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Monoclonal Antibodies Versus Reverse Transcription-PCR for Detection of Respiratory Viruses in a Patient Population With Respiratory Tract Infections Admitted to Hospital

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In the winter season 2001–2002, 239 nasopharyngeal aspirate and 15 bronchoalveolar lavage samples from 208 patients (135 pediatric and 73 adults, including 19 lung transplant recipients) admitted to hospital because of an acute respiratory tract infection were examined for rapid diagnosis of respiratory viruses by two diagnostic approaches: immunological, using specific monoclonal antibodies (MAb); and molecular, using specific reverse transcription (RT)-PCR assays. Both methods detected influenza viruses A (H1N1 and H3N2) and B, human parainfluenza virus types 1 to 3, human respiratory syncytial virus (hRSV) types A and B, and human adenoviruses. In addition, human coronavirus (hCoV) groups I (229E-like) and II (OC43-like), as well as the new human metapneumovirus (hMPV), types A and B, were searched for by RT-PCR alone. When results obtained by both methods were added, the overall percentage of patients positive for at least one respiratory virus peaked at 44.2%, involving 92/208 patients (81 pediatric, and 11 adults), while 116 patients (55.8%) were negative for any respiratory virus tested. The most common circulating virus was hRSV, infecting 54 (25.9%) patients (24 type A, and 30 type B strains), followed by hMPV, infecting 12 (5.8%) patients (7 type A and 5 type B strains). Coinfections by two respiratory viruses interested 11 (5.3%) patients, and 9 (81.8%) of these were infected by hRSV in association with another respiratory virus. In the great majority of infected children, hRSV and hMPV were associated with lower respiratory tract infections. In lung transplant recipients, viruses present in bronchoalveolar lavage appeared to be associated frequently with lower respiratory tract infections. In conclusion: the combination of immunological and molecular assays is the most sensitive approach to the diagnosis of respiratory viral infections; and infections caused by the less investigated hCoVs and hMPVs represent a fair proportion of respiratory infections. ***J. Med. Virol. 75:336–347, 2005.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: human metapneumovirus; human coronavirus; human respiratory syncytial virus; respiratory coinfections; monoclonal antibody; RT-PCR

INTRODUCTION

Until three decades ago, the diagnosis of respiratory virus infection required virus isolation and identification in embryonated hens eggs or in cell cultures, and this procedure was cumbersome and time-consuming. At that time, rapid diagnosis of respiratory virus infections in cells from respiratory tract started by using immunofluorescence and cross-absorbed animal immune sera [Gardner and Mc Quillin, 1974]. In the 1990s, monoclonal antibodies (MAb) specific for the different conventional respiratory viruses became available, thus rendering rapid diagnosis of respiratory tract infections in nasopharyngeal aspirates more reliable [Stout et al., 1989; Navarro-Mari et al., 1999; Fong et al., 2000]. In parallel, new cell culture systems with increased susceptibilities to respiratory viruses were identified, in addition to primary monkey kidney cell cultures. Thus, Madin–Darby canine kidney (MDCK) cells were found to be highly susceptible to influenza

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viruses A and B, LLC-MK2 to human parainfluenza viruses [Frank et al., 1979; Shih et al., 1999], human lung carcinoma cells (A549) to human adenoviruses [Woods and Young, 1988; Smith et al., 1991], and mink lung epithelial (Mv1Lu) cells to several influenza viruses A and B as well as other respiratory viruses [Huang and Turchek, 2000]. Thus, at the moment, fluorescent MAbs and appropriate cell culture systems, including mixed Mv1Lu and A549 cell cultures [Huang and Turchek, 2000], are widely used for optimal detection of respiratory viruses in nasopharyngeal secretions [Percivalle et al., 1989; Navarro-Mari et al., 1999; Shih et al., 1999; Zavattoni et al., 2003].

More recently, the polymerase chain reaction (PCR) and the reverse transcriptase-PCR (RT-PCR) have contributed substantially to rapid molecular diagnosis of acute respiratory tract infections [Freymuth et al., 1997; Fan et al., 1998; Kehl et al., 2001; Mullins et al., 2004; Williams et al., 2004]. However, whether the immunological and the molecular approach to diagnosis of respiratory tract infections are to be considered complementary or alternative, and which one of the two techniques is more cost-effective, remains to be defined.

In addition, the difficulty in virus isolation and cell culture propagation of conventional human respiratory coronaviruses (hCoVs) [Lai and Holmes, 2001] as well as human metapneumoviruses (hMPV) [van den Hoogen et al., 2001], and the consequent lack of specific and reliable immunological reagents have limited our knowledge on the circulation of these viruses in some geographic areas, which have been only investigated recently by molecular methods. Finally, the rate and the role of respiratory virus coinfections in respiratory tract pathology remains substantially undefined.

In the present report, the prevalence and the pathogenic role of conventional viruses (detected by both immunologic and molecular methods) as well as the new viruses detected only by molecular methods, along with the role of respiratory virus coinfections, were investigated in a patient population (both infants and adults) admitted to hospital with acute respiratory infections.

MATERIALS AND METHODS

Patients and Specimens

In the winter–spring season 2001–2002 (October, 2001 through May, 2002), 239 nasopharyngeal aspirates from 208 (135 pediatric and 73 adult) patients, admitted to hospital because of an episode of acute respiratory tract infection, were collected and examined for presence of respiratory viruses. Of the 73 adult patients (median age 51, range 16–81 years), 19 were lung transplant recipients (median age 45, range 24–65 years), from whom 15 bronchoalveolar lavage and 14 nasopharyngeal aspirate samples were taken during follow-up. Upon specimen reception and following homogenization, each respiratory sample was divided into four aliquots: one was used immediately for direct fluorescent antibody staining of respiratory cells present in respiratory secretions; the second was also used immediately and inoculated onto cell cultures for virus isolation; the third was frozen immediately and then used retrospectively for molecular assays; and the fourth was subaliquoted and stored frozen as a back-up sample for future testing or studies. All samples were examined by two diagnostic approaches: immunological and molecular. The immunological approach was adopted prospectively and based on use of MAb for virus identification in both respiratory secretion cells and inoculated cell cultures. The molecular approach was adopted retrospectively and was based on use of RT-PCR, which was performed directly on nucleic acids extracted from respiratory samples. Following reverse transcription, cDNA was used for all RT-PCR assays as well as repeat testing. Viruses sought for by both methods were influenza viruses A (H1N1 and H3N2) and B, human parainfluenza viruses types 1 to 3 (hPIV 1-3), human respiratory syncytial virus (hRSV), and human adenoviruses (hAdVs). In addition, hCoV, groups I (229E-like) and II (OC43-like), as well as hMPV, types A and B, were sought by RT-PCR only. The hCoVs were grouped by sequencing and hMPVs were typed by sequencing and phylogenetic analysis.

