Journal of Medical Virology 80:716–723 (2008)

Two-Year Prospective Study of Single Infections and Co-Infections by Respiratory Syncytial Virus and Viruses Identified Recently in Infants With Acute Respiratory Disease

Filippo Canducci,[1]* Maurizia Debiaggi,[2] Michela Sampaolo,[1] Maria Chiara Marinozzi,[1] Stefano Berrè,[1] Cristina Terulla,[3] Gianluigi Gargantini,[4] Patrizia Cambieri,[5] Egidio Romero,[2,3] and Massimo Clementi[1]

[1]*Laboratorio di Microbiologia e Virologia, Università "Vita-Salute" San Raffaele, and Istituto Scientifico San Raffaele, Milan, Italy*
[2]*Dipartimento di Scienze Morfologiche, Eidologiche e Cliniche, Sezione di Microbiologia, Università di Pavia, Pavia, Italy*
[3]*Servizio Analisi Microbiologiche, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico, Policlinico San Matteo, Pavia, Italy*
[4]*Unità Operativa di Pediatria e, Azienda Ospedaliera di Melegnano, Presidio di Vizzolo Predabissi, Melegnano, Italy*
[5]*Servizio Analisi Chimico Cliniche e Microbiologiche, Azienda Ospedaliera di Melegnano, Presidio di Vizzolo Predabissi, Melegnano, Italy*

A prospective 2-year analysis including 322 infant patients with acute respiratory disease (ARD) hospitalized in a pediatric department in northern Italy was carried out to evaluate the role as respiratory pathogens or co-pathogens of recently identified viruses. The presence of respiratory syncitial virus (RSV), human Metapneumoviruses (hMPVs), human Bocaviruses (hBoVs), and human Coronaviruses (hCoVs) was assayed by molecular detection and clinical symptoms evaluated. Nasopharyngeal aspirates from 150 of the 322 infants (46.6%) tested positive for at least one pathogen. Ninety samples (28.0%) tested positive for RSV RNA (61.5% genotype A and 38.5% genotype B), 46 (14.3%) for hMPV RNA (71.7% subtype A and 28.3% subtype B), 28 (8.7%) for hCoV RNA (39.3% hCoV-OC43, 35.7% hCoV-NL63, 21.4% hCoV-HKU1, and 3.6% hCoV-229E), and 7 (2.2%) for hBoV DNA (of the 6 typed, 50% subtype 1 and 50% subtype 2); 21/150 samples revealed the presence of 2 or more viruses. Co-infection rates were higher for hMPVs, hCoVs, and hBoV (38.3%, 46.4%, and 57.1%,) and lower for RSV (23.3%). RSV was associated with the presence of complications ($P < 0.001$) and hypoxia ($P < 0.015$). When the presence of RSV alone and the RSV-hMPV co-infections were considered, RSV mono-infected patients resulted to have longer hospitalization and higher hypoxia ($P < 0.001$). The data highlight that (i) RSV has a central role as a respiratory pathogen of infants, (ii) the wide circulation of recently identified viruses does not reduce the clinical and epidemiological importance of RSV, and that (iii) recently identified agents (hMPVs, hBoVs, and hCoVs) act as primary pathogens or co-pathogens. ***J. Med. Virol. 80:716–723, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: respiratory viruses; RSV; hMPV; coronavirus; bocavirus

INTRODUCTION

Viral agents are a worldwide leading cause of respiratory diseases in infants with high morbidity and mortality. From 45% to 60% of all acute respiratory diseases (ARDs) in infants and young children are associated with infections by respiratory syncytial virus (RSV), rhinovirus, adenovirus, and parainfluenza viruses [Kuiken et al., 2003; Choi et al., 2006; Medici et al., 2006]. In addition, other viruses are presently emerging or have been identified recently as important respiratory pathogens. Firstly, a novel paramyxovirus,

*Correspondence to: Canducci Filippo, MD, PhD, Laboratorio di Microbiologia e Virologia, Università "Vita-Salute" San Raffaele, Milan, Italy. E-mail: canducci.filippo@hsr.it

Accepted 20 November 2007
DOI 10.1002/jmv.21108
Published online in Wiley InterScience (www.interscience.wiley.com)

© 2008 WILEY-LISS, INC.

WILEY InterScience® DISCOVER SOMETHING GREAT

named human metapneumovirus (hMPV), has been isolated and characterized; it is presently believed that hMPV infects children and adults, accounting for 5–10% of all ARDs [van den Hoogen et al., 2001]. Secondly, novel pathogens belonging to the family *Coronaviridae* have then been isolated in respiratory samples of patients with ARD [Pyrc et al., 2007]; even if the previously identified human coronaviruses (hCoV) OC43 and 229E were mainly associated with mild upper ARDs in adults, and only rarely with infections with severe symptoms in children [Tyrrell and Bynoe, 1965; Hamre and Procknow, 1966], particular attention was directed toward this group of viruses after the SARS-CoV epidemics. These studies led to the discovery of novel hCoV strains, namely hCoV-NL63, which was identified in a child with bronchiolitis in 2004 [van der Hoek et al., 2004], and hCoV-HKU1, that was discovered in an adult patient with chronic pulmonary disease in 2005 [Woo et al., 2005]. After identification, these pathogens were found in respiratory samples in many countries [Pyrc et al., 2007]. Thirdly, a novel human parvovirus was identified in clinical specimens from infants with ARDs and designated human bocavirus (hBoV) [Allander et al., 2005]; hBoV is being detected worldwide in samples from children with a wide range of respiratory diseases, from upper ARDs to severe bronchiolitis and pneumonia [Arden et al., 2006; Arnold et al., 2006; Ma et al., 2006; Sloots et al., 2006; Maggi et al., 2007; Pierangeli et al., 2007].

