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Use of a multiplex real-time PCR to study the incidence of human metapneumovirus and human respiratory syncytial virus infections during two winter seasons in a Belgian paediatric hospital

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ABSTRACT

Viruses are an important cause of acute respiratory tract infection (ARTI) in children. This study aimed to develop and evaluate a rapid molecular diagnostic test (duplex real-time PCR) for human respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV), and to determine the frequency of these two viruses as causative agents of ARTI in Belgium. Nasopharyngeal aspirates were collected over two winter and spring seasons (November 2003 to May 2004 and November 2004 to May 2005) from children aged <5 years with ARTI (n = 778). The duplex real-time PCR showed a linear range of 10^4–10^{10} copies/mL for both hMPV and hRSV. Analysis of the stability of the hRSV and hMPV genomes revealed that nasopharyngeal aspirates could be stored at room temperature for up to 1 month without significant loss of detection. hRSV was detected by antigen testing and by real-time PCR; hMPV was detected by real-time PCR only. The hRSV antigen test was less sensitive than PCR, and failed to detect one-third of the hRSV infections. Overall, 54 (6.9%) and 306 (39.3%) of the 778 samples were positive for hMPV and hRSV, respectively. Both viruses infected young infants, but the mean age of infants infected by hRSV was lower than that of infants infected by hMPV (12 months vs. 17 months, respectively).

Keywords Acute respiratory tract infection, antigen testing, children, human metapneumovirus, human respiratory syncytial virus, real-time PCR

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INTRODUCTION

Acute viral respiratory tract infections (ARTIs) are a leading cause of illness in both children and adults. Most such infections are mild and self-limiting, and are confined to the upper respiratory tract. However, in infants and children, upper respiratory tract infections may spread to the lower respiratory tract, where they can cause more severe infections and even death. The causative agents are a variety of genetically diverse viruses, including the influenza viruses, human respiratory syncytial virus (hRSV), adenoviruses, rhinoviruses and coronaviruses. It is accepted that hRSV is the most common cause of lower respiratory tract infections among children aged ≤3 years. Nevertheless, 15–35% of cases of bronchiolitis and pneumonia in paediatric patients are of unknown aetiology [1–4], suggesting that other infectious agents may exist in the respiratory tract and remain to be identified.

In 2001, van den Hoogen *et al*. [5] reported the isolation of a new respiratory virus, human metapneumovirus (hMPV), from nasopharyngeal aspirates (NPAs) obtained from children. Since its discovery, hMPV infection has been reported worldwide, accounting for *c*. 7–10% of ARTI in children [6–16]. The clinical symptoms observed most frequently in hMPV-positive children are cough, wheezing and dyspnoea [17]. Thus, based on the clinical picture of ARTI in paediatric populations, it is currently impossible to distinguish between hRSV and hMPV infection [11].

In previous years, using hRSV and influenza antigen testing only, a pathogen was identified in OLV Hospital, Aalst, Belgium for <30% of cases of ARTI in children. The high incidence of hRSV infections, the relatively high incidence of hMPV

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infections described in the literature, and the possibly severe illness associated with both viruses, emphasised the need for a reliable, sensitive and user-friendly diagnostic test for the detection of both these viruses in NPAs. Testing for the presence of both pathogens would also enable co-infections to be documented [18]. Accordingly, the present study describes the development of a real-time PCR for screening NPAs for both hRSV and hMPV. Using this assay, NPAs from paediatric patients with symptoms of ARTI were analysed in retrospective (2003–2004) and prospective (2004–2005) studies, and the effectiveness of antigen testing and the multiplex real-time PCR were compared for the detection of hRSV.

MATERIALS AND METHODS

Specimen collection and non-molecular detection assays

Between 1 November 2003 and 31 May 2004, and between 1 November 2004 and 31 May 2005, NPAs were collected from all infants (aged ≤5 years) who presented with symptoms of ARTI at the OLV Hospital (54 paediatric beds). All infants included in the study suffered from a respiratory infection and had respiratory distress. Each infant was examined by a paediatrician, and an NPA was obtained from each infant with dyspnoea and/or wheezing whose respiratory condition was sufficiently poor to require evaluation of the degree of hypoxaemia by pulse oxymetry. A decision with respect to hospitalisation or ambulatory treatment was then made on the basis of the degree of hypoxaemia and the clinical and respiratory status of each child.

