Journal of Medical Virology 84:162–169 (2012)

Clinical Evaluation of Multiplex Real-Time PCR Panels for Rapid Detection of Respiratory Viral Infections

Sonali K. Sanghavi,[1] Arlene Bullotta,[1] Shahid Husain,[2] and Charles R. Rinaldo[1]*

[1]*Clinical Virology Laboratory, Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania*

[2]*Transplant Infectious Diseases, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania*

Respiratory viral infections are one of the leading causes of morbidity and mortality, particularly in children, the elderly and immunocompromised persons. Rapid identification of viral etiology is critical in ruling out non-viral infections, initiating antiviral treatment and limiting the spread of the infection. Multiplex assays of more than one viral gene target in a single tube have the advantage of rapid screening of a large number of potential viral pathogens in a short time. A multiplex real-time PCR assay was used in this study for detection of respiratory RNA and DNA viral infections in 728 specimens received from 585 adult and pediatric patients comprised of symptomatic and asymptomatic organ transplant recipients and non-recipients for diagnosis of respiratory illnesses and for routine clinical monitoring. Multiplex PCR was more sensitive than the multiplex immunofluoresence culture assay (R-mix) and also detected additional respiratory viruses that were not covered by the R-mix panel. The number of respiratory viruses detected in symptomatic patients was significantly higher than asymptomatic patients in both adult and pediatric patients. Herpesviral infections were the predominant cause of lower respiratory tract infection in the organ transplant recipients, whereas respiratory syncytial virus was the most common pathogen in non-transplant patients particularly children. Multiplex real-time PCR for detection of respiratory viruses has the potential for rapid identification of viral pathogens. In this era of emerging viral infections, addition of newer viral targets to the multiplex PCR panels will be beneficial in determining both patient management and public health epidemiology. ***J. Med. Virol. 84:162–169, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: organ transplant recipients; viral culture; real-time PCR

INTRODUCTION

Respiratory viral infections are responsible for a spectrum of disease severity ranging from mild, uncomplicated illness to severe, complicated viral pneumonias. Disease severity can be affected by various host and viral predisposing factors such as age, immune-status of the host, single or mixed infections and virulence mechanisms of the viral pathogen. The common respiratory viruses causing infections are influenza A and B viruses, respiratory syncytial virus (RSV), parainfluenza viruses (PIV), coronaviruses (CoV), and adenovirus (AdV). Newly emerging respiratory viral pathogens include swine origin influenza A H1N1, human metapneumovirus (hMPV), and human bocavirus (hBoV) particularly in pediatric patients [Lu et al., 2006; Dare et al., 2007; Gordon, 2009; Scalera and Mossad, 2009]. Immunocompromised hosts such as organ transplant recipients are often infected with opportunistic herpesviruses. Therefore, rapid screening for respiratory viral pathogens is critical to allow timely therapeutic decisions and limit pathogenesis.

A commercial, rapid viral culture and direct fluorescence antigen (viral culture/DFA) multiplex detection method (R-mix) is used commonly by many clinical diagnostic laboratories including ours for respiratory viral detection [St. George et al., 2002]. However,

Sonali K. Sanghavi present address is King Edward Memorial Hospital, Microbiology Laboratory, Sardar Moodliar Road, Rasta Peth, Pune 411011, India.

Shahid Husain present address is Assistant Professor of Medicine; Director, Multi-Organ Transplant Infectious Diseases, University Health Network, NCSB 11C-1206, 585 University Avenue Toronto, ON Canada M5G 2N2.

*Correspondence to: Charles R. Rinaldo, UPMC Presbyterian, Clinical Virology Laboratory, A-Wing, Room A912, 200 Lothrop Street, Pittsburgh, PA 15213, USA. E-mail: rinaldocr@upmc.edu

Accepted 27 June 2011

DOI 10.1002/jmv.22186

Published online in Wiley Online Library (wileyonlinelibrary.com).

though relatively rapid, this technique is limited to detection of only a few of the common respiratory viruses. One of the advancements of the polymerase chain reaction (PCR) assay is multiplex real-time PCR which allows detection of multiple RNA and DNA targets in a single tube [van Elden et al., 2004]. In this study, a multiplex real-time PCR assay was developed targeting respiratory RNA and DNA viruses to assess its utility as a rapid molecular screening tool for clinical diagnosis. With this assay, a variety of respiratory viruses causing symptomatic and asymptomatic infections was identified in organ transplant and non-organ transplant adult and pediatric patients.

