Journal of Medical Virology 79:463–468 (2007)

Detection and Typing by Molecular Techniques of Respiratory Viruses in Children Hospitalized for Acute Respiratory Infection in Rome, Italy

Alessandra Pierangeli,[1]* Massimo Gentile,[1] Paola Di Marco,[1] Paolo Pagnotti,[1] Carolina Scagnolari,[1] Simona Trombetti,[1] Lelia Lo Russo,[2] Valeria Tromba,[2] Corrado Moretti,[2] Fabio Midulla,[2] and Guido Antonelli[1]

[1]*Department of Experimental Medicine, Virology Section, Rome, Italy*
[2]*Pediatric Department, "La Sapienza" University, Rome, Italy*

Detection of a broad number of respiratory viruses is not undertaken currently for the diagnosis of acute respiratory infection due to the large and always increasing list of pathogens involved. A 1-year study was undertaken on children hospitalized consecutively for acute respiratory infection in a Pediatric Department in Rome to characterize the viruses involved. Two hundred twenty-seven children were enrolled in the study with a diagnosis of asthma, bronchiolitis, bronchopneumonia, or laringo-tracheo bronchitis. A molecular approach was adopted using specific reverse transcription (RT)-PCR assays detecting 13 respiratory viruses including metapneumovirus (hMPV) and the novel coronaviruses NL63 and HKU1; most amplified fragments were sequenced to confirm positive results and differentiate the strain. Viral pathogens were detected in 97 samples (42.7%), with 4.8% of dual infections identified; respiratory syncytial virus (RSV) was detected in 17.2% of children, followed by rhinovirus (9.7%), parainfluenza virus type 3 (PIV3) (7.5%), and influenza type A (4.4%). Interestingly, more than half the patients (9/17) that have rhinovirus as the sole respiratory pathogen had pneumonia. HMPV infected children below 3 years in two peaks in March and June causing bronchiolitis and pneumonia. One case of NL63 infection is described, documenting NL63 circulation in central Italy. In conclusion, the use of a comprehensive number of PCR-based tests is recommended to define the burden of viral pathogens in patients with respiratory tract infection. ***J. Med. Virol. 79: 463–468, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: respiratory tract infection; pneumonia; bronchiolitis; RT-PCR; co-infection

INTRODUCTION

Detection of viral respiratory agents has improved in the last decade due to new molecular techniques and availability of monoclonal antibodies for a number of different viral species [Garbino et al., 2004; Legoff et al., 2005; Rovida et al., 2005]. Nonetheless, the majority of all episodes of acute respiratory infection have none of the pathogen identified in pediatric as well as adult populations [Griffin et al., 2004; Louie et al., 2005]. It is likely that the prevalence of viral infection is underestimated because of the large number of respiratory viruses that are eventually involved in respiratory tract infection. There is also a continuously increasing list of new respiratory pathogens, identified by molecular techniques that can contribute significantly to the burden of acute respiratory infection. Indeed, human metapneumovirus (hMPV), detected in 2001 [van den Hoogen et al., 2001], is now considered an important lower respiratory tract pathogen, second only to respiratory syncytial virus (RSV) judged by the incidence rates among infants and children [van den Hoogen et al., 2003; Crowe, 2004]. By the end of 2004, following SARS coronavirus outbreaks, two independent research groups reported the discovery of a fourth human coronavirus, that was named NL63 [van der Hoek et al., 2004] or NH [Esper et al., 2005a]. Early in 2005, again the detection of a fifth human coronavirus, HKU1, was published by a research group in Hong Kong [Woo et al., 2005a]. Several reports confirmed retrospectively that these new human coronaviruses were

Grant sponsor: Università "La Sapienza" (fondi Ateneo).