All NPAs were tested for hRSV using the hRSV Respistrip (Coris Bioconcept, Gembloux, Belgium). In addition, at the request of the clinician and in line with the national surveillance data, the presence of influenza A virus antigen was investigated for a small proportion of the samples (Virion; Serion Immundiagnostica, Würzburg, Germany). The samples collected between 1 November 2003 and 31 May 2004 were stored at −70°C until batch testing. The samples collected between 1 November 2004 and 31 May 2005 were analysed on a daily basis for the presence of hRSV and hMPV RNA by the real-time PCR described in this study (see below).

RNA extraction, reverse transcription, real-time PCR and sequencing

Virus RNA was extracted from 140 μL of NPA with a QIAamp Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands), used according to the manufacturer's protocol. The resulting RNA (10 μL) was then reverse-transcribed with random hexamer primers in 20-μL volumes using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA).

hRSV and hMPV cDNA sequences were amplified and detected in a multiplex real-time PCR on the ABI Prism 7000 sequence detection system (Applied Biosystems). The hMPV primers and probe (Table 1) were designed to cover most of the known genetic variants (based on Maertzdorf *et al.* [19], adapted according to recent sequence information available in GenBank). Primers and probe targeted a part of the hMPV nucleoprotein (N) gene (163 bp). The hRSV primers and probe were designed using Primer Express software (Applied Biosystems) as part of the present study, and target the nucleoprotein (N) genes of both hRSV-A and hRSV-B (163 bp). Each 25-μL reaction contained 12.5 μL Universal Mastermix (Applied Biosystems), 5 μL cDNA, 300 nM each hRSV primer and 200 nM hRSV probe (VIC-TAMRA-labelled), and 600 nM each hMPV primer and 200 nM hMPV probe (FAM-TAMRA-labelled). PCR amplification conditions comprised 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

Amplicon sequencing for confirmation of identity was performed on a random selection of positive samples (n = 5) with a CEQ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Analis, Suarlée, Belgium). Sequencing reactions were analysed on a CEQ 8000 Capillary Sequencer (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions. The resulting sequences were aligned with each other and with sequences in GenBank (accession numbers: AY297749, hMPV; M11486, hRSV-A; and D00736, hRSV-B) with the use of BioEdit software (Ibis Therapeutics, Carlsbad, CA, USA).

Construction of positive controls

A positive control was created by ligating the hMPV and hRSV real-time amplification products into the pCRII-TOPO cloning vector (TOPO TA Cloning Kit; Invitrogen, Paisley, UK)

Table 1. Nucleotide sequences of the primers and probes used in this study

Primer or probe	Sequence[a]
hMPV[b]	
Forward primer	5′-CATATAAGCATGCTATATTAAAAGAGTCTCA-3′ (35)
Reverse primer	5′-CCTATTTCTGCAGCATATTTGTAATCAG-3′ (197)
Probe	5′-FAM-CAACHGCAGTGACACCCTCATCATTGCA-TAMRA-3′ (95)
hRSV[c]	
Forward primer	5′-GCTCTTAGCAAAGTCAAGTTRAATGATACA-3′ (15)
Reverse primer	5′-GTTTTTGCACATCATAATTRGGAGT-3′ (138)
Probe	5′-VIC-CTGTCATCYAGCAAATACACTATCCAACGTAGCACAGG-TAMRA-3′ (63)

hMPV, human metapneumovirus; hRSV, human respiratory syncytial virus.
H, mix of T, C and A; R, mix of A and G; Y, mix of C and T.
[a]The location of the primers and probes is indicated in parentheses (the reference sequence for hMPV is AY145286, and that for hRSV is X00001).
[b]Maertzdorf *et al.* [19], adapted according to the recent sequence information available in GenBank.
[c]Designed as part of the present study.

according to the manufacturer's instructions. The identity of the inserts was confirmed by cycle sequencing with the CEQ DTCS Quick Start Kit on the CEQ 8000 Capillary Sequencer.

