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Human metapneumovirus infection

Adilia Warris and Ronald de Groot

Department of Pediatrics, Radboud University Nijmegen Medical Centre, and the Nijmegen University Center for Infectious Disease, Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

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Abstract

Initially, human metapneumovirus (hMPV) was isolated from children with clinical symptoms of respiratory syncytial virus (RSV) infection in whom RSV could not be detected. Since then, numerous reports have described the detection of hMPV in clinical specimens from children, adults and the elderly (both immunocompetent and immunocompromised patients), diagnosed with an acute respiratory illness all over the world. hMPV is associated with a substantial number of respiratory tract infections in otherwise healthy children, with clinical illnesses similar to those associated with other common respiratory viruses. Serological surveys have shown that hMPV is a ubiquitous virus that infects all children by the age of 5–10 years and has been circulating in humans for at least 50 years. hMPV is a member of the *Metapneumovirus* genus of the *Paramyxoviridae* family, a group of negative-stranded RNA viruses. Genetic studies on hMPV have demonstrated the presence of two distinct hMPV serotypes each divided in two subgroups. Diagnosis is made by RT-PCR assays on respiratory secretions. Rapid antigen detection tests are not yet available and its growth in cell cultures is fastidious. No vaccines, antibodies (monoclonal or polyclonal), or chemotherapeutic agents are currently licensed for use to prevent or treat hMPV infections. The contribution of hMPV to pediatric respiratory tract infections suggests that it will be important to develop a vaccine against this virus in combination with those being developed for RSV and parainfluenza viruses. Reverse genetics technology is currently used to develop multivalent vaccines against hMPV and a variety of other important respiratory viruses such as RSV. Additional research to define the pathogenesis of this viral infection and the host' specific immune response will enhance our knowledge to guide the search for preventive and therapeutical strategies.

Background

In 2001, a new infectious agent, human metapneumovirus (hMPV), was isolated from nasopharyngeal aspirates of young children with respiratory tract illness from The Netherlands [1]. Initially, hMPV was isolated from children with clinical symptoms of respiratory syncytial virus (RSV) infec-

tion in whom RSV could not be detected. Since then, numerous reports have described the detection of hMPV in clinical specimens from children, adults and the elderly (both immunocompetent and immunocompromised patients), diagnosed with an acute respiratory tract infection (RTI) all over the world.

hMPV is an enveloped virus with a genome that is a single strand of RNA of approximately 13 kb [1]. Its genome contains eight genes that presumably code for nine different proteins [2, 3]. The genomic organization for hMPV is similar but not identical to that for RSV being a member of the *Pneumovirus*. In contrast to the *Pneumovirus*, the *Metapneumovirus* lacks the NS1 and NS2 genes and has a different positioning of the other common genes, i.e., the N (nucleocapsid RNA binding protein), P (phosphoprotein), M (matrix protein), F (fusion glycoprotein), L (major polymerase subunit), G (major attachment protein), M2 (transcription elongation and RNA synthesis regulatory factor), and SH (small hydrophobic surface protein). The absence of open reading frames (ORFs) between the M and F genes in the hMPV virus and the lack of NS1 and NS2 genes is in agreement with it being the first identified non-avian member of the *Metapneumovirus* genus [2, 4]. Genetic analysis of the N, M, P and F genes revealed that hMPV showed a higher sequence homology to the *Metapneumovirus* genus (average of 66%) as compared to the genus *Pneumovirus* (average of 30%) [1, 5]. On the basis of the organization of the viral genome and sequence identity to the *Metapneumovirus* avian pneumovirus, also known as turkey rhinotracheitis virus, hMPV was assigned to be a member of the *Metapneumovirus* genus of the *Paramyxoviridae* family. The *Metapneumovirus* and the *Pneumovirus* genera are two genera within the subfamily of *Pneumovirinae* (Fig. 1). The *Pneumovirinae* and the *Paramyxovirinae* belong to the *Paramyxoviridae* family, a group of negatively stranded RNA viruses including several major pathogens of humans and animals. hMPV does not infect chickens or turkeys, and the virus is unlikely to be a zoonotic source.

RT-PCR analyses using primer sets for specific paramyxoviruses (parainfluenza virus, mumps virus, measles virus, RSV, simian virus type 5, Sendai virus and Newcastle disease virus) did not react with the newly identified hMPV, indicating no close genetic relatedness to these viruses. hMPV-specific antisera did not react in immunofluorescence (IF) assays with cells infected with a panel of paramyxoviruses and orthomyxoviruses (parainfluenza viruses, influenza virus A and B, RSV) [1].

Although genetically not closely related, hMPV shares many biological properties with RSV. The hMPV isolates replicate slowly in tertiary monkey kidney (tMK) and rhesus monkey kidney (LLC-MK2) cells, very poorly in Vero cells and A549 cells, and could not be propagated in Madin Darby canine kidney (MDCK) cells or chicken embryo fibroblasts (CEF) [1]. The cytopathic effects are indistinguishable from those caused by RSV, although they occurred slightly later, 10–17 days post inoculation. Electron microscopy revealed paramyxovirus-like pleiomorphic particles of 150–600 nm,

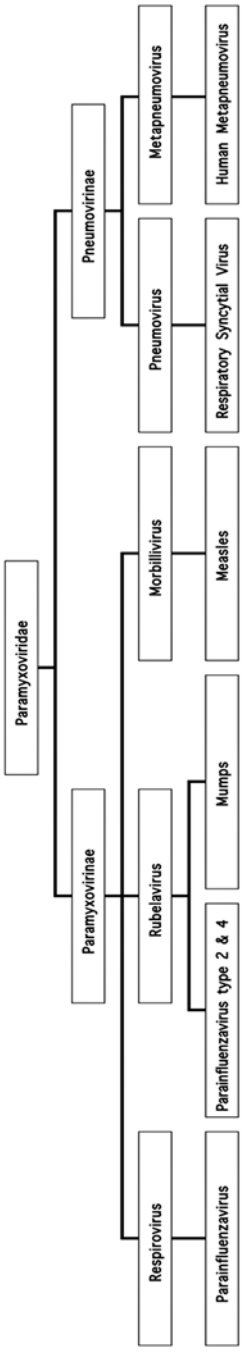

Figure 1. Classification of viral pathogens of the Paramyxoviridae family that infect humans.

with short envelope projections of 13–17 nm, indistinguishable from RSV [1]. hMPV is chloroform sensitive, and replicates optimally in a trypsin-dependent manner, in contrast to RSV, in tMK cells. No hemagglutinating activity with turkey, chicken or guinea pig erythrocytes was displayed. These combined virological data indicate that the hMPV is indeed a member of the *Paramyxoviridae* family.