MATERIALS AND METHODS

Specimen Collection

This concurrent prospective study was approved by the University of Pittsburgh Institutional Review Board. During December 2006–March 2007, residual respiratory specimens received for viral culture/DFA at the University of Pittsburgh Medical Center, Clinical Virology Laboratory, were stored in a lysis buffer for nucleic acid isolation and multiplex PCR testing. A total of 728 specimens were collected from 585 adult and pediatric patients, of whom 258 were organ transplant recipients. Clinical information on all patients was collected for correlation with laboratory data. Upper respiratory tract infection was defined as clinical symptoms of rhinorrhea, sore throat or cough with or without fever, without evidence of dyspnea and abnormalities in chest X-ray. Lower respiratory tract infection was defined as presence of dyspnea and or clinical evidence of pulmonary pathology on chest X-ray along with fever or cough [Machado et al., 2003]. Patients presenting without apparent respiratory symptoms were defined as asymptomatic. Most of these latter specimens were obtained from organ transplant recipients as routine surveillance cultures. The pediatric age group included specimens from neonates (0–4 weeks) to children up to 18 years of age.

Viruses and Positive Control Nucleic Acid Material

Prequantitated influenza A and influenza B viruses and human cytomegalovirus (CMV) and human herpesvirus 6 (HHV-6) DNA were obtained from Advanced Biotechnologies (Columbia, MD). Coronaviruses 229E (hCoV-229E) and OC43 (hCoV-OC43) with known $TCID_{50}$ titers were purchased from ATCC (Manassas, VA) (VR-740 and VR-1558). Clinical isolates of PIV-1, PIV-2, PIV-3, AdV, RSV, and herpes simplex virus 1 (HSV-1) were derived in the Clinical Virology Laboratory and titered for 50% tissue culture infectious dose ($TCID_{50}$) [Reed and Muench, 1938]. The plasmid construct of hBoV DNA was kindly provided by Dr. Dean Erdman (CDC, Atlanta, GA) and that for hMPV RNA was generated in the laboratory as previously described [Dare et al., 2007].

Nucleic Acid Extraction

Isolation of viral nucleic acid from control material and patient specimens was done using an EasyMag automated extractor (bioMérieux, Durham, NC) according to the manufacturer's instructions. Briefly, 200 μl of specimen was mixed with lysis buffer (bioMerieux) for 15 min, to which a fixed volume and concentration of equine arteritis virus (EAV; $10^{6.25}$ $TCID_{50}$/ml) was added as an internal control for RNA extraction and amplification, followed by addition of magnetic silica as per the manufacturer's instructions. The nucleic acid was eluted in 60 μl elution volume and stored at −80°C until further processing.

Multiplex Real-Time PCR

Multiplex one-step PCR was performed in six panels as shown in Table I. Previously published, virus-specific primer and probe nucleotide sequences were used for PIV-1, PIV-2, PIV-3, hMPV, EAV (internal control), hCoV-229E, hCoV-OC43, CMV-US17, HHV-6, and hBoV [Locatelli et al., 2000; Balasuriya et al., 2002; Templeton et al., 2004; van Elden et al., 2004; Kuypers et al., 2006; Lu et al., 2006; Dare et al., 2007; Sanghavi et al., 2008]. Influenza A, influenza B, influenza A-H1, and influenza A-H3 primer and probe sequences were available from the CDC through the Laboratory Response Network (http://www.bt.cdc.gov/lrn/). The probes were labeled with either 6-FAM, CAL Fluor Red 590 or Quasar 670 reporter dyes (Biosearch Technologies, Novato, CA). Primer and probe sequences are as shown in Table I. Each PCR reaction consisted of 25 μl volume with 12.5 μl of 2× Quantitect multiplex RT-PCR NR master mixes with RT-enzyme, for RNA viral targets and 2× Quantitect multiplex PCR NR master mixes for DNA viral targets (Qiagen, Valencia, CA). Concentrations of primer and probes for viral targets in each panel were optimized for sensitivity and are shown in Table I. Multiplex PCR reaction mixes were supplemented with 0.1 μl/reaction of AmpliTaq Gold DNA polymerase. PCR thermal cycling conditions for RNA PCR were optimized to the following conditions: 20 min at 50°C for reverse transcription, 15 min at 95°C and 45 cycles of 45 sec at 94°C, and 1.15 min at 60°C. PCR thermal cycling conditions for DNA PCR were: 2 min at 50°C, 15 min at 95°C and 45 cycles of 1 min at 94°C and 1.3 min at 60°C. Detection was performed using the ABI 7500 Real-Time PCR Instrument (Applied Biosystems, Foster City, CA).