*Correspondence to: Alessandra Pierangeli, Sezione Virologia, Dipartimento Medicina Sperimentale, Università La Sapienza, Viale di Porta Tiburtina, 28 00185 Roma, Italy.
E-mail: alessandra.pierangeli@uniroma1.it

Accepted 20 November 2006
DOI 10.1002/jmv.20832
Published online in Wiley InterScience (www.interscience.wiley.com)

circulating in different countries worldwide [Bastien et al., 2005; Esper et al., 2005a; Woo et al., 2005b; Vabret et al., 2006]. Therefore, a surveillance program for viral agents was conducted from October 2004 to September 2005, in children hospitalized for acute respiratory infection in a pediatric department at the University "La Sapienza" hospital of Rome. A molecular approach using specific reverse transcription (RT)-PCR assays to detect nearly all respiratory viruses including the novel coronaviruses NL63 and HKU1 was adopted. The main aim of this study was to estimate the incidence of respiratory viral agents in children suffering from respiratory tract infection and to characterize the different species in their pathogenic role.

MATERIALS AND METHODS

Patients and Specimens

The study was conducted from October 2004 to September 2005 in children hospitalized consecutively for acute respiratory infection or related conditions at the "Umberto I" Hospital Pediatric Department (University "La Sapienza," Rome). Patients enrolled had diagnosis of asthma, asthma with consolidation, bronchiolitis, bronchopneumonia, or laringo-tracheo bronchitis; exclusion criteria were underlying medical problems (e.g., prematurity, congenital diseases, cistis fibrosis). Informed consent was obtained from the parents of the children. One to 3 days after hospitalization, all patients underwent nasal washing, obtained with 3 ml of sterile saline physiological solution injected into each nostril and collected with a syringe. All samples were delivered on ice within 1–2 hr to the virology laboratory and on arrival, if needed, they were vortexed with beads to solve mucus. They were divided into two aliquots: one was treated for nucleic acid extraction; the second was sub-aliquoted and stored at −80°C.

Nucleic Acid Extraction and Reverse Transcription

Two hundred microliters of respiratory specimens were subjected to nucleic acid extraction with use of a Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany), eluting into 50 μl of the supplied elution buffer. To test for most respiratory viruses, 20 μl of RNAs were denatured for 10 min at 65°C, added to 20 μl of RT-reaction mixes containing random hexamers (Amersham Biosciences, Buckinghamshire, UK), and MuMLV-RT (Roche) and incubated for 1 hr at 42°C, followed by 10 min at 95°C. On the other hand, the RT reactions for detection of hMPV, NL63, NH, and HKU1 were performed adding to the reaction mix the proper, specific antisense primer.

PCR Assays for Respiratory Viruses

A panel of PCR or nested PCR assays, some of which in a multiplex format, were developed for detection of 13 respiratory viruses including influenza A, influenza B, RSV, hCoV OC43, hCoV 229E, adenovirus, rhinovirus, PIV1, PIV2, PIV3, hMPV, hCoV NL63, and hCoV HKU1 (Table I). Primers specific for each virus were selected from published protocols and reaction conditions were optimized to run different reactions with the same thermal profile whenever possible [Grondahl et al., 1999].

Viral nucleic acid was extracted, as described above, from reference viruses when available in the virology laboratory (influenza A strain Valorn, RSV type A, coronaviruses OC43 and 229E, adenovirus type 1). PIV3 and rhinovirus were isolated from respiratory samples

TABLE I. PCR Reactions Used for Detection of Respiratory Viruses, With References, Gene Products and Amplification Conditions

