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Changing circulation rate of human metapneumovirus strains and types among hospitalized pediatric patients during three consecutive winter-spring seasons

Brief Report

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Summary. From 2001 through 2004, 808 pediatric patients admitted to hospital because of acute respiratory infections were examined for presence of respiratory viruses by either direct fluorescent staining using monoclonal antibodies or RT-PCR during three consecutive winter-spring seasons. On the whole, 336 (42%) patients were detected as positive for one or more respiratory viruses. The most widely circulating virus was human respiratory syncytial virus (hRSV) infecting 50% of positive patients, followed by human metapneumovirus (hMPV) found in 13% of patients, and then by influenza virus type A, human parainfluenzaviruses and coinfections. Significant variations in the circulation rate of hRSV, hMPV and influenzavirus type A were observed during the individual seasons. In addition, the circulation rates of the different types of hMPV changed yearly. In 2001–2002 and 2002–2003 hMPV circulated at a significant lower proportion than hRSV, while in 2003–2004 the circulation rates of the two viruses were closer. In conclusion, the 4 hMPV subtypes circulated yearly in Northern Italy flanking hRSV as major respiratory pathogens in the infantile patient population.

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Human metapneumovirus (hMPV), the most recently identified human respiratory viral pathogen [17] along with the new human coronaviruses (hCoVs) Sars-CoV, NL63 and New Haven virus [6, 21], is the etiologic agent of upper and lower respiratory tract infections in both the immunocompetent and the immunocompromised hosts [1, 4, 5, 7, 11, 20, 22]. In particular, infants and

young children are among patients most severely affected by hMPV infections [2, 12, 22].

However, several epidemiological features of hMPV infections remain to be investigated. In particular, differences in the circulation rate of hMPV strains as well as different hMPV types and subtypes during different seasons, and the relative pathogenicity of hMPV with respect to human respiratory synctial virus (hRSV) remain to be defined.

In this study, we examined: i) the circulation rate of hMPV among the other respiratory viruses during 3 consecutive winter-spring seasons; ii) the relative circulation of the 2 types and the 4 subtypes of hMPV during the same 3-year period; iii) the relative impact of hMPV as compared to hRSV in determining admission to the hospital of infants with acute respiratory infections.

From December 2001 through November 2004, 808 infants and young children were admitted to hospital because of an acute respiratory virus infection. All patients had fever, cough, and lower respiratory tract involvement. Nasopharyngeal aspirates (NPA) were collected upon admission and then divided into 4 aliquots: one was used for molecular assays (RT-PCR), the second for direct fluorescent antibody (DFA) staining of respiratory cells, the third for short-term and long-term virus isolation in cell cultures, and the fourth was frozen as a back-up sample [13]. Specimens were examined for influenzaviruses A and B, parainfluenzaviruses (hPIV) 1 to 3, hRSV, and human adenoviruses (hAdV) by DFA and virus isolation in cell cultures. In addition, hCoVs, group I (229E-like) and II (OC43-like), and hMPV types A and B were sought by RT-PCR. Typing of hMPV strains was achieved by sequencing and phylogenetic analysis. Similarly, grouping of hCoVs was achieved by sequencing. The study was approved by the IRCCS Policlinico San Matteo Ethics Committee. The simultaneous detection of 2 respiratory viruses in the same NPA was referred to as coinfection.

Rapid virus recovery was achieved in cell cultures by inoculating NPA samples onto 2 shell vials of a mixture of A549 and Mv1Lu (1:1) cells [9], and 2 shell vials of both LLC-MK2 and MDCK cells, which were stained with MAbs at 2 and, if required, 7 days p.i. Conventional virus recovery was obtained following inoculation of 2 tubes of LLC-MK2 and MDCK cells, which were stained with MAbs 7 days p.i. or, if required, during the following 2 weeks.

A pool of fluorescein-labeled MAbs to conventional respiratory viruses (influenzavirus A and B, hPIV 1–3, hRSV, and hAdV), as well as the single fluorescein-conjugated MAbs included in the pool (purchased from Chemicon International, Inc., Temecula, CA) were used for both DFA and virus identification in cell cultures.