Viral Culture and Antigen Detection by Viral Culture/DFA

Two R-Mix shell vials containing a mixed monolayer of mink lung cells (strain Mv1Lu) and human adenocarcinoma cells (strain A549) were inoculated

TABLE I. Primer-Probe Sequences Along With Final Concentrations in Nanomoles Used in Multiplex PCR Reaction

Virus	Forward primer (5′–3′) [final concentration]	Reverse primer (5′–3′) [final concentration]	Taqman probe (5′–3′) –BHQ [final concentration]
Panel 1			
Flu A	CAT GGA RTG GCT AAA GAC AAG ACC [650 nM]	AGG GCA TTT TGG ACA AAK CGT CTA [650 nM]	TGC AGT CCT CGC TCA CTG GGC ACG [650 nM]
Flu B	TCC TCA ACT CAC TCT TCG AGC G [250 nM]	CGG TGC TCT TGA CCA AAT TGG [250 nM]	CCA ATT CGA GCA GCT GAA ACT GCG GTG [250 nM]
RSV	CACWGAAGATGCWAATCATAAATTCA [650 nM]	GTATYTTTATRGTGTCTTCYCTTCCTAACC [650 nM]	TAATAGGTATGTTATATGCKATGTC [650 nM]
Panel 2			
PIV-1	ACAGATGAAATTTTCAAGTGCTACTT-TAGT [650 nM]	GCCTCTTTTAATGCCATATTATCATTAGA [650 nM]	ATGGTAATAAATCGACTCGCT [650 nM]
PIV-2	CCATTTACCTAAGTGATGGAA [250 nM]	CGTGGCATAATCTTCTTTTT [250 nM]	AATCGCAAAAGCTGTTCAGTCAC [250 nM]
PIV-3	TTA CARATAGGGA TAATAACTGT [650 nM]	TTAGGAGTGCTAGAGAACAT [650 nM]	AAACTCAGAC TTGGTACCTG ACTTAAAT [650 nM]
Panel 3			
hCoV 229E	CAGTCAAATGGGCTGATGCA [250 nM]	AAAGGGCTATAAAGAGAATAAGGTATTCT [250 nM]	CCCTGACGACCACGTTGTGGTTCA [250 nM]
hCoV OC43	CGATGAGGCTATTCCGACTAGGT [250 nM]	CCTTCCTGAGCCTTCAATATAGTAACC [250 nM]	TCCGCCTGGCACGGTACTCCCT [250 nM]
Panel 4			
hMPV	CATCAGGTAATATCCCACAAAATCAG [500 nM]	GTGAATATTAAGGCACCTACACATAA-TAARA [500 nM]	TCAGCACCAGACACAC [500 nM]
EAV	GGCGACAGCCTACAAGCTACA [150 nM]	CGGCATCTGCAGTGAGTGA [150 nM]	TTGCGGACCCGCATCTGACCAA [150 nM]
Panel 5			
AdV	GGACGCCTCGGAGTACCTGAG [250 nM]	AC/ideoxyI/GTGGGGTTTCTGAACTTGTT [250 nM]	CTGGTGCAGTTCGCCCGTGCCA [250 nM]
hBoV	TGC AGA CAA CGC YTA GTT GTT T [200 nM]	CTG TCC CGC CCA AGA TAC A [200 nM]	CCA GGA TTG GGT GGA ACC TGC AAA [200 nM]
Panel 6			
CMV	CGA TCA AGA ACG CGA TAA CG [250 nM]	ACC GTC GAT GGC AGG TCA T [250 nM]	CGA TCA CAA ACA GCG [250 nM]
HHV-6	CGC TAG GTT GAG GAT GAT CGA [200 nM]	CAA AGC CAA ATT ATC CAG AGC G [200 nM]	CAC CAG ACG KCA CAC CCG AAG GAA T [200 nM]
HSV-1	CATCACCGACCCGGAGAGGGAC [250 nM]	GGGCCAGGCGCTTGTTGGTGTA [250 nM]	CCGCCGAACTGAGCAGACACCCGCGC [250 nM]
Flu A-H1	AGG CAA ATG GAA ATC TAA TAG CGC [200 nM]	CCA TTG GTG CAT TTG AGG TGA TG [200 nM]	TGA YCC AAA GCC “T”CT ACT CAG TGC GAA AGC [200 nM]
Flu A-H3	AAG CAT TCC YAA TGA CAA ACC [200 nM]	ATT GCR CCR AAT ATG CCT CTA GT [200 nM]	CAG GAT CAC ATA TGG GSC CTG TCC CAG [200 nM]
Flu A-N1	ATGGTAATGGTGTTTGGATAGGAAG [200 nM]	AATGCTGCTCCCACTAGTCCAG [200 nM]	TGATTTGGGATCCTAATGGATGGACAG [200 nM]
Flu A-N2	AAGCATGGCTGCATGTTTGTG [200 nM]	ACCAGGATATCGAGGATAACAGGA [200 nM]	TGCTGAGCACTTCCTGACAATGGGCT [200 nM]

