Thesis

Detection of human metapneumovirus in the routine diagnostic laboratory

submitted by

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1 Abstract

1.1 German

Hintergrund: Derzeit gilt die Reverse Transkriptase (RT)-PCR als Goldstandard für den Nachweis von humanem Metapneumovirus (hMPV) in nasopharyngealen Proben. Mit kürzlich entwickelten monoklonalen Antikörpern können Oberflächenantigene von hMPV mittels eines direkten Immunfluoreszenztests (IF) nachgewiesen werden.

Ziele der Studie: In dieser Studie wurde der neue IF-Test evaluiert und die Ergebnisse mit denen der real-time RT-PCR verglichen.

Methoden: Insgesamt wurden 97 nasopharyngeale Proben sowohl mit dem neuen IF-Test, als auch mit der real time RT-PCR auf hMPV getestet. Zusätzlich wurden die Proben auf andere häufige Erreger von Atemwegsinfektionen getestet.

Ergebnisse: Sechs Proben (6.2%) konnten auf Grund einer zu geringen Anzahl an Zellen im IF-Test nicht ausgewertet werden und wurden daher von der Untersuchung ausgeschlossen. Von den 91 gültigen Proben ergaben 3 (3.3%) ein positives Ergebnis für hMPV im IF-Test und der real time RT-PCR. Weiters zeigten 50 Proben (55%) im IF-Test eine unspezifische Färbung. Acht Proben wurden positiv auf RSV getestet (8.2%), zwei auf *Bordetella pertussis* (2.1%), eine auf *Chlamydia pneumoniae* (1.0%), und jeweils eine Probe ergab eine Koinfektion mit hMPV/RSV (1.0%) bzw. RSV/*Chlamydia pneumoniae* (1.0%).

Zusammenfassung: Obwohl die Durchführung des IF-Tests größtenteils manuell erfolgt und die endgültige Beurteilung subjektiv ist, könnte der neue IF-Test auf Grund der Einfachheit der Handhabung und der kürzeren Dauer eine gute Alternative zur real time RT-PCR darstellen.

Schlüsselwörter: humanes Metapneumovirus (hMPV); Immunfluoreszenztest (IF Test); real time reverse transcription Polymerasekettenreaktion (RT-PCR); Atemwegsinfektion.

1.2 English

Background: Currently, reverse transcription (RT)-PCR is the method of choice for the detection of human metapneumovirus (hMPV) in nasopharyngeal samples. Monoclonal antibodies for detection of hMPV surface antigens by a direct immunofluorescence (IF) test were described recently.

Goals of the study: To evaluate the performance of a new IF test for the detection of hMPV and to compare results with those obtained by the routinely used real-time RT-PCR.

Methods: A total of 97 nasopharyngeal samples were tested for hMPV with both, the new direct IF test and the real time RT-PCR as gold standard. Additionally, all samples were tested for other common respiratory pathogens.

Results: Six samples (6.2%) gave an invalid result with the IF test and were therefore excluded from further investigations. Out of the 91 valid samples, 3 samples (3.3%) tested positive for hMPV with the new direct IF test and the routinely used RT-PCR. Fifty samples (55%) showed non-specific staining in the IF test. Eight samples tested positive for RSV (8.2%), two for *Bordetella pertussis* (2.1%), one for *Chlamydia pneumoniae* (1.0%), one for hMPV/RSV co-infection (1.0%), and one for RSV/*Chlamydia pneumoniae* co-infection (1.0%).

Conclusions: The IF test is mainly manual, lacks automation, and the final interpretation remains subjective. Yet, because of the ease of the method and the shorter overall time, the IF test might be preferable.

Key words: human metapneumovirus (hMPV); immunofluorescence (IF) test; real time reverse transcription polymerase chain reaction assay (real time RT-PCR); respiratory tract disease (RTD).

2 Introduction

Respiratory tract disease (RTD) is one of the leading causes of morbidity and mortality in infants and young children worldwide. The World Health Organization ranks RTD as the second leading cause of death worldwide in children younger than 5 years.

Viruses such as the respiratory syncytial virus (RSV), influenza A and B viruses, adenoviruses, rhinoviruses, and coronaviruses play the major role as etiologic agents. However, the etiology of a large number of respiratory infections remains unknown.

In 2001, the human metapneumovirus (hMPV) was isolated from children suffering from RTD in the Netherlands (van den Hoogen et al, 2001). The newly discovered respiratory virus shows clinical and epidemiological features similar to those of RSV. From its initial description onwards, hMPV has been associated with acute upper and lower respiratory tract infections in Europe, America, Australia, Asia, and South Africa. Infections produced by the hMPV have been found in individuals of all ages and the virus seems to be responsible for a significant proportion of respiratory infections in early infancy and childhood (Boivin et al., 2003; Peiris et al., 2003; Foulongne et al., 2006).

2.1 The human metapneumovirus (hMPV)

The hMPV was isolated initially from respiratory specimens in tertiary monkey kidney cells (van den Hoogen et al., 2001) (**Table 1**). Viral gene sequences were decoded by polymerase chain reaction (PCR). Based on the similarities in both genome organization and amino acid sequence to avian pneumovirus, hMPV was classified in the *Metapneumovirus* genus of the sub-family *Pneumovirus*, family *Paramyxoviridae*, of the order *Mononegavirales* and given the provisional name of human metapneumovirus. It turned out to be the first member of the metapneumovirus genus that was capable of infecting humans. Because of the

close relation to the avian pneumovirus the hMPV was suggested to originate from birds. Serological studies showed that antibodies had been circulating for at least half a century indicating that the possible zoonotic event must have taken place before 1958 (van den Hoogen et al., 2001).

Table 1

Classification of hMPV

Group	Group V (negative single-stranded RNA)
Order	Mononegavirales
Family	Paramyxoviridae
Subfamily	Pneumovirinae
Genus	Metapneumovirus

The hMPV was detected only recently because of three reasons (van den Hoogen et al., 2001): First of all, the virus does not seem to replicate efficiently in continuous cell lines many virology laboratories use. Second, hMPV shows very slow replication kinetics *in vitro*. Third, the hMPV replication is trypsin dependent which was not used in earlier studies.

2.1.1 Morphology

The morphology of hMPV is similar to that of paramyxoviruses and of RSV in electron microscopy (van den Hoogen et al., 2001; Freymuth et al., 2004) (**Figures 1, 2**). Infected tertiary monkey kidney cells reveal pleomorphic particles with 150-600 nm in diameter, spherical particles with a mean diameter of 209 nm, and filamentous particles with an average size of 282 x 62 nm. The viral particles show short envelope projections in the range of 13-17 nm. Nucleocapsids were observed rarely (van den Hoogen et al., 2001).

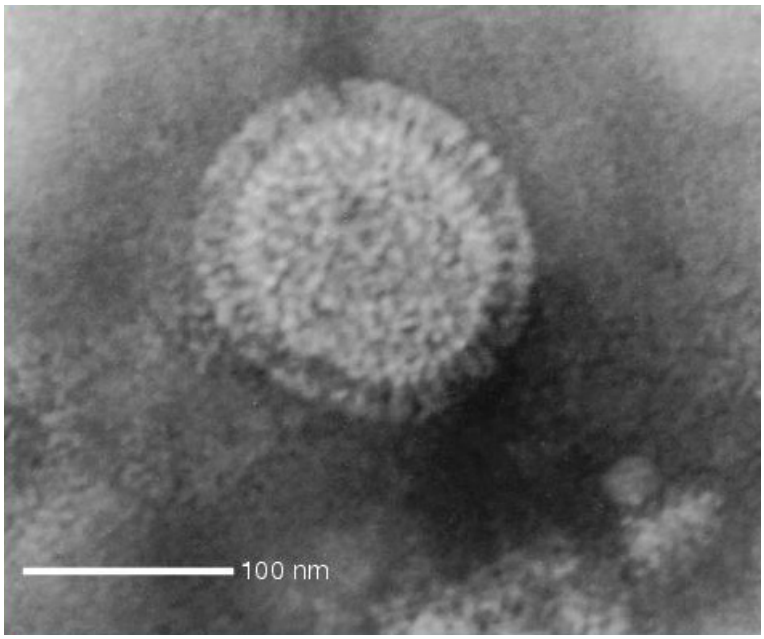

Figure 1. Electron microscopic image of a viral particle.

The viral particles are pleomorphic, spherical, and filamentous (Peret et al., 2002; van den Hoogen et al., 2001) (**Figures 2, 3**). Because of its lipid layer it is very susceptible to organic solutions (Peret et al, 2002).

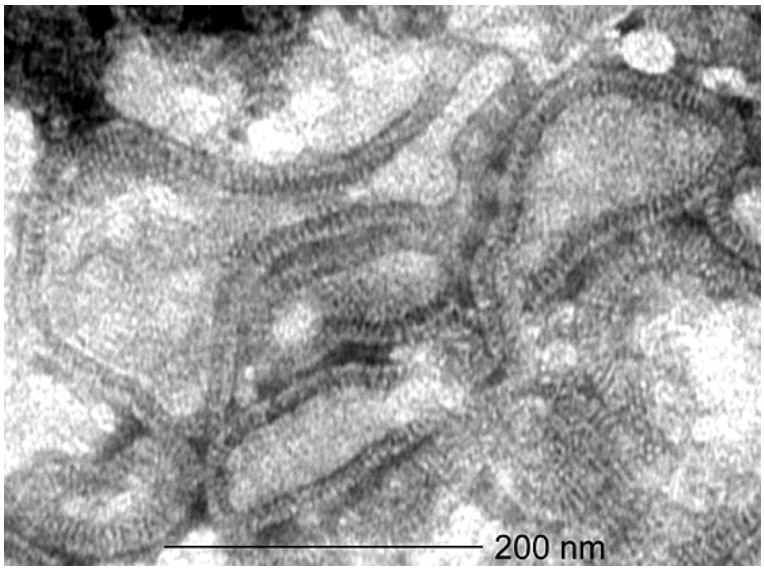

Figure 2. Electron micrograph of hMPV particles with magnification ×92 000. Virus concentrated from infected tMK cell culture supernatants visualized by negative contrast electron microscopy.

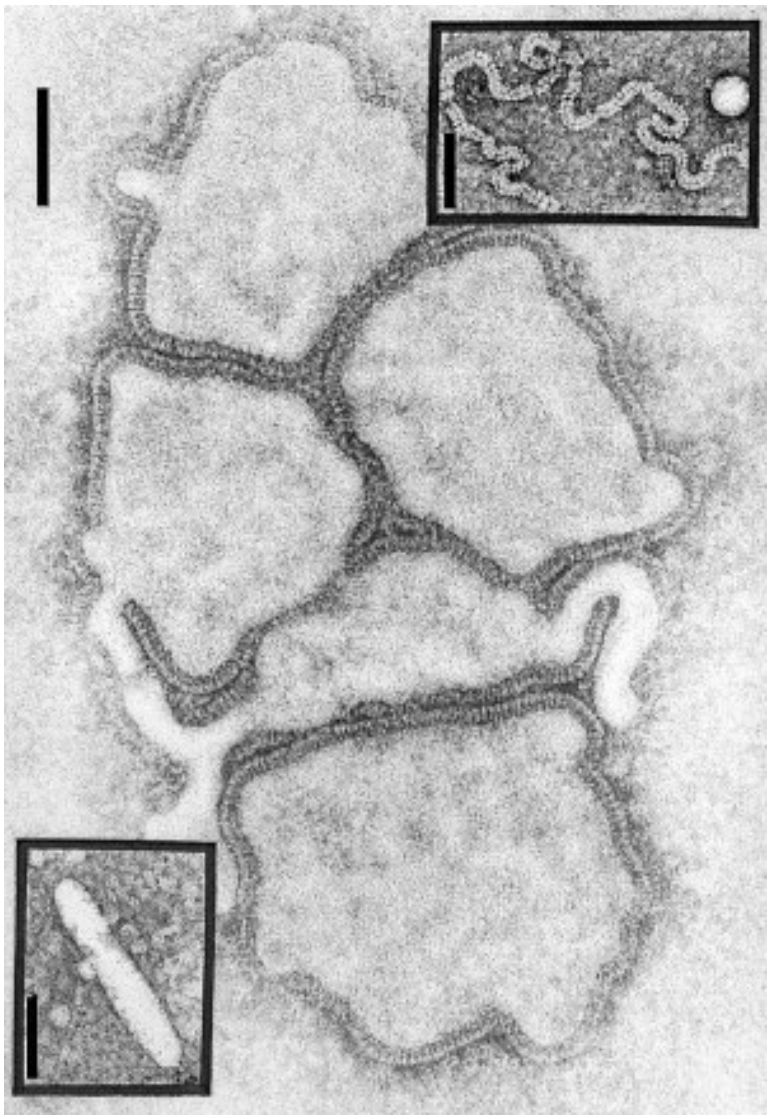

Figure 3. Negative strain electron micrographs of hMPV. The center image shows five pleomorphic hMPV particles with projections along the periphery of the viruses. The images in the upper right and lower left corners are, respectively, of the nucleocapsid and filamentous rod-like particle. Staining was done with 2% phosphotungstic acid, the bar markers represent 100 nm.