Following RT reaction, PCR assays for the identification of hCoVs and hMPV strains were optimised to detect at least 10 input plasmid copies. To this purpose, primers for hCoV (groups I and II) were selected from a published protocol [12a], whereas primers for hMPV types A and B (genes N and F) were originally designed from GenBank published virus sequences (Table 1). Amplification products were cloned in PCR2.1 plasmid vector (TA Cloning Kit, Invitrogen, Carlsbad, CA) and quantitative standards were obtained following titration

Table 1. PCR parameters used for detection of hMPV in NPA samples

Virus	Target gene (nt–nt)	Amplicon size	Thermal profile	Cycle no.	Primer pair
hMPV, subtypes A and B	F (574–1103)	529 bp	94 °C/1′ 60 °C/1′ 72 °C/1′	5	Forw: 5′-agcttcagtcaattcaacagaag-3′
			94 °C/1′ 50 °C/1′ 72 °C/1′	50	Rev: 5′-tgtcttcctgtgctractttgc-3′
hMPV, subtypes A and B	N (1–726)	726 bp	94 °C/1′ 60 °C/1′ 72 °C/1′	5	Forw: 5′-atgtctcttcaagggattcacc-3′
			94 °C/1′ 50 °C/1′ 72 °C/1′	50	Rev: 5′-actttctgctttgctmcctg-3′

of insert-containing plasmids [13]. Thus, defined amounts of target sequences could be amplified in parallel with clinical samples. Nucleic acids were extracted using Nuclisens® Iso Kit (BioMerieux, Lyon, France). RT and PCR reactions were performed as reported [13]. PCR products were examined on 3% agarose gel.

Classification of hMPV strains of this study into types A and B, and subtypes A1–A2, and B1–B2, was achieved by phylogenetic analysis of 2 fragments of both genes F and N of hMPV strains, respectively. Viral sequences of the amplified gene F (nt 661 to1094), and gene N (nt 121 to 562) fragments of hMPV isolates as well as reference strains were aligned with the Clustal W program version 1.7, whereas sequence similarity comparisons were carried out with the Megalign program (DNAstar Inc., Madison, WI). The Philips (njplot) program was used to construct phylogenetic trees with nucleotide sequences by means of the neighbour-joining method from the same distance matrices. Bootstrap support was determined by 100 resamplings of the sequences.

Statistical differences in the circulation rate of respiratory virus infections among the 3 consecutive seasons were determined by the chi square test.

The relative distribution of different respiratory viruses causing severe infections requiring admission to the hospital of infants and young children in three consecutive winter-spring seasons from 2001 through 2004 is reported in Table 2. In all three consecutive seasons the proportions of patients with diagnosed respiratory viral infections ranged from 37.6% (n = 98/261) in 2001–2002, and 41.9% (n = 101/241) in 2002–2003 to 50.0% (n = 153/306) in 2003–2004 (P = 0.01). In the same period, the number of viral coinfections (Table 3) was 11 (11.2%) in 2001–2002 (including 9 hRSV and 1 hMPV coinfections), 7 (6.9%) in 2002–2003 (including 4 hRSV and 3 hMPV coinfections), and 15 (9.8%) in 2003–2004 (including 5 hRSV and 9 hMPV coinfections).

Within the aliquot of patients found positive for some respiratory virus, no difference in the circulation rate was observed for respiratory virus infections caused by influenzavirus B, hPIVs, hAdVs, hCoVs, and coinfections along the three years studied. On the contrary, significant differences in the circulation rate

Table 2. Incidence of patients admitted to the hospital and positive for respiratory viruses in 3 consecutive winter-spring seasons

Respiratory virus[a]	Seasonal incidence: no. pts (%)				
	2001–2002	2002–2003	2003–2004	Total	P[b]
Flu A	1 (1.0)	13 (12.9)	21 (13.7)	35 (9.9)	0.002
Flu B	4 (4.1)	1 (1.0)	2 (1.3)	7 (2.0)	ns
hPIV	11 (11.2)	9 (8.9)	11 (7.2)	31 (8.8)	ns
hRSV	54 (55.1)	59 (58.4)	57 (37.3)	170 (48.3)	0.006
hAdV	2 (2.1)	2 (2.0)	8 (5.2)	12 (3.4)	ns
hCoV	4 (4.1)	2 (2.0)	8 (5.2)	14 (4.0)	ns
hMPV	11 (11.2)	8 (7.9)	31 (20.3)	50 (14.2)	0.014
Coinfections	11 (11.2)	7 (6.9)	15 (9.8)	33 (9.4)	ns
Total pos pts	98 (37.6)	101 (41.9)	153 (50.0)	352 (43.6)	ns
Total neg pts	163 (62.4)	140 (58.1)	153 (50.0)	456 (56.4)	ns
Total pts	261	241	306	808	ns