In the present study, the role of the emerging viral pathogens in infants with ARD was addressed and a 2-year prospective analysis in patients (all less than 2 years old) hospitalized in a pediatric department in northern Italy was planned. The impact of a well known culprit in pediatric respiratory diseases such as RSV was then compared to that of hMPVs, hBoVs, or hCoVs (hCoV-OC43, hCoV-229E, hCoV-NL63, and hCoV-HKU1). On all samples, molecular detection, genotyping of all viral strains by sequence analysis were performed and the associated clinical symptoms recorded.

MATERIALS AND METHODS

Patients and Clinical Records

From October 2004 to September 2006, 322 infants (177 males and 145 females) with a diagnosis of ARD and hospitalized in Milan (Italy), Azienda Ospedaliera in Melegnano were enrolled consecutively in the present study. All children were <24-month old, with a median age in the study population of 3 months. Three hundred and two patients were <1-year old, and 20 were between 1 and 2 years. Main reasons for hospitalization were bronchiolitis, pneumonia, bronchospasm or wheezing, rinitis, bronchitis, and laryngitis. Presence of comorbidities or subsequent bacterial infections, the duration of children hospitalization, the presence of hypoxia (oxygen saturation <92%), fever >38°C, or diarrhoea at the time of diagnosis were also recorded.

Pathogen Detection, Genotyping, and Phylogenetic Analysis

All samples were split into two aliquots (200 μl each) and frozen at −80% if nucleic acids were not immediately extracted. Only one aliquot per patient was used to perform all the analyses. Viral genomes were extracted from 200 μl of sample by using Qiagen Mini extraction kits and eluted in 60 μl of EB buffer following manufacturer' instructions. All extracts were subjected to the following diagnostic panel, including RSV RNA, hMPV RNA, hCoV RNA (by family-specific RT-PCR protocols), and hBoV DNA. For hCoV, hMPV, and RSV detection, 10 μl of extract for each pathogen were previously reverse transcribed, by using random hexamers and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) in a 20 μl reaction mixture. After reverse transcription (RT), family- or strain-specific PCR protocols were performed (Table I) by combining 10 μl of RT reaction mixture with 40 μl PCR mixtures and using 5U AmpliTaq Gold DNA polymerase (Applied Biosystems). In particular, RT-PCR amplification for RSV RNA and subsequent genotyping by nested PCR, was performed using previously described protocols [Coiras et al., 2003]; hMPV RNA was amplified using primer pairs encompassing a 150-bp region of the N-gene of all known hMPV subtypes, as described elsewhere [Debiaggi et al., 2006]; a novel hCoV amplification strategy was designed in order to allow the detection of both hCoV-NL63 and hCoV-229E, as well as the amplification of SARS-CoV, hCoV-NL63, and hCoV-HKU1. In particular four degenerated primers (Forward primers: PanCoVfw and PanCoVfw2, reverse primers: PanCoVr and PanCoVr2, respectively) were included in the hCoV family specific PCR reaction. The hMPV amplification protocol ensured the detection of 5 copies with comparable sensitivity for all hMPV subtypes; similarly, a sensitivity level of less than 10 copies for all hCoV genotypes (SARS-CoV included) was observed (data not shown). Finally, to detect hBoV DNA, a previously published PCR protocol was used [Allander et al., 2005]; to further define the subtype of positive samples, a second PCR targeting a more variable region of the viral genome was performed using a second couple of primers (BocaGenoFW and BocaGenoRW) (Table I). These primers were designed on the alignments based on GenBank-published virus sequences. The sensitivity of all amplification protocols, was determined by cloning each amplified target region into pCR2.1 plasmid vector (TA Cloning Kit; Invitrogen), serially diluted from 10^6 copies to 1 copy processed in parallel. To reduce the impact of known or possible base mismatches with virus variants, annealing temperatures allowing hybridization of primers even in the presence of minimal sequence variations were used.

RSV genotyping was possible by direct observation on agarose gel after electrophoresis of the nested PCR products. All positive viral amplification products (except for RSV) were sequenced in both directions by using BigDye Terminator v3.1 and an ABI

TABLE I. Oligonucleotides and Thermal Profile of Amplification Protocols Used in This Study

Primer name	Oligonucleotide sequence	Target gene	Origin
PanCoVfw	TTATGGGTTGGGATTATCCYAARTGTGAT	Polymerase	This study
PanCoVfw2	ATGGGATGGGACTATCCTAAGTGTGATAGAG		
PanCoVr	GTACTAGCRTCACCAGAAGTYGTACCACC		
PanCoVr2	TTGCATCACCACTRCTAGTRCCACCAGGC		
PanCoV	PCR Thermal profile: 96°C 30 sec, 63–54°C 30 sec, 72°C 30 sec (10 cycles); 96°C 30 sec, 60°C 30 sec; 72°C 30 sec (35 cycles)		
PanMPVfw	GAAATGGGCCCTGAATCTGGRCTTCTACA	Nucleoprotein	Debiaggi et al. [2006]
PanMPVr	TTGGYACTCTCCCTCGATACATACCGATTATGC		
PanMPVr2	TTGGTACTCTTCCTCTGTACATTCCGATTATAC		
FwseqMPV	TGAATCTGGGCTCCTACATTTAAGGCAAAG		This study
RevseqMPV	GAGCCCAGATTCAGGGCCCATTTCTC		
PanHMPV	PCR Thermal profile: 96°C 30 sec; 53°C 30 sec; 72°C 30 sec (45 cycles)		
188F	GACCTCTGTAAGTACTATTAC	NP1	Allander et al. [2005]
542R	CTCTGTGTTGACTGAATACAG		
BocaGenoFW	CCAAAAAAGACACTTTTACTTTGCTAACTCA	VP2	This study
BocaGenoRW	TGGACGCCAGTTCTTTGTTGCGTATCTTTC		
BoV	PCR Thermal profile: 96°C 30 sec, 55–51°C, 72°C 30 sec (5 cycles); 96°C 30 sec, 53°C 30 sec, 72°C 30 sec (40 cycles)		
RSVAB1	ATGGAGYTGCYRATCCWCARRRCAARTGCAAT	F	Coiras et al. [2003]
RSVAB2	AGGTGTWGTTACACCTGCATTRACACTRAATTC		
RSVA3	TTATACACTCAACAATRCCAAAAAWACC		
RSVA4	AAATTCCCTGGTAATCTCTAGTAGTCTGT		
RSVB3	ATCTTCCTAACTCTTGCTRTTAATGCATTG		
RSVB4	GATGCGACAGCTCTGTTGATTTACTATG		
RSV	PCR Thermal profile: 96°C 30 sec, 56°C 30 sec, 72°C, 30 sec (45 cycles)		

Each primer was used to a final 100 mM concentration in all PCR mixtures.