2.1.2 Genome, proteins, and replication

The hMPV genome consists of a single negative strand RNA of approximately 13 kb containing 8 genes coding for 9 different proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), transcription elongation

factor (M2.1), RNA synthesis regulatory factor (M2.2), small hydrophobic protein (SH), major attachment glycoprotein (G), and major polymerase subunit (L) (van den Hoogen et al., 2001; Biacchesi et al., 2003, Freymuth et al., 2004) (**Figure 4**). Sequence analysis revealed that F genes were relatively well conserved (Bastien et al., 2003). Limited sequence diversity has been observed in this gene making it a good target for detection by nucleic acid amplification tests (van den Hoogen et al., 2004; Kuypers et al., 2005; Winther at al., 2005; Agapov et al., 2006; Galiano et al., 2006).

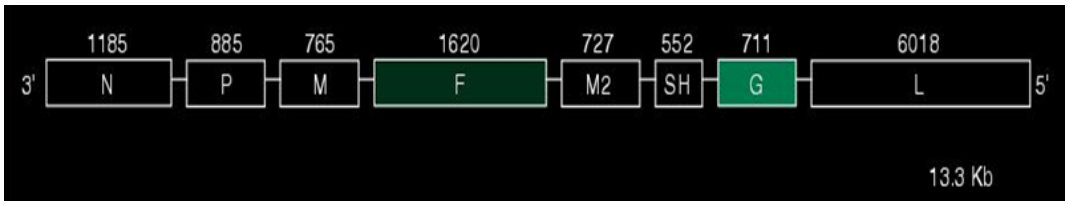

Figure 4. The hMPV genome consists of a single negative strand RNA of 13.3 kb containing 8 genes, i.e. 3'-N-P-M-F-M2-SH-G-L-5'.

The virus can be classified into two different genotypes (A and B), with two subtypes (A1, A2, B1, B2) according to sequence analysis of the F, G and P genes. The two genotypes A and B show a similarity of 80 to 88% at the nucleotide level, while the similarity within each subtype ranges from 93% to almost 100% (van den Hoogen et al., 2001, 2004; Peret et al., 2002). The majority (72%) of hMPV isolates belongs to subtype A1 (Bastien et al., 2003; Foulongne et al., 2006). However, the existence of further subtypes is very likely (Schildgen et al., 2004).

In phylogenetic analysis, the comparison of the isolates from the 2001-2002 season with those from previous seasons reveal a shift in the predominant genotype, possibly indicating evolution of hMPV. Furthermore, the phylogenetic comparison of hMPV isolates from Canadian provinces and The Netherlands suggests that this evolution correlates with temporal rather than geographical variations (Bastien et al., 2003) (**Figure 5**).

The viral genome heterogeneity seems to be the reason of incomplete immunity and repeated. There seems to be no cross-protection among the different subtypes and re-infections have been reported (Pelletier et al., 2002; Peret et al., 2002; Williams et al., 2004; Principi et al., 2006).

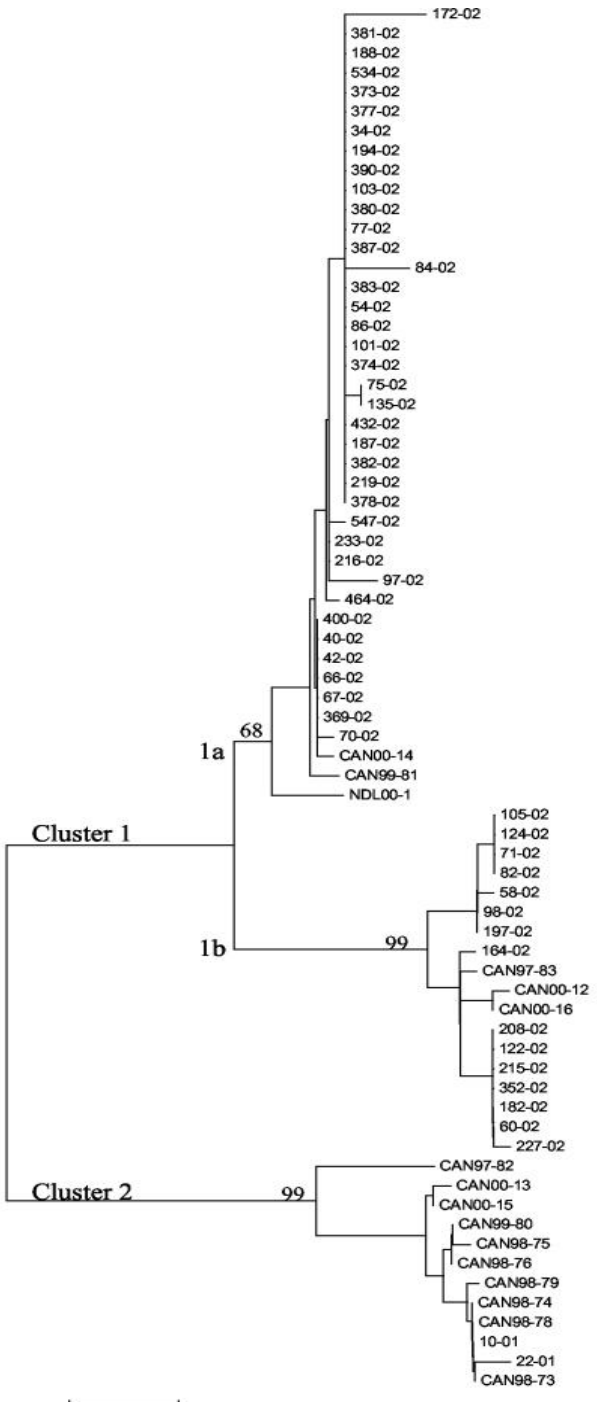

Figure 5. The phylogenetic analysis of hMPV isolates shows sequences of nucleotides 688 to 1032 of the F gene. The corresponding gene sequences of previously reported Canadian and Dutch hMPV isolates are also included. The phylogenetic analysis was performed by the neighbor-joining method of the MEGA program. The previous Canadian isolates are indicated by CAN followed by the year of collection and the isolate number (e.g., CAN97-83). The isolates presented in study performed by Bastien et al. are indicated by the isolate number and the year (e.g., 172-02). The Dutch isolate is indicated by NDL00-1. Canadian and Dutch isolates from 1997, 1999, and 2000 were found in both clusters, whereas isolates from 1998 were found only in cluster 2. The two isolates from 2001 were found in cluster 2. Cluster 1 contains all isolates reported in 2002.

Studies about an association of severe cases of RTD with a specific hMPV subtype have not been conclusive yet. However, a recent study revealed that the viral load may play a greater role than the viral subtype regarding the severity of hMPV infection in children (Bosis et al., 2008).

2.1.3 Epidemiology

Based on recent studies, 4 – 9% of RTDs are related to hMPV (Boivin et al. 2003; Peiris et al., 2003; IJpma et al., 2004; Mullins et al., 2004). The hMPV is a ubiquitous respiratory pathogen. It has been detected in patients from all ages but almost all children at the age of five years show antibodies indicating that hMPV is acquired early in life and suggesting that young children are particularly susceptible to hMPV infection (van den Hoogen et al., 2001; Crowe et al., 2004) (**Table 2**). Seropositivity rises to approximately 100% by the age of ten (Ebihara et al., 2004).

Table 2

Seroprevalence of hMPV

	Immunofluorescence assays		Virus neutralization 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 (refs. 16,17).

The median age for the primary hMPV infection has been reported to be between 6 and 12 months (Mullins et al., 2004; Kuypers et al., 2005; Williams et al., 2004). In a recent study, children with hMPV infection had a median age of 6 months, while RSV- and rhinovirus-infected children had a median age of less than 4 months (Manoha et al., 2007) (**Figure 6**).

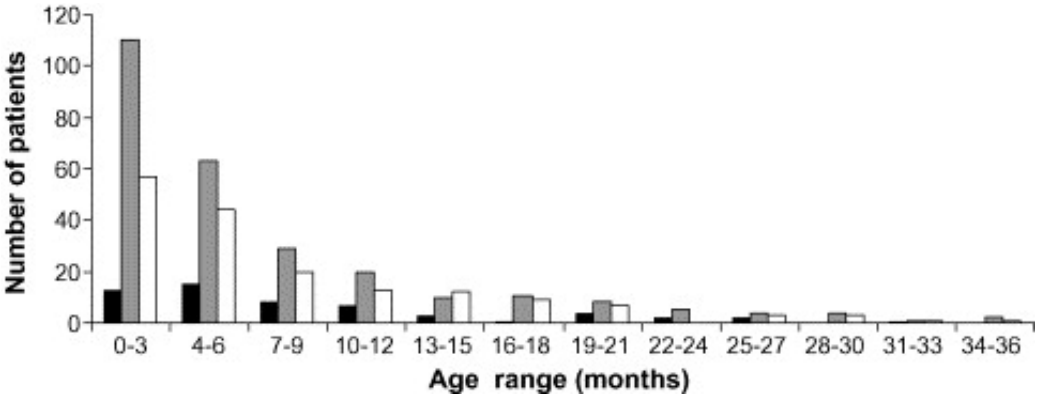

Figure 6. Age distribution of patients infected with hMPV (black), RSV (grey) or rhinoviruses (white).

With regard to prevalence data, results obtained by several studies have not been conclusive yet. The prevalence of hMPV infection varies between 3% and 30% in children hospitalized due to acute respiratory tract infection (Wilkesmann et al., 2007). Incidence of hMPV infection was shown to be 1.5 – 20% in different studies (Foulongne et al., 2006; Principi et al., 2006).

The socio-economic impact of hMPV-infected children seems to be significant, presenting a substantial public health problem. Recent studies showed that households with hMPV-positive children had significantly more illness, needed significantly more medical visits, received more antipyretics, and missed significantly more work or school days than those of the RSV-positive children (Bosis et al., 2005) (**Table 3**).

Table 3

Clinical and socioeconomic impact of different viral infections among the household contacts of the children in whom a single infectious agent was demonstrated

Characteristics	Households of hMPV-positive children (n = 128)	Households of RSV-positive children (n = 507)	Households of influenza-positive children (n = 806)
Disease similar to that of the infected child (%)	16 (12.5)*	24 (4.7)	78 (9.7)*
Additional medical visits (%)	16 (12.5)**	16 (3.2)	78 (9.7)**
Antipyretic prescriptions (%)	14 (10.9)*	18 (3.6)	104 (12.9)**
Antibiotic prescriptions (%)	6 (4.7)	11 (2.2)	36 (4.5)
Hospitalization (%)	0	0	3 (0.4)
Lost working days, median (range)	4 (2–10)*	2.5 (2–7)	4 (1–10)*
Lost school days, median (range)	4 (3–15)*	2 (2–4)	5 (1–15)*

No other statistically significant differences. hMPV, human metapneumovirus; RSV, respiratory syncytial virus.
*$P < 0.05$.
**$P < 0.0001$ versus households of RSV-positive children.

2.1.3.1 Seasonal distribution

The hMPV has been detected throughout the year at low levels (Kahn, 2003). The seasonal distribution of hMPV infections has been identified with major infections in winter and early spring months, i.e. from December to April, in temperate climates (van den Hoogen et al., 2003; Esper et al., 2004; Williams et al., 2004; Robinson et al., 2005; Foulogne et al., 2006) (**Figure 7**). It thus overlaps with the season of other respiratory viruses, possibly leading to co-infection with RSV, parainfluenza virus, adenovirus, and influenza virus. However, it is not known whether co-infection results in more severe RTD than infection with a single virus only.