[a]Flu, influenzavirus; hPIV, human parainfluenzavirus; hRSV, human respiratory syncytial virus; hAdV, human adenovirus; hCoV, human coronavirus; hMPV, human metapneumovirus; neg pts, patients negative for respiratory viruses; pos pts, patients positive for respiratory viruses

[b]Chi square test; ns = not significant (>0.05)

Table 3. Coinfection (dual infection) rate in the 3 consecutive seasons

Coinfection[a]	No. patients with coinfection			Total no. (%) (n = 33)
	2001–2002 (n = 11)	2002–2003 (n = 7)	2003–2004 (n = 15)	
hRSV + hPIV	4	1	0	5 (15.2)
hRSV + FluA	2	0	2	4 (12.1)
hRSV + FluB	1	0	0	1 (3.0)
hRSV + hAdV	1	1	1	3 (9.2)
hRSV + hCoV	1	2	1	4 (12.1)
hMPV + hPIV	1	0	0	1 (3.0)
hMPV + FluA	0	1	2	3 (9.2)
hMPV + hAdV	0	1	3	4 (12.1)
hMPV + hCoV	0	1	3	4 (12.1)
hMPV + hRSV	0	0	1	1 (3.0)
FluA + FluB	1	0	0	1 (3.0)
FluA + hAdV	0	0	1	1 (3.0)
FluB + hCoV	0	0	1	1 (3.0)

[a]See legend of Table 2

were found for influenzavirus A, hRSV, and hMPV (Table 2). However, while hRSV and hMPV infections predominated during the first 3 months of age, the other infections were evenly distributed during the first 5 years of age.

In more detail, influenzavirus A-infected patients increased significantly in 2002–2003 and 2003–2004 with respect to 2001–2002 ($P = 0.002$). On the other hand, hRSV, although remaining the major etiologic agent of respiratory infections among infants and young children along the entire study period, dropped significantly ($P = 0.006$) in 2003–2004 with respect to the two preceding seasons. Finally, the circulation rate of hMPV increased significantly ($P = 0.014$) in 2003–2004 compared to the two preceding seasons (Table 2).

Surprisingly, in the group of 33 coinfections, simultaneous infection of the same patient by hMPV and hRSV was observed in a single case in 2003–2004 (Table 3).

The circulation rate of hMPV reached its peak in 2003–2004. In detail, the aliquot of patients with single hMPV infection was 11.2% ($n = 11$) in 2001–2002, 7.9% ($n = 8$) in 2002–2003, reaching 20.3% ($n = 31$) in 2003–2004 (Fig. 1). As shown in Table 3, if patients with coinfections by hMPV and some other respiratory virus were included, the aliquot rose ($P = 0.0018$) from 11.2% to 12.2% in 2001–2002 ($n = 12$), from 7.9% to 9.9% in 2002–2003 ($n = 11$), and from 20.3% to 26.1% in 2003–2004 ($n = 40$).

Different types and subtypes of hMPV were identified by sequencing and phylogenetic analysis of the indicated fragments of genes F and N (Figs. 2,

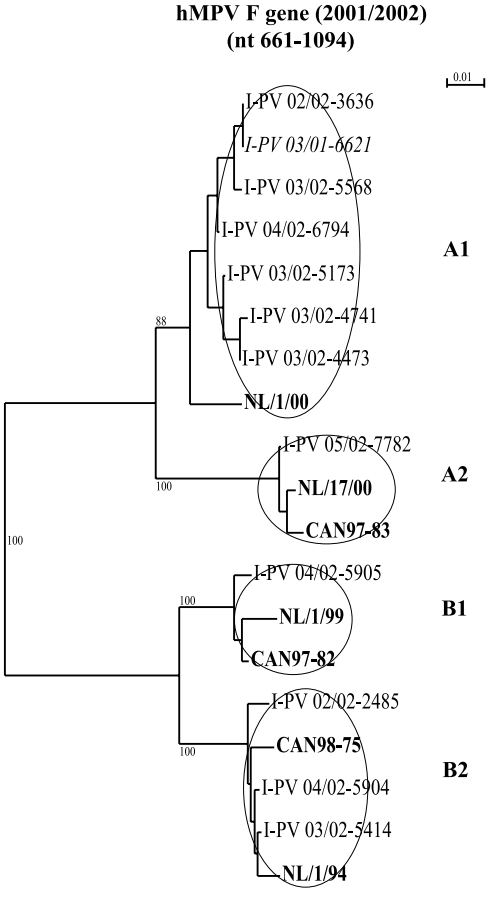

Fig. 1. Phylogenetic analysis of 12 hMPV strains circulating in the winter-spring season 2001–2002 based on nucleotide sequence of a portion of gene F (nt 661 to 1094). Reference strains are in bold. Local reference A1 strain is in italics

