Human Metapneumovirus

Discovery, Characterisation and Associated Disease

Bernadette G. van den Hoogen

Cover illustration: T. Kuiken en F. van der Panne
Demonstration of hMPV antigen (stained by immunohistochemistry) in bronchial epithelium of an experimentally infected macaque.

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Aan Anne, Ron en mijn vader
ter nagedachtenis aan mijn moeder

Abbreviations

aa	amino acid
ALL	acute lymphoblastic leukaemia
APV	avian pneumovirus
ARI (ARTI)	acute respiratory tract illnesses
BAL	broncho-alveolar lavage
CI	confidential interval
CPE	cytopathic effects
DIF	direct immuno-fluorescence assay
dpi	days post infection
ELISA	enzyme-linked immunosorbent assay
F	fusion protein
G	attachment protein
HA	haemagglutinin
HI	histology
HMPV	human metapneumovirus
HRA	heptad repeat region A
HRB	heptad repeat region B
HSCT	haematopoietic stem cell transplantation
IF(A)	immuno-fluorescence (assay)
ILI	influenza-like illnesses
IHC	immunohistochemistry
L	large (polymerase) protein
LRT	lower respiratory tract
M	matrix protein
M2-1	transcription anti-termination factor
M2-2	RNA regulatory protein involved in replication and transcription
N	nucleocapsid protein
NA	neuraminidase
NASBA	nucleic acid sequence-based amplification
NPA	nasopharyngeal aspirate
NS	non-structural protein
nt	nucleotide
ORF	open reading frame
P	phosphoprotein
PIV	parainfluenza virus
RAP PCR	RNA arbitrary primed PCR
RSV	respiratory syncytial virus
RTI(s)	respiratory tract illnesses
RT-PCR	reverse transcriptase polymerase chain reaction
SH	small hydrophobic protein
$TCID_{50}$	50% tissue culture infectious doses
tMK	tertiary monkey kidney
TRTV	turkey rhinotracheitis virus
URT	upper respiratory tract

Contents

1

General Introduction

General introduction

Acute respiratory tract infections are responsible for considerable morbidity and mortality and costs attributable to acute respiratory tract illnesses (ARI) are an important burden on national health care budgets[13,165]. A variety of viruses, bacteria and fungi are associated with ARI[174]. This thesis only focuses on viral causes of ARI

Diagnosis of respiratory tract infections is important for adequate treatment of patients and community surveillance studying the spread of respiratory viruses forms the basis for preventive strategies. Despite the use of a variety of diagnostic assays, 15-50% of the samples obtained from persons suffering from ARI do not yield positive diagnoses, which is at least in part due to limitations of diagnostic procedures. However, a proportion of ARI may be caused by still unknown pathogens, providing an additional explanation for the relatively high proportion of negative laboratory diagnoses. This thesis describes the identification and characterisation of a hitherto unknown virus as the causative agent of ARI, which was subsequently found to be responsible for a high portion of the unexplained ARI. In this introduction background information on characteristics, diagnostics and epidemiology of known respiratory viruses will be provided.

Viruses associated with ARI

A large number of viral pathogens, belonging to a variety of virus families, have been associated with ARI in humans. Viruses detected most frequently in association with ARI belong to the families *Paramyxoviridae*, *Orthomyxoviridae*, *Picornaviridae*, *Adenoviridae*, and *Coronaviridae*, although other viruses such as members of the family *Herpesviridae*, and Hantavirus (*Bunyaviridae*) have also been associated with respiratory tract disease in humans (Table 1). Furthermore many viral pathogens, most of them related to human viral pathogens, are associated with respiratory disease in animals. In the next paragraphs only the most important respiratory viruses of humans will be described in detail.

Members of the family *Paramyxoviridae*

The *Paramyxoviridae* family is among the most costly in terms of disease burden and economic impact caused by its members in humans and animals[142,155,272]. Moreover, a large number of new candidate members have been identified in humans and animals over the past decade. The family is divided into two subfamilies, the P*aramyxovirinae*, with five genera, and the *Pneumovirinae*, with two genera (Figure 1). The classification of these viruses is based on morphological criteria, genome organisation, the biological activities of the proteins, the sequence relationship of the encoded proteins and finally on overall biological criteria. The pneumoviruses can be distinguished from the *Paramyxovirinae* members morphologically because they contain narrower nucleocapsids. In addition, pneumoviruses have differences in genome organisation, the number of encoded proteins and an attachment protein that is different from that of members of the subfamily *Paramyxovirinae*[142].

Subfamily Paramyxovirinae

Two of the genera (*Respirovirus* and *Rubulavirus*) within this subfamily include major causes of human ARI: the parainfluenza viruses (PIV), genetically and antigenically divided into serotypes 1 to 4[112,142]. As a group PIV 1, 2 and 3 are second to respiratory syncytial virus (RSV) as important causes of lower respiratory tract (LRT) infections in young children[41,56,112]. Most children have been infected by PIV-3 by the age of two years and by PIV 1 and 2 by the age of five years. Pneumonia and bronchiolitis resulting from PIV-3 infection occur primarily in the first six months of life, as is the case for RSV infection, but with lower frequency. Croup is the most presented clinical manifestation of infection with PIV, especially type 1, and is the main cause of hospitalisation from PIV infections in children two to six years of age[112]. Although little is known about infections in adults, immunity to PIV appears to be incomplete and repeated infections occur throughout life. Infection with PIV 4 is relatively rare and it is therefore often not included in epidemiological studies (reviewed in 112,105).

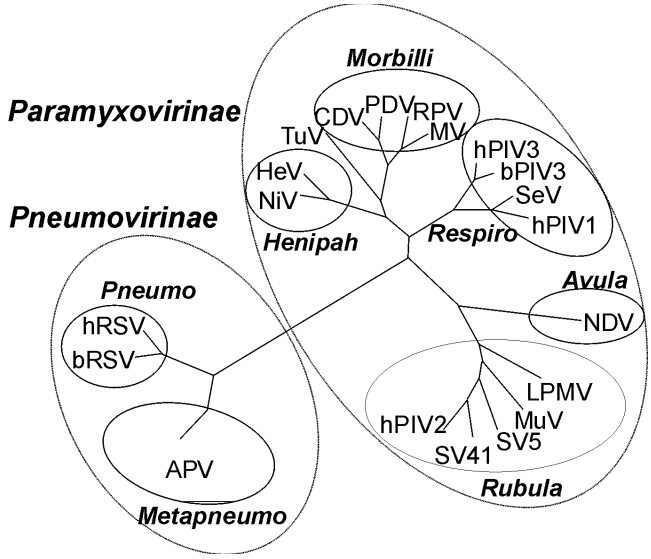

Figure 1:
Schematic diagram of known members of the Paramyxoviridae family based on phylogenetic analysis of the nucleotide sequences for the polymerase gene of these viruses.
Abbreviations: NiV: Nipah virus; HeV: Hendra virus; TuV: Tupaia virus; CDV: Canine distemper virus; PDV: Phocine distemper virus; MV: Measles virus; MuV: Mumps virus; SV5: Simian virus 5; SV41: Simian virus 41; APV: Avian pneumovirus; PVM: Pneumonia virus of mice; RSV: Respiratory syncytial virus; b: bovine; h: human.

The genus *Rubulavirus* includes mumps virus (MuV) infections with which are characterised by salivary gland swelling. This virus infects the respiratory tract, but clinical manifestations usually relate to infection of the parotid gland, without respiratory tract illness[276].
The genus *Morbillivirus* includes one human virus: measles virus (MV). Infection with MV is characterised by fever, cough, and conjunctivitis followed by the appearance of generalised maculopapular rash. LRT illnesses have been reported, but through the wide application of a live attenuated vaccine, measles virus is no longer a major pathogen of ARI in the developed world[102].
The *Avulavirus* genus contains only avian viruses. The genus *Henipahvirus*, with the human viruses Nipah and Hendra, has recently been established (see below).

Newly identified members of the subfamily Paramyxovirinae
In the last decade a large number of hitherto unknown paramyxoviruses have been identified in animals and humans. Most of these were identified in animals such as snakes[88,210], salmon[140], horses (Salem virus)[209], tree shews (Tupaia virus)[247], and pigs and bats (Mapuera, Menangle and Tioman virus)[35,111,193]. Not all of these viruses have been associated with illness, several of them were detected in the hunt for the reservoir for two of the newly emerging human viruses, Hendra virus (isolated from horses and humans) and Nipah virus (isolated from pigs and humans)[34,178]. These two viruses are associated with severe ARI and/or encephalitis in humans with a high mortality rate. They are genetically related and have recently been classified in a new genus: the *Henipahvirus* genus. Both viruses caused relatively small outbreaks, with high case fatality rates, restricted to Australia, Malaysia and Singapore in 1995-1999.

Subfamily Pneumovirinae
The subfamily *Pneumovirinae* contains two genera: the pneumoviruses and the metapneumoviruses. RSV is the type species of the genus *Pneumovirus* and was until recently the only human pathogen identified in the subfamily *Pneumovirinae*. RSV is a ubiquitous pathogen circulating in humans of all ages around the globe. Two subgroups (A and B) have been identified based on differences in nucleotide sequences, reactivity patterns with monoclonal antibodies and in vitro neutralisation assays with subgroup-specific antisera[7,37,95,176]. Some studies have suggested that viruses belonging to the two subgroups differ in pathogenicity, although this has not been found by other groups[116,164,246,263].
RSV infects approximately 90% of all children under the age of 2. Almost 1% of these children develop bronchiolitis and/or pneumonia severe enough to require hospital admission, and of these about 1% die, particularly those with congenital heart defects, bronchopulmonary dysplasia, very low birth weight, prematurity or immunodeficiency[234,272]. Re-infections with both homologous and heterologous viruses during life are common. However, the severity of disease generally decreases with subsequent re-infections, and the frequency of re-infections decreases with age[46]. Unlike the severe infections seen in infants, RSV infections in healthy adults are usually self-limiting and induce only mild to moderate symptoms[55,78]. The virus is however an important pathogen among elderly and immunocompromised patients (see later sections).

Newly identified members of the subfamily Pneumovirinae

In 1976 a respiratory tract disease was observed in turkeys, which was demonstrated to be caused by a virus tentatively named turkey rhinotracheitis virus (TRTV)[26,27,96,129,163,274,278]. Examination of the proteins and genes revealed similarities with pneumoviruses which established the classification in the *Pneumovirinae* subfamily and resulted in the name avian pneumovirus (APV)[31,40,118,150,281]. Additional genomic sequencing of APV revealed a distinct genomic order and content of genes as compared to RSV: APV does not encode NS1 and NS2 genes, and has a different order between the matrix and polymerase genes[151,204,282]. These differences formed the basis for subdivision of the subfamily *Pneumovirinae* into two genera: the pneumoviruses with RSV as type species and the metapneumoviruses with APV as the sole member[118,200]. Further comparison of nucleotide and amino acid sequences of the attachment protein G, and differences in antigenicity, led to classification of the different APV isolates into two distinct subgroups: A and B[39,130]. New subgroups or subtypes of APV have been recently identified, such as APV C in the United States[222,224] and APV D in France[11] and this subdivision is again based on sequence differences, in particular for the attachment protein G, and on differences in antigenicity. Although there is only limited information on APV D, it has become clear from genetics, serology and protection studies that APV A and B are more closely related to each other than any of them to APV C[51, 109]. It has been suggested that APV C constitutes a separate serotype of APV[51,223].

In 1986, sero-epidemiological studies reported that adult human sera exhibited moderate to high neutralising activity against pneumonia virus of mice (PVM). This virus, a member of the genus *Pneumovirus* within the subfamily *Pneumovirinae*, causes respiratory tract disease mostly in laboratory mice and rats. The suggestion that PVM or an antigenically related virus circulates in the human population has so far not lead to the identification of such a virus[201].

Members of other virus-families

The family *Ortomyxoviridae* includes the three influenza virus types A, B and C, divided on basis of antigenic properties of internal proteins of influenza viruses. Influenza virus type A is associated with annual influenza epidemics and intermittent pandemics. Influenza virus type B is associated with influenza epidemics only and influenza virus type C is clinically irrelevant. Type A influenza virus can be further divided into antigenic subtypes based on differences of the haemagglutinin (HA) and the neuraminidase (NA) glycoproteins, so far 15 subtypes of HA and 9 of NA have been described in birds and some are found in various combinations in humans and other mammals[143]. Influenza pandemics occur when new influenza A subtypes, carrying antigenically distinct HA and/or NA, are introduced into the human population (antigenic shift). After the introduction of such a new subtype, it causes recurrent annual epidemics, during which the HA and NA reveal gradual antigenic changes (antigenic drift), supposedly as the result of herd immunity. In temperate climates, influenza epidemics occur annually in the winter months and usually last 6 to 10 weeks. The severity of an epidemic depends on the circulating virus and the pre-existing immunity to the virus in the community. During an outbreak of influenza type A, up to 5-10% of the general population may become infected[100,174]. Influenza associated severe disease is most often seen in young children, elderly, and those with underlying illnesses. ARI caused by influenza can develop into pneumonia, often accompanied by bacterial infections, and may result in death[114]. Despite the availability of vaccines and antiviral drugs, influenza continues to be a significant cause of illness in all age groups.

Members of the family *Adenoviridae* have a typical icosahedral shell with fibre-like projections[228]. Today there are 51 known serotypes, divided into the six species A-F, recognised to infect humans. The incidence of adenovirus infection peaks between the ages of 6 months and 5 years, where they account for approximately 5-10% of the LRT infections[53,71]. Certain serotypes such as 1,2,3,5,7 and 21, can cause severe LRT disease in children[135]. The respiratory symptoms caused by adenovirus infection include common cold like syndromes, but sporadic cases might be indistinguishable from other viral ARI, such as infections with influenza virus, PIV, and RSV. Adenovirus infections can result in pneumonia requiring hospitalisation, but they are usually not of great concern in older individuals[170]. Of great concern are the 5-20% of adenovirus infections occurring in patients undergoing stem cell transplantation, where they are associated with mortality rates of up to 50%[262].

The major groups within the family *Picornaviridae* causing ARI are the rhinoviruses, consisting of more than 110 serotypes, and the enteroviruses, comprising 68 serotypes[216]. Whereas human enteroviruses (Coxsackieviruses and ECHOviruses) cause 5-15% of viral ARI, rhinoviruses have been associated with 25-50% of common colds. The general mildness of human picornavirus-associated illness is reflected by the low percentage of patients seeking medical care[213]. However, rhinovirus infections have been reported to account for 22% of patients of all age groups with influenza-like symptoms consulting their general physician[14] and may also cause complications involving the URT and LRT, such as exacerbations of asthma[93,206]. Re-infections with antigenically different rhinoviruses are common since there is no cross-immunity among rhinovirus serotypes[213]. Due to incomplete and short-lived immunity after infection, homologous re-infection with rhinoviruses is common too. Enterovirus infections are often accompanied by mild ARI, although they are also detected in patients suffering from croup, bronchiolitis or pneumonia[155].

The *Coronaviridae* family comprises the genera *Coronavirus* and *Torovirus*, and within the genus *Coronavirus* three groups have been recognised based on genetic data and serological assays. The majority of human coronaviruses are serologically related to one of two reference strains, 229E and OC43 (group I and group II, respectively). Although coronaviruses infect people of all age groups, they may cause severe LRT illnesses in frail patients, such as young children and elderly individuals[72,79,97,254]. The symptoms associated with human coronavirus infections are similar to those associated with rhinovirus infections, accounting for approximately 35% of common colds in adults[79]. Coronaviruses have been reported to account for 2% of patients of all age groups with influenza-like symptoms consulting their general physician[14,254]. In 2003, a worldwide outbreak occurred of a life threatening febrile respiratory illness that has been named severe acute respiratory syndrome (SARS). A causal association between SARS and a newly identified coronavirus has been demonstrated by a number of research groups working together to identify the cause of SARS[66, 67, 86, 138, 139, 189]. Where the classical coronaviruses usually cause mild symptoms in healthy persons, individuals infected with SARS coronavirus may experience severe respiratory illness and sometimes require mechanical ventilation. In particular, patients with underlying disease and elderly are susceptible to severe disease, whereas children less than 15 years of age do not seem to be particular vulnerable to severe disease caused by this newly discovered virus[138].

Table 1: Characteristics of selected human viral respiratory pathogens

Virus family	virus	Serotypes/ subgroups (n=)	genome	virion structure	seasonality
Paramyxoviridae	Parainfluenza virus	4	negative sense RNA	Enveloped 150-350 nm	Type 1 and 2: autumn-winter, type 3: spring-summer
	Respiratory syncytial virus	2[(a)]	negative sense RNA	Enveloped 150-350 nm	Late fall to early spring
Orthomyxoviridae	Influenza virus	3[(b)]	negative sense RNA (segmented)	Enveloped 80-120 nm	Winter–early spring
Adenoviridae	Adenovirus	51 [(c)]	double-stranded DNA	Non-enveloped 65-80 nm	All year, time of epidemics depends on the serotype
Coronaviridae	Coronavirus	4 [(d)]	positive sense RNA	Enveloped 60-200 nm	Late fall to early spring
Picornaviridae	Rhinovirus	>110	positive sense RNA	Non-enveloped 27-30 nm	Early fall and spring, rarely in summer
	Enterovirus	68	positive sense RNA	Non-enveloped 27-30 nm	All year, peaks in summer and fall

Footnotes: (a) two subgroups have been defined, which have not fulfilled the criteria for serotypes (b) 3 types of influenza have been identified, with those of type A divided in subtypes (c) 9 of the serotypes are associated with ARI in humans (d) 3 antigenic groups have been associated with human ARI.

General features of respiratory viruses

Seasonality

The seasonal fluctuation of the prevalence of numerous respiratory viruses has been established in many epidemiological studies. Worldwide, the seasonality of respiratory agents varies geographically, largely according to climate zones. The reasons for seasonality are not fully understood. Indoor crowding, relative humidity and temperature, overall immunological status and a variety of other factors may all account for this phenomenon. RSV and influenza viruses cause epidemics mainly in the winter to early spring in temperate regions and in the late spring-summer season in the (sub) tropics[266]. The start of the influenza season typically follows that of the RSV season. Rhinoviruses circulate in all months of the year with peaks of illness in the fall and in the spring, where fall-outbreaks are a result of children returning to school[213,214]. Parainfluenza-viruses are most prevalent in the summer months but also may be present during the spring and fall. Spring and summer infections most often are associated with outbreaks of PIV- 3 infections, whereas PIV-1 and 2 more

frequently are isolated during fall months[113]. Coronaviruses cause well-defined epidemics, with peak incidence in the late fall to early spring in temperate climate zones[148].

Nosocomial spread

Nosocomial infections are infections that spread in the hospital or instutions, such as elderly homes. Transmission of nosocomial respiratory viruses is usually seasonal, with the peak incidence occurring in the winter months, mirroring the disease activity in the community. Paediatric units, wards with elderly and immunosuppressed individuals, and institutions such as nursing homes are particularly prone to seasonal introductions and nosocomial spread of viral infection. RSV is one of the most common agents of nosocomial infections followed by influenza viruses, PIV, rhinoviruses, and adenoviruses. Cross infection with RSV within paediatric wards is a common problem, with over 40% of the children becoming infected if hospitalised during the winter months for more than 7 days, and each year approximately 50% of paediatric staff acquire the infection. RSV outbreaks in oncology and bone marrow transplant wards and nursing homes have also been detected. Several studies detected RSV in 2 to 89% of the nursing home patients during epidemics[2]. For influenza numerous nosocomial outbreaks involving long-stay facilities for the elderly have been reported. These outbreaks are often but not exclusively associated with poor vaccine coverage. In addition, outbreaks of influenza involving medical, paediatric, and bone marrow transplant units have been reported[250]. The prevention of nosocomial spread of viral infections depends on timely diagnosis, and effective preventive procedures such as patient isolation, wearing of protective clothing, and limiting contact between patients and symptomatic staff or visitors. Besides vaccination of health care workers (for influenza), the prophylatic use of antiviral drugs is an option for some of the viruses involved (see next section)[2].

Prevention and intervention

The most effective and cost-effective intervention strategy for known respiratory virus infections is the use of vaccines. After the eradication of smallpox and the ongoing efforts for poliovirus, measles virus is on the list of the world health organisation to be eradicated through vaccination. In developed countries, influenza vaccines are used to limit the impact of the disease, primarily in the elderly and other risk-groups for severe disease. The development of PIV and RSV vaccines has been a high priority for decades, however so far without satisfying results. Major complications for vaccine development are the antigenic variability of certain viral respiratory pathogens and the potential enhanced disease associated with the use of certain inactivated vaccines, such as seen for RSV[132,134].

Only for RSV and influenza viruses antiviral drugs are available for prophylactic or therapeutic use. For RSV hyper-immune-globulins (RSV-Ig) and humanised monoclonal antibodies against the F protein (palivizumab) are used in high-risk patients to protect against severe disease[233]. For influenza, a number of antiviral drugs are available. Amantadine and rimantadine function by blocking the M2 ion channel protein of the virus. Both drugs may be of substantive benefit in treating influenza A but not without side effects on the central nervous system, the liver and kidneys and by the rapid emergence of drug resistance. More recently zanamir and osteltamivir have been developed, which block the action of neuraminidase to prevent release of newly formed virus from the infected host cell and its spread within the host. These neuraminidase inhibitors, which inhibit both influenza A and B are associated with little side effects or emergence of resistant viruses. Both drugs have been

known to be effective in preventive and therapeutic use, reducing clinical disease and complications[57,114,250].

The antiviral drug Pleconaril appears to be a promising drug for the treatment of enteroviral and rhinoviral infections. Clinical studies have reported a reduction in the duration and intensity of symptoms in children and adults with enteroviral meningitis and in adults with rhinoviral respiratory tract infections treated with pleconaril. Lastly, pleconaril has demonstrated efficacy in the treatment of severe life-threatening enteroviral infections of the newborn and in immunosuppressed individuals[84,212].

Several other drugs, for instance ribavirin, which prevents replication of certain viruses, are used preventive and therapeutic against a wide variety of viral respiratory viruses. However, clinical use of this compound is contentious, because ribavirin may cause side effects, treatment is expensive and there is some question about its practical utility and effectiveness against RSV infections[65,277].

Laboratory diagnosis of viral ARI

Virus isolation

Virus isolation from nasal washes or nasopharyngeal aspirates has long been the gold standard for diagnosis of viral ARI. Alternative samples (throat swabs, broncho-alveolar lavages, etc) are also commonly used. However, such procedures can require many days or even weeks for virus detection and identification, providing diagnostic outcomes to clinicians in a period of time that may not be clinically useful. A variety of specific serological assay (such as IF, haemagglutination assays, etc) is used to characterise positive viral cultures. Centrifugation of the clinical specimens onto cell monolayers (shell vial cultures) followed by immuno-fluorescence (IF) detection to identify viral antigens allows a much earlier diagnosis of infection. However, depending on the virus involved, these techniques may still not establish a diagnosis within a clinically useful time period[185,202]. A serious problem with virus isolation is that different respiratory viral pathogens may require a wide range of cell substrates in which they can replicate. Potentially, some viruses may not replicate in commonly used in-vitro cell cultures at all.

Antigen detection

For rapid diagnosis, most laboratories use virus specific antibodies to detect the respiratory viral antigens directly in cells in clinical specimens. These rapid testing methods frequently use direct immuno-fluorescence assay (DIF) and/or membrane enzyme-linked immunosorbent assay techniques in various testing formats, with results obtained within 10 to 60 minutes[157,229]. Nasopharyngeal, tracheal or bronchial aspirate samples are the preferred specimens for DIF, since throat or nose swabs usually contain a limited number of intact infected cells[157]. Not all virus infections can be detected by DIF since, either suitable specimens cannot be taken, or the variety of antigenic types precludes making appropriate reagents or reagents are not available at all[157,229,244]. At last, the results obtained in studies using DIF, depend on the balance between sensitivity and specificity of the applied test. Very high sensitivity may result in false positive identification, and a high specificity usually results in lower sensitivity. In most studies DIF resulted in 70-95% sensitivity compared with conventional culture techniques[157,208]. While rapid testing plays an important role in

individual diagnosis, comparative studies have shown that these procedures should be used in conjunction with conventional culture and serological tests or genome detection to yield the highest identification rate for epidemiological surveys. The culture of samples negative by direct assays not only increases the yield of viruses for which direct testing was attempted but also allows the recovery of agents not covered by direct-testing protocols, including rare or even unknown agents[16,64,127,157,264].

Genome detection

Virus genome detection by nucleic acid amplification assays (for instance PCR, RT-PCR, real time PCR and NASBA) is now widely used in the modern laboratories. A major advantage of these assays is the possibility to screen for several respiratory viruses at the same time[74,80,103,152]. PCR methods are particularly useful for detecting viruses that are difficult to isolate, such as rhinoviruses and coronaviruses. Unlike cell culture techniques, PCR assays do not require viable pathogens. PCR assays also provide materials for storage and further analysis. However, PCR tests do not discriminate between "live" and non-replicating (e.g., neutralised) virus. In addition, RNA viruses in particular have highly variable genomes; therefore reverse transcriptase PCR (RT-PCR) assays have to use primers that are designed against highly conserved regions. Monitoring genomic changes in these viruses is of utmost importance to keep the designed primers up to date with the circulating genotypes. Attempts to isolate virus from samples negative by PCR may result in the identification of viruses with sequence variation in the primer-binding sites and may also allow the detection of other agents, including rare or unknown agents[16,64,127,264]. Moreover, PCR based assays are not widely available or standardised yet and not all laboratories have implemented such costly techniques in their routine screening for respiratory viruses.

Antibody detection

Direct diagnosis of ARI is not always successful, since acute infection when viral load is usually high, may have occurred before the patient seeks care. Therefore, serological methods to detect virus-specific antibodies can contribute to the identification of viral pathogens causing ARI. Since antibodies are usually detected after the acute phase of disease, this technique is only useful for epidemiological studies or retrospective diagnoses. Enzyme-linked immunosorbent assays (ELISA), immune fluorescence assays (IFA), neutralisation and haemagglutination inhibition tests to detect viral antibodies are commonly used. A fourfold rise in antibody titer is generally considered significant to identify acute infection if paired acute- and convalescent-phase serum samples are tested[36,183,207]. Although, not used standard for respiratory viruses, a rapid diagnosis can be made on the basis of a single acute-phase serum by demonstrating virus-specific antibody of the IgM class, as for instance used for the diagnosis of measles virus infection[271].

However, serologic tests in infants are less usefull, particularly in those younger than 4-8 months[22]. Moreover, one must be aware of serological cross-reactions between different viruses, even when the same serological assays do not detect such cross-reactivity using animal antisera. For instance, although the three parainfluenza virus types belong to two different genera of the *Paramyxovirinae,* antigenic determinants present on the H site of the HN proteins of the PIV 1, 2 and 3 cross react in complement-fixation, haemagglutination-inhibition or virus neutralisation assays[41,145].

Epidemiological studies

Epidemiological studies are crucial for our understanding of the behaviour of the different pathogens involved and their impact on the burden of disease. Such studies can detect changes in spread or virulence of respiratory viruses and are the basis for preventive strategies such as vaccination.

Most of the studies have been hampered by incomplete study approaches. First, the number of aetiologic agents tested for are often limited. The majority of studies are restricted to either virology or bacteriology, although studies focussing on bacteria sometimes include major viral pathogens such as influenza virus and RSV, and virology-based studies sometimes include a small number of non-viral pathogens. Secondly, criteria for patient inclusion are usually restricted, based on illness manifestation and age. In studies restricted to patients visiting their physician with only influenza-like or febrile illnesses (not general ARI) RSV and influenza viruses and rhinoviruses – if tested for - account for the highest percentages of illnesses, but this underestimates the circulation of other respiratory viruses (not causing fever) in the population. In addition, studies limited to adults describe relatively low percentages of RSV and PIV infections and relatively high percentages of influenza virus compared to studies among children. The majority of hospital-based studies are limited to patients suffering from pneumonia, excluding those patients who are hospitalised with other severe ARI[131,147,148,251,279].Third, diagnostic methods used might underestimate the true incidence of the particular pathogen. As mentioned above, virus isolation is used as the gold standard, but in combination with IF, antibody detection and PCR techniques detection rates of viral pathogens can be increased[131].

There are few reports describing the detection of all known aetiological agents of ARI in the same set of patients, and even fewer studies have combined different diagnostic techniques to identify the maximum number of pathogens. One of the most comprehensive studies on the causative pathogens (both viral and non-viral) of community-acquired pneumonia identified a causative agent in 85% of the patients[131]. This hospital-based study used a combination of conventional culture with IF, PCR assays and serological tests and detected 62% of the patients with a viral, 53% with a bacterial and 30% with a mixed infection (Figure 2) In another study among patients attending their physician for ARI, a causative agent was identified in 60% of the cases, using conventional culture, IF and PCR techniques (Figure 2)[109]. Despite the use of combinations of diagnostic assays and targeting at both bacteria and viruses, 15-50% of the patients thus remain undiagnosed. This may be due to reasons such as inappropriate sample collection, transport, storage and processing, or to poor sensitivities of the diagnostic tests. However, unidentified aetiological agents may account for a substantial percentage of the undiagnosed cases of ARI.

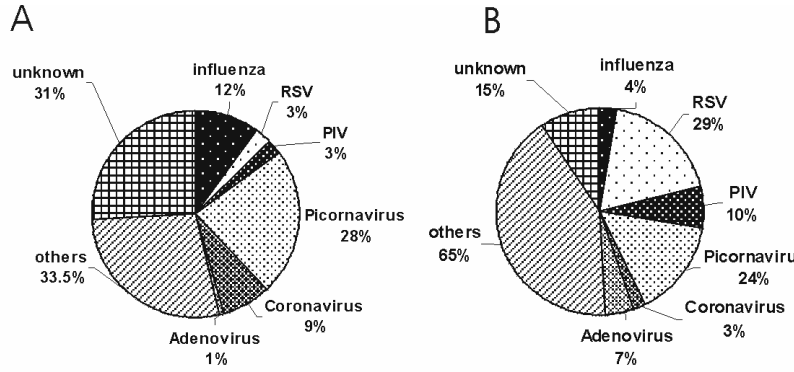

Figure 2:
Percentages of viruses and other agents detected in A) 540 patients visiting their physician for ARI[109] and B) 254 hospitalised patients with pneumonia[131]

ARI in the general community.

The most prevalent acute respiratory tract illnesses in the general community are generally benign, self-limiting events following primarily infections of the URT[169]. These illnesses, often referred to as common colds, are the leading cause of patient visits to physicians, as well as work and school absenteeism. Primary pathogens associated with common colds are the rhinoviruses and coronaviruses[160,171]. URT infection may precede LRT illnesses of which bronchitis, bronchiolitis and pneumonia are the most common clinical manifestations. These manifestations are usually mild and self-limiting, but can be severe enough to require the attention of a physician or admission to hospital.

Viral ARI affect persons of all ages, but their incidence is higher among children than among adults. The incidence in adults is 2 to 4 colds per year and in children 6 to 8 per year[173,253]. In 1998, about 3.5 million deaths occurred worldwide from ARI, of which 55% were children under the age of five years old (WHO 1999, http://www.who.int/infectious-disease-report/pages/graph5). Bronchiolitis, one of the main clinical syndromes in hospitalised children, accounts for up to 60% of all LRT illnesses during the first year of life and for up to 32% of hospitalisations for LRT illnesses in this age group[144]**.** The main causative agents of severe LRT in children were, until recently, RSV, followed by PIV, influenza viruses and adenoviruses**.**

ARI in adults are responsible for a large percentage of physician office visits, days lost from work, and for a smaller percentage of the utilisation of emergency centre facilities, and hospitalisations. Adults with ARI usually suffer from common cold like symptoms and are rarely in need of medical attention. Epidemiological studies among patients who are visiting their physician, have shown that rhinoviruses and influenza virus are detected most frequently[109,147,148,171].

ARI in the elderly

Respiratory viral infections have been recognised as important contributors to death and disability in older adults in the community or in long-term care facilities. Waning immunity and decreasing immunocompetence may play a crucial role, but the specific reasons for the enhanced disease associated with ARI in the elderly is still subject of research. Influenza has received the greatest attention and remains a significant problem in the elderly. Although only about one-fourth of hospitalisations for influenza occur in individuals over the age of 65, 75% of influenza-associated deaths occur in this age group. RSV and PIV have also been recognised as cause of serious illness in these individuals, especially among institutionalised elderly. The severity of RSV infection in these patients is variable; serious complications are well-recognised with rates of pneumonia ranging from 5 to 67%, and mortality from 0-53%[78,250].

ARI in hospitalised patients

Severe LRT illnesses such as bronchiolitis, bronchitis and pneumonia are the most prevalent clinical manifestations for ARI-associated hospitalisation. Children constitute the largest group of such hospitalised patients, followed by elderly and adults with underlying disease. In North America each year approximately 3% of all children under the age of 1 are admitted to the hospital with moderate to severe LRT infections[21,227]. RSV is detected in 50-80% of these children, with the high percentages detected during the winter months[227]. Epidemiological studies among hospitalised patients suffering from ARI demonstrated that RSV and occasionally influenza viruses are the leading cause of viral ARI, followed by PIV and adenoviruses, although these numbers depend on the study population and clinical illnesses studied[131,235,251]. In a substantial proportion of these patients however, no aetiological agent is identified.

ARI in high risk patients

Next to frail elderly, patients with weakened immunity due to for instance intensive chemotherapy, corticosteroid use, or iatrogenic immunosuppression for bone marrow or solid organ transplantation have been identified as being at the highest risk for developing severe ARI. In addition, patients with chronic lung conditions such as asthma or chronic obstructive pulmonary disease and patients with congenital heart disease are a well-defined risk groups for severe ARI. Viral respiratory infections among immunocompromised patients are characterised by persistence of infection and viral shedding beyond the time period reported for immunocompetent patients, and high frequencies of pneumonia and death in association with the infection[55,83,113]. The types of viral respiratory infections in these patients often correspond with those circulating in the community at that particular time point[97]. The clinical illnesses range from self-limited upper respiratory illness to fatal pneumonia, depending on the type of virus and the nature of immunosuppression. The direct cause of pneumonia may be viral, bacterial/fungal, or mixed. The highest frequency of progression to fatal viral pneumonia has been reported for RSV infections in recently transplanted bone marrow transplant recipients and immunosuppressed patients with leukaemia[269]. Next to RSV, and PIV, influenza virus infections can be especially severe in immunocompromised patients, with progression to pneumonia observed in over 50% of patients and mortality rates of 28-75% [20,78,153,270].