The mode of transmission has not been formally studied, but is likely by large particle respiratory secretions and fomites, based on its relatedness to other pneumoviruses. Nosocomial transmission does occur [6] and warrants contact isolation and scrupulous hand washing by health care providers.

Epidemiology

Genetic studies on hMPV have demonstrated the presence of two distinct hMPV groups each divided in two subgroups [4, 5, 7–9]. Representative strains of the four subgroups are hMPV/NL/1/00 (subgroup A1), hMPV/NL/1/99 (subgroup B1), hMPV/CAN/97/83 (subgroup A2), and hMPV/CAN/98/75 (subgroup B2). A nearly complete genome sequence was determined for the prototype NL/1/00 strain of hMPV, and complete genome sequences were determined for two Canadian strains, CAN97/83 and CAN98/75 [2, 3]. These studies also confirmed that the 3'–5' hMPV gene order is N-P-M-F-M2,1-M2,2-SH-G-L. None of these proteins have been identified or characterized by direct biochemical means, and their functions still need to be confirmed [10].

Bastien and colleagues [5] determined the complete nucleotide sequences of the N, P, M, and F genes of Canadian hMPV isolates. Comparison of the deduced amino acid sequences for the N, M, and F genes of the different isolates revealed that all three genes were well conserved with 94.1–97.6% identity between the two distinct clusters. The P gene showed more diversity with 81.6–85.7% amino acid identity for isolates between the two clusters, and 94.6–100% for isolates within the same cluster. The Canadian cluster 1 isolates show over 96% amino acid identity with the NL/1/00 isolates for all the viral proteins analyzed [2].

Analysis of the F and G protein genes of the four subgroups show that the F protein is highly conserved and demonstrated low variability within the four groups [7]. With an amino acid identity of 93–96% between the subgroups, the F protein becomes attractive as a principal target of protective antibodies. In contrast, the G gene shows high sequence diversity. Furthermore, these phylogenetic analyses showed that the hMPV strains obtained from different years and from different countries were randomly distributed over all four sublineages. To address the antigenic relationship between the different lineages, virus neutralization assays were performed showing a difference in antigenicity between lineage A and B. On the basis of these results it was proposed to define the two main lineages of hMPV as

serotypes A and B. Although each serotype can be divided into two genetic subgroups, these subgroups did not reflect major antigenic differences.

To characterize the extent of genetic diversity among hMPV strains in Australia and worldwide, comparative nucleotide- and predicted amino acid-sequence studies were performed with the N and P genes [11]. Comparison of aligned sequences revealed an 11.9–17.6% nucleotide variation, which divided the viral strains into two main lineages. In addition, two distinct subtypes were apparent within each lineage, which were defined as hMPV types A1, A2, B1, and B2. The variability of the P gene permitted a reliable classification of hMPV into its four subtypes, indicating that the P gene is a valuable target for phylogenetic studies. To confirm that this classification, based on both the N and the P gene, agreed with that proposed in other studies, similar sequencing and analyses of all available M, F, G, and L gene sequences were performed. The same lineages were found and thus the P gene seems a useful single target for genotyping and for the creation of a global classification scheme for hMPV. A large community-based phylogenetic study of hMPV for both surface glycoproteins F and G provides the evidence for the presence of multiple genotypes within each subgroup of hMPV [12]. This evidence came from the topology of the phylogenetic trees and bootstrap values in which sequences were arbitrarily considered a genotype if they clustered together with bootstrap values of 70–100%. This resulted in nine genotypes and six possible genotypes in the four subgroups together.

Strains from both hMPV groups may co-circulate in a particular year as shown in South Africa, but at the same time not all four subgroup viruses are detected in a single year [12]. Limited data indicate that both hMPV groups can circulate in a single season with the possibility of the predominant group switching in successive seasons [4, 11, 13]. Agapov et al. [13] showed furthermore that, within each genotype, the F and N genes were conserved, but that the G and SH genes showed marked variation. Despite the genetic variability, no difference in the severity of illness caused by various hMPV isolates was noted. In contrast, a recent study [14] suggests that genotype A causes a more severe acute RTI in small children compared to genotype B. Although the number of cases was small, but comparable to the study of Agapov et al. [13], significant differences were found in parameters reflecting greater severity (diagnosis of pneumonia) as well as the severity index combining clinical data (hypoxemia, intensive care admission) [14]. These different results might be related to the patient groups studied.

Many studies reported the detection of hMPV serogroup 1 as the only or the predominant serotype circulating. In an Israeli study, hMPV serogroup 1 also had the highest circulation rate (92% of the sequenced samples). Of the four subgroups, only three were identified (1A 65%, 1B 25%, and 2B 10%) [15].

Williams et al. [16] showed that the four genetic lineages of hMPV have persisted over the last 20 years in the community. More than one lineage

was present concurrently during some seasons, whereas a single lineage dominated others. In their study the B2 lineage was most common, whereas the B1 lineage seems to circulate periodically. This is in contrast with others studies, were the A1 and A2 lineage accounts for the majority of clinical isolates. This might be due to primers that have shown to be less sensitive for detection of the B lineages.

The circulation of multiple lineages and the changes of the dominant group of virus may suggest an attempt at evasion of preexisting immunity, as has been seen also for RSV. Studies performed in very different geographic areas showed that specific strains coexist across geographic areas [11, 12].

Preliminary evidence of the existence of hMPV other than the four known major genetic lineages of hMPV comes from the isolation of hMPV in a child with an acute asthma exacerbation [17]. Positive PCR results were obtained using primers derived from the N gene [18] and this amplified fragment was cloned in a plasmid vector and sequenced. This confirmed the specificity of the PCR, although the nucleotide sequence differed significantly from the representative hMPV strains of the known four lineages. This might indicate that the heterogenicity of hMPV is higher than recorded till now, and this has important consequences in the optimization of RT-PCR protocols.

hMPV has circulated in humans for at least 50 years; a 100% seroprevalence was found in 72 serum samples obtained from individuals 8–99 years old, collected in 1958 in the Netherlands [1]. There appears to be two periods of acquisition of hMPV in childhood [19]. The first period occurs within the first 3 years of life, the second period occurs in children >48 months of age. The percentage of seropositive children increases in these age categories from 35–45% to about 75%, while it is >90% in children >5 years of age. Seroprevalence studies in children from Japan and Israel showed a 100% seropositivity in children above 8–10 years of age, while only 52% of children up to 2 years of age had hMPV-specific antibodies [15, 20, 21]. In the Netherlands, the seroprevalence of hMPV in children reaches 100% by the age of 5 years [1]. In South African children a lower seropositivity rate was observed up to the age of 10 months (22%), which can be partially explained by the clearance of maternal-derived antibodies. From 10 months onwards the seropositive rate increased to 92% in children aged 24–36 months [22]. The seroprevalence studies indicate that virtually all children are infected in early childhood.