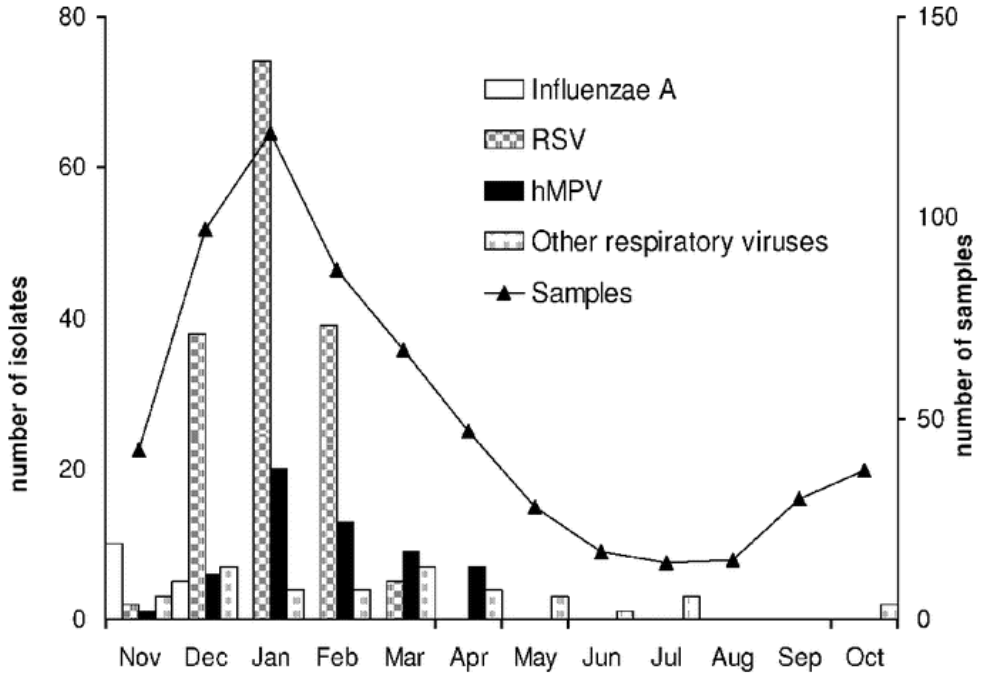

Figure 7. Seasonal distribution of hMPV, RSV, influenza A virus and other respiratory viruses (parainfluenza virus type 3, adenovirus and rhinovirus) infections during the 1-year study period.

However, the seasonal distribution shows variability in intensity and peak activity over the course of successive seasons (Falsey et al., 2003; Gerna et al., 2005; Ludewick et al., 2005; Mackay et al., 2004; Williams et al., 2006; Manoha et al., 2007). Additionally, there is evidence that different hMPV subtypes co-circulate during the same season (van den Hoogen et al., 2004; Manoha et al., 2007).

2.1.4 Transmission, pathogenesis, and pathomorphological features

Both the shedding period and the mode of transmission are not known yet but they are suggested to be similar to other Paramyxoviruses, i.e. transmission by aerosols (Hamelin et al., 2005; Semple et al., 2007). The tenacity remains unknown as well (Wilkesmann et al., 2007). The incubation period has been estimated to be 4-6 days (Ebihara et al., 2004).

There seems to be only a transient immunity after wild-type hMPV infections according to animal experiments (Herfst et al., 2008). Re-infections through alternative subtypes may occur frequently in humans; children more than twelve years of age have higher virus neutralizing antibody titers than those aged five years (van den Hoogen et al., 2001). Furthermore, serological studies revealed that antibody titers measured by IF assays showed a greater range in individuals aged more than 2 years than in patients aged 6-24 months, suggesting a booster effect as a consequence of re-infection with the same or a closely related virus (Principi et al., 2006).

Data concerning the pathology and pathogenesis of the hMPV infection are rare and have been collected predominantly for patients with underlying lung disease (Crowe, 2004; Principi et al., 2006). The hMPV mainly affects the airway epithelium (Vargas et al., 2004). Recent animal experiments have shown that hMPV is able to replicate in the upper and lower respiratory tract, and that the viral replication is polarized to the apical surface of ciliated respiratory epithelial cells (van den Hoogen et al., 2002; Alvarez et al., 2004; Kuiken et al., 2004; Skiadopoulos et al., 2004; Hamelin et al., 2005) (**Figure 8**).

Infection is associated with airway epithelial cell changes and increased inflammatory cell infiltrate. The infiltrate predominately consists of mononuclear cells in the lung interstitium (Alvarez et al., 2004; Skiadopoulos et al., 2004; Wyde et al., 2005).

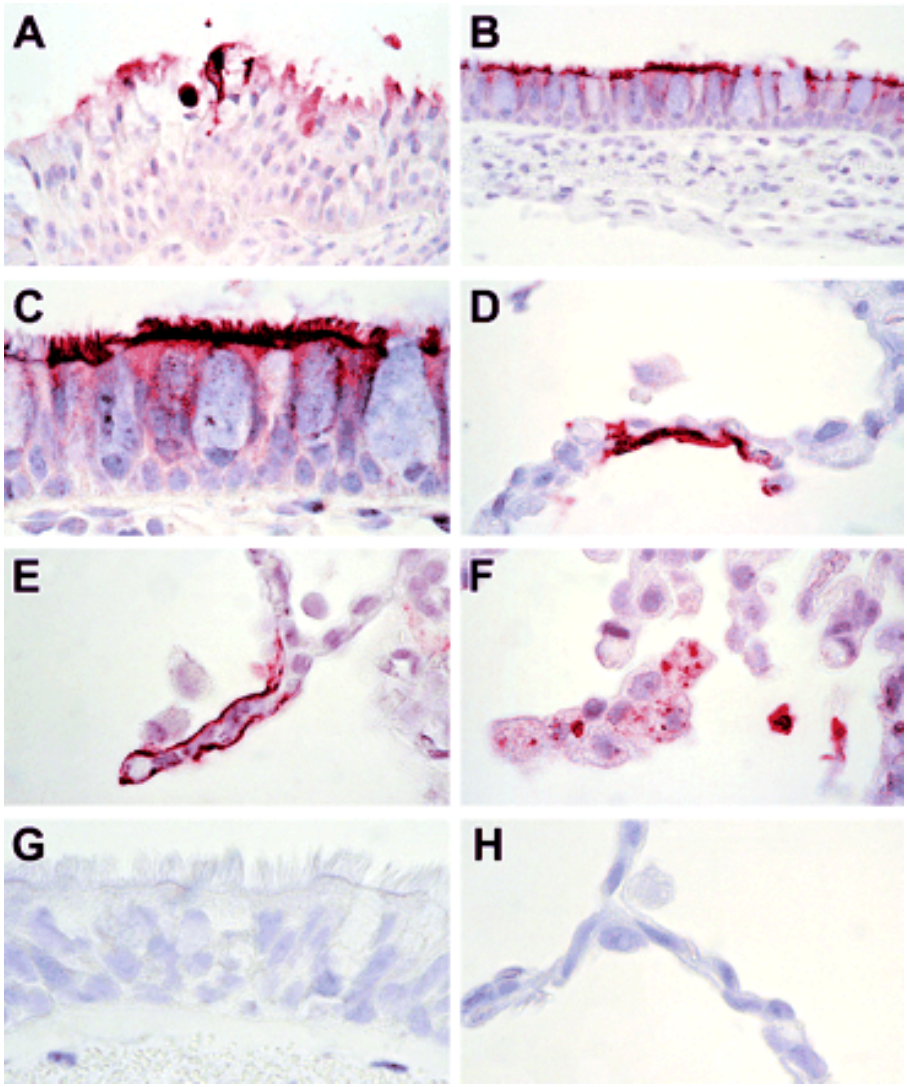

Figure 8. Immunohistochemistry of experimental hMPV infection in cynomolgus macaques. A: Section of respiratory mucosa from nasal septum: Expression of hMPV occurs in the cytoplasm of degenerate epithelial cells and in cell debris (Immunoperoxidase stain for hMPV, original magnification, x100). B: Bronchial section: Expression of hMPV occurs in the cytoplasm of morphologically normal ciliated epithelial cells (Immunoperoxidase stain for hMPV, original magnification, x100). C: Bronchial section: Detail of panel B. Expression of hMPV is most pronounced in the cilia and apical plasma membrane of ciliated epithelial cells (Immunoperoxidase stain for hMPV, original magnification, x250). D and E: Pulmonary section: 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: Expression of hMPV occurs in the cytoplasm of alveolar macrophages and in intraluminal cell debris. (Immunoperoxidase stain for hMPV, original magnification, x250). G: Bronchial section of negative control macaque (Immunoperoxidase stain for hMPV; original magnification, x250). H: Pulmonary section of negative control macaque (Immunoperoxidase stain for hMPV, original magnification, x250).

The early stage of the infection results in cell degeneration and/or necrosis, red cytoplasmic inclusions caused by hemosiderin-laden macrophages, abundant neutrophils, and prominent mucus (Alvarez et al. 2004; Vargas et al., 2004; Mahalingham 2006) (**Figure 9**). Biopsies taken at a later stage of the infection (i.e. at least one month after detection of hMPV from nasal secretion) have shown expansion of peribronchiolar lymphoid tissue, squamous metaplasia, hemosiderin, and accumulation of intra-alveolar foamy macrophages (Vargas et al., 2004).

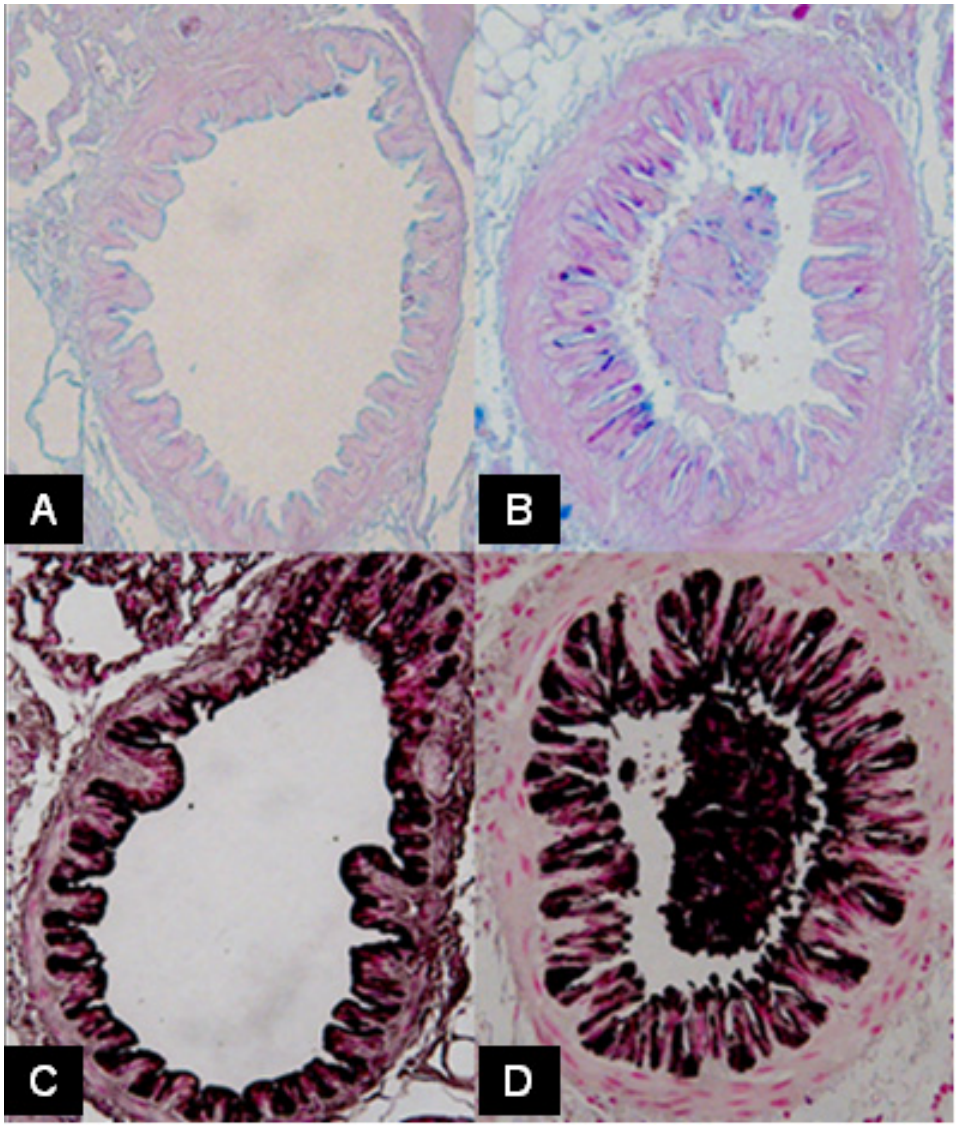

Figure 9. The histopathology of a murine airway epithelium reveals that hMPV infection is associated with airway remodelling. Mucus production peaks on day 2 (B) and is still detectable at day 4. Mucus production is not apparent after day 4 (C). The black staining is associated with altered airway epithelial morphology like increased myofibroblast thickening and staining of cell debris in the airway lumen (D).