3100 sequencer (Applied Biosystems) to confirm amplification specificity and to allow subsequent phylogenetic analysis. PCR products were sequenced with the same primers used in the amplification protocols, except for hMPV, where primers FwseqMPV and RevseqMPV were designed for resequencing.

To subtype hMPV and hBoV strains, alignments of all amplified and reference sequences were generated using ClustalW (available at: http://www.ebi.ac.uk/clustalw), and corrected manually with the BioEdit (version 5.0.6; available at: http://www.mbio.ncsu.edu/bioedit/bioedit.html). Phylogenetic relationships were estimated using MEGA software (version 3.1; available at: http://www.megasoftware.net) (neighbor-joining method by using the model estimated by using Modeltest [available at: http://darwin.uvigo.es/software/modeltest.html]; the α value used in MEGA was estimated directly from the data by using HYPHY [available at: http://www.hyphy.org]).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software version 11. Univariate and bivariate analysis were performed.

RESULTS

Clinical Symptoms at Admission

Diagnosis at admission was lower acute respiratory disease (L-ARD) in 230 of the 322 patients (71.4%) [bronchiolitis in 166 (51.6%) and pneumonia in 64 (19.9%)], mild upper respiratory infection (U-ARD; including rinitis, bronchitis or laryngitis) in 40 (12.4%), bronchospasm or wheezing in 26 (8.0%). Other minor causes of admission were fever (2%), and bacteraemia (3%).

Detection of Viral Pathogens in Nasopharyngeal Aspirates of Infants With Acute Respiratory Disease

Nasopharyngeal aspirates from the 322 consecutive patients with a diagnosis of ARD were analyzed in the present study. Samples from 150 of the 322 infants (46.6%) tested positive for at least one pathogen. In particular, 90 of the 322 samples (28.0%) were positive for RSV RNA, 46 (14.3%) for hMPV RNA, 28 (8.7%) for hCoV RNA, and 7 (2.2%) for hBoV DNA. Twenty-one out of the 150 samples tested positive for two or more viral agents (see below).

Genotyping and Phylogenetic Analysis

All hMPV and hCoV positive samples were genotyped by sequencing of the PCR products and subsequent phylogenetic analysis; in six out of the seven hBoV DNA-positive samples, the VP2 coding region allowing virus subtyping could be amplified. RSV subtyping was possible by strain specific nested PCR only for 65 out of the 90 RSV positive samples due to samples availability. For RSV, 40 of the 65 samples (61.5%) resulted to be genotype A, and 25 (38.5%) genotype B. For hMPV infection, 33 out of the 46 hMPV RNA positive patients

(71.7%) resulted to be infected by hMPV subtype A (25 subtype A1, and 8 subtype A2) and 13 (28.3%) by hMPV subtype B (8 subtype B1, and 5 subtype B2). Of note, during the first year of study, we found only 2 subtype A2 strains out of the 12 infected subjects, and no subtype B2 out of the 6 B-strains. During the second year, all subtype B-strains were B2, and 5 out of the 13 subtypes A resulted to be A2. For the 28 hCoV infections, 11 samples (39.3%) tested positive for hCoV-OC43, 10 (35.7%) for hCoV-NL63, 6 (21.4%) for hCoV-HKU1, and 1 (3.6%) hCoV-229E, documenting a substantial co-circulation of the first three types. For the hCoV-HKU1, two distinct subtypes were detected: two of the six were genotype A and four genotype B strains, both circulating in the same season. Finally, three out of the six hBoV VP2 sequences resulted to belong to hBoV subtype 1 and three to subtype 2; the two subtypes were found with equal proportion in both years. Figure 1a and b shows the phylogenetic relationships of the hMPV and hCoV strains detected in the present study, respectively.

Co-Infections by Two or More Viral Pathogens

Co-infection by at least 2 of the viral pathogens under study was observed in 21 of the 322 patients (6.5% of the total number of cases, and 14.0% of the 150 tested positive for the viral agents investigated); in only two cases a triple infection was observed. In particular (Table II), 21 out of the 90 RSV infected patients (23.3%) were co-infected with an additional virus. Out of the 21, (13; 61.9%) were co-infected with hMPV, 6 with hCoVs (4 hCoV-OC43, 1 hCoV-NL63, and 1 hCoV-HKU1), and 2 with hBoV. Among the 47 hMPV RNA positive samples, 18 (38.3%) were also positive for another viral pathogen [13 RSV, 4 hCoV (2 hCoV-OC43, 1 hCoV-NL63, and 1 hCoV-HKU1), and 1 hBoV]. Of note, the rate of co-infections with other pathogens was also high for hCoVs (13/28; 46.4%); in particular, hCoV-OC43 positive samples were co-infected in six cases (four with RSV and two with hMPV), hCoV-NL63 samples in four cases (three with RSV and one with hMPV) and hCoV-HKU1 in two (one with RSV and one with hMPV) (Table II). No co-infection could be detected in the single hCoV-229E infection. Finally, co-infections were revealed in four out of the seven hBoV infected patients (57.1%), including two RSV, one hMPV, and one hCoV-OC43 infection.