Since the first description of hMPV infection in children by van den Hoogen et al. [1], hMPV has been found in most parts of the world (Tabs 1 and 2): North America, Europe, Asia, and Australia [8, 9, 15, 16, 18, 23–41]. The virus has also been identified in HIV-infected and non-immunocompromised children from South Africa [8]. hMPV infections account for at least 4–8% of RTI in hospitalized children, although some studies report much higher prevalences (Tab. 1). In the general community hMPV infections account for at least 3% of children who visit a general practitioner or

outpatient clinic for RTI (Tab. 2). The relative role of hMPV in respiratory syndromes of adults has not been well studied.

In a large study of patients with RTI, the diagnostic outcomes for 685 specimens sent specifically for respiratory pathogen testing were compared. RSV was detected most frequently, in 126 (18%) of 685 samples obtained with patients with RTI. hMPV was the second-most-detected viral pathogen, found in 7% of the samples, and was isolated more frequently than parainfluenza viruses, adenovirus, rhinovirus, and influenza viruses types A and B [9]. In almost 200 premature infants and young children <2 years of age with chronic lung disease or congenital heart disease in Buenos Aires, the impact of hMPV among other respiratory viruses causing RTIs was only 2%. RSV and parainfluenza virus were detected in 25% and 4%, respectively in this patient group [42]. In spite of the low number of infections caused by hMPV, severe lung disease was seen in some cases. hMPV has been isolated in 51–55% of patients with severe acute respiratory syndrome (SARS), but its contribution to that illness remains uncertain [43, 44].

Pathogenesis and host response

Experimental animal models of hMPV infection have been reported, including both primates and rodents. The first published experimental hMPV infection model in cynomolgus macaques (*Macaca fascicularis*) confirmed that hMPV is a primary pathogen of the respiratory tract in primates [45]. The hMPV-infected macaques showed mild clinical signs of rhinorrhea corresponding with a suppurative rhinitis at pathological examination. In addition, mild erosive and inflammatory changes in the mucosa and submucosa of conducting airways, and an increased number of alveolar macrophages in bronchioles and pulmonary alveoli were observed. A close association between these lesions and the specific expression of hMPV antigen was shown by immunohistochemistry. Based on the antigen expression, viral replication mainly took place at the apical surface of ciliated epithelial cells throughout the respiratory tract. Pharyngeal excretion of hMPV showed a peak at day 4 post infection (p.i.) decreasing to zero by day 10, concomitant with a reduction in the number of infected epithelial cells. The mild upper respiratory tract disease as observed in these macaques corresponds to that in immunocompetent adults. Due to the fact the hMPV can replicate in the lower respiratory tract of cynomolgus macaques, more severe disease can be expected in immunocompromised patients. Some investigators have also shown that hMPV can replicate in the lungs of hamsters and cotton rats without producing recognizable clinical signs, although transient histopathological pulmonary changes were noted in cotton rats [46–50].

hMPV infection in other small-animal models such as ferrets and rabbits has been reported to induce a strong immune response [10], but the level of virus replication in these animals has not been reported. The study of

Table 1. Incidence of human metapneumovirus (hMPV) infections in hospitalized children with respiratory tract disease

	Country	Study period	Population	Method	hMPV-positive/ total number of patients	Prevalence	Peak age
Regev et al. [15]	Israel	Nov. 02–May 03 Nov. 03–May 04	<5 years, RTI	RT-PCR(1)	42/338	10.8%	1–2 year
Wilkesmann et al. [23]	Germany	Oct. 02–May 03 Oct. 03–May 04	Children; RTI	RT-PCR(2)	114/637*	17.9%	<24 months
Foulongne et al. [24]	France	Nov. 03–Oct. 04	<5 years; RTI	RT-PCR(2)	50/589	8.5%	
Bouscambert-Duchamp et al. [25]	France	Sept. 01–June 02	Infants; <24 months	RT-PCR(2)	6/94	6.4%	2–6 months
IJpma et al. [22]	South Africa	June–Aug. 2002	Children; RTI	RT-PCR(2)	8/137	5.8%	2–24 months
König et al. [26]	Germany	Nov. 99–Oct. 01	<3 years; RTI <6 months; apneu admitted to ICU	PCR	15/87	18%	
McAdam et al. [27]	USA	Oct. 00–Sept. 02	≤18 years	RT-PCR(1)	54/868	6.2%	3–24 months
Jartti et al. [28]	Finland	Sept. 00–June 02	3 months–16 years; acute expiratory wheezing	RT-PCR(2)	12/291	4%	3–11 months
Døllner et al. [29]	Norway	Nov. 02–Apr. 03	Children; RTI	PCR(2)	50/236	21%	≤12 months
Mullins et al. [30]	USA	Aug. 00–Sept. 01	<5 years; RTI	RT-PCR(2)	26/641	4%	6–24 months
Esper et al. [31]	USA	Nov. 01–Nov. 02	<5 years	RT-PCR(3)	54/668	8.1%	<12 months
Madhi et al. [8]	South-Africa	Mar. 00–Oct. 00	Infants	RT-PCR(3)	14/196	7.1%	
van der Hoogen et al. [32]	Netherlands	Oct. 00–Feb. 02	All ages; RTI	RT-PCR(1)	48/685*	6.5%	4–6 months
Viazov et al. [18]	Germany	Jan. 02–May 02	<2 years; RTI	RT-PCR(2)	11/65	17.5%	

Maggi et al. [33]	Italy	Jan. 00–May 02	<2 years; RTI	RT-PCR(4)	23/90	25%	≤3 months
Peiris et al. [9]	Hong Kong	Aug. 01–Mar. 02	≤18 years; RTI	RT-PCR(2)	32/587	5.5%	
Thanasugarn et al. [34]	Thailand	Mar. 01–Sept. 02	<14 years; RTI	RT-PCR(2)	5/120	4.2%	
Rawlinson et al. [35]	Australia	2 summers & 2 winters 00-02	<12 years; URTI <17 years; asthma	PCR(2) PCR(2)	9/150 3/179	6% 2%	
Freymuth et al. [36]	France	Nov. 00–Mar. 01 Nov. 01–Feb. 02	Children	RT-PCR(3)	19/337*	6.6%	<1 year

(U)RTI: (upper) respiratory tract infection.