ARI and asthma

Acute respiratory, wheezing illnesses (bronchiolitis, wheezy bronchitis and asthma) are the major causes of hospitalisation of children. Asthma is defined as recurrent episodes of airway obstruction caused by inflammation of the airway and increased susceptibility to bronchoconstriction in response to irritants or allergens (airway hyperresponsiveness) [94]. Many respiratory viruses may provoke acute asthma symptoms, but rhinoviruses are most often implicated, especially during spring and fall[93,206]. In fact, peaks in hospitalisations for asthma closely coincide with patterns of rhinovirus isolation within the community[126]. A possible link between RSV-induced wheezing in infancy and later development of persistent asthma is still the subject of discussion in the RSV field[115,232,238]. ARI may produce dual effects pertaining to asthma. First, it has been suggested that vigorous stimulation of the immune system by a severe viral or bacterial infection can affect the development of the immune system and modify the subsequent risk of allergy or asthma. Secondly, in children and adults with established asthma, viruses such as rhinovirus and RSV may trigger acute symptoms of asthma. The precise direct and indirect mechanisms of viral contribution to inception and exacerbation of asthma remain to be elucidated[92,252].

Scope of the present thesis

Over the last 20 years, the virus diagnostic laboratory of Erasmus Medical Centre collected specimens from patients suffering from ARI, in which no pathogen could be detected. These samples were obtained primarily in the winter months and the majority of the samples were collected from young children suffering from clinical illnesses resembling those caused by RSV. Upon inoculation of in-vitro cell cultures with a number of these clinical specimens, cytopathic effects (CPE) were observed resembling the CPE caused by RSV. A variety of diagnostic assays failed to detect any known respiratory virus. Examination of these viral cultures under the electron microscope revealed virus particles with a paramyxovirus-like structure, and classical virological techniques revealed that the virus contained an envelope, and did not agglutinate erythrocytes.

We identified this virus as a new paramyxovirus within the genus *Metapneumovirus* of the subfamily *Pneumovirinae* (chapters 2 and 3). Based on this proposed classification we named the virus human metapneumovirus (hMPV). Chapter 4 describes the genetic and antigenic variation of hMPV, revealing the presence of two serotypes of hMPV. To collect evidence that this virus is an aetiologic agent of ARI, we studied hMPV infections in cynomolgus macaques (chapter 5). After identification and characterisation of the virus, studies were undertaken to investigate the prevalence and clinical impact of the virus in patients suffering from ARI (Chapters 6, 7, 8). In chapter 9 the findings presented in this thesis, and results from other groups that followed-up on our initial discovery, are discussed.

2

A newly discovered human pneumovirus isolated from young children with respiratory tract disease

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Abstract

From 28 young children in The Netherlands, we isolated a paramyxovirus that was identified as a tentative new member of the *Metapneumovirus* genus based on virological data, sequence homology and gene constellation. Previously, avian pneumovirus was the sole member of this recently assigned genus, hence the provisional name for the newly discovered virus: human metapneumovirus. The clinical signs of the children from whom the virus was isolated were similar to those caused by human respiratory syncytial virus infection, ranging from upper respiratory tract disease to severe bronchiolitis and pneumonia. Serological studies showed that by the age of five years, virtually all children in The Netherlands have been exposed to human metapneumovirus and that the virus has been circulating in humans for at least 50 years.

Introduction

The *Paramyxovirinae* and the *Pneumovirinae* subfamilies of the *Paramyxoviridae* family include several major pathogens of man and animals. The *Pneumovirinae* are taxonomically divided in the *Pneumovirus* and the *Metapneumovirus* genera[118]. Human respiratory syncytial virus (hRSV), the type species of the *Pneumovirus* genus, is the single most important cause of lower respiratory tract infections (RTIs) during infancy and early childhood worldwide[65,226]. Other members of the *Pneumovirus* genus include bovine respiratory syncytial virus (bRSV), ovine respiratory syncytial virus (oRSV) and pneumonia virus of mice (PVM). Avian pneumovirus (APV), previously known as turkey rhinotracheitis virus (TRTV), is the aetiologic agent of an upper RTI of turkeys[50,96] and the sole member of the recently assigned *Metapneumovirus* genus[118]. Until now, metapneumoviruses have not been associated with infections or disease in mammals. The classification of the two genera is based primarily on their gene constellation: metapneumoviruses lack non-structural proteins NS1 and NS2 and the gene order is different from that of pneumoviruses (RSV: '3-NS1-NS2-N-P-M-SH-G-F-M2-L-5'; APV: '3-N-P-M-F-M2-SH-G-L-5')[151,204,282].

In the past decades, many aetiologic agents of respiratory tract illnesses (RTI) have been identified[76,142]. However, a proportion of these illnesses can still not be attributed to known pathogens. We have now isolated a previously undiscovered paramyxovirus from nasopharyngeal aspirate samples taken from 28 epidemiologically unrelated children in The Netherlands suffering from RTI during the past 20 years. Of the patients, 27 were below the age of five years and 13 of these were infants between the ages 0 and 12 months old. The clinical symptoms of these children were largely similar to the RTI caused by hRSV, ranging from mild respiratory problems to severe cough, bronchiolitis and pneumonia, often accompanied by high fever, myalgia and vomiting. Some of these patients were hospitalised and needed mechanical ventilation. The virus isolates exhibited a paramyxovirus-like morphology in negative contrast electron microscopy and were closely related both antigenically and genetically. Based on sequence homology and gene constellation, the viruses seemed to be a tentative new member of the *Metapneumovirus* genus that we have provisionally named human metapneumovirus (hMPV). Serological surveys showed that by the age of five years virtually all children in The Netherlands have been exposed to hMPV and that the virus has been circulating in humans for at least half a century.

Results

Virus isolation and characterisation

Within our diagnostic virology setting, we isolated 28 unidentifiable viruses from patients in The Netherlands with RTI over the past 20 years. Of the 26 patients for whom personal information was available, eight (31%) were females, 13 (50%) were between the age of 0 and 12 months old, and 12 (46 %) were between 1 and 5 years. All viruses were isolated from samples collected in the winter months. The unidentified virus isolates replicated slowly in tertiary monkey kidney (tMK) cells, very poorly in Vero cells and A549 cells and could not be propagated in Madin Darby kidney (MDCK) cells or chicken embryo fibroblasts (CEF). The cytopathic effects induced by these virus isolates were virtually indistinguishable from those caused by hRSV, with characteristic syncytia formation followed by rapid internal disruption of the cells and subsequent detachment from the culture plate. The cells usually displayed cytopathic effects at day 10-14 post inoculation, slightly later than the cytopathic effects induced by hRSV-containing specimens (data not shown). After 14 days of virus propagation, the viral titers in the cultures were on average 10,000 $TCID_{50}$ (50% tissue culture infectious doses) per ml. Supernatants of infected tMK cells were used for electron microscopy analysis, which revealed the presence of paramyxovirus-like pleiomorphic particles in the range of 150-600 nm, with short envelope projections in the range of 13-17 nm (Figure 1). Similar to some other paramyxoviruses such as selected hRSV and parainfluenza virus isolates, nucleocapsids were rarely observed.

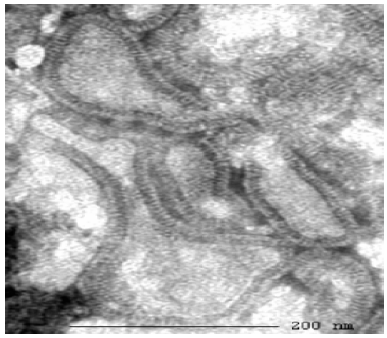

Figure 1:
Electron micrograph of hMPV particles. Virus concentrated from infected tMK-cell culture supernatants were visualised by negative contrast electron microscopy after PTA staining.
Magnification, x 92,000.

Consistent with the biochemical properties of enveloped viruses such as the *Paramyxoviridae*, standard chloroform treatment[187] resulted in 10,000-fold reduction of the $TCID_{50}$ for tMK cells. Virus-infected tMK cell-culture supernatants did not display haemagglutinating activity with turkey, chicken or guinea pig erythrocytes. In tMK cell cultures, virus replication was found to be dependent on trypsin. These combined virological data indicated that the newly identified virus is a member of the *Paramyxoviridae* family.

We isolated RNA from the supernatant of tMK cells infected with 15 of the unidentified virus isolates for reverse transcription (RT)-PCR analyses using primer sets specific for known paramyxoviruses (human parainfluenza virus (hPIV) types 1-4, mumps virus, measles virus, hRSV, simian virus type 5 (SV-5), Sendai virus and Newcastle disease virus (NDV)). We carried out RT-PCR assays at low stringency in order to detect potentially related viruses and used RNA isolated from homologous virus stocks as positive controls. Whereas the controls all reacted positive with the respective virus-specific primers, the newly identified

virus isolates did not (data not shown), indicating the virus was not closely related to the viruses for which primer sets had been selected.
We next inoculated ferrets and guinea pigs intranasally with two of the virus isolates (without prior purification) to raise virus-specific antisera. The animals did not display any clinical symptoms. The antisera did not react in immune fluorescence assays (IFA) with cells infected with a panel of paramyxoviruses and orthomyxoviruses (hPIV types 1-4, hRSV, influenza virus type A and B). All animals seroconverted as shown in virus neutralisation assays and IFA against the homologous viruses. We next tested the 28 unidentified virus isolates with the guinea pig and ferret pre- and post-infection sera. tMK cell cultures infected with each of the 28 virus isolates reacted positive in IFA with the post-infection animal sera, indicating that they were serologically related or identical.

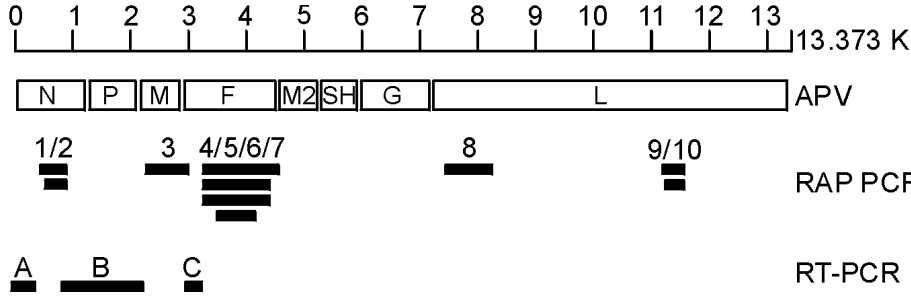

Figure 2:
Schematic representation of genomic fragments obtained from hMPV. A schematic diagram of APV (3'to 5'end, left to right) is shown above the fragments obtained with RAP-PCR and RT-PCR on virus isolate 00-1. Fragments 1-10 were obtained using RAP-PCR. Fragment A was obtained with a primer in RAP-PCR fragment 1 and 2 and a primer designed on basis of alignment of leader and trailer sequences of APV and RSV[204]. Fragment B was obtained with primers designed in RAP-PCR fragments 1 and 2 and RAP-PCR fragment 3. Fragment C was obtained with primers designed in RAP-PCR fragment 3 and RAP-PCR fragments 4-7.

RAP-PCR
To obtain sequence information on the unknown virus isolates, we used a random PCR amplification strategy known as RAP-PCR[203,267]. To this end, we infected tMK cells with one of the virus isolates (isolate 00-1) or with hPIV-1 that served as a control. We sequenced 20 differentially displayed bands specific for the unidentified virus. When we used the deduced amino acid sequences to search for homologies against sequences in the Genbank database using the BLAST software (www.ncbi.nlm.nih.gov/BLAST), 10 out of 20 fragments displayed resemblance to APV/TRTV sequences. These 10 fragments were located in the genes coding for the nucleoprotein (N; fragments 1 and 2), the matrix protein (M; fragment 3), the fusion protein (F; fragments 4, 5, 6, 7,) and the polymerase protein (L; fragments 8, 9

and 10) (Figure 2). We can exclude the possibility of contamination of the human samples with APV/TRTV as we do not have this virus in our laboratories. We next designed PCR primers to complete the sequence information for the 3' end of the viral genome based on our RAP-PCR fragments as well as published leader and trailer sequences for the *Pneumovirinae*[204] (Figure 2).

Figure 3 :
Nucleotide and deduced amino-acid sequences for selected regions of the hMPV genome. *A* and *B,* The nucleotide sequences for the 3'end of the viral genome and the intergenic region between M and F ORFs. Note that the underlined sequence in A refers to the primer used for PCR amplification, and therefore does not necessarily reflect the actual hMPV leader squence. C-F, Comparison of the amino acid sequences of the putative N (panel C), P (panel D), M (panel E) and F (panel F) ORFs of hMPV, aligned with those of APV and RSV. Residues that differ between isolate 00-1 and the other viruses are shown, identical amino acids are represented by periods, gaps are represented by dashes.

In total, we have generated 5.7 kilobases of sequence information of the viral genome. Analyses of the sequences of these fragments revealed the absence of NS1 and NS2 open reading frames (ORFs) at the extreme 3' end of the viral genome (Figure 3A) and positioning of the putative F ORF immediately adjacent to the putative M ORF (Figure 3B). This genomic organisation resembles that of the metapneumovirus APV, which is consistent with the sequence homology. It should be noted however that the genomic organisation of hMPV cannot be simply deduced from that of APV, and requires further analysis. The translated sequences for the N, P, M and F ORFs were aligned with the amino-acid sequences of hRSV and APV (Figure 3 C-F). For N, these alignments revealed a 20% homology with hRSV and 52% homology with APV. The amino-acid sequences for P revealed 25% homology with hRSV and 67% with APV; for M, 37% with hRSV and 87% with APV; and for F, 32% with hRSV and 80% homology with APV.

Phylogeny

As an indication for the relationship between the newly identified virus isolates and members of the *Pneumovirinae*, phylogenetic trees were constructed based on the N, P, M and F ORFs of these viruses. In all four phylogenetic trees, the newly identified virus isolate was most closely related to APV (Figure 4). From the four serotypes of APV that have been described [11,51], APV serotype C, the metapneumovirus found primarily in birds of the United States, showed the closest resemblance to the newly identified virus; however, only limited sequence information for APV serotype D is available.

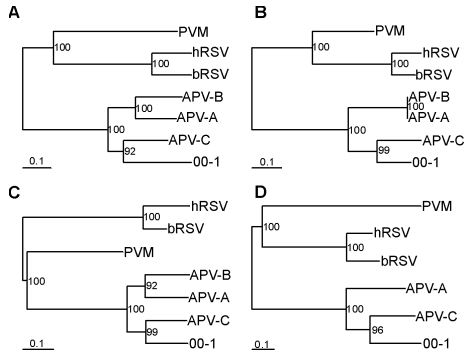

Figure 4:
Phylogenetic analysis of ORFs of hMPV and selected pneumoviruses. A-D, The F (panel A), N (panel B), M (panel C), and P (panel D) ORFs of hMPV isolate 00-1 were aligned with those of other members of the genus *Pneumovirinae*, and phylogenetic trees were generated by maximum likelihood analyses using 100 bootstraps and 3 jumbles. The scale representing the number of nucleotide changes is shown for each tree. Bootstrap values are based on the consensus trees.

Experimental infection of birds and cynomolgus macaques

Since hMPV appeared to be closely related to APV, we wished to test whether hMPV is a primary human pathogen or an avian pathogen that can also infect humans. We inoculated four juvenile turkeys, four juvenile chickens and four juvenile cynomolgus macaques with 50,000 $TCID_{50}$ of hMPV on their conjunctivae and in their respiratory tracts. We collected cloaca and/or throat samples and tested them for the presence of hMPV by RT-PCR. During the 3 weeks follow-up, none of the birds showed clinical signs or virus replication as determined by RT-PCR using RNA isolated from the throat and cloaca swabs. In contrast, the virus replicated efficiently in the respiratory tract of all four monkeys as shown by RT-PCR of RNA isolated from the throat swabs. Whereas vial RNA was not detectable in samples taken at day 1, virus replication peaked between days 2 and 8 after inoculation. Two of the monkeys presented with mild upper respiratory tract signs that upon histological analysis proved to be associated with suppurative rhinitis (data not shown). Although the detailed pathological, virological and immunological analyses are incomplete at present, these preliminary data indicate that hMPV is a primate pathogen associated with respiratory disease.

Genetic heterogeneity of hMPV isolates

We next amplified small parts of the N, M, F and L ORFs (71, 143, 142 and 102 nucleotides respectively) for 9 of the 28 virus isolates by RT-PCR, then sequenced these fragments directly to minimize the possibility of errors introduced by the amplification procedure. Phylogenetic analyses revealed significant sequence variation and the existence of two

potential genetic clusters of hMPV isolates obtained in The Netherlands (Figure 5). The nucleotide sequence identity between isolates within a cluster was 90-100 % for all four fragments, whereas the sequence identity for isolates between the two clusters was 81-88 %. Variation was similar for the N, M, F and L fragments and did not seem to correlate with the year of virus isolation (indicated by the first to numbers in the name of the isolates). The genetic clusters might represent subgroups of the newly identified virus in analogy to other pneumoviruses such as RSV.

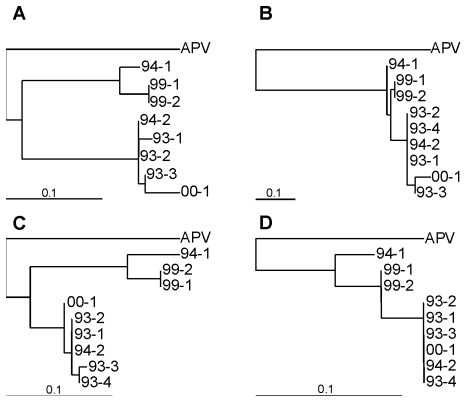

Figure 5:
Phylogenetic analysis of 9 hMPV isolates. A-D, Parts of the F (panel A), N (panel B), M (panel C) and L (panel D) ORFs of the indicated hMPV isolates were amplified by PCR and sequenced directly (fragments of 142, 71, 143, and 102 nucleotides, respectively). Phylogenetic trees were generated as described in Figure 4. The first 2 numbers in the isolate names refer to the year from which the isolate originated. We were unable to amplify the F fragent for isolate 93-4.

Seroprevalence

To study the prevalence of this virus in the human population, we tested sera from humans in different age categories by indirect IFA using tMK cells infected with one of the unidentified virus isolates. This analysis revealed that 25% of the children between 6 and 12 months had antibodies to the virus, and towards the age of 5 virtually all of the children had become seropositive (Table 1). In addition, 56 serum samples tested by indirect IFA were tested by virus neutralisation assays. For 51 (91%) of the samples, the results of the virus neutralisation assay (titer >8) correlated with the results obtained with indirect IFA (titer>32). Four samples that were positive in IFA (titer >32) were negative by virus neutralisation tests (titer <8), and one serum sample that reacted negative in IFA (titer<32) was positive in the virus neutralisation test (titer >16). Indirect IFA conducted with 72 sera taken from humans in 1958 (ages 8-99 years)[162,177] revealed a 100% seroprevalence, indicating that the virus has been circulating in the human population for more than 43 years. In addition, testing of a number of these sera in virus neutralisation assays confirmed the IFA data (Table 1).

The range of antibody titers appeared higher for individuals older than 2 years as compared to children between 6 and 24 months. This observation could be related to boosting of antibody responses as a consequence of reinfection with the same or a closely related virus. However, more detailed serological and virological analyses will be required to substantiate this speculation.

Table 1: Seroprevalence of hMPV antibodies in humans categorised by age group.

	Immunofluorescence assays		Virus neutralisation assays		
Age (Years)	n tested	n positive (%)	n tested	n positive (%)	Titer range
0.5-1	20	5 (25)	12	3 (25)	16-32
1-2	20	11 (55)	13	4 (31)	16-32
2-5	20	14 (70)	8	3 (38)	16-512
5-10	20	20 (100)	4	4 (100)	32-256
10-20	20	20 (100)	4	3 (75)	32-128
> 20	20	20 (100)	4	3 (75)	32-128
8-99*	72	72 (100)	11	11 (100)	16-128

*, Sero-archeological analysis using sera collected in 1958 (Mulder, J. *et al*., 1958; Masurel, N. *et al*., 1969)

Discussion

Here, we describe the characterisation of a new member in the *Paramyxoviridae* family, isolated from 28 children with RTI in The Netherlands. The children presented with clinical symptoms reminiscent of those caused by hRSV infection, ranging form mild upper respiratory tract disease to severe bronchiolitis and pneumonia. As is seen with hRSV-infected children, very young hMPV-infected children appeared to require hospitalisation and mechanical ventilation. Whether hMPV infection causes RTI cannot be formally concluded from the present data. Most importantly, acute and convalescent paired sera were not available for the children from whom hMPV was isolated. However, considering that no other virus was identified in any of the 28 hMPV-infected children and that hMPV was not isolated from 400 samples taken from children under two years of age without respiratory symptoms (not shown), a causal relationship seems likely. The experimental infection of cynomolgus macaques supports this conclusion.

Based on the data here we propose the name human metapneumovirus (hMPV). hMPV proved to have a paramyxovirus-like morphology in electron microscopy, to be chloroform-sensitive and to replicate optimally in a trypsin-dependent manner in tMK cells. The clinical symptoms of the hMPV- infected children, the characteristic cytopathic effects and the lack of haemagglutinating activity further suggested that this virus might be related to hRSV; although most paramyxoviruses have haemagglutinating activity, most of the pneumoviruses do not[199]. Genetic analyses of the putative N, M, P and F genes revealed that hMPV showed a higher sequence homology to the recently proposed genus *Metapneumovirus* (average of 66%) as compared to the genus *Pneumovirus* (average of 30%) and a genomic organisation that is most similar to APV/TRTV. In contrast to the genomic organisation of the pneumoviruses ('3-NS1-NS2-N-P-M-SH-G-F-M2-L-5'), metapneumoviruses lack NS1 and

NS2 genes and have a different positioning of the genes between M and L ('3-N-P-M-F-M2-SH-G-L-5'). The absence of ORFs between the M and F genes in this virus and the lack of NS1 and NS2 genes adjacent to N is in agreement with it being the first identified non-avian member of the *Metapneumovirus* genus. However, until sequence information becomes available for the entire genomic organisation of hMPV, a definitive classification of this virus is not possible.

Of the four serotypes of APV that have been described, serotype C was most closely related to hMPV based on the N, P, M and F genes. However, for serotype D only limited sequence information for the F gene is available from Genbank and for serotype B, only M, N and F sequences are available. For both hRSV and APV, different genetic and serological subtypes have been described. We also found evidence for genetic variation between hMPV isolates. Although it is tempting to speculate that these subgroups of hMPV isolates represent different serotypes of hMPV, sequence analyses of other parts of the hMPV genome and of isolates obtained from different geographical locations as well as detailed serological studies will be needed to support this hypothesis.

Our serological survey revealed that hMPV commonly infects children. The repeated isolation of this virus from clinical samples from children with RTI indicates that hMPV infection may have considerable clinical and economical impact. New diagnostic assays based on virus detection and serology should allow a more detailed analysis of the incidence and both clinical and economical impact of this newly identified human virus. To get an idea of the potential impact of this virus for RTI we have tested nasopharyngeal aspirate sample collected during the 2000 winter season from children presenting with RTI, at Erasmus University Medical Center that were negative for RSV, influenza A and B viruses, parainfluenza virus types 1-3, human rhinovirus and corona virus (HCV-229E and HCV-OC43). Seven of the 68 samples tested proved to be positive for hMPV by RT-PCR, indicating that for the 2000 winter season approximately 10% of the unexplained RTI may be associated with hMPV.

The minor differences between our IFA and virus neutralisation results (5 samples) may be due to several factors: only IgG serum antibodies are detected in IFA, virus neutralisation antibodies are generally directed against surface glycoproteins, the sensitivities of the two assays may differ and different serotypes of hMPV may exist. The latter suggestion is of particular interest, since only one isolate (00-1) was used for IFA and virus neutralisation, potentially giving rise to false negative results using sera raised against genetically distinct hMPV isolates such as those shown in Figure 5.

As hMPV seems most closely related to APV, we speculate that the human virus has originated from birds. Analysis of serum samples taken from humans in 1958 revealed that hMPV has been widespread in the human population for at least half a century, indicating that a possible zoonotic event must have taken place before 1958.

The question than arises: Why has this virus remained unidentified for so many years? First of all, many virology laboratories use continuous cell lines for virus isolation, in which hMPV does not seem to replicate efficiently. Second, hMPV displays very slow replication kinetics *in vitro*, and may therefore have remained undetected. Third, replication of hMPV *in-vitro* appeared to be trypsin dependent and many of the earlier studies to find respiratory pathogens did not use trypsin. Finally, the apparent lack of cross-reactivity in the serologic assays we performed and the low nucleotide sequence homology to known human viral pathogens make the detection of hMPV unlikely with standard serological or molecular

techniques. Pringle and Eglin have described serological evidence for an unidentified "PVM-like" pneumovirus that frequently infects humans[201]. We feel it is unlikely that hMPV is the PVM-like-virus described by Pringle and Eglin for a number of reasons. First, high titer sera specific for several paramyxoviruses, including PVM and RSV, failed to react in immunofluorescence studies with hMPV-infected tMK cells as targets (data not shown). Second, most of our hMPV-positive human serum samples did not have PVM-specific antibodies as measured by ELISA. Furthermore, PVM (and supposedly a PVM-like virus) has haemagglutinating activity, whereas we have no evidence that hMPV does. Finally, the genomic organisation of PVM is similar to that of RSV and thus different from that of hMPV.
The identification of this tentative new member of the *Metapneumovirus* genus may aid in the development of diagnostic assays, vaccines and antiviral agents for viral RTIs.

Methods

Virus isolation
Over the past decades our virus diagnostic hospital-based laboratory has collected nasopharyngeal aspirates from children suffering from RTI. These samples were routinely tested for the presence of viruses by direct immune fluorescence assays (DIF) using antibodies against viruses known to cause RTI (hPIV types 1-3, hRSV, influenza virus type A and B) and by virus isolation using rapid shell-vial techniques on tMK cells and CEF, as well as various cell lines such as Vero, A549 and MDCK cells[23,215]. Samples showing cytopathic effects after two or three passages that were negative in DIF were tested by indirect IFA using virus-specific antibodies against influenza virus types A, B and C, hRSV, hPIV types 1-4, measles virus, mumps virus, Sendai virus, SV-5, and NDV. Although for many cases the aetiologic agent could be identified, some specimens yielded cytopathic effects in cell cultures but were negative for all viruses tested.

Animal immunisation
To raise virus-specific antisera, 1.0 ml volumes of virus-infected tMK cell- culture supernatants of two different virus isolates containing approximately 1 x 10^4 $TCID_{50/}$ ml were used to inoculate guinea pigs and ferrets intranasally without prior purification. Samples were taken at day 0 and at day 14 or 28 post infection the animals were bled by cardiac puncture, and their pre-and post-infection sera were used as negative and positive reference sera respectively. The sera were tested for reactivity against all previously described viruses with DIF and IFA as described above.

Virus characterisation
We cultured the virus isolates in tMK cells in Eagle's MEM with Hanks's salt, supplemented with 0.02% trypsin and without serum. After inoculation, the plates were incubated at 37 °C for a maximum of 14 days during which time the medium was changed once a week and cultures were checked daily for cytopathic effects. Because the degree of cytopathic effects differed slightly for each isolate, all cultures were tested at days 10-14 with indirect IFA using ferret antisera raised against one of the isolates. For electron microscopy analyses, virus was concentrated from infected cell-culture supernatants in a micro-centrifuge at 4 °C

at 17,000*g*, after which the pellet was resuspended in PBS and after potassium tungstat acid (PTA) staining, inspected by negative contrast electron microscopy[156]. Standard haemagglutination assays, chloroform sensitivity tests, DIF and IFA were performed as described[23,145,187,215]. Virus neutralisation assays were performed on tMK cells with serial 2-fold dilutions of human or animal sera (starting at 1:8) and with 100 $TCID_{50}$ of hMPV, following procedures as described[145]. Since cytopathic effects were not always easy to detect, IFA was used to determine the virus neutralisation titer. The virus neutralisation titer was defined as the reciprocal of the highest dilution of the serum sample resulting in negative IFA and inhibition of cytopathic effects in cell cultures.

RAP-PCR and RT-PCR

RNA was isolated from the supernatant of infected cell cultures or sucrose gradient fractions using a High Pure RNA Isolating kit according to instructions from the manufacturer (Roche Diagnostics, Almere, The Netherlands). RT-PCR assays were performed according to established techniques. Primers specific for the panel of paramyxoviruses tested and conditions are available upon request. For RAP-PCR, virus was concentrated from infected tMK-cell supernatants by ultracentrifugation on a 20-60% sucrose gradient. Gradient fractions were inspected for the presence of virus-like particles by electron microscopy and poly-acrylamide gel electrophoresis followed by silver staining. The approximately 50% sucrose fractions that appeared to contain nucleocapsids were used for RNA isolation and RAP-PCR. RAP-PCR was performed essentially as described[203,267] and primer sequences used are available upon request. Differentially displayed fragments specific for hMPV were purified from the gel with a Qiaquick Gel Extraction kit (Qiagen, Leusden, The Netherlands) and cloned in vector pCR2.1 (Invitrogen, Groningen, The Netherlands) according to instructions from the manufacturer.

Sequence analysis

RAP-PCR products cloned in vector pCR2.1 were sequenced with M13-specific oligonucleotides. DNA fragments obtained by RT-PCR were purified from agarose gels using a Qiaquick Gel Extraction kit (Qiagen, Leusden, The Netherlands) and sequenced directly with the same oligonucleotides used for RT-PCR assays. Sequence analyses were performed using a Dyenamic ET terminator sequencing kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and an ABI 373 automatic DNA sequencer (PE Applied Biosystem, Nieuwerkerk aan den IJssel, The Netherlands), according to the instructions of the manufacturer.

Phylogenetic analyses

For all phylogenetic trees, DNA sequences were aligned using the ClustalW software package and maximum likelihood trees were generated using the DNA-ML software package of the Phylip 3.5 program using 100 bootstraps and 3 jumbles. Bootstrap values were computed for consensus trees created with the consense package[82]. hMPV sequences are available from Genbank under accession numbers AF371330 through AF371367. All other sequences used here are available from Genbank under accession numbers NC001781 (hRSV all ORFs); NC001989 (bRSV all ORFs); D10331 (PVM N); U39295 (APV-A N); U39296 (APV-B N); AF176590 (APV-C N); U09649 (PVM P); U22110 (APV-A P); AF176591 (APV-C P); U66893 (PVM M); X58639 (APV-A M); U37586 (APV-B M); AF262571 (APV-C M); D11128 (PVM F); D00850 (APV-A F); Y14292 (APV-B F); AF187152 (APV-C F).

Acknowledgments

We thank M.H. Malim and R. Oakey for providing oligonucleotides and for stimulating discussions on RAP-PCR and J. Habova, T. Bestebroer, B. Niemeyer, G. Aaron and C. Burghoorn for excellent technical assistance. R.F. is a fellow of the Royal Dutch Academy of Arts and Sciences.

3

Analysis of the genomic sequence of a human metapneumovirus

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Abstract

We recently described the isolation of a novel paramyxovirus from children with respiratory tract disease in The Netherlands. Based on biological properties and limited sequence information the virus was provisionally classified as the first non-avian member of the *Metapneumovirus* genus and named human metapneumovirus (hMPV). This report describes the analysis of the sequences of all hMPV open reading frames (ORFs) and intergenic sequences as well as partial sequences of the genomic termini. The overall percentage amino acid sequence identity between APV and hMPV N, P, M, F, M2-1, M2-2 and L ORFs was 56 to 88%. Some nucleotide sequence identity was also found between the noncoding regions of the APV and hMPV genomes. Although no discernable amino acid sequence identity was found between two of the ORFs of hMPV and ORFs of other paramyxoviruses, the amino acid content, hydrophilicity profiles and location of these ORFs in the viral genome suggest that they represent SH and G proteins. The high percentage sequence identity between APV and hMPV, their similar genomic organisation (3'-N-P-M-F-M2-SH-G-L-5'), and phylogenetic analyses provide evidence for the proposed classification of hMPV as the first mammalian metapneumovirus.

Introduction

Recently we reported the isolation of human metapneumovirus (hMPV) from nasopharyngeal aspirate samples taken from young children in The Netherlands[256]. The clinical symptoms of these children were largely similar to the respiratory tract illnesses caused by the human respiratory syncytial virus (hRSV), ranging from mild respiratory problems to severe cough, bronchiolitis and pneumonia. The newly discovered virus displayed high percentage sequence identity with and probably a genomic organisation similar to avian pneumovirus (APV), the aetiologic agent of an upper respiratory tract disease in turkeys[50,96]. APV, also known as turkey rhinotracheitis virus (TRTV) belongs to the *Metapneumovirus* genus, which together with the *Pneumovirus* genus constitutes the *Pneumovirinae* subfamily within the *Paramyxoviridae* family[118]. The *Pneumovirus* genus contains the mammalian respiratory syncytial viruses (human, ovine, bovine RSV) and pneumonia virus of mice (PVM). Until the discovery of hMPV, APV was the sole member of the *Metapneumovirus* genus. The classification of the two genera is based primarily on the gene constellation; metapneumoviruses lack nonstructural proteins NS1 and NS2 and the gene order is different from that of pneumoviruses (RSV, 3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5'; APV, 3'-N-P-M-F-M2-SH-G-L-5')[42,151,204,282].