Seasonal Distribution of the Cases and Clinical Syndromes Associated to the Different Agents

During the first year, from October 2004 to September 2005, 190 patients were enrolled; during the second year, from October 2005 to September 2006, 133 patients were studied. Peak incidence was observed from December to January in the first year of observation, and from January to February in the second year (Fig. 2). While RSV, and the other respiratory infections had a sharp decline after winter months, with almost no positive samples from May to November, sporadic cases of hMPV positive samples were detected all over the year during the first season.

RSV was the most common respiratory pathogen in all age groups. Although observed in all groups of age, RSV and hCoV cases were mainly detected in the first year of life; no hBoV infection was observed before the third month of age and a partial protection from

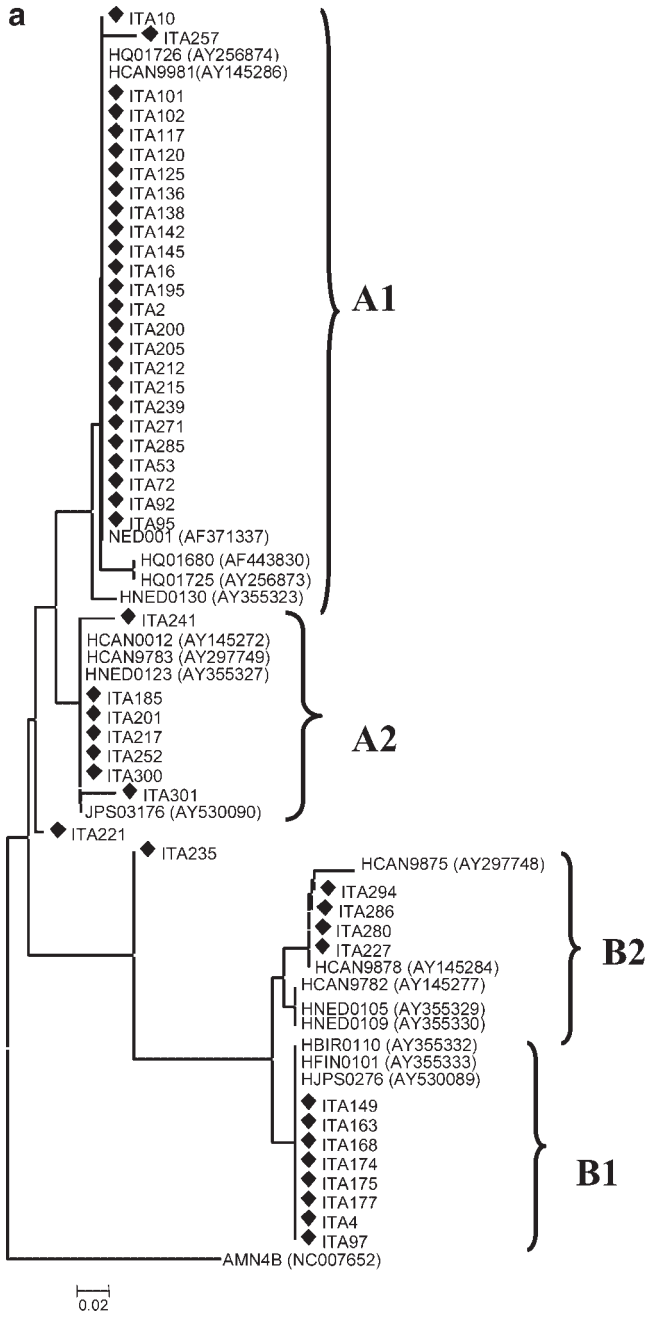

Fig. 1. Neighbor-joining trees (Tamura-Nei method, with Γ distribution) showing phylogenetic relationships between hMPVs (**a**), hCoVs (**b**), and hBoVs (**c**) sequences. Viral sequences amplified from patient-s'samples are indicated as filled symbols: ◆ for hMPVs, ▲ for hCoVs, and ■ for hBoVs sequences. GenBank accession numbers of hMPV sequences amplified from enrolled patients are: EF549622 to EF549667; for 229E sequence is EF549594; for hCoV-HKU1 sequences EF549595 to EF549600; for hCoV-NL63 sequences are EF549601 to EF549610; for hCoV-OC43 sequences are EF549611 to EF549621. GenBank accession numbers of reference sequences are specified in the phylogenetic trees.