(1) All respiratory specimens obtained; (2) nasopharyngeal aspirates; (3) on common respiratory viruses negative nasopharyngeal aspirates; (4) nasal swabs.

* Number of samples.

Table 2. Incidence of hMPV infections in non-hospitalised children with RTI

	Country	Study period	Population	Method	hMPV-positive/ total number of patients	Prevalence	Peak age
Williams et al. [16]	USA	1982–2001	<5 years; URTI	RT-PCR(3)	118/2384	5%	
König et al. [26]	Germany	Oct. 00–Apr. 01	<3 years; RTI <6 months; apneu	PCR(3)	2/620	<1%	
Laham et al. [37]	Argentina	June 02–Sept. 02	<1 year; RTI	RT-PCR(4)	22/373	6%	
Principi et al. [38]	Italy	Nov. 02–Apr. 03	<15 years; RTI	PCR	41/1331	3.1%	
Williams et al. [39]	USA	1976–2001	<5 years; RTI or AOM	RT-PCR(3)	49/248	20%	6–12 months
Bastien et al. [40]	Canada	Oct. 01–Apr. 02	RTI	RT-PCR(1)	66/445	14.8%	<5 years, >50 years
Falsey et al. [41]	USA	Nov. 99–Apr. 00 Nov. 00–Apr. 01	Fit elderly >65 years;RTI Young adults;RTI	RT-PCR(2) serology	4/233 11/167	1.7% 6.6%	

(U)RTI: (upper) respiratory tract infection; AOM: acute otitis media.
(1) All respiratory specimens obtained; (2) nasopharyngeal aspirates; (3) on common respiratory viruses negative nasopharyngeal aspirates; (4) nasal swabs.

Skiadopoulos et al. [49] extended these observations to show that members of both hMPV lineages replicated efficiently in hamsters and that infection induced a high level of neutralizing antibodies and resistance to challenge that was effective against both homologous and heterologous strains. In addition, two species of nonhuman primates were also identified as useful models for the development of respiratory tract disease (chimpanzees) and for viral replication (African green monkeys). Chimpanzees developed a robust immune response, although the level of virus shedding was low. They were protected from disease following re-challenge with either strain. Therefore, chimpanzees may provide a useful nonhuman primate model for hMPV disease but are less ideal for studying virus replication. In contrast, rhesus macaques are not ideal animal models for the quantitation of hMPV replication, although they developed serum neutralizing antibodies following hMPV infection. hMPV replicated most efficiently in the respiratory tract of African green monkeys and the infected animals developed high level of hMPV serum-neutralizing antibodies effective against both lineages. A high degree of genetic relatedness and cross-protection was shown mediated by immunity to the highly conserved F protein. An human parainfluenza virus 1 (hPIV1) vector bearing the hMPV F protein provided protection against hPIV1 as well as both lineages of hMPV, indicating that such vectors might be useful as vaccines to protect against disease caused by both hPIV1 and hMPV.

BALB/c mice and cotton rats are considered a good and convenient experimental model to study the pathogenesis of human RSV, another paramyxovirus. For hMPV, the BALB/c mouse has been described as a convenient animal model, with efficient viral replication and significant histopathological changes in the lungs associated with systemic and respiratory signs when large intranasal inocula are used [50–52]. A small animal experimental model of hMPV infections in BALB/c mice was developed to study mechanisms contributing to immunity and disease pathogenesis [51]. A biphasic kinetics of hMPV replication in lung tissue was shown with peak titers on days 7 and 14 p.i. Viable virus could be recovered from the lungs up to 60 days p.i., while genomic hMPV-RNA was detected up to 180 days p.i. The lung histopathology was modest and characterized by mononuclear cell infiltration in the interstitium starting on day 2, peaking on day 4 and decreasing on day 14 p.i, associated with bronchial and bronchiolar inflammation. This low pulmonary inflammatory response may contribute to the persistence of the virus. Hamelin et al. [50] did not detect any infectious virus in the lungs of BALB/c mice by day 21 after hMPV infection, although histopathological changes were still significant at that time, compared with those in sham-infected mice. Both duration and severity of inflammation around the alveoli was more limited in cotton rats compared to the BALB/c mice. Clinical symptoms of respiratory distress and weight loss were observed between days 4 and 10 p.i. in mice, but not in infected cotton rats. Recently, Alvarez and Tripp reported that hMPV RNA could

still be detected ≥180 days p.i. in the lungs of hMPV-infected mice and that such persistence results in an aberrant immune response [53]. The duration of pulmonary inflammation associated with a single hMPV challenge and the characterization of the consequences of this viral infection with respect to respiratory functions was further evaluated by Hamelin et al. [54]. The results showed that small amounts of viral RNA are still present in 33% in the lungs of hMPV-infected mice for at least 154 days p.i. and are associated with significant peribronchiolitis and perivasculitis. During the first 2–3 weeks, the inflammation mostly consisted of interstitial inflammation and the presence of alveolitis, as reported previously [50]. Over time, the inflammation became characterized by a prominent peribronchiolar and perivascular infiltrate, which was still significant on day 154. An increased number of PAS-positive cells in the central and peripheral airways up to day 12 p.i. were seen, suggesting increased mucus production. Concurrently with the time of maximal viral replication and histopathological score, the airway obstruction was most severe, followed by a gradually decrease but was still significant on day 70 p.i. Such inflammation seems to be responsible for chronic obstruction and hyperresponsiveness of the airways, which persist for >2 months. These results reinforce the concept that severe paramyxovirus infections early during childhood can be associated with the development of asthma in children.

Overall, these data suggest that BALB/c mice are more susceptible to hMPV infection than cotton rats on the basis of higher virus titers and levels of lung inflammation, combined with the absence of clinical signs. The absence of clinical signs has also been reported in hamsters and ferrets. These experimental models of hMPV infection show similarities with the pathogenesis, as far as studied, of RSV infection in humans.