Sequence analyses of the nucleoprotein (N), phosphoprotein (P), matrix protein (M) and fusion protein (F) genes of hMPV revealed the highest percentage of sequence identity with APV serotype C, the avian pneumovirus found primarily in birds in the United States. These analyses also revealed the absence of NS1 and NS2 at the 3' end of the viral genome and positioning of F immediately adjacent to M. Here we present the sequences of the putative 22K protein (M2), the small hydrophobic protein (SH), the attachment glycoprotein (G) and the large polymerase protein (L) genes, the intergenic regions, and the trailer sequence. In combination with the sequences described previously the sequences presented here complete

the genomic sequence of hMPV with the exception of the extreme 12-15 nt of the genomic termini and establish the genomic organisation of hMPV. Side by side comparisons of the sequences of the hMPV genome with those of APV subtype A, B and C, hRSV subgroup A and B, bRSV, PVM and other paramyxoviruses provides strong evidence for the classification of hMPV in the *Metapneumovirus* genus.

Results

Sequence strategy

hMPV isolate 00-1[256] was propagated in tertiary monkey kidney (tMK) cells and RNA isolated from the supernatant 3 weeks after inoculation was used as template for RT-PCR analyses. Primers were designed on the basis of the partial sequence information available for hMPV 00-1[256] as well as the leader and trailer sequences of APV and RSV[167,204]. Initially, fragments between the previously obtained products, ranging in size from 500 to 4000 bp in length, were generated by RT-PCR amplification and sequenced directly. The genomic sequence was subsequently confirmed by generating a series of overlapping RT-PCR fragments ranging in size from 500 to 800 bp that represented the entire hMPV genome. For all PCR fragments, both strands were sequenced directly to minimize amplification and sequencing errors. The nucleotide and amino acid sequences were used to search for related sequences in the Genbank database using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST). Putative protein names were assigned to open reading frames (ORFs) based on sequence identity with known viral genes as well as their location in the genome. Based on this information, a genomic map for hMPV was constructed (Figure 1). The hMPV genome was found to be 13,378 nt in length and its organisation was found to be similar to the genomic organisation of APV. It should be noted that the sequence of the genomic termini reflect primer sequences and therefore these parts of the genome are still uncertain with respect to size and sequence. Below, we present a comparison between the ORFs and noncoding sequences of hMPV and those of other paramyxoviruses and discuss the important similarities and differences.

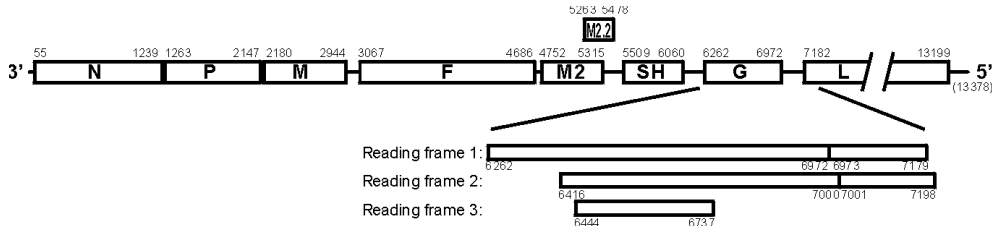

Figure 1:
Genomic map of hMPV isolate 00-1. Putative ORFs and the nt positions of the start and stop codons are indicated. The double lines crossing the L ORF indicate the shortened representation of the L gene. Note that the length of the genome is still uncertain since the sequence of the genomic termini reflects primer sequences. The three reading frames below the map indicate the primary G ORF (nt 6262-6972) and overlapping potential secondary ORFs.

Table 1: Lengths of the putative ORFs of hMPV and other paramyxoviruses

	N [a]	P	M	F	M2-1	M2-2	SH	G	L
hMPV	394	294	254	539	187	71	183	236	2005
APV A	391	278	254	538	186	73	174	391	2004
APV B	391	279	254	538	186	73	-[b]	414	-[b]
APV C	394	294	254	537	184	71	-[b]	-[b]	-[b]
APV D	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	389	-[b]
hRSV A	391	241	256	574	194	90	64	298	2165
hRSV B	391	241	256	574	195	90	65	299	2166
bRSV	391	241	256	574	186	90	81	257	2162
PVM	393	295	257	537	176	98	92	396	-[b]
Others[C]	418-542	225-709	335-393	539-565	-[d]	-[d]	-[d]	-[d]	2183-2262

a. Lengths in amino acid reidues. *b*. Sequences not available. *c*. Human parainfluenza virus types 2 and 3, Sendai virus, Measles virus, Nipah virus, phocine distemper virus, and Newcastle disease virus. *d*. ORF absent in viral genome

The nucleoprotein (N) ORF

As shown previously[256] the first gene in the genomic map of hMPV codes for a 394-aa protein, which resembles the N protein of other pneumoviruses. The length of the N ORF is identical to the length of the N ORF of APV-C (Table 1) and is smaller than those of other paramyxoviruses[8]. Analysis of the aa sequence revealed the highest percentage of sequence identity with APV-C (88%), and only 7-11% with other paramyxoviruses (Table 2).

Table 2: Amino acid sequence identity between the putative ORFs of hMPV and those of other paramyxoviruses[a]

	N	P	M	F	M2-1	M2-2	L
APV A	69	55	78	67	72	26	64
APV B	69	51	76	67	71	27	-[b]
APV C	88	68	87	81	84	56	-[b]
hRSV A	41	24	38	33	36	18	44
hRSV B	41	23	37	33	35	19	44
bRSV	7-11	22	38	34	35	13	44
PVM	41	26	37	38	34	12	-[b]
Others[c]	45	4-9	7-10	10-18	-[d]	-[d]	13-1

a. Percentage sequence identity could not be determined for the G and SH proteins because these proteins could not be properly aligned with known G and SH proteins.
b. Sequence not available. *c.* Human parainfluenza virus types 2 and 3, Sendai virus, measles virus, Nipah virus, phocine distemper virus, and Newcastle disease virus.
d. ORF absent in viral genome.

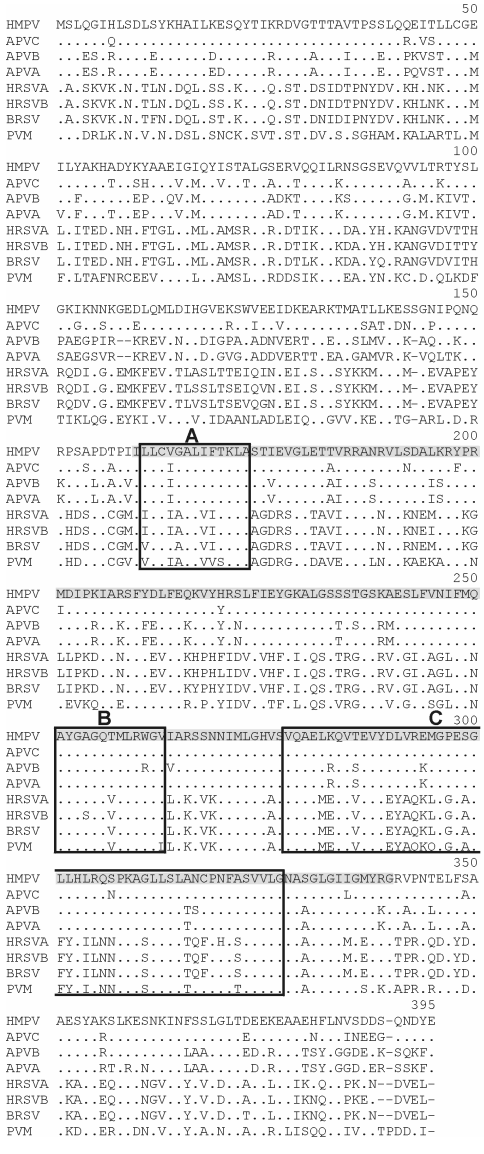

Figure 2:
Amino acid sequence comparison of the putative nucleoprotein ORF of hMPV and other pneumoviruses. The conserved regions (Barr *et al*., 1991)[8] are represented by boxes and are labeled A, B, and C. The conserved region among pneumoviruses (Li *et al*., 1996)[146] is shown shaded in gray. Gaps are represented by dashes and periods indicate the positions of identical aa residued compared to hMPV.

Barr *et al.* (1991)[8] identified 3 regions of similarity between viruses belonging to the order *Mononegavirales*: A, B and C (Figure 2). Here, aa residues are regarded as similar when they are replaced by aa residues from a group sharing some physical or chemical properties: hydrophobic/aliphatic (I, L, V, M and A); small/neutral (G, P, S, T and A); acidic/polar (D, E, Q and N); aromatic (F, Y, W and H); basic (K and R); thiol (C). Although similarities are highest within a virus family, these regions are highly conserved between virus families. In all three regions hMPV revealed 99,3% aa sequence similarity with APV-C, 98,6% with APV-A, 95,3% with APV-B, and 78-92% with RSV and PVM.
The region between aa residues 160 and 340 appears to be highly conserved among metapneumoviruses and to a somewhat lesser extent the *Pneumovirinae*[8,146,168].
This is in agreement with hMPV being a metapneumovirus, showing 99% similarity with APV-C.

The phosphoprotein (P) ORF
The second ORF in the genome map codes for a 294-aa protein which shares 68% aa sequence identity with the P protein of APV-C, and only 22-24% with the P protein of RSV (Table 2). The putative P gene of hMPV contains one substantial ORF and in that respect is similar to P from many other paramyxoviruses (Reviewed in[142,225]).
In contrast to APV-A and -B and PVM and similar to RSV and APV-C the hMPV P ORF lacks cysteine residues. The C-terminus of the hMPV P protein is rich in glutamate residues as has been described for APVs[149]. Ling *et al*. (1995)[146] suggested that a region of high similarity between all pneumoviruses (aa 185-241) plays a role either in the RNA synthesis process or in maintaining the structural integrity of the nucleocapsid complex. This conserved region may represent the domain that interacts with the polymerase protein. This

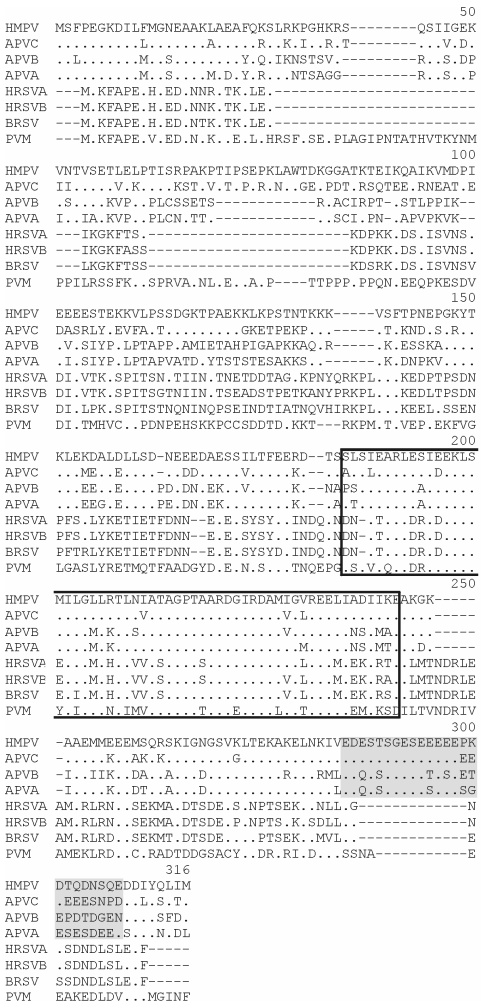

Figure 3:
Amino acid sequence comparison of the putative phosphoprotein ORF of hMPV and other pneumoviruses. The region of high similarity (Ling et al., 1995)[149] is boxed, and the glutamine-rich region is shaded in gray. Gaps are represented by dashes and periods indicate the position of identical aa residues compared to hMPV

region of high similarity is also found in hMPV (Figure 3), showing 100% similarity with APV-C, 93% with APV-A and -B, and approximately 81% with RSV (see the section on the N ORF for the definition of similarity).

The matrix protein (M) ORF

The third ORF of the hMPV genome encodes a 254-aa protein, which resembles the M ORFs of other pneumoviruses. The putative M ORF of hMPV has exactly the same size as the M ORFs of other metapneumoviruses (Table 1) and shows high aa sequence identity with the matrix proteins of APV (76-87%), lower identity with those of RSV and PVM (37-38%) and 10% or less identity with those of other paramyxoviruses (Table 2).

Easton and Chambers (1997)[33] compared the sequences of matrix proteins of all pneumoviruses and found a conserved hexapeptide at residue 14 to 19 that is also conserved in hMPV (Figure 4). For RSV, PVM and APV small secondary ORFs within or overlapping with the major ORF of M have been identified (52 aa and 51 aa in bRSV, 75 aa in hRSV, 46 aa in PVM and 51 aa in APV)[69,220,282]. We noticed two small secondary ORFs in the M ORF of hMPV. One small ORF of 54 aa residues was found within the major M ORF, starting at nt 2281, and one small ORF of 33 aa residues was found overlapping with the major ORF of M, starting at nt 2893 (data not shown). Similar to the secondary ORFs of RSV and APV there is no significant sequence identity between these secondary ORFs and secondary ORFs of the other pneumoviruses, and apparent start and stop signals are lacking. Evidence for the synthesis of proteins corresponding to these secondary ORFs of APV and RSV has not been reported.

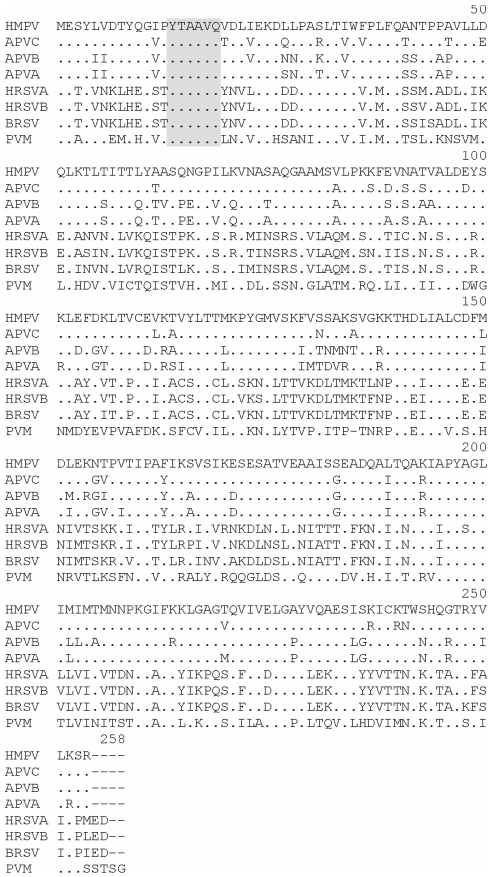

Figure 4:
Amino acid sequence comparison of the putative matrix protein ORF of hMPV and other pneumoviruses. The conserved hexapeptide sequence (Easton and Chambers, 1997)[69] is shaded in gray. Gaps are represented by dashes and periods indicate the position of identical aa residues relative to hMPV.

The fusion protein (F) ORF

The putative F ORF of hMPV is located adjacent to the putative M ORF, which is characteristic for members of the *Metapneumovirus* genus. The F gene of hMPV encodes a 539-aa protein, which is 2 aa residues longer than F of APV-C (Table 1). Analysis of the aa sequence revealed 81% sequence identity with APV-C, 67% with APV-A and -B, 33-38% with other pneumovirus F proteins, and only 10-18% with other paramyxoviruses (Table 2). One of the conserved features among F proteins of paramyxoviruses and also seen in hMPV is the distribution of cysteine residues[175,280]. The metapneumoviruses share 12 cysteine residues in F1 (7 are conserved among all paramyxoviruses), and two in F2 (1 is conserved among all paramyxoviruses). Of the three potential *N*-linked glycosylation sites present in the F ORF of hMPV, none are shared with RSV and two (position 66 and 389) are shared with APV. The third, unique, potential *N*-linked glycosylation site for hMPV is located at position 206 (Figure 5).

Despite the relatively low percentage sequence identity with other paramyxoviruses, the F protein of hMPV revealed typical fusion protein characteristics consistent with those described for the F proteins of other *Paramyxoviridae* family members[175]. F proteins of *Paramyxoviridae* members are synthesised as inactive precursors (F0) that are cleaved by host cell proteases, which generate N-terminal F2 subunits and large C-terminal F1 subunits. The cleavage site is conserved among all members of the *Paramyxoviridae* family[46]. The cleavage site of hMPV contains the residues RQSR. Both arginine residues are shared with APV and RSV, but the glutamine and serine residues are shared with other paramyxoviruses such as human parainfluenza virus type 1, Sendai virus and morbilliviruses (data not shown). In RSV a second cleavage site (RARR) was found which is separated from the first cleavage site immediately upstream of the fusion peptide by a stretch of 27 aa. Cleavage at both sites is required for the acquisition of membrane fusion potential of the F protein.

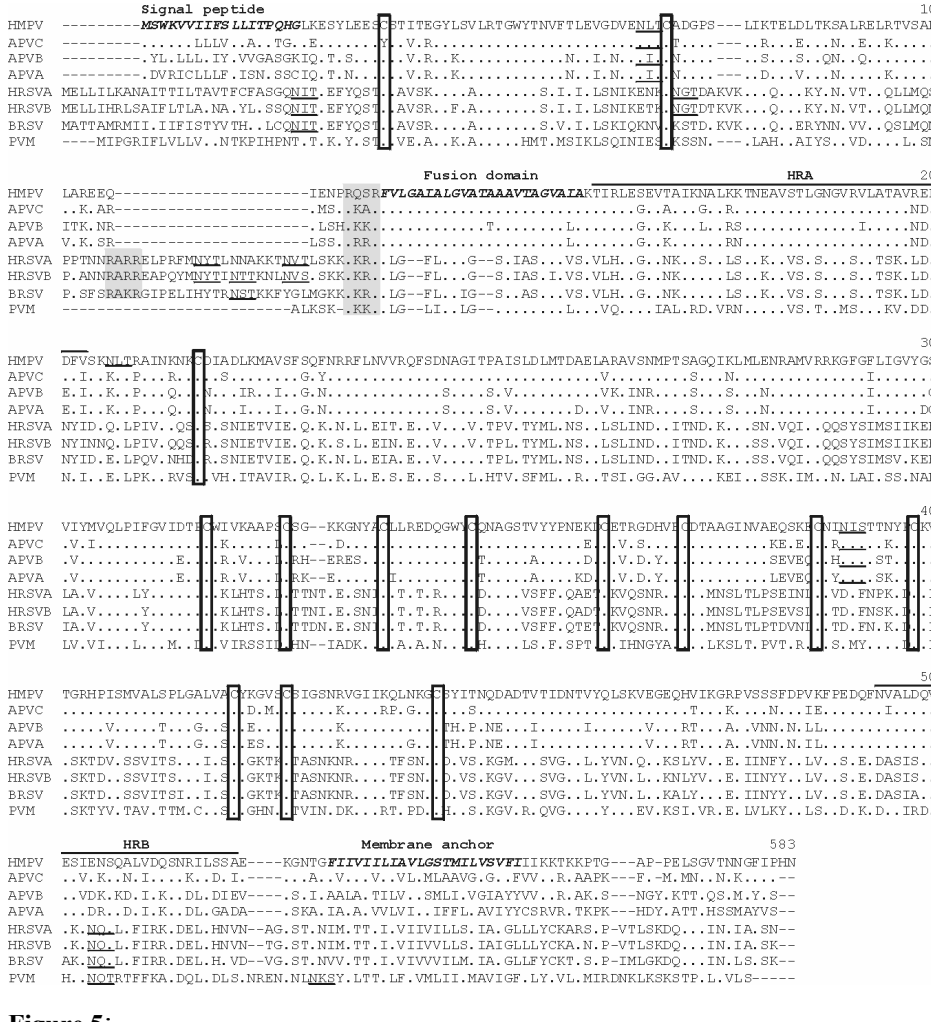

Figure 5:
Amino acid sequence comparison of the putative fusion protein ORF of hMPV and other pneumoviruses. The cleavage sites are shown shaded in gray and three hydrophobic domains (the putative signal peptide, fusion domain, and membrane anchor domain) are shown italicalised in boldface type. The conserved cysteine residues are boxed, putative N-linked glycosylation sites are underlined, and domains HRA and HRB are indicated with a line above the sequence. Gaps are represented by dashes and periods indicate the position of identical aa residues relative to hMPV.

The F proteins of other pneumoviruses such as APV and PVM as well as other paramyxoviruses lack the sequence between the two cleavage sites[99,286]. As can be seen in Figure 5, this second cleavage site and the connecting peptide are absent in the hMPV F protein. The hydrophobic region at the N-terminus of F1 functions as the membrane fusion domain and displays a high degree of sequence similarity among paramyxoviruses and

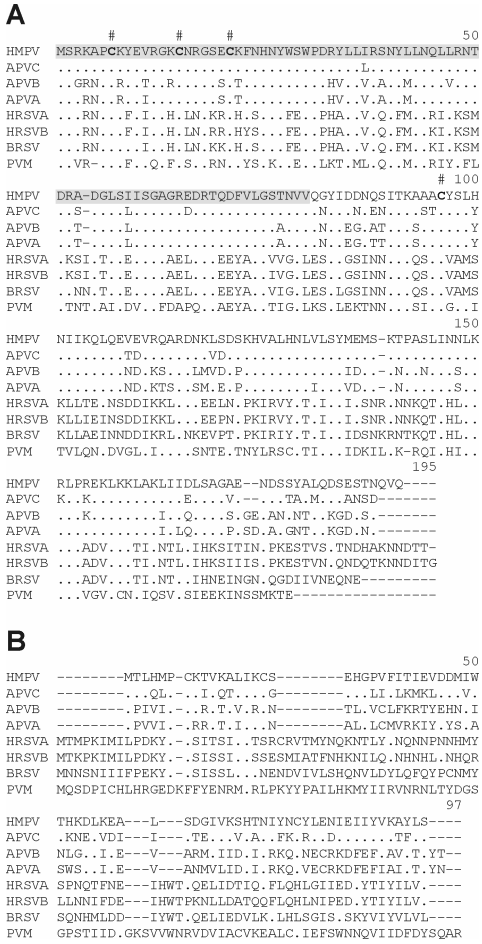

Figure 6:
Amino acid sequence comparison of the putative M2 ORFs of hMPV and other pneumoviruses. (A) Alignment of M2-1 ORFs, with the conserved N-terminus (Collins et al., 1990; Zamora and Samal, 1992)[45,248] shown shaded in gray. The conserved cysteine residue are printed in boldface type and are indicated by pound signs. (B) Alignment of M2-2 ORFs. Gaps are represented by dashes and periods indicate the position of identical aa residues relative to hMPV.

morbilliviruses and to a lesser extend the pneumoviruses[117,175]. This hydrophobic region (position 137-159, Figure 5) are conserved between hMPV and APV-C, which is in agreement with this region being highly conserved among the metapneumoviruses[179,224]. Adjacent to the fusion peptide and transmembrane segment are two regions that contain heptad repeats (HRA and HRB) which are relatively poor in glycines, contain no helix-breaking prolines, and contain charged aa side chains in all heptad positions except a and d. These heptad regions that are necessary for viral fusion[25,33,141,217] are also found in the hMPV F gene. Whereas a high percentage sequence identity is found between HRA of hMPV and all other pneumoviruses (especially in positions a and d of the repeats), a high level of sequence identity in HRB is restricted to the metapneumoviruses (Figure 5).
Furthermore, for RSV and APV, the signal peptide and anchor domain were found to be conserved within subtypes and displayed high variability between subtypes[179,195]. At the N-terminus of F2 of hMPV, 11 out of 18 aa residues are identical to those of APV-C and lower sequence identity is observed with the signal peptides of other APV or RSV F ORFs. Much more variability is seen in the membrane anchor domain at the C-terminus of F1, although some sequence identity is still seen with APV-C.

The 22K protein (M2) ORF

The M2 gene is unique to the members of the *Pneumovirinae* subfamily and two overlapping ORFs have been observed in all pneumoviruses. The first major ORF represents the M2-1 protein which enhances the processivity of the viral polymerase[43,46] and its readthrough of intergenic regions[81,108]. The putative M2-1 gene for hMPV, located adjacent to the F gene, encodes a 187-aa protein (Table 1) and reveals the highest percentage of sequence

identity with M2-1 of APV-C (84%, Table 2). Comparison of all pneumovirus M2-1 proteins revealed the highest level of conservation in the N-terminal half of the protein[1,45,284], which is in agreement with the observation that hMPV displays 100% similarity with APV-C in the first 80 aa residues of the protein (Figure 6A). The hMPV M2-1 protein contains 3 cysteine residues located within the first 30 aa residues that are conserved among all pneumoviruses. Such a concentration of cysteines is frequently found in zinc-binding proteins[1,61].

The secondary ORFs (M2-2) that overlap with the M2-1 ORFs of pneumoviruses are conserved in location but not in sequence and are thought to be involved in the control of the switch between virus RNA replication and transcription[1,3,10,12,45,49,73,151,284]. For hMPV, the putative M2-2 ORF starts at nt 512 in the M2-1 ORF (Figure 1), which is exactly the same start position as for APV-C. The length of the M2-2 ORFs are the same for APV-C and hMPV, 71 aa residues (Table 1). Sequence comparison of the M2-2 ORF (Figure 6B) revealed 56% aa sequence identity between hMPV and APV-C and only 26-27% aa sequence identity between hMPV and APV-A and B (Table 2).

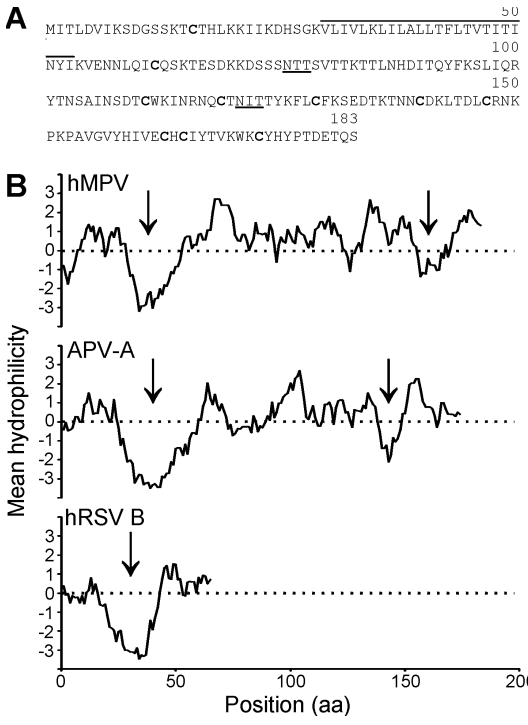

Figure 7:
Amino acid sequence analysis of the putative SH ORF of hMPV. (A) Amino acid sequence of the putative SH ORF of hMPV, with cysteine residues in boldface type, potential N-lined glycosylation sites underlined, and the most hydrophobic region represented by a line above the sequence. (B) Alignment of the hydrophilicity plots of the SH proteins of hMPV, APV-A, and hRSV-B. The procedure of Kyte and Doolittle (1982) was used with a window size of 17 aa residues. Arrows indicate strong hydrophobic domains.

The small hydrophobic protein (SH) ORF

The gene located adjacent to M2 of hMPV probably encodes a 183-aa SH protein (Figures 1 and 7). There is no discernible sequence identity between this ORF and other RNA virus genes or gene products. This is not surprising since sequence similarity between pneumovirus SH proteins is generally low. The putative SH ORF of hMPV is the longest SH ORF known to date (Table 1). The aa composition of the SH ORF is relatively similar to that of APV, RSV and PVM, with a high percentage of threonine and serine residues (22, 18, 19, 20, 21 and 28% for hMPV, APV, RSV A, RSV B, bRSV and PVM respectively). The SH ORF of hMPV contains 10 cysteine residues, whereas APV SH contains 16 cysteine residues. The SH ORF of hMPV contains two potential *N*-linked glycosylation sites (aa 76 and 121), whereas APV has one, RSV has two or three and PVM has four. The hydrophilicity profiles for the putative hMPV SH protein and SH of APV and

RSV revealed similar characteristics (Figure 7B). The SH ORFs of APV and hMPV have a hydrophilic N-terminus, a central hydrophobic domain which can serve as a potential membrane-spanning domain (aa 30-53 for hMPV), a second hydrophobic domain (aa 155-170), and a hydrophilic C-terminus. In contrast, RSV SH appears to lack the C-terminal part of the APV and hMPV ORFs. In all pneumovirus SH proteins the hydrophobic domain is flanked by basic aa residues, which are also found in the SH ORF for hMPV (aa 29 and 54).

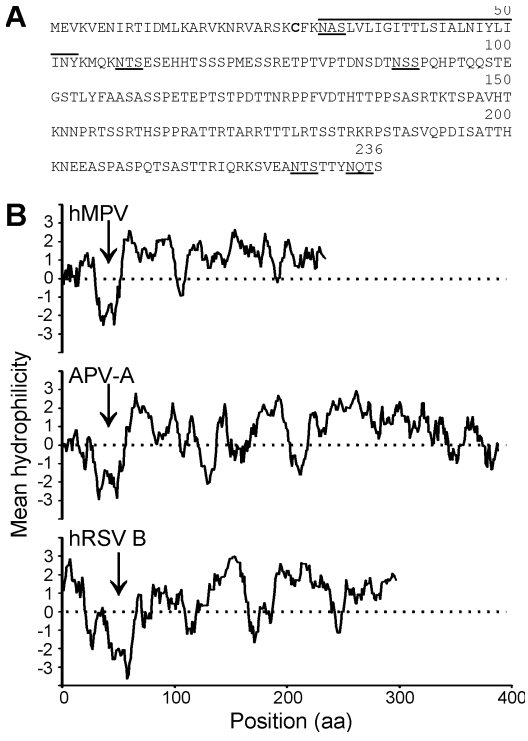

Figure 8:
Amino acid sequence analysis of the putative G ORF of hMPV. (A) Amino acid sequence of the putative G ORF of hMPV, with the cysteine residue in boldface type, potential N-linked glycosylation sites underlined, and the hydrophobic region represented by a line above the sequence. (B) Alignment of the hydrophilicity plots of the G proteins of hMPV, APV-A, and hRSV-B. The procedure of Kyte and Doolittle (1982) was used with a window size of 17 aa residues. Arrows indicate hydrophobic domains.

The attachment glycoprotein (G) ORF

The putative G ORF of hMPV is located adjacent to the putative SH gene and encodes a 236-aa protein (nt 6262-6972, Figure 1). A secondary small ORF is found immediately following this ORF, potentially coding for 68 aa residues (nt 6973-7179) but lacking a start codon. A third potential ORF in the second reading frame of 194 aa residues is overlapping with both of these ORFs but also lacks a start codon (nt 6416-7000). This ORF is followed by a potential fourth ORF of 65 aa residues in the same reading frame (nt 7001-7198), again lacking a start codon. Finally, a potential ORF of 97 aa residues (but lacking a start codon) is found in the third reading frame (nt 6444-6737, Figure 1).

Unlike the first ORF, the other ORFs do not have apparent gene start or gene end sequences (see below). Although the 236 aa G ORF probably represents at least a part of the hMPV attachment protein it can not be excluded that the additional coding sequences are expressed as separate proteins or as part of the attachment protein through some RNA editing event. It should be noted that for APV and RSV no secondary ORFs after the primary G ORF have been identified but that both APV and RSV have secondary ORFs within the major ORF of G. However, evidence for expression of these ORFs is lacking and there is no sequence identity between the predicted aa sequences for different viruses[151].

The secondary ORFs in hMPV G do not reveal characteristics of other G proteins and whether the additional ORFs are expressed requires further investigation.

BLAST analyses with all ORFs revealed no discernible sequence identity at the nucleotide or aa sequence level with other known virus genes or gene products. This is in agreement with the low percentage sequence identity found for other G proteins such as those of hRSV A and B (53%)[125] and APV-A and- B (38%)[130].

Whereas most of the hMPV ORFs resemble those of APV in both length and sequence, the putative G ORF of 236 aa residues of hMPV is considerably smaller than the G ORF of APV (Table 1). The aa sequence revealed a serine and threonine content of 34%, which is even higher than the 32% for RSV and 24% for APV. The putative G ORF also contains 8.5% proline residues, which is higher than the 8% for RSV and 7% for APV. The unusual abundance of proline residues in the G proteins of APV, RSV and hMPV has also been observed in glycoproteins of mucinous origin where it is a major determinant of the proteins three-dimensional structure[48,121,268]. The G ORF of hMPV contains five potential *N*-linked glycosylation sites, whereas hRSV has seven, bRSV has five and APV has three to five.

The predicted hydrophilicity profile of hMPV G revealed characteristics similar to the other pneumoviruses. The N-terminus contains a hydrophilic region followed by a short hydrophobic area (aa 33-53 for hMPV) and a mainly hydrophilic C-terminus (Figure 8B). This overall organisation is consistent with that of an anchored type II transmembrane protein and corresponds well with these regions in the G protein of APV and RSV. The putative G ORF of hMPV contains only 1 cysteine residue in contrast to RSV and APV (5 and 20 respectively). Of note, only two of the

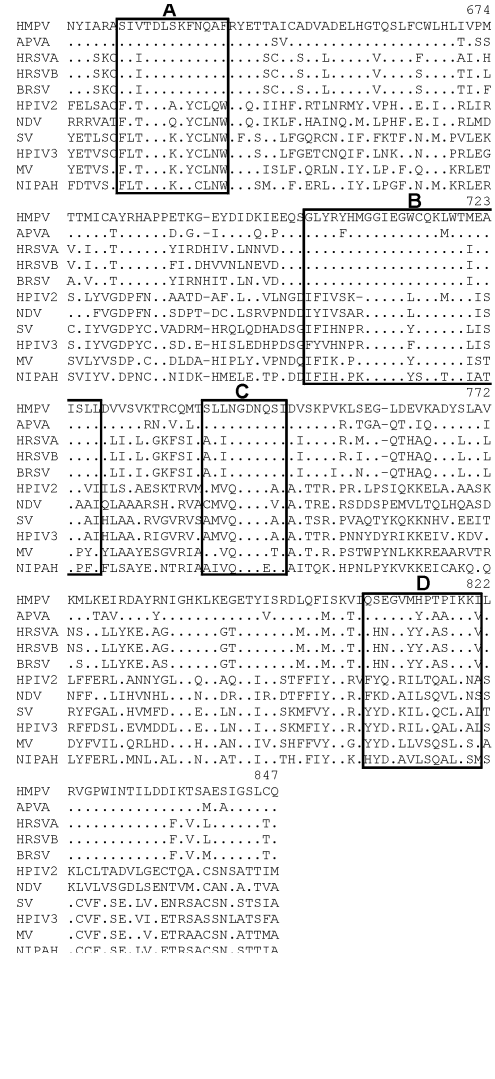

Figure 9:
Amino acid sequence comparison of a conserved domain of the putative polymerase ORF of hMPV and other paramyxoviruses. Domain III is shown with the four conserved polymerase motifs (A, B, C, and D) boxed (Poch *et al*., 1989, 1990). Gaps are represented by dashes and periods indicate the position of identical aa residues relative to hMPV. HPIV2 and HPIV3, human parainfluenza virus types 2 and 3; SV, Sendai virus; NDV, Newcastle disease virus; MV, measles virus; NIPAH, Nipah virus.

four secondary ORFs in the G gene contained one additional cysteine residue and these four potential ORFs revealed 12-20% serine and threonine residues and 6-11% proline residues.