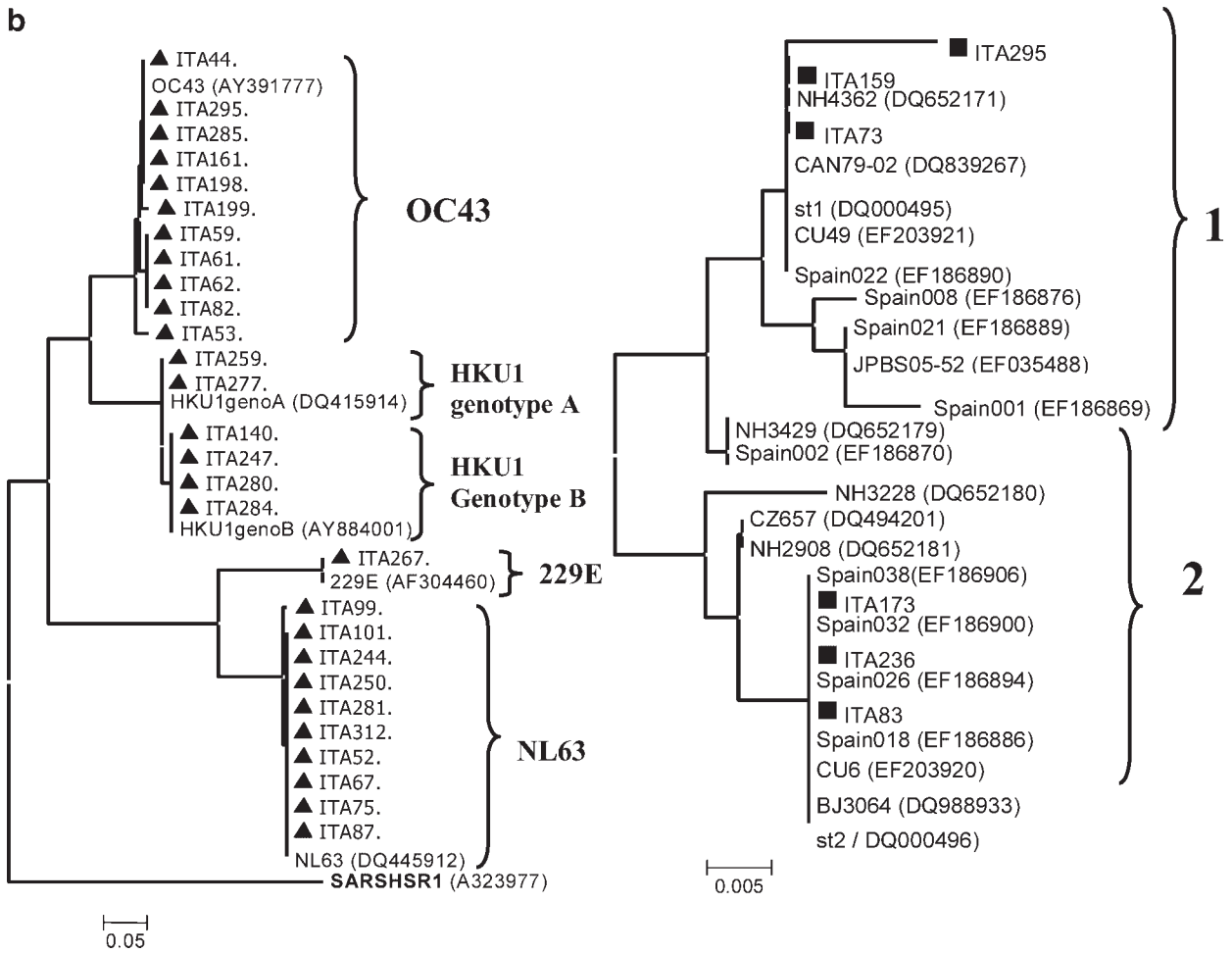

Fig. 1. (*Continued*)

maternal antibodies can be hypothesized. Both hCoV-OC43 and hCoV-HKU1 cases were detected mainly before 6 months of age suggesting a high prevalence of these pathogen in the general population. A higher prevalence of male subjects (five out of seven) were found to be infected by hBoV compared to females.

Among the 90 patients infected with RSV, 81 (90%) were hospitalized with a diagnosis of L-ARD. In particular, 67 RSV-positive patients (74.4%) were hospitalized with a diagnosis of bronchiolitis, 9 (10%) with a diagnosis of bronchopneumonia, and 5 (5.5%) bronchospasm or wheezing. Out of the 46 hMPV infected patients, 18 (39.1%) had bronchiolitis at admission, 14 (30.4%) had bronchopneumonia, and 2 (4.3%) were admitted to the hospital for bronchospasm or wheezing. Of note, RSV was the only pathogen significantly associated with the presence of respiratory complications such as bacterial pneumonia ($P < 0.001$) and hypoxia ($P < 0.015$); hypoxia was the only symptom significantly correlated with a prolonged hospitalization. Only one

TABLE II. Schematic Representation of the Co-Infections Observed in the Study

	Co-pathogens					
	RSV	hMPV	hCoV-OC43	hCoV-NL63	hCoV-HKU1	hBoV
RSV		13/90 (14%)*	4/90 (4%)*	1/90 (3%)	3/90 (1%)	2/90 (2%)*
hMPV	13/46 (28%)		2/46 (4%)	1/46 (2%)	1/46 (2%)	1/46 (2%)
hCoV-OC43	4/11 (36%)	2/11 (18%)		—	—	1/11 (2%)
hCoV-NL63	3/10 (30%)	1/10 (10%)	—		—	—
hCoV-HKU1	1/6 (16%)	1/6 (16%)	—	—		—
hBoV	2/7 (29%)	1/7 (14%)	1/7 (14%)	—	—	

*Two cases of triple infections were identified and are included in the table: in one patient RSV, hBoV and hCoV-OC43 genomes were found, and RSV, hCoV-OC43 and hMPV were detected in another patient.