with 200 μl of clinical specimen according to the manufacturer's instructions (Diagnostic Hybrids, Athens, OH). Briefly, following incubation at 35°C the R-Mix shell vials were screened by staining coverslips with a pool of respiratory virus fluorescent antibodies (AdV, influenza A and B viruses, RSV, PIV 1, 2, and 3) (Bartels, Issaquah, WA) according to the manufacturer's instructions. Positive specimens were further identified with the second R-Mix shell vial by staining with virus-specific monoclonal antibodies.

For CMV culture and staining, sterile one-dram vials containing MRC-5 human lung fibroblast cells on coverslips (DHI) were used as described [St. George and Rinaldo, 1994]. Briefly, the vials were inoculated with 200 μl of prepared sample, centrifuged and incubated at 30–36°C. After incubation, coverslips were washed, fixed and the cell monolayers were stained with 0.2 ml anti-CMV monoclonal antibody (MAB810; Millipore, Billerica, MA) and counterstained with fluorescein isothiocyanate-labeled goat antimouse immunoglobulin G conjugate with Evans blue dye (B1029-86B; Bartels Trinity Biotech, Jamestown, NY). Positive samples exhibited an apple green fluoresence detailing the nuclei of CMV.

For HSV cultures, tubes containing MRC5 (human fetal lung fibroblast) or MRHF (human foreskin fibroblast), and A549 (human lung carcinoma) cells, were used (DHI). The tubes were inoculated with 0.2–0.5 ml of prepared sample, placed in horizontal position and incubated (adsorption step) at 35–37°C for 60–90 min. The inoculum was then removed and the tubes fed with fresh maintenance media (Eagle's minimum essential medium with 2% fetal bovine serum). The tube cultures were returned to their horizontal position in the 35–37°C incubator, and observed for cytopathic effect (CPE) for 24 hr up to 7 days post-inoculation. HSV cultures were observed for characteristic CPE as rounded and ballooned cells, with or without syncytia.

Statistical Analysis

Student's *t*-test was used to determine statistical significance ($P < 0.05$).

RESULTS

Analytical Sensitivity and Specificity

Assay performance and sensitivities were determined by using serial dilutions of known quantities of viruses and viral RNA. The sensitivity for each target in multiplex PCR assays was similar to that obtained by singleplex PCR for individual viral targets. The lower limit of detection of multiplex PCR was as follows: influenza A = 50 viral particle units per milliliter (VPU/ml); influenza B = 100 VPU/ml; RSV = 5 $TCID_{50}$/ml; PIV-1 = $5 \times 10^{1.5}$ $TCID_{50}$/ml; PIV-2 = $5 \times 10^{-0.75}$ $TCID_{50}$/ml; PIV-3 = $5 \times 10^{-1.25}$ $TCID_{50}$/ml; hCoV 229E = $5 \times 10^{-1.5}$ $TCID_{50}$/ml; hCoV OC43 = $5 \times 10^{-0.5}$ $TCID_{50}$/ml; AdV = 0.5 $TCID_{50}$/ml; HSV-1 = $5 \times 10^{-0.75}$ $TCID_{50}$/ml; hMPV = 30 RNA copies/ml; CMV = 15 DNA copies/ml; HHV-6 = 45 DNA copies/ml; hBoV = 1 DNA copy/ml. Amplification was specific for target viral genes, and non-specific amplification was not observed when tested with viruses in the same panel and those in other panels, as well as with HIV-1. Randomly selected 10 PCR positive/R-mix negative discrepant specimens were confirmed by DNA sequencing of purified PCR products. In addition, an alternate multiplex nucleic acid detection kit (Multicode-PLx System, Eragen Biosciences, Madison, WI) using the Luminex (Austin, TX) detection platform was used to assay 200 specimens in parallel with our multiplex PCR assay. The Eragen assay detects influenza A, influenza B, RSV, PIV-1-4, hCoV 229E, hCoV OC43, hCoV NL63, AdV, hMPV, and rhinovirus. Both of these multiplex PCR assays detected five influenza A, one influenza B, 28 RSV, two PIV-2, four PIV-3, three hMPV, one CoV-229E, five CoV OC43, and seven AdV in the same specimens. Our multiplex PCR also detected one RSV, one PIV-1, six hMPV, two CoV-229E, and five AdV positive specimens, and missed detection of one PIV-2 infection, compared to the Eragen assay.