RESULTS

Optimisation of real-time PCR

The concentrations of probe and primers in the real-time PCR for hRSV and hMPV were initially optimised in a single real-time PCR, and were then combined in a multiplex PCR and further optimised using ten-fold dilution series (covering a range of six $\log_{10}$ dilutions) of the hMPV and hRSV constructs (Table 2). Sensitivities and efficiencies of the two formats were comparable, since the cut-off positive cycle threshold (Ct) values of the multiplex assay were within 1.2 cycles of those of the single assays. Analysis of hMPV- or hRSV-positive NPAs showed no cross-reaction between the hMPV and the hRSV PCR.

The specificity of the multiplex real-time PCR was determined in two stages. First, an analysis of all primers and probes by BLAST in GenBank revealed no homology with sequences derived from other organisms. Second, samples positive for influenza virus A or B, adenovirus, parainfluenza viruses 1, 2 or 3, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, parvovirus B19, *Bordetella* or *Moraxella catarrhalis* were all negative when analysed with the hMPV/hRSV multiplex PCR. Sequencing of amplicons derived from five hRSV antigen-negative but PCR-positive samples revealed that the amplified fragments contained hRSV sequences.

Table 2. Cut-off positive cycle threshold (Ct) values obtained for the analysis of the human respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV) constructs in single and multiplex real-time PCRs

	Ct single PCR	Ct multiplex PCR	Delta Ct[a]
Concentration hRSV			
10^{10} copies/mL	16.29	16.39	0.10
10^{9} copies/mL	20.74	21.22	0.48
10^{8} copies/mL	25.68	24.49	−1.19
10^{7} copies/mL	27.62	27.86	0.24
10^{6} copies/mL	31.25	31.61	0.36
10^{5} copies/mL	34.52	35.45	0.93
10^{4} copies/mL	39.85	40.20	0.35
R^2	0.9958	0.9968	
Efficiency (%)	84.20	82.60	
Concentration hMPV			
10^{10} copies/mL	16.02	16.56	0.54
10^{9} copies/mL	18.98	19.82	0.84
10^{8} copies/mL	22.46	22.39	−0.07
10^{7} copies/mL	25.87	26.28	0.41
10^{6} copies/mL	29.57	30.02	0.45
10^{5} copies/mL	32.88	33.00	0.12
10^{4} copies/mL	35.83	35.90	0.07
R^2	0.9992	0.9978	
Efficiency (%)	98.00	101.50	

[a]Delta Ct = Ct multiplex PCR minus Ct single PCR.

Stability of hMPV and hRSV viral RNA in NPAs

Knowledge concerning the stability of virus RNA in NPAs is important in order to determine the time-frame for analysis and the transport conditions of the aspirates. Therefore, positive aspirates ($n = 5$) were kept at room temperature for 1 month and RNA was extracted at different time-points. The RNA was converted to cDNA and analysed by multiplex real-time PCR. The samples could be stored for up to 1 month at room temperature without degradation of the virus RNA (data not shown).

Comparison of hRSV antigen testing and hRSV real-time PCR

All the NPAs collected during the two winter and spring seasons were analysed with the hRSV Respistrip. Of 778 NPAs analysed, 200 (25.7%) were positive. Real-time PCR indicated the presence of the hRSV genome in all 200 of these samples, together with an additional 106 (13.6%) samples that were negative according to the hRSV Respistrip.

Testing NPAs for hRSV and hMPV by real-time PCR

Overall, 54 (6.9%) and 306 (39.3%) of 778 samples were positive for hMPV and hRSV, respectively. Whereas hRSV infection peaked in December, hMPV infections were observed throughout the entire study period (Table 3). The median age of the hRSV-infected children was 8 months (range 1–58, mean 12 months), with the majority (66.6%; 204/306) of the hRSV-positive children being aged <1 year (Table 4; Fig. 1). The hMPV-infected children had an overall median age of 8 months (range 1–54 months, mean 17 months) (Table 4). Upper respiratory tract infections with hMPV occurred predominantly during the first 2 years of life; 72.2% (39/54) of the hMPV-infected children were aged <2 years, and 53.7% (29/54) were aged <1 year (Fig. 1). No significant

Table 3. Detection of human metapneumovirus (hMPV) and human respiratory syncytial virus (hRSV) in nasopharyngeal aspirates collected between November 2003 and May 2004 and between November 2004 and May 2005