Immunological Method

A pool of MAbs (MAbP), allowing simultaneous detection of influenza viruses A and B, hPIV 1-3, hRSV, and hAdV, as well as individual MAbs identifying single respiratory viruses were used for diagnosis of respiratory virus infections by direct fluorescent antibody staining of both respiratory cells and shell vial or conventional cell cultures. The MAbP used in the study (SimulFluor Respiratory Screen, Chemicon International, Inc., Temecula, CA) contains two components. One component is a pool of fluorescein-conjugated MAbs staining bright apple-green influenza viruses A and B, hPIV 1-3-, hRSV-, and hAdV-infected cells without differentiating among these viruses. The other component is a rhodamine-conjugated MAb directed against hRSV and staining yellow-gold hRSV-infected cells, thereby differentiating hRSV from the other respiratory viruses. The fluorescein-conjugated MAbs to individual respiratory viruses were obtained from the same commercial source (Chemicon). MAb to hCoV OC43 (Chemicon) was preliminarily found to perform satisfactorily for virus identification of the reference strain as well as cells from respiratory secretions positive by RT-PCR, whereas MAb to hCoV 229E was not able to identify the relevant prototype virus. No MAb to hMPV was found to be available commercially.

Direct Fluorescent Antibody Staining

An aliquot of each respiratory sample was vortexed and washed repeatedly for direct staining of respiratory cells, as reported previously [Zavattoni et al., 2003]. Cell pellet was suspended in 100–200 μl PBS and cells were spotted onto two slides. Slides were air-dried and fixed with acetone. The first slide was stained with MAbP (two spots). A yellow-gold fluorescence identified hRSV-

positive cells in the nasopharyngeal aspirate. If an apple-green fluorescence pattern was observed, the second slide was stained with individual MAbs, except for MAb to hRSV. Slides containing no epithelial respiratory cells or an insufficient number of epithelial respiratory cells were scored as inadequate. All slides were read blindly by two independent observers.

Cell Lines

A549 and Mv1Lu cells were obtained from ATCC (Rockville, MD) at passage 77 and 44, respectively. A mixture (MIX) of A549 and Mv1Lu was used for shell vial culture (SVC) at a ratio of 1:1. HEp-2, LLC-MK2, and MDCK cell lines were originally obtained from the same source and subcultured in the laboratory. Cell culture medium was E-MEM supplemented with penicillin–streptomycin (1%), glutamine (1%), and fetal calf serum (FCS).

Shell Vial Cultures

Nasopharyngeal secretion samples were processed immediately upon arrival in the laboratory, and an aliquot was placed in viral transport medium [consisting of E-MEM supplemented with penicillin–streptomycin, gentamycin and neomycin (500 μg/ml), fungizone (25 μg/ml), glutamine 1%, and FCS 0.5%] for 1 hr prior to inoculation onto cell cultures, as reported [Zavattoni et al., 2003]. As a rule, 200 μl of each specimen processed for cell cultures were inoculated onto each of two shell vials of LLC-MK2, MDCK, and MIX cell cultures. Shell vials were centrifuged at 1,800 rpm for 45 min and incubated at 33°C in a 5% CO_2 atmosphere. After 48 hr, one shell vial for each cell type was fixed with methanol-acetone and stained with the MAbP. hRSV was identified if a yellow-gold pattern of fluorescence was detected. If an apple-green pattern of fluorescence was observed, the second shell vial was trypsinized, cells spotted onto one slide (eight spots) and stained with individual MAbs, except hRSV, for final identification. In case of negative results at 48 hr, the second shell vial was trypsinized and cells stained 96 hr p.i.

Conventional Virus Isolation and Identification by Cell Cultures

All types of cell cultures used (HEp-2, LLC-MK2, and MDCK) were inoculated with 200 μl of specimen (two tubes for each cell line), incubated at 33°C in a stationary position and observed daily for cytopathic effect up to 7 days, as reported [Zavattoni et al., 2003]. Both cell cultures positive and negative for cytopathic effect were trypsinized, washed, and resuspended in PBS, and finally spotted onto two slides: the first slide had two spots, and the second slide eight spots. Slides were then air-dried and fixed with cold acetone. The first slide was stained with the MAbP (both spots). hRSV was identified in the presence of yellow-green fluorescence. If an apple-green fluorescence was observed, the second slide was stained with all individual fluorescent MAbs except hRSV. In case of negative results, cultures were kept under observation for 2 additional weeks.

Optimized RT-PCR Protocols

Optimized RT-PCR protocols for the identification of respiratory viruses were developed, as shown in Table I. Primers were either selected from published protocols or originally designed from GenBank published virus sequences. Reference strains were propagated in cell cultures and virus RNA or DNA was extracted by using the NucliSens® Isolation Reagents procedure (BioMérieux, Boxtel, NL) for the simultaneous recovery of both

TABLE I. PCR Parameters Used for Detection of Different Respiratory Viruses in Nasopharyngeal Aspirate Samples