The polymerase protein (L) ORF

In analogy to other negative-strand viruses, the last ORF of the hMPV genome is the RNA-dependent RNA polymerase component of the replication and transcription complexes. The L gene of hMPV encodes a 2005-aa protein, which is one residue longer than the APV-A L protein (Table 1). The L protein of hMPV shares 64% aa sequence identity with APV-A, 44% with RSV, and 13-15% with other paramyxoviruses (Table 2). Poch *et al.*[196,197] identified six conserved domains within the L proteins of non-segmented negative strand RNA viruses, from which domain III contained the four core polymerase motifs that are thought to be essential for polymerase function. These motifs (A, B, C and D) are well conserved in the hMPV L protein: hMPV shares nearly 100% aa sequence similarity with other pneumoviruses. For the entire domain III (aa 625- 847 in the hMPV L ORF), hMPV shares 83% aa sequence identity with APV, 67-68% with RSV and 26-30% with other paramyxoviruses (Figure 9). In addition to these polymerase motifs the pneumovirus L proteins contain a sequence which conforms to a consensus ATP-binding motif $K(X)_{21}GEGAGN(X)_{20}K$[237].The hMPV L ORF contains a similar motif as APV, in which the spacing of the intermediate residues is off by one: $K(X)_{22}GEGAGN(X)_{19}$ K.

Phylogenetic analyses

As an indicator for the relationship between hMPV and members of the *Pneumovirinae*, phylogenetic trees based on the N, P, M, and F ORFs have been constructed previously[256] and revealed a close relationship between hMPV and APV-C. Because of the low sequence identity of the hMPV SH and G genes with those of other paramyxoviruses, reliable phylogenetic trees for these genes could not be constructed. In addition, the distinct genomic organisation between members of the *Pneumovirus* and *Metapneumovirus* genera make it difficult to generate phylogenetic trees based on the entire genomic sequence. We therefore only constructed phylogenetic trees for the M2-1 and L genes in addition to those previously published. Both these trees confirmed the close relation between APV and hMPV within the *Pneumovirinae* subfamily (Figure 10).

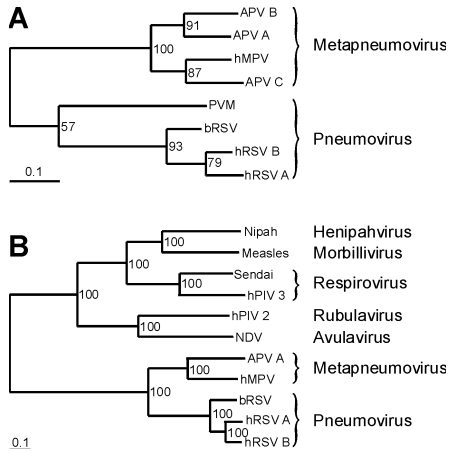

Figure 10:
Phylogenetic analyses of the M2-1 and L ORFs of hMPV and selected paramyxoviruses. The putative M2-1 (A) and L (B) ORFs of hMPV were aligned with those from selected paramyxoviruses (see legend Figure 9) and phylogenetic trees were generated by maximum-likelihood analyses using 100 bootstraps and three jumbles. The most probable trees are shown with numbers representing the boostrap values calculated from the consensus trees. The scale bars roughly represent 10% of nucleotide changes between close relatives.

hMPV noncoding sequences

The noncoding regions between the ORFs of pneumoviruses contain gene end signals, intergenic regions and gene start signals, and they vary in size and sequence[17,48,62]. The noncoding sequences between the hMPV ORFs range in size from 23 to 209 nt (Figure 11A) and do not reveal significant sequence identity with those of APV and RSV.

The region between the M and F ORFs of hMPV contains part of a potential secondary ORF, which starts in the primary M ORF (see above).

A

Pos.,ORF	Stop	Non-coding sequence	Gene start	Start	Pos.,ORF
1,Le		ACGAGAAAAAAACGCGUAUAAAUUAGAUUCCAAAAAAAUAU.....	GGGACAAGUGAAA	AUG	55,N
1237,N	UAA	UUAAAAAAGU.....	GGGACAAGUCAAA	AUG	1263,P
2145,P	UAG	UUUAAUAAAAAAUAAACAAU.....	GGGACAAGUAAAA	AUG	2180,M
2942,M	UAA	CAACCAAGCACCUUGGCCAAGAGCUACUAACCCUAUCUCAUAGAUCAUAAAGUCACCAUUCUAGUUAUAU AAAAAUCAAGUUAGAACAAGAAUUAAAUCAAUCAAGAAC.....	GGGACAAAUAAAA	AUG	3067,F
4684,F	UAG	UUAAUUAAAAAUAAAGUAAAUUAAAAUAAAUUAAAAUUAAAAAUAAAAAUUU.....	GGGACAAAUCAUA	AUG	4752,M2
5476,M2	UAG	UAAAAACACAUCAGAGU.....	GGGAUAAAUGACA	AUG	5509,SH
6058,SH	UAA	AUGUUAACACCAGAUUAGGAUCCAUCCAAGUCUGUUAGUUCAACAAUUUAGUUAUUUAAAAAUAUUUUGA AAACAAGUAAGUUUCUAUGAUACUUCAUAAUAAUAAGUAAUAAUUAAUUGCUUAAUCAUCAUCACAACAU UAUUCGAAACCAUAACUAUUCAAUUUAAAAAGUAAAAAACAAUAACAU.....	GGGACAAGUAGUU	AUG	6262,G
6970,G	UAA	CAAAAAAUACAAAAUAACUCUAAGAUAAACCAUGCAGACACCAACAAUGGAGAAGCCAAAAGACAAUUCA CAAUCUCCCCAAAAAGGCAACAACACCAUAUUAGCUCUGCCCAAAUCUCCCUGGAAAAAACACUCGCCCA UAUACCAAAAAUACCACAACCACCCCAAGAAAAAAACUGGGCAAAACAACACCCAA.....	GAGACAAAUAACA	AUG	7182,L
13197,L	UGA	AAAAUGAUAAAAAUGAUAAAAUAGGUGACAACUUCAUACUAUUCCAAAGUAAUCAUUUGAUUAUGCAAUU AUGUAAUAGUUAAUUAAAAACUAAAAAUCAAAAGUUAGAAACUAACAACUGUCAUUAAGUUUAUUAAAAA UAAGAAAUUAUAAUUGGAUGUAUACGGUUUUUUUCUCGU			13378,Tr

B

```
hMPV Le: 3' UGCUCUUUUUUUGCGCAUAUUUAAUCUAAGGUUUUUUUAUACCCU
            |||||||||||| |||||| ||   |||  ||     |||   |
hMPV Tr: 5' ACGAGAAAAAAAC CGUAUACAUCCAAUUAUAAUUUCUUAUUUUU


hMPV Tr: 5' ACGAGAAAAAAACCGUAUACAUCCAAUUAUAAUUUCUUAUUUUUA
            |||||||||||||||||| |||| |||| | |  | ||| ||||
APV  Tr: 5' ACGAGAAAAAAACCGUAUUCAUCAAAUUUUUAGCUUUUAGUUUUU


hMPV Le: 3' UGCUCUUUUUUUGCGCAUAUUUAAUCUAAGGUUUUUUUAU-ACCC
            |||||||||||||| | || || ||| | ||||||| ||||
APV  Le: 3' UGCUCUUUUUUUUGCGUA-AGUUCGUCCAAGAUCUUUUUUAUUACCC
```

Figure 11:

Noncoding sequences of hMPV isolate 00-1. (A) The noncoding sequences between the putative ORFs ant at the genomic termini are shown in the positive sense. From left to right, stop codons of indicated ORFs are shown, followed by the noncoding sequences, the gene start signals, and start codons of the indicated subsequent ORFs. Numbers indicate the first position of start and stop codons in the hMPV map. Sequences that display similarity to published gene end signals are underlined and sequences that display similarity to UAAAAAU/A/C are indicated with a line above the sequence. (B) Nucleotide sequences of the genomic termini of hMPV. The genomic termini of hMPV are aligned with each other and with those of APV. Underlined regions represent the primer sequences used in RT-PCR assays, which are based on the 3'and 5'end sequences of APV and RSV (Randhawa et al., 1997; Mink et al., 1991). Boldface italicalised nucleotides are part of the gene start signal of the N gene. Le, leader; Tr, trailer.

The region between SH and G ORFs of 201 nt does not appear to have coding potential based on the presence of numerous stop codons in all three reading frames.
The region between G and L of 209 nt may encode additional ORFs in two reading frames (see above). Interestingly, the start of the L ORF is located in these secondary ORFs. Whereas the L gene of APV does not start in the preceding G ORF, the L ORF of RSV starts in the preceding M2 gene. Comparison of the noncoding sequences between the ORFs of hMPV revealed a consensus sequence for the gene start signal of the N, P, M, F, M2 and G genes: GGGACAA A/G U (Figure 11A), which is similar to the consensus gene start signal of the APV[11,146,151,282]. The gene start signals for the SH and L genes of hMPV were found to be slightly different from this consensus (SH: GGGAUAAAU, L: GAGACAAAU). For APV the gene start signal of L was also found to be different from the consensus: AGGACCAAT (APV-A)[205] and GGGACCAGT(APV-D)[11]. In contrast to the similar gene start sequences of hMPV and APV, the consensus gene end sequence of APV, UAGUUAAUU[33,205], could be found only in the F and L genes of hMPV. Variants of this motif, with 1 to 3 nt substitutions were found in the N, P, M, M2 and SH genes, but no homologue was found in G. Another repeated sequence (UAAAAA U/A/C) was found downstream each of the hMPV ORFs except G. The gene end signal described for G of APV Fr/85/1[11] was found also at the end of the G ORF of hMPV (Figure 11A).Although these sequences may represent (part of) the gene end signal, sequence analyses of viral mRNA is required to formally delineate the hMPV gene end signals.
Short extragenic regions at the 3' and 5' extremities of the genome of paramyxoviruses are referred to as the leader and trailer sequences, and approximately the first 12 nt of the leader and last 12 nt of the trailer are complementary, probably because each of them contain basic elements of the viral promoter[17,62,167]. The 3' leader of hMPV and APV are both 41 nt in length, and some sequence identity is seen in the region between nt 16 and 41 of both viruses (18 out of 26 nt) (Figure 11B). As mentioned before, the first 15 nt of the hMPV genomic map are based on a primer sequence derived from the APV genome. At the 5' end of the viral genome of hMPV after the stop codon of the L ORF 179 nt of noncoding sequence is found, including the trailer sequence. Alignment of the APV trailer sequence with the extreme 45 nt of the hMPV genome revealed 24 out of 33 nt to be identical, excluding the extreme 12 nt which represent primer sequences based on the genomic sequence of APV (Figure 11B).

Discussion

Previously we proposed the classification of hMPV in the *Metapneumovirus* genus based on limited sequence information for this virus. The sequence information described here is in agreement with this classification. Our sequence analyses revealed the absence of NS1 and NS2 genes at the 3' end of the genome and a genomic organisation resembling the organisation of metapneumoviruses (3'-N-P-M-F-M2-SH-G-L-5'). The high percentage sequence identity found between hMPV and APV ORFs further emphasises the close relationship between these two viruses. For the N, P, M, F, M2-1 and M2-2 ORFs of hMPV an overall aa identity of 80% is found with APV-C. In fact, for these genes APV-C and hMPV revealed percentages sequence identity which are in the same range as those found

between subgroups of other genera, such as RSV- A and -B or APV-A and -B. This close relationship between APV-C and hMPV is also seen in the phylogenetic analyses, which revealed hMPV and APV-C to be always in the same branch, separate from the branch containing APV-A and B. The identical genomic organisation, the high percentage sequence identity, and phylogenetic analyses are all in favor of the classification of hMPV as the first mammalian member in the *Metapneumovirus* genus.

It should be noted that previously found sequence variation between different virus isolates of hMPV in the N, M, F and L genes revealed the possible existence of different genotypes[256]. Here we describe the genomic sequence of virus isolate 00-1, the prototype of one of the subgroups. Sequence analyses of the genome of a member of the other subgroup is in progress to further investigate the relationship between distinct hMPV isolates and APV.

The close relationship between hMPV and APV-C is not reflected in the host range, since APV infects birds in contrast to hMPV[256]. This difference in host range may be determined by the differences between the SH and G proteins of both viruses. The SH and G proteins of hMPV did not reveal significant aa sequence identity with SH and G proteins of any other virus. Although the aa content and hydrophilicity plots are in favor of defining these ORFs as SH and G, experimental data are required to formally prove this. Such analyses will also shed light on the role of the additional overlapping ORFs in the putative G gene. Sequence analysis of a second hMPV isolate that belongs to a distinct genetic lineage[256] revealed the presence of a similar primary G ORF and the presence of secondary ORFs (data not shown). However, the positions and sizes of the secondary ORFs were not identical to those of isolate 00-1. Sequence analyses of the SH and G genes of APV-C might provide more insight in the function of the putative SH and G proteins of hMPV and their relationship with those of APV-C.

Some of the noncoding regions of hMPV were found to be similar to those of APV. The 3'leader and 5' trailer sequences of APV and hMPV displayed a high percentage of sequence identity. Although the lengths of the intergenic regions were not always the same for APV and hMPV, the consensus gene start signals of most of the genes were found to be identical and some similarity may exist between potential gene end signals of APV and hMPV. Sequence analysis of viral mRNAs is required to formally delineate the gene end sequences of hMPV. It should also be noted that sequence information for 15 nt at the extreme 3' end and 12 nt at the extreme 5' end are still missing. This information will be of crucial importance for the development of reverse genetics systems for hMPV, as was shown recently for RSV and APV [161]. Nevertheless, the sequence information provided here will be of importance for the generation of diagnostic tests, vaccines, and antivirals for hMPV.

Materials and Methods

Sequence analysis

Virus isolate 00-1 was propagated to high titers (approximately 10,000 $TCID_{50}$/ml) on tertiary monkey kidney cells as described previously[256]. Viral RNA was isolated from supernatants from infected cells using a High Pure RNA Isolating Kit according to instructions from the manufacturer (Roche Diagnostics, Almere, The Netherlands). Primers were designed based on sequences published previously[256] in addition to sequences

published for the leader and trailer of APV/RSV[167,204] and are available upon request. RT-PCR assays were conducted with viral RNA, using a one-tube assay in a total volume of 50µl with 50 mM Tris pH 8.5, 50 mM NaCl, 4.5 mM $MgCl_2$, 2 mM DTT, 1 µM forward primer, 1µM reverse primer, 0.6 mM dNTP's, 20 units RNAsin (Promega, Leiden, The Netherlands), 10 U AMV reverse transcriptase (Promega, Leiden, The Netherlands), and 5 units Taq Polymerase (PE Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Reverse transcription was conducted at 42°C for 30 min, followed by 8 min inactivation at 95°C. The cDNA was amplified during 40 cycles of 95°C for 1 min, 42°C for 2 min, 72°C for 3 min, with a final extension at 72°C for 10 min. After examination on a 1% agarose gel, the RT-PCR products were purified from the gel using a Qiaquick Gel Extraction kit (Qiagen, Leusden, The Netherlands) and sequenced directly using a Dyenamic ET terminator sequencing kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and an ABI 373 automatic DNA sequencer (PE Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), according to the instructions of the manufacturer.
Sequence alignments were made using the Clustal software package available in the software package of BioEdit version 5.0.6.[107] (http://jwbrown.mbio.ncsu.edu/Bioedit/bioedit.html).

Phylogenetic analysis
To construct phylogenetic trees, DNA sequences were aligned using the ClustalW software package and maximum likelihood trees were generated using the DNA-ML software package of the Phylip 3.5 program using 100 bootstraps and three jumbles. Bootstrapping is a method for deriving confidence values for the groupings in a tree, which involves making random samples of sites from the alignment, drawing trees from each sample, and counting how many times each grouping from the original tree occurs in the sample trees. Bootstrap values were computed for consensus trees created with the consense package[82].
The hMPV genomic sequence is available from Genbank under accession No. AF371337. All other sequences used here are available from Genbank under accession Nos. AB046218 (measles virus, all ORFs), NC001796 (human parainfluenza virus type 3, all ORFs), NC001552 (Sendai virus, all ORFs), X57559 (human parainfluenza virus type 2, all ORFs), NC002617 (Newcastle Disease virus, all ORFs), NC002728 (Nipah virus, all ORFs), NC001989 (bRSV, all ORFs), M11486 (hRSV A, all ORFs except L), NC001803 (hRSV, L ORF), NC001781 (hRSV B, all ORFs), D10331 (PVM, N ORF), U09649 (PVM, P ORF), U66893 (PVM, M ORF), U66893 (PVM, SH ORF), D11130 (PVM, G ORF), D11128 (F ORF), AF176590 (APV-C, N ORF), U39295 (APV-A, N ORF), U39296 (APV-B, N ORF), AF262571 (APV-C, M ORF), U37586 (APV-B, M ORF), X58639 (APV-A, M ORF), AF176591 (APV-C, P ORF), AF325443 (APV-B, P ORF), U22110 (APV-A, P ORF), AF187152 (APV-C, F ORF), Y14292 (APV-B, F ORF), D00850 (APV-A, F ORF), AF176592 (APV-C, M2 ORF), AF356650 (APV-B, M2 ORF), X63408 (APV-A, M2 ORF), U65312 (APV-A, L ORF), S40185 (APV-A, SH ORF). The PVM M2 ORF was taken from Ahmadian *et al.* (1999)[1].

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4

Antigenic and genetic variability of human metapneumoviruses

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Abstract

Human metapneumovirus (hMPV) is a member of the subfamily *Pneumovirinae* within the family *Paramyxoviridae*. Other members of this subfamily, respiratory syncytial virus and avian pneumovirus, can be divided in subgroups based on genetic and/or antigenic differences. For hMPV the existence of different genetic lineages has been described based on variation in a limited set of available sequences. In the present study we addressed the antigenic relationship between the different genetic lineages in virus neutralisation assays. In addition, we analysed the genetic diversity of hMPV by phylogenetic analysis of sequences obtained for part of the fusion protein (n=84) and the complete attachment protein open reading frames (n=35). On the basis of sequence diversity between attachment protein genes and the differences in virus neutralisation titers, two hMPV serotypes were defined. Each serotype could be divided in two genetic lineages, but these did not reflect significant antigenic differences.

Introduction

The human metapneumovirus (hMPV) has recently been identified as a causative agent of respiratory tract illnesses (RTI) in humans world-wide[77,90,186], and is a member of the *Pneumovirinae* subfamily within the *Paramyxoviridae* family[259]. The *Pneumovirinae* subfamily consists of two genera: the pneumoviruses and the metapneumoviruses. Human respiratory syncytial virus (hRSV) the major viral cause of severe RTI in children, is the type species of the pneumoviruses[46]. Avian pneumovirus (APV), the causative agent of RTI in turkeys and chickens[96] was the sole member of the *Metapneumovirus* genus until the discovery of hMPV[118].

For most pneumoviruses different subgroups or subtypes have been identified. For hRSV, two subgroups have been identified based on differences in nucleotide sequences, reactivity patterns with monoclonal antibodies and in vitro neutralisation assays with subgroup-specific antisera[7,37,95,176]. Additional genotypes have been identified within subgroups, largely based on the high variability of the attachment protein gene[29,241]. The fusion (F) and the attachment (G) proteins are the main targets for the neutralising and protective antibody response[6,125,184], with F being one of the most conserved proteins and G the most variable[123,124,130,179]. For APV two different subgroups (A and B) have been defined on the basis of nucleotide sequences of the G protein and neutralisation tests using monoclonal antibodies that also recognise the G protein, but these subgroups belonged to one serotype[52]. APV type C, a possible second serotype, was identified based on the lack of cross reactivity with antisera specific for groups A and B and the nucleotide sequences also proved to be significantly different from strains belonging to group A or B[222,224]. In addition, there is evidence for a subgroup D containing isolates from France that are not neutralised by monoclonal antibodies raised against viruses belonging to either subgroup A, B or C[11].

For hMPV two major genetic lineages have been identified world-wide based on analysis of a limited set of sequences[192,192,239,256]. One of the features of hMPV that poses a challenge to future vaccine development is that infections may occur in the presence of pre-existing immunity. Very young children (<1 year) were shown to be infected by the virus, and re-infections have also been demonstrated[191]. hMPV might cause repeated infections throughout

life similar to RSV, which could be due either to incomplete immunity or to genetic heterogeneity of the virus. For the development of vaccines it is important to develop a thorough understanding of the extent of genetic and antigenic variability of the different hMPV transmembrane glycoproteins. In the present study we analysed the genetic diversity of hMPV by phylogenetic analyses of sequences obtained for part of the F (n=84) and the complete G open reading frames (ORFs) (n=35). In addition, we addressed the antigenic relationship between the different lineages with virus neutralisation (VN) assays using lineage-specific antisera raised in ferrets. Classical virology studies have used a definition of a homologous-to-heterologous VN titer ratio of more than 16 as a definition for serotypes[87]. Based on the results shown in the present work, and based on the described definition, we now define the two major lineages of hMPV as serotype A and B. In accordance with the definition and the obtained results the sublineages within each serotype are not identified as different serotypes. The presence of at least two serotypes of hMPV in the human population has implications for the development of intervention strategies, such as immunisation and vaccination.

Materials and Methods

Sample collection, RNA isolation, RT-PCR assays and sequencing

HMPV positive nasopharyngeal aspirate samples were obtained from different cohort studies: 61 samples from The Netherlands, 11 samples from Finland, 8 samples from England, one from Hong-Kong and two from Brazil. Clinical samples had been obtained between 1981 and 2002. Samples were obtained from young children, infants, adults, elderly and immune compromised individuals and these patients suffered from mild RTI to severe RTI. Epidemiological and clinical data for most isolates have been described elsewhere[30,120,258].

Similar to the influenza nomenclature, sequences are identified by the country of origin, an identification number and the year of isolation. RNA isolation was performed as described previously[256]. cDNA was synthesised at 42°C for 60 minutes with random hexamer primers (Promega, Leiden, The Netherlands) and superscript II RNase H-reverse transcriptase (Invitrogen, Merelbeke, Belgium). An aliquot of cDNA was used in a PCR assay to amplify the full-length G ORF or a fragment of the F ORF. Primers: SH7: 5'-TACAAAACAAGAACATGGGACAAG-3' and SH-8 5'-GAGATAGACATTAACAGTG-GATT-3' (G ORF), BF100 5'-CAATGCAGGTAT AACACCAGCAATATC-3' and BF101 5'-GCAACAATTGAACTGATCTTCAGGAAAC-3' (F ORF). Thermocycling conditions: 94 °C for 1 min., 40 °C for 2 min., 72 °C 3 min. (40 cycles). When necessary a nested PCR was performed using 5 µl of PCR product with primers SH7 and SH8 for the G ORF or primers BF 103 5'-ACATGCCAACATCTGCAGGACAAA TAAAAC-3' and BF104 5'-ACATGCTGTTCACCTTCAACTTTGC-3' for the F ORF. PCR products were sequenced directly on both strands with multiple primers as described before[256]. When identical sequences were obtained (suspicious of laboratory contamination), and to confirm sequence uncertainties such as frame shifts, we repeated the RNA isolation, RT-PCR and subsequent sequencing using the original materials.

Phylogenetic analysis
Nucleotide sequences were aligned using the Clustal W program running within the Bioedit software package, version 5.0.9. Maximum likelihood trees were generated with the Seqboot and Dnaml packages of Phylip version 3.6 using 100 bootstraps and 3 jumbles. The consensus tree was calculated using the Consense package of Phylip 3.6 and was subsequently used as usertree in Dnaml to recalculate the branch lengths from the nucleotide sequences. Finally, the trees were rerooted at midpoint using the Retree software of Phylip 3.6. Trees were visualised with the Treeview 1.6.6 program distributed with Bioedit version 5.0.9[82]. Sequences are available from Genbank under accession numbers AY295930 to AY296012 (F partial) and AY304360 to AY304362 (complete F for NL/17/00, NL/1/99 and NL/1/94 respectively), AF371337 (complete genome NL/1/00) and AY296014 to AY296047 (complete G regions).

Virus preparations and titration's
Viruses were isolated on tertiary monkey kidney (tMK) cells as described before[256]. For each genetic lineage a prototype virus isolate was chosen on the basis of its ability to grow to high titers on tMK cells, and to reflect the specific genotype for the lineage. Virus titrations were cultured for 7 days and infected wells were identified by immune fluorescence assays (IFA) with hMPV specific polyclonal antiserum raised in guinea pigs. Titers were expressed in 50% tissue culture infectious dose ($TCID_{50}$).

Antisera
Lineage-specific polyclonal hMPV antisera were raised by inoculating ferrets with 1 ml of virus-infected tMK supernatants containing approximately 10^4-10^5 $TCID_{50}$ virus. All inoculations were performed in duplo, and the animals with the highest antibody responses are shown. Sera were collected at days 0 and 28 post infection (ferret I and II) or at days 0 and 21 (ferret III to VI). Inoculations were performed as follows: ferrets I and III: hMPV NL/1/00, prototype virus for lineage A1. Ferrets II and V: hMPV NL/1/99, prototype virus for lineage B1. Ferret IV: hMPV NL/17/00, prototype virus for lineage A2 and ferret VI: hMPV UK/5/01 a virus from lineage B2. Ferrets were housed in isolator cages to avoid cross-infections.
HMPV-specific polyclonal antisera were raised in guinea pigs as described before[256]. Antisera raised in separate guinea pigs against viruses from the two main genetic lineages (A and B) were mixed 1:1, and this mixture tested positive against all hMPV isolates in IFA.

Virus neutralisation assays
VN assays of heat-inactivated (30 min. 56 °C) ferret sera were performed as described before[256]. Briefly, twofold serial serum dilutions starting at 1:8 were incubated with approximately 30 $TCID_{50}$ virus. Seven days after infection of tMK cells with the antibody/virus mixture, IFA with the guinea pig antiserum was performed. The VN titer was defined as the reciprocal of the highest serum dilution at which no positive IFA signal was obtained (depicted as means of duplicate measurements). Each experiment included virus titrations of the working solution of the virus, using two-fold dilutions, and a range of 10 to 100 $TCID_{50}$/well was considered acceptable.

Results

Variation in the fusion protein gene

Partial F gene sequences (nucleotide (nt) 780-1221 in the F ORF) were obtained from clinical samples collected from 84 hMPV-infected patients. Phylogenetic analysis of these sequences confirmed the presence of two main genetic lineages, A and B. Each of these lineages appeared to consist of two sublineages, which were tentatively named A1, A2, B1 and B2 (Figure 1A). Comparison of the sequences revealed high percentage identities between members of the same sublineage (nt: 97-100%, aa: 99 to 100%), members of the two different sublineages within each main lineage (nt: 94 –96%, aa: 97-99%) and even between members of the two different main lineages A and B (nt: 84 –86%, aa: 94-97%). Whereas no specific amino acid (aa) residue substitutions could be found between sequences from subgroups A1 and A2, there were 5 specific aa substitutions between sequences from genotypes A and B, and one substitution between B1 and B2 (Table 1). The low variability was also observed when complete F protein genes from prototype viruses for each sublineage were sequenced (Figure 2).

Table 1: Lineage-specific amino acid substitutions between the four sublineages in the fusion open reading frame between position 260 and 407.

Sublineage	AA286	AA296	AA312	AA348	AA404
A1	V	K	Q	K	N
A2	V	K	Q	K	N
B1	I	N	K	R	P
B2	I	D	K	R	P

Variation in the attachment protein gene

Nucleotide sequences of the region between the start codons of the G and the polymerase (L) open reading frame (ORF) were obtained for 35 samples. Phylogenetic analysis revealed the same clustering of the sequences over the four sublineages as seen for the F protein gene (Figure 1B). The G region showed some variation in length, ranging from 860-908 nt. The first 657-708 nt have been described as the putative primary G ORF[259]. Alignment of the primary G ORFs revealed a variation in length of these ORFs even for members of the same sublineage, due to single nt substitutions that resulted in premature termination codons (Figure 3). For two samples a change in reading frame was observed as a result of an addition (BR/2/01: G pos. 519) or a deletion (NL/2/93: C at position 243) of a single nt. These mutations resulted in relatively short G ORFs (NL/2/93 110 aa; BR/2/01: 193 aa) because of premature termination and in drastic changes in the deduced amino acid sequences of the carboxy-terminus of the G proteins.

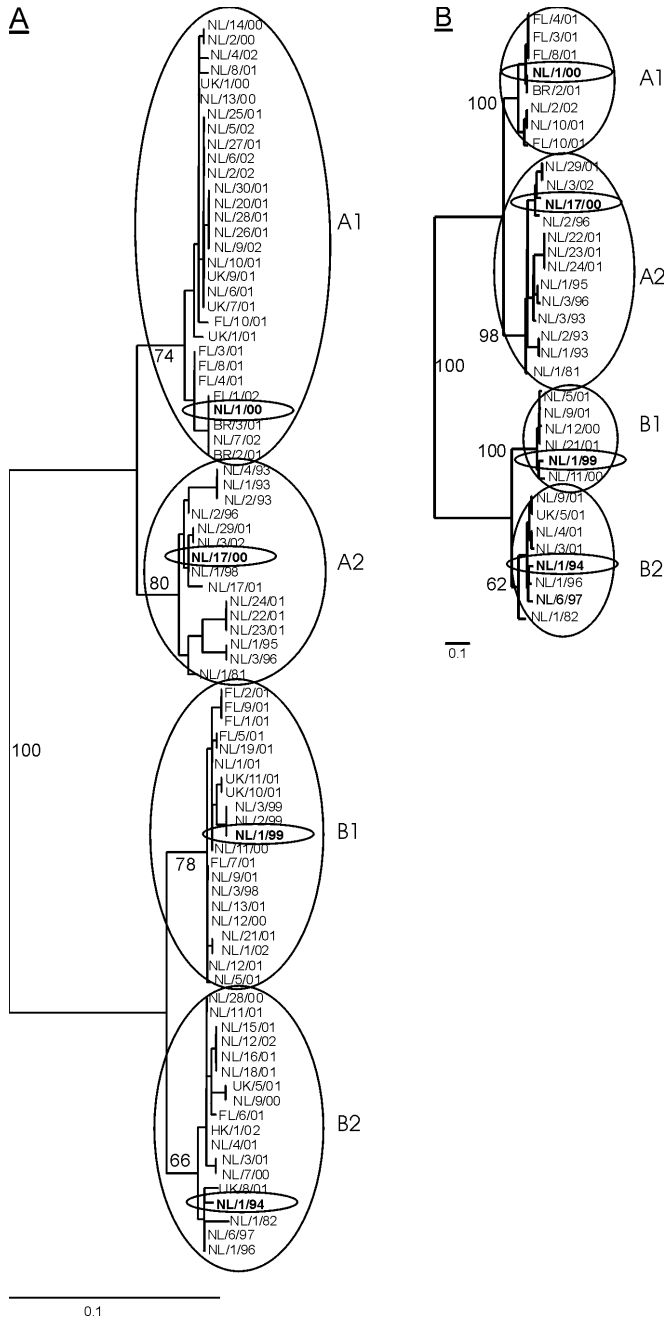

Figure 1:
Phylogenetic trees constructed based on the (A) partial F gene (ORF position 780-1221, n=84) or (B) the complete G coding region (start G ORF to start L ORF, n=35). Trees were generated by maximum likelihood analysis using 100 bootstraps and 3 jumbles. The scale representing the percentage of nucleotide changes is shown for each tree. Bootstrap values are based on the consensus trees, and relevant numbers are shown in the tree. The four prototype viruses are shown in boldface, with ovals drawn around them. NL = viruses from the Netherlands; FN= viruses obtained from Finland; UK=

Comparison of the primary G ORF sequences, excluding sequences of NL/2/93 and BR/2/01 because of the putative frameshifts, revealed a relatively high percentage identity between members of the same sublineage (nt: 93-100%, aa: 75 to 99.5%), less identity between members of the two different sublineages within each main lineage (nt: 76-83%; aa: 60-75%) and low sequence identity between members of the two different main lineages A and B (nt: 50-57%, aa: 30-37%).

The position of the hydrophobic domain, a high percentage of proline, serine and threonine residues and a cysteine residue at position 27 are features shared by all hMPVs. Whereas the cytoplasmic tail was conserved among all members (58-70% aa identity), the proposed ectodomains (start aa 51) were quite variable (18 –25% aa identity between lineage A and B). The number and position of potential sites for *N*-linked glycosylation sites varied even within each sublineage, ranging from 2 to 6 potential sites, with one located at the proposed cytoplasmic tail conserved among all lineages.