Period	No. samples tested by hRSV/hMPV PCR (aged <60 months)	No. hRSV-Ag positive	No. hRSV-PCR positive (%)	No. hMPV positive (%)
2003–2004				
November	29	3	10 (34.5)[a]	2 (6.9)
December	122	47	76 (62.3)	3 (2.5)[b]
January	45	22	25 (55.6)	4 (8.9)
February	27	9	13 (48.1)	6 (22.2)
March	26	3	3 (11.5)	4 (15.4)
April	13	0	0 (0)	3 (23.1)
May	17	0	0 (0)	0 (0)
Subtotal	279	84	127 (45.5)	22 (7.8)
2004–2005				
November	45	10	18 (40.0)	0 (0)
December	135	60	93 (68.9)	6 (4.4)
January	82	28	37 (45.1)	4 (4.9)
February	88	11	22 (25.0)	3 (3.4)
March	62	2	3 (4.8)	7 (11.3)
April	43	2	2 (4.9)	5 (11.6)
May	44	3	4 (9.1)	7 (15.9)
Subtotal	499	116	179 (35.9)	32 (6.4)
Total	778	200	306 (39.2)	54 (6.9)

Ag, antigen.
[a]Two individuals had two hRSV-positive specimens obtained <5 days apart; these were considered to be a single infection.
[b]One individual had two hMPV-positive specimens obtained <4 days apart; these were considered to be one infection.

Table 4. Comparison of the age and gender of children infected with human metapneumovirus (hMPV) and human respiratory syncytial virus (hRSV)

		hMPV-positive			hRSV-positive		
	All	All	2003–2004	2004–2005	All	2003–2004	2004–2005
No. samples	780	54	22	32	306	127	179
Male	399	23	9	14	147	66	81
Female	381	31	13	18	159	61	98
Age (months)							
Mean	15	17	16	17	12	11	13
Median	10	8	7	9	8	7	8
Range	1–59	1–54	1–47	1–54	1–58	1–58	1–55

differences in age, gender, frequencies or time of infection were observed between the two study periods (2003–2004 and 2004–2005). Paediatricians affirmed that, clinically, no aetiological distinction between hRSV- and hMPV-infected infants was possible.

DISCUSSION

Virus infections are an important cause of ARTI in children. The aims of the present study were to develop and evaluate a molecular test for hRSV and hMPV, and to determine the frequency of these two viruses as causative agents of ARTI. A multiplex real-time PCR using the ABI Prism 7000 system was preferred, since this approach was already incorporated in the routine laboratory as a diagnostic tool for other infectious agents, and multiplexing reduces workloads.

The optimised duplex real-time PCR had a linear range of 10^4–10^{10} copies of hMPV or hRSV/mL. The efficiency of detection was high and was comparable with that of single-locus PCRs. The apparent stability of the hRSV and hMPV genomes in NPAs indicated that samples could be stored at room temperature for at least 1 month without significant loss of detection. This eliminates the need for special storage and transport conditions, and eases the incorporation of the test into the routine laboratory workflow.

In the present study, 778 NPAs were collected during two winter and spring seasons. The samples collected between November 2004 and May 2005 were analysed prospectively on a daily basis, and results were communicated to the paediatricians, generally within 24 h of sample collection. Overall, 54 (6.9%) and 306 (39.3%) of the samples were positive for hMPV and hRSV, respectively. These data are consistent with previous reports on hMPV-positivity obtained using molecular detection methods for hospitalised children with ARTI. In general, incidences of 5–10% have been found in this specific patient population [7,10–13,15,20–24].

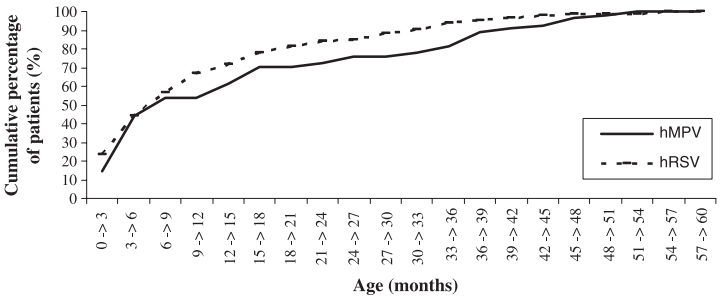

Fig. 1. Cumulative age distribution of patients with human metapneumovirus (hMPV) or human respiratory syncytial virus (hRSV) infection.