Virus	Target gene (nt-nt)	Thermal profile	Cycle no.	Reference/primer pair
Influenza A H1N1	HA (44–1,058)	94°C/1′ 60°C/1′ 72°C/1′	10[a]	Stockton et al. [1998]
		94°C/1′ 52°C/1′ 72°C/1′	40	
Influenza A H3N2	HA (291–897)	94°C/1′ 60°C/1′ 72°C/1′	10[a]	Forward: 5′-ccttttttgttgaacgcagcaa-3′
		94°C/1′ 52°C/1′ 72°C/1′	40	Reverse: 5′-gcttccatttggagtgatgcat-3′
Influenza B	HA (154–1,053)	94°C/1′ 60°C/1′ 72°C/1′	10[a]	Stockton et al. [1998]
		94°C/1′ 52°C/1′ 72°C/1′	40	
hPIV-1	HN (7,692–7,936)	94°C/1′ 55°C/1′ 72°C/1′	40	Forward: 5′-ggaacaaggggttatcagtt-3′ Reverse: 5′-ggagttgttaagccaaagta-3′
hPIV-2	HN (7,498–7,733)	94°C/1′ 55°C/1′ 72°C/1′	40	Forward: 5′-ggaatcaatcgcaaaagctgt-3′ Reverse: 5′-cctagatgatagatcccgctt-3′
hPIV-3	HN (762–1,239)	94°C/1′ 55°C/1′ 72°C/1′	40	Echevarria et al. [1998]
hRSV subtype A + B (I step)	F (1–705)	94°C/30″ 50°C/2′ 68°C/1′	45	Coiras et al. [2003]
hRSV subtype A (II step)	F (347–682)	94°C/30″ 55°C/1′ 72°C/1′	35	Coiras et al. [2003]
hRSV subtype B (II step)	F (30–614)	94°C/30″ 55°C/1′ 72°C/1′	35	Coiras et al. [2003]
hAdV	Hexon (21–321)	94°C/30″ 55°C/30″ 72°C/30″	35	Allard et al. [1991]
hMPV subtype A	L (4,121–4,290)	94°C/30″ 47°C/30″ 72°C/30″	40	Van den Hoogen et al. [2002]
hMPV subtype B	L (4,121–4,290)	94°C/30″ 47°C/30″ 72°C/30″	40	Forward: 5′-tatgcctactataaaaggtcag-3′ Reverse: 5′-caccccagtctttcctaaag-3′
hCoV (229E and OC43)	ORF 1b (14,321–14,536)	94°C/15″ 60°C/15″ 72°C/30″	10	Poutanen et al. [2003]
		95°C/15″ 56°C/15″ 72°C/30″	40	

[a]Annealing temperature decreasing by 1°C/cycle.

nucleic acids, which were submitted to RT-PCR. Amplification products were then cloned in PCR2.1 plasmid vector (TA Cloning Kit, Invitrogen, Carlsbad, CA) to achieve quantitative standards for the determination of the analytical sensitivity of each RT-PCR assay. In initial experiments, carried out according to amplification protocols reported in the literature, a wide variability in the analytical sensitivity of individual amplification assays was observed (range 10^0–10^5 input copies). Therefore, all RT-PCR reactions were optimized to detect at least 10 input plasmid copies. To this purpose, while the reported thermal profiles or reaction mixtures had to be modified for some viruses, new sets of primers were needed for other viruses (Table I). The RT reaction was performed in a final volume of 120 μl, using 12 μl of extracted nucleic acids, 600 pmol of random examer primers (New England Biolabs, Beverly, MA) and 240 U of SuperScript™II RNase H-Reverse Transcriptase (Invitrogen). PCR reactions were performed in a final volume of 50 μl using 10 μl cDNA (containing 1 μl extracted DNA for hAdVs amplification), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (for hRSV, hAdV, hMPV, and hCoV), 2.0 mM $MgCl_2$ (for influenza A H1N1 and H3N2, and influenza B), and 3.0 mM $MgCl_2$ (for hPIV-1, hPIV-2, and hPIV-3), 0.001% gelatin, 250 mM dNTP, 25 pmol each primer and 1.5 U of Taq polymerase (Roche Diagnostics GmbH, Mannheim, Germany). Table I reports the amplified fragment of the target gene, the thermal profiles, the cycle number, and when appropriate, the relevant reference for each virus. PCR products were examined on 3% agarose gel.

hMPV Phylogenetic Analysis

Strains of hMPV were classified into types A and B according to a recent report [van den Hoogen et al., 2004a]. Viral sequences of hMPV isolates from 12 patients as well as 3 reference strains (NL/1/00, CAN97-83, CAN98-75) 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). Distances between pairs of nucleotide sequences were calculated by using the DNAdist (with Kimura's two-parameter method) modules in the Philip package, version 3.572 (Felsenstein, Department of Genetics, University of Washington, Seattle, WA). The percent genetic distances within the L gene were calculated between the two major branches of the tree. The Philip (njplot) program was used to construct phylogenetic trees with nucleotide sequences by means of the neighbor-joining method from the same distance matrices. Bootstrap support was determined by 100 resampling of the sequences.

RESULTS

Optimized RT-PCR Assays

RT-PCR assays used in this study consisted of a single RT reaction followed by PCR thermal profiles specific for each of the following groups of viruses: (i) influenza A (H1N1), influenza A (H3N2) and influenza B; (ii) hPIV-1, hPIV-2, and hPIV-3; (iii) hRSV (subtypes A and B); (iv) hMPV (subtypes A and B); and (v) hCoV (groups I 229E-like, and II OC43-like). The sole DNA virus amplified by PCR was hAdV (Table I). However, being genomic DNA unmodified by the RT step, the same volume of RT reaction used for detection of all other viruses was also utilized for amplification of hAdV. Amplification of cells infected with reference respiratory virus strains showed absence of both non-specific amplification products and cross-reactions. In addition, all amplification reactions were able to detect at least 10 input plasmid copies for each virus (Fig. 1).

Immunological Detection

MAbs were applied to virus detection by direct fluorescent staining, shell vial culture, and conventional cell cultures. A total of 239 respiratory secretion samples from 208 patients were examined for detection of conventional respiratory viruses. In addition, 15 bronchoalveolar lavage samples from lung transplant recipients were examined using the same procedure. As mentioned, hCoV 229E and hMPV were excluded from the immunological detection due to the unavailability of specific MAbs. The ratio of positive to examined nasopharyngeal aspirate samples for each method was 53/144 (36.8%) for direct fluorescent staining, 76/236 (32.2%) for shell vial culture, and 67/210 (31.9%) for conventional cell cultures. Thus, direct fluorescent staining appeared slightly more sensitive than shell vial culture and conventional cell cultures. Examples of direct staining of respiratory cells positive for different respiratory viruses in nasopharyngeal secretions are given in Figure 2, panels 1–6.

