPV associated illness in these eight children presented as LRTI. The ages of the children at first diagnosis ranged from 5.8 months to 26.5 months, with the second illness occurring between 30-307 days apart.

Adequate samples were only available for further hMPV genotyping analyses in 10 of these 17 episodes and genotypes for recurrent episodes were only available for 4 children (1 HIV infected and three HIV uninfected).

6.2 Phylogenetic analysis

Phylogenetic analysis performed on partial F gene sequences for the 10 strains are shown in Figure 6.1 and genotypes in Table 6.1. Nine of the 10 strains clustered with subgroup B2 and the other with subgroup A1. Identities between the subgroup B2 viruses ranged from 98.9%-100%. Four of the 8 patients had genotypes for both initial and subsequent infections.

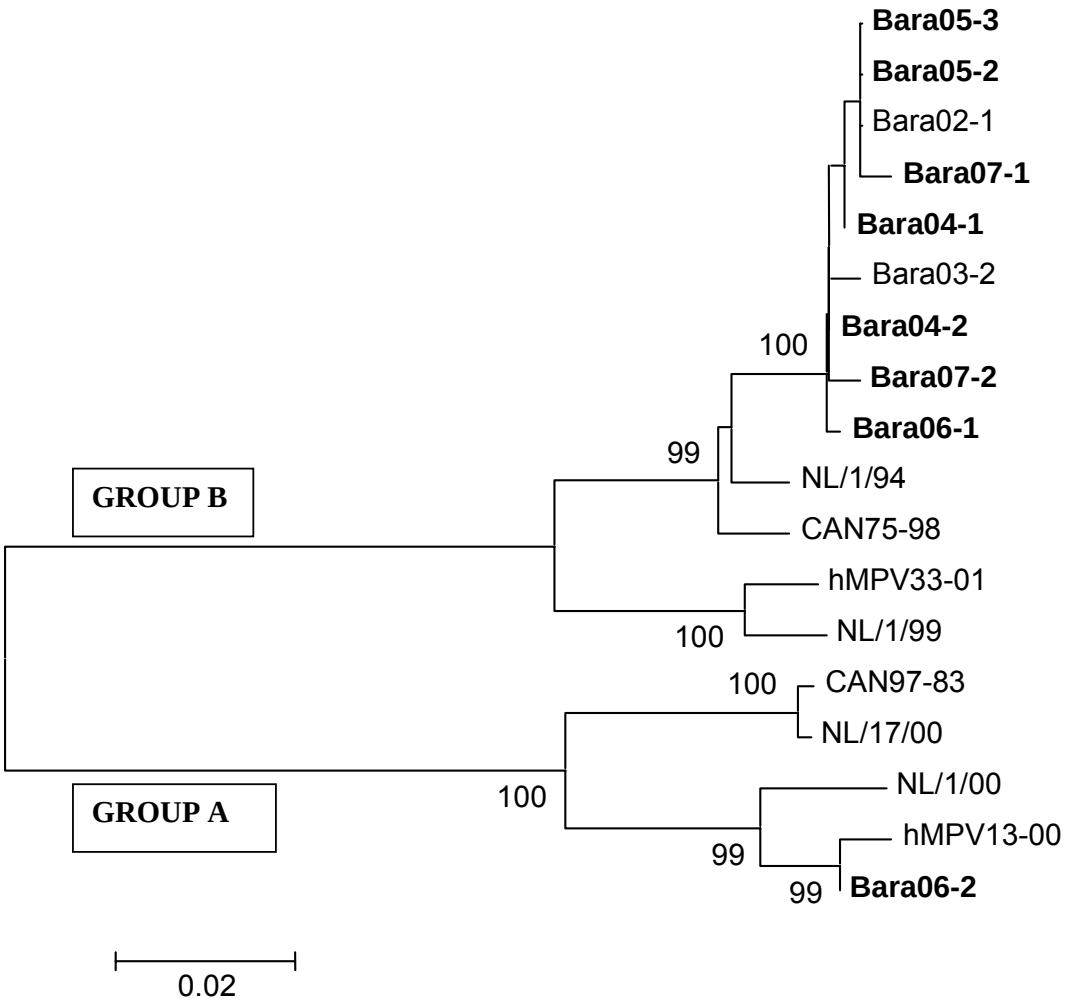

Figure 6.1. Neighbour-joining trees based on nucleotide sequences from the partial F gene 10 South African hMPV isolates. The trees were computed with MEGA version 2.1 using the Nucleotide: Kumira 2-parameters (Kumar et al., 2001a). Bootstrap probabilities for 500 replicas are shown at the branch nodes. Only values of 70-100% are indicated. Isolates used in the analysis for repeated infections are indicated by Bara (e.g. Bara01-1 refers to subject 1 in Table 6.1 and the virus from the 1st illness, with Bara01-2 indicating the virus from the 2nd illness from the same subject, etc). The viruses from Canada (CAN97-83, hMPV13-00, CAN75-98 and hMPV33-01) and the Netherlands (NL/1/00, NL/17/00, NL/1/99 and NL/1/94) are prototypes for each subgroup.