Histopathological assessment of hMPV infection on lung tissue obtained by open or transbronchial biopsies from five immunocompromised patients showed acute and organizing lung injury [55]. More specifically, areas of diffuse alveolar damage with hyaline membrane formation and foci of bronchiolitis obliterans/organizing pneumonia-like reactions were seen. In each sample, enlarged type II pneumocytes with smudged hyperchromatic nuclei resembling smudge cells found in adenovirus infection were detected. In contrast, smudge cells were not detected in lung tissue samples of four patients with lower RTIs due to RSV, rhinovirus, or parainfluenza virus. This might be a characteristic histopathological pattern of hMPV lower RTI. The histopathological pattern shown in this study with humans was distinct from those found in experimental infection of nonhuman primates, in which erosive and inflammatory changes were confined to the conducting airways [45].

Little is known about the nature of cytokine responses to hMPV. Human peripheral blood mononuclear cells in culture stimulated by hMPV revealed that classical CD4 T cell activation depending on antigen presentation and CD86-mediated co-stimulation occurred, comparable to

stimulation by RSV [56]. In a study using BALB/c mice, it was shown that the indolent pulmonary inflammatory response was characterized by minimal innate immune and CD4 T cell trafficking, with low-level interferon (IFN)-γ expression, induction of Th2-type interleukin (IL)-10 expression later during the infection, and delayed cytotoxic lymphocyte (CTL) activity [53]. Peak expression of macrophage inflammatory protein 1α, IFN-γ, IL-4 and RANTES (regulated upon activation, normal T cell expressed and secreted) was related to the severity of the pulmonary inflammation in BALB/c mice [50]. hMPV was a weaker inducer of IFN-γ, IL-10 and CCL5 than RSV, but induced higher levels of IL-6 instead. When looking at cytokine releases at the respiratory epithelial surfaces, hMPV, in contrast with RSV, seemed to be a poor inducer but elicited identical symptoms of similar severity [37]. Levels of the inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-10, and IL-12 in respiratory secretions of infants <1 year with an acute RTI, were two- to sixfold lower in those infected with hMPV compared to RSV. The higher levels of IL-6, inhibiting Th1 differentiation, combined with the lower levels of IFN-γ induced by hMPV, are responsible for a weaker antiviral response leading to lower memory cells upon viral recall. This mechanism underlies the life-long, typically symptomatic re-infection with hMPV. IL-8 and RANTES in nasal secretions of children <16 year admitted to hospital with acute expiratory wheezing were different from that reported in infections with RSV [57]. Patients with RSV infection had high concentrations of RANTES and varying levels of IL-8, whereas children with hMPV infection had lower concentrations of RANTES and higher levels of IL-8. It seems that mechanisms other than those known for RSV elicit symptomatic disease after infection with hMPV. Other mechanisms may include, although they are not limited to, (1) direct viral damage to the airways; (2) Th1 vs. Th2 polarization of the pulmonary immune response, leading to different clinical symptoms; and (3) chemokine-mediated inflammation. Further research is needed to elucidate the exact mechanisms of illnesses caused by hMPV.

The fusion F surface glycoprotein has been identified as a major cross-protective antigen [48, 49]. In addition to the F protein, the subfamily *Pneumovirinae* of the paramyxoviruses also have a separate surface glycoprotein that is involved in attachment and is called the G protein. The F and G surface glycoproteins are the only significant neutralization antigens, and are major independent protective antigens [58]. hMPV virions appear to have three surface glycoproteins, the F, G and SH protein [59]. To analyze the contribution of these three glycoproteins in neutralizing and protective antibodies, hamsters were immunized intranasally with recombinant PIV type 1 expressing each glycoprotein individually from an added gene [60]. The F glycoprotein was shown to be the major contributor to the induction of neutralizing antibodies and protective immunity. The G and SH glycoproteins did not induce detectable neutralizing antibodies, and the contributions to protection were minor or negligible, respectively. This is in contrast

with other paramyxoviruses (including RSV) in which the G protein stimulates high levels of neutralizing and protective antibodies.

Cleavage of the precursor of the F glycoprotein is a prerequisite for infectivity and is an important determinant of virulence for most *Paramyxoviridae*. The contribution of the trypsin-dependent cleavage site R-Q-S-R in hMPV to its growth *in vitro* is well known. This requirement for trypsin *in vitro* raises the possibility that hMPV virulence is restricted by the inefficient cleavage of the F protein. Using recombinant hMPV in which the naturally occurring cleavage sequence was replaced by sequences not depending upon added trypsin *in vitro*, it was shown that replication in hamsters and African green monkeys was not changed. These results suggest that cleavage activation is not a major determinant of hMPV virulence [61]. Similar results were reported by others using a point mutation in the F gene that conferred intracellular cleavability of hMPV in a hamster model [62].

Diagnosis

Four principal methods are used for the diagnosis of respiratory virus infections: virus isolation by culture, antigen detection, RNA or DNA detection, and serological study. For a virus that is not easily detected by virus isolation in the laboratory, it is of great importance to develop rapid, sensitive and reproducible diagnostic tests. The identification of the two hMPV serotypes, A and B, with each serotype divided into genetic sublineages, 1 and 2, has implications for the development of RT-PCR assays and serological diagnostic tests. Because of the unavailability of rapid antigen detection tests and because of its fastidious growth in cell cultures, RT-PCR has become the method of choice. RT-PCR procedures have proved to be more sensitive than virus isolation, and can detect genetically distinct hMPV strains [32].

The cytopathic effect is variable, with RSV-like syncytia formation or focal rounding and cell destruction. The search by van den Hoogen et al. [63] of a cell line with similar susceptibility for the four hMPV lineages and with enhanced detection of the virus by cytopathic effects, resulted in the generation of a subclone of Vero cells (Vero cell clone 118). This cell line is now used routinely for virus isolation in the Netherlands. Commercially available antibodies are not yet available. Monoclonal antibodies (mAb) recognizing conserved epitopes will be useful for rapid viral diagnostics using immunofluorescence (IF) or direct IF techniques as currently used for diagnosing RSV. Confirmation of hMPV causing the cytopathic effect is achieved by RT-PCR testing of the viral culture.

Most RT-PCR protocols reported to date have relied on amplification of the L, N, or F gene with primer sequences mainly derived from the prototype strain 001 from the Netherlands. A comparative evaluation of RT-PCR assays performed in a LightCycler instrument for detection of

hMPV in infected cell cultures showed positivity rates of 100%, 90%, 75%, 60%, and 55% using primers for the N, L, M, P, and F genes [64]. A second evaluation in the same study on nasopharyngeal aspirates positive for the hMPV N gene, the PCR positivity rate for the L, M, P, and F genes were 90%, 60%, 30% and 80%, respectively. From this study it can be concluded that RT-PCR assays aimed at amplifying the N and L genes, which code for two internal viral proteins and seemed to be more conserved regions of the genome, appear particularly suitable for detecting hMPV from both lineages [32, 64, 65]. Rapid and sensitive RT-PCR assays for the N gene (detection limit of 100 copies) have been developed allowing rapid amplification and detection of hMPV sequences directly from clinical samples in <2 h [64, 65]. However, if inadequate primers are selected for PCR amplification, the hMPV detection might be underestimated.