The hMPV can persist in the lungs for several weeks, suggesting that the virus uses strategies to overcome host defense (Vargas et al. 2004). Recent animal experiments have shown that hMPV persists despite the presence of neutralizing antibodies, suggesting that humoral immunity to hMPV may be insufficient in eliminating the virus. Furthermore, T-cell or natural killer (NK) cell deletion results in increased titers of hMPV in the lungs of infected mice indicating that at least some T-cell-mediated as well as antibody-mediated immune control of viral persistence may exist (Alvarez et al., 2004; Herd et al., 2006).

2.1.5 Clinical features of infection

The hMPV is predominantly associated with respiratory infections in early infancy and childhood. The clinical symptoms of an infection with hMPV are similar to those caused by other respiratory viruses, thus making it impossible to distinguish between these viral infections on the basis of seasonality and clinical manifestations.

The hMPV causes both upper and lower respiratory tract infections. Patients may therefore present with a spectrum of disease severity ranging from coryza with cough to failure of gas exchange requiring mechanical ventilation (Semple et al., 2007).

HMPV infections may produce clinical symptoms such as fever, cough, flu-like symptoms, sore throat, tachypnea, wheezing, prolonged exspirium, and gastrointestinal symptoms such as nausea and vomiting (**Figure 10**). Less frequent symptoms include headache, muscle ache, rash, and conjunctivitis (Bastien et al., 2003; Wolf et al., 2006).

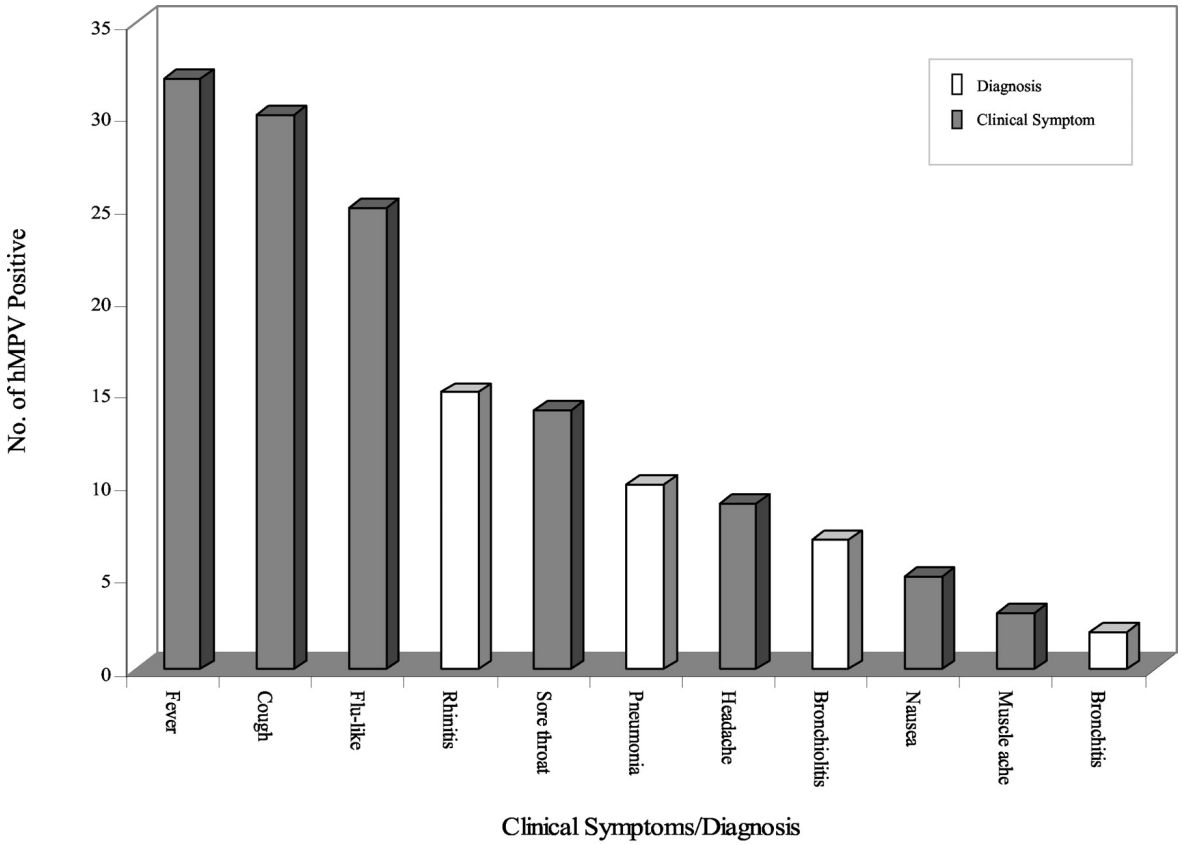

Figure 10. Clinical symptoms and diagnoses reported in hMPV-positive patients.

Severe lower respiratory tract disease is most frequently reported in very young, very old and immunosuppressed patients (Fenwick et al., 2007). Infants run the risk of dehydration due to difficulties in feeding, increased *perspiratio insensibilis*, and fever. Apnoes related to hMPV have been reported with maximum occurrence in the prematurely born (Wilkesmann et al., 2007). Furthermore, severe and even fatal cases of hMPV infections have been described in immunosuppressed patients (Cane et al., 2003; Larcher et al., 2005; Sivaprakasam et al., 2007).

Pre-existing risk factors such as immunosuppression, cardiopulmonary problems or prematurity may contribute to a complicated progress of disease. Furthermore, HMPV infection may stimulate bronchial asthma in patients with a medical history of asthma, and it may be related to the onset and exacerbation of childhood bronchial asthma (Hamelin et al., 2005; Principi et al., 2006). However, it is not clear if an hMPV infection of the lower respiratory tract in early infancy leads to constant bronchial hyperreagibility or bronchial asthma in later life.

2.1.6 Diagnosis

Clinical symptoms and radiological signs do not permit exact conclusions on the causative agent. In fact, rates of cough, pharyngitis, tachypnea, wheezing, and prolonged expirium among patients with hMPV are statistically similar to those found in RSV patients (Wolf et al., 2006) (**Table 4**). This underlines the significance of specific diagnostics in patients suffering from RTD (Wilkesmann et al., 2007).

Table 4

Comparison of clinical presentation and findings in children with hMPV, RSV and influenza A infections and hMPV co-infection

Characteristic	HMPV (N = 48)	RSV (N = 79)	FluA (N = 53)	HMPVco (N = 14)	*P*
Temperature >38°C	35/42 (83)*	**48/69 (70)**†	**43/48 (90)**	11/13 (85)	**0.01; Flu vs RSV**
Cough	44/48 (92)	70/78 (90)	38/51 (75)	12/13 (92)	NS
URI	24/46 (52)	25/70 (36)	22/48 (46)	7/13 (54)	NS
Pharyngitis	**6/46 (13)**	16/76 (21)	**18/53 (34)**	**1/14 (7)**	**0.01; HMPV vs. FluA** **0.04; HMPVco vs. FluA**
GI symptoms	20/47 (43)	**19/69 (28)**	19/47 (40)	**9/13 (69)**	**0.004; HMPVco vs. RSV** **0.08; HMPVco vs. HMPV** **0.06; HMPVCo vs. FluA**
Otitis media	12/46 (26)	20/76 (26)	19/51 (37)	2/13 (15)	NS
Tachypnea	31/46 (67)	47/79 (60)	26/53 (49)	5/13 (39)	NS
Wheezing	**26/47 (55)**	**45/77 (58)**	**13/52 (25)**	5/13 (38)	**0.002; HMPV vs. FluA** **<0.01; RSV vs. FluA**
Prolonged expirium	**18/47 (38)**	**36/76 (47)**	**11/53 (21)**	5/14 (43)	**0.05; HMPV vs. FluA** **0.002; RSV vs. FluA**
Rales	17/45 (38)	27/77 (35)	21/53 (40)	6/14 (43)	NS
Mean WBC/mm^3	14,090 ± 6112 (N = 47)	13,500 ± 5469 (N = 77)	13,288 ± 6331 (N = 52)	14,219 ± 4868 (N = 14)	NS
Oxygen saturation <94%	**27/47 (57)**	44/78 (56)	**17/53 (32)**	5/14 (36)	**0.01; HMPV vs. FluA**
Abnormal radiographic findings	41/47 (87)	64/76 (84)	34/42 (81)	13/13 (100)	NS
Infiltrates	31/47 (66)	38/76 (50)	22/42 (52)	10/13 (77)	NS
Air trapping	**9/48 (19)**	**30/79 (38)**	17/53 (32)	4/14 (29)	**0.02; HMPV vs. RSV**
Atelectasis	**6/15 (40)**	**5/38 (13)**	**4/30 (13)**	2/5 (40)	**0.01; HMPV vs. FluA** **0.03; HMPV vs. RSV**

*Numbers in parentheses, percent.
†Significant differences are highlighted in bold.
FluA indicates influenza A; NS, nonsignificant; URI, upper respiratory infection; GI, gastrointestinal; WBC, white blood cells.

2.1.6.1 Radiological diagnosis

More than 50% of hospitalized patients with an hMPV infection show pathological X-rays such as hyperexpansion, peribronchitis, and atelectasis. Several respiratory viruses produce lobar or segmental pneumonias that are difficult to

distinguish from bacterial lower respiratory tract infection (Wolf et al., 2006; Wilkesmann et al., 2007) (**Figure 11**). In fact, neither clinical features nor radiological images permit a tentative differentiation between viral and bacterial pneumonias in infants and young children.

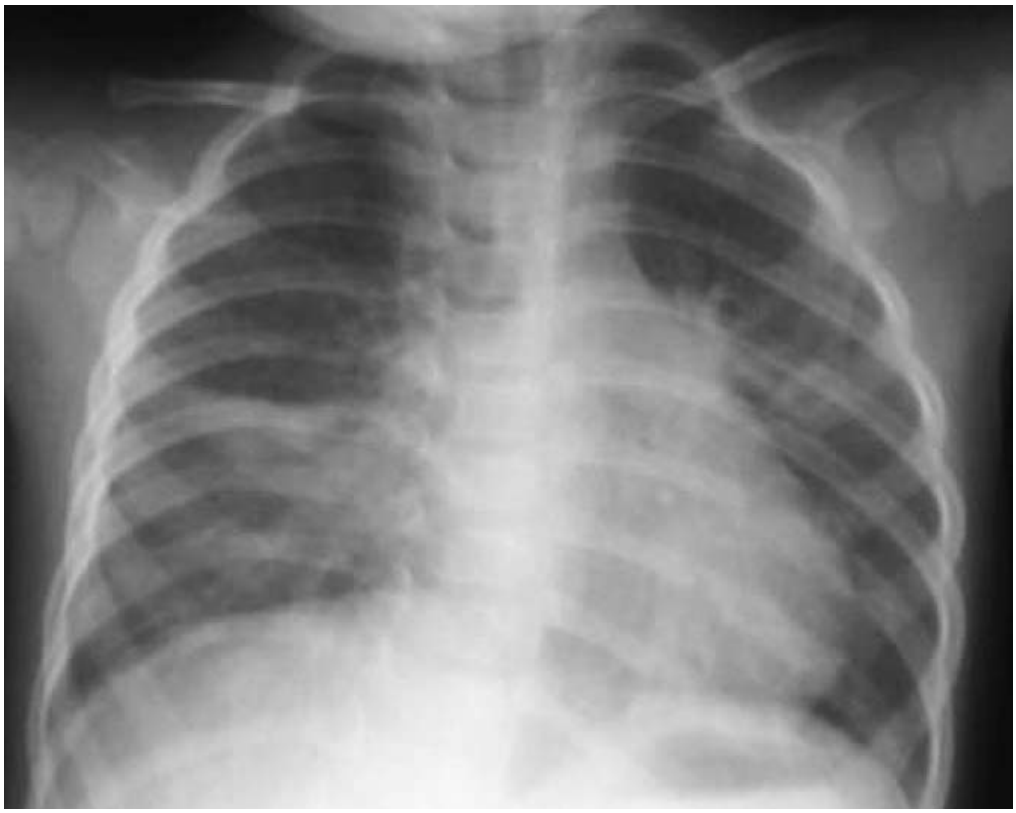

Figure 11. Chest X-ray from a 13 months old female patient showing lobar pneumonia. The patient was tested positive for hMPV, negative for RSV.