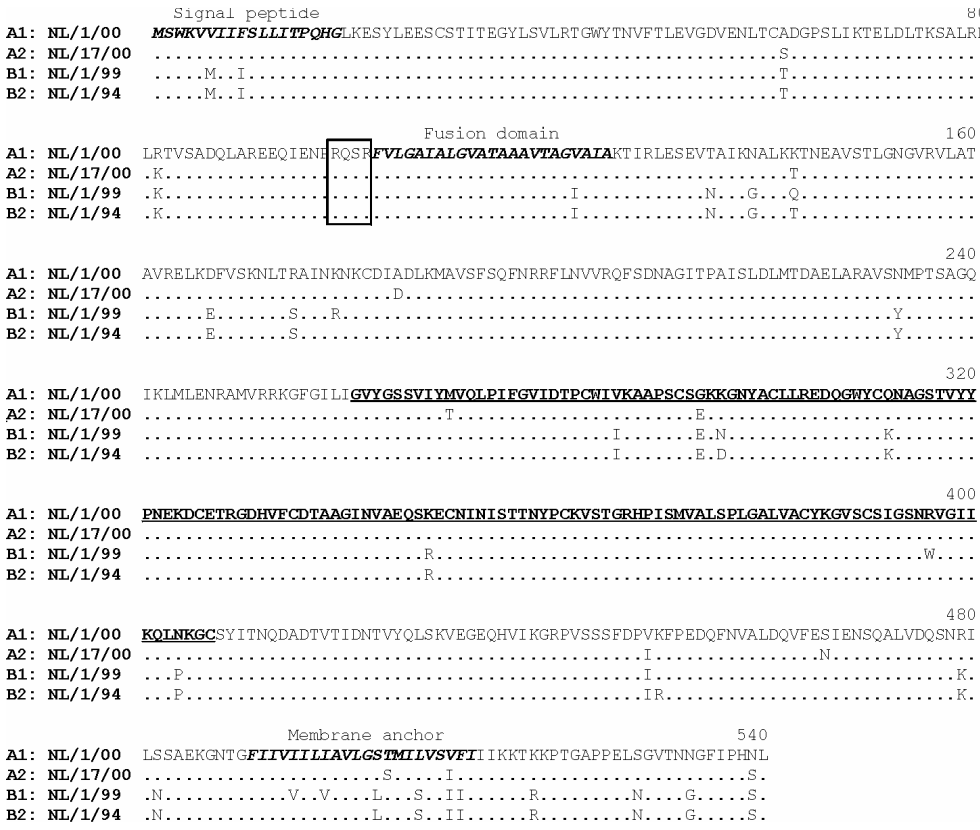

Figure 2:
Amino acid sequence comparison for the fusion genes of prototype hMPV isolates of each sublineage. The predicted signal peptide, fusion domain and membrane anchor are shown in italics in boldface type, the cleavage sites are boxed and the region sequenced for 84 samples is underlined in boldface type. Periods indicate the position of identical aa residues relative to isolate NL/1/00.

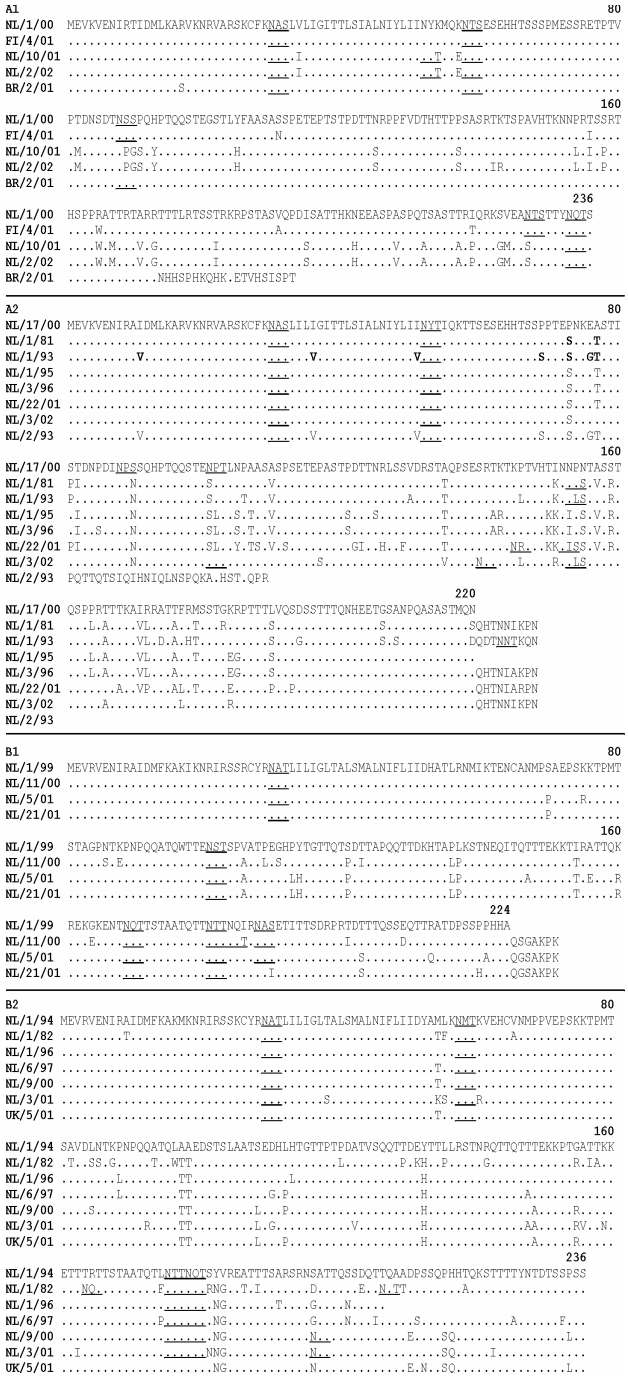

Figure 3:
Amino acid sequence comparison of the putative attachment (G) protein of hMPV strains per genetic lineage. For each lineage only representative samples are depicted, resulting in 24 sequences. Representative sequences were chosen, so that form each year in which samples were obtained at least one sequence is depicted, and sequences with only a few amino acid substitutions were omitted. Potential *N*-linked glycosylation sites are underlined, and periods indicate the position of identical aa residues relative to the first sequence in each subgroup. Numbers indicate the nucleotide position in the primary G ORF.

Geographical and temporal distribution

Analysis of hMPV sequences obtained from samples received from different countries revealed that sequences from Finland, the United Kingdom, and the limited sequences from Asia and Southern America, were found on branches between the Dutch sequences in the F tree, and not as a separate lineage. The variation between sequences obtained from samples from a single country was found in the same range as the variation found between samples obtained from different countries. In agreement with the genetic lineages of hMPV observed world wide, which usually includes sequences similar to those of isolate NL/1/00 or NL/1/99, geographical clustering does not appear to apply to hMPV. The different hMPV samples were obtained over the last 20 years with most of them in the years 2000 until 2002 and fourteen from the 1990's. As indicator for possible fixation of aa variation over time, we analysed the G ORF aa sequence of members in sublineage A2 and B1 (containing samples from 1981 to 2002) in more detail. The aa sequence variation between the viruses from 1981 and 2001 was in the same range as the variation found between viruses from 2001, and alignments of the sequences did not reveal fixation of aa changes between 1981 and 2002. Thus, antigenic drift, as observed for influenza A and B viruses, does not appear to be an important phenomenon for hMPV.

A

Experiment	Virus used in VN	$TCID_{50}$/ well	Ferret I NL/1/00 [A1]	Ferret II NL/1/99 [B1]
1	NL/1/00	12	1024	32
	NL/1/99	9	16	512
2	NL/1/00	30	1024	8
	NL/1/99	20	8	768
3	NL/1/00	40	768	24
	NL/1/99	25	16	768
Ratio A-B			48-128	
Ratio B-A				16-96

B

Virus used in VN	Ferret III NL/1/00 [A1]	Ferret IV NL/17/00 [A2]	Ferret V NL/1/99 [B1]	Ferret VI UK/5/01 [B2]
NL/ 1/00	256	256	6	32
NL/17/00	512	768	12	24
NL/ 1/99	16	32	256	384
UK/ 5/01	12	64	256	512
Ratio A-B	16-21	12-24		
Ratio B-A			21-43	16-21

Homologous VN titers are underlined, values are average of duplicate measurements, ratio's are given as the homologous VN titer divided by the heterologous titers.

Table 2:
Homologous and heterologous VN antibody titers of sera obtained from ferrets infected with hMPV viruses belonging to different genetic sublineages. (A) VN antibody titers obtained in three independent experiments for sera collected 28 days post infection from ferrets I and II (infected with NL/1/00 and NL/1/99, respectively). (B) VN antibody titers obtained for sera collected 21 days post infection from ferrets III to VI (infected with NL/1/00, NL/17/00, NL/1/99 and UK/5/01, respectively).

Antigenic variation

To address the antigenic variation between the genetic lineages A and B, we raised antisera in ferrets against isolate NL/1/00, the prototype virus for lineage A1 and against isolate NL/1/99, the prototype virus for lineage B1. The sera were collected 28 days post infection and tested in VN assays against the homologous and heterologous viruses. In three independent experiments, the virus titer used per well varied between 10 and 50 $TCID_{50}$, which variation did not affect the measured VN titers (Table 2A). Ferret I, infected with the lineage A prototype virus (NL/1/00), showed a 48 to 128 –fold higher VN titer against the homologous virus NL/1/00 than to the heterologous virus NL/1/99. Similarly, ferret II, infected with the lineage B prototype virus NL/1/99, had a 16 to 96-fold higher homologous than heterologous VN titer. In a second experiment, ferret antisera were raised to viruses from all four sublineages, and to measure the most specific serological response, sera were collected 21 days post infection after which homologous and heterologous VN titers were measured (Table 2B).

Within each main genetic lineage, a high degree of cross-neutralisation was observed between viruses from the two sublineages (e.g. A1 vs A2 and B1 vs. B2), which is reflected in the low ratio between homologous to heterologous VN titers: 0.5 to 3.0. Although sera from ferret III to VI had slightly lower homologous VN titers than those of ferrets I and II, sera raised against viruses from the main lineage A still showed a 12 to 24 fold higher VN titer against the lineage A viruses than to lineage B viruses. Similarly, sera raised against viruses from lineage B had a 16 to 43 fold higher VN titer against the lineage B viruses than to lineage A viruses.

Discussion

In the present study the genetic heterogeneity of hMPV was addressed by analysis of the nucleotide and predicted amino acid sequences of part of the F (n=84), complete F (n=4), and the complete G (n=35) protein genes. Phylogenetic analysis of these sequences revealed the presence of two main lineages (A and B) with each divided into two sublineages (1 and 2). As for RSV and APV, the F protein was highly conserved, which is in agreement with F proteins of pneumoviruses having structural and functional constraints for amino acid mutations[236]. Based on the high percentage sequence identity for the complete F proteins of the prototype viruses of the four lineages, sequences for the complete F proteins of all 84 samples would probably demonstrate similar low variability. In contrast to the F protein, the nucleotide and predicted amino acid sequences of the complete G coding regions revealed high sequence diversity (as low as 30-37% aa identity). Besides the high amino acid sequence variation, we observed variation in length of the different G proteins. Where most of the length variation was due to nucleotide substitutions, two of the samples revealed a change in reading frame due to deletion or addition of single nucleotides. Frameshift mutations and use of alternative reading frames have been described for RSV[28,28,91,243,243]. As described for isolate NL/1/00, the G coding region of the 35 samples sequenced in the present work revealed long alternative reading frames. However these secondary ORFs varied in length and position compared to the ones described[259]. Whether the presence of premature stopcodons and the incidence of frame shift mutations and possible use of

alternative reading frames have influence on the antigenic properties of the viruses needs to be examined in more detail.

Phylogenetic analyses revealed that the hMPV samples obtained from different years and from different countries were randomly distributed over all four sublineages. For RSV it has also been reported that very similar viruses were isolated at different times and from geographical distant sites[28]. Different lineages within RSV subgroup A and B have been found based on the variation in the G protein. Within each subgroup progressive accumulation of amino acid changes was noted, suggesting that the G protein of RSV might be susceptible to immune pressure[28]. Analysis of the amino acid sequences of the hMPV samples described in the present study did not reveal indications of such accumulation over time. However the observations that I) most of the amino acid sequence variation was found in the extracellular domain of the G protein and II) the variation found at the amino acid sequence level was higher than that at the nucleotide sequence level and III) the number and position of potential glycosylation sites were not conserved and IV) deletions, additions and substitutions of single nucleotides resulted in premature stopcodons and drastic changes of the carboxy terminal of the protein, indicate that the variation of the hMPV G protein might occur as a result of immunogenic pressure in a same manner as was postulated for the RSV G protein[28,124,124]. Until a larger number of more chronologically diverse hMPV samples have been examined this issue remains inconclusive.

To address the antigenic relationship between members of the different hMPV lineages, we tested ferret sera raised against viruses from the four sublineages in VN assays. Serological responses upon infections tend to broaden over time. Based on the relatively close genetic relationship between sublineages A1 and A2 or B1 and B2, we decided to collect sera at an early time point, in order to obtain large antigenic differences between the four sublineages. The low homologous VN titers in sera collected 21 dpi may explain the lower ratio between homologous and heterologous VN titers as compared to sera collected 28 dpi. The studies with sera collected at 21 dpi revealed that viruses within one main lineage (e.g. A1 and A2 or B1 and B2) were antigenically closely related. The difference in homologous and heterologous VN titers between members of the two different lineages A and B (12 to 128 fold higher homologous titer than heterologous titer) indicate a difference in antigenicity between lineage A and B. Classical virology studies have used a definition of a homologous-to-heterologous VN titer ratio of more than 16 for defining serotypes. This same definition notes that if neutralisation shows a certain degree of cross-reaction between two viruses in either or both directions (homologous-to-heterologous titer ratio of 8 or 16), distinctiveness of serotype is assumed if substantial differences in sequences are observed[87]. Based on the presented results, and based on the described definition, we here propose to define the two main lineages of hMPV as serotypes A and B. The hMPV samples were obtained from different study populations, from different countries and from patients with a wide spectrum of clinical signs. So far, we have no indication for an association between infection with either of the serotypes and a specific study group or with severity of disease. More epidemiological studies are needed to address this issue.

The circulation of two serotypes of hMPV might have implications for the development of vaccines. Studies in cynomolgous macaques revealed that re-infection is suppressed by high titers of VN antibodies against the homologous virus and far less by heterologous VN antibodies (data not shown). So far, for humans only one –heterologous- reinfection has been reported[191]. However, it has been shown that children around the age of five have higher VN

antibody titers than those at the age of one or two[256] suggesting that re-infections may occur frequently, most likely with the viruses from the heterologous serotype. For RSV, the significance of difference in antigenicity between the two subgroups regarding protective immunity and vaccine development is still subject of discussion. However, in animals and in humans the neutralising capacity against homologous viruses is higher than that against heterologous viruses and in animals high homologous VN titers protect against re- infection. In humans it has been shown that re-infection often occurs with a strain from the heterologous group, and high homologous VN antibodies titers protect against severe infection (reviewed in 241). The two serotypes of hMPV might resemble the two subgroups of hRSV in immunogenic properties, although more extensive epidemiological and immunological studies have to prove this. It may well be that the cross-reactive immunity provided by the F protein is sufficient to overcome the effects of changes in the G protein. For hRSV the immune response against the F protein is cross reactive between subgroup A and B, whereas the response against the G protein is subgroup (and sometimes even genotype) specific[125,128,128,184,184]. The prophylactic use of a VN monoclonal antibody preparation directed against the RSV-F protein has been shown to decrease the severity of lower respiratory tract diseases caused by both subgroups of RSV[104,110,110,231,231]. In a similar way, the conserved F protein of hMPV could be a target for the development of monoclonal antibodies for treatment of hMPV-infected individuals.

The data presented in this work support a technical description of two serotypes of hMPV in experimentally infected ferrets. It will be of interest to identify the existence and relevance of these serotypes in other animal species, including humans.

Our results in combination with data published by others[18,192,239192,239] demonstrate that hMPV clusters in two globally distributed serotypes. However, the identification of two serotypes does not exclude the possible existence of more serotypes or sublineages. The described viruses were all identified by using primers against conserved regions in the genome of the four prototype viruses, but in order to allow identification of more diverse hMPV strains, virus isolation of original materials is a standard procedure in our laboratory.

Acknowledgments

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5

Experimental human metapneumovirus infection of cynomolgus macaques (*macaca fascicularis*) results in virus replication in ciliated epithelial cells and pneumocytes with associated lesions throughout the respiratory tract

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Abstract

A substantial proportion of hitherto unexplained respiratory tract illnesses is associated with human metapneumovirus (hMPV) infection. This virus also was found in patients with severe acute respiratory syndrome (SARS). To determine the dynamics and associated lesions of hMPV infection, six cynomolgus macaques (*Macaca fascicularis*) were inoculated with hMPV and examined by pathological and virological assays. They were euthanised at 5 (n = 2) or 9 (n = 2) days post infection (dpi), or monitored until 14 dpi (n = 2). Viral excretion peaked at 4 dpi and decreased to zero by 10 dpi. Viral replication was restricted to the respiratory tract and associated with minimal to mild, multifocal erosive and inflammatory changes in conducting airways, and increased numbers of macrophages in alveoli. Viral expression was seen mainly at the apical surface of ciliated epithelial cells throughout the respiratory tract, and less frequently in type 1 pneumocytes and alveolar macrophages. Both cell tropism and respiratory lesions were distinct from those of SARS-associated coronavirus infection, excluding hMPV as the primary cause of SARS. This study demonstrates that hMPV is a respiratory pathogen and indicates that viral replication is short-lived, polarised to the apical surface, and occurs primarily in ciliated respiratory epithelial cells.

Introduction

A substantial proportion of hitherto unexplained respiratory tract illnesses in human beings is associated with infection by a recently discovered paramyxovirus, provisionally named human metapneumovirus (hMPV)[256]. It is most closely related to avian pneumovirus type C (APV), the aetiologic agent of rhinitis and sinusitis in turkeys[256,259]. Human metapneumovirus was first identified in The Netherlands, where serologic studies indicate that it has been circulating in the human population since at least 1958 and that most children are infected by 5 years of age[256]. Since its discovery in The Netherlands, hMPV infection also has been reported elsewhere in Europe[90,120,239,260] and in North America[75,192], Asia[70,190], and Australia[182]. Respiratory tract disease associated with hMPV infection occurs both in children and adults, suggesting that hMPV is capable of causing clinically important reinfection of individuals later in life. Clinically, hMPV-associated disease includes rhinitis, pharyngitis, bronchitis, bronchiolitis, and pneumonia, and resembles that of human respiratory syncytial virus (RSV) infection[255]. Severity of disease varies from common cold to death, with very young children, the elderly, and immunocompromised patients being predisposed to severe lower respiratory tract disease[255]. In the recent epidemic of severe acute respiratory syndrome (SARS), the role of hMPV as a primary pathogen or co-pathogen was considered[198,255]. Although a newly discovered virus, SARS-associated coronavirus (SCV), proved to be the primary cause of the disease[86,189], 12% (41/335) of SARS patients also were infected with hMPV[139], so that the role of hMPV as a copathogen cannot be ruled out at this time. Until now, pathologic confirmation that hMPV is a primary respiratory pathogen is lacking[18]. Diagnosis of hMPV as the aetiologic agent of respiratory illness in the above studies was based on virus isolation, reverse transcription-polymerase chain reaction (RT-PCR), seroconversion to hMPV, or a combination of these methods, combined with the

failure to detect other known respiratory pathogens. In order to characterise the virus excretion, virus distribution, and associated lesions of hMPV infection, and to determine whether they differ from those of SCV infection, we experimentally inoculated six cynomolgus macaques (Macaca fascicularis) with the prototype hMPV isolate NL/1/00. They were euthanised at 5 (n = 2) or 9 (n = 2) days post infection (dpi), or monitored until 14 dpi (n = 2). Here, we report the pathological, immunohistochemical, virological, serological, and molecular biological findings of this experiment.

Materials and methods

Virus preparation

The prototype hMPV isolate NL/1/00 was propagated three times on tertiary monkey kidney (tMK) cells and used to make a virus stock on tMK cells as previously described[256]. Virus was harvested 7 dpi and frozen in 25 % sucrose at –70 °C. The infectious virus titer of this stock was $10^{4.5}$ median tissue culture infective dose ($TCID_{50}$) per ml by titration on tMK cells.

Experimental protocol

Five days before infection, six juvenile, colony-bred cynomolgus macaques were placed in a negatively pressurised glove box in pairs of one male and one female. The macaques were infected with 5.0 x 10^4 $TCID_{50}$ of hMPV, which was suspended in 5 ml of phosphate-buffered saline (PBS). Approximately 4 ml was applied intratracheally by use of a catheter, 0.5 ml on the tonsils, and 0.25 ml on each of the conjunctivae.The macaques were observed daily for the occurrence of malaise, coughing, exudate from the eyes or nose, forced respiration, and any other signs of illness. Macaques #1 and #2 were euthanised at 5 dpi and macaques #3 and #4 at 9 dpi by exsanguination under ketamine anesthesia, while macaques #5 and #6 were monitored until 14 dpi. The body temperatures of macaques #5 and #6 were measured by telemetry (IMAG, Wageningen, The Netherlands) every 3 min. To this end, a transponder was placed in their abdomens 30 days before inoculation. Just before infection and daily from 2 dpi until euthanasia or 10 dpi, the macaques were anesthetised with ketamine and pharyngeal swabs were collected in 1 ml transport medium [85] and stored at –70 °C until RT-PCR and/or virus isolation. In addition, blood was collected from an inguinal vein just before infection in all macaques and at 14 dpi in macaques #5 and #6. Blood samples were collected in EDTA tubes and centrifuged at 2500*g* for 15 min, the plasma was collected and stored at –70 °C until immunofluorescence assay.

Pathologic examination

Necropsies were carried out according to a standard protocol. Samples for histological examination were stored in 10% neutral-buffered formalin (lungs after inflation with formalin), embedded in paraffin, sectioned at 4 mm, and stained with hematoxylin and eosin (H & E) for examination by light microscopy. The following tissues were examined by light microscopy: adrenal gland, brain stem, cerebellum, cerebrum, heart (left and right ventricle), kidney, larynx, lung (left and right; cranial, medial and caudal lobes), liver, nasal septum (posterior section covered by respiratory epithelium), pancreas, primary bronchus (left and right), small intestine, spleen, stomach, tonsil, trachea, tracheo-bronchial lymph node, upper

eyelid (left and right), urinary bladder. Tissue sections of a clinically healthy juvenile male cynomolgus macaque that had not been infected with hMPV were used as a negative control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 4 mm-thick sections of the same tissues examined by light microscopy were stained using an immunoperoxidase method. Tissue sections were mounted on coated slides (Klinipath, Duiven, The Netherlands), deparaffinised, rehydrated and boiled for 15 min in citric acid buffer (10mM, pH6.0) using a microwave oven. Endogenous peroxidase was revealed with 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO), yielding a blue-black precipitate. Sections were subsequently washed with PBS containing 0.05% Tween 20 (Fluka, Chemie AG, Buchs, Switzerland) and incubated with a polyclonal guinea pig antiserum (dilution 1:200) to hMPV prepared as described previously or with a negative control guinea pig serum for 1 hr at room temperature. After washing, sections were incubated with a horseradish peroxidase (HRP) labeled rabbit-anti-guinea pig Ig (DAKO, Glostrup, Denmark) for 1 hr at room temperature. HRP activity was revealed by incubating the sections in 3-amino-9-ethylcarbazole (Sigma Chemical Co.) solution for 10 min, resulting in a red precipitate. Sections were counterstained with hematoxylin. Formalin-fixed, paraffin-embedded tMK cells infected with hMPV were included as a positive control. Tissue sections of a clinically healthy juvenile male cynomolgus macaque that had not been infected with hMPV were used as a negative control. Selected lung sections were stained with monoclonal antibody AE1/AE3 (Neomarkers, Fremont, California) for the identification of epithelial cells according to standard immunohistochemical procedures.

RT-PCR

Tissue samples of brain, heart, kidney, lung (cranial and caudal lobes), liver, nasal septum, primary bronchus, spleen, tonsil, trachea, and tracheo-bronchial lymph node were weighed and homogenised in minimal essential medium (10 ml per g tissue) by use of Potter tissue grinders. The homogenates were incubated with lysis buffer (2 ml per ml homogenate) containing proteinase K for 1 hr at room temperature, and RNA was isolated by use of a High Pure RNA Isolating Kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's instructions, resulting in 50 µl RNA. RNA was isolated from 200 µl of transport medium from the pharyngeal swabs according to the same method. A Taqman real-time PCR developed in-house[158] was performed in triplo on 5 µl of isolated RNA from each sample, using serial dilutions of a titrated stock of the same virus as the calibration curve. The virus titer was expressed as $TCID_{50}$ per mg tissue or ml transport medium from pharyngeal swabs.

Virus isolation

Virus isolation on pharyngeal swabs was performed on Vero cells, clone 118, in the absence of fetal calf serum in the presence of 0,02% trypsin and 0,3 % bovine albumin Fraction V (Invitrogen, Groningen, The Netherlands). The Vero-118 cell line, developed in house, has equal susceptibility and sensitivity to all known genetic lineages of hMPV. The Vero-118 cells had been grown in Iscove's Modified Dulbecco's medium (Biowhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (Biowhittaker) and 2 mM glutamine (Biowhittaker). The identity of the virus was confirmed by RT-PCR and automatic sequencing[258].

Immune fluorescence assay

96-Well plates coated with Vero-118 cells and infected with hMPV NL/1/00 were incubated with serial dilutions (up to 1:64) of plasma samples for 1 hr at 37°C. After washing with PBS, plates were incubated with fluorescein isothiocyanate-labelled anti-human IgG (DAKO, 1:60) for 1 hr at 37°C. After washing with PBS and background staining with eriochrome black (Sigma Chemical Co.) for 1 min, plates were read under an ultraviolet microscope. Non-infected Vero-118 cells were used as a negative control. Titers were expressed as the reciprocal of the last positive dilution.

Results

Clinical findings

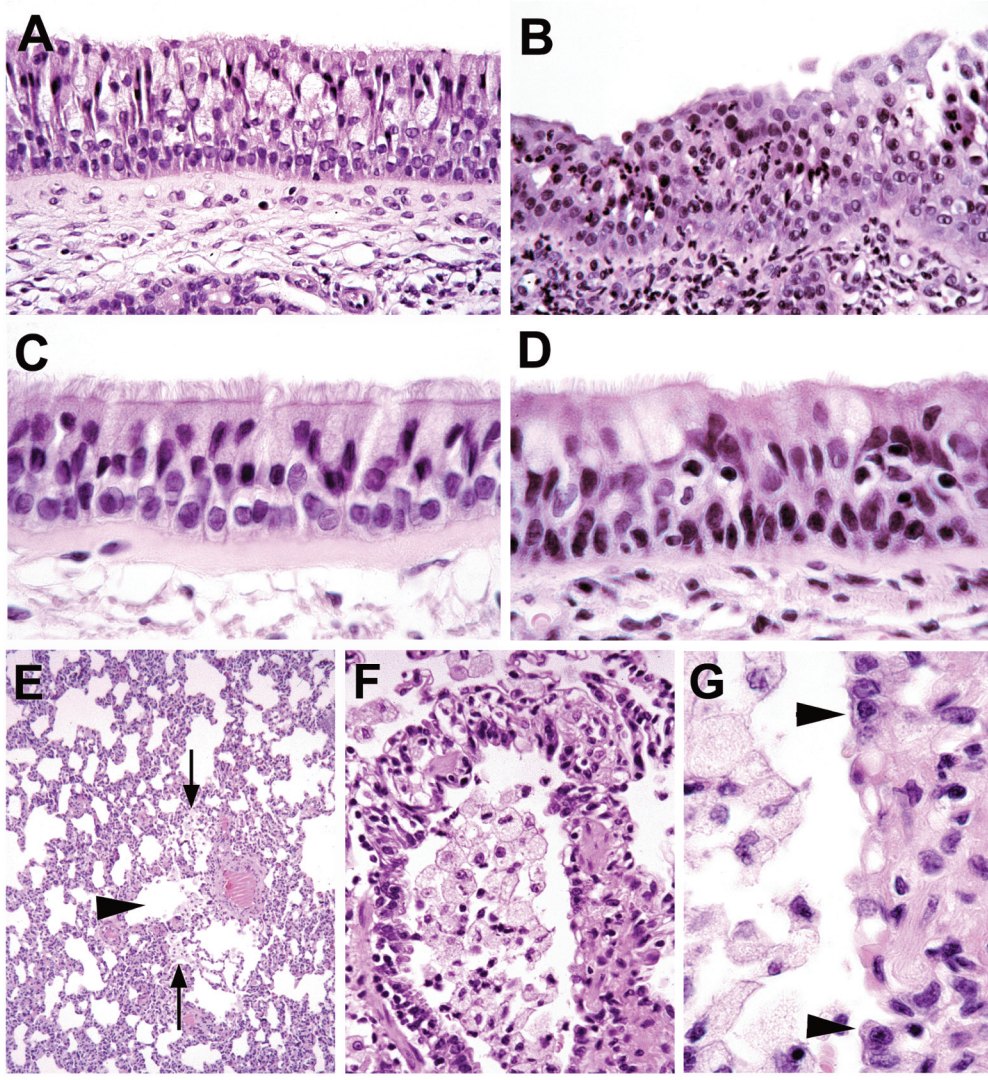
Rhinorrhea was seen in macaque #4 and #5 at 8 dpi. No clinical signs, including increased body temperatures in macaques #5 and #6, were seen in the other macaques.

Gross pathology

Inspissated purulent exudate was present in the nasal cavity of macaques #2 and #4. In the former, the lining mucosa was reddened. Macaque #4 had aspirated food remains immediately prior to euthanasia and was excluded from further laboratory examination because histological lesions of hMPV infection were masked and aspirated debris reacted non-specifically by immunohistochemistry. No other gross lesions were seen in these two macaques or in the other macaques.

Figure 1→:

Histopathology of experimental human metapneumovirus infection in cynomolgus macaques. **A:** Section of respiratory mucosa from nasal septum of negative control macaque. The pseudostratified epithelium consists of ciliated cells with cilia on apical surface, goblet cells with clear cytoplasm, and basal cells lying on the basement membrane. (H&E. Original magnification X100) **B:** Section of respiratory mucosa from nasal septum of macaque # 1. Suppurative rhinitis. There is loss of cilia, intercellular edema, architectural disruption, erosion, and infiltration with many neutrophils in the epithelium and submucosa. (H&E. Original magnification X100) **C:** Section of tracheal mucosa of negative control macaque. The pseudostratified epithelium has the same constituent cells as in panel A. (H&E. Original magnification X250) **D:** Tracheal section of macaque #1. Suppurative tracheitis. There is multifocal loss of cilia, intercellular edema, architectural disruption, and infiltration with a few neutrophils in the epithelium and submucosa. (H&E. Original magnification X250) **E:** Pulmonary section of macaque #3. Bronchiolitis. The bronchiolar lumen (arrowhead) is partly filled with macrophages. An increased number of macrophages also is present in alveolar lumina (arrowheads) adjacent to the bronchiole. (H&E. Original magnification X25) **F:** Pulmonary section of macaque #3. Erosive bronchiolitis. There is loss of bronchiolar epithelium, and the bronchiolar lumen is filled with macrophages. (H&E. Original magnification X100) **G:** Pulmonary section of macaque #3. Erosive bronchiolitis. Detail of panel F. Cuboidal cells (arrowheads) line the bronchiolar wall. The bronchiolar wall in between the arrowheads is denuded. (H&E. Original magnification X250).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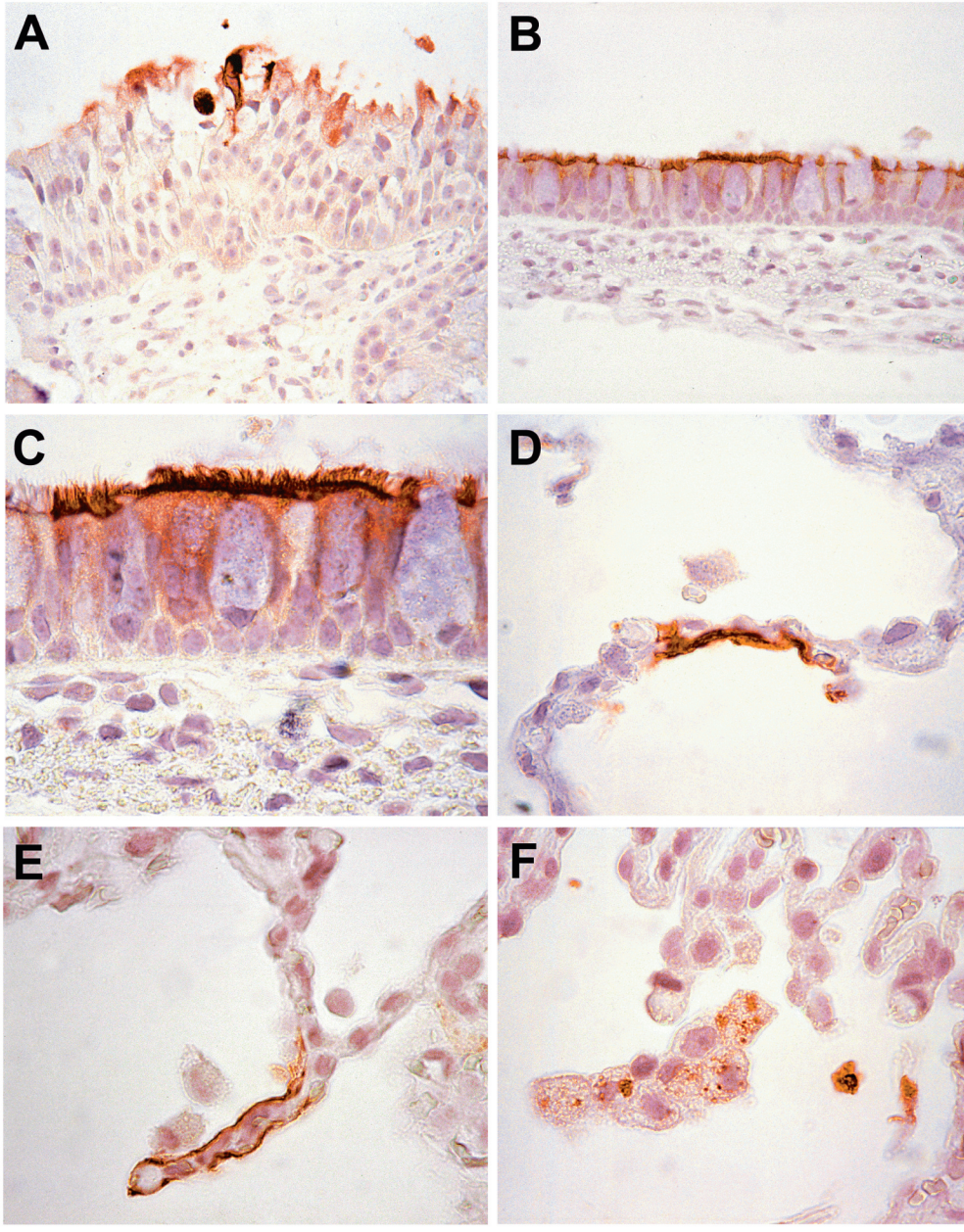
Histopathology

Macaques #1 to 3 had a mild rhinitis, characterised in the epithelium by loss of ciliation, architectural disruption, intra- and intercellular edema, and transmigration of a few neutrophils (Figure 1B). There was edema and infiltration with a few neutrophils in the underlying submucosa. All three macaques had minimal multifocal lesions in the conducting airways, variable in extension from larynx to bronchioles (Table 1; Figure 1, D to F). Epithelial lesions consisted of loss of ciliation, architectural disruption, erosion, intercellular edema, and transmigration of neutrophils. There was infiltration with a few neutrophils in the underlying submucosa. The lumen of some bronchi contained a few sloughed ciliated epithelial cells admixed with scant cellular debris and mucus. The lumen of some bronchioles contained a few alveolar macrophages, rare multinucleated giant cells and neutrophils, admixed with scant cellular debris and fibrin. Similar material was present in the alveoli around affected bronchioles. No significant histological changes were seen in sections of other tissues examined. Above lesions were not seen in the tissues of the negative control macaque.