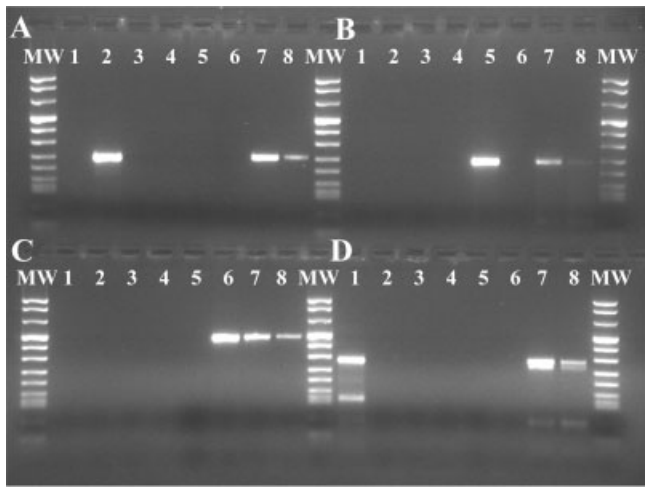

Fig. 1. Agarose gel detection of PCR products obtained using the same thermal profile, following reverse transcription of viral RNA extracted from nasopharyngeal aspirates nos. 1–6. **A**: PCR for hPIV-1. **Lanes 1–6**: Sample no. 2 positive for hPIV-1; **lanes 7–8**, 100 and 10 copies of hPIV-1 plasmid. **B**: PCR for hPIV-2. **Lanes 1–6**: sample no. 5 positive for hPIV-2; **lanes 7–8**, 10 and 1 copy of hPIV-2 plasmid. **C**: PCR for hPIV-3. **Lanes 1–6**: Sample no. 6 positive for hPIV-3; **lanes 7–8**, 100 and 10 copies of hPIV-3 plasmid. **D**: PCR for hAdV. **Lanes 1–6**: Sample no. 1 positive for hAdV; **lanes 7–8**, 100 and 10 copies of hAdV plasmid. MW, molecular weights markers.

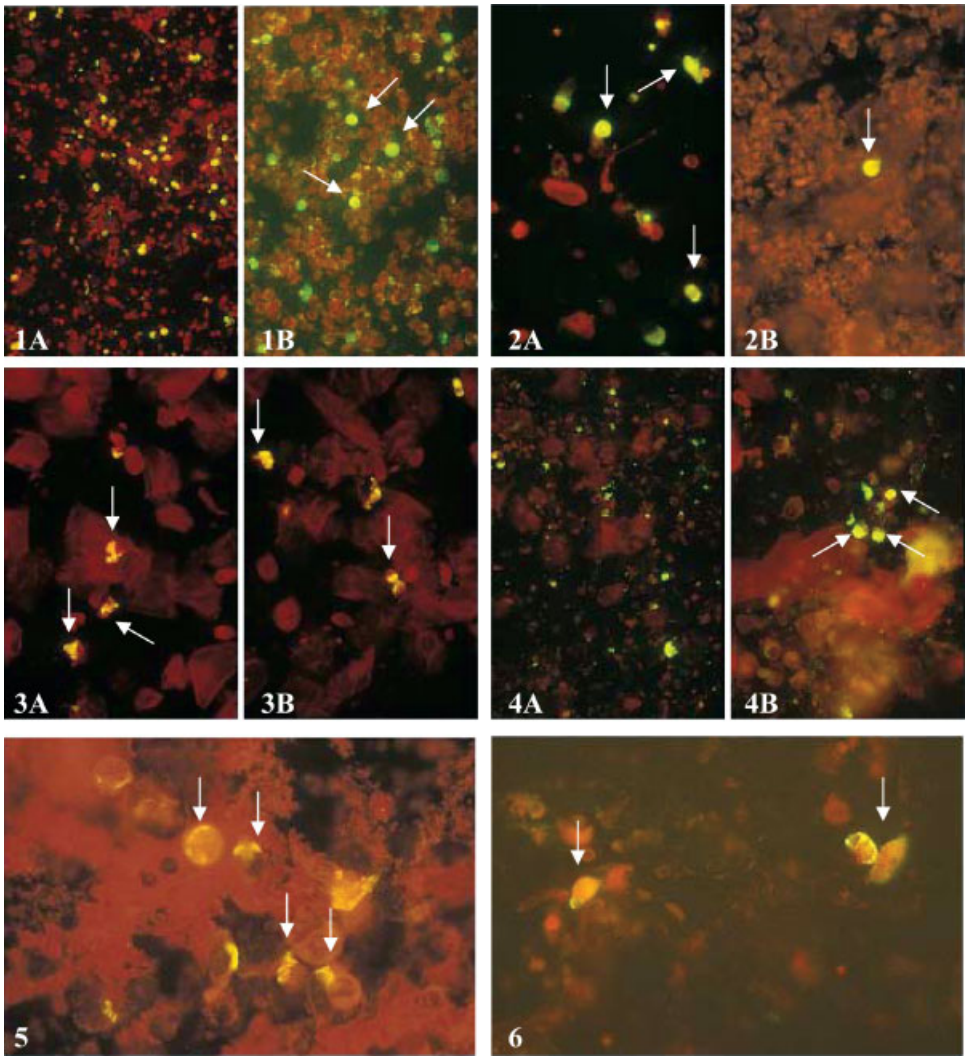

Fig. 2. Direct fluorescent antibody detection of respiratory viruses in respiratory cells from nasopharyngeal aspirates using specific monoclonal antibodies. **1A**: Several influenza virus A-infected fluorescent cells are scattered throughout the smear (low magnification); **1B**: multiple influenza virus A-infected fluorescent cells (arrows) are spread among uninfected respiratory cells (higher magnification). **2A**: Influenza virus B-infected fluorescent cells (arrows) are interspersed among few uninfected cells. **2B**: A single influenza virus B-infected fluorescent cell (arrow) at the centre of the smear. **3A**: Four hRSV-infected fluorescent cells (three indicated by arrows). **3B**: hRSV-infected fluorescent cells (arrows) are spread among uninfected cells. **4A**: Several hPIV-positive respiratory cells are visible throughout the smear (low magnification). **4B**: hPIV-infected fluorescent cells (arrows) at the centre of the smear (higher magnification). **5**: hAdV-infected fluorescent cells (arrows) in the central area of the smear. **6**: A few hCoV OC43-positive cells (arrows) show fluorescent cytoplasm.