Patient Characteristics

This study was comprised of both adult (n = 337) and pediatric (n = 248) patient populations. A total of 728 specimens were collected from total of 585 patients. Of this 585 patients, 258 were stem cell and solid organ transplant recipients (adults = 232; pediatric = 26) while the remaining 327 patients did not undergo organ transplantation (adults = 105; pediatric = 222) and comprised of both hospitalized patients and out-patients. Among organ transplant recipients, most specimens were from lung transplant (n = 149) followed by stem cell transplant (n = 36) recipients. Clinical symptoms ranged from asymptomatic to mild upper respiratory tract infection to severe lower respiratory tract infection.

Comparison of Multiplex PCR With R-Mix

The multiplex PCR was more sensitive than R-mix and also detected additional respiratory viruses that were not included in the R-mix panel (Table II). Out of 405 total positives, multiplex PCR detected 126 (27.9%) more positives than R-mix and an additional 147 (36%) viral infections not covered by the R-mix culture.

Among community acquired viral infections, RSV (n = 78) was the most common etiologic agent, followed by AdV (n = 36) and influenza A virus (n = 23). All 23 influenza A virus positives were further tested for human influenza A strain H1, H3, N1, and N2 subtypes of which 21 were H1/N1 and two were H3/N2 positives. Among the respiratory viruses not covered by R-mix, significant infections were caused by

TABLE II. Respiratory Viruses Detected by Multiplex RT-PCR and Culture and DFA (Total Number of Specimens: 728)

Virus	Positive by		
	R-mix (or tube culture[a]) only	Multiplex RT-PCR only	Both [Multiplex RT-PCR and R-mix (or tube culture[a])]
Flu A	0	6	17
Flu B	0	0	6
RSV	0	65	13
PIV-1	0	2	1
PIV-2	0	0	0
PIV-3	0	11	4
AdV	0	29	7
CMV[a]	0	61	15
HSV-1[a]	0	18	3
Total	0	192	66
hMPV	NA	21	NA
hCoV-229E	NA	4	NA
hCoV-OC43	NA	27	NA
hBoV	NA	24	NA
HHV-6	NA	71	NA
Total	NA	147	NA

NA, not applicable.
[a]For CMV and HSV-1, tube culture was not performed on all specimens.

hMPV, CoV and the recently identified hBoV. CMV and HHV-6 were detected most frequently in organ transplant recipients.

Viral Infections in the Adult Population

Clinical significance of the viral infections was determined by analyzing the clinical data from symptomatic and asymptomatic patients. In adult organ transplant recipients, CMV, HHV-6, and HSV-1 were found to be significantly associated with lower respiratory tract infection (Table III). Surprisingly, in this group, RSV was also found to have a strong association with upper and lower respiratory tract infections. Influenza A virus and AdV also caused significant infections in organ transplant recipients. In the non-transplant recipient adult patients, in addition to influenza A virus, RSV and AdV, hMPV and CoV were significantly associated with mild or severe respiratory infections. CMV, HHV-6, and HSV-1 were also significantly associated with symptomatic respiratory infections in apparently immunocompetent adults.

Viral Infections in Children

In contrast to the adult patient population, herpesviruses CMV, HHV-6, and HSV-1 were not associated with respiratory infection in transplant and non-transplant recipient pediatric patients. In non-transplant recipients, community acquired viruses such as influenza A virus, RSV, and hBoV were the predominant etiologic agents of respiratory infections, while none were significantly associated with respiratory infections in organ transplant recipients (Table III).