Figure 2→:

Immunohistochemistry of experimental human metapneumovirus (hMPV) infection in cynomolgus macaques. **A:** Section of respiratory mucosa from nasal septum of macaque #2. Expression of hMPV occurs in the cytoplasm of degenerate epithelial cells and in cell debris. (Immunoperoxidase stain for hMPV. Original magnification X100) **B:** Bronchial section of macaque #1. Expression of hMPV occurs in the cytoplasm of morphologically normal ciliated epithelial cells. (Immunoperoxidase stain for hMPV. Original magnification X100) **C:** Bronchial section of macaque #1. Detail of panel B. Expression of hMPV is most pronounced in the cilia and apical plasma membrane of ciliated epithelial cells. Mucus cells and basal cells stain negative. (Immunoperoxidase stain for hMPV. Original magnification X250) **D, E:** Pulmonary section of macaque #1. Expression of hMPV occurs diffusely in the cytoplasm of type 1 pneumocytes lining the alveolar walls. (Immunoperoxidase stain for hMPV. Original magnification X250) **F:** Pulmonary section of macaque #1. Expression of hMPV occurs in the cytoplasm of alveolar macrophages and in intraluminal cell debris. Staining of alveolar macrophages is multifocal and granular. (Immunoperoxidase stain for hMPV. Original magnification X250)


A
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Immunohistochemistry

Expression of hMPV occurred mainly in ciliated respiratory epithelium from the nasal cavity to the bronchioles in both macaques euthanised at 5 dpi, but not in the macaque euthanised at 9 dpi (Table 1; Figure 2, A to C). It occurred multifocally in individual or groups of adjacent ciliated epithelial cells, and was seen both in morphologically normal (Figure 2B) and in degenerate or sloughed ciliated cells (Figure 2A). Expression of hMPV was visible as dark red staining of the cilia and apical plasma membrane, and diffuse lighter red staining of the cytoplasm. Neither goblet cells nor basal cells stained positively, even where they were located immediately adjacent to positively staining ciliated cells.

Expression of hMPV occurred occasionally in alveoli of all three macaques (Figure 2, D to F). It occurred multifocally in type 1 pneumocytes, individual or small clusters of adjacent alveolar macrophages, and in intraluminal cellular debris. Expression in type 1 pneumocytes was visible as diffuse cytoplasmic staining (Figure 2, D and E). These cells were identified as type 1 pneumocytes because they lined the alveolar walls, were squamous, and expressed keratin in serial sections. In alveolar macrophages, it consisted of multiple distinct dark red granules in the cytoplasm (Figure 2F). Multinucleated giant cells did not stain positively. No positive staining was observed in any of the other tissues examined, nor in tissues of the negative control macaque.

RT-PCR and virus isolation

After an incubation period of 2 days at most, excretion of hMPV increased rapidly to a peak of 1.3 x 10^6 TCID50/ml at 4 dpi, and then decreased gradually to 0 by 10 dpi (Figure 3). The results of RT-PCR were confirmed by virus isolation: hMPV was re-isolated from pharyngeal swabs collected at the peak of virus excretion of all six macaques.

At necropsy, hMPV was detected by RT-PCR throughout the respiratory tract, from the nasal cavity to the lungs, and virus titers were generally higher at 5 dpi than at 9 dpi (Table 1). The virus titers (mean ± standard deviation $TCID_{50}$ per mg tissue) in the tonsil (26 ± 6.4 in macaque #1, 14 ± 3.2 in macaque #2, and 0 in macaque #3) and tracheo-bronchial lymph node (192 ± 42 in macaque #1, 2.1 ± 0.7 in macaque #2, and 3.2 ± 2.5 in macaque #3) corresponded to this temporal pattern. Human metapneumovirus was not detected by RT-PCR in brain, heart, kidney, liver, or spleen of any of the macaques.

Table 1:

Microscopic lesions and presence of hMPV antigen in various tissues of the respiratory tract of cynomolgus macaques experimentally infected with hMPV.

Animal no.	dpi	Nasal septum			Trachea			Bronchus			Cranial lung			Caudal lung		
		HI	IHC	PCR	HI	IHC	PCR	HI	IHC	PCR	HI	IHC	PCR	HI	IHC	PCR
1	5	+*	+	73 ± 9.5†	+	+	377 ± 41	+	+	1675 ± 32	+	+	32 ± 9.3	+	+	73 ± 18
2	5	+	+	47 ± 5.8	-‡	-	4 ± 1.7	-	+	98 ± 16	+	+	115 ± 30	+	+	68 ± 7.4
3	9	+	-	5 ± 2.3	+	-	0 ± 0.2	-	-	25 ± 7.8	+	+	45 ± 5.9	+	+	15 ± 7.5

*:positive; †:mean ± standard deviation $TCID_{50}$/mg tissue; -‡ negative; dpi: days post infection; HI: histology; IHC: immunohistochemistry; PCR: RT-PCR

Immune fluorescence assay
Before inoculation, anti-hMPV antibodies were not detected in plasma samples of any of the six macaques. At 14 dpi, both macaques #5 and #6 had seroconverted with an anti-hMPV antibody titer of > 64.

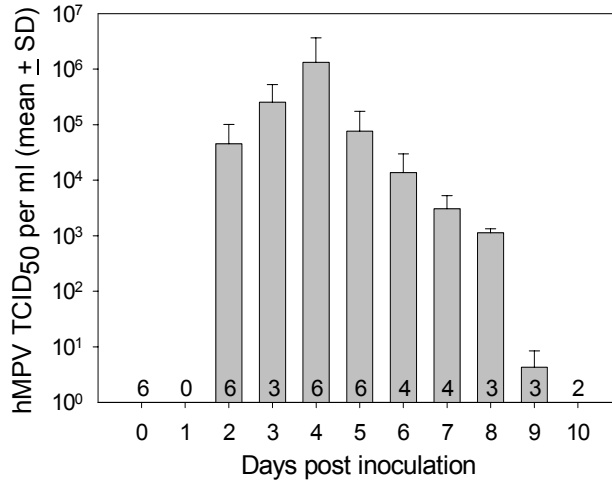

Figure 3:
Pharyngeal excretion of human metapneumovirus by experimentally infected macaques. The numbers in the bars indicate the number of macaques sampled on each day.

Discussion

This experimental infection confirms that hMPV is a primary pathogen of the upper and lower respiratory tract in cynomolgus macaques. Clinical signs in hMPV-infected macaques were limited to rhinorrhea, and corresponded with a suppurative rhinitis at pathologic examination. Additional histological lesions in the respiratory tract were minimal to mild erosive and inflammatory changes in mucosa and submucosa of conducting airways, and an increased number of alveolar macrophages in bronchioles and pulmonary alveoli. The close association between the respiratory lesions and the specific expression of hMPV antigen by immunohistochemistry, together with the absence of these lesions in the negative control tissues, support our conclusion that hMPV infection was the cause of these lesions.
Based on expression of hMPV by immunohistochemistry, viral replication in ciliated epithelial cells was widespread throughout the respiratory tract and more sporadic in type 1 pneumocytes. Viral antigen also was detected in alveolar macrophages, but the distinct granular character of cytoplasmic staining suggests phagocytosis of viral material rather than viral replication. The strong reduction in the distribution of hMPV-infected cells in the respiratory tract between 5 and 9 dpi (Table 1) corresponds with the reduction in viral

excretion, as measured by RT-PCR, from the peak at 4 dpi to zero by 10 dpi (Figure 3). The absence of hMPV expression by immunohistochemistry in other tissues indicates that hMPV replication is restricted to the respiratory tract. The above conclusions were corroborated by the results of virus isolation and RT-PCR. These findings substantiate the claim of van den Hoogen et al. that hMPV infection causes respiratory tract illness in human beings.1 The subclinical or mild character of the disease associated with hMPV infection in these macaques corresponds to that in immunocompetent middle-age adults[255]. Based on the ability of hMPV to replicate in the bronchioles and alveoli of cynomolgus macaques, one may expect more extensive viral replication and an associated increased severity of lesions in the lower respiratory tract of immunocompromised human beings, resulting in the severe bronchiolitis and pneumonia diagnosed clinically in such patients[30,191].The predominant tropism of hMPV for ciliated epithelial cells in the conducting airways and the mildness of the associated lesions, as seen in this study, contrasts with the predilection of SCV for alveolar epithelial cells and the severity of the associated pneumonia in SARS patients[89,138,181,189] and experimentally infected macaques[86,139]. These differences confirm that SCV and not hMPV is the primary aetiologic agent of SARS.

The pathogenesis of hMPV infection in macaques is similar in many ways to that of RSV in human beings, as far as it has been studied. As in hMPV, incubation period and excretion period are short, although RSV excretion may be prolonged in infants and immunocompromised individuals[58]. Both initial infection and subsequent shedding of RSV are restricted to ciliated epithelial cells, based on an in vitro study using recombinant RSV expressing green fluorescent protein[285]. Similar to the localisation of RSV in that study, the results of immunohistochemistry in these macaques show that hMPV infection is polarised to the apical surface of ciliated respiratory epithelium. In immunocompetent individuals, the most common clinical manifestation of RSV infection is mild upper respiratory tract disease[275]. However, viral distribution and associated lesions of this mild disease have not been reported. In very young or immunocompromised individuals, RSV replication occurs in epithelial cells of bronchus, bronchiole, and in alveolar macrophages[180,230], and is associated with severe bronchiolitis, interstitial pneumonia, or giant cell pneumonia[166]. Although clinical studies indicate that hMPV infection may cause similar lesions in this category of patients, confirmation of fatal bronchiolitis or pneumonia from hMPV infection awaits pathologic assessment of biopsy or autopsy samples[18]. The pathogenesis of hMPV infection also is similar to that of APV, its closest known relative[259.] As for hMPV infection in cynomolgus macaques, APV infection in turkeys has a short incubation and excretion period (2 and 8 days, respectively), occurs primarily in ciliated respiratory epithelial cells, and is associated with superficial erosive and inflammatory changes. In contrast to hMPV infection, APV antigen is not present in alveolar macrophages or alveolar walls. However, this difference may be explained, at least in part, by the intratracheal application of hMPV in macaques (this study) compared to the conjunctival and intranasal application of APV in experimentally infected turkeys[122]. The results of this study provide the first characterisation of the viral excretion, viral distribution and associated lesions of hMPV infection in cynomolgus macaques, and help to understand the pathology of this infection in human beings. The immunohistochemical method described above may be useful for retrospective analysis of respiratory tissues of human patients with respiratory disease of unknown viral origin, and to study the possible role of hMPV as a co-pathogen in patients with SARs.

6

Prevalence and Clinical Symptoms of Human Metapneumovirus Infection in Hospitalised patients

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Abstract

During a 17-month period, we performed retrospective analyses of the prevalence of and clinical symptoms associated with human metapneumovirus (hMPV) infection, among patients in a university hospital in The Netherlands. All available nasal-aspirate, throat swab, sputum and bronchoalveolar-lavage samples (N = 1515) were tested for hMPV RNA by reverse-transcriptase polymerase chain reaction. HMPV RNA was detected in 7% of samples from patients with respiratory tract illnesses (RTIs) and was the second- most-detected viral pathogen in these patients during the last 2 winter seasons. hMPV was detected primarily in very young children and immunocompromised individuals. In young children, clinical symptoms associated with hMPV infection were similar to those associated with human respiratory syncytial virus (hRSV) infection, but dyspnea, feeding difficulties and hypoxemia were reported more frequently in hRSV-infected children. Treatment with antibiotics and corticosteroids was reported more frequently in hMPV-infected children. From these data, we conclude that hMPV is an important pathogen associated with RTI.

Introduction

Acute respiratory tract illnesses (RTIs) are the most common diseases experienced by people of all ages worldwide[171]. In young children, human respiratory syncytial virus (hRSV) is the most common cause of RTI[98,133,171]. Recently, a previously unknown pneumovirus was isolated from nasopharyngeal-aspirate samples obtained from children with RTIs in The Netherlands[256]. Those preliminary data indicated that the clinical symptoms of the children were similar to those of patients with RTI caused by hRSV ranging from mild respiratory problems to severe cough, bronchiolitis and pneumonia, often accompanied by high fever, myalgia and vomiting. Some of these patients were hospitalised and required mechanical ventilation. On the basis of the organisation of the viral genome and sequence identity to the *Metapneumovirus* avian pneumovirus, also known as turkey rhinotracheitis virus[63,224], the virus was named human metapneumovirus (hMPV)[256,259]. As a result the *Pneumovirus* and *Metapneumovirus* genera within the subfamily *Pneumovirinae* (family *Paramyxoviridae*) now contain the human pathogens hRSV and hMPV, respectively. Serological surveys have indicated that the prevalence of hMPV in the Dutch population is high, because virtually all children tested were seropositive before the age of 6 years[256]. Recently, hMPV was also detected in children, adults, elderly individuals, and immunocompromised individuals with RTI, in Australia, North America, the United Kingdom, and Finland[18,120,182,191,192,239], indicating that it is a common and ubiquitous human pathogen.

In the present study, all respiratory samples obtained from patients in the university hospital in Rotterdam over a period of 17 months, were tested for the presence of hMPV RNA by reverse transcriptase polymerase chain reaction (RT-PCR). We compared the clinical symptoms of hMPV-infected patients with those of patients infected with other respiratory viruses. Our data indicate that, the prevalence and clinical severity of hMPV infections in an academic hospital setting are slightly lower than those of hRSV infections. Nevertheless, we conclude that hMPV is an important cause of RTI, primarily in infants and immunocompromised individuals, during the winter months.

Subjects, Materials and Methods

Data collection

Between 28 September 2000 and 16 February 2002, 645 throat-swab, 844 nasopharyngeal-aspirate, 18 sputum and 297 bronchoalveolar-lavage samples were obtained at the different wards of the university hospital in Rotterdam and were sent to the diagnostic virology laboratory. For 1515 of these samples (573 throat-swab, 661 nasopharyngeal-aspirate, 12 sputum, and 269 bronchoalveolar-lavage samples), sufficient material was available for hMPV testing. Of these samples, 45% were sent specifically for testing for respiratory viruses, including influenza A and B viruses, hRSV, human parainfluenza virus (PIV) types 1-4, adenovirus, and rhinovirus. Routine virological testing for respiratory pathogens was performed using a combination of direct immunofluorescence (DIF) on cells present in the respiratory specimen, virus isolation in cell cultures, and immunofluorescence (IF). Cell lines used for virus isolation included human embryonal kidney cells, tertiairy monkey kidney cells, Madine Darby canine kidney cells, Vero cells, and Hep-2 cells. After diagnosis of enterovirus infection by virus isolation, IF, and/or DIF, a PCR was performed with rhinovirus-specific primers[194]. Of the throat-swab samples, 622 were obtained from transplant recipients and were submitted for testing for herpes simplex virus (HSV) types 1 and 2, cytomegalovirus and Epstein-Barr virus and were used here as controls. Throat-swab samples were collected in virus transport media[85], and other samples were stored without virus transport media. All samples were kept at 4ºC during processing, and subsequently stored for prolonged periods at -70ºC.

Detection of hMPV RNA by RT-PCR

RNA was isolated from 40-200 µl sample by use of a high pure RNA isolation kit (Roche Diagnostics), according to the manufacturer's instructions. RT-PCRs were performed by use of a 1-tube reaction with primers L6 (5' CAT GCC CAC TAT AAA AGG TCA G 3') and L7 (5' CAC CCC AGT CTT TCT TGA AA 3'), amplifying a conserved fragment of 170 nt in the polymerase gene. This RT-PCR was optimised with respect to enzymes, buffer components and cycling parameters and was found to be 10-100-fold more sensitive than inoculation of tertiary monkey kidney cells with titrated virus stocks (data not shown). Moreover, the assay can detect the genetically diverse hMPV isolates described elsewhere[256]. The L gene was chosen as target for RT-PCR because of nucleotide-sequence conservation, thereby reducing the chance of missing genetic lineages of hMPV that have not been detected previously. PCRs were performed in a volume of 50-µl volume containing 50 mmol/L Tris Cl (pH 8.5), 50 mmol/L NaCl, 4.5 mmol/L $MgCl_2$, 0.2 µmol/L each primer, 0.6 mmol/L each dNTP, 20 U RNAsin, 10 U avian myeloblastosis virus RT, and 5 U of Taq DNA polymerase (all enzymes from Promega). Thermocycling was performed in an MJ PTC-200 apparatus (MJ Research) with the following cycling parameters: 45 min at 42ºC and 5 min at 95ºC once; 1 min at 95ºC, 2 min at 45ºC and 3 min at 72ºC repeated 40 times; 10 min at 72ºC once; and storage at 4ºC. PCR products were analysed by dot-blot hybridisation, as described elsewhere[85], by use of a biotinylated oligonucleotide (5' CTG TTA ATA TCC CAC ACC AGT GGC ATG C 3')

Clinical evaluation and statistical analysis

The medical files of hMPV-infected children were scored by pediatricians, as described elsewhere[136]. In brief, scoring lists included demographical data (sex, age, number of children and parents in the family, gestational age, weight at birth, breast-feeding, and underlying disease of the patient or in the family, etc), clinical symptoms (cough, rhinitis, body temperature, dyspnea, wheezing, feeding difficulties, retractions, respiratory rate, pulse, and cyanosis), laboratory testing (oxygen saturation, pCO2, hemoglobin, hematocrit, platelet counts, levels of C-reactive protein, and chest X-ray results), and intervention and follow-up (artificial respiration, administration of oxygen, use of bronchodilators, and administration of antibiotics and corticosteroids). The medical files for 25 hRSV-infected children were scored for comparison. For each hMPV-infected child, an hRSV-infected child of the same sex who was hospitalised during the same time period (2-3 weeks range) and was closest in age to the hMPV-infected child was selected. Demographic and clinical variables were calculated for the hMPV and the hRSV groups, respectively, and were expressed as point estimates using percentages for dichotomous variables or means with 95% confidence interval (CI) for continuous variables, by use of SPSS for Windows (version 9.0; SPSS). For each variable, the difference between both point estimates with 95% CIs (hMPV vs. hRSV) was calculated by use of the software program Confidence Interval Analysis (version 1.0; BMJ Publishing Group)[4]. If the CI of the difference did not include zero, the corresponding point estimates were regarded as significantly different.

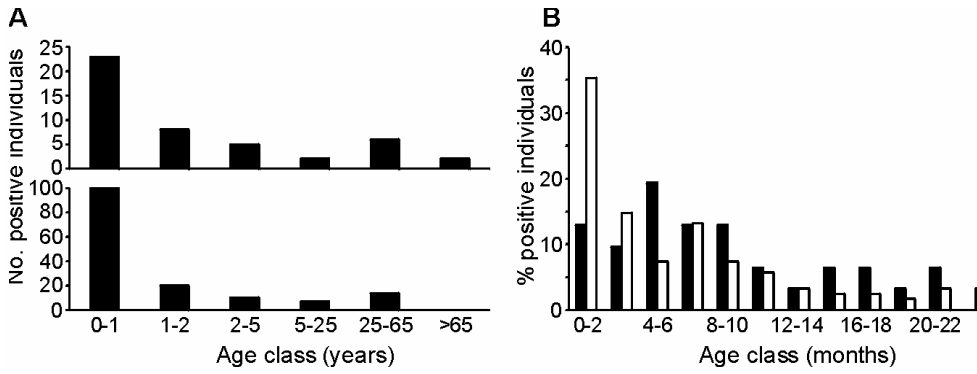

Figure 1:

A. Age distribution of patients infected with human metapneumovirus (hMPV) (*top*) or human respiratory syncytial virus (hRSV) (*bottom*). B. Age distribution of hMPV-infected (*black bars*) and hRSV-infected (*white bars*) children<2 years old. The proportion of children in each age group was calculated relative to the no. of children <2 years old.

Results

Prevalence of hMPV

Fifty-two samples obtained from 46 patients (28 males and 18 females) tested positive for hMPV by RT-PCR. For 44 of these 46 hMPV-positive patients, the samples had been obtained specifically because of respiratory symptoms, including fever, cough, rhinorrhoea, dyspnea, tachypnea, rhinitis, bronchitis, bronchiolitis, and pneumonia (see below). The throat-swab samples obtained from the 2 other patients were sent for HSV testing, but these patients may also have experienced a mild or subclinical RTI. Of the 46 hMPV-infected individuals, 37 were hospitalised in the wards of the children's hospital, 4 were hospitalised in the nursing rooms of the cancer center, and 5 were hospitalised in other departments of Erasmus Medical Center (including the departments of neurology, hematology and internal medicine). The age distribution of the patients with RTI who tested positive for hMPV is depicted in Figure 1.

Most of the hMPV-positive patients were children <2 years old who did not have illnesses other than RTI. Of the hMPV-positive patients who were >5 years old, most had other diseases (e.g. cystic fibrosis, leukemia, and non-Hodgkin lymphoma) or had recently received bone-marrow or kidney transplantation. These latter hMPV infections may therefore, have been associated with host immunosuppression. Although the overall age distribution of hMPV-positive patients was found to be fairly similar to that of hRSV-positive patients in this same cohort (Figure 1A), hMPV was found significantly less frequently in children <2 months old than was hRSV. Of the 31 hMPV-positive children <2 years old, only 4 (13%) were <2 months old, whereas 43 (35%) of the 122 hRSV-positive children <2 years old were also <2 months old (rate difference: -22.3%, 95%CI: -36.9% to -7.8%). We obtained, from 4 hMPV-infected patients, >1 sample that tested positive for hMPV RNA. Three of these patients were 2-, 11-, and 16-month-old children, and virus was detected in nasal-aspirate samples obtained from them 6, 3 and 6 days apart, respectively. The fourth patient was a 36-year-old bone marrow-transplant recipient who was recovering from a varizella-zoster pneumonia and subsequent infection with influenza B virus infection; 4 of this patient's respiratory-tract specimens obtained during a 19-day period, tested positive for hMPV.

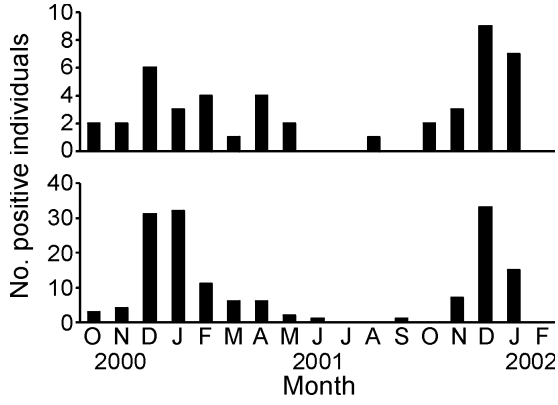

Figure 2:
Detection of human metapneumovirus (hMPV) (*top*) and human respiratory syncytial virus (hRSV) (*bottom*) per month, between September 2000 and March 2002.

hMPV was detected predominantly in samples obtained during the winter months (Figure 2). The seasonal distribution of hMPV-positive samples was found to be largely similar to that of hRSV-positive samples, with the peak of virus detection in December and January, in both the 2000-2001 and 2001-2002 winter seasons. hMPV might be slightly less seasonal than hRSV, since the number of hRSV diagnoses in these 2 seasons was higher than the number of hMPV diagnoses.

To compare the effect of hMPV to that of other respiratory viral pathogens more quantitatively, we compared the diagnostic outcome for 685 specimens sent to the diagnostic virology laboratory specifically for respiratory pathogen testing and for which sufficient material was available to complete all tests. The routine testing for respiratory pathogens in our diagnostic virology laboratory included influenza virus types A and B, hRSV, PIV types 1-4, rhinovirus, and adenovirus. hRSV was detected most frequently, in 126 (18 %) of 685 samples obtained from patients with RTI, and hMPV was the second-most-detected viral pathogen, in 48 (7%) samples (Figure 3).

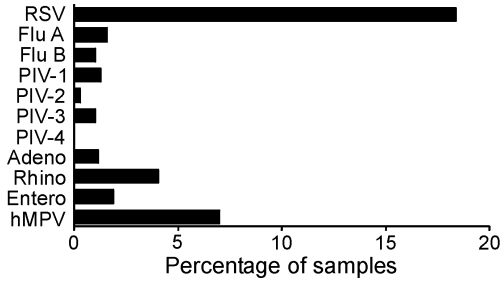

Figure 3:
Comparison of the frequency of detection of human metapneumovirus (hMPV) with those of other respiratory pathogens, in 685 samples obtained from patients with respiratory tract illness. Adeno, adenovirus; Entero, enterovirus; Flu, influenza virus; PIV, parainfluenza virus; Rhino, rhinovirus; RSV; respiratory syncytial virus.

Thus, from patients with RTI, hMPV was isolated more frequently than the PIV (9, 2, 7 and 0 samples were positive for PIV types 1-4, respectively), adenovirus (8 samples), rhinovirus (28 samples), and influenza viruses types A and B (11 and 7 samples, respectively). It is important to note that the sensitivities of detection methods for the range of viral pathogens may be different, making it difficult to compare the contribution of each viral pathogen to the RTI quantitatively. It should also be noted that the influenza virus epidemics in these seasons were milder than those in previous years. From 6 of the hMPV-positive samples, we isolated another respiratory virus: hRSV was isolated from 3 samples, and rhinovirus, influenza A virus, or adenovirus each was isolated from 1 sample. hMPV RNA was detected in only 2 of 622 samples obtained from patients who did not have RTI, suggesting that subclinical hMPV infection is rare in hospitalised patients.

Table 1: Data from medical files of patients infected with human metapneumovirus (hMPV) and human respiratory syncytial virus (hRSV)

Characteristics	hMPV		RSV		95% CI of difference[b]
	No.of patients	Point estimate[a]	No. of patients	Point estimate[a]	
Demographics					
Sex , % male	25	64.0%	25	56.0%	-19.1 to 35.1
Age, months	25	15.0 (8.3 to 21.6)	25	11.2 (5.4 to 16.9)	-4.8 to 12.4
Patient with asthma	25	**16.0%**	25	**0.0%**	1.6 to 30.4
Asthma in family	9	66.7%	20	30.0%	-0.1 to 73.4
Clinical symptoms and laboratory tests					
Cough	25	72.0%	25	76.0%	-28.3 to 20.3
Rhinitis	25	80.0%	25	72.0%	-15.6 to 31.6
Fever	18	61.1%	23	47.8%	-17.1 to 43.7
Dyspnea	25	**28.0%**	25	**76.0%**	-72.3 to –23.7
Wheezing	25	24.0%	25	32.0%	-32.8 to 16.8
Feeding difficulties	25	**36.0%**	25	**76.0%**	-65.2 to –14.8
Retractions	25	60.0%	25	64.0%	-30.9 to 22.9
Hyperventilation	19	42.1%	24	37.5%	-24.9 to 34.1
Tachycardia	17	23.5%	23	52.2%	-57.3 to 0.1
Cyanosis	25	8.0%	25	20.0%	-30.9 to 7.0
Hypoxemia	19	**47.4%**	22	**81.8%**	-62.1 to –6.8
Hypercapnia	14	42.9%	17	41.2%	-33.2 to 36.6
Decreased Hemoglobin	22	22.7%	23	13.0%	-12.6 to 32.0
Decreased Hematocrit	21	33.3%	21	19.0%	-12.0 to 40.5
Trombocytosis	21	9.5%	19	31.6%	-46.4 to 2.3
Illness score[c]	25	**0.38 (0.33 to 0.44)**	25	**0.50 (0.44 to 0.56)**	-0.20 to –0.03
C-reactive protein	18	35.9 (2.8 to 69.1)	20	39.2 (15.7 to 62.7)	-41.8 to 35.3
Chest x-ray performed	25	60.0%	25	64.0%	-30.9 to 22.9
Atelectasis	15	40.0%	16	18.8%	-10.1 to 52.6
Hyperinflation	15	33.3%	16	43.8%	-44.5 to 23.6
Infiltrate	15	33.3%	16	31.3%	-30.9 to 35.0
Bronchial thickening	15	0.0%	16	12.5%	-28.7 to 3.7
Intervention and follow-up					
Artificial respiration	25	12.0%	25	8.0%	-12.6 to 20.6
Oxygen administration	25	**36.0%**	25	**64.0%**	-54.6 to –1.4
Bronchodilators	25	36.0%	25	24.0%	-13.2 to 37.2
Antibiotics	25	**60.0%**	25	**12.0%**	25.0-71.0
Corticosteroids	25	20.0%	25	4.0%	-1.5 to 33.5
Time in hospital, days[d]	15	6.6 (4.6 to 8.6)	19	6.2 (4.6 to 7.7)	-1.9 to 2.8

Clinical symptoms

We next examined the medical files of hMPV-infected children. Because the underlying disease for most hMPV-infected adults could have obscured the hMPV-related RTI, we limited these analyses to otherwise healthy children. Medical files were available for 25 of these children, and the medical files for 25 selected hRSV-infected children were used as controls. Demographic data were similar for the hMPV- and the hRSV-infected groups (Table 1). There were slightly more boys than girls in both groups. Although not statistically significant, the mean age was slightly higher in the hMPV-infected group than in the hRSV-infected group. Virtually all the children in both groups who had siblings were the youngest children in the family, and the household compositions (numbers of children and adults) were similar. The pre- and perinatal data for the 2 groups (e.g. gestational age, birth weight and breast-feeding status) were also not significantly different. In contrast, history of asthma was more often associated with hMPV than with hRSV infection: 16% of the hMPV-infected patients had asthma and 67% of them had a family member with asthma, whereas none of the RSV-infected patients had asthma and only 30% of them had a family member with asthma.The clinical symptoms observed for the hMPV-infected children included cough (72%), rhinitis (80%), fever (61%), dyspnea (28%), wheezing (24%), feeding difficulties (36%), retractions (60%), hyperventilation (42%), tachycardia (23%) and cyanosis (8%). Most of these symptoms were observed with similar frequency in the group of hRSV-infected children.

Footnotes to table 1:

The groups of hMPV- and hRSV-infected children both included 25 patients, but, because some of the files were incomplete, the no. of patients used for comparison may be lower. The following dichotomous variables were calculated from continuous variables by use of reference tresholds derived from healthy children: fever (body temperature >38.5°C), hyperventilation (respiratory rate >60 or >40 breaths/min for children <1 or >1 year old, respectively), tachycardia (pulse >175, >177, >163 and >143 for children 0-3, 3-6, 6-12 and >12 months old, respectively), hypoxemia (oxygen saturation <95%), hypercapnia (pCO2 >5.6 kPa), decreased hemoglobin (<8.1 or <6.6 mmol/L for children <1 or >1 month old, respectively), decreased hematocrit (<0.42 or <0.33 L/L for children <1 or >1 month old, respectively), and trombocytosis (>390 x 10^9 or >473 x 10^9 platelets/L for children <1 or >1 month old, respectively). CI, confidence interval.

[a] .Point estimates are given as percentages, for dichotomous variables, or as mean (95% CI), for continuous variables.

[b] .If the 95% CI did not include 0, the corresponding point estimates were considered to be statistically significant and appear in boldface

[c] .Each of the 15 clinical symptoms above was scored (1 and 0 for presence and absence, respectively), and the sum was divided by the no. of recorded symptoms. Thus, the illness score could range from 0.00 (no symptoms present) to 1.00 (all symptoms present).

[d] .Three cases of hMPV infection were excluded from this comparison because of additional morbidity unrelated to the hMPV infection.

However, dyspnea, feeding difficulties (primarily, decreased intake of food), and hypoxemia were reported significantly more frequently in hRSV-infected children than in hMPV-infected children. Platelet counts, relative white blood cell counts, and levels of C-reactive protein were similar for both groups. For 62% of the children, an X-ray of the lungs revealed atelectasis, hyperinflation, and infiltrates as the most common abnormalities, in both groups of patients.