Virus/PCR format	References	Target gene/bp fragments	Thermal profile
Influenza A and B/multiplex	Claas et al. [1992]	Segment 8/192 (influenza A) 241 (influenza B)	94°C, 60 sec; 54°C, 60 sec; 72°C, 60 sec
RSV	Paton et al. [1992]	F1 subunit/232	94°C, 60 sec; 54°C, 60 sec; 72°C, 60 sec
PIV1	Fan and Henrickson [1996]	*HN* gene/180	94°C, 60 sec; 55°C, 45 sec; 72°C, 45 sec
PIV2/nested	Echevarria et al. [1998]	*HN* gene/200 (II round)	94°C, 60 sec; 50°C, 60 sec; 72°C, 60 sec; 94°C, 60 sec; 58°C, 60 sec; 72°C, 60 sec
PIV3/nested	Echevarria et al. [1998]	*HN* gene/100 (II round)	94°C, 60 sec; 50°C, 60 sec; 72°C, 60 sec; 94°C, 60 sec; 58°C, 60 sec; 72°C, 60 sec
Rhinovirus	Deffernez et al. [2004]	*5′UTR*/200	94°C, 45 sec; 55°C, 45 sec; 72°C, 60 sec
229E, OC43/multiplex	Pitkaranta et al. [1997]	*N* gene/294(229E);370(OC43)	94°C, 60 sec; 60°C, 60 sec; 72°C, 60 sec
Adenovirus	Osiowy [1998]	*Hexon*/210	94°C, 60 sec; 60°C, 60 sec; 72°C, 60 sec
hMPV I assay	van den Hoogen et al. [2001]	*L* gene/170	94°C, 45 sec; 54°C, 45 sec; 72°C, 60 sec
hMPV II assay	Peret et al. [2002]	*F* gene/450	94°C, 60 sec; 54°C, 60 sec; 72°C, 60 sec
NL63 I assay/nested	van der Hoek et al. [2004]	*1b* gene/170 (II round)	94°C, 60 sec; 55°C, 60 sec; 72°C, 90 sec; 94°C, 60 sec; 55°C, 60 sec; 72°C, 90 sec
NL63 II assay/nested	van der Hoek et al. [2004]	*1a* gene/520 (II round)	94°C, 60 sec; 55°C, 60 sec; 72°C, 120 sec; 94°C, 60 sec; 55°C, 60 sec; 72°C, 120 sec
NH	Esper et al. [2005a]	*1a* gene/215	94°C, 45 sec; 55°C, 60 sec; 72°C, 60 sec
CoV-HKU1	Woo et al. [2005a]	*1a-b* gene/440	94°C, 45 sec; 55°C, 45 sec; 72°C, 75 sec

before starting the study and propagated on T25 flasks of MRC5 to have virus stocks for RNA extraction. The viral cDNA (or DNA for adenovirus) obtained as described above, at low copy number and a negative control of nuclease-free water were run in each specific PCR assay. Separation of an aliquot of amplified material along with molecular weight markers was undertaken by electrophoresis for 30–60 min on 2–3% agarose gel. PCR reactions that produced proper bands were purified on Qiaquick PCR purification column (Qiagen, Hilden, Germany) and sequenced to confirm positivity. Amplification products were then cloned using Topo TA Cloning Kit (Invitrogen, Carlsbad, CA) to have plasmid DNA to use as a positive control in PCR reactions. All PCR reactions were optimized to detect at 10–100 input plasmid copies. A RT-PCR assay specific for hMPV *L* gene was validated using RNA transcribed from the proper cDNA clone [Maggi et al., 2003] (a kind gift from Prof. Bendinelli, University of Pisa, Italy). To detect hMPV types A and B, in addition to the widely used primers (L6/7) [van den Hoogen et al., 2001] that do not hybridize to type B [Sarasini et al., 2006], a second PCR test was used (Table I).

HCoV NL63 was sought for using three different RT-PCR reactions because of the lack of a positive control to evaluate the amplification sensitivity (Table I).

Prior to testing for respiratory virus, the random primer-directed RT reaction from each sample, performed as described, was subjected to PCR amplification of the invariant β-actin gene to rule out the occurrence of PCR inhibitors; if β-actin PCR was negative, specimens were re-extracted from a frozen aliquot.