Monoclonal Antibodies Versus RT-PCR

When the 208 patients were stratified according to conventional respiratory viruses searched for by both immunological and molecular methods (thus excluding hCoV and hMPV), 70 were found to be positive by both MAbs and RT-PCR, 3 by MAbs only, 4 by RT-PCR only, while 131 were negative by both methods (Table II). Thus, MAbs were able to detect one or more respiratory viruses in 73 (35.1%), and RT-PCR in 74 (35.6%)

TABLE II. Ability of MAbs and PCR to Detect Conventional and Unconventional Respiratory Viruses in Nasopharyngeal Aspirate Samples From 208 Patients

Respiratory viruses	No. (%) positive patients			No. (%) negative patients
	MAbs	PCR	Total	
Conventional (influenza + hPIV + hRSV + hAdV)	73 (35.1)	74 (35.6)	77 (37.0)	131 (63.0)
Unconventional (hMPV + hCoV)	NA[a]	15 (7.2)	15 (7.2)	193 (92.8)
Total	73 (35.1)	89 (42.8)	92 (44.2)	116 (55.8)

[a]NA, not applicable.

patients, while the overall ability of both methods to detect respiratory virus infections interested 77 (37.0%) patients. When hCoV and hMPV (detected by RT-PCR only) were included in the overall evaluation of patients positive for one or more respiratory viruses, the number of RT-PCR-positive patients went up to 89 (42.8%), and the total number of patients positive for respiratory viruses was 92/208 (44.2%).

In more detail, comparison of the immunological and the molecular approach for the diagnosis of individual conventional respiratory virus infections in the 197 hospitalized patients in whom no double infection was detected, showed that 59/66 positive patients (89.4%) had the same virus identified by both methods, while of the 7 patients with discrepant results, 1 was found positive for influenza A by RT-PCR only, and 6 were found positive for hRSV by either method (3 by MAbs, and 3 by RT-PCR), 131 patients remaining consistently negative (Table III).

Testing of the same 197 patients by RT-PCR for hCoV and hMPV revealed 3 additional patients positive for hCoV (one 229E-like, and two OC43-like strains), and 12 additional patients positive for hMPV (7 strains belonging to type A, and 5 to type B). Thus, the total number of patients positive for a single respiratory virus was 81/197 (41.1%). As already mentioned, the unavailability of valuable MAbs specific for these two groups of viruses prevented the comparison of the two methods for the detection of these two respiratory viruses.

TABLE III. Detection of Respiratory Viruses by MAb Versus RT-PCR in 197 Patients (226 Nasopharyngeal Aspirate Samples) With Single Acute Respiratory Infection

	No. of patients (nasopharyngeal aspirates) examined by RT-PCR	
MAb detection	Positive	Negative
Influenza A		
Positive	0	0
Negative	1 (1)	196 (225)
Influenza B		
Positive	4 (4)	0
Negative	0	193 (222)
hPIVs		
Positive	6 (7)	0
Negative	0	191 (219)
hRSV		
Positive	48 (55)	3 (3)
Negative	3 (4)	143 (164)
hAdV		
Positive	1 (1)	0
Negative	0	196 (225)
hCoV		
ND[a]	3 (3)	194 (223)
hMPV		
ND	12 (12)	185 (214)

[a]ND, not done.

Coinfections

Among the 208 patients examined, 11 cases of coinfection or double infection (5.3%) were detected (6 infants and 5 adults, of whom 3 were lung transplant recipients). The 11 cases were numbered and reported in Table IV. Thus, in our series, the overall number of patients infected by one or two respiratory viruses was 92 (44.2%), as mentioned above. A single case of coinfection (patient no. 5) was detected by using MAbs (1/11, 9.1%), whereas as many as 6/11 (54.5%) cases were identified by using PCR ($P = 0.06$, Chi square exact Fisher test). As reported in Table IV, while RT-PCR detected a coinfection in patients nos. 1–6, MAbs detected a double infection only in patient no. 5. Of the 11 patients with coinfection, as many as 9 (5 children and 4 adults) were infected by hRSV in association with another respiratory virus. Patient no. 10 was coinfected by both influenza viruses A and B. Of the five adult patients with coinfection and a lower respiratory tract infection, as many as three were lung transplant recipients. Finally, patient no. 3 had a coinfection by hRSV and hCoV 229E-like, while patient no. 7 had a double infection by hPIV and hMPV type A.

Incidence of Respiratory Virus Infections and Their Distribution by Month and Age

On the whole, 92/208 (44.2%) patients were positive for one or more respiratory viruses (81 infants and 11 adults) by either the molecular or the immunological detection, while 116 (55.8%) were negative. In detail, hRSV (24 type A, and 30 type B strains) was detected in 54 (25.9%) patients and was the most highly circulating virus, followed by hMPV (12 patients, 5.8%) and coinfections (11 patients, 5.3%). During the winter season October 2001 through May 2002, the maximal incidence of respiratory viral infections occurred between January and April 2002 with the peak in March 2002 (Fig. 3A). In addition, the distribution of respiratory infections within the first year of age showed that of 68 infants and young children aged $\leq$12 months (out of the 81 children with respiratory infections of the study) as many as 49 were affected by hRSV and, of these, 32 (65.3%) were in the first 4 months of life, while 6/8 (75.0%) children affected by hMPV were included in the range of 5–10 months of age (Fig. 3B). No major age-related peaks were observed for the other respiratory viruses within the first year of age. This data seems to indicate a trend towards an earlier predominant pathogenicity of hRSV in early infancy with respect to hMPV.

hMPV Infection

Following sequencing, phylogenetic analysis of hMPV strains circulating in 2001–2002 winter season indicated the simultaneous presence of the two major types (A and B) of hMPV in Northern Italy (Fig. 4). Among the 208 patients studied, 12 had a single hMPV infection. Of these, seven were positive for type A hMPV, and five for type B. As for patients infected with type A hMPV strains, five were infants within the first year of life, and two were adults. Of the five infants, one had bronchiolitis and three pneumonia (no clinical data were available for one), while the two adults both had

TABLE IV. Respiratory Virus Coinfections (Double Infections) in 11 Patients

Patient no.	Age (months)	MAb	RT-PCR	Coinfection
1	16 years	Influenza B	Influenza B + hRSV	Influenza B + hRSV
2	17	hRSV	hPIV-3 + hRSV	hRSV + hPIV-3
3	4	hRSV	hRSV + hCoV	hRSV + hCoV[a]
4	8	hRSV	hRSV + hPIV-1	hRSV + hPIV-1
5	5	hRSV + Influenza A	Influenza A (H1N1) + hRSV	hRSV + influenza A (H1N1)
6[c]	36 years	Influenza A	Influenza A (H3N2) + hRSV	hRSV + influenza A (H3N2)
7	12	hPIV	hMPV	hPIV + hMPV[b]
8	4	hAdV	hRSV	hRSV + AdV
9[c]	39 years	hPIV	hRSV	hRSV + hPIV
10	43 years	Influenza B	Influenza A (H3N2)	Influenza A (H3N2) + influenza B
11[c]	63 years	hPIV	hRSV	hRSV + hPIV