Overall, reports of the prevalence of hMPV differ markedly, ranging from 1.5% to 25%. These differences might be related to the population (age group, inpatient vs. outpatient, negativity for other viruses) and the period (winter and spring vs. year-round) studied. Previous reports have also suggested year-to-year variations in the frequency of hMPV [13,21,25]. Furthermore, the individual real-time PCR assays that have been described target a variety of regions (i.e., the N, M, L, F and P genes), implying the possibility of different performance characteristics. The primers and probe used in the present study targeted a part of the hMPV N-gene. Cote *et al*. [26] reported that PCR assays that amplify the N gene have superior specificities and sensitivities, and the primers and probe were adapted to cover all hMPV variants found in GenBank.

hMPV and hRSV circulated in the present study population throughout the study periods (except for May 2004), confirming the seasonal circulation described previously [5,7,18,27,28]. In both years, hMPV infections could be found during the entire study period, with a slight increase in frequency from February onwards, but no significant peak in hMPV infection was detected. In contrast, the frequency of hRSV infection was higher in the winter months, with significant peaks during December 2004 and December 2005 (Table 3). Boivin *et al*. [20] and McAdam *et al*. [29] reported hMPV outbreaks in shorter periods compared with hRSV. The present study did not support this observation, as the outbreaks of hRSV and hMPV were distributed equally during both periods (Table 3). Several previous studies have documented hMPV infection during the winter and spring periods [10,28–31], but most studies published previously cover only one season, which does not allow a good definition of the seasonality of hMPV. In the present study, hRSV activity peaked in both periods during December, suggesting that this seasonal distribution is an annual occurrence [7,29].

The present study analysed NPAs obtained from children aged <60 months. This selection was based on seroprevalence surveys indicating that virtually all children are infected by the age of 5–10 years [5,9,32]. ARTI with hMPV was found to occur predominantly during the first 2 years of life, with 72.2% of the hMPV-infected children being aged <2 years, and 53.7% being aged <1 year. Several reports have suggested that hospitalised children experience an hMPV infection at an older age compared to hRSV [20,22,24], and this tendency was also observed in the present study, with 66.6% (204/306) of the detected hRSV infections occurring in children aged <1 year, compared with only 53.7% (29/54) of the hMPV infections. A greater proportion of hRSV infections (23.6% vs. 14.8% hMPV infections) also occurred in very young infants (aged <3 months). The mean age for hRSV infection was 12 months, compared with 17 months for hMPV.

All samples ($n = 778$) were tested for both hMPV and hRSV in order to detect co-infections with these viruses. Greensill *et al*. [18] reported a high frequency of hMPV-positive children with severe hRSV infection, raising the possibility that hMPV might influence the severity of hRSV disease. A report from Germany, focusing on children in the intensive care unit, reported a total hMPV prevalence of 18% ($n = 15$); of these children, 60% were also hRSV-positive [33]. However, in the present study, only three of the 54 hMPV-positive samples were also positive for hRSV by real-time PCR, and one of these samples was positive for hMPV, hRSV and influenza A virus. In another patient, hMPV and hRSV were found 2 weeks apart (data not shown). The present study also found that the male:female ratio for hMPV-positive patients was 1:1.4 in 2003–2004 and 1:1.3 in 2004–2005. These data are in contrast to results from other studies, and to the fact that males are associated with an increased risk of respiratory tract disease caused by respiratory viruses [15,34].

Using the routine hRSV antigen test, a pathogen was identified in only 26% (200/778) of the individuals tested. The real-time multiplex PCR revealed a causative viral agent in an additional 160 (20.6%) specimens. hMPV was present in 9.3% (54/578) of all cases of ARTI with no other virological diagnosis. It was concluded that hRSV and hMPV are both significant causes of ARTI in children aged <5 years. These viruses co-circulate at high frequencies in winter and spring, and rapid and sensitive detection is required in order to give optimal care to patients. The fact that the real-time PCR assay is relatively rapid and is more sensitive than the hRSV antigen test makes it a valuable tool for the clinician. Further studies are needed to reveal the impact of rapid diagnosis of viral infections on clinical decision-making and antibiotic prescription patterns.

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