Table 6.1: Repeat episodes of human metapneumovirus (hMPV) associated hospitalizations in children

Number	Age at 1st episode (months)	Days between episodes	Sex	GA[1]	HIV[2] Infection status	PCV [3] vaccination status	Virus Subgroup 1st/ 2nd/3rd episode	First Illness Diagnosis	Second Illness diagnosis	Underlying or concurrent illness
1	5.8	30	M	40	Negative	Placebo	NA/NA[4]	Bronchiolitis	Bronchiolitis	None
2	26.5	68	M	35	Infected	Placebo	B2/NA	Bronchopneumonia	Lobar pneumonia	Ex-prem
3	5.1	81	F	40	Negative	PCV	NA/B2	Bronchiolitis and gastroenteritis	Bronchiolitis	None
4	10.9	92	M	40	Negative	Placebo	B2/B2	Lobar pneumonia	Bronchiolitis	None
5[5]	14.0	121 and 163	F	40	Infected	PCV	NA/B2/B2	Bronchopneumonia and gastroenteritis	Bronchopneumonia and gastroenteritis	Pulmonary tuberculosis
6	17.4	244	F	40	Negative	PCV	B2/A1	Bronchiolitis	Bronchopneumonia	Concurrent RSV during 1st illness
7	12.8	304	F	40	Negative	PCV	B2/B2	Lobar pneumonia	Bronchiolitis	None
8	7.4	307	M	34	Negative	PCV	NA/NA	Bronchiolitis	Asthma	Ex-prem and concurrent RSV during 1st illness

Table 6.1 footnote: [1]GA=gestational age at birth. [2]HIV: Human immunodeficiency virus type-1. [3]PCV=pneumococcal conjugate vaccine. [4]NA=sample not available for genotyping. [5]Subject had three episodes of which sample were only available for the 2nd and 3rd for genotyping. Intervals are between the 1st and 2nd and 2nd and 3rd episodes of hospitalization when hMPV was identified. Diagnosis at time of 3rd episode was lymphocytic interstitial pneumonitis and pulmonary tuberculosis.

Patient 4 a male at age 10.9 months had lobar pneumonia caused by a subgroup B2 virus and 92 days the second episode of hMPV associated illness (diagnosis bronchiolitis) was also due to a virus from subgroup B2. Patient 5 had three episodes of hospitalization for hMPV associated illnesses. The first episode occurred at age 14 months with the second and third 121 and 163 from first episode respectively. The hMPV strain causing the first episode could be genotyped and genotypes were only available for second and third episodes. The virus recovered from these 2 episodes clustered with subgroup B2. In addition this patient was HIV seropositive diagnosed with bronchopneumonia, gastroenteritis and was concurrently diagnosed with pulmonary tuberculosis. Patient 6, a female first episode was at age 17.4 months caused by B2 virus was diagnosed with bronchiolitis and bronchopneumonia 244 days was the second episode caused by an A1 virus. This patient was also co-infected with RSV during the first illness.

Patient 7 also a female was infected 304 days apart on both occasions with a B2 virus.

6.5 Discussion

Animal studies have shown that cross protective immunity between major lineages was possible (MacPhail et al., 2004; Skiadopoulos et al., 2004; van den Hoogen et al., 2004a). The mechanism by which re-infection occurs is not know but may be due to waning of immunity or the initial lack of developing immunity following the primary infection. Alternatively there may be prolonged shredding of the hMPV.

The present study shows that reinfection can occur within 3 months with either homologous or heterologous strains in both HIV uninfected and HIV infected children. The finding that at least two HIV uninfected children were hospitalized for recurrent episodes of LRTI associated with subgroup B2 virus with only minor differences in the percent sequence identity of the F protein indicate that immunity following a primary infection may be incomplete. This may be a result of a poor primary immune response to the viral infection, or of waning immunity as these episodes occurred between 92 and 304 days apart. Another possibility is prolonged shedding in the subject in whom the episodes occurred 92 days apart - for which there was 99.8% identity in the nucleotide sequence of studied F protein of the virus identified in each of the episodes. The subject with repeat hMPV episodes that occurred 304 days apart, for which there was 98.9% identity of the F protein nucleotide sequence studied, had two additional episodes of hospitalization for respiratory tract infections between these episodes for which nasopharyngeal samples tested negative for hMPV, making repeat infections rather than prolonged shedding more likely in this subject. The repeat episodes of hMPV-associated hospitalization in an HIV infected child with 3 episodes (subject 5 in Table 6.1), especially documentation of subgroup B2 virus on the second and third episodes for which there was 100% homology in the nucleotide sequence of the F protein indicates that identification of hMPV in this child may have been due to prolonged shedding of the virus for at least 163 days. Falsey et al. have in contrast reported that hMPV was not detected more than 14 days after onset of illness in otherwise healthy individuals (Falsey et al., 2006).

Re-infection with heterologous strains of hMPV has been reported in an immunocompromized child (Pelletier et al., 2002) and recently in a 9 month old child who had repeat infections one month apart (Ebihara et al., 2004a). Williams et al. reported heterologous hMPV re-infections in a 20 year follow-up study of children with upper respiratory tract infections, as well as two children with homologous re-infection due to subgroup B1 and A2 hMPV (Williams et al., 2006). Williams et al. also suggested that primary immunity induced following hMPV infection of the LRTI reduced subsequent infections in lower respiratory tract and limited the replication of hMPV to the upper respiratory tract. In contrast the present study which is limited to children with LRTI shows that subsequent infections may also occur in the lower respiratory tract. Although the immune response to hMPV infections is unclear for humans, animal studies using BALB/c mouse model indicate that the cytotoxic T lymphocyte and antibody response to hMPV infection is delayed (Alvarez and Tripp, 2005).

In conclusion, the data suggest that similar to RSV (Scott et al., 2006) re-infection can occur with homologous and heterologous strains of hMPV and that prior infection with hMPV is not adequate to protect against subsequent infections in some children.