[a]Human coronavirus 229E-like (group I).
[b]Human metapneumovirus type A.
[c]Lung transplant recipient.

bronchopneumonia. Of these, one was a 61-year-old single lung transplant recipient, and the other was a 73-year-old woman admitted to the Intensive Care Unit. As for the 5 type B hMPV strains, 2 were detected in old patients with bronchitis and pneumonia, respectively, and 3 interested infants <1-year-old with bronchiolitis. In addition, one infant had a double infection by both hMPV type A and hPIV.

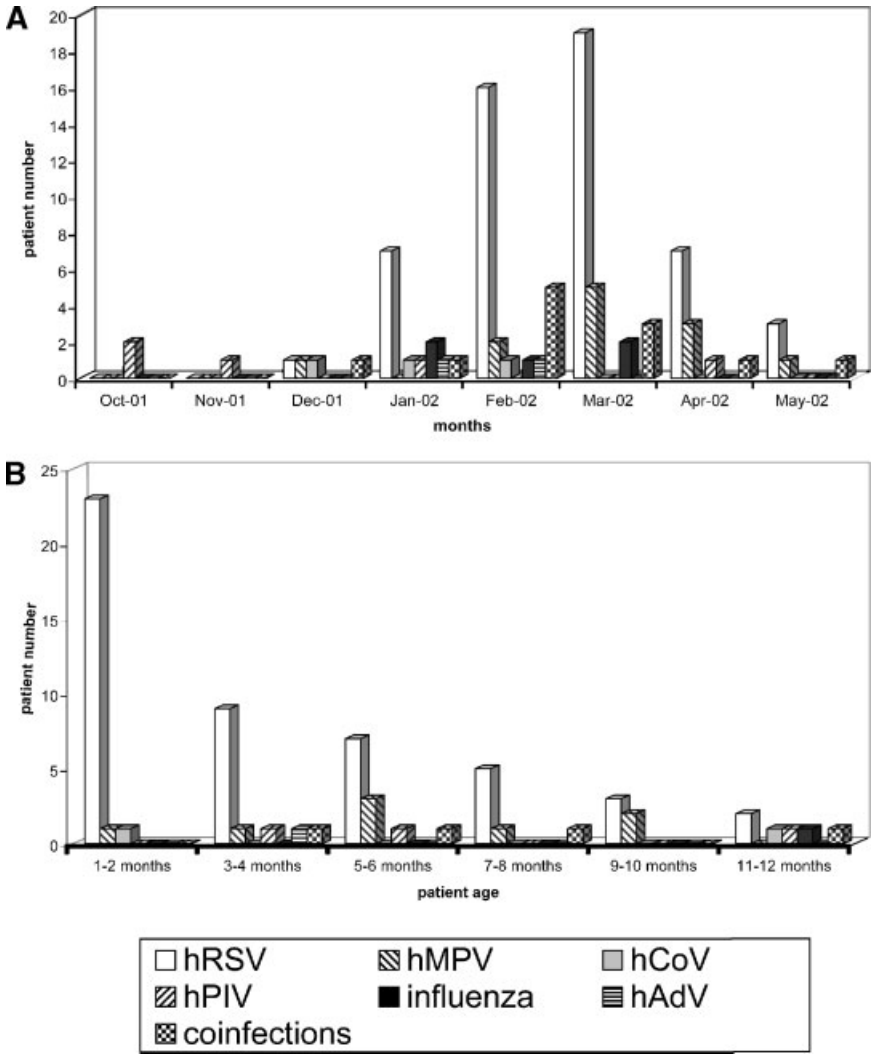

Fig. 3. **A**: Monthly incidence of viral respiratory infections in the period October 2001 through May 2002.
B: Incidence of viral respiratory infections during the first year of life.

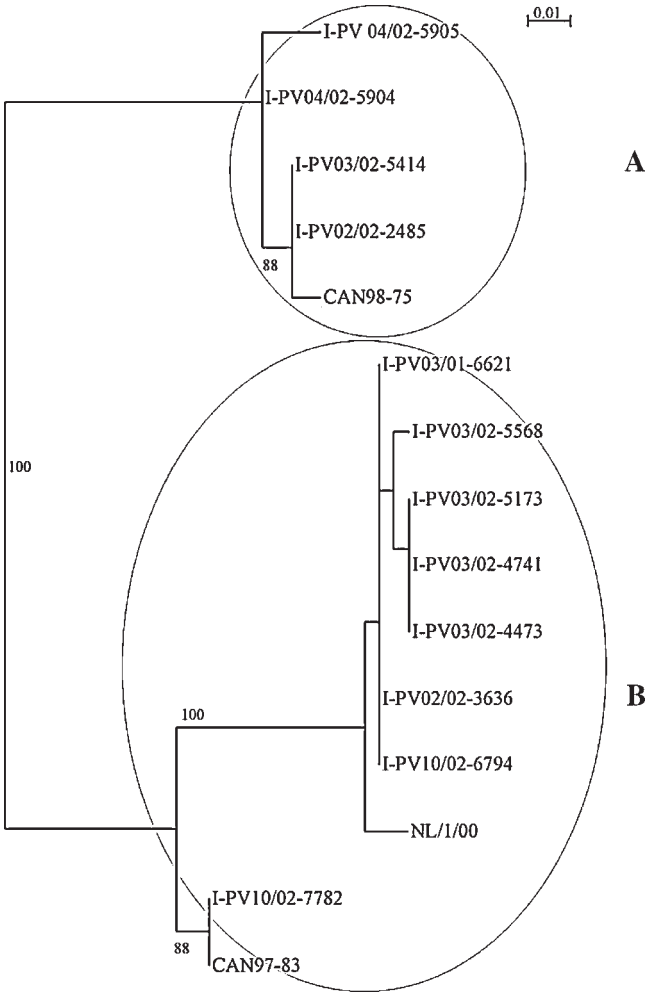

Fig. 4. Phylogenetic analysis of hMPV strains circulating in Northern Italy in the winter–spring season 2001–2002. The two major types of hMPV are indicated using the L gene and the Netherlands (NL/1/00) and Canada (CAN98-75 and CAN97-83) reference strains. The two types are designated **A** and **B** in agreement with the recent report by Van den Hoogen et al. [2004a].

hCoV Infection

Three patients (two infants and one adult) had a single hCoV infection. Following sequencing, two patients were found to be infected by an OC43-like strain, and one by a 229E-like strain. Both infants (4-, and 11-month-old) had a lower respiratory tract infection (one had bronchiolitis and one bronchopneumonia), whereas the adult patient was a lung transplant recipient with an upper respiratory infection. In addition, a 1-month-old infant had cough associated with a double infection by hCoV (229E-like strain) and hRSV.