2.1.6.2 Laboratory diagnosis

For detection of hMPV, virus isolation in cell culture is used rarely because hMPV is difficult to detect due to its selectivity, slow growth, and mild cytopathicity (Boivin et al., 2002; Chan et al., 2003; Williams et al., 2004; Deffrasnes et al., 2005). Currently, diagnosis of hMPV is based on molecular assays including conventional reverse transcription (RT)-PCR (van den Hoogen et al., 2004). The superior sensitivity and specificity of RT-PCR has been shown; it may detect as few as 5-10 viral genomes and is actually considered as the gold standard method of

diagnosis (Reina et al., 2007). Yet, it may not be useful for many routine diagnostic laboratories because of the highly-sophisticated methodology (Fenwick et al., 2007). One alternative to RT-PCR and virus isolation might be direct antigen detection which shows a sensitivity of 70-75% when compared to RT-PCR (Ebihara et al., 2005; Barry-Murphy et al., 2006; Ingram et al., 2006).

2.1.6.2.1 Real-time RT-PCR

The development of real-time PCR significantly simplified routine molecular diagnostics as it enables simultaneous detection and quantification of a specific sequence in a DNA sample. In comparison with conventional PCR, the real time PCR combines amplification and detection of the target DNA in the same closed vessel resulting in a reduced rate of contamination. Furthermore, real-time PCR offers the advantages of a shorter analytical turnaround time and a greater quantization range (i.e. 5 to 6 logs in real-time PCR compared to 2 to 3 logs in conventional PCR-assays) (Kessler, 2007).

Modern molecular assays are based on the two major steps: First, the targeted nucleic acid is extracted, followed by the real-time PCR including hybridization and detection of the amplification products. Real-time PCR utilizes fluorescence to detect the PCR product generated. The linear correlation between PCR product generation and fluorescence intensity can thus be monitored and the amplified product can be measured after each PCR cycle. Instruments that combine thermal cycler, fluorimeter, optics for fluorescence excitation and emission collection, and software for processing and analyzing data are used for real-time PCR (Kessler, 2007).

Compared to conventional RT-PCR, real-time RT-PCR is more sensitive, faster, and more cost-effective (Principi et al., 2006). Usually, real-time RT-PCR allows amplification and detection of hMPV in less than 2 hours (Mackay et al., 2003; Hamelin et al., 2005; Leung et al., 2005).

2.1.6.2.2 Immunofluorescence test

Monoclonal antibodies have been described recently, allowing the detection of hMPV in nasopharyngeal samples (Percivalle et al., 2005; Gerna et al., 2006). The direct immunofluorescence (IF) technique utilizing specific monoclonal antibodies may permit an even faster diagnosis of hMPV. Therefore, the IF test may be a good alternative for a rapid and safe diagnosis of hMPV.

2.1.6.2.3 Serological tests

Serological tests allow a retrospective diagnosis only. To confirm a recent infection, seroconversion or, in case of pre-existing antibodies, a ≥ 4-fold increase in antibody titers must be demonstrated (Crowe et al., 2004; Hamelin et al., 2004, 2005; Mejìas et al., 2004; van den Hoogen et al., 2004).

2.1.7 Prevention and treatment

Currently, there are neither specific prevention procedures nor causative treatment for hMPV infection available (Principi et al., 2006). Retrospective studies show that 26-44% of patients hospitalized because of hMPV infection were treated with systemic steroids and more than 50% with bronchodilatators (Boivin et al., 2002; Døllner et al., 2004; von Linstow et al., 2004; Wilkesmann et al., 2007). Antibacterial chemotherapy was given in 22-67% leading to an increase of nosocomial multiresistant bacteria strains (Cunha, 2004; Low et al., 2004). Patients with fever are usually treated with antipyretics.

In vitro investigations showed that ribavirin, polyclonal antibodies or their combination may inhibit the replication of hMPV (Wyde et al., 2003). Because of

substantial limitations of these medications such as severe side effects, difficult administration, and high cost, they should be considered only for therapy of immunosuppressed patients with severe hMPV infection (Principi et al., 2006).

Infants showing bronchiolitis must be hospitalized and monitored due to the possibility of apnea. Most severe cases showing hypoxia may require supplemental oxygen administration, mechanical ventilation, and admission to intensive care unit.

Currently, studies on the development of a specific hMPV vaccine are undertaken (Principi et al., 2006; Herfst et al., 2008). Antibodies against the F protein which is highly conserved in the two genotypes have been shown to achieve sufficient immunization in animal models (Tang et al., 2005; Skiadopoulos et al., 2006). The F protein may thus be a good substrate for inclusion in a subunit vaccine. Furthermore, inactivated viruses may be useful to boost existing immune response in immunosuppressed individuals and the elderly, while live attenuated viruses may be useful to prevent hMPV infections in young naïve children (Herfst et al., 2008). Immunization of naïve children with inactivated virus or purified protein vaccines may induce enhanced disease upon subsequent infections (Kim et al., 1969). A recent study showed that peptide immunization with hMPV cytotoxic T-lymphocyte epitopes reduces viral load and immunology in the lungs of hMPV-challenged mice, enhances the expression of Th1-type cytokines (i.e. gamma interferon and interleukin-12) in lungs and regional lymph nodes, and reduces the Th2-type (i.e. interleukin-10 and interleukin-4) cytokine levels. This might demonstrate the efficacy of an hMPV vaccination in the control of hMPV infection in a murine model for the first time (Herd et al., 2006).

2.2 Aims of the study

Currently, RT-PCR is the method of choice to detect hMPV. Recently, monoclonal antibodies have been described which can be employed for detection of hMPV in nasopharyngeal samples by using the direct IF technique.

This study was performed to investigate the performance of a new direct IF test for the detection of hMPV and to compare results with those obtained by the routinely used real-time RT-PCR.

3 Materials and Methods

3.1 Study design

This study was conducted at the Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Austria. From December 2006 to January 2007, routine nasopharyngeal samples were collected from 97 hospitalized patients (53 females, 44 males; mean age, 35.6 months; age range, 0.4 to 204 months) with symptoms compatible with RTD at the University Hospital of Graz. All samples were tested for hMPV with both the standard molecular assay which served as gold standard and a new direct IF test, the IMAGEN^{TM} hMPV (Oxoid, Cambridge, UK). Results obtained by both of the tests were compared. Parameters evaluated included discrepant results and user-friendliness.

All samples were tested additionally on infection with adenovirus, *Bordetella pertussis*, *Chlamydia pneumoniae*, influenza A, influenza B, *Legionella spp.*, *Mycobacteria spp.*, *Mycoplasma pneumoniae,* and RSV.

3.2 Samples

3.2.1 Collection of samples

Samples were collected with the Muco-Safe^{TM} Mucus Extractor (Unomedical, Birkerød, Denmark).

The nasopharyngeal samples were collected at the University Clinic of Pediatrics and Adolescent Medicine, University Hospital of Graz, and sent to the Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, immediately after collection.

3.2.2 Preanalytical handling of samples

After receipt, the suction vessel was left at room temperature for 10 minutes to allow sedimentation of nasopharyngeal cells.

For the IF test, cells were collected from the bottom of the vessel by using a sterile swab and brought onto a Teflon coated glass microscope slide included in the test package. After this, the specimens were air dried at room temperature and fixed in fresh acetone at room temperature for 10 minutes. Thereafter, the slide was stored at -20° C until tested.

For the molecular assay, the remaining sample in the vessel was tested for hMPV RNA immediately or frozen at -20°C for at maximum seven days until tested.

3.3 The IF test

The IF test detects antigens *in situ* based on the interaction between antibodies and specific antigens. Fluorescent dyes such as fluorescein can be coupled to antibodies, the conjugates can then complex with antigen and be visualized via fluorescence microscopy. The pattern of fluorescence is characteristic for each tissue antigen.

3.3.1 Principle of the test

Cells are incubated with an antibody conjugated to a fluorochrome and applied directly to a monolayer of cells or frozen tissue on a slide (**Figure 12**).

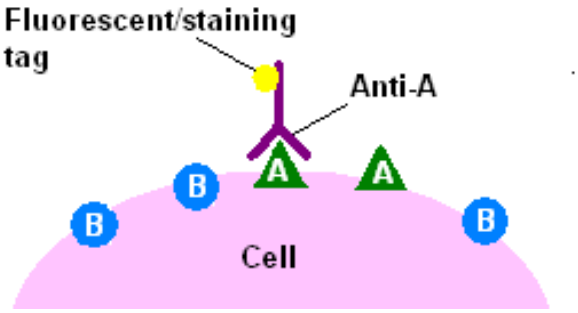

Figure 12. The direct IF test uses one labeled antibody, which binds directly to the antigen being stained for.

The IMAGEN™ hMPV is a qualitative test (**Figure 13**). It is based on a one-step direct IF technique that utilizes monoclonal antibodies conjugated to a fluorescent dye. The conjugated antibodies bind specifically to viral surface antigens, i.e. specific structural proteins present in all strains of hMPV.

Figure 13. The IMAGEN™ hMPV test.

3.3.2 Test procedure

A total of 25 µl of the IMAGEN™ hMPV reagent were added to the fixed cell preparation on the slide or to the positive control. The reagent covered the entire

well area.

For the first incubation, the samples were incubated with the reagent containing fluorescein isothiocyanate (FITC) conjugated antibodies for 15 minutes. The excess reagent was then washed off with phosphate buffered saline (PBS) and the slide was gently washed in an agitating bath containing PBS for 5 minutes. One drop of IMAGEN^{TM} hMPV mounting fluid was added to the center of each well and a cover slip was placed over the slide ensuring that no air bubbles were trapped.

For the examination of the entire well containing the stained sample, an epifluorescence microscope with a filter system for FITC (mean emission wavelength 520 nm) was used. Fluorescence was visible at 200-fold to 500-fold magnifications. For obtaining optimal results, the samples were read immediately after staining.

3.3.3 Interpretation of test results

HMPV was considered present if one or more cells showed typical fluorescence. In the case of a negative result, at least 20 respiratory epithelial cells must be seen clearly. If less than 20 cells were present, the test was considered invalid.

The FITC reagent may non-specifically stain *Staphylococcus aureus* strains because of large amounts of protein A produced by a non-immune interaction between protein A and the Fc region of the monoclonal antibody (Krech et al., 1985). However, this does not show the typical fluorescence and should thus be interpreted as non-specific staining.

A negative test result does not exclude the possibility of an hMPV infection. False negative results may be caused by factors such as collection of the samples at an inappropriate time of disease, improper sampling and handling of the specimen or failure of cell culture. The interpretation of the IF test results must be done with regard to epidemiological data and clinical presentation of the patient.

3.3.3.1 Controls

The positive control slide shows cells with apple green fluorescent intracellular cytoplasmic granules contrasting a red background of counterstained specimen (**Figure 14**). These cells are slightly larger than respiratory epithelial cells but show similar cytoplasmic fluorescence when infected with hMPV. Positive control slides were used to check that the staining procedure has been performed satisfactorily.

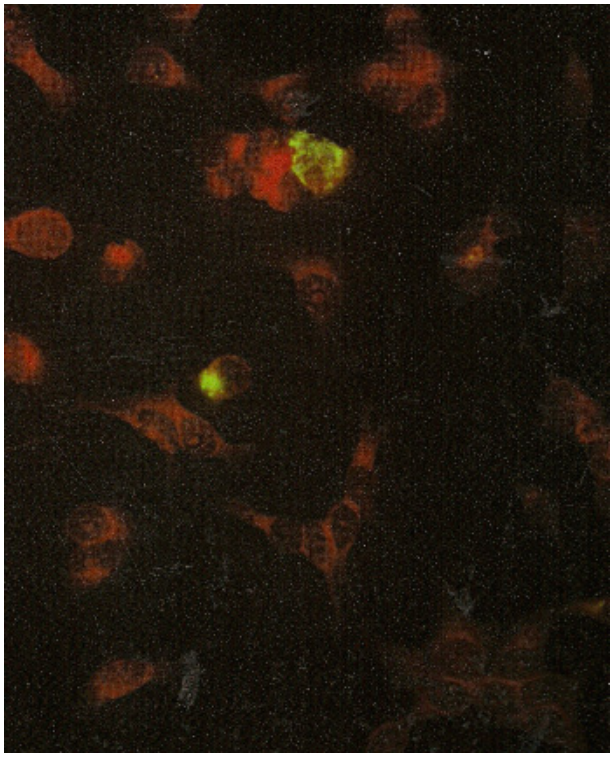

Figure 14. Positive Control

3.3.3.2 Clinical samples

HMPV infected respiratory epithelial cells show the typical apple green fluorescent intracellular cytoplasmic granules or filaments (**Figures 15, 16**). Uninfected cells appear red.