Co-Infections

Out of 728 specimens tested, dual viral infections were detected in 76 (10.4%) specimens (Table IV), while triple viral infections were found in 10 (1.3%) specimens (data not shown). Most of these co-infections were associated between RSV and AdV (n = 11), CMV and HHV-6 (n = 10), and RSV and CMV (n = 6). Triple infections between CMV/HHV-6/HSV-1 (n = 4) were more common among adult transplant recipients (data not shown).

DISCUSSION

Respiratory infections are one of the major causes of morbidity and mortality. With newly emerging respiratory viral infections, about 20–30% of viral etiologies go undetected either due to lack of assay sensitivity or a limited number of viruses detectable by conventional methods [Freymuth et al., 1987]. In this study, the use of an in-house, multiplex real-time PCR assay was evaluated as a diagnostic tool for rapid detection of viral respiratory tract infections and correlated the findings with clinical symptoms and viral culture.

The multiplex PCR assay had several advantages over the conventional viral culture/DFA (R-mix) and tube culture detection methods. First, similar to other studies [Kim et al., 2009; Murali et al., 2009], the multiplex PCR detected more viruses than viral culture/DFA. The sensitivity of this multiplex PCR was found to be comparable to singleplex PCR for individual viruses and higher than viral culture/DFA detection. The reason for this higher sensitivity is that PCR detects lower titers of viral genomes as well as viruses that are not replication competent [Liolios et al., 2001]. Second, viruses that were not included in

TABLE III. Respiratory Viral Infections in Symptomatic and Asymptomatic Patient Populations

Specimens	Organ transplant recipients				Non-transplant recipients			
Adults (n = 429)	Symptomatic (n = 105) URTI	Symptomatic (n = 105) LRTI	Asymptomatic (n = 176)	*P*-value	Symptomatic (n = 79) URTI	Symptomatic (n = 79) LRTI	Asymptomatic (n = 69)	*P*-value
Flu A	1	2	0	0.01	1	2	0	0.05
Flu B	0	0	0	NA	1	1	0	NS
RSV	8	9	3	<0.001	3	1	0	0.03
PIV-1	0	0	0	NA	0	0	0	NA
PIV-2	0	0	0	NA	0	0	0	NA
PIV-3	2	1	1	NS	1	1	1	NS
hMPV	1	3	2	NS	2	1	0	0.05
hCoV-229E	0	0	2	NS	0	0	1	NS
hCoV-OC43	3	2	3	NS	2	2	0	0.03
AdV	4	2	2	0.01	1	2	0	0.05
hBoV	0	0	4	NS	1	1	2	NS
CMV	0	42	12	<0.0001	0	15	5	0.02
HHV-6	0	29	11	<0.0001	0	20	3	<0.001
HSV-1	0	7	1	<0.001	0	10	3	0.04

	Symptomatic (n = 31)				Symptomatic (n = 202)			
Pediatric 299	URTI	LRTI	Asymptomatic (n = 7)	*P*-value	URTI	LRTI	Asymptomatic (n = 59)	*P*-value
Flu A	0	0	0	NA	14	3	0	<0.001
Flu B	0	0	0	NA	2	2	0	0.02
RSV	2	2	0	NS	19	25	6	<0.01
PIV-1	1	0	0	NS	0	2	0	NS
PIV-2	0	0	0	NA	0	0	0	NA
PIV-3	1	0	0	NS	4	2	1	NS
hMPV	0	1	0	NS	4	6	1	NS
hCoV-229E	0	0	0	NA	1	0	0	NS
hCoV-OC43	1	1	0	NS	6	4	3	NS
AdV	1	0	2	NS	10	7	5	NS
hBoV	2	0	0	NS	10	3	1	0.02
CMV	0	1	0	NS	1	0	0	NS
HHV-6	0	1	0	NS	1	3	3	NS
HSV-1	0	0	0	NA	0	0	0	NA

NA, not applicable; NS, not significant; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection.

the R-mix culture panel such as hMPV, hBoV, HHV-6, and hCoV could also be detected by the multiplex PCR assay. Since this study was carried out before the emergence of the swine-origin influenza A H1N1 virus pandemic, our multiplex PCR did not include primers for this target. However in 2009, the multiplex PCR assay was modified for detection of swine-origin influenza A H1N1. We found it to be highly sensitive and specific for detection of the three predominant influenza viruses, that is, using the