Association of Respiratory Viruses With Upper or Lower Respiratory Tract Infections

As many as 45/49 (95.8%) hRSV-infected children had lower respiratory tract infection, including bronchiolitis and pneumonia often associated to X-ray infiltrates. Similarly, 7/8 (87.5%) hMPV-infected children (four by type A and three by type B strains) had lower respiratory tract infection. Among children, other cases of lower respiratory infections were detected in association with hPIV infection (2/5, 40%), and hCoV infection (2/2, 100%). Among adult patients, only 3/11 (27.2%) had lower respiratory infections (1 by influenza virus B, 1 by hMPV, and 1 by hCoV). Of these, two were lung transplant recipients, and one was an elderly man with chronic lung disease. In the group of coinfections, as many as nine included hRSV. Of these, five involved children with lower respiratory infections, while four involved adults with upper respiratory infections.

Respiratory Viral Infections in Lung Transplant Recipients

Of 19 lung transplant recipients examined, 9 were positive for one or two respiratory viruses. In detail, 3/15 bronchoalveolar lavage samples from 3/11 patients, and 7/14 nasopharyngeal aspirate samples from 6/9 patients were positive for respiratory viruses. The three bronchoalveolar lavage-positive samples allowed detection of two hMPV strains (1 type A, and 1 B) and one coinfection by hPIV and hRSV in the three patients with lower respiratory infection, while the seven nasopharyngeal aspirate-positive samples revealed presence of 1 hCoV (OC43-like), 2 influenza viruses B, 1 hRSV, 1 coinfection by influenza A + hRSV, and 1 coinfection by hPIV and hRSV in the six patients, respectively, affected by upper respiratory infection. Unfortunately, no simultaneous samples from both sites were taken.

DISCUSSION

A major objective of this study was to investigate whether, in a diagnostic virology laboratory, the molecular approach to the diagnosis of acute respiratory tract infections in a patient population admitted to hospital was more convenient than the immunological approach. Both strategies may provide clinicians with most results of viral assays the same day of sample collection. However, the immunologic approach is not based only on direct staining of cells from nasopharyngeal secretions (thus providing results within a few hours after specimen collection), but also on virus isolation in both short-term and long-term cell cultures, followed by virus identification with MAbs. Thus, in this case, although the majority of infected patients may be detected by direct staining, a fair aliquot of acute respiratory infections may only be diagnosed following cell cultures, i.e., at later times. In addition, reading of directly stained slides requires expertise in immunofluorescence and dedicated personnel for interpretation of results, while handling and testing of a large number of samples is not feasible. In addition, nasopharyngeal samples may not be adequate and, according to our protocol, influenza viruses could not be subtyped, hPIVs were not typed, and, finally, both hCoVs 229E and hMPVs could not be detected.

RT-PCR may be better standardized and allows direct typing of respiratory viruses. Obviously, the PCR performance bears the risk of carry-over contamination, which must be prevented by adopting strict containment measures (separate rooms, disposable tips, adequately educated personnel). In addition, multiple negative and positive PCR controls were included in each test run. Recently, several multiplex PCR and RT-PCR systems have been developed, which have been claimed to have the same, and even a greater, sensitivity than conventional viral culture or antigen detection methods [Gilbert et al., 1996; Ellis et al., 1997; Freymuth et al., 1997; Osiowy, 1998; Coiras et al., 2003]. In this respect, we decided not to use multiplex PCR to amplify different respiratory viruses to achieve maximal sensitivity. Instead, we grouped identical thermal profiles for PCR detection of respiratory viruses belonging to the same group, such as influenza viruses or hPIVs, which could therefore be amplified in the same amplification run. Specific amplification runs were carried out for the other viruses requesting individual thermal profiles. In addition, influenza A viruses could be subtyped, hPIVs typed and both hCoVs and hMPVs detected. Thus, the complete panel of the most important respiratory viruses could be sought. In addition, PCR was more efficient than MAbs in detecting coinfections.

In this study, results provided by molecular and immunologic procedures on the same samples are grossly comparable in number, and discrepant results are restricted to a minimal number of specimens. The predominant incidence of hRSV (25.9%) infections was followed by infections caused by the following groups of viruses: (i) hMPV; (ii) two viruses (coinfections); (iii) influenza viruses A and B, and hPIV; and (iv) hCoV and hAdV. Thus, the observed prevalence of different respiratory virus infections in our study is close to that recently reported among children hospitalized with acute respiratory illness in two US cities [Mullins et al., 2004]. The predominant incidence of hRSV infection occurred between January and April with the peak in March. In addition, hRSV appeared to be responsible for the great majority of respiratory infections during the very first months of life, while hMPV seemed to play a major role in the subsequent months, as already reported by others [van den Hoogen et al., 2004b].

However, the conclusion that immunological and molecular methods are comparable in sensitivity can only be applied to conventional respiratory viruses detectable by both approaches. If diagnosis is extended to non-conventional respiratory viruses, such as hMPVs and hCoVs, for which MAbs indicated for reliable diagnostic use are not yet available or not yet widely tested, then only molecular methods may be used. Apart from the recently identified hCoV responsible for the worldwide outbreak of severe acute respiratory syndrome [Ksiazek et al., 2003; Kuiken et al., 2003; Lee et al., 2003; Peiris et al., 2003a; Poutanen et al., 2003], two distinct antigenic groups of hCoV were first detected in the 1970s, having the 229E and the OC43 strains as prototypes, referred to as prototypes of antigenic groups I and II, respectively [Lai and Holmes, 2001]. At that time, hCoV infections could be diagnosed only by serologic testing and hCoVs were considered responsible, along with rhinoviruses, for most of the common cold syndromes [McIntosh et al., 1970; Hamre and Been, 1972]. Recently, the development of molecular methods allowed to revisit the circulation and the spectrum of clinical syndromes caused by hCoVs [El-Sahly et al., 2000; Falsey et al., 2002a], including lower respiratory tract infections of infants as well as the elderly and the immunocompromised patients [Pene et al., 2003; Vabret et al., 2003]. Our study documents and confirms the sporadic association of hCoVs with lower respiratory tract infections of both infants and transplanted patients.