For amplification, 5 μl of cDNA template (or 2 μl of nucleic acid for adenovirus detection) was added to 45 μl of a reaction mix containing 25 pmol of each primer, 200 mM dNTP, 1.5 mM $MgCl_2$ (2.5 mM $MgCl_2$ in PIV2 and PIV3 first and second round reactions), and 1.25 U of Taq polymerase (Roche). Positive and negative controls were run in each reaction and the PCR products were then electrophoresed; most amplified fragments were purified and sequenced as described. The sequences obtained were compared with those in the National Center for Biotechnology Information database (NCBI Genebank) and several viral strains were typed accordingly. The amplification products not sequenced were confirmed as true positives by testing a second specimen aliquot.

RESULTS

This study was carried out during 1 year on 227 children that were hospitalized for acute respiratory infection or related condition, in a Pediatric Department for at least 3 days; the subjects median age was 15 months (range: 0–139 months).

In 97/227 children (42.7%), at least one viral pathogen was identified; 86/227 (37.9%) had an infection with one of the virus investigated; 11/227 (4.8%) had a dual infection, with a total of 108 viruses identified, while 130 patients (57.3%) were negative for any respiratory virus tested. Table II shows the results and percentages of cases.

Considering the virus-positive cases, the most common agent was RSV (40.2%), followed by rhinovirus (22.6%), and PIV3 (17.3%). Approximately half of the PCR-amplified fragments were sequenced, randomly along the study period, to confirm positive results and to eventually type the viruses. Twenty out of a total of 39 RSV isolates were sequenced; sequence homologies showed all of them to be subtype B. Multiplex PCR for influenza revealed 10 positives for influenza A and 1 for influenza B, that were confirmed by sequencing. HMPV were present in eight cases that were positive to both PCR assays we used; sequencing analysis revealed that they were all type A. Six samples positive in the multiplex PCR for hCoV OC43 and 229E were all confirmed by sequencing to be hCoV OC43. Sequencing identified and confirmed one NL63 case, amplified with both NL63 couple of primers in 1a and 1b genomic regions [van der Hoek et al., 2004], but not with NH primers [Esper et al., 2005a]. HCoV HKU1 was sought retrospectively, on frozen aliquots of samples from October 2004 to May 2005, the time when the original paper was published [Woo et al., 2005a]. No positive HKU1 was found either in frozen or in fresh samples; nonetheless, the RT-PCR assay used in this study was not properly validated due to the lack of a positive control.

Dual infections contributed more than 10% of total positive cases: PIV3 was present in 6 out of 11 cases of co-infections, RSV and rhinovirus in 5 cases each, including 2 RSV-rhinovirus double infections (Table III).

Seasonal and age distribution of respiratory viruses detected in this study was in accord with that of previous reports [Griffin et al., 2004; Monto, 2004; Nicholson et al., 2006]. RSV circulated during winter, with little activity outside these months. The circulation of influenza viruses was similar to RSV but no cases were detected outside the winter peak; the case of influenza B was detected apparently at the end of influenza A circulation in early spring. HMPV distribution was not limited to the winter months but presented a second

TABLE II. Detection Rates and Distribution by Pathogen of Viral Infections in 227 Children Hospitalized for Acute Respiratory Infection (October 2004–September 2005)

Cases of	Number (%)[a]
Total positives	97 (42.7%)
Single infections	86 (37.8%)
Dual infections	11 (4.8%)
RSV	39 (17.2%)
Rhinovirus	22 (9.7%)
PIV3	17 (7.5%)
Influenza A	10 (4.4%)
HMPV	8 (3.5%)
OC43	6 (2.6%)
ADENO	5 (2.2%)
Influenza B	1 (0.4%)
NL63	1 (0.4%)

[a]% are on total number of patients.

TABLE III. Occurrence of Dual Respiratory Virus Infection in Virus Positive Children

No. of cases	Viruses
2	RSV-PIV3
2	RSV-Rhino
1	RSV-OC43
2	PIV3-Rhino
1	PIV3-OC43
1	PIV3-Ad
1	Influenza A-OC43
1	hMPV-Rhino

peak of infection in June. The other respiratory viruses were detected all over the year. Co-infections were detected more frequently, but not exclusively, in the winter months.