TABLE IV. Dual Respiratory Viral Infections Detected by Multiplex Real-Time RT-PCR

	Flu A	Flu B	RSV	PIV-1	PIV-2	PIV-3	AdV	hMPV	hCoV 229E	hCoV OC43	CMV	HHV6	HSV-1	hBoV
Flu A	NA	0	0	0	0	0	0	0	0	1	0	1	0	2
Flu B		NA	0	0	0	0	0	0	0	0	0	0	0	0
RSV			NA	0	0	1	11	2	0	4	2	5	0	5
PIV-1				NA	0	1	0	0	0	0	0	0	0	0
PIV-2					NA	0	0	0	0	0	0	0	0	0
PIV-3						NA	0	0	0	2	1	1	0	0
AdV							NA	1	0	2	0	2	0	1
hMPV								NA	0	0	1	0	0	2
hCoV 229E									NA	0	1	1	0	0
hCoV OC43										NA	3	1	0	0
CMV											NA	10	4	3
HHV-6												NA	2	1
HSV-1													NA	0
hBoV														NA

NA, not applicable.

multiplex PCR assay 2,992 swine H1N1 virus, 324 influenza A H3N2 virus and 300 influenza B virus infections were detected in 18,920 specimens from April, 2009 through April, 2011 (unpublished results). Thus, this multiplex assay can be modified according to the needs of the clinicians and the laboratory to add new viruses in the detection panel following simple optimization and validation studies.

A lower number of respiratory viral infections were noted in immunocompetent compared to immunocompromised adults. It has been suggested that respiratory viral infections in immunocompromised patients differ from immunocompetent patients by higher viral replication leading to increased viral shedding [Leung et al., 2005; Camps Serra et al., 2008]. Compared to some other studies [Templeton et al., 2004; Hindiyeh et al., 2005], our multiplex PCR had relatively lower sensitivity. However, more respiratory viral infections were detected in the symptomatic group than in asymptomatic immunocompetent adults, indicating true active infections. The overall positivity rate of multiplex PCR was 46.5%, which is comparable to that reported by others (Gadsby et al., 2010). Among the positives, 78.4% were from symptomatic patients and 25% from asymptomatic patients. A significant association was also observed between symptomatic respiratory illness and positive viral detection.

Most of the adult patients comprised of immunocompromised organ transplant recipients, while the pediatric patients were mostly non-organ transplant recipients with respiratory complaints. The spectrum of viral isolates differed between adult and pediatric patients as well as between organ transplant and non-transplant recipients. Herpesviral infections CMV, HHV-6, and HSV-1 were more predominant in adult organ transplant recipients, whereas RSV was present predominantly in pediatric non-transplant patients. These opportunistic herpesviruses were also associated significantly with lower respiratory tract infection in non-organ transplant recipients and apparently immunocompetent hosts. The significance of these herpesviral infections, which are known to cause life-long, latent/reactivated infections, needs to be evaluated further. Detection of RNA transcripts and/or antigens or determining viral load using quantitative PCR for these viruses will help in detecting replicating viruses. These herpesviruses have been reported previously to cause pneumonitis in immunocompetent adults, underlying their role in community-acquired pneumonias [Karakelides et al., 2003; Merk et al., 2005; Rafailidis et al., 2008]. In the present study, RSV was also significantly associated with symptomatic respiratory infections in organ transplant recipients. RSV and other community viral infections have been reported to be important causes of morbidity and mortality in immunocompromised transplant recipients [Muir and Pillay, 1998; Nichols et al., 2001]. It is therefore possible that the conventional viral culture/DFA (R-mix) method missed detection of some viruses causing severe infections in immunocompromised and immunocompetent patients.

An advantage of the PCR-based assay was detection of co-infections with two or more respiratory viruses which are usually missed by viral culture/DFA (R-mix). A range of 5–40% multiple infections have been reported [Guittet et al., 2003] but the clinical significance of these infections is not well understood. One plausible reason for this could be that residual, persistent viral nucleic acid, particularly viral DNA, from past infection was co-detected by the highly sensitive multiplex PCR assay. In the present study, RSV was found to be associated more often with co-infections in community acquired viral infections. It has been suggested that RSV-associated dual infections usually lead to increased severity of clinical illness [Aberle et al., 2005]. In the adult organ transplant recipients, CMV and