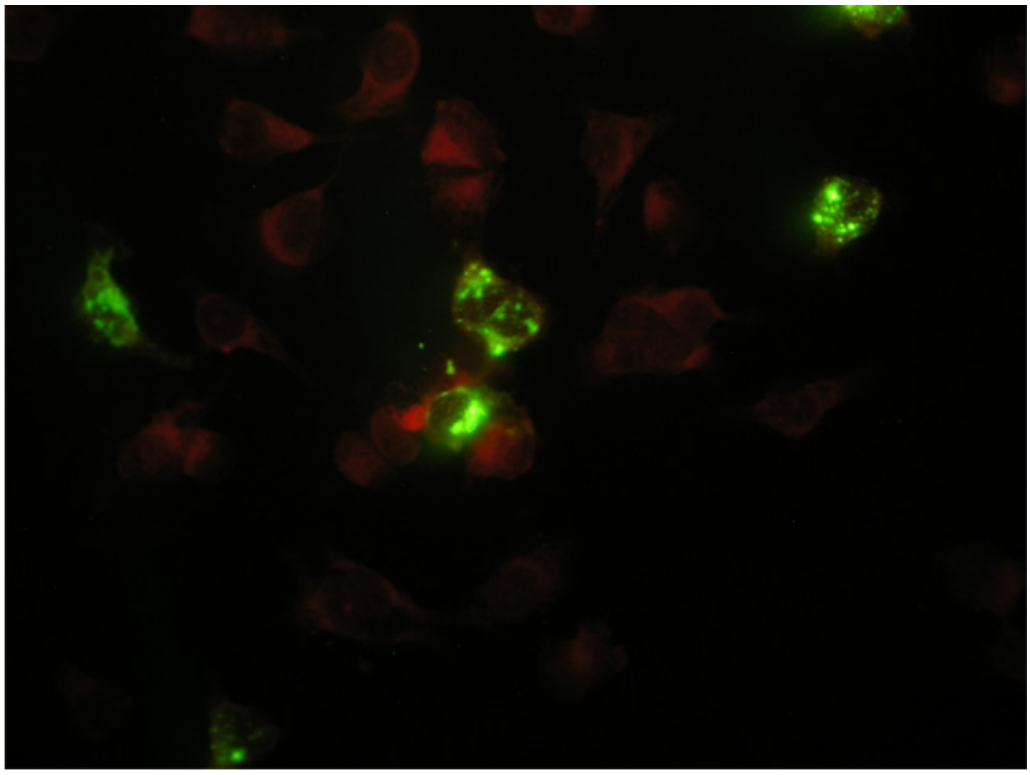

Figure 15. HMPV-positive cells show typical apple green fluorescence.

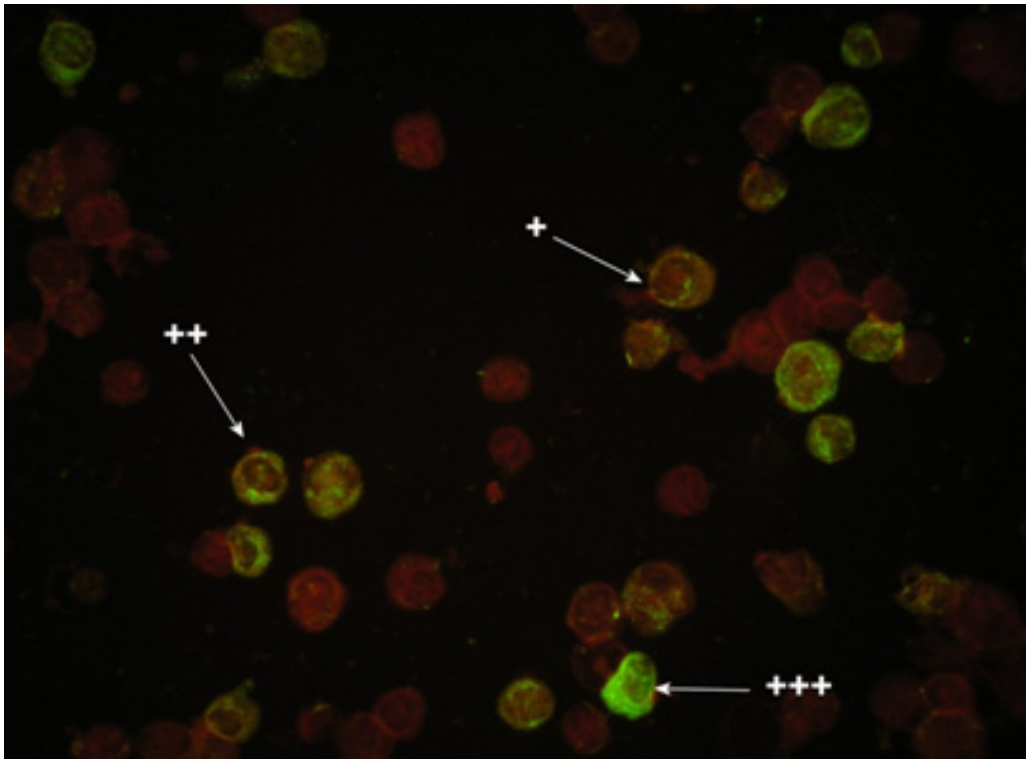

Figure 16. Positive cells are evaluated as +, ++, and +++ depending on the intensity of fluorescence.

3.4 The molecular assay

3.4.1 Principle of the test

The molecular assay for detection of hMPV consisted of three major steps. After extraction of hMPV RNA, cDNA was generated by reverse transcription. Amplification and detection of amplification products was done by means of the real-time PCR technique.

3.4.1.1 Automated RNA extraction

HMPV RNA was extracted on the NucliSens® easyMAG™ instrument (bioMérieux, Marcy l'Etoile, France) (**Figure 17**).

Figure 17. NucliSens® easyMAG™

The NucliSens® easyMAG™ allows automated extraction of nucleic acids from clinical samples based on the nucleic acid binding property of silica (**Figure 18**).

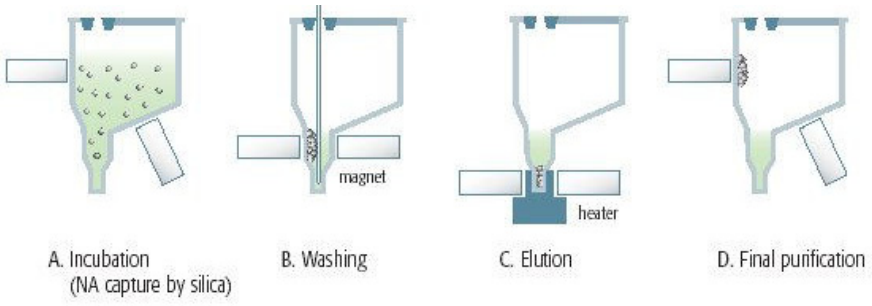

Figure 18. Extraction principle of NucliSens® easyMAG™. A: Incubation: During the incubation of the lysed samples, all the target nucleic acid is captured by magnetic silica particles. B: Washing: The NucliSens® easyMAG™ magnetic device attracts all the magnetic silica, enabling the system to purify the nucleic acid through several washing steps. C: Elution: The heating step releases the nucleic acids from the silica. D: Final purification: At the final step, the magnetic silica particles are separated from the eluate by the magnetic device.

3.4.1.2 Reverse transcription

The single-stranded RNA of hMPV was reverse transcribed into utilizing the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The kit includes a recombinant reverse transcriptase expressed in *E. coli* as the core component. The enzyme synthesizes long cDNA products up to 14 kb, is highly sensitive and highly thermostable, thus can be used for temperatures up to 65°C. The reverse transcriptase completes first-strand cDNA synthesis within 30 minutes.

3.4.1.3 Amplification and detection by real-time PCR

After reverse transcription, cDNA was amplified and amplification products detected on the LightCycler® 2.0 CE/IVD instrument (Roche, Basel, Switzerland) (**Figure 19**). The target sequence is within the N gene of hMPV. A

280 bp fragment of the N gene is amplified with specific primers. By using both the Lightmix® for detection of human Metapneumovirus (TIB MOLBIOL, Berlin, Germany) and the LightCycler®, PCR results can be obtained within one hour. According to the manufacturer, the detection limit is 10 copies of hMPV cDNA. The linear range of the assay is $1x10^1$ to $1x10^6$ of hMPV cDNA.

Figure 19. Lightcycler®.

For detection of amplification products, the hybridization probe technology was used (**Figure 20**). This detection format is based on two different fluorescence-labeled oligonucleotides. The donor probe carries a fluorescein label at its 3' end, while the acceptor probe is labeled with a different fluorescein label at the 5' end. When the fluorescein label of the donor probe is excited it emits light at a certain wavelength. Due to certain sequences both probes hybridize to the amplified DNA fragment in a head to toe manner, bringing the two probes into close proximity. As soon as the fluorescent dyes of both probes are in that close proximity, the emitted energy excites the fluorescein of the acceptor probe. This fluorescence resonance energy transfer (FRET) only takes place when both probes are hybridized to the target DNA (i.e. just after the annealing step) and results in the emission of fluorescent light at a longer wavelength. The increase of this measured signal thus is proportional to the increasing amount of the PCR product. After the annealing step, the temperature is raised and the probes are displaced by the polymerase. At the end of the elongation step, the PCR product is double stranded and the probes are too far apart to allow FRET (Kessler, 2007).

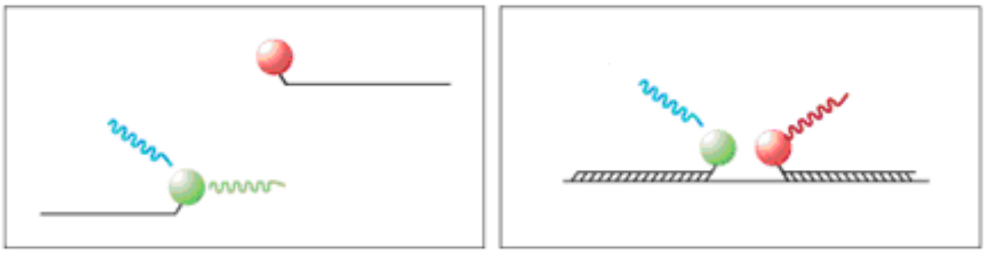

Figure 20. Binding of two single-labeled probes in a head-to-toe manner, also known as "kissing probes", or HybProbe. The FRET induced fluorescence of the acceptor dye is detected and measured.

To identify the PCR product, a melting curve analysis is done. Depending on the length and the sequence (i.e. mainly GC content) double-stranded DNA separates at a specific temperature. After completing the amplification of the target DNA, the temperature is steadily increased while monitoring the fluorescence. Fluorescence decreases as the temperature increases. At a certain temperature, fluorescence abruptly decreases caused by the melting of the product. The melting temperature, however, is defined as the temperature at which half of the DNA is single stranded and represents the steepest decrease of fluorescent signal (Kessler, 2007).

3.4.2 Test procedure

For the nucleic acid extraction performed on the NucliSens® easyMAG™, the sample data including sample matrix, sample size, and elution volume were programmed. After this, 500 µl of each sample were loaded into the 8-well sample vessel and the run was started (**Figure 21**). The lysis reagent was automatically added by the NucliSens® easyMAG™ followed by an incubation for 10 minutes and by addition of the magnetic silica particles. After completion of the different automated steps, the extracted RNA was resolved in 50 µl of elution buffer.

Figure 21. Loading of the samples into the 8-well sample vessels.

The reverse transcription of hMPV RNA was performed using the Transcriptor First Strand cDNA Synthesis Kit on the LightCyler® 2.0 CE/IVD instrument. Therefore, all reagents were centrifuged briefly before used. In the next step, the template-primer mixture was prepared in a sterile, nuclease-free, thin walled PCR tube on ice, by adding 10 µL of hMPV RNA, 2 µL of the random hexamer primers, and 1 µL of PCR grade water. To ensure the denaturation of RNA secondary structures, the reagents were incubated at 65°C for 10 minutes in the thermal block cycler; then the tube was immediatedly placed on ice. Subsequently, 4 µL of Transcriptor Reverse Transcriptase Reaction Buffer, 0.5 µL of Protector RNase Inhibitor 40 U/µL, 2 µL of Deoxynucleotide Mix, and 0.5 µL of Transcriptor Reverse Transcriptase 20 U/µL were added, resulting in a final volume of 20 µL in the tube. The reagents were mixed carefully and the tube was centrifuged briefly to collect the sample on the bottom. After this, the mix was first incubated at 25 °C for 10 minutes, then incubated for another 60 minutes at 50°C. In the last step the Transcriptor Reverse Transcriptase was inactivated by heating the mix to 85°C for 5 minutes and the reaction was stopped by placing the tube on ice (**Figure 22**). The cDNA could then be added to the PCR without purification.