In agreement with the finding of a high number of hRSV- infected patients with hypoxemia, oxygen was administered to significantly more hRSV-infected patients than hMPV-infected patients (64% vs. 36%). In contrast, hMPV-infected patients were treated more often with bronchodilators (36% vs. 24%), antibiotics (60% vs. 12%, statistically significant), and corticosteroids (20% vs. 4%) than were hRSV-infected patients (Table 1). The mean duration of hospital stay was similar for both patient groups (6-7 days). It should be noted that, for this latter analysis, 3 hMPV-infected children were excluded because of additional morbidity that was presumably unrelated to the hMPV infection, such as subsequent infection with other pathogens.

Discussion

Since the discovery of hMPV several studies have provided important information on hMPV epidemiology, clinical symptoms associated with hMPV infection, and the patient groups that are at risk for hMPV infection[18,120,182,191,192,239,256]. From these studies, it is now becoming increasingly clear that hMPV is an important human pathogen associated with RTI in young children, immunocompromised individuals, elderly individuals, and, to a lesser extent, other populations. In the present study, we analysed the relative contribution of hMPV to RTI, in a university hospital setting, and compared the clinical signs with those caused by infection with the related pathogen, hRSV.

In the 2000-2001 and 2001-2002 winter seasons in The Netherlands, hMPV and hRSV were both found primarily in December and January; they were rarely detected in the summer months. It should be noted that, during these years, hMPV infections were temporally distributed somewhat more equally than were hRSV infections. The populations of patients most affected by the two viruses- children, immunocompromised individuals, and elderly individuals- were also quite similar, as was the overall age distribution of the infected patients. However, it is interesting to note that hRSV was detected more frequently in very young children than was hMPV; 35% of hRSV-infected children <2 years old were also <2 months old, compared with only 13% of the hMPV-infected children <2 years old. Whether this observed difference is a true reflection of the differences in biological properties of the respective viruses requires further confirmation. During the 2 winter seasons under study, the 2 genetic lineages of hMPV that were identified previously[256]were cocirculating in The Netherlands (data not shown), as has been described recently for other countries[18,192,239]. Differences in the pathogenicity of viruses belonging to the hRSV subgroups A and B have been described by some research teams but not by others [106,116,164,246,263]. Unfortunately, at present, our dataset is too limited at present to compare the relative prevalence and pathogenicity of the distinct lineages of hMPV in a similar fashion.

Comparison of the medical files of hMPV-infected children with those of hRSV- infected children revealed that the clinical symptoms associated with these viruses were quite similar.

Dyspnea, feeding difficulties, and hypoxemia were recorded more frequently in hRSV-infected children than in hMPV-infected children, but all other recorded symptoms were found at the same frequency in both groups. For each of the children in both groups, we calculated an illness score, defined as the number of clinical symptoms the children had, divided by the total number of symptoms that were recorded for each child (Table 1). This analysis revealed that the hMPV-infected children had, on average, 38% of the recorded symptoms, whereas hRSV-infected children had 50% of the symptoms (statistically significant), suggesting that hRSV may be slightly more pathogenic than hMPV. The higher proportion of children <2 months old in the hRSV group did not have a serious effect on this observation; reanalysis of the data, excluding children <2 months old, still resulted in a statistically significant difference for the illness scores.

It is important to note that subclinical hMPV infections appear to be rare; we detected hMPV RNA in only 2 of 622 samples obtained from patients who did not have RTI. This finding, as well as the observation that hMPV may cause mild RTI in experimentally infected macaques[256], is indicative for hMPV being a causative agens of RTI. In agreement with these observations, we did not detect any other pathogens in the majority of patients with hMPV-related RTI. Three hMPV-infected patients were also positive for hRSV, and 3 were positive for either influenza A virus, adenovirus or rhinovirus. On the basis of the similar seasonal distribution of hMPV and hRSV infections (Figure 2), dual infection with these 2 viruses is not unlikely.

The statistical significance of the difference between the proportion of children with asthma in the hMPV group and that of children with asthma in the hRSV group requires confirmation in larger scale studies in the future. Numerous studies have provided evidence for a link between hRSV in early childhood and subsequent manifestations of asthma[232,238], although this issue is still being studied[137]. The link between hMPV infection and asthma appears to be different from that suggested for hRSV infection. It will be of interest to further elucidate the underlying mechanisms for the possible association between asthma and either hRSV or hMPV infection, in future studies.

It was not surprising that antibiotics and corticosteroids were administrated to hMPV-infected children more often than to hRSV-infected children. Because hRSV diagnostics were performed in real time, physicians were able to choose not to give antibiotics and corticosteroids after a positive hRSV diagnosis. However, because, at the time of hospitalisation, no aetiologic agent had been identified in children with RTI associated with hMPV infection, physicians continued treatment with antibiotics and corticosteroids, to control potentially unidentified bacterial infections. This indicates that the inclusion of hMPV in diagnostic testing of patients with RTI may reduce unnecessary use of antibiotics and corticosteroids. Moreover, hMPV diagnostic testing may reduce virus transmission between children in hospital wards, through isolation or alternative measures currently used to limit the spread of hRSV. For a virus that is not easily detected by virus isolation in the laboratory, it will be of great importance that rapid, sensitive, and reproducible diagnostic tests be developed. Our RT-PCR procedure, which is based on the amplification of a conserved sequence in the polymerase gene, proved to be more sensitive than virus isolation and can detect genetically distinct hMPV strains. However, RT-PCRs based on the N gene may take advantage of the transcriptional gradient used by paramyxoviruses and, therefore, may be even more sensitive. In addition, monoclonal antibodies recognising conserved hMPV epitopes will be useful for rapid virus diagnostics by use of IF or DIF techniques

currently used for diagnosing infections with other virus pathogens, including hRSV. It will be important to conduct a wide range of prospective and retrospective studies to obtain a better estimate of the incidence, prevalence, and clinical effect of hMPV in different populations, the full spectrum of hMPV diseases, and risk factors that may be associated with severe hMPV disease.

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Metapeumoviruses and acute wheezing in children

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Abstract

A new respiratory virus, human metapneumovirus, was recently identified. We detected this virus by PCR in ten (8%) of 132 consecutive children admitted to Turku Hospital, Finland, for acute expiratory wheezing (median age 7 months, range 4–25). The mean duration of hospital stay was 2.5 days (SD 1.6) and mean duration of respiratory symptoms was 19 days (8). The white blood cell count, C-reactive protein, and regulated upon activation, normal T-cell-expressed and T-cell-secreted (RANTES) concentrations in nasal secretion remained low, whereas interleukin 8 concentrations in nasal secretion were high. Human metapneumovirus is a clinically important causative agent of acute wheezing in young children.

Report

Investigators from The Netherlands[256] and North America[192] have identified an earlier unknown virus from the *Paramyxoviridae* family, human metapneumovirus. Identification of this virus was based on virological data, sequence homology, and gene constellation. Clinically and cytopathologically, infection with human metapneumovirus resembles that of respiratory syncytial virus, ranging from mild upper respiratory tract disease to severe bronchiolitis and pneumonia in children. Results of a serological survey[256] showed that human metapneumovirus usually infects young children; all 80 children in the survey in The Netherlands at the age of 5 years were seropositive for the virus. We investigated the frequency of human metapneumovirus in children admitted to the paediatric department of Turku University Hospital, Finland for acute expiratory airway obstruction.

Between Sept 1, 2000, and May 31, 2001, 132 consecutive children participated in the continuing study of the efficacy of systemic glucocorticoids in acute expiratory wheezing in the Department of Paediatrics, Turku University Hospital, Finland. We included children if they were aged 3 months to 16 years, had been admitted for acute expiratory wheezing, and if their parents had provided written informed consent. Children were excluded if they had a chronic disease other than asthma or allergy, had taken systemic glucocorticoids 4 weeks or less before the start of the study, or had severe wheezing and treatment in an intensive care unit. The median age of the children was 2.0 years (range 4.0 months to 13.5 years). 25 children were diagnosed with bronchiolitis, 59 with wheezy bronchitis, and 48 with acute asthma. The study protocol was approved by the ethics committee of Turku University Central Hospital.

On admission, we obtained nasopharyngeal aspirates and assessed them for presence of human metapneumovirus with a reverse transcriptase PCR assay using primers in the L gene that detect both genotypes of the virus[256]. The primers were tested for specificity against members of the respective paramyxovirus genera and the assay detected 0.01 tissue culture infective dose 50/mL virus. We cultured the aspirates and assessed the presence of antigens of influenza A, influenza B, adenovirus, respiratory synctial virus, and parainfluenza types 1, 2, and 3, and did PCR for coronavirus, rhinovirus, and enterovirus[160]. Primer sequences and experimental methods are available from the authors. Symptoms were recorded by the parents using a home diary for 2 weeks after discharge.

We identified a potential causative viral agent in 116 (88%) of the 132 children; 31 (27%) had rhinovirus, 26 (22%) had enterovirus, 19 (16%) had non-typable picornavirus, 16 (14%) had respiratory syncytial virus, and ten (9%) had human metapneumovirus. Seven of these ten children had human mettapneumovirus alone; the other three children also had other respiratory viruses. Human metapneumovirus was detected in children only from from January to April 2001. The median age of the children with human metapneumovirus was 7 months (range 4–25). Five children had bronchiolitis, four wheezy bronchitis, and one newly detected asthma. No child had previously used glucocorticoids.
On admission, in children with human metapneumovirus, cough had lasted for a mean of 9 days (SD 10), rhinitis for 4 days (5), expiratory wheezing for 3 days (2), and fever for 3 days (2). Two of the seven children with human metapneumovirus alone were diagnosed with acute otitis media. The mean axillary temperature was 37.8°C (1.0) and the mean oxygen saturation was 96% (2). Children had a mean of 9.5×10^9 white blood cells/L (3.0×10^9) and a mean serum C-reactive protein concentration of 9 mg/L (11). The mean interleukin 8 concentration in nasopharyngeal aspirates obtained from six children was 480 ng/L (360) and the mean concentration of regulated by activation, normal T-cell expressed and secreted (RANTES) was 50 ng/L (31). All patients were given nebulised salbutamol. The patients were randomly allocated to receive 2 mg/kg oral prednisolone per day for 3 days or placebo. The study code has not yet been opened. The mean duration of hospital stay was 69 h (45). After hospital stay, the cough had lasted for a mean of 7 days (4), dyspnea for 6 days (5), and low fever for 0.4 days (0.9). One of the seven patients was readmitted to hospital within 2 weeks because of recurrent breathing difficulties.

In our study, the clinical diagnosis and age of the patients was closely similar to those seen in studies of infection with respiratory syncytial virus[218,256]. However, human metapneumovirus arose at a different time from respiratory syncytial virus infections. Human metapneumovirus was identified from the middle of winter to spring—a time when a limited spring epidemic of respiratory syncytial virus had just started in Finland. From January to April, 2001, we identified human metapneumovirus in ten (32%) of the 31 children with wheezing.
The chemokine profile of interleukin 8 (mainly a chemotactic factor for neutrophils) and RANTES (chemotactic factor for eosinophils) in nasal secretions was different to that reported in infections with respiratory syncytial virus[219]. Patients with respiratory syncytial virus had high concentrations of RANTES and varying concentrations of interleukin 8[219], whereas in our study, children with human metapneumovirus had low concentrations of RANTES and high concentrations of interleukin 8. The cytokine concentrations and blood samples were not confounded by use of prednisolone because the samples were taken before treatment. Clinically, the efficacy of systemic glucocorticoids in bronchiolitis remains unknown.

Our results suggest that human metapneumovirus is a causative agent of acute wheezing in young children. During the study period, this virus was identified in childen at a time when other common causative agents, rhinovirus and respiratory syncytial virus, were not epidemic. Although the white blood cell counts and concentrations of C-reactive protein remained low, as usually found in viral infection[218], the inflammatory response to human metapneumovirus was different from that seen in respiratory syncytial virus infection.

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Human metapneumovirus in a haematopoietic stem cell transplant recipient with fatal lower respiratory tract disease

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Abstract

Respiratory viruses are increasingly recognised as a cause of pneumonitis following haematopoietic stem cell transplantation (HSCT). However, frequently, no pathogen is identified in cases of suspected viral pneumonia. Recently, a previously undescribed paramyxovirus, designated 'human metapneumovirus' (hMPV), was isolated from children with respiratory illness. We have detected hMPV as the sole pathogen in the nasopharyngeal aspirate of an HSCT recipient who succumbed to progressive respiratory failure following an upper respiratory prodrome. This report highlights the importance of further studies to elucidate the role of hMPV in causing respiratory illnesses in the HSCT population.

Introduction

Interstitial pneumonia remains an important cause of mortality following haematopoietic stem cell transplantation (HSCT). With better understanding and management of cytomegalovirus infections, community respiratory viruses account for most of these cases. The most common viruses detected are human respiratory syncytial virus (RSV) and parainfluenza viruses. They account for about 30% of the acute respiratory illnesses post transplant and 50% of them progress to pneumonia with 30-100% mortality, depending on the clinical setting[20,32,270]. However, a significant minority of HSCT recipients present with a clinical condition indistinguishable from respiratory illness caused by respiratory viruses, which cannot be attributed to a known pathogen. We have an ongoing surveillance programme for post-transplant respiratory virus infection and no aetiologic agent is identified in 40% of upper respiratory illnesses and 30% of cases of suspected viral pneumonia[32].
Recently, a previously undescribed virus was isolated from children with RSV-like disease in The Netherlands[256]. This has been designated human metapneumovirus (hMPV) as it resembles avian pneumovirus (also known as turkey rhinotracheitis virus), in both gene order and sequence[259]. Preliminary data indicate that this newly described virus is found in about 10% of childhood lower respiratory disease cases in which no other virus was identified. HMPV has now also been identified in respiratory samples from individuals aged 2 months to 87 years with acute respiratory tract illness in Canada[192]. Little is known about the significance of this newly described pathogen in immunocompromised patients. Recently, fatal pneumonia owing to hMPV was described in a child on treatment for acute lymphoblastic leukaemia (ALL)[191]. We describe the detection of hMPV for the first time in a fatal case of pneumonitis in an allogeneic HSCT patient and discuss the possible implications.

Case report

A 33-year-old lady with ALL received a T-cell-depleted peripheral blood stem cell graft (6 $x10^6$/kg of CD 34+ cells) in August 2000, from her HLA-matched sibling, following conditioning with cyclophosphamide and total body irradiation. Cyclosporine A alone was used as graft-versus-host disease (GVHD) prophylaxis. She had engraftment with donor cells by 14 days post transplant. She had a secondary graft failure 80 days after the transplant and

received a second transplant ($2.6x10^6$/kg of CD 34+ cells) conditioned with ATG. However, she remained pancytopenic 21 days following this and received a further dose of donor cells ($5.6x10^6$/kg of CD 34+ cells) after conditioning with fludarabine and melphalan. Seven days after her third transplant she developed pyrexia with cough and coryzal symptoms with negative microbial culture from blood, sputum, stool, and urine. She was initially treated with broad-spectrum antibiotics. Amphotericin and clarithromycin were introduced 5 days later. The respiratory symptoms progressively worsened and the chest X-ray and CT scan showed both interstitial and alveolar opacities. A nasopharyngeal aspirate carried out at that stage (12 January 2001) was negative for RSV, influenza A and B, adenovirus and parainfluenza virus by direct immunoflorescence and culture. PCR assays for CMV were carried out on peripheral blood twice weekly and remained negative. She deteriorated progressively with worsening hypoxia and lung infiltrates coincident with recovery of the white cell count and was treated with a combination of imipenem, clarithromycin and ambisone. The patient refused any further intervention in the form of broncho-alveolar lavage (BAL) and ventilation. She succumbed to progressive respiratory failure on 17 January 2001. The family did not consent to a post-mortem examination.

Detection of hMPV

The stored nasopharyngeal aspirate taken 5 days before the demise of the patient was retrospectively examined for hMPV using RT-PCR and sequencing of PCR product undertaken as previously described[256,259]. This was found to be positive for hMPV. Nucleotide sequence analysis of part of the fusion (F) gene of the virus from this patient showed that it was most closely related to the ned/00/01 strain previously described[256,259].

Discussion

This patient presented with an upper respiratory prodrome and progressed to develop pneumonia and respiratory failure. Microbiological investigations for bacteria and viruses were consistently negative. Although the clinical features were compatible with a respiratory virus illness, the NPA sample was negative for the known viruses. Worsening of the respiratory illness in this patient was coincident with white cell recovery, compatible with the pattern noted with other respiratory viruses. The detection of this newly identified respiratory virus, hMPV, in this patient is significant in that light and in the given clinical situation, hMPV can be considered as a possible aetiology for the respiratory illness. Whether or not hMPV was the cause of the respiratory symptoms cannot be definitively determined without demonstrating viral replication in the lung tissue and it is possible that the virus was simply an innocent bystander or a copathogen. A recent report implicated this virus in the causation of recurrent lower respiratory tract infections in a child with ALL[191]. In this case, hMPV was identified retrospectively and it was not demonstrable in the BAL specimen. However, the demonstration of hMPV in the NPA samples from both episodes in the absence of other isolates suggests that hMPV was probably the pathogen responsible for the fatal pneumonia in this child. Although this child was on intensive treatment and immunosuppressed, the immunodeficiency in allograft recipients is more severe and global. The similarities between this case[191] and our findings would warrant further investigation into the role of hMPV in respiratory illnesses following HSCT.

The implications of such a finding could be quite profound in the setting of HSCT. If hMPV is indeed a cause of lower respiratory infection in transplant recipients, further understanding of the epidemiology of this virus would be essential for the prevention of its spread within this vulnerable population, particularly within BMT units. Moreover, there might be a case for exploring the susceptibility of hMPV to antiviral agents such as ribavirin, both *in vitro* and *in vivo*.

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9

General Discussion

Adapted from

Clinical impact and diagnosis of hMPV infections

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Prevalence of hMPV infections

After the discovery of hMPV as an aetiologic agent of respiratory tract illnesses (RTI)[256] several retrospective studies randomly surveyed specimens collected during the past 10 years from patients with RTIs of unknown aetiology[18,182,192]. HMPV was detected primarily in samples collected during the winter months. Samples were generally obtained from young children, but also from adults. Most of the hMPV-positive samples were obtained from children younger than 5 years of age with mild to severe RTI. Serological studies showed the presence of antibodies to the virus in virtually all children age 5 years or older, and the virus has been circulating at least 43 years in the human population[256]. Since the discovery of hMPV in The Netherlands, the virus has been detected worldwide.

hMPV in hospitalised children

Several studies have confirmed the original observation that children younger than 5 years of age are most susceptible to hMPV infection (Table 1). A recent study by Peiris, et al. evaluated the impact of hMPV infection in hospitalised children younger than 18 years with RTI within a 13-month period[190]. They demonstrated the presence of hMPV in 5.5% of the children. These hMPV-positive patients ranged from 3 months to 5 years of age, with a mean age of 32 months. Our study on samples collected during a 17-month period from hospitalised patients in all age categories demonstrated the presence of hMPV in 6.5% of the patients suffering from RTI[258]. Most of the hMPV infections were found in children younger than 5 years, with a peak in children between 4 and 6 months of age. As found by Peiris, et al., hMPV-infected patients proved to be slightly older than respiratory syncytial virus (RSV)-infected children: 35% of RSV-infected children <2 years of age were <2 months old, compared to only 13% of the hMPV-infected children <2 years of age[190,258]. Because these studies demonstrated that children <2 years of age are at the greatest risk, it is not surprising to find 17.5%[260] or 25%[159] of samples from hospitalised children younger than 2 years with RTI to be hMPV-positive (Table 1). If slightly older children are included, lower percentages of patients are hMPV-positive. Among hospitalised children younger than 3 years[19,261] 5.5% and 4.1% were hMPV-positive , and in two studies of children younger yhan 5 years of age[75,90], 6.4% and 6.6% were positive. Again, however, most of the hMPV-positive patients in these studies were under the age of 2 years.

The similar seasonality of several respiratory virus infections may result in frequent co-infection of hMPV with other respiratory viruses such as RSV and influenza virus[9,18,19,75,77,159,190,258,260]. This might lead to underestimates of the percentage of hMPV-positive samples identified in studies in which only samples negative for other respiratory viruses were tested[75,90]. The data thus far illustrate that hMPV accounts for roughly 5 to 7% of the RTI in hospitalised young children, with children <2 years of age being most at risk for serious hMPV infections (Figure 1).

hMPV in the elderly and immunocompromised individuals

Like RSV and influenza virus infections hMPV infections also account for RTIs in the elderly population and in patients with underlying disease[57,78,113]. In our study most of the hMPV-positive patients between the ages of 5 and 65 years (n = 13) either had an underlying disease, such as cystic fibrosis, or received immunotherapy[258]. In other studies[19,18,75] 25 to 50% of the hMPV- positive patients had an underlying disease. One study among the elderly indicated that hMPV caused more severe disease in frail elderly patients, such as nursing home residents, than in elderly or younger adults who are "fit"[77]. This same study included 309 hospitalised elderly patients or patients with underlying disease; 6.5% of these patients were positive for hMPV. Although comprehensive surveys for hMPV infections among immunocompromised and elderly individuals have not yet been performed, these combined data indicate that hMPV infection, just like RSV and influenza virus infections[119,234] may be responsible for mild to severe RTI in the elderly and immunocompromised (Figure 1).

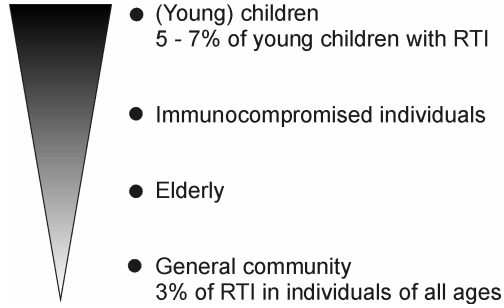

Figure 1:
Prevalence of hMPV in different populations. Percentages are probably underestimates, because of the choice of inclusion criteria and diagnostic tests (see text for details).

hMPV in the general community

Although hMPV has also been detected among individuals suffering from respiratory illnesses in the community, the incidence varied by study group. In the UK, 1.3% of patients presenting to family physicians with influenza-like illnesses (ILI) were hMPV-positive[239]. This is likely an underestimate, because the study did not include all RTI but was limited to samples from ILI patients who were negative for other viruses. In studies in which all respiratory viruses were assayed in samples obtained from patients suffering from acute RTI (ARTI), samples from 3% of the patients tested positive[19,273]. In fact, in the study conducted in The Netherlands, fewer hMPV-positive samples were observed among patients with ILI than among patients with ARTI[273]. A study evaluating 167 adult volunteers younger than 40 years of age during home visits for respiratory disease detected hMPV in 6.6% of the patients using a combination of reverse transcriptase (RT)-PCR and serological testing during a 2- to 4-month period[77]. Samples from 50% of the seroconverters were shown to be positive by RT-PCR. These serological tests also detected seroconversion during a 5- to 6-month period in samples obtained from healthy adult volunteers in the community. When samples obtained from patients hospitalised for other diseases or from patients attending a physician for complaints other than RTI were tested, only two of 1282 samples were found positive for hMPV[19,258,273], indicating that asymptomatic or subclinical infections are rare.

The failure to detect hMPV in healthy individuals in combination with the described observations in patients suffering from RTI give strong indications for the causal relationship

between hMPV infection and RTI, according to Koch's postulates, as modified by Rivers for viral diseases[211]: isolation of the virus from diseased hosts, cultivations of the virus in host cells, and seroconversion upon infections. Additional criteria, such as production of comparable disease in the original host species or a related one, re-isolation of the virus, and detection of a specific immune response to the virus, were obtained by studies in experimentally infected cynomolgus macaques (chapter 4). These macaques demonstrated viral shedding as measured by RT-PCR and virus culture, and seroconverted upon infection as measured by IFA and ELISA. The criterion to produce a comparable disease in the original host species or a related one has not been completely fulfilled. Pathologic examination of the respiratory tract of these infected monkeys revealed virus replication in epithelium cells in both the upper and lower respiratory tract, however these results cannot be compared with pathological examinations of respiratory tracts of hMPV infected humans, since these have not (yet) been conducted. In addition, the subclinical or mild character of the disease associated with hMPV infection in these macaques corresponds to that in immunocompetent middle-age adults, while hMPV causes primarily severe disease in young infants. Where the obtained results established hMPV as an aetiologic agent of RTI, additional experiments have to reveal whether cynomolgus macaques (or other animals) are suitable as animal model to mimic severe disease caused by hMPV in young infants.
Studies for such animal models have been undertaken, and have demonstrated that Syrian golden hamsters, ferrets, and African Green Monkeys are suitable animals to study hMPV infections, candidate vaccines and intervention strategies (M. MacPhail, et al, submitted).

Seasonality of hMPV infections

Whereas some of the respiratory viruses, such as parainfluenza viruses and rhinoviruses, may circulate throughout the year, others, such as RSV and influenza viruses, circulate mainly during the winter season in temperate regions and in the late spring-summer season, also called the respiratory season, in the subtropical areas[172]. Most of the initial hMPV-positive samples[18,182,192,256] were collected in the respiratory season. Studies focusing on specimens collected in this season, often within a 2-month period, found high percentages of hMPV-positive specimens (Table 1). Year round surveillance studies[159,190,258] confirmed that hMPV circulates primarily during the respiratory season in the temperate regions, peaking between December and February in the winters of 2000 and 2001 (Table 1). The virus was primarily found in the spring and summer months in Hong Kong, where RSV and sometimes influenza virus infections may have the same seasonality[190]**.** However, in the temperate regions, patient samples have tested positive for hMPV from October until May, with an occasional virus isolated in August[19,258]. In addition, as observed for RSV and influenza virus infections, the incidence of hMPV infections may vary by year or location. In a North American study among adults in the general community, hMPV was detected more frequently in 2001 than in 2000 (7% *vs*. 1.5%)[77]. In an Italian study, hMPV was detected considerably less frequently in hospitalised children younger than 2 years of age with RTI in 2001 than in 2000 and 2002 (7% *vs*. 37% and 43%)[159]. More comprehensive and long-term studies are needed to determine the seasonality of hMPV infections around the world.

Clinical manifestations of hMPV infections

A wide spectrum of clinical symptoms associated with hMPV infection has been reported in patients of all ages, ranging from mild upper RTI to severe disease requiring hospitalisation (Table 2). In the general community, hMPV-infected adults usually suffer from relatively mild common cold-like respiratory symptoms such as cough, rhinorrhea, hoarseness, sore throat and sometimes fever[77,239,273]. In hospitalised children, patients with underlying disease, immunocompromised individuals, and fragile elderly, hMPV disease tends to be more severe. A spectrum of symptoms involving both the lower and upper respiratory tract has been reported from this group of patients, including cough, rhinorrhea, wheeze and dyspnea. Resulting diagnoses may range from rhinopharyngitis to bronchitis and pneumonia, and some patients may be admitted to intensive care units. In addition, diarrhea, vomiting, rash, febrile seizures, feeding difficulties, conjunctivitis and otitis media have been reported[18,19,75,190,258] (Table 2). The wide spectrum of hMPV-induced illnesses reported thus far are similar to those caused by RSV and influenza virus infections.

Comparison of hMPV and RSV infections

A number of studies have compared the clinical symptoms associated with hMPV infection in hospitalised patients with those associated with RSV and influenza virus infections[19,75,182,190,192,258,260]. Although hMPV infections tend to be slightly milder than RSV infections, in many studies a statistically significant difference was not observed. When we compared the clinical symptoms of 25 hMPV-infected children with age-matched RSV-infected children, we could not discriminate between clinical symptoms caused by RSV or hMPV, although dyspnea, hypoxemia and feeding difficulties were found more often in RSV-infected individuals than in hMPV-infected patients[258]. In addition, hMPV patients had 38% of all recorded symptoms compared to 50% for the RSV-infected children, indicating that hMPV infections are slightly milder than RSV infections. Two studies in hospitalised patients showed that hMPV patients did not need intensive care, in contrast to some of the RSV- and influenza infected patients. The study also showed that pneumonia was more often associated with RSV than with hMPV[19,260]. Another study comparing hMPV-infected children to age-matched RSV or influenza virus–infected children revealed that hMPV infection was more frequently associated with lower respiratory tract involvement, thus prompting more X-rays[190]. In agreement with this observation, hMPV infection was diagnosed more frequently in children with lower RTI than in children with upper RTI in two studies[18,159].

Table 1: Detection of hMPV in studies conducted on samples obtained in different study groups and different seasons.

Study[1]	study period	Country[2]	Remark[3]	study group	number	hMPV positive	Peak age	peak period
Hospital								
18	Dec 01- Apr 02	CAN	b	All ages; RTI	862	20 (2.3%)	35% <5 yr 46% >65 yr	Feb - 02
190	Aug 01 - Mar 02	HK	c	<18 yr; ARTI	587	32 (5.5%)	3 - 72 mo	Resp. Season
258	Okt 00 - Mar 02	NL	c	all ages; RTI	681	47 (7%)	4 - 6 mo	Dec - Jan 02
260	Jan 02 - May 02	DEU	d	<2 yr; RTI	63	11 (17.5%)		Jan - Apr 02
159	Jan 00 - May 02	ITA	c*	<2 years; ARTI			7.8 ± 5.5 mo	Jan - Jun
	2000				19	7 (37%)		
	2001				41	3 (7 %)		
	2002				30	13 (43%)		
19	Dec 01- Apr 02	CAN	e	<3 yr; ARTI	208	12 (5.8%)	3 - 5 mo	Mar - Apr 02
				<3 yr; healthy	51	0		
261	Nov 00 - Feb 01 Nov 01 - Feb 02	SPA	b	<3 years; ARTI				
				neg for other	147	6 (4.1%)	7 - 20 mo	ND
				pos for other	100	0		
75	Nov 01- Feb 02	USA	a	<5 yr; RTI	296	19 (6.4%)	8.7 mo	Jan - Feb 02
90	Nov 01- Feb 02	FRA	b	children; RTI	337	26 (6.6%)	3 mo -12 yr	Dec - Jan 02
77	Nov 99 - Apr 00 Nov 00 - Apr 01	USA	e	fit elderly >65 yr RTI	233	4 (1.7%)		Feb 00 and 01
				high risk adults RTI	238	7 (2.9%)		
				healthy<40 yrs RTI	167	11 (6.6%)		
				nursing homes RTI	37	2 (5.5%)		
				hospitalised RTI	309	20 (6.5%)		
				asymptomatic	217	44 (4.5%)		
general community								
19	Jan 02 - Jun 02	CAN	f	RTI	1505	36 (2.9%)	67% <2yr	Mar - May 02
239	Okt 00 - Mar 01	UK	e	ILI	405	9 (1.3%)	0 >65 yr	Dec - 00
273	2001	NL	c	ARI/ILI	448	13 (2.9%)	4 - 74 yr	ND
				Healthy	397	0		

1.See references for study groups.

2.CAN: Canada; HK: Hong Kong; NL: The Netherlands; DEU: Germany; ITA: Italy; SPA: Spain; USA: United States of America; FRA: France; UK: United Kingdom.

3.a; samples negative for other viruses were tested, no documentation on other viruses in that period. b; samples negative for other viruses were tested, but other viruses are also documented. c; samples were tested for other viruses also (* but not differentiated in report). d; samples only tested for RSV and hMPV. e; samples tested for hMPV, RSV and influenza virus. f; no information. ND= not defined. Resp.season indicates winter season in modern climate zones and late spring to summer in the (sub) tropics.

Table 2: Clinical symptoms, signs and laboratory findings reported for hMPV infection in different studies.

	Studygroup[1]							
	18 n=6 15-65 yr	**18 n=12 <5 yr**	**18 n=10 >65 yr**	**190 n=32 <18 yr**	**258 n=25 8-22 mo**	**19 n=12 <3 yr**	**75 n=19 <5 yr**	**90 n=19 children**
Cough	83	75	100	90	72	100	69	-
Fever	83	92	80	100	61	67	63	16
Rhinorrhea	-	-	-	-	-	92	69	-
Hypoxia	-	-	-	-	47	-	31	-
Wheeze		50			24	83	50	-
Dyspnoea	50	83	70	-	28	-	-	-
Retractions	-	-	-	-	60	92	-	-
Diarrhea	-	-	-	6	-	8	-	10
Vomiting	-	-	-	-	-	25	-	-
Hoarseness	-	-	-	6	-	-	-	-
Truncal rash	-	-	-	13	-	-	-	-
Sore throat	50	-	-	-	-	-	-	-
Lacrymation	-	-	-	-	-	25	-	-
Febrile seizures	-	-	-	16	-	-	-	-
Feeding difficulties	-	-	-	-	36	-	-	-
Hyperventilation	-	-	-	-	42	-	-	-
Cyanosis	-	-	-	-	8	-	-	-
Otitis media	-	25	-	-	-	50	-	21
Lymphopenia	-	-	-	0	-	-	-	-
Rhinitis	-	-	-	-	80	-	-	-
Rhinopharyngitis	-	-	-	-	-	-	-	5
Pharyngitis	-	-	-	-	-	0	-	-
Laryngitis	-	-	-	-	-	-	-	5
Tachycardia	-	-	-	-	23	-	-	-
Conjunctivitis	-	-	-	-	-	-	-	5
Asthma	-	-	-	23	-	-	11	21
Pneumonititis	50	67	40	-	-	17	-	-
Bronchiolitis	-	58	60	10	-	67	47	62
Bronchitis/ Bronchospasm	-	-	60	-	-	0	-	5
Influenza like illness	50	-	-	-	-	0	-	-
Upper RTI	-	-	-	-	-	-	16	-
Other	-	-	-	-	-	8	21	-
Pneumonia	-	-	-	36	-	-	-	-

1. See references for study groups.
Numbers are given as percentages of the study group.

hMPV infection in the elderly and immunocompromised individuals

Although the data presented above may suggest that hMPV tends to cause slightly milder disease than RSV, the virus may cause severe problems in patients with underlying disease and in fragile elderly. In three patients with acute lymphoblastic leukemia who suffered from RTI and subsequently died (a 7-month old girl[191], a 33-year-old woman[30] and a child younger than 5 years[18]), hMPV was the sole pathogen detected. Although there was no pathological examination in these cases, no other pathogens were identified, suggesting that hMPV infection was the cause of death. In contrast, a 5-year-old child diagnosed with acute lymphoblastic leukemia and infected with hMPV recovered uneventfully[190]. Our study revealed that hMPV-positive patients above the age of 5 (n = 13) had underlying disease and thus presented with more severe clinical signs than generally observed in the younger population[258]. In a study among young and elderly adults, hMPV caused more severe disease in fragile elderly than in healthy elderly or young adults[77]. Whereas young children have been studied in detail, studies on elderly and immunocompromised individuals thus far have been relatively weak. However, they may reveal an impact for hMPV similar to those for RSV and influenza virus infections[78].