CHAPTER 7

STUDY LIMITATIONS

The limitations of this study may be due either to molecular or clinical aspects and will be discussed below.

7.1 Molecular limitations

The prevalence of the hMPV-associated LRTI may be underestimated in this study probably due the collections, transport as well as the method for identification of hMPV used in this study. RNA is unstable and it is possible that the transport and storage of NPAs could have compromised the RNA yields and detection of true positives despite the use of a sensitive nested RT-PCR approach. Using a nested PCR approach you also run the risk of detecting false positives due to contamination. To reduce the risk of contamination, RNA isolation, primary RT-PCR and nested PCR were performed in separate laboratories. Further to control for false positives and to examine the specificity of the primers, hMPV positives were sequenced.

Although a significant number (40% of all hMPV positives) of hMPV were sequenced it still possible that the distribution of the genotypes may be biased thus affecting the conclusions on the circulation pattern over the course of the study. Another limitation may be the design of the primers, however, it is unlikely that this could have any effect on the study as similar results were obtained from sequencing and phylogenetic analysis of the both surface

glycoproteins, i.e. for example, samples that were genotyped as belonging to A1 for the F gene were confirmed as A1 for the G gene.

The present study also describes the F gene to be highly conserved based on the identities from the partial sequences of this gene. Although the entire F gene was not sequenced a study by van den Hoogen et al. showed that similar identities could be obtained from partial and full length sequencing of the F gene (van den Hoogen et al., 2004a).

7.2 Clinical limitations

Although samples were available for hMPV testing in the majority of HIV infected and HIV uninfected children, the differences observed between LRTI episodes for which samples were and were not available may have some implications regarding the study conclusions. These include that younger children with hMPV may have been missed and this may have underestimated the incidence of hMPV-LRTI in our population since hMPV illness is more common during infancy (Williams et al., 2004). The reasons for the unavailability of samples in this study may be due to: i. smaller volumes of saline being used and/or retrieved when that aspirates were performed in very young children; ii. most of the retrieved sample may have been used for identifying specific viruses once the pooled respiratory virus screening test indicated the presence of one of the viruses which were being studied prospectively; and this more commonly occurred in younger children.

The prevalence of co-infections with other respiratory viruses may have been under-reported in this thesis and may be due to the different methods used for detection of other viruses. Co-infection may be particularly important regarding the duration of hospitalization and outcome of subjects since, although controversial,(Lazar et al., 2004) dual infections with RSV have been found to result in more severe disease (Greensill et al., 2003; Konig et al., 2004). The lack of any difference in the duration of hospitalization or case fatality rate of those LRTI episodes for which samples were and were unavailable among HIV infected and HIV-uninfected children in this study however makes this unlikely. Nevertheless, the differences observed in this study between tested and untested episodes of LRTI highlights the problem associated with drawing conclusions and making comparisons with the many other retrospectively conducted studies which may also have been biased in their findings based on the samples available for hMPV testing.

Limitations of the study include differences in the ages of subjects and in the prevalence of other respiratory viruses in those LRTI episodes for which nasopharyngeal samples were unavailable for hMPV testing. It is, therefore possible that the vaccine efficacy in younger children may be lower than that observed in the overall population, as suggested by the age group analysis in Table 5.3. Possible explanations for this lesser effect in young children may be due to the following: 1) hMPV is an independent cause of severe LRTI in very young infants; 2) PCV is less able to protect against pneumococcal pneumonia in very young infants, since some may not have completed their full series of primary vaccination; and 3) coinfections with other viruses,

especially RSV which commonly infects infants <6.0 months of age may also result in severe LRTI (Cuevas et al., 2003; Greensill et al., 2003). That the latter may occur independently of pneumococcal coinfection is indirectly supported by in this study, in children hospitalized with hMPV-associated LRTI, the prevalence of coinfection with other respiratory viruses was 4.1 greater among PCV recipients than placebo, suggesting that viral coinfection may have been involved in the pathogenesis of severe hMPV-LRTI among the PCV recipients. Since some specimens from younger children were unavailable for hMPV testing, the bias introduced by the exclusion of those samples may have led to a conservative estimate of the association between hMPV and other viral pneumonias.

CHAPTER 8

CONCLUDING REMARKS

Human metapneumvirus was initially isolated from nasopharyngeal aspirates collected from 28 children in the Netherlands with respiratory tract infections (van den Hoogen et al., 2001). The virus was illusive prior to 2001 due to its specific cell culture requirements, but has been circulation in humans for at least 50 years (van den Hoogen et al., 2001).

Although not an emerging pathogen in the true sense, the hMPV is still new and the epidemiology is still being investigated. Following, its initial isolation the hMPV has been shown to circulate worldwide and isolated as the major cause of upper and lower respiratory tract infections. Clinically it is indistinguishable from RSV, and is second to RSV as the cause of RTI. Its seasonality overlaps with RSV and appears to cause infections in children that are older than RSV.

The common respiratory viruses such as RSV, influenza A/B, parainfluenza types 1-3 have been shown to have a major impact on the morbidity and mortality of HIV in South Africa (Madhi et al., 2001). The present thesis describes the molecular epidemiology of the hMPV in South Africa and examines the impact it has on the burden of disease in HIV infected and uninfected children.