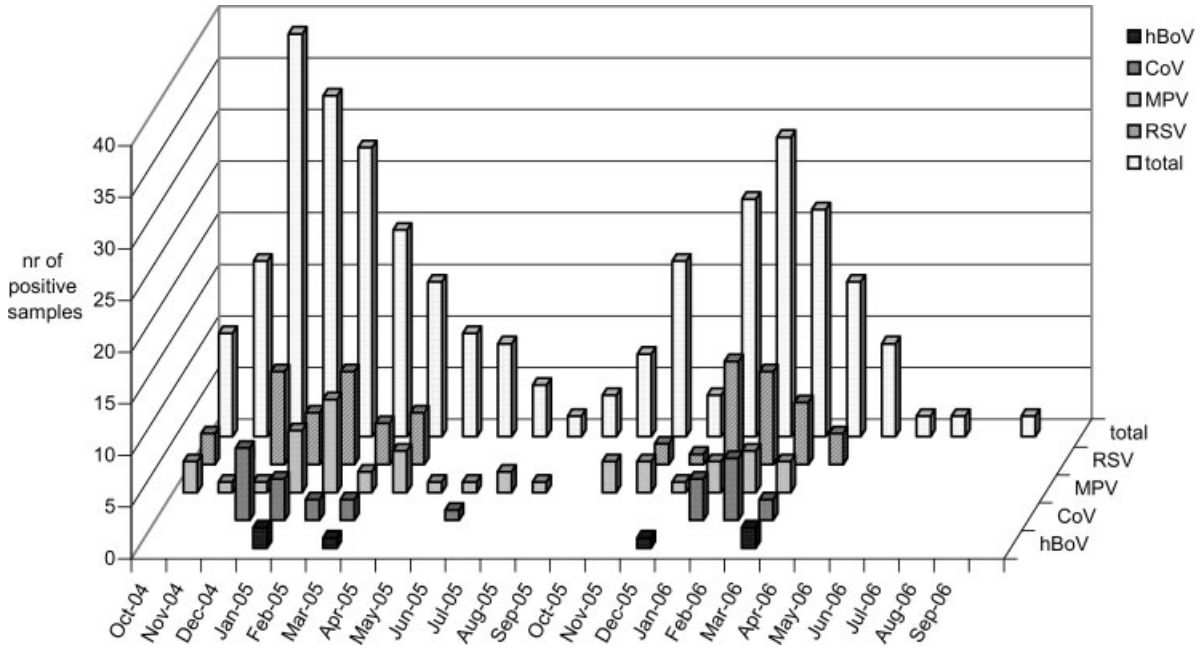

Fig. 2. Monthly distribution of respiratory samples analyzed in this study from October 2004 to September 2006.

case of U-ARD was observed associated with a hCoV-229E infection (1/1), seven cases of L-ARD and four U-ARD were recorded for hCoV-OC43, nine cases of L-ARD and one case of wheezing were observed for hCoV-NL63. Among the cases associated with hCoV-HKU1 infection, two cases of U-ARD and four L-ARD were recorded. Interestingly, when hMPV subtype B infected patients were compared to hMPV subtype A infections, a significant association between hypoxia and subtype B infection was observed ($P = 0.001$). Indeed, part of the hMPV subtype B infected patients 3 out of the 10 (30.0%) developed bacterial respiratory complications, but this was not statistically significant ($P > 0.05$). Clinical presentation and subtype associations are shown on Table III.

A minority of the patients (21/322; 6.5% of the total, and 14.0% of the 150 tested positive for the viral agents investigated) documented infection by two or more viral agents. These co-infections resulted to be more prevalent between 4 and 6 months of age ($P = 0.02$). However, when the presence of RSV alone and the co-infections by both RSV and hMPV were considered, the RSV mono-infected patients resulted to have a longer hospitalization and a higher prevalence of hypoxia ($P < 0.001$). The same analysis was not significant when RSV mono-infection and co-infections with any of the other pathogen were considered.

DISCUSSION

In this study, the molecular epidemiology of recently identified respiratory viruses and their role as co-pathogens in ARDs of infant patients infected by RSV were addressed. The relevant characteristics of this analysis are (i) a high number of consecutive infants with ARD analyzed for a period of 2 years in a metropolitan area, (ii) a strict homogeneity in age distribution of the population (94% under 1 year of age with a median of 3 months), and (iii) a panel of viral agents investigated that includes newly identified viral pathogens. Since the study was focused on the respiratory infections associated with RSV and other recently identified viral respiratory agents, the data supply a picture of the circulation of these viruses. From an epidemiological point of view, it should be underlined that other viral agents not included in the present evaluation (influenza A and B viruses rhinoviruses and adenoviruses) could have had a role in the cases that tested negative for the agents under evaluation.

Several aspects of the data shown require a comment. Firstly, the role of RSV as a major respiratory pathogen of infants is not influenced by the co-circulation of other emerging viral agents with similar seasonal distribution. Indeed, 90 out of 322 cases of ARD tested in this 2-years study (28.0% of the total, and 60.0% of the 150 cases diagnosed etiologically) were associated with RSV infection, in most of the patients as a unique pathogen. Secondly, the study confirms previous observations that hMPV infection may be associated in infants with L-ARD, but the severity of the disease associated with this viral agent is generally lower than that observed with RSV [van Woensel et al., 2006]. Notably, hMPV type B was associated to more clinically severe disease than hMPV type A in our study. Thirdly, the data shown extend the evaluation on the role as respiratory pathogens in infants, to the recently identified hCoVs and hBoVs. While the molecular epidemiology of these recently identified agents, as appears by

TABLE III. Clinical Characteristics of Infants Infected by Respiratory Viruses Detected in This Study

	Main illness		Sex		Age (month) (median)	Days hospitalized (median)	Hypoxia (<92%)	Fever (>38°)	Diarrhoea	Comorbidity	
	U-ARD	L-ARD	M	F						RD	Other
RSV	9/90 (10%)	81/90 (90%)	42/90 (47%)	48/90 (53%)	3	4	51/84 (61%)	40/84 (48%)	4/84 (5%)	24/84 (28%)	7/84 (8%)
RSV gen A	4/41 (10%)	36/41 (90%)	19/41 (46%)	21/41 (53%)	3	4.5	27/37 (73%)	21/37 (58%)	1/37 (3%)	1/37 (3%)	—
RSV gen B	3/25 (12%)	22/25 (88%)	10/25 (40%)	15/25 (60%)	4	3	16/23 (70%)	10/23 (45%)	2/23 (9%)	4/23 (17%)	2/23 (7%)
hMPV	7/46 (15%)	34/46 (85%)	23/46 (51%)	23/46 (49%)	3	3	13/41 (32%)	24/41 (59%)	1/41 (2%)	3/41 (7%)	5/41 (12%)
hMPVgen A	6/33 (18%)	25/33 (94%)	19/33 (54%)	16/33 (46%)	3	3	6/31 (19%)	17/31 (55%)	1/31 (3%)	—	5/31 (16%)
hMPVgen B	1/13 (10%)	9/13 (90%)	4/ 13 (40%)	6/13 (60%)	3	5	7/10 (70%)	7/10 (70%)	—	3/10 (50%)	-
HCoV	9/28 (68%)	19/28 (68%)	15/28 (54%)	12/28 (43%)	4	3	5/25 (20%)	16/25 (64%)	2/25 (8%)	4/25 (16%)	3/25 (12%)
hCoV OC43	4/11 (36%)	7/11 (64%)	6/11 (60%)	4/11 (40%)	4	3	2/11 (18%)	8/11 (73%)	1/11 (9%)	2/11 (18%)	—
hCoV NL63	2/10 (20%)	8/10 (80%)	4/10 (40%)	6/10 (60%)	3.5	3	1/8 (12%)	4/8 (50%)	—	—	2/8 (25%)
hCoV 229E	1/1 (100%)	—	—	1/1 (100%)	1	—	—	—	—	—	—
hCoV HKU1	2/6 (33%)	4/6 (67%)	5/6 (83%)	1/6 (17%)	4	3	2/6 (33%)	4/6 (67%)	1/6 (17%)	2/6 (33%)	1/6 (3%)
HBoV	1/8 (12%)	7/8 (87%)	5/8 (62%)	3/8 (37%)	7	5.5	4/8 (50%)	6/8 (75%)	1/8 (12%)	2/8 (20%)	—
hBoV gen 1	—	4/4 (100%)	3/4 (75%)	1/4 (25%)	5.5	5.5	1/4 (25%)	3/4 (75%)	1/4 (25%)	1 /4 (25%)	—
hBoV gen 2	1/3 (33%)°	2/3 (67%)	1/3 (33%)	2/3 (67%)	8	3	2/3 (67%)	2/3 (67%)	—	1/3 (50%)	—