In Table IV, the distribution of detected viruses according to patient age is shown. RSV was far the most common pathogen affecting newborns up to 3 months, present in about 70% of the virus-infected infants, followed by hMPV and PIV3 (11% of infections). Incidence of RSV infection decreased dramatically with age, from 72.2% of those aged 0–3 months to 31.2% of those aged 1–3 years and 17.4% of older children. Rhinovirus was the pathogen identified more frequently in children older than 3 years (39.1%).

DISCUSSION

The present study describes virological testing by molecular methods of 227 nasal washings from hospitalized children suffering from acute respiratory infection. Nasal lavage was chosen because it is the simpler and less invasive for obtaining a respiratory specimen in pediatric age and it is significant also for viral lower tract respiratory infections. Indeed, nasal washings were taken in the acute phase of the illness and, though not proven with seroconversion, it is reasonable to consider the detected virus as the principal pathogen involved.

The present study did not include bacterial pathogens or atypical respiratory viral pathogens (e.g., cytomegalovirus); nevertheless, a comprehensive array of amplification-based tests has been performed to determine the burden of respiratory viruses.

As expected in a pediatric population, RSV was the most common agent (39 cases, 40.2% of all positive cases) detected mainly in bronchiolitis (18 cases) and pneumonia (15 cases). In the present study, RSV infection started in December and ended in July. A recent study described a different seasonal distribution of RSV infection, diagnosed by enzyme immunoassays in Italian children (Medici et al., 2006); the RSV epidemic started in October–November and ended in May, showing a peak of incidence in February. The peak of incidence is similar (January–March) but in the present study, some cases were detected also in June and July. The different sensitivity of the assays used may possibly explain such a discrepancy. Detection of sporadic RSV and other respiratory virus infections could contribute to the survival of these viruses during summer.

Rhinovirus was the second most common viral pathogen detected and was present all the year. Rhinovirus is represented in 45% of co-infection cases and this figure probably reflects its high prevalence in upper respiratory infection in children [Legg et al., 2005]. Some authors reported that respiratory picornaviruses are one of the leading etiological causes of bronchiolitis in French infants (Jacques et al., 2006). In the present study, more than half patients that have rhinovirus as the sole respiratory pathogen were affected by pneumonia (9/17 = 53%); in addition, it was detected in four children hospitalized for asthma episodes. Recent studies indicate that rhinovirus is responsible for direct infection of the lower airway epithelium [Papadopoulos et al., 2000]; furthermore, it is able to replicate in the bronchial epithelium both in vitro and in vivo [Mosser et al., 2002]. Consistent with those reports, this study suggests the involvement of rhinovirus in cases of pneumonia. The actual proportion of lower airways pathogenicity of rhinovirus, following natural infections, remains to be assessed.

Overall, in children up to 3 years, hMPV infections represented about 11% of all viral illness, a value slightly lower than that (13.1%) reported recently in Northern Italy [Sarasini et al., 2006]. In this study in Rome, only hMPV type A was detected, in contrast to the report that identified both types in the winter–spring season 2003–2004 [Sarasini et al., 2006]. In this study population, hMPV cases occurred in two peaks of infections, the former in winter and the latter in June;

TABLE IV. Distribution by Children Age of Cases of Respiratory Viruses Infection

Pathogen[a]	0–3 months, n = 18	4–12 months, n = 28	1–3 years, n = 28	>3 years, n = 23
RSV	13 (72.2%)	13 (46.4%)	9 (31.2%)	4 (17.4%)
Rhinovirus	1 (5.5%)	5 (17.8%)	7 (25.0%)	9 (39.1%)
PIV-3	2 (11.1%)	6 (21.4%)	5 (17.8%)	4 (17.4%)
Influenza	1 (5.5%)	1 (3.2%)	5 (17.8%)	4 (17.4%)
hMPV	2 (11.1%)	3 (10.7%)	3 (10.7%)	0
OC43	0	0	3 (10.7%)	3 (13.0%)
Adeno	0	3 (10.7%)	1 (3.2%)	1 (4.3%)
Dual infection	1 (5.5%)	3 (10.7%)	5 (17.8%)	2 (8.7%)

[a]% are on total number of virus positive cases in the corresponding age class.

interestingly, in winter they were associated with bronchiolitis (children younger than 6 months), whereas in June with pneumonia (patients 6 months to 3 years old).