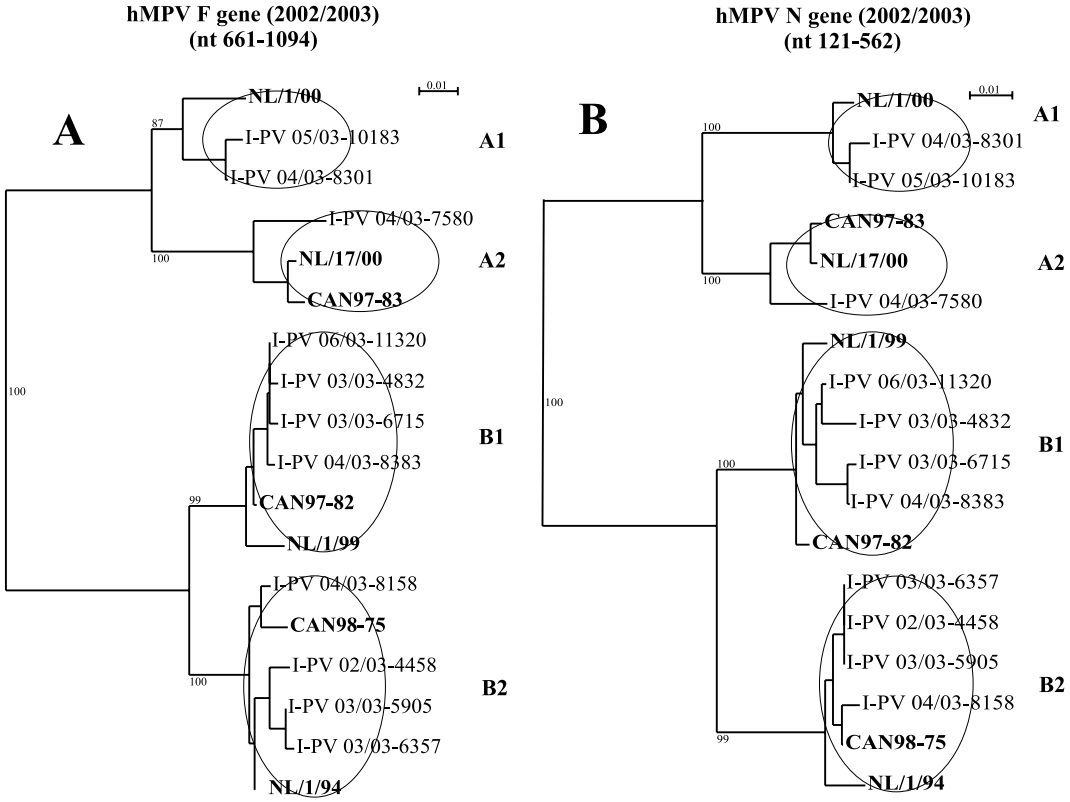

Fig. 2. Phylogenetic analysis of 11 hMPV strains circulating in the winter-spring season 2002–2003 based on nucleotide sequence of a fragment of gene F (nt 661 to 1094) and gene N (nt 121 to 562). Reference strains are in bold

3A and B, and 4). Although reported only for the 2002–2003 season, results of the comparative phylogenetic analysis were consistently overlapping following sequencing of either gene (Fig. 3A and B). In addition, in each season all hMPV strains detected clustered into two distinct lineages (types A and B), while each type included two distinct subtypes (A1–A2, and B1–B2).

Although all 4 subtypes were represented during each season, the relative distribution changed significantly ($P < 0.0001$) in different years. In fact, in 2001–2002 subtype A1 was predominant (59%, 7/12 strains), while subtypes B1 and B2 were circulating at a predominant comparable proportion (36.5%, 4/11 strains each) in 2002–2003, and subtype A2 was by far predominant (72%, 26/36 strains) in 2003–2004 (Fig. 4).