Bronchiolitis, pneumonia and bronchospasm or wheezing are included together in the LARDs; laryngitis and rinitis in the UARD category. In 6 out of 90 RSV positive patients and 5 out of 46 hMPV positive patients data were not collected properly during hospitalization and were excluded from the analysis.

the present analysis, confirms their widespread circulation as seasonal respiratory pathogens (or, in a large proportion of the cases, as co-pathogens), the data indicate a lower pathogenic potential than that exhibited by RSV, that was the only virus associated with hypoxia and respiratory complications (clinically evaluated). All the other viruses (hMPV, hCoVs and hBoVs) were detected in a high proportion of infants in this cohort as co-pathogens. Notably, RSV-hMPVs co-infections were significantly observed in less severe respiratory diseases when compared to unique RSV infections. This observation is in agreement with other analyses documenting that co-infections are normally not observed in mechanically ventilated children, where unique RSV infections are frequently detected [Lazar et al., 2004; van Woensel et al., 2006]. On the other hand, this finding is in contrast with other studies, where the dual-infection with RSV and hMPV was associated with severe diseases [Greensill et al., 2003; Semple et al., 2005]. Whether our observation is due to a different epidemiological distribution of pathogens, or to the virus specific virus-host interactions remains to be determined. Indeed, respiratory viruses, including the recently described emerging agents are widespread agents, as demonstrated by their very early age of infection, and the high proportion of co-infections observed in the cases described here. Theoretically, several host and viral features may influence the clinical outcome in these co-infections. These features may include, on the one hand, a modified cytokine induction profile, and on the other hand, interferon susceptibility of the viral agents [Huck et al., 2007]. As an example, during the SARS-CoV epidemics, a large proportion of hMPV co-infections were observed, but no significant association with a worse prognosis was demonstrated, and an opportunistic role was suggested [Chan et al., 2003]. Whether this feature is linked to specific SARS-CoV characteristics, including the high degree of resistance to alpha interferon [Scagnolari et al., 2004], or if the same type of interactions could be observed in other co-infections should be clarified in the near future. However, the data show that co-infections with these agents are not rare and occasional events.

Other information obtained from the analysis of the data presented is as follows. A high proportion of patients infected by hCoV-NL63 (8/10), hCoV-HKU1 (3/6), and hCoV-OC43 (6/11), at hospital admission presented a diagnosis of bronchiolitis or pneumonia, in agreement with previous observations [Tyrrell and Bynoe, 1965; van den Hoogen et al., 2001; Sloots et al., 2006]. Furthermore, previous reports have shown a high prevalence of underlying diseases and a high frequency of gastrointestinal diseases in hCoV-HKU1 infected patients [Vabret et al., 2006], but this study cannot confirm this observation, since only one case of diarrhea was observed and no predisposing conditions were documented. The same observation was done for hBoV infections, that has been reported in patients with gastroenteritis and ARD, but that in only one out of the seven cases of this cohort of patients was associated with

diarrhea at diagnosis or during hospitalization. For hCoV-HKU1, phylogenetic analysis documented the co-circulation of two distinct genotypes; similarly, for the newly identified hBoVs, two co-circulating subtypes were also detected [Lau et al., 2006; Bastien et al., 2007]. For hMPV infection, the amplified region of the hMPV genome offered enough phylogenetic signal to identify at least four subtypes correctly. The proportion between subtype B and A did not change during the two epidemic seasons, ranging between 24% and 33%; the B strains seem to circulate at alternate seasons, even if within subtype cross-neutralization has been described in vitro and in animal studies. Further study may clarify this crucial issue for vaccines production or passive immunotherapeutic strategies.

Overall, the study document the molecular epidemiology of classical (RSV, hCoV-NL63, hCoV-229E) and recently identified (hMPV, hCoV-HKU1, hCoV-229E, and hBoVs) viral pathogens in a 2-years study of ARDs in patients with less than 2 years of age living in a metropolitan area of northern Italy. Using molecular detection, sequencing, and phylogenetic analysis, the study has evaluated the clinical impact of infections with single pathogens and co-infections with two or more agents. The data highlight that RSV maintains a distinct role as a respiratory pathogen of infants, that the wide circulation of other viruses does not reduce the clinical relevance of this virus, and that the recently identified agents (including hMPVs, hBoVs, and hCoV) may act as primary pathogens or co-pathogens. The bio-pathology of co-infections by respiratory viruses should be addressed further with specific in vitro and in vivo analyses.

REFERENCES

Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci USA 102:12891–12896.

Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM. 2006. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol 78:1232–1240.