Association of hMPV with asthma

The role of viral respiratory tract infections in acute and chronic asthma has been a subject of much debate and research. Viruses such as RSV and rhinoviruses in particular have been suggested as the principal trigger of asthma exacerbation in older children and adults[38,188]. Several studies indicated a possible association between hMPV infection and asthma[60,75,90,190,258]. In children with asthma, hMPV was found more frequently than RSV, and asthma exacerbation accounted for a larger proportion of wheezing in hMPV-infected children than in RSV-infected children[190,258]. A study of children hospitalised with acute expiratory wheezing demonstrated hMPV in 8% of the 132 children tested, suggesting that hMPV is a causative agent of acute wheezing in young children[120]. In contrast other studies have found asthma to be more frequently associated with rhinoviruses than with hMPV [206] (T. Jarti, et al., personal communication). Studies aiming at the identification of an association between RSV and/or hMPV and asthma are problematic because asthma is a difficult clinical diagnosis in children younger than 2 years of age, the most susceptible population for both hMPV and RSV. Nevertheless, these preliminary results on the association between asthma and hMPV infections warrant further research.

Co-infection of hMPV and other respiratory viruses

Many respiratory viruses share seasonality and susceptible populations; therefore, it is not surprising that coinfections are detected at a rate of 1% to 3% in various sample sets. However, it is uncertain whether coinfections predispose for more severe disease [148]. Greenshill, et al. reported a high frequency of coinfections by hMPV and RSV[101]; 90% of RSV-infected infants with severe bronchiolitis were coinfected with hMPV, suggesting that coinfection with hMPV and RSV may predispose for severe disease. In contrast other studies that reported coinfections of hMPV with RSV or influenza virus did not report more severe disease in those patients. Care should be taken with the diagnosis of coinfections. Sensitive RT-PCR assays may enable the detection of viral genomes during two consecutive infections, which may be mistaken for double infections. Based on the detection of hMPV in severe acute respiratory syndrome (SARS) patients in different parts of the world, hMPV has

also been suggested to play a role in SARS[198]. However, a novel coronavirus was isolated from far more SARS patients studied[66,138] and was thus identified as the primary causative agent[86,139,189]. In addition studies in macaques have revealed that hMPV did not cause the lesions associated with SARS, whereas the SARS coronavirus did. This same study showed that in cynomolgus macaques, the disease caused by the SARS corona virus was not enhanced upon subsequent inoculation with hMPV[86,139]. Further research may identify a role of secondary pathogens in SARS patients.

Classification of hMPV

Based on gene constellation and sequence homology with avian pneumovirus (APV), hMPV has been tentatively classified as the first human virus within the genus *Metapneumovirus*, subfamily *Pneumovirinae*[259]. RSV is a member of the other genus in the subfamily *Pneumovirinae*, the *Pneumovirus* genus. The separation between the two genera is based primarily on the difference in gene constellation[151,204,282]. High amino acid sequence identity (56% to 87%) is shared between hMPV and APV in all but two genes (G and SH), and < 50% identity is shared with RSV[259].

For APV two subgroups, circulating mainly in Europe, have been identified: APV A and B, based on sequence variation of the attachment protein G, and differences in antigenicity[39,130]. In 1999, a new type –APV C- has been identified in the United States based on genetic and antigenic differences[51,222,248]. For most of the genes (N, P, M, F, M2) and intergenic regions, hMPV shares the highest sequence homology with APV C, in fact for those genes and regions hMPV and APV C are genetically more closely related to each other than both of them to either APV A and B[259].

The glycoprotein G and the small hydrophobic (SH) protein of hMPV did not reveal sequence homologies with those of RSV and APV A and B, and some similarity to, but also extensive sequence divergence with APV C[5,249,283]. Since new APV types, such as type D, are being detected it might be possible that hMPV is even closer related to other, undetected, APV types. Despite the close genetic relationship between APV C and hMPV antigenic relationships have not yet been described. Our own observation revealed that hMPV antibodies could be detected with commercially available ELISAs for the detection of APV antibodies, and APV can be detected in immune fluorescence assays using polyclonal hMPV antibodies. However, virus neutralisation assays have to reveal the true antigenic relationship between APV and hMPV. The close relationship between hMPV and APV C led to the hypothesis that either of the two viruses can cross infect, i.e. that hMPV might infect turkeys and/or APV C infects humans. Preliminary studies in which juvenile turkeys, chickens and cynomolgus macaques were inoculated with hMPV resulted in virus replication in the monkeys but not in the chickens and turkeys. The high sequence differences between the attachment proteins of hMPV and APV may form the basis of differences in host restriction.

Serological assays demonstrated that the virus circulated in the human population for at least 45 years. In addition, after identification of hMPV, many diagnostic laboratories tested samples collected over the last decades that were suspected of an unknown respiratory virus, retrospectively and hMPV was detected in samples dating 25 years ago[60]. These results indicate that hMPV is not a newly emerging virus, but a newly detected virus.

Heterogeneity of hMPV

When hMPV was first described as the causative agent of RTI in children, at least two genetic lineages of hMPV were identified[256]. Several groups have subsequently confirmed the presence of these lineages around the globe[9,18,77,159,191,192,239,260]. We recently showed that, based on phylogenetic analysis of sequences obtained for part of the fusion protein (n = 83) and the complete attachment protein (n = 32), each of these lineages can be divided in two sublineages (Figure 2)[257]. Where the fusion protein revealed approximately 95% amino acid sequence identity between viruses from the two lineages, the G protein shared only 30% identity. Sequences obtained for the complete genome of viruses from the two different lineages, revealed an overall 80-81% nucleotide sequence identity, with a 92-93% identity between strains belonging to the same main lineage. The G and SH proteins are the most divergent between the two main lineages, and the G protein is even more divergent than observed between human RSV A and B (unpublished data and[15]).

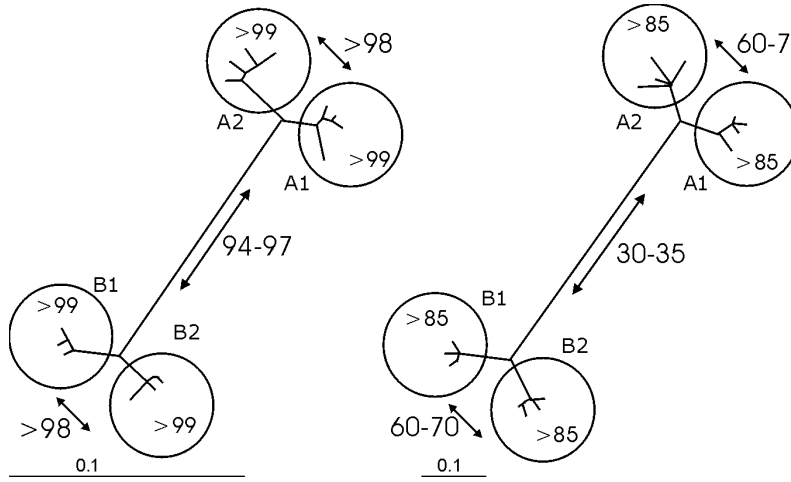

Figure 2:
Phylogenetic trees for the fusion (F) and attachment (G) genes of selected hMPV isolates. From each of the four genetic lineages (van den Hoogen, *et al.*, 2004)[257] four representative isolates were selected, and maximum likelihood trees were generated for the G gene (right) and 451 nucleotides in the F gene (left). Numbers in trees represent percentages amino acid identity between virus isolates.

Virus neutralisation assays using lineage-specific ferret anti-sera in our study demonstrated a twelve- to a > 100-fold difference in virus neutralisation titers between viruses from the two main lineages. Classical virology studies have used a definition of a homologous-to-heterologous virus neutralisation titer of more than 16 as a definition of serotypes. On the basis of this definition, the antigenic differences and the high sequence divergence between the two main lineages, we defined two serotypes of hMPV. In accordance with both the RSV and APV nomenclature, we named these two main lineages serotype A and B, with each serotype divided into genetic sublineages 1 and 2[257]. In addition, virus neutralisation assays conducted with sera obtained 28 days post infection from Syrian golden hamsters demonstrated similar antigenic differences between viruses from the two main lineages (M.MacPhail, submitted). Although antigenic differences between sera collected from one animal species is sufficient for a technical description of serotypes, the relevance of these findings for infection of humans has still to be defined.
The identification of two serotypes of hMPV has direct implications for the development of diagnostics assays (see below) and possibly also for the development of vaccines and other intervention strategies.

Laboratory diagnosis of hMPV infections

The identification of two serotypes of hMPV has implications for the development of both serological diagnostic tests and RT-PCR assays. Serological tests based on prototype viruses from one serotype were found to be less sensitive in detecting viruses belonging to the other serotype (Van den Hoogen, et al., unpublished data), indicating that mixtures of antigens should be used in developing diagnostic tests. For RT-PCR assays, it is important to design primers based on regions that are conserved between viruses of the various genetic lineages.

Because hMPV was relatively difficult to isolate using standard procedures, hMPV has circulated unnoticed for some time. On the basis of antibody prevalence, it has circulated in the human population for at least 45 years and more likely longer. After the original isolation of hMPV on tMK cells, more cell lines have been explored for culturing hMPV and these days some laboratories use LLC-tMK2 or Vero cells successfully. Because virus isolation is time-consuming and sometimes difficult and serological tests have not yet been standardised, RT-PCR assays are usually the test of choice.

Virus isolation
Our own observations indicate that the different hMPV strains vary in growth kinetics and that cytopathic effects may be hard to observe. For instance, viruses closely genetically related to prototype strain NL/1/00 (serotype A) revealed cytopathic effects more clearly on tMK cells than viruses related to prototype strain NL/1/99 (serotype B). In search of a cell line similarly susceptible for both types, we generated a subclone of Vero cells. Vero cell clone 118 was permissive for infection with viruses from all four lineages, and cytopathic effects were easy to observe (Van den Hoogen, et al., unpublished data). This cell line is now used routinely for virus isolation in our laboratory, and similar subclones may be generated from other established cell lines to enhance detection of hMPV by virus isolation.

RT-PCR assays

The design of RT-PCR assays for hMPV diagnostics should take in account the genetic variation between hMPV isolates. Thus far in conventional RT-PCR assays, only primers targeting the polymerase protein gene (L)[256] and the fusion protein gene (F)[191] have been demonstrated to detect viruses from both serotypes efficiently. Because conventional PCR is time consuming, real time PCR assays are preferable. The first reported primers and probes located in the nucleoprotein (N) or L genes[54,154] for a LightCycler assay were developed based on sequences available for NL/1/00, the prototype virus for serotype A and similar viruses. Comparison of all nucleoprotein gene sequences available from Genbank and sequences obtained for 53 hMPV-positive specimens in our laboratory revealed that the N-based primers[154] probably have a lower sensitivity for serotype B viruses because of mismatches[158]. We were able to design primers and probes in the N gene for a real time PCR assay with a minimum number of mismatches for both serotypes. The newly designed assay was demonstrated to detect viruses belonging to the genetic sublineages described to date with equal specificity and sensitivity, and is now used in our laboratory for diagnosis of hMPV in samples from patients suffering from RTI[158]. Besides RT-PCR based diagnostics, isolation of hMPV from clinical specimens should be carried out since virus culture may allow the identification of hMPV lineages that have so far remained undetected.

Development of antibody therapy and vaccination

The clinical impact of hMPV has resulted in efforts to generate (live attenuated) hMPV vaccines as well as neutralising hMPV monoclonal antibodies by a number of research institutions worldwide. For the pneumoviruses, such as RSV, the F and G proteins are the main targets for the neutralising and protective antibody response, with F being one of the most conserved proteins and G the most variable[6,123-125,184]. For RSV, the immune response against the F protein is cross reactive between subgroup A and B, whereas the response against the G protein is subgroup specific[6,125,184,240,242]. Immunisation with individual F or G proteins in animals has shown that the F protein is broadly cross protective whereas the G protein provides a more group-specific protection (reviewed in 241). The circulation of two serotypes of hMPV has to be taken in account for the development of both therapeutics and vaccines as the effectiveness of the intervention may vary by serotype or genetic lineage.

Antibody therapy

For RSV the prophylactic use of a virus neutralising monoclonal antibody preparation directed against the F protein has been shown to decrease the severity of lower respiratory tract (LRT) diseases by both subgroups of the virus. In a similar way, the conserved F protein of hMPV could be a target for the development of monoclonal antibodies for treatment of hMPV-infected individuals. However, future experiments still have to elucidate the correlates of protection for hMPV.

Vaccines

Vaccine development for RSV has been the goal of multiple studies for the last 20 years. Several strategies have been used, although only inactivated, subunit and live attenuated

RSV vaccines have been evaluated in clinical trials to date[68]. The use of a formaline inactived RSV vaccine and the enhanced disease observed upon subsequent natural infection has been a major complication of RSV vaccine development, and has led to the exploration of different vaccine candidates[134]. Several candidate subunit vaccines based on the RSV-F and/or G proteins, the main targets for induction of neutralising antibodies, have been developed. These subunit products are not particularly immunogenic in young infants, however they are suitable for immunisation of previously infected patients who are at high-risk of severe disease or the elderly (reviewed in 24,59).
Several different strategies for the development of a live attenuated RSV vaccine were originally explored, including the creation of host range mutants, cold-passaged mutants, and temperature-sensitive mutants. Importantly, enhanced disease was not observed when infants who were immunised with these candidate vaccines were naturally infected with wild type RSV. To date, a second generation, genetically stable through multiple attenuating mutations, live attenuated RSV vaccine candidates are tested in clinical trials (reviewed in[24,59,68]). Recently, investigators have demonstrated the ability to recover infectious virus from cDNA clones of RSV[44]. These reverse genetics systems provide a powerful tool for the generation of vaccine candidates, including live-attenuated vaccines, because point-mutations, deletions and insertions can be engineered to suit specific needs. The use of reverse genetics resulted in a variety of genetically designed RSV vaccine candidates that harbour mutations in essential RSV genes or deletions of nonessential RSV genes in an effort to attenuate virus replication without compromising immunogenicity[47].

hMPV vaccine development might encounter similar problems as RSV vaccine development (for instance enhanced disease upon wild type infection after inactivated virus vaccination). At present, live-attenuated virus vaccines receive the most attention, which can be achieved by the use of reverse genetics systems. Recently our laboratory has established a reverse genetics system for the rescue of both serotypes of hMPV (S.Herfst, submitted). With this establishment, recombinant hMPV strains can be constructed that harbour the surface glycoproteins of both serotype A and B isolates of hMPV, which may induce a broad antibody response in infected hosts. Using reverse genetics, one can also envisage creating chimeric viruses between hMPV and other human respiratory pathogens (RSV, PIV) to combat RTI in humans. Chimeric live-attenuated vaccines based on the hMPV genome, in which genes of RSV and or PIV are inserted, may be useful as multivalent vaccine candidates. Recently, a reciprocal vector in which an hMPV gene was inserted in a b/hPIV3 vector has been described, and was found to induce protective antibody titers in a hamster model[245]. The rescue of RSV, PIV and RSV in reverse genetics systems allows the use of different backbones for creating chimeric viruses which might be advantageous for circumventing specific immune responses.
Next to vaccine development, reverse genetics systems provide a powerful tool for studies of molecular biology, viral pathogenesis, and the host immune response.

Conclusion

hMPV is a new member of the *Metapneumovirus* genus within the subfamily *Pneumovirinae*, family *Paramyxoviridae*. This proposed classification is based on a similar genomic constellation and sequences to APV, so far the sole member of the *Metapneumovirus* genus, which causes respiratory tract disease in chickens and turkeys.
The failure to detect hMPV in healthy individuals in combination with the isolation of hMPV from patients suffering from RTI and the results obtained in experimentally infected macaques corroborates with the causal relationship between hMPV infection and RTI.
hMPV accounts for a significant portion of RTI in hospitalised children, with high incidences during epidemics in the winter months in moderate climate zones and late spring-early summer in the subtropics. Only RSV, and occasionally influenza virus, was detected more frequently in the studies that included surveys for other respiratory viruses. Very young children (< 2 years of age), people with underlying disease, the immunocompromised, and fragile elderly are most at risk from hMPV infections; thus the virus shares its susceptible population with RSV. Surveillance in the general community found approximately 3% of samples from individuals attending physicians for RTI positive for hMPV, and serological studies indicate that hMPV may cause a self-limiting "common cold" among adults. The spectrum of clinical symptoms observed in hMPV-infected individuals are comparable to those in RSV-infected individuals, ranging from common cold-like symptoms in the general community to severe pneumonia in hospitalised patients. So far, RSV and hMPV infections cannot be discriminated on the bases of clinical signs.
At this moment, at least two circulating serotypes of hMPV have been identified, and this variability must be taken into account in the development of diagnostic tests and possibly in the development of intervention strategies and vaccines.

Future perspectives

It will be important to conduct a wide range of prospective and retrospective studies to obtain a better understanding of the clinical impact of hMPV infections in different populations, the full spectrum of hMPV associated diseases and risk factors that may be associated with severe hMPV disease. In addition, the preliminary results on the association between asthma and hMPV infections, the role of hMPV in SARS, and the possible consequences of co-infection of hMPV and RSV (or other respiratory viruses) on the severity of disease warrant further research. To conduct these epidemiological studies it is important to develop diagnostic assays to detect both serotypes of hMPV with equal specificity and sensitivity.
The implications and relevance of the identification of two serotypes of hMPV has to be further elucidated, with an emphasis on the impact on intervention strategies. The development of vaccines and therapeutics demands the use of well-defined animal models, for which only preliminary results are known to date.

Within the genus *Metapneumovirus*, new viruses have been identified, such as APV C and D and both types of hMPV. It would be interesting to investigate the ancestral relationships of APV and hMPV and whether the two serotypes of hMPV are the result of independent introductions in the human population. It can not be ruled out that new viruses belonging to this genus will be identified in humans and animals.

Epidemiological studies for the detection of aetiologic agents of respiratory tract illnesses detect a causative agent in 50-85% of the specimens, primarily depending on the skills of the laboratory involved. Studies so far have shown that hMPV accounts for a high proportion of these unidentified samples. It is highly likely, that other agents are responsible for a proportion of the remaining unidentified samples. The detection of hMPV has shown that virus isolation on a broad range of cells (such as tertiary monkey kidney cells, not routinely used in most laboratories) and for a longer period than standard, allows the detection of viruses, which cannot easily be detected with techniques currently used in diagnostic laboratories. The combination of classical virological techniques and the use of state of the art techniques, such as molecular techniques, might identify new pathogens. Molecular techniques may include the use of degenerate primer sets in PCR assays to identify new members of a known virus family, or for instance the random amplification PCR (RAP PCR) technique described in this thesis. Genomics tools (such as, the recently described DNA microarray-based platform for novel virus identification[265]) might identify virus specific proteins or genome sequences directly. Alternatively these tools may allow identification of specific host gene expression profiles associated with viral infection.

S

Summary/samenvatting

Summary

Acute respiratory tract infections are responsible for considerable morbidity and mortality, and costs attributable to acute respiratory tract illnesses (RTI) are an important burden on national health care budgets. A variety of viruses, bacteria and fungi are associated with RTI. **Chapter 1** provides background information on the most important viral causes of RTI in humans.

We have isolated a previously undiscovered paramyxovirus from samples obtained from children with RTI (**chapter 2**). The newly discovered paramyxovirus was identified as a tentative new member of the *Metapneumovirus* genus within the subfamily *Pneumovirinae* based on virological data, sequence homology and gene constellation. Previously, avian pneumovirus was the sole member of this recently assigned genus, hence the provisional name for the newly discovered virus: human metapneumovirus (hMPV). The clinical symptoms of the children from whom the virus was isolated were similar to those caused by human RSV infection, ranging from mild upper RTI to severe bronchiolitis and pneumonia. Serological studies showed that by the age of five years, virtually all children in The Netherlands have been exposed to hMPV and that the virus has been circulating in humans for at least 45 years. Analysis of the complete genomic sequence of hMPV confirmed the classification of this new paramyxovirus in the genus *Metapneumovirus* of the subfamily *Pneumovirinae* (**chapter 3)**. The overall percentage amino acid sequence identity between APV and hMPV ORFs was 56 to 88 %. Some nucleotide sequence identity was also found between the noncoding regions of the APV and hMPV genomes.

In **chapter 4**, analyses of a large number of hMPV sequences obtained for the fusion and attachment glycoprotein genes, revealed the presence of two main genetic lineages (A and B), each consisting of two sublineages (A1, A2, B1, B2). Virus neutralisation assays with ferret sera raised against viruses from the four sublineages revealed a 12 to 128 fold higher homologous than heterologous virus neutralisation titer for viruses belonging to the two main lineages A and B. We thus conclude that two hMPV serotypes, each of which can be divided in two genetic lineages, are circulating in humans around the world.

The dynamics and associated lesions of hMPV infection were studied in experimentally infected cynomolgus macaques (**chapter 5**). Viral excretion in the nose of these macaques peaked at 4 days post infection and decreased to zero by 10 days post infection. Viral replication was restricted to the respiratory tract and associated with minimal to mild, multifocal erosive and inflammatory changes in conducting airways, and increased numbers of macrophages in alveoli. Viral expression was seen mainly at the apical surface of ciliated epithelial cells throughout the respiratory tract, and less frequently in type 1 pneumocytes and alveolar macrophages. The animals demonstrated a subclinical or mild disease associated with hMPV infection, which corresponds to that in immunocompetent middle-age adults. The obtained results, in combination with isolation of the virus from humans with RTI and not from healthy individuals, established hMPV as an aetiologic agent of RTI.

After identification and molecular characterisation of the virus, we performed retrospective analyses of the prevalence of and clinical symptoms associated with hMPV infection among patients in a university hospital in The Netherlands, during a 17-month period (**chapter 6**). hMPV was detected in 7% of samples obtained from patients with RTI and in only 2 patients without RTI. hMPV was the second-most-detected viral pathogen in these patients during the

winter seasons of 2000 and 2001 and was detected primarily in very young children and in immunocompromised individuals. In young children, the clinical symptoms associated with hMPV infection were similar to those associated with RSV infection, perhaps slightly milder. Whereas hMPV-infections were most frequently detected in children between 4 and 6 months of age, hRSV infections were detected most frequently in children below 2 months of age. A potential link between hMPV infection and exacerbation of asthma was also described in chapter 6. In agreement with this, 8% of 132 Finish children admitted for acute expiratory wheezing were found to be positive for hMPV (**chapter 7**). In chapter 6, most of the hMPV-positive patients between the ages 5 and 65 years either had an underlying disease or received immunotherapy. The relevance of hMPV infection in immunocompromised individuals is illustrated by the study described in **chapter 8**. Here, hMPV was detected as the sole pathogen in a hematopoietic stem cell transplant recipient with a fatal respiratory illness.

After the initial discovery of hMPV, several surveys on the burden of disease of hMPV infection have been conducted in various study groups and with different diagnostic assays. We reviewed these surveys to establish the burden of disease of hMPV infection in **chapter 9.** We concluded that hMPV infections occur worldwide and account for at least 5 to 7% of the RTI in hospitalised children, but that immunocompromised and elderly individuals are also at risk. Surveillance studies in the general community revealed that approximately 3% of samples from individuals attending physicians for RTI were positive for hMPV, and serological studies indicate that hMPV may cause a self-limiting "common cold" among adults.

Future perspectives

It will be important to conduct a wide range of prospective and retrospective studies to obtain a better understanding of the clinical impact of hMPV infections in different populations, the full spectrum of hMPV diseases and risk factors that may be associated with severe hMPV disease. The implications and relevance of the identification of two serotypes of hMPV has to be further elucidated, with an emphasis on the impact on intervention strategies. The development of vaccines and therapeutics demands the use of well-defined animal models, for which only preliminary results are known to date.

Epidemiological studies for the detection of aetiologic agents of RTI detect a causative agent in 50-85% of the specimens, and studies so far have shown that hMPV accounts for a high proportion of these unidentified samples. It is highly likely that other agents are responsible for a proportion of the remaining unidentified samples, which, with the aid of classical virological assays and state-of the art molecular techniques, can be identified in the future.

Nederlandse samenvatting

Luchtweginfecties (LWI) zijn verantwoordelijk voor aanzienlijke ziekte- en sterftecijfers. Ze vormen een belangrijke uitgavenpost op het budget van het ministerie van VWS. LWI kunnen worden veroorzaakt door een groot aantal verschillende pathogenen, zoals virussen, bacteriën en schimmels. **Hoofdstuk 1** geeft een overzicht van de belangrijkste virale luchtweginfecties bij de mens.

Wij hebben een onbekend virus geïsoleerd uit monsters die werden verkregen van kinderen met LWI. Dit is beschreven in **hoofdstuk 2**. Met behulp van klassieke virologische experimenten hebben we aangetoond dat dit virus tot de familie der *Paramyxoviridae* behoort. De virusfamilie *Paramyxoviridae* is onderverdeeld in twee subfamilies, de *Paramyxovirinae* en de *Pneumovirinae*. Deze laatste subfamilie is vervolgens ingedeeld in twee genera, het genus *Pneumovirus* en het genus *Metapneumovirus*.

Het respiratoir syncytieel virus (RSV), het prototype virus van het genus *Pneumovirus*, is over de hele wereld de belangrijkste veroorzaker van lagere LWI bij baby's en peuters. Het aviaire pneumovirus (APV), veroorzaker van hogere LWI bij kalkoenen, was tot de ontdekking van het nieuwe virus het enige lid van het genus *Metapneumovirus*. Van het ontdekte paramyxovirus is op basis van virologische data en de organisatie van het virale genoom vastgesteld dat het een lid van het genus *Metapneumovirus* is. Daarom hebben wij het de naam 'humaan metapneumovirus' (hMPV) gegeven. Onderzoek naar de aanwezigheid van antistoffen tegen hMPV toonde aan dat kinderen vóór het bereiken van de 5-jarige leeftijd bijna allemaal in aanraking zijn geweest met hMPV, en dat het virus al minstens 45 jaar onder mensen circuleert.

De analyse van het complete genoom van hMPV is beschreven in **hoofdstuk 3**. De resultaten van deze analyse bevestigen de classificatie van het virus als een nieuw lid van het genus *Metapneumovirus*. De leesramen in de genomen van APV en hMPV waren 56 tot 88 % identiek. Ook in de niet-coderende delen van het genoom werden op nucleotideniveau grote overeenkomsten gevonden.

Hoofdstuk 4 beschrijft de genetische verscheidenheid van hMPV aan de hand van analyses van sequenties van het fusie-eiwit en het aanhechtingseiwit van een groot aantal isolaten. De hMPV-isolaten werden ingedeeld in twee hoofdgroepen (A en B), waarna elke hoofdgroep verder werd verdeeld in twee subgroepen (A1, A2, B1 en B2). Alleen tussen virussen van de twee hoofdgroepen werden vervolgens grote antigene verschillen waargenomen. Op basis van de genetische verschillen en de verschillen in antigene reactiviteit hebben wij twee hMPV-serotypen gedefinieerd.

De dynamiek en de gevolgen van een hMPV-infectie zijn bestudeerd in experimenteel geïnfecteerde Java-apen (**hoofdstuk 5**). Deze apen werden pathologisch en virologisch onderzocht. Dit onderzoek heeft aangetoond dat hMPV een respiratoir pathogeen is, dat de virusreplicatie van korte duur is, en dat deze voornamelijk plaatsvindt in trilhaar-epitheelcellen. Het subklinische of milde ziektebeeld dat werd waargenomen bij deze apen is vergelijkbaar met het hMPV-gerelateerde ziektebeeld in immuuncompetente volwassenen van gemiddelde leeftijd. In combinatie met het feit dat het virus wel werd geïsoleerd bij mensen met LWI maar niet bij gezonde mensen, vormen de verkregen resultaten het bewijs dat hMPV een veroorzaker is van LWI.

Na identificatie en moleculaire karakterisering van het virus, is onderzoek verricht naar de prevalentie en klinische symptomen die in verband worden gebracht met hMPV-infecties.

Dit staat beschreven in **hoofdstuk 6**. Voor dit onderzoek zijn monsters getest die zijn verzameld van ziekenhuispatiënten die werden opgenomen voor LWI maar ook voor andere klachten. In 7% van de monsters die werden afgenomen van patiënten met LWI werd hMPV aangetoond, terwijl maar 2 van de patiënten zonder LWI hMPV-positief waren. hMPV was het op één na vaakst waargenomen virale pathogeen gedurende de winterseizoenen van 2000 en 2001. Het werd voornamelijk aangetroffen bij zeer jonge kinderen en individuen met een verzwakt immuunsysteem. De meeste hMPV-positieve patiënten tussen de 5 en 65 jaar hadden een onderliggende ziekte of werden met immuunsuppressieve middelen behandeld. Bij jonge kinderen met een hMPV-infectie waren de symptomen vergelijkbaar met die bij kinderen met een RSV-infectie, hoewel het ziektebeeld van een hMPV-infectie iets milder was. hMPV werd meestal waargenomen bij kinderen van 4 tot 6 maanden oud, terwijl RSV vooral werd waargenomen bij kinderen van 0 tot 2 maanden oud.
Hoofdstuk 7 levert verdere aanwijzingen voor het een verband tussen hMPV-infectie en astma dat al werd beschreven in hoofdstuk 6. In dit Finse onderzoek was 8% van de monsters, afgenomen van 132 kinderen met astmaverschijnselen, hMPV-positief. De relevantie van hMPV-infecties in individuen met een verzwakt immuun systeem wordt ook aangetoond in een studie beschreven in **hoofdstuk 8**. In dit onderzoek bij een beenmergtransplantatiepatiënte met een ziekte van de hogere luchtwegen, met fatale afloop, werd hMPV als enig pathogeen waargenomen
Na de ontdekking van hMPV hebben een groot aantal onderzoeksgroepen over de hele wereld onderzoek gestart naar het klinische belang van hMPV. **Hoofdstuk 9** bevat een van deze studies. Hieruit concluderen wij dat hMPV wereldwijd circuleert en minimaal 5 tot 7% van de acute LWI bij in het ziekenhuis opgenomen kinderen veroorzaakt. Tevens bleek dat individuen met een verzwakt immuunsysteem en ouderen ook tot de risicogroepen behoren. Van patiënten met LWI die hun huisarts bezochten bleek ongeveer 3% met hMPV geïnfecteerd te zijn. Serologische onderzoeken hebben aangetoond dat hMPV-infecties ook een gewone verkoudheid kunnen veroorzaken bij volwassenen, zonder dat deze medische zorg nodig hebben.

Toekomstperspectief
Het is wenselijk dat er uitgebreide prospectieve en retrospectieve onderzoeken naar hMPV-infecties worden verricht. Hiermee kan het klinische belang van hMPV-infecties in verschillende populaties verder worden aangetoond. Tevens moeten alle ziekten en risicofactoren worden geïdentificeerd die in verband kunnen worden gebracht met ernstige hMPV-infecties. De implicaties en de relevantie van de identificatie van twee verschillende hMPV-serotypes zullen verder onderzocht moeten worden. Bij dit toekomstige onderzoek zal de nadruk moeten liggen op het belang voor interventiestrategieën. Voor het ontwikkelen van vaccins en therapeutische middelen zijn goed gedefinieerde proefdiermodellen nodig. Momenteel zijn hiervoor slechts beperkte resultaten beschikbaar.
Met de identificatie van hMPV is het nu mogelijk om in monsters van patiënten met LWI, waarin voorheen geen pathogeen gedetecteerd kon worden, een oorzaak voor de ziekte aan te wijzen. De verwachting is dat met een combinatie van klassiek virologische en moderne moleculaire technieken nog meer respiratoire pathogenen zullen worden gevonden, zodat in een groter percentage van de monsters van patiënten met LWI een oorzaak kan worden aangewezen.

R

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Curriculum Vitae/Publications

Curriculum Vitae

Bernadette van den Hoogen werd op 22 oktober 1967 geboren te Oudewater. In 1985 behaalde zij haar HAVO-diploma aan het Sint Antonius College te Gouda. Van 1985 tot 1989 volgde zij een H.L.O opleiding in de richting botanie aan de Rijks Hogere Agrarische School (R.H.A.S.) te Wageningen. Van 1989 tot 1995 werkte zij als researchanalist bij Zaadveredelingsbedrijf Royal Sluis te Enkhuizen. Tijdens een verblijf van 3 jaar in de Verenigde Staten als research technician aan de University of Pennsylvania in Philadelphia, onder begeleiding van Prof. Dr. S.R. Ross, werkte zij aan de karakterisering van de cellulaire receptor van het muizenborstkanker virus (MMTV). Vanaf 1999 heeft zij gewerkt aan de ontdekking en karakterisatie het in dit proefschrift beschreven hMPV, alsmede aan de ziekte verschijnselen die met hMPV-infectie zijn geassocieerd. Dit werk werd uitgevoerd op de afdeling Virologie van het Erasmus MC te Rotterdam, onder begeleiding van Prof. Dr. A.D.M.E. Osterhaus.

Publicaties

Ross,S.R., Dzuris,J.L., Golovkina,T.V., Clemmons,W.C. & **van den Hoogen, B.G** Mouse mammary tumor virus (MMTV), a retrovirus that exploits the immune system. Genetics of susceptibility to MMTV infection.
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