Due to the high similarity of the pathology caused in newborns and infants, the circulation rates of hMPV and hRSV were analysed by comparing the relevant number of infected patients admitted to hospital in the winter-spring (December through May) and summer-fall (June through November) seasons of each year. Both viruses were nearly absent during the summer-fall seasons, whereas they were highly circulating, at a different proportion, during the winter-spring seasons. Although the incidence of hRSV infections in pediatric patients admitted to the

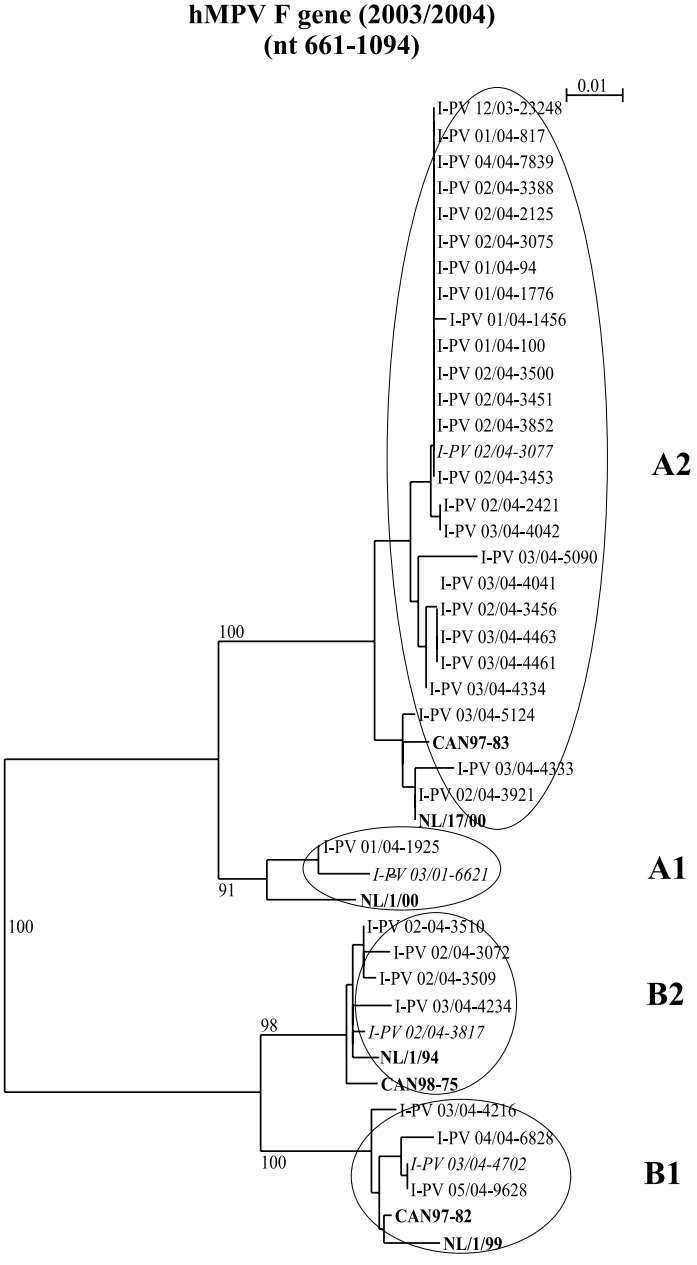

Fig. 3. Phylogenetic analysis of 36/40 hMPV strains circulating in the winter-spring season 2003–2004 based on nucleotide sequence of a fragment of gene F (nt 661 to 1094). Reference strains are in bold. Local reference strains for the 4 hMPV subtypes are in italics

hospital was consistently greater than that of hMPV, the latter increased significantly ($P = 0.0002$) in 2003–2004, so that the relative proportion of hRSV-infected patients with respect to hMPV-infected patients (including coinfections) dropped from >80% in 2001–2002 and 2002–2003 (54 *vs* 12, and 59 *vs* 11 patients, respectively) to 58.8% in 2003–2004 (57 *vs* 40 patients).