Arnold JC, Singh KK, Spector SA, Sawyer MH. 2006. Human bocavirus: Prevalence and clinical spectrum at a children's hospital. Clin Infect Dis 43:283–288.

Bastien N, Chui N, Robinson JL, Lee BE, Dust K, Hart L, Li Y. 2007. Detection of human bocavirus in Canadian children in a 1-year study. J Clin Microbiol 45:610–613.

Chan PK, Tam JS, Lam CW, Chan E, Wu A, Li CK, Buckley TA, Ng KC, Joynt GM, Cheng FW, To KF, Lee N, Hui DS, Cheung JL, Chu I, Liu E, Chung SS, Sung JJ. 2003. Human metapneumovirus detection in patients with severe acute respiratory syndrome. Emerg Infect Dis 9:1058–1063.

Choi EH, Lee HJ, Kim SJ, Eun BW, Kim NH, Lee JA, Lee JH, Song EK, Kim SH, Park JY, Sung JY. 2006. The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000–2005. Clin Infect Dis 43:585–592.

Coiras MT, Perez-Brena P, Garcia ML, Casas I. 2003. Simultaneous detection of influenza A, B, and C viruses, respiratory syncytial virus, and adenoviruses in clinical samples by multiplex reverse transcription nested-PCR assay. J Med Virol 69:132–144.

Debiaggi M, Canducci F, Sampaolo M, Marinozzi MC, Parea M, Terulla C, Colombo AA, Alessandrino EP, Bragotti LZ, Arghittu M, Goglio A, Migliavacca R, Romero E, Clementi M. 2006. Persistent symptomless human metapneumovirus infection in hematopoietic stem cell transplant recipients. J Infect Dis 194:474–478.

Greensill J, McNamara PS, Dove W, Flanagan B, Smyth RL, Hart CA. 2003. Human metapneumovirus in severe respiratory syncytial virus bronchiolitis. Emerg Infect Dis 9:372–375.

Hamre D, Procknow JJ. 1966. A new virus isolated from the human respiratory tract. Proc Soc Exp Biol Med 121:190–193.

Huck B, Neumann-Haefelin D, Schmitt-Graeff A, Weckmann M, Mattes J, Ehl S, Falcone V. 2007. Human metapneumovirus induces more severe disease and stronger innate immune response in BALB/c mice as compared with respiratory syncytial virus. Respir Res 8:6.

Kuiken T, Fouchier R, Rimmelzwaan G, Osterhaus A. 2003. Emerging viral infections in a rapidly changing world. Curr Opin Biotechnol 14:641–646.

Lau SK, Woo PC, Yip CC, Tse H, Tsoi HW, Cheng VC, Lee P, Tang BS, Cheung CH, Lee RA, So LY, Lau YL, Chan KH, Yuen KY. 2006. Coronavirus HKU1 and other coronavirus infections in Hong Kong. J Clin Microbiol 44:2063–2071.

Lazar I, Weibel C, Dziura J, Ferguson D, Landry ML, Kahn JS. 2004. Human metapneumovirus and severity of respiratory syncytial virus disease. Emerg Infect Dis 10:1318–1320.

Ma X, Endo R, Ishiguro N, Ebihara T, Ishiko H, Ariga T, Kikuta H. 2006. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J Clin Microbiol 44:1132–1134.

Maggi F, Andreoli E, Pifferi M, Meschi S, Rocchi J, Bendinelli M. 2007. Human bocavirus in Italian patients with respiratory diseases. J Clin Virol 38:321–325.

Medici MC, Arcangeletti MC, Rossi GA, Lanari M, Merolla R, Paparatti UD, Chezzi C. 2006. Four year incidence of respiratory syncytial virus infection in infants and young children referred to emergency departments for lower respiratory tract diseases in Italy: The "Osservatorio VRS" Study (2000–2004). New Microbiol 29:35–43.

Pierangeli A, Gentile M, Di Marco P, Pagnotti P, Scagnolari C, Trombetti S, Lo Russo L, Tromba V, Moretti C, Midulla F, Antonelli G. 2007. Detection and typing by molecular techniques of respiratory viruses in children hospitalized for acute respiratory infection in Rome, Italy. J Med Virol 79:463–468.

Pyrc K, Berkhout B, van der Hoek L. 2007. The novel human coronaviruses NL63 and H KU1. J Virol 81:3051–3057.

Scagnolari C, Vicenzi E, Bellomi F, Stillitano MG, Pinna D, Poli G, Clementi M, Dianzani F, Antonelli G. 2004. Increased sensitivity of SARS-coronavirus to a combination of human type I and type II interferons. Antivir Ther 9:1003–1011.

Semple MG, Cowell A, Dove W, Greensill J, McNamara PS, Halfhide C, Shears P, Smyth RL, Hart CA. 2005. Dual infection of infants by human metapneumovirus and human respiratory syncytial virus is strongly associated with severe bronchiolitis. J Infect Dis 191:382–386.

Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, Mackay IM. 2006. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol 35:99–102.

Tyrrell DA, Bynoe ML. 1965. Cultivation of a novel type of common-cold virus in organ cultures. Br Med J 1:1467–1470.

Vabret A, Dina J, Gouarin S, Petitjean J, Corbet S, Freymuth F. 2006. Detection of the new human coronavirus HK U1: A report of 6 cases. Clin Infect Dis 42:634–639.

van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD. 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 7:719–724.

van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B. 2004. Identification of a new human coronavirus. Nat Med 10:368–373.

van Woensel JB, Bos AP, Lutter R, Rossen JW, Schuurman R. 2006. Absence of human metapneumovirus co-infection in cases of severe respiratory syncytial virus infection. Pediatr Pulmonol 41:872–874.

Woo PC, Lau SK, Chu CM, Chan KH, Tsoi HW, Huang Y, Wong BH, Poon RW, Cai JJ, Luk WK, Poon LL, Wong SS, Guan Y, Peiris JS, Yuen KY. 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J Virol 79:884–895.