Serological testing only permits a retrospective diagnosis. Because infection is almost universal in childhood, a seroconversion or a ≥fourfold increase in antibody titers must be demonstrated to confirm recent infection. The serological survey performed in the Netherlands was based on an indirect IF assay using hMPV-infected cells [1]. A homemade ELISA method has also been developed using cell lysates of hMPV [41]. To conduct large serological surveys, simpler ELISA tests using viral proteins possibly derived from the two serotypes will be needed.

mAbs used for diagnostic purposes can be directed against whole hMPV proteins or against individual proteins. Ishiguro et al. [66] used specific antibodies against nucleocapsid (N) and matrix (M) proteins in 97 serum samples, and these were tested by Western blot using recombinant N and M proteins of hMPV expressed in *Escherichia coli*. Results indicate that the antibodies against N and M proteins are highly specific (100%) but less sensitive (42.1% N protein; 40.8% M protein) when compared with immunofluorescence antibody (IFA) detecting whole proteins of hMPV. Western blot analysis using recombinant P protein was not successful due to nonspecific binding to human sera. The hMPV IFA-positive sera reacted with the F protein of hMPV by SDS-PAGE, but the signal was weak, suggesting that they were probably directed to conformational-type epitopes of the F protein [67]. Most of the antibodies detected by hMPV IFA were suspected to reaction with the F protein. These authors developed a baculovirus (Bac)-expressed hMPV protein IFA and showed that it was more sensitive than hMPV IFA. An ELISA using the N protein of hMPV has been developed recently [50] and was reported to detect in 58 (81.6%) of 71 adults antibodies against the N protein of hMPV. In previous studies, 20 (100%) of 20 adults aged >20 years had antibodies detected by both hMPV IFA [1, 20], and Bac-F IFA [67]. In this Bac-F IFA study, 192 of 200 serum samples of Japanese subjects between 1 month and 41 years of age showed concordant results with conventional IFA based on hMPV-infected LLC-MK2 cells [67]. The titers obtained by Bac-F were equal or higher than those obtained by the conventional IFA. From the Bac-F IFA study it can be concluded that the

availability of large quantities of Bac-expressed hMPV F protein offers an opportunity to use this recombinant protein as a diagnostic reagent (EIA, IFA, immunoblot) and to study antigenic and immunogenic characteristics of the F protein. Studies like these are important and urgently needed to be able to develop an hMPV vaccine in the near future.

Leung et al. [19] used vesicular stomatitis virus (which infects animals and seldom humans) to produce recombinant hMPV F protein in a seroepidemiological study. The ELISA-based system has many advantages over the methods used in previous studies. The amount of hMPV-specific antigen can be standardized for each assay, antibody to genotype-specific viral glycoproteins can be measured, and the results are based on defined criteria rather than subjective determinations of a positive result in an IFA.

Two rapid antigen detection methods are available: an IFA test and an ELISA. This study compared the rate of virus detection in nasopharyngeal secretions by an indirect IFA with that by RT-PCR, and showed that the IFA with an anti-hMPV mouse mAb could detect hMPV in nasopharyngeal secretions with 73.3% sensitivity and 97.0% specificity compared with the results of RT-PCR [20]. ELISA is easier to perform in daily clinical practice and provides results that are more objective than IFA.

Immunofluorescence staining of clinical specimens and shell vial centrifugation cultures (SVCC) are methods commonly used in clinical virology laboratories for rapid diagnosis, but need sensitive and specific mAbs. Landry et al. [68] evaluated mAb-8 to hMPV M protein for its utility in the rapid diagnosis of hMPV by both IF and SVCC methods. Detection of hMPV was similar in A549, Hep-2, and LLC-MK2 SVCC, and mAb-8 staining was optimal on day 2 post inoculation. The ability to detect positive results by 1 or 2 days after inoculation is a great advantage over present conventional culture methods. The use of mAb-8 in IF staining of clinical specimens was, however, not successful due to nonspecific background staining. mAb-8 is commercially available (MAB8510, Chemicon International, Temecula, CA) and the results of its utility in the diagnosis of viral RTIs are awaited.

Clinical characteristics

The first description of hMPV in children with lower RTI has been reported by a Dutch group that identified the virus in respiratory secretions [1]. Clinical symptoms were similar to those caused by RSV, ranging from upper RTI, severe bronchiolitis and pneumonia during the winter season. All 28 children observed were <5 years of age, and 46% were <1 year old. Asymptomatic carriage seems to be rare in children; no hMPV was detected in 400 infants without respiratory symptoms.

The prevalence and clinical symptoms of hMPV-infected patients, identified by RT-PCR in respiratory samples obtained from patients in a

university hospital, indicated that the prevalence and clinical severity due to hMPV infections are slightly lower than those of RSV infections during the winter season [32]. Most of the hMPV-positive patients were children <2 years old without any underlying illnesses. hMPV was found significantly less frequently than RSV in children <2 months old. Of the 31 hMPV-positive children <2 years old, only 4 (31%) were <2 months old, whereas 43 (35%) of the 122 hRSV-positive children <2 years old were also <2 months old. Others have found that the mean age of patients infected with hMPV was slightly lower than that compared to RSV [39]. 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, indicating an association with immunosuppression. Two severely immunocompromised patients died due to progressive respiratory failure with hMPV as the sole pathogen detected [69]. In studies involving young and elderly adults, hMPV caused more severe disease in fragile elderly than in healthy elderly or young adults [4, 41]. Clinical symptoms in children <10 years of age ($n=238$) due to hMPV infection include cough (82%), rhinitis (67%), fever (72%), respiratory distress (71%), wheezing (59%), and retractions (54%) [29, 30–32, 37, 39, 69]. Specific clinical syndromes caused by hMPV seem to differ from that caused by other respiratory viruses. Williams et al. [39] tested respiratory specimens over a 25-year period in the US from previously healthy children. Infection due to hMPV was more likely to be associated with bronchiolitis and less likely to be associated with croup than infection due to (para)influenza virus. hMPV infection was less likely to be associated with pneumonia than was infection with RSV or influenza virus. Various studies show frequent involvement (16–24%) of hMPV in acute bronchiolitis in infants, a percentage only second to RSV [18, 33, 70]. hMPV is associated with a substantial number of URTI episodes in otherwise healthy outpatient children with clinical illnesses similar to those associated with other common viruses, including frequent acute otitis media [16].