Human coronaviruses OC43 and 229E are considered agents of upper respiratory tract diseases, including common cold; nonetheless, they are reported to cause more serious respiratory illnesses in children below the age of 1 year. In our coronavirus cases caused by OC43 serotype, five were affected with pneumonia and one with laryngitis, and patients were more than 1 year old. The child infected with NL63, hospitalized for acute respiratory infection, had a subsequent clinical diagnosis that met some criteria for attribution to Kawasaki disease. Obviously, only one case cannot contribute to the association of NL63 infection with Kawasaki disease [Esper et al., 2005b; Shimizu et al., 2005]; in any case, this report confirms the circulation of NL63 in Italy, first reported when this manuscript was in preparation [Gerna et al., 2006] in Northern Italy, where it was detected only in the winter–spring season of 2004–2005.

It is well known that infants with severe bronchiolitis have an increased risk of developing recurrent wheezing later in life and viruses other than RSV and multiple viral infections may contribute to the severity of bronchiolitis. It was reported [Papadopoulos et al., 2002; Semple et al., 2005] that dual infections with hMPV and RSV, or rhinovirus and RSV, confer a 5- to 10-fold increase of severe disease in children admitted to pediatric intensive care units. In contrast, other studies reported that co-infection with two respiratory viruses were not significantly associated with disease severity [van Woensel et al., 2006; Wolf et al., 2006]. In the present study, dual infections were detected in 10% of virus-positive cases, mainly in children with pneumonia. Clinical data available from co-infected children did not show significant differences from other patients. Larger studies are needed to clarify the issue whether multiple infections could be involved with the severity of low respiratory tract infections.

Respiratory virus infections continue to be undiagnosed or misdiagnosed for the lack of reliable immunoassays (with the notable exceptions of RSV and influenza) and the complexity of monoclonal antibodies- and PCR-based tests. The seasonal and geographical distribution and the pathogenic role of newly identified viral pathogens and of viruses considered mild pathogens such as rhinovirus and coronavirus, can be assessed given that a reliable identification is undertaken. The recent outbreaks of SARS-CoV and the possible emergence of a recombinant avian-human influenza virus, given the non-specific initial signs and symptoms of patients, indicate the advantages of rapid diagnosis of respiratory infections. The availability of new classes of drugs active against both influenza A and B, and the forthcoming use of drugs targeted at RSV, rhinovirus and adenoviruses should encourage further reliable detection of respiratory viruses.

ACKNOWLEDGMENTS

This study was supported by grants to G.A. from Università "La Sapienza" (fondi Ateneo).

REFERENCES

Bastien N, Robinson JL, Tse A, Lee BE, Hart L, Li Y. 2005. Human coronavirus NL63 infections in children: A 1-year study. J Clin Microbiol 43:4567–4573.

Claas EC, Sprenger MJ, Kleter GE, van Beek R, Quint WG, Masurel N. 1992. Type-specific identification of influenza viruses A, B and C by the polymerase chain reaction. J Virol Methods 39:1–13.

Crowe JE, Jr. 2004. Human metapneumovirus as a major cause of human respiratory tract disease. Pediatr Infect Dis J 23:S215–S221.

Deffernez C, Wunderli W, Thomas Y, Yerly S, Perrin L, Kaiser L. 2004. Amplicon sequencing and improved detection of human rhinovirus in respiratory samples. J Clin Microbiol 42:3212–3218.

Echevarria JE, Erdman DD, Swierkosz EM, Holloway BP, Anderson LJ. 1998. Simultaneous detection and identification of human parainfluenza viruses 1, 2, and 3 from clinical samples by multiplex PCR. J Clin Microbiol 36:1388–1391.