In addition, by using molecular methods, the hMPV was identified for the first time in the Netherlands in 2001 [van den Hoogen et al., 2001], and then shown to circulate in Canada [Peret et al., 2002; Boivin et al., 2003], Australia [Nissen et al., 2002], the United Kingdom [Stockton et al., 2002], France [Freymuth et al., 2003], Italy [Maggi et al., 2003], Hong Kong [Peiris et al., 2003b], the United States [Esper et al., 2003; Mullins et al., 2004], and South Africa [Ijpma et al., 2004]. hMPV has been shown to cause upper and lower respiratory tract infections, the latter occurring more frequently in infants, much like hRSV, and immunocompromised patients [Boivin et al., 2002, 2003; Pelletier et al., 2002; Falsey et al., 2002b, 2003; Freymuth et al., 2003]. The circulation of hMPV among infants and young children admitted to the hospital because of an acute respiratory infection suggests and confirms that hMPV may be associated with a severe lower respiratory tract infection, according to clinical and radiological patterns already recognized for hRSV. Both genotypes (or antigenic types) A and B were found to cocirculate in the same winter season including subgenotypes (or subtypes) A1 and A2, and B1 and B2 [van den Hoogen et al., 2004a]. hMPV circulation in Italy has been already reported [Maggi et al., 2003]. However, a very original finding of the Italian study was the detection of hMPV in blood of some infants with acute respiratory infection. This finding awaits confirmation from other laboratories. In our experience, no hMPV RNA could be detected by RT-PCR in blood of six infants with lower respiratory infection associated with hMPV during the acute phase of the infection (unpublished data). Whether the presence of hMPV in blood may be the result of a primary infection in an immunogically susceptible infant remains to be investigated. Recently, human rotavirus antigenemia was detected in blood of infants with acute gastroenteritis [Blutt et al., 2003], while influenza virus A RNA and hAdV DNA were detected in blood of patients with acute encephalopathy associated with influenza A virus infection [Steininger et al., 2003].

In the present study, as many as 15 patients were diagnosed as infected by either hMPV of group A ($n = 7$) or B ($n = 5$) or hCoV ($n = 3$) strains by RT-PCR only. Thus, while respiratory viruses present in

nasopharyngeal secretions examined by both procedures were detected for the great majority by both MAbs and PCR, molecular methods contributed significantly in increasing the diagnostic rate of respiratory virus infections not detected by MAbs.

Furthermore, of the 11 cases of respiratory virus coinfection diagnosed in this study, as many as 10 would have been missed without using RT-PCR. This result may somewhat be difficult to interpret. One can speculate that in cell cultures the presence of one virus may interfere with growth of another virus. On the other hand, PCR may detect nucleic acid sequences in respiratory samples belonging to viruses unable to grow in cell cultures. However, it may be difficult to discriminate whether the detection of two viruses is the expression of two simultaneous infections occurring in the same patient, or one virus represents the residual virus of a previous infection detected concomitantly with the virus causing the current infection. Virus quantification in nasopharyngeal aspirates might help in discriminating the etiologic role of each of the two viruses. Although varying among different studies, the coinfection rate is generally low [Coiras et al., 2003; van den Hoogen et al., 2003] ranging from 1 to 3% [van den Hoogen et al., 2004b]. However, whether coinfection represents a risk factor for more severe disease is uncertain. It has been reported that coinfection with hMPV and hRSV may worsen prognosis of a severe respiratory disease [Greensill et al., 2003], whereas others reported a comparable rate of bronchopneumonia in children infected with hMPV alone or with mixed infections by hMPV and another respiratory virus [Maggi et al., 2003]. The role of respiratory virus coinfections in determining the severity of respiratory infections remains to be substantially determined. In our study, coinfections did not seem to worsen the prognosis of respiratory infections, because the most severe coinfections were those caused by hRSV in association with some other respiratory virus. Since hRSV is known to be significantly more associated with lower than upper respiratory tract infections and has been confirmed in this study, it appears reasonable to attribute the severity of coinfections involving hRSV in infants and young children to hRSV itself rather than the coinfecting virus.

As a preliminary conclusion of our study carried out prospectively for the immunological part, and retrospectively for the molecular part, it is suggested that in a diagnostic laboratory receiving a limited number of specimens per day (<10 samples), MAbs may be used for detection of conventional viruses by direct staining and RT-PCR for unconventional viruses. By using this approach, only viruses scarcely represented in respiratory samples would be lost, but this would not have a major impact on the clinical management of respiratory infections. However, if a single procedure can be used for diagnosis of acute respiratory infections, the molecular one should be preferred over the immunological approach because it is characterized by: greater overall rapidity; greater cost-effectiveness; and greater sensitivity in detecting respiratory viruses. A recent report showed the superiority of RT-PCR as compared to conventional viral culture in the diagnosis of acute respiratory infections in children by documenting an increased yield of viral identification by twofold [Weinberg et al., 2004].

Finally, the role of respiratory viruses deserves special attention in solid-organ recipients and, particularly, in lung transplant recipients. Our data seem to suggest preliminarily that in this patient population the presence of a respiratory virus in bronchoalveolar lavage samples is associated frequently with a lower respiratory tract infection. Thus, attribution of clinical and prognostic significance to the detection of a respiratory virus in respiratory secretions would require bronchoalveolar lavage examination in a lung transplanted patient population affected by lower respiratory tract infection.

In conclusion: immunologic and molecular methods may be used in combination to achieve the highest rate of identification of respiratory infection burden; all respiratory viruses may cause severe lower respiratory tract infections in infants and young children as well as in the elderly and the immunocompromised host; and the pathogenic role of coinfections remains to be elucidated.

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