Studies examining the role of hMPV with respect to exacerbations of asthma have yielded conflicting results [35, 39, 57]. Two studies in adult patients with chronic obstructive pulmonary disease (COPD) showed that hMPV could be detected in 2.5% of the hospitalized COPD patients with an acute exacerbation [14, 71], while no hMPV was detected in stable COPD patients. Although there is no doubt that some patients with asthmatic exacerbations have hMPV infection, whether or not the virus is associated more frequently than other respiratory viruses with these exacerbations is not yet clear. Remarkably, a history of asthma or a family member with asthma was more often associated with hMPV (16% and 67%, respectively) than with RSV (0% and 30%, respectively) [9].

The similar seasonality and susceptible population shared by several respiratory viral infections will result in prevalent co-infection of hMPV with other respiratory viruses. This might lead to an underestimation of the

percentage of hMPV-positive samples identified in studies in which only samples negative for other respiratory viruses were tested (see also Tabs 1 and 2). Co-infection rates of 5–10% with one or more respiratory viruses have been demonstrated in several studies searching for the causative pathogen of RTI. Because the epidemic seasonality for RSV coincides with that for hMPV, the potential exists for RSV/hMPV co-infections. Several studies have identified cases of lower RTI in which evidence for the presence of both RSV and hMPV has been detected [23, 33, 39, 72]. Dual infection with RSV and hMPV was more frequent in infants with severe disease (i.e., those who needed supplementary oxygen) and even more frequent in infants with severe disease admitted to the intensive care unit for mechanical ventilation [26, 72]. Foulongne et al. [24] showed that another respiratory virus was detected in 32% of hMPV-positive samples obtained from children <5 years of age with RTI, and all but one of these co-infections involved RSV. Duration of hospitalization and requirement for supplemental oxygen was significantly higher in hMPV/RSV co-infected children. Greensill et al. [73] collected non-bronchoscopic bronchoalveolar samples from 30 infants <48 weeks of age ventilated with RSV bronchiolitis diagnosed by antigen testing. Detection of hMPV was performed by RT-PCR of the M, F, and N genes. In 16 of the 24 infants with a positive RT-PCR for RSV in the bronchoalveolar lavage sample, genomic hMPV was also detectable. This high rate of co-infection raises the possibility that co-infection with RSV and hMPV is a determinant of disease severity. These results were confirmed by others studying the association between severe bronchiolitis and dual infection by RSV and hMPV in children <2 years of age who were admitted to the hospital. Co-infection with both viruses conferred a tenfold increase in relative risk of admission to a pediatric intensive care unit for mechanical ventilation. A high case incidence (52%) of hMPV infection has been described in association with hospital admission of patients with severe acute respiratory syndrome in Hong Kong [43].

In contrast, others found a similar rate of bronchopneumonia in infants infected with hMPV alone as in dual infections [33]. Wilkesmann et al. [23] did not find a lower illness severity when comparing hMPV-infected children with matched RSV-infected children without hMPV co-infections. On the other hand, the seasonal distribution of hHMP and RSV may differ in specific geographic areas as demonstrated in studies from Argentina and Hong Kong where co-infections were not or infrequently observed [9, 37]. The peak of hMPV in these countries becomes prevalent in late winter and early spring. It is likely that by the development of more sensitive detection methods, dual or mixed infections will be increasingly recognized, and do not necessarily result in more severe infection. A positive RT-PCR test result does not differentiate between active infection and prolonged shedding after a recent acute infection that has been terminated.

It is currently not known whether hMPV infection leads to an increased susceptibility to secondary bacterial infections. The absence of sensitive

tools to diagnose bacterial pneumonia has been an obstacle to defining the role of bacterial co-infection in children with virus-associated pneumonia. In a hypothesis-generating study involving a cohort of children randomized to receive the 9-valent pneumococcal vaccine or placebo, children were tested for the presence of hMPV by a nested RT-PCR when admitted to the hospital with a lower RTI. In both HIV-uninfected and HIV-infected children the incidence of hMPV-associated lower RTI was reduced by 46%, and the incidence of clinical pneumonia was reduced by 58% [74]. These data, combined with comparable finding for other respiratory viruses [75], suggest that respiratory viral infections as caused by hMPV predispose to pneumococcal co-infection and that bacterial-viral co-infections are important in the pathogenesis of virus-associated pneumonia in children.

The socioeconomic impact of hMPV infection on children and their households is not well known. It is reported that household contacts of hMPV-infected children, like influenza-infected children, fell ill significantly more frequently, required more medical visits, received more anti-pyretic prescriptions, and were also absent more frequently from work or school, than those of RSV-infected children [76]. These findings suggest that hMPV infection in children considerably affects their families.

Vaccination

No vaccines, antibodies (monoclonal or polyclonal), or chemotherapeutic agents are currently licensed for use to prevent or treat hMPV infections. However, ribavirin and polyclonal antibody preparations (IVIG), used in the therapy and prevention of RSV infections in children, are known to have broad-spectrum activity and can inhibit different viruses. In tissue culture-based assays ribavarin and IVIG preparations containing high titers of hMPV-neutralizing antibodies were found to inhibit hMPV replication [77]. The clinical utility of these findings needs to be tested.

Ulbrandt et al. [78] describe the generation of a panel of neutralizing mAbs that bind to the hMPV F protein (like palivizumab for RSV). A subset of these antibodies has the ability to neutralize prototypic strains of both the A and B hMPV subgroups *in vitro*. Two of these antibodies exhibited high-affinity binding to the F protein and were shown to protect hamsters against infection with hMPV. Studies so far have not shown that mAbs to the F protein alone can protect animals from virus challenge. This might be the first step to use such an mAb prophylactically to prevent lower RTI caused by hMPV. Two of the antibodies found, mAb 234 and mAb 338, have characteristics comparable to palivizumab that make them appealing for further studies. Despite similarities in structure of hMPV and RSV, the F proteins of these two viruses share only a 33% amino acid sequence identity; consequently, antisera generated against either RSV or hMPV do not neutralize across the *Pneumoviridae* group [77].