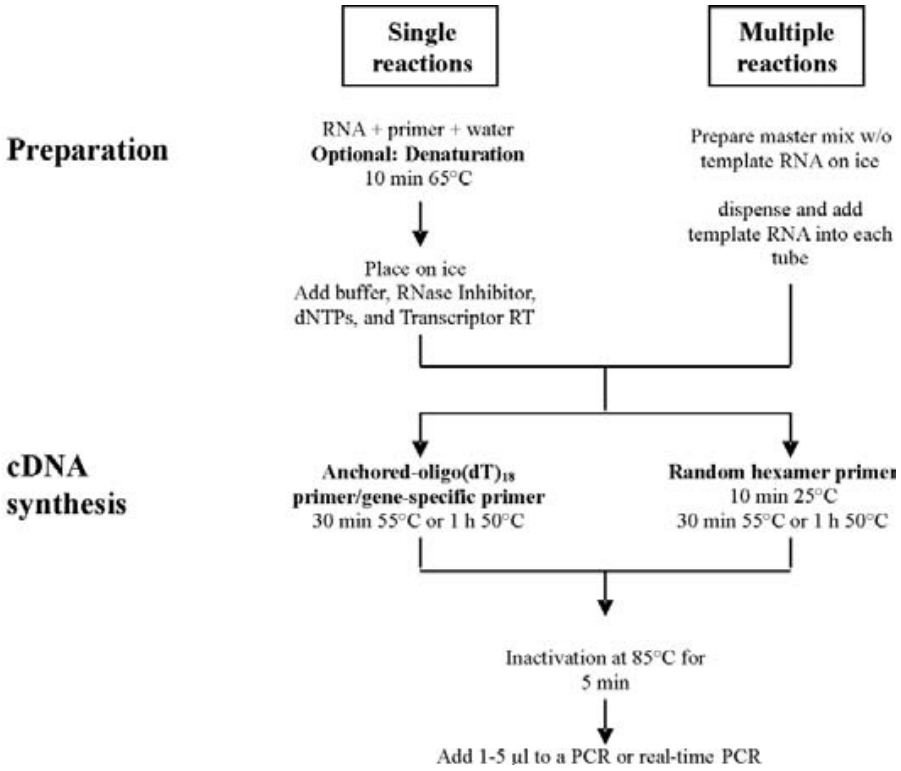

Figure 22. Overview of cDNA synthesis procedures in single and multiple reactions. Figure 22 shows the standard procedure for cDNA synthesis used for single reactions and the simplified one if multiple reactions should be performed. For the synthesis of hMPV cDNA the single reactions pathway was followed using random hexamer primers.

The amplification and detection of hMPV cDNA was performed by real-time PCR with the LightCyler® 2.0 using LightMix®. The solutions were prepared as follows: For the reagent mix, 66 µL of PCR-grade water were added to a blue clipped vial containing all primers and probes to run 16 LightCyler® reactions for hMPV. For the heterologous internal control, 66 µL were added to a white clipped vial containing all primers, probes, and DNA for 16 LightCyler® reactions. The vials were mixed and spun down. In the next step the standard row for the quantification was prepared: The target DNA was provided in 6 different concentrations from 10^1 to 10^6 target molecules per reaction. 40 µL of PCR-grade water were added to each vial of the row and the target DNA was mixed by pipetting the solution up and down ten times. For the preparation of the LightCyler® reaction the following components were added in a tube cooled below 4° C: 2.6 µL of PCR-grade water provided with FastStart kit, 2.4 µL of Mg^{2+} solution, 4.0 µL of reagent mix, 4.0 µL of the internal control mix, and 2.0 µL of Fast start mix. The components of 15µL volume were mixed, spun down, and transferred to a LightCyler® capillary. In the last step, 5 µL of cDNA or standard were added to each capillary to obtain a final

volume of 20µL.

Subsequently, the run was started. The programming of the the LightCycler® involves four steps. The first step represents the denaturation of the sample and the activation of the enzyme. In the second step the PCR-amplification of the target DNA and the detection of hMPV DNA are carried out. HMPV DNA is detected by measuring the increase of fluorescence resulting from the FRET of both the hybridization probes for the target (channel 640/530) and those for the heterologous internal control (channel 705/530). The third step involves generation of a melting curve for the identification of hMPV DNA (**Figure 23**). In the last step, the LightCycler® is cooled.

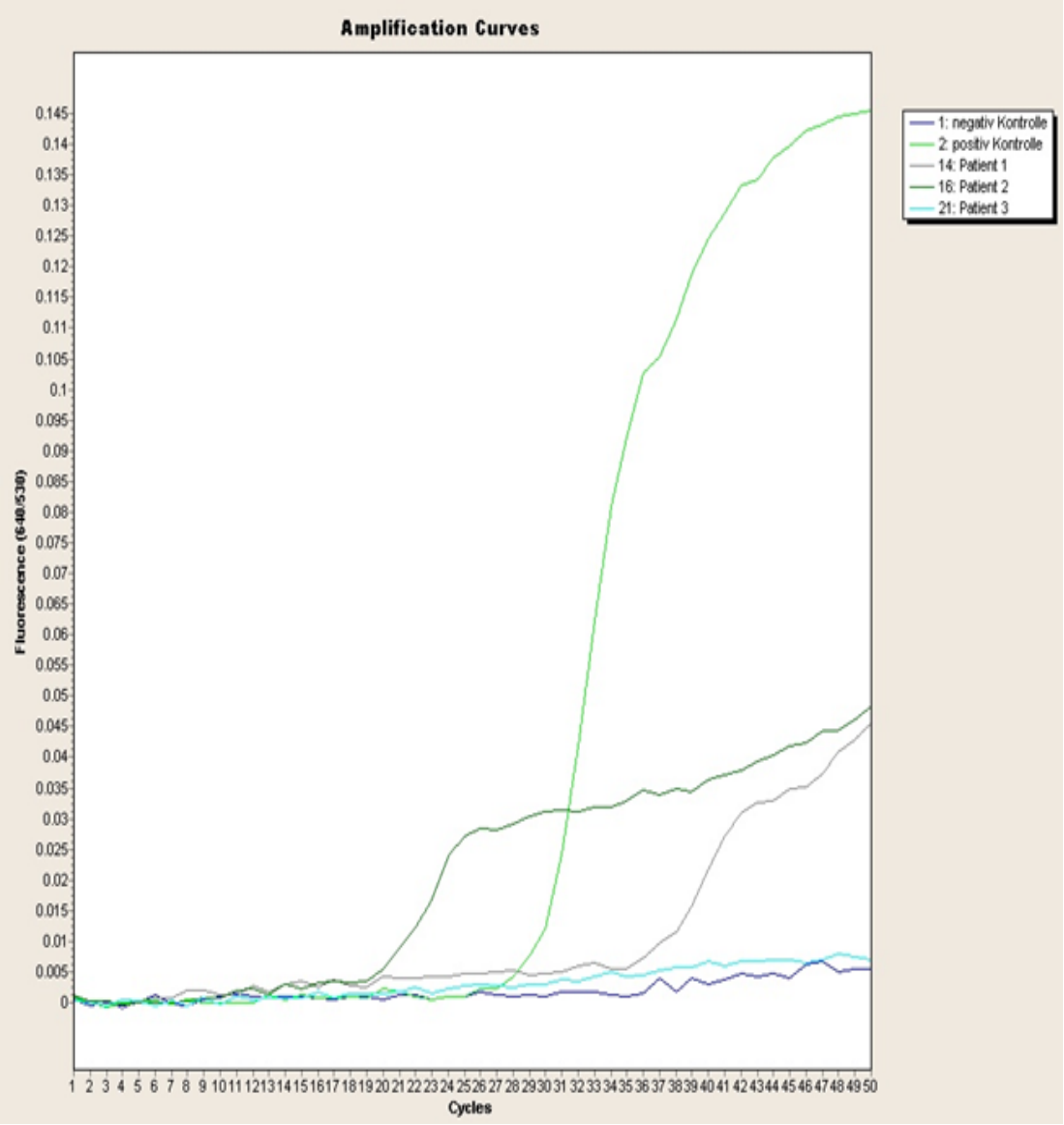

Figure 23. Melting curve analysis of clinical samples, positive, and negative controls to identify the presence of a 280 bp fragment of the N gene of hMPV.

The specific melting point of the amplified N gene with a maximum of 280 bp fragment of hMPV is 62°C. An additional PCR product of 278 bp is formed from the internal control DNA. The specific melting point of this control is about 67 to 69°C.

The amplification of the internal control DNA will usually fail in the presence of higher concentrated hMPV samples but it will display an amplification signal in negative and low concentrate samples.

3.5 Testing on other respiratory pathogens

For adenovirus, the samples were tested with a fully automated molecular assay based on automated sample preparation with the MagNA Pure Compact System (Roche Applied Sciences, Mannheim, Germany) and real-time PCR with the LightCycler® 2.0 instrument (Roche, Basel, Switzerland) as published by Koidl et al., 2005. *Legionella spp.*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* DNAs were detected by a fully automated DNA extraction protocol on the MagNA Pure LC system (Roche Applied Sciences, Mannheim, Germany) and real-time PCR on the LightCycler® instrument (Roche, Basel, Switzerland) as published by Raggam et al., 2005.

Bordetella pertussis, influenza A and B viruses, and *Mycobacteria spp*. were detected by commercial PCR-assays (RealArt Bordetella LC RT-PCR Kit, QIAGEN, Hilden, Germany; RealArt Influenza LC RT-PCR Kit, QIAGEN, Hilden, Germany; RealArt Mycobacterium diff. LC PCR Kist, QIAGEN, Hilden, Germany) according to the manufacturer's package insert. For detection of RSV, a commercially available antigen-ELISA was employed (Directigen RSV, BD, Sparks, MD).

4 Results

A total of 97 samples were tested for hMPV with both the new direct IF test and the molecular assay. Six samples (6.2%) gave an invalid result with the direct IF test because of an insufficient number of cells and were excluded from further evaluations. Corresponding real-time PCR results were found to be negative. Three (3.3%) of the remaining 91 samples were found positive for both the direct IF test and the molecular assay (**Figure 24**; **Table 5**). Eigthy-eight (96.7%) samples were found to be negative for hMPV with both tests. The heterologous internal control included in the molecular assay was consistently detected throughout the study. Out of 91 samples, 50 (55.0%) showed non-specific staining of *Staphylococcus aureus* with the IF test.

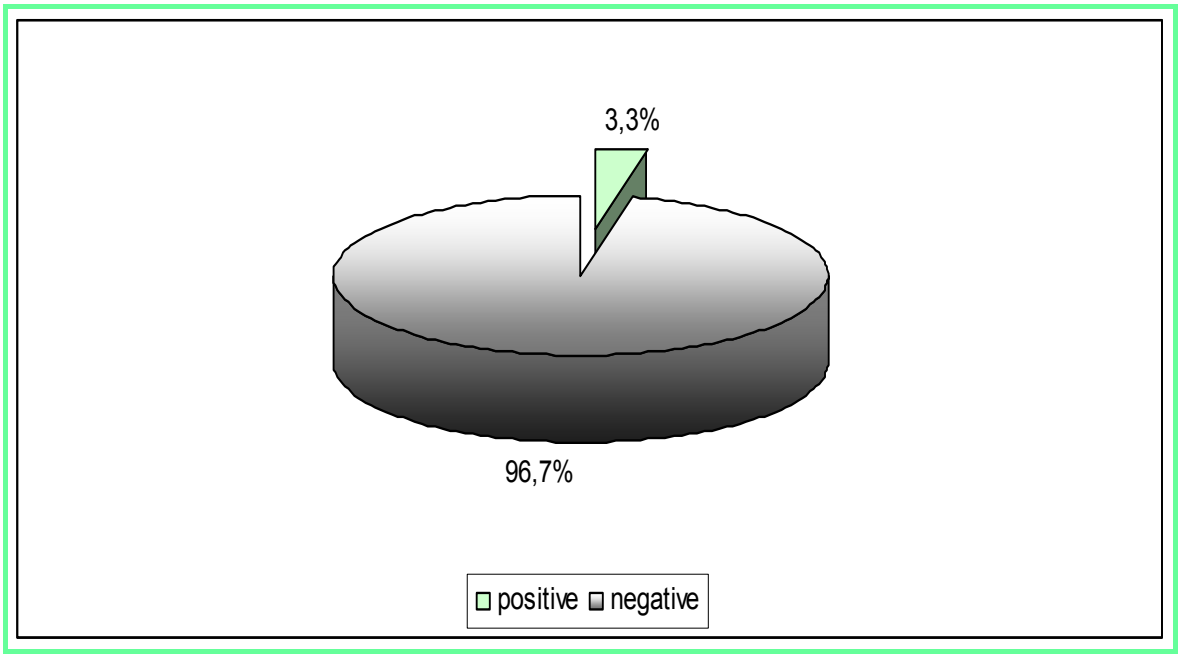

Figure 24. Results obtained from testing for hMPV with the direct IF test and the molecular assay (*n*=91).