Using PCR as a diagnostic tool, the hMPV RNA was detected overall in 7.4% of children hospitalized with LRTI's, of which 9.1% where HIV uninfected and 4.5% HIV infected. The mean age of children with hMPV-LRTI was 13.3 months and hMPV-LRTI occur at an early age in HIV uninfected (12.3 months) than HIV infected children (17.6 months; P=0.007). The overall measured incidence rate of hMPV-LRTI was higher for HIV infected children than HIV uninfected despite hMPV being a less common cause of LRTI in HIV infected children compared to HIV uninfected children, a finding in keeping with studies of other respiratory viruses in South Africa (Madhi et al., 2000). Similarly, hMPV disease was shown be more severe in HIV infected children than HIV uninfected children. There was higher mortality rate (4.4 vs 0%; P=0.05) and longer duration of hospitalization (5.8 vs. 4.1 days, P=0.003) for HIV infected children compared to HIV uninfected. HIV infected children were more likely to have concurrent bacteremia (16.3% vs. 0%, P<0.0001), had higher median CRP levels (P=0.01) therefore more likely to be co-infected with bacteria and were more likely to present with pneumonia than bronchiolitis (P=0.0001). In addition, *Pneumocystis jiroveci* pneumonia (PCP) was diagnosed in two (25%) of eight HIV infected children with hMPV-LRTI in whom an immunofluorescence assay was performed to identify *Pneumocystis jiroveci* cysts.

hMPV was as common as RSV in being identified in HIV infected children with pneumonia and was second to RSV as the most frequent virus identified in HIV uninfected children. Similar to RSV, it was shown capable of causing repeated infections as described in Chapter 6. This chapter shows that prior

infection with hMPV is not sufficient to prevent from subsequent infections, even within the same season. The study reports that the hMPV is capable causing repeated infections in a space of 3 months by either homologous or heterologous strains.

Pneumococcal co-infections were also shown to be an essential part of the pathogenesis of most severe hMPV infections progressing to pneumonia. Using PCV as a probe to define the role of pneumococcocal coinfections as described in Chapter 5. The study observed a 58% reduction (58%) in clinical pneumonia among hMPV infected children that were given PCV compared to hMPV infected children that were given the placebo (P-0.0001). PCV also had an impact on hMPV associated bacterial pneumonia as overall a 56.0% (P=0.02) reduction in hMPV pneumonia associated with CXR-AC was observed and a 65% (P=0.007) reduction in the incidence of hMPV pneumonia with a CRP level of ≥ 40 mg/l in fully vaccinated recipients was also observed. The present study also demonstrates that coinfection of hMPV with pneumococci in children >6 months of age progressed to pneumonia that necessitates hospitalization. An implication of this observation is that children hospitalized for hMPV-associated pneumonia should be treated with antibiotics.

In South Africa, hMPV had a perennial circulation, peaking during the autumn-winter months after the RSV peak season and prior to the influenza and parainfluenza peak season. The molecular epidemiology as examined in Chapter 4 provides evidence for the ability of the hMPV to survive and cause

annual epidemics. The study shows that all subgroups circulate in South Africa and that annual epidemics were caused by the switching of predominating hMPV strains in subsequent years (B2 in 2000 to A2 in 2001). Further sequence of the two surface glycoproteins demonstrated that the fusion gene protein (F gene) was conserved whereas the attachment glycoprotein (G) was highly variable particularly in the extracellular domain suggestive of positive selection probably due to immunological pressure. Therefore, the F gene may be a target for a vaccine as it is conserved and has been shown to be the major neutralization antigen that confers substantial neutralization and protection across lineages (Skiadopoulos et al., 2006).

In conclusion the hMPV is also a major cause of LRTI among children in South Africa and can be added to the vast list of pathogens that can cause pneumonia thus impacting on the HIV epidemic in sub-Saharan Africa.

APPENDICES

APPENDIX 1: AMINO ACID ALIGNMENT OF FUSION PROTEIN

Alignment of the F proteins of representative samples of SA hMPV isolates and prototype sequences from the Netherlands (NL/1/00, NL/1/99, NL/1/94 and NL/17/00) and Canada (hMPV13-00, CAN97-83, hMPV33-01 and CAN75-98). Only amino acid residues that differed from the Netherlands prototypes for each subgroup are shown and identical amino acids are represented by periods and dashes indicate gaps. The proposed fusion domain is indicated in bold. Potential glycosylation sites are underlined. Numbers indicate the amino acid position in the F open reading frames corresponding to the Netherlands prototype isolates

A1.1 hMPV Group A1

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NL/1/00         YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
hMPV13-00       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/9/02        YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/39/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/14/02       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/10/02       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/11/02       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/1/02        YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/44/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/7/02        YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/53/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/20/02       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/21/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/3/02        YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/8/02        YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/33/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/19/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/4/02        YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/11/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/34/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/24/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/51/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/22/02       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/46/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/30/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/45/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/15/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/17/02       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/36/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/31/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
RSA/54/01       YTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRELRTVSADQLAREEQIENPRQSRF 60
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NL/1/00         VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
hMPV13-00       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/9/02        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/39/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/14/02       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/10/02       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/11/02       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/1/02        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/44/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/7/02        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/53/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/20/02       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/21/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/3/02        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/8/02        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/33/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/19/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/4/02        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/11/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/34/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/24/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/51/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/22/02       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/46/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/30/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/45/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/15/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/17/02       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/36/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/31/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
RSA/54/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLATAVR 120
                ************************************************************