The contribution of hMPV to pediatric RTIs suggests that it will be important to develop a vaccine against this virus in combination with those being developed for RSV and parainfluenza viruses. The circulation of two serotypes of hMPV might have implications for the development of vaccines. Studies in cynomolgous macaques showed that re-infection is suppressed by high titers of virus neutralization antibodies against the homologous virus and far less by heterologous virus neutralization antibodies [7]. Others report cross-protection and reciprocal cross-neutralization studies in experimental models of hMPV infection, showing that cross-protection is induced at a high level, consistent with a single serotype [10]. The most relevant test of the importance of genetic diversity is whether or not viruses of one genotype induce greater protection against the homologous virus than against the heterologous one. Although difficult to assess, the extent of cross-protection is important to estimate to ultimately develop a monovalent or bivalent vaccine formulation. One of the difficulties in assessing the cross-protection is the occurrence of re-infections. Virus neutralization antibody titers in children >5 years of age are higher than in those of 1–2 years of age, which suggests that re-infections may occur frequently.

Before the discovery of hMPV in 2001, several groups were working with molecular systems that allow the generation of recombinant paramyxoviruses from plasmid DNA copies of virus genes and virus genome. Similar strategies using this technique referred to as reverse genetics, have been rapidly employed to study the replication of hMPV and to generate live attenuated hMPV vaccine candidates. Foreign genes such as the reporter gene for green fluorescence protein were inserted into the hMPV genome and expressed, which effectively defined the transcription start and gene end signals [59]. Reverse genetics has been used to rescue both strains from Canada and the Netherlands entirely from complementary DNA (cDNA). Because the viruses are made from DNA copies, chimeric viruses can be made with the use of the antigenic protein of one virus inserted into the genome of another virus. Neutralizing antibody responses can be induced by such a chimeric virus, protecting the host against challenge with hMPV strains.

MacPhail et al. [48] identified both small-animal and primate models for evaluation of vaccine candidates. These kind of models are not only wanted to evaluate the effectiveness and safety of vaccine candidates, but also for future hMPV antiviral drugs, and therapeutic and prophylactic mAbs. Their results showed that Syrian golden hamsters, ferrets and African green monkeys supported hMPV replication in the lower and upper respiratory tract efficiently, resulting in high levels of hMPV neutralizing antibodies.

More recent work by Biacchesi et al. [79] investigated the function of the SH and G gene to develop a live-attenuated vaccine. Previously, it was shown that deletion of a number of RSV genes such as the SH and G gene was not deleterious to the virus and such RSV mutants have been evalu-

ated in primates as putative live attenuated vaccine candidates [80]. The recovered recombinant hMPV was analyzed *in vitro* and by experimental infection in hamsters [78]. Deletion of a single gene, either SH or G, showed similar replication as wild-type virus in cell culture. This means that the F protein alone is sufficient to mediate attachment and fusion to cells in the absence of the other two surface proteins. In addition, hMPV G and SH are not required for the efficient assembly or release of progeny virus. Mutant hMPV strains lacking the SH and/or G genes were immunogenic and highly protective against hMPV challenge and represent promising vaccine candidates. The mutants lacking G (both ΔG and ΔSH/G) showed reduced replication, in contrast to the mutants lacking only the SH gene, and represent promising vaccine candidates that needed to be studied further in nonhuman primates such as African green monkeys. This was performed in a following study of the same group. Experiments were performed with recombinant hMPV in which the SH, G, or M2-2 gene or ORF was deleted by reverse genetics [81]. These mutants were evaluated for replication and vaccine efficacy following intranasal and intratracheal administration to the respiratory tract of African green monkeys. Each gene-deletion virus was highly immunogenic and protective against wild-type hMPV challenge. The ΔG and ΔM2-2 viruses showed a markedly reduced replication, in contrast to ΔSH virus, and are promising vaccine candidates appropriate for clinical evaluation. Deletion of the hMPV M2-2 protein resulted in a decrease in RNA replication and an increase in gene expression in cell culture [82]. The consequence of this might be that this mutant provides greater antigen synthesis and immunogenicity *in vivo*.

Tang et al. [83] used a different approach to generate an hMPV vaccine candidate. They utilized an attenuated PIV type 3 (PIV3) vector to deliver the hMPV F protein with the aim of inducing both humoral and cellular immunity against hMPV infection. The use of this vector is not new, and has been used in the development of RSV and other respiratory pathogens vaccine candidates [84, 85]. In this study, the chimeric bovine/human PIV3/hMPV F2 was shown to elicit hMPV-specific as well as virus-specific antibodies and T cell responses in African green monkeys. The bovine/human PIV3 vectored hMPV vaccine might, therefore, function as a bivalent vaccine for immunization against both hMPV and PIV3 infections. The development of a bovine/human PIV3 vector-based vaccine expressing both the F protein of hMPV and RSV should provide protection against these three respiratory pathogens that cause significant disease in young children. However, the genetic stability of such a vaccine should be addressed first. The possibility of the host developing immunity to the vector itself is a matter of concern especially when there is a need to boost the primary vaccination. A recent trial in young infants showed that multiple doses of an attenuated PIV3 did not result in inhibitory vector immunity when the intervals between the vaccinations were timed appropriately [86].

Conclusions

The epidemiology and clinical manifestations associated with hMPV have been found to be reminiscent of those of the RSV, with most severe RTI occurring in young infants, elderly subjects, and immunocompromised hosts. The seasonal distribution resembles that of RSV and influenza virus infections, with recurrent epidemics during the winter months. hMPV is the second most important cause, after RSV, of viral lower RTI in children. hMPV infections account for at least 4–8% of the RTI in hospitalized children. In the general community, hMPV infections account for at least 3% of patients who visit a general practitioner for RTI. Interestingly, the rates of detection of hMPV have been generally higher in retrospective than prospective studies, an observation consistent with some selection bias. Larger prospective studies, not limited to the typical respiratory virus season, not limited to testing respiratory samples negative for the other respiratory viruses, and using appropriate controls need to be conducted. Diagnosis is made by RT-PCR assays aimed at amplifying the N or L gene. Additional research to define the pathogenesis of this viral infection and the host' specific immune response will enhance our knowledge to guide the search for preventive and therapeutical strategies.

The development of a simple direct IF assay on nasopharyngeal samples in the near future will certainly enhance our understanding of the role of hMPV in RTIs in humans. Reverse genetics technology is currently being used to develop multivalent vaccines against hMPV and a variety of other important respiratory viruses such as RSV.

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