Table 5

Comparison of results obtained

Molecular assay	IF test		Total
	No. positive	No. negative	
No. positive	3	0	3
No. negative	0	88	88
Total	3	88	91

4.1 Comparison of user friendliness

The comparison of user friendliness including parameters such as method of sample preparation, hands-on time, overall time, and limitations is shown in **Table 6**.

Table 6

Comparison of the molecular assay and the IF test regarding user friendliness

	Molecular assay	IF test
Principle of the test	RNA extraction, reverse transcription, real-time PCR	Antigen detection
Sample preparation	Automated	Manual
Hands-on time	30 min	45 min
Overall time	240 min	100 min
Limitations	Inhibition	Insufficient number of cells; non-specific staining of *Staphylococcus aureus*

4.2 Testing on other respiratory pathogens

All samples were tested additionally for other frequent etiologic agents capable of producing RTD including adenovirus, *Bordetella pertussis*, *Chlamydia pneumoniae*, influenza A, influenza B, *Legionella spp.*, *Mycobacteria spp.*, *Mycoplasma pneumoniae* and RSV. Eight samples tested positive for RSV (8.2%), another two for *Bordetella pertussis* (2.1%), one for *Chlamydia pneumoniae* (1.0%), one for hMPV/RSV co-infection (1.0%), and one for RSV/*Chlamydia pneumoniae* co-infection (1.0%) (**Figure 25**).

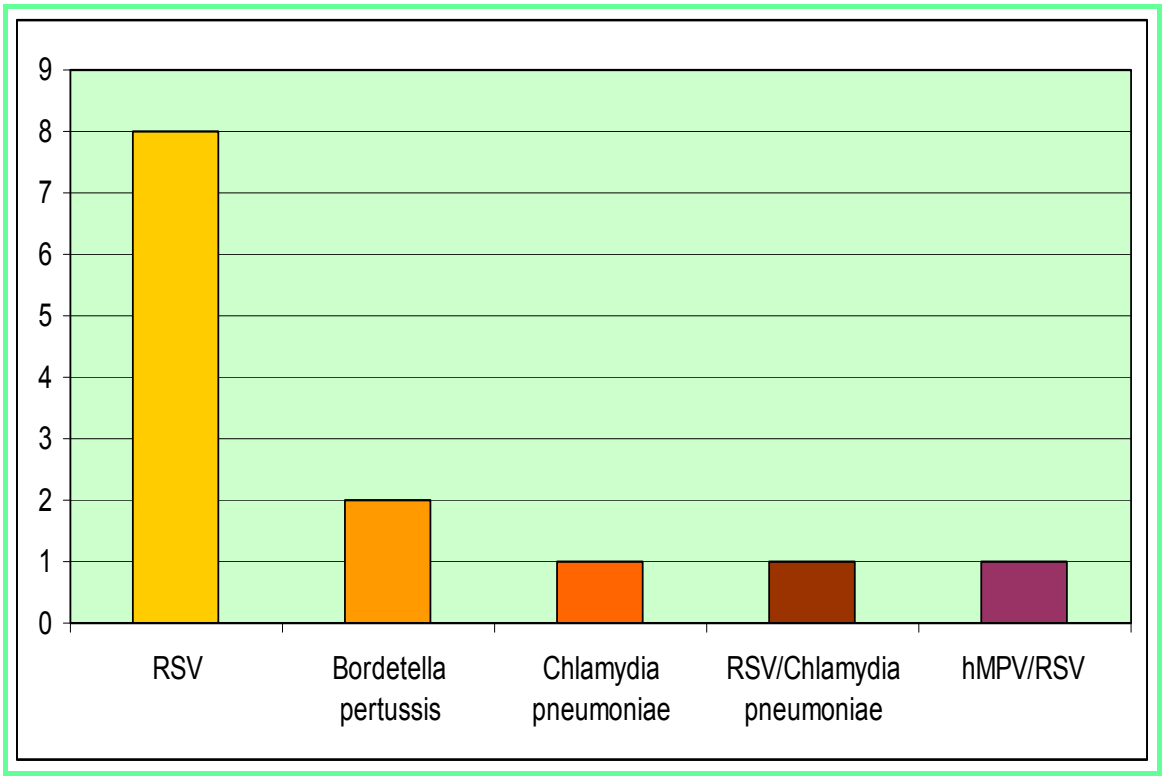

Figure 25. Other respiratory pathogens detected in this study.

5 Discussion

According to the WHO, RTDs are among the leading causes of morbidity and mortality in children worldwide. Yet, the etiology of a large number of RTDs is still unknown.

The recently discovered hMPV seems to play a significant role as an etiologic agent for RTD. Between 4 and 9% of RTDs may be related to hMPV (Boivin et al. 2003; Peiris et al., 2003; IJpma et al., 2004; Mullins et al., 2004). Infections have been reported in patients of all age groups, with infants, young children, and patients with underlying risk factors appearing to be particularly susceptible to hMPV infections (van den Hoogen et al., 2001; Crowe et al., 2004, Fenwick et al., 2007). HMPV infections show a wide spectrum of clinical symptoms ranging from mild RTDs with coryza and cough to failure of gas exchange requiring mechanical ventilation. Even fatal hMPV infections with a rapid onset and severe progress have been reported in patients with underlying risk factors (Sivaprasakam et al., 2008). Difficulties in distinguishing infections produced by hMPV from those produced by other respiratory pathogens underline the importance of a safe and rapid diagnostic method for hMPV in the clinical practice. Furthermore, as reported recently, 22-67% of patients with hMPV infections were treated blindly with antibacterial chemotherapy (Cunha, 2004; Low et al., 2004). This may result in an increase of multiresistant bacteria strains representing a substantial, yet partially avoidable global health problem. The incidence, morbidity, and socio-economic impact of hMPV infections thus justify adding hMPV to the list of common respiratory viruses routinely screened for by clinical laboratories (Chano et al., 2005).

Diagnostic methods for the detection of hMPV have relied upon molecular assays because hMPV replicates slowly in cell lines most commonly used for the detection of respiratory viruses. Molecular assays are generally more sensitive than other methods for detection of viruses, can be automated, and are suitable for high-volume testing. However, inhibition of amplification due to PCR inhibitors may be a limiting factor of molecular assays (Kessler et al., 2007). PCR inhibitors may interact directly with DNA, block the enzyme activity of DNA polymerase or interfere with co-factors of the DNA polymerase. The combination of correct

sample handling and processing techniques with extraction systems proven to provide inhibitor-free DNA or RNA are essential for avoiding inhibition. In this study, the NucliSens® easyMAG™ was used for automated RNA extraction. The automated extraction procedure results in highly purified hMPV RNA. Furthermore, a heterologous IC was included in the PCR mix to check for inhibitions. The IC was consistently detected, suggesting that there were no PCR inhibitors.

However, molecular assays are still demanding. In the conventional hospital laboratory, there is usually neither special equipment nor trained staff for performing molecular assays.

Recently, monoclonal antibodies were developed that are able to detect surface antigens of hMPV in nasopharyngeal samples and thus may provide an alternative option for the diagnosis of hMPV infection by IF testing.

In this study the performance of the new direct IF test, the IMAGEN™ hMPV, was evaluated. Results were compared with those obtained by the routinely used molecular assay based on real time RT-PCR. Ninety-seven nasopharyngeal samples were collected and tested for hMPV with both of the tests. Six samples (6.2%) gave an invalid result with the direct IF test because of an insufficient number of epithelial cells. In fact, for the new IF test, we identified two major performance limiting factors, i.e. an insufficient number of cells and unspecific staining. According to the manufacturer's package insert, a minimum of 20 epithelial cells must be visible on the slide before a negative result is reported. Therefore, the samples should be obtained during peak time of viral shedding. However, as the shedding period is not known yet, collection of the samples at an inappropriate time of disease may easily result in a too low number of cells in the sample (Hamelin et al., 2005; Semple et al., 2007). Further factors associated with failure to detect hMPV with the IF test may include improper sampling, handling, storage and/or transportation of the samples, and failure of cell culture. Therefore, to avoid invalid results and to increase the sensitivity of the IF test, it is advisable to centrifuge the collection devices. However, this was not done in this study because of the comparative design. Otherwise, it has been shown that the removal of the cellular content of nasopharyngeal samples for other purposes such as IF tests did not affect the quantification of hMPV viral load (Semple et al., 2007). This may be explained with the presence of a rather large amount of free virus in the sample.

Non-specific staining may be an additional limitation of the IF test. It is caused by an interaction of the protein A of *Staphylococcus aureus* and the Fc region of the monoclonal antibodies. In this study, non-specific staining was found in 50 samples (55.0%). However, this unspecific staining is distinguishable from the specific one because it does not show the typical intracellular fluorescence pattern seen in cells infected with hMPV.

In this study, 3 (3.3%) out of 91 valid samples tested positive for hMPV, the remaining 88 samples (96.7%) tested negative with both tests. This indicates that hMPV plays a role as pathogen for RTDs in Austria where the frequency of hMPV infections may be lower when compared to other parts of the world (Boivin et al. 2003; Peiris et al., 2003; IJpma et al., 2004; Mullins et al., 2004). However, this study was undertaken only in December and January. An increased occurrence at alternative time points cannot be excluded. This may be supported by several publications showing that hMPV infections peek between December and April and may vary in intensity and peek activity from one season to another (van den Hoogen et al., 2003; Esper et al., 2004; Williams et al., 2004; Robinson et al., 2005).

The results of this study show a 100% concordance between the new IF test and the molecular assay. According to previous studies, direct antigen tests have a sensitivity of 70-75% when compared to RT-PCR as gold standard (Ebihara et al., 2005; Barry-Murphy et al., 2006; Ingram et al., 2006). Yet, further studies with larger study populations are needed in order to obtain data regarding specificity and sensitivity of the IF test.

When the user friendliness of the two tests was compared, an extended hands-on time was found for the IF test because of the lack of automation. In fact the main IF test procedure is still manual. Furthermore, the microscopic interpretation remains subjective and can be complicated by non-specific staining and adherent mucus (Landry et al., 2008). Yet, because of the ease of the method the IF test might be preferable in the clinical routine. Furthermore, reports from immunosuppressed patients with a dramatically rapid progress of hMPV infections underline the need for rapid detection of the virus. Because the IF test requires a shorter overall time, it might be a better option in such situations.

However, the presence of hMPV in the clinical samples does not necessarily exclude co-infection with other etiologic agents of RTD. Test results thus should

always be interpreted regarding information about the seasonal diseases in the area, the clinical diagnosis, and additional diagnostic procedures. However, it is not clear yet, whether co-infections lead to more severe RTDs. In this study, all samples were tested for infections with other common etiologic agents of RTD. RSV was found to be the most common pathogen producing RTD in this study. With a frequency of 3.3%, hMPV seems to be the second commonest etiologic agent for seasonal RTD.

In conclusion the new IF test was found to be easy to handle providing reliable results. In comparison with the molecular assay, it requires a shorter overall time and may be a good option for use in the routine diagnostic laboratory.

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List of figures and tables

Abbreviations

cDNA	complementary DNA
DNA	deoxyribonucleic acids
F	fusion protein
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
G	major attachment glycoprotein
GC	guanine-cytosine
hMPV	human metapneumovirus
IF	immunofluorescence
L	major polymerase subunit
M	matrix protein
M2.1	transcription elongation factor
M2.2	RNA synthesis regulatory factor
N	nucleoprotein
NK cell	natural killer cell
P	phosphoprotein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acids
RSV	respiratory syncytial virus
RT	reverse transcription
RTD	respiratory tract disease
SH	small hydrophobic protein
Th	T helper

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12/2008 – 04/2009	Practical training at the Department of Trauma Surgery, Landeskrankenhaus Bruck a. d. Mur, Austria (10 weeks)
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Scholarship

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Extracurricular Activities

Since 2006	Member of the Austrian Medical Students' Association
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Other qualifications

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	Portuguese	basic
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Diving	Rescue Diver – Certification Participation at the Course "Emergency First Response Primary and Secondary Care", Dahab, Egypt	

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Swimming and other sports
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