Esper F, Weibel C, Ferguson D, Landry ML, Kahn JS, 2005a. Evidence of a novel human coronavirus that is associated with respiratory tract disease in infants and young children. J Infect Dis 191:492–498.

Esper F, Shapiro ED, Weibel C, Ferguson D, Landry ML, Kahn JS. 2005b. Association between a novel human coronavirus and Kawasaki disease. J Infect Dis 191:499–502.

Fan J, Henrickson KJ. 1996. Rapid diagnosis of human parainfluenza virus type 1 infection by quantitative reverse transcription-PCR-enzyme hybridization assay. J Clin Microbiol 34:1914–1917.

Garbino J, Gerbase MW, Wunderli W, Deffernez C, Thomas Y, Rochat T, Ninet B, Schrenzel J, Yerly S, Perrin L, Soccal PM, Nicod L, Kaiser L. 2004. Lower respiratory viral illnesses: Improved diagnosis by molecular methods and clinical impact. Am J Respir Crit Care Med 170:1197–1203.

Gerna G, Campanini G, Rovida F, Percivalle E, Sarasini A, Marchi A, Baldanti F. 2006. Genetic variability of human coronavirus OC43-, 229E-, and NL63-like strains and their association with lower respiratory tract infections of hospitalized infants and immunocompromised patients. J Med Virol 78:938–949.

Griffin MR, Walker FJ, Iwane MK, Weinberg GA, Staat MA, Erdman DD, New Vaccine Surveillance Network Study Group. 2004. Epidemiology of respiratory infections in young children: Insights from the new vaccine surveillance network. Pediatr Infect Dis J 23:188–192.

Grondahl B, Puppe W, Hoppe A, Kuhne I, Weigl JA, Schmitt HJ. 1999. Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: Feasibility study. J Clin Microbiol 37:1–7.

Jacques J, Bouscambert-Duchamp M, Moret H, Carquin J, Brodard V, Lina B, Motte J, Andreoletti L. 2006. Association of respiratory picornaviruses with acute bronchiolitis in French infants. J Clin Virol 35:463–466.

Legg JP, Warner JA, Johnston SL, Warner JO. 2005. Frequency of detection of picornaviruses and seven other respiratory pathogens in infants. Pediatr Infect Dis J 24:611–616.

Legoff J, Guerot E, Ndjoyi-Mbiguino A, Matta M, Si-Mohamed A, Gutmann L, Fagon JY, Belec L. 2005. High prevalence of respiratory viral infections in patients hospitalized in an intensive care unit for acute respiratory infections as detected by nucleic acid-based assays. J Clin Microbiol 43:455–457.

Louie JK, Hacker JK, Gonzales R, Mark J, Maselli JH, Yagi S, Drew WL. 2005. Characterization of viral agents causing acute respiratory infection in a San Francisco University Medical Center Clinic during the influenza season. Clin Infect Dis 41:822–828.

Maggi F, Piferi M, Vatteroni M, Fornai C, Tempestini E, Anzilotti S, Lanini L, Andreoli E, Ragazzo V, Pistello M, Specter S, Bendinelli M. 2003. Human metapneumovirus associated with respiratory tract infections in a 3-year study of nasal swabs from infants in Italy. J Clin Microbiol 41:2987–2991.

Medici MC, Arcangeletti MC, Rossi GA, Lanari M, Merolla R, Paparatti UD, Chezzi C, Osservatorio VRS Study Group. 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.

Monto AS. 2004. Occurrence of respiratory virus: Time, place and person. Pediatr Infect Dis J 23:S58–S64.

Mosser AG, Brockman-Schneider R, Amineva S, Burchell L, Sedgwick JB, Busse WW, Gern JE. 2002. Similar frequency of rhinovirus-infectible cells in upper and lower airway epithelium. J Infect Dis 185:734–743.