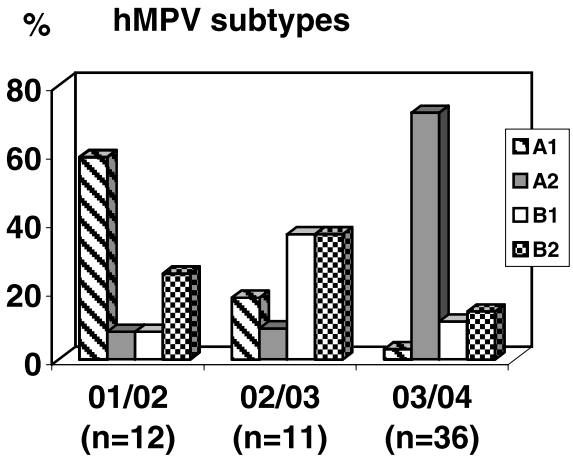

Fig. 4. Different distribution of the 4 hMPV subtypes in 3 consecutive winter-spring seasons

An epidemiological survey on the circulation rate of the most important respiratory viruses responsible for admission to the hospital of infants and young children in a defined geographical area during the 3-year period from 2001 through 2004 was conducted using NPA samples as clinical specimens. In order to contain costs, on the basis of recently acquired results showing a comparable sensitivity of the molecular (RT-PCR) and the immunological (monoclonals) diagnostic approaches [13], influenzaviruses A and B, hPIV 1–3, hRSV, and hAdVs were detected by monoclonal antibodies, while hCoVs and hMPVs were detected by RT-PCR. While no significant difference was observed in the circulation rate of the majority of respiratory viruses, a significant difference in the circulation of influenzavirus A, hRSV and hMPV, was found during the 3 consecutive winter-spring seasons. Apart from influenzaviruses A, which are known to circulate differently in different years, hMPV and hRSV circulated at a stable rate in the first 2 seasons examined. On the contrary, the relative proportion of patients infected by hRSV decreased sharply in the most recent season, while that of patients infected by hMPV increased significantly. As a consequence, the number of young patients admitted to the hospital because of lower respiratory tract infections caused by hMPV increased accordingly. Yearly variation in the circulation rate of hMPV has been recently reported [7, 8, 11, 19].

Besides a striking variation in the overall circulation rate of hMPV, a significant variation in the circulation of the 4 hMPV subtypes was observed during the study period. In fact, subtype A1 was predominant in 2001–2002, subtype A2 in 2003–2004, while the 2 subtypes B predominated in 2002–2003. This indicates that multiple strains co-circulate in the same area in different years.

Whether different hMPV types and subtypes may be differentiated only on the basis of nucleotide sequence (phylogenetic analysis) or may represent true serotypes or subserotypes remains to be determined. In fact, while ferret immune sera have been reported to identify two distinct serotypes (A and B) showing a $\geq$16fold dilution difference between homologous and heterologous neutralizing antibody titer [18], several primate as well as hamster immune sera did not show

any discriminating capacity [15]. Similarly, in our experience, as already reported by others [16], guinea pig immune sera were not able to identify two distinct serotypes. Thus, at the moment, the terms types and subtypes indicate genotypes and subgenotypes rather than serotypes and subserotypes. In a recent study by our group, using a fragment of gene N (nt 80 to 596), it was possible to identify 8 amino acid substitutions at positions 14, 31, 52, 56, 63, 66, 70, and 96, that might be used to differentiate type A from type B strains [14]. Monoclonal antibodies reactive with epitopes containing these differential amino acids might discriminate between the two hMPV types.

The simultaneous circulation of hRSV and hMPV in the same period of the year (winter-spring season) and in the same patient population (infants and young children), along with a comparable degree of pathogenicity, could hypothetically facilitate coinfections by these two members of the *Paramyxovirinae* subfamily. In a recent population-based prospective multicenter study of the children requiring intensive care conducted in Germany over 2 years, 18% had hMPV infections, and 60% of these children were infected with hMPV in combination with hRSV, whereas hMPV was not detected in a large series of hRSV-positive children not requiring intensive care support. This data support the hypothesis that coinfections with hRSV and hMPV are more severe than infections with either hRSV or hMPV alone, at least in children less than 3 years of age [10]. In our study, outside the intensive care unit, surprisingly a single case of coinfection by hMPV and hRSV was found, thus suggesting that this coinfection is very infrequent in the immunocompetent host.

With reference to the Italian pediatric population admitted to hospital during the winter-spring season, the following conclusions can be drawn from our epidemiological study: i) hMPV strains circulate at a different rate in different years; ii) changes in the prevalence of hMPV types and subtypes occur yearly; iii) the prevalence of hMPV versus hRSV infections may also change significantly in different years.

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