NL/1/00         ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
hMPV13-00       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRKVLNVVRQFSDNAGITPAISLDLMT 180
RSA/9/02        ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/39/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/14/02       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/10/02       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/11/02       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/1/02        ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/44/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/7/02        ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/53/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/20/02       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/21/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/3/02        ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/8/02        ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/33/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/19/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/4/02        ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/11/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/34/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/24/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPSISLDLMT 180
RSA/51/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/22/02       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/46/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/30/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/45/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/15/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/17/02       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/36/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/31/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/54/01       ELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
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NL/1/00         DAELARAVSNMPT 193
hMPV13-00       DAELARAVSNMPT 193
RSA/9/02        DAELARAVSNMPT 193
RSA/39/01       DAELARAVSNMPT 193
RSA/14/02       DAELARAVSNMPT 193
RSA/10/02       DAELARAVSNMPT 193
RSA/11/02       DAELARAVSNIAT 193
RSA/1/02        DAELARAVSNMPT 193
RSA/44/01       DAELARAVSNMPT 193
RSA/7/02        DAELARAVSNMPT 193
RSA/53/01       DAELARAVSNMPT 193
RSA/20/02       DAELARAVSNMPT 193
RSA/21/01       DAELARAVSNMPT 193
RSA/3/02        DAELARAVSNMPT 193
RSA/8/02        DAELARAVSNMPT 193
RSA/33/01       DAELARAVSNMPT 193
RSA/19/01       DAELAIAVSNMPT 193
RSA/4/02        DAELARAVSNMPT 193
RSA/11/01       DAELARAVSNMPT 193
RSA/34/01       DAELARAVSNMPT 193
RSA/24/01       DAQLARAVSNMPT 193
RSA/51/01       DAELARAVSNMPT 193
RSA/22/02       DAELARAVSNMPT 193
RSA/46/01       DAELARAVSNMPT 193
RSA/30/01       DAELARAVSNMPT 193
RSA/45/01       DAELARRVSNMPT 193
RSA/15/01       DAELARAVSNMPT 193
RSA/17/02       DAELARAVSNMPT 193
RSA/36/01       DAELARAVSNMPT 193
RSA/31/01       DRELARAVSNMPT 193
RSA/54/01       DAELARAVSNMPT 193
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A1.2 hMPV GROUP A2

```
NL/17/00        YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
CAN97-83        YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/7/00        YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/7/01        YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/5/00        YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/26/00       YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/20/01       YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/44/00       YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/27/00       YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/20/00       YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/48/00       YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/49/00       YTNVFTLEVGDVENLTCSDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
                ************************************************************

NL/17/00        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
CAN97-83        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/7/00        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/7/01        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/5/00        VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/26/00       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/20/01       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/44/00       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/27/00       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/20/00       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/48/00       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
RSA/49/00       VLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKTTNEAVSTLGNGVRVLATAVR 120
                ************************************************************

NL/17/00        ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
CAN97-83        ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/7/00        ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/7/01        ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/5/00        ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/26/00       ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/20/01       ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/44/00       ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/27/00       ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/20/00       ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/48/00       ELKDFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/49/00       ELKNFVSKNLTRAINKNKCDIDDLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
                *** ********************************************************

NL/17/00        DAELARAVSNMPT 193
CAN97-83        DAELARAVSNMPT 193
RSA/7/00        DAELARAVSNMPT 193
RSA/7/01        DAELARAVSNMPT 193
RSA/5/00        DAELARAVSNMPT 193
RSA/26/00       DAELARAVSNMPT 193
RSA/20/01       DAELARAVSNMPT 193
RSA/44/00       DAELARAVSNMPT 193
RSA/27/00       DAELARAVSNMPT 193
RSA/20/00       DAELARAVSNMPT 193
RSA/48/00       DAELARAVSNMPT 193
RSA/49/00       DAELARAVSNMPT 193
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A1.3 hMPV Group B1

```
RSA/21/02       YTYVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/23/02       YTYVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
NL/1/99         YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
hMPV33-01       YINVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
                *  ************************************************************

RSA/21/02       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKQTNEAVSTLGNGVRVLATAVR 120
RSA/23/02       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKQTNEAVSTLGNGVRVLATAVR 120
NL/1/99         VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKQTNEAVSTLGNGVRVLATAVR 120
hMPV33-01       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKQTNEAVSTLGNGVRVLATAVR 120
                ************************************************************

RSA/21/02       ELKEFVSKNLTSAINRNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/23/02       ELKEFVSKNLTSAINRNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
NL/1/99         ELKEFVSKNLTSAINRNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
hMPV33-01       ELKEFVSKNLTSAINRNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
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RSA/21/02       DAELARAVSYMPT 193
RSA/23/02       DAELARAVSYMPT 193
NL/1/99         DAELARAVSYMPT 193
hMPV33-01       DAELARAVSYMPT 193
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A1.4 hMPV Group B2

```
NL/1/94         YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
CAN75-98        YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/1/00        YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/4/00        YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/91/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/19/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/16/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/15/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/71/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/93/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/29/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/55/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/23/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/12/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/90/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/54/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/52/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/24/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/17/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/3/00        YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/58/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
RSA/37/00       YTNVFTLEVGDVENLTCTDGPSLIKTELDLTKSALRELKTVSADQLAREEQIENPRQSRF 60
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NL/1/94         VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
CAN75-98        VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/1/00        VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/4/00        VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/91/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/19/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/16/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/15/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/71/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/93/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/29/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/55/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/23/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/12/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/90/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/54/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/52/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/24/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKTTNEAVSTLGNGVRVLATAVR 120
RSA/17/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKKTNEAVSTLGNGVRVLATAVR 120
RSA/3/00        VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKKTNEAVSTLGNGVRVLATAVR 120
RSA/58/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKKTNEAVSTLGNGVRVLATAVR 120
RSA/37/00       VLGAIALGVATAAAVTAGIAIAKTIRLESEVNAIKGALKKTNEAVSTLGNGVRVLATAVR 120
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NL/1/94         ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
CAN75-98        ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/1/00        ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/4/00        ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/91/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/19/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/16/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/15/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/71/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/93/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/29/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/55/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/23/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/12/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/90/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/54/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/52/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/24/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/17/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/3/00        ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/58/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
RSA/37/00       ELKEFVSKNLTSAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMT 180
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NL/1/94         DAELARAVSYMPT 193
CAN75-98        DAELARAVSYMPT 193
RSA/1/00        DAELARAVSYMPT 193
RSA/4/00        DAELARAVSYMPT 193
RSA/91/00       DAELARAVSYMPT 193
RSA/19/00       DAELARAVSYMPT 193
RSA/16/00       DAELARAVSYMPT 193
RSA/15/00       DAELARAVSYMPT 193
RSA/71/00       DAELARAVSYMPT 193
RSA/93/00       DAELARAVSYMPT 193
RSA/29/00       DAELARAVSYMPT 193
RSA/55/00       DAELARAVSYMPT 193
RSA/23/00       DAELARAVSYMPT 193
RSA/12/00       DAELARAVSYMPT 193
RSA/90/00       DAELARAVSYMPT 193
RSA/54/00       DAELARAVSYMPT 193
RSA/52/00       DAELARAVSYMPT 193
RSA/24/00       DAELARAVSYMPT 193
RSA/17/00       DAELARAVSYMPT 193
RSA/3/00        DAELARAVSYMPT 193
RSA/58/00       DAELARAVSYMPT 193
RSA/37/00       DAELARAVSYMPT 193
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APPENDIX 2: AMINO ACID ALIGNMENT OF THE G PROTEIN

Alignment of the G proteins of representative samples of SA hMPV isolates and prototypes sequences from the Netherlands (NL/1/00, NL/1/99, NL/1/94 and NL/17/00) and Canada (hMPV13-00, CAN97-83, hMPV33-01 and CAN75-98). Only amino acid residues that differed from the Netherlands prototypes for each subgroup are shown and identical amino acids are represented by periods and dashes indicate gaps. The proposed intracellular, transmembrane and extracellular domains are indicated by arrows above the alignment. Potential N-linked glycosylation sites are underline. Numbers indicate the amino acid position in the G open reading frames corresponding to the Netherlands prototype isolates

A2.1 hMPV subgroup A1

```
                    Intracellular ><    Transmembrane  >< Extracellular
NL/1/00         IDMLKARVKNRVARSKCFKNASLVLIGITTLSIALNIYLIINYKMQKNTSESEHHTSSSP 70
RSA/54/01       .......L.....I.....................E..... 70
RSA/30/01       ..........................................K..... 70
RSA/8/02        .....................E..... 70
RSA/20/02       .....................E..... 70
RSA/22/02       ..... 70
RSA/33/01       ..... 70
RSA/7/02        ..... 70
RSA/53/01       ..... 70
RSA/17/02       ..... 70
RSA/3/02        ..... 70
RSA/4/02        ..... 70
hMPV13-00       .......V.....T.....T..... 70
RSA/21/01       .....K..... 70
RSA/19/01       ..... 70
RSA/31/01       ..... 70
RSA/34/01       ..... 70
RSA/36/01       .....H..... 70
RSA/10/02       .....YT..... 70
RSA/14/02       ..... 70
RSA/11/02       ..... 70
RSA/9/02        ..... 70
RSA/1/02        ..... 70
RSA/44/01       ..... 70
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NL/1/00         MESSRETPTVPTDNSDTNSSPQHPTQQSTEGSTLYFAASASSPETEPTSTPDTTNRPPFV 130
RSA/54/01       ..................PGS.Y..........H..................AS..L.. 130
RSA/30/01       ..................................................... 130
RSA/8/02        .....................................................L..... 130
RSA/20/02       .....................................................P..... 130
RSA/22/02       ............................G.....P..................... 130
RSA/33/01       ............................E.....F.................T..... 130
RSA/7/02        ..................................................... 130
RSA/53/01       ..................................................... 130
RSA/17/02       ...............................F..................... 130
RSA/3/02        ..................................................... 130
RSA/4/02        ..............................S...................... 130
hMPV13-00       ..........................................................P.. 130
RSA/21/01       ....................................V..................L.. 130
RSA/19/01       ...................................A..................... 130
RSA/31/01       ......................H.............................. 130
RSA/34/01       ..............................................I.......... 130
RSA/36/01       ..........L...........Y.....................T.......... 130
RSA/10/02       ..........PM..........H...........S...T...G......S......PL. 130
RSA/14/02       ..................................................... 130
RSA/11/02       ..................................................... 130
RSA/9/02        ..........................................................A.. 130
RSA/1/02        ......................Y............F.......E.............PF. 130
RSA/44/01       ...................................A.................. 130
                ***********  ******   * ****** * **   ** *** *** ** **  **  *

NL/1/00         DTHTTPPSASRTKTSPAVHTKNNPRTSSRTHSPPRATTRTARRTTTLRTSSTRKRPSTAS 190
RSA/54/01       ............R..........L.I.P......W.M...V.G........I.....A.. 190
RSA/30/01       ..................................................... 190
RSA/8/02        .....................................................T..... 190
RSA/20/02       ..................................................... 190
RSA/22/02       ........................P.................................L.... 190
RSA/33/01       ........................L...........................I...P.... 190
RSA/7/02        ..................................................... 190
RSA/53/01       ..................................................... 190
RSA/17/02       ..................................................... 190
RSA/3/02        .....................................................T..... 190
RSA/4/02        ........................P...........................I..... 190
hMPV13-00       ........................LK..............................T.. 190
RSA/21/01       .......................R.............................. 190
RSA/19/01       ...................Y....P....K........................... 190
RSA/31/01       ...................H....L.T..R...................H...T..... 190
RSA/34/01       .....................................................R..... 190
RSA/36/01       ........................P.............................. 190
RSA/10/02       ..........................I......S...T.................... 190
RSA/14/02       ................................P..................... 190
RSA/11/02       ..................................................... 190
RSA/9/02        ..................................................... 190
RSA/1/02        .....................................................I..... 190
RSA/44/01       ............K............T.............................. 190
                ************ ***** ****   *  *** * * *** * **** *** *** * **

NL/1/00         VQPDISATTHKNEEASPASPQTSASTTRIQRKSVEANTSTTYNQT 235
RSA/54/01       ....S......H....SV........A.P...------------- 222
RSA/30/01       ................P.............------------- 222
RSA/8/02        ..................................------------- 222
RSA/20/02       ..................................------------- 222
RSA/22/02       ..................................------------- 222
RSA/33/01       ..................................------------- 222
RSA/7/02        ......................-N.......------------- 221
RSA/53/01       ......................AS.......------------- 222
RSA/17/02       ..............................------------- 222
RSA/3/02        ..................................------------- 222
RSA/4/02        ..................................------------- 222
hMPV13-00       ......T.......................GMEASTSTTHNQT 235
RSA/21/01       .R....A.....................------------- 222
RSA/19/01       .Q..........................------------- 222
RSA/31/01       ..............................------------- 222
RSA/34/01       ..................................------------- 222
RSA/36/01       ......................P.........------------- 222
RSA/10/02       ......................S.......GR------------- 222
RSA/14/02       ..................................------------- 222
RSA/11/02       ...........Q...................------------- 222
RSA/9/02        ...........H...................------------- 222
RSA/1/02        ..................................------------- 222
RSA/44/01       ..............................------------- 222
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A2.2 hMPV subgroup A2

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                 Intracellular ><    Transmembrane  >< Extracellular
NL/17/00          VKNRVARSKCFKNASLILIGITTLSIALNIYLIINYTIQKTTSESEHHTSSPPTEPNKEA 77
RSA/7/00          ...........................................................S.... 77
RSA/26/00         ...........M.....................................................  77
RSA/27/00         ...........I.....................................................  77
RSA/7/01          ..................................................................P.... 77
RSA/20/01         ...........................................................S.... 77
RSA/44/00         .....................................................................  77
RSA/5/00          .....................................................................  77
RSA/48/00         .....................................................................  77
RSA/49/00         .....................................................................  77
CAN97-83          ...................................S.................................  77
RSA/20/00         ..........................................M...T.....................  77
                  ************************ *********** *** ************* ****

NL/17/00          STISTDNPDINPSSQHPTQQSTENPTLNPAASASPSETEPASTPDTTNRLSSVDRSTAQP 137
RSA/7/00          ...........N...S.....................................................  137
RSA/26/00         .....................................................................  137
RSA/27/00         .....N...............................................................  137
RSA/7/01          ....................................I.............A.....  137
RSA/20/01         ....................................T.............V.....  137
RSA/44/00         ..........................................................S.....  137
RSA/5/00          ..................Y.....................................R.....  137
RSA/48/00         ..................S.....................I........P....  137
RSA/49/00         .....................................T........S....  137
CAN97-83          ..............P.....................V.....................  137
RSA/20/00         ....................................A...................A.....  137
                  ***** ****** *** *** ********** **** ******** ***** *  ****

NL/17/00          SESRTKTKPTVHTINNPNTASSTQSPPRTTTKAIRRATTFRMSSTGKRPTTTLVQSDSST 197
RSA/7/00          ..........A..R...S....I.....A..........L.....................  197
RSA/26/00         .......E..V.....T.L.................................  197
RSA/27/00         ...................................F.....................  197
RSA/7/01          ...................M.....................P......I  197
RSA/20/01         ..................F.....T.....................S......T  197
RSA/44/00         ....A.....T.....S.....................L.....  197
RSA/5/00          ....T..K.....S..S.....P.....................S.....  197
RSA/48/00         ...............A.....Q.....................L.....  197
RSA/49/00         ...................SP.....................  197
CAN97-83          ...............P.....................S.....  197
RSA/20/00         ........L.....................L.T..........P....I..  197
                  **** **  * ** **  * ** *  ** * ******** * ********** **** *

NL/17/00          TTQNHEETGSANPQASASTMQN--------- 219
RSA/7/00          ..............V.....QHTNNTKPN 211
RSA/26/00         ..................QHTNNTKPN 211
RSA/27/00         ..................QHTNNTKPN 211
RSA/7/01          ..............A.....QHTNNTKPN 211
RSA/20/01         ..........T.....V.....QHTNNTKPN 211
RSA/44/00         ..........A..........QHTNNIKPS 211
RSA/5/00          ..............A.....QHTNNIKPN 211
RSA/48/00         ..................QHTNNIKPN 211
RSA/49/00         ................I..QHTNNIKPN 211
CAN97-83          ..............V..M..HTNN-IKPN 210
RSA/20/00         ..............A.---------- 202
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A2.3 hMPV subgroup B1

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                Intracellular ><    Transmembrane   >< Extracellular
NL/1/99          SRCYRNATLILIGLTALSMALNIFLIIDHATLRNMIKTENCANMPSAEPSKKTPMTSTAG 84
hMPV33-01        .....................................................  84
RSA/23/02        .....................................................  84
RSA/21/02        .....................................P.........I....  84
                 ****************************************** ********* ****

NL/1/99          PNTKPNPQQATQWTTENSTSPVATPEGHPYTGTTQTSDTTAPQQTTDKHTAPLKSTNEQI 144
hMPV33-01        .S..................A..L......E....P...........Y..LS.......  144
RSA/23/02        ................................G................H...P.......  144
RSA/21/02        .N.....................P....H.............................  144
                 * ****************** ** **** * **** ********** **  *******

NL/1/99          TQTTTEKKTIRATTQKREKGKENTNQTTSTAATQTTNTTNQIRNASETITTSDRPRTDTT 204
hMPV33-01        .......K.T...P.R.K.......Q..........................I.I.  204
RSA/23/02        .......R.....TPKKG.R.....P.....................T.T.  204
RSA/21/02        .......K......QRRE.......Q.....................K..I.....S.  204
                 ******* * ***     * ***** ********************** ********** * *

NL/1/99          TQSSEQTTRATDPSSPPHHA------- 224
hMPV33-01        ..........E.GF.....RRGAGPR 231
RSA/23/02        ..........A...SS.Y..RRGAGPR 231
RSA/21/02        ..........TD.S.P.HR.QGSAKPK 231
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A2.4 hMPV subgroup B2

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                 Intracellular ><      Transmembrane  >< Extracellular
NL/1/94          SSKCYRNATLILIGLTALSMALNIFLIIDYAMLKNMTKVEHCVNMPPVEPSKKTPMTSAV 60
CAN75-98         ..........S.............TS.......................... 60
RSA/4/00         ..........L.............L..V....................... 60
RSA/29/00        ..........................................................
RSA/90/00        ..........................................W.................
RSA/71/00        ..........................................................
RSA/12/00        ..........................................C...........L..... 60
RSA/23/00        ..........................................................
RSA/54/00        ..........................M.................P..... 60
RSA/93/00        ..........................................................
RSA/24/00        ..........................................................
RSA/3/00         ..........M.....I.................
RSA/17/00        ..........................................................
RSA/37/00        ..........................................V.................
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NL/1/94          DLNTKPNPQQATQLAAEDSTSLAATSEDHLHTGTTPTPDATVSQQTTDEYTTLLRSTNRQ 120
CAN75-98         .P...........TT.........L.....................H......T... 120
RSA/4/00         .L...L.................S.N.......Q.S...I.............I... 120
RSA/29/00        ..........................................................
RSA/90/00        ....................................I..................... 120
RSA/71/00        ..........................................................
RSA/12/00        ..............................................K.......... 120
RSA/23/00        ..............................................E.......... 120
RSA/54/00        .......L.........................P..................... 120
RSA/93/00        .I...........N.................................................
RSA/24/00        YL.....P......T............D.....................................
RSA/3/00         D.........................L.....IP...V...A..........T... 120
RSA/17/00        ....................................T..................... 120
RSA/37/00        .S.........................S......TS.T.....T.............. 120
                   *** * ******  ** **  ** * *  ****   * * *** **  ****** ***

NL/1/94          TTQTTTEKKPTGATTKKETT-----RTTSTAATQTLNTTNQTSYVREATTTSARSRNSAT 175
CAN75-98         .....A.....R....KETT----------.....L.......NGR...........N.. 175
RSA/4/00         .....T.....G.I..KEKE---TT.........P.........K.......G...G.. 177
RSA/29/00        ..............KEKE---TT.....................................R...... 177
RSA/90/00        ..............KEKE---TT.....................................I..... 177
RSA/71/00        I.............KEKE---TT.....................................T..... 177
RSA/12/00        T.............KEKE---TT.....................................
RSA/23/00        ..............KEKE---TT.....................................
RSA/54/00        ..............KEKE---TT.....................R.............
RSA/93/00        ..............KEKEKETTT.....................
RSA/24/00        ..............KEKE---TT....I.....................K.... 177
RSA/3/00         ..............-EKE---TT....T.....L.....................R..V. 176
RSA/17/00        ..............KEKE---TT.....................................
RSA/37/00        .......N......A.---E---TT..........P.............K...P..GA. 174
                  **** * *** *  *         **** ***** *******   **** **   *  *

NL/1/94          .QSSDQTTQAADPSSQPHHTQKSTTTTYNTDTSSPSS 212
CAN75-98         ..S.............SQ.........H......... 212
RSA/4/00         ..N....I.......KPH..................... 214
RSA/29/00        ..................................... 214
RSA/90/00        ..................................... 214
RSA/71/00        ..................................... 214
RSA/12/00        ..................................... 214
RSA/23/00        ............................Y......... 214
RSA/54/00        ............S..............H......L.. 214
RSA/93/00        ............P.......................... 217
RSA/24/00        .......T..................Y......P.. 214
RSA/3/00         .........T.....Q.Y................SN. 213
RSA/17/00        ..................................... 214
RSA/37/00        .R..........Q.....................S. 211
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APPENDIX 3. Ethics clearance certificate

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