Nicholson KG, McNally T, Silverman M, Simons P, Stockton JD, Zambon MC. 2006. Rates of hospitalisation for influenza, respiratory syncytial virus and human metapneumovirus among infants and young children. Vaccine 24:102–108.

Osiowy C. 1998. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J Clin Microbiol 36:149–154.

Papadopoulos NG, Bates PJ, Bardin PG, Papi A, Leir SH, Fraenkel DJ, Meyer J, Lackie PM, Sanderson G, Holgate ST, Johnston SL. 2000. Rhinoviruses infect the lower airways. J Infect Dis 181:1875–1884.

Papadopoulos NG, Moustaki M, Tsolia M, Bossios A, Astra E, Prezerakou A, Gourgiotis D, Kafetzis D. 2002. Association of rhinovirus infection with increased disease severity in acute bronchiolitis. Am J Respir Crit Care Med 165:1285–1289.

Paton AW, Paton JC, Lawrence AJ, Goldwater PN, Harris RJ. 1992. Rapid detection of respiratory syncytial virus in nasopharyngeal aspirates by reverse transcription and polymerase chain reaction amplification. J Clin Microbiol 30:901–904.

Peret TC, Boivin G, Li Y, Couillard M, Humphrey C, Osterhaus AD, Erdman DD, Anderson LJ. 2002. Characterization of human metapneumoviruses isolated from patients in North America. J Infect Dis 185:1660–1663.

Pitkaranta A, Arruda E, Malmberg H, Hayden FG. 1997. Detection of rhinovirus in sinus brushings of patients with acute community-acquired sinusitis by reverse transcription-PCR. J Clin Microbiol 35:1791–1793.

Rovida F, Percivalle E, Zavattoni M, Torsellini M, Sarasini A, Campanini G, Paolucci S, Baldanti F, Revello MG, Gerna G. 2005. Monoclonal antibodies versus reverse transcription-PCR for detection of respiratory viruses in a patient population with respiratory tract infections admitted to hospital. J Med Virol 75:336–347.

Sarasini A, Percivalle E, Rovida F, Campanini G, Genini E, Torsellini M, Paolucci S, Baldanti F, Marchi A, Revello MG, Gerna G. 2006. Detection and pathogenicity of Human Metapneumovirus respiratory infections in pediatric Italian patients during a winter-spring season. J Clin Virol 35:59–68.

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.

Shimizu C, Shike H, Baker SC, Garcia F, van der Hoek L, Kuijpers TW, Reed SL, Rowley AH, Shulman ST, Talbot HK, Williams JV, Burns JC. 2005. Human coronavirus NL63 is not detected in the respiratory tracts of children with acute Kawasaki disease. J Infect Dis 192:1767–1771.

Vabret A, Dina J, Gouarin S, Petitjean J, Corbet S, Freymuth F. 2006. Detection of the new human coronavirus HKU1: 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 den Hoogen BG, van Doornum GJ, Fockens JC, Cornelissen JJ, Beyer WE, de Groot R, Osterhaus AD, Fouchier RA. 2003. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. J Infect Dis 188:1571–1577.

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.

Wolf DG, Greenberg D, Kalkstein D, Shemer-Avni Y, Givon-Lavi N, Saleh N, Goldberg MD, Dagan R. 2006. Comparison of human metapneumovirus, respiratory syncytial virus and influenza A virus lower respiratory tract infections in hospitalized young children. Pediatr Infect Dis J 25:320–324.

Woo PC, Lau SKP, 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. 2005a. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J Virol 79:884–895.

Woo PC. Lau SKP, Tsoi HW, Huang Y, Poon RW, Chu CM, Lee RA, Luk WK, Wong GK, Wong BH, Cheng VC, Tang BS, Wu AK, Yung RW, Chen H, Guan Y, Chan KH, Yuen KY. 2005b. Clinical and molecular epidemiological features of coronavirus HKU1-associated community-acquired pneumonia. J Infect Dis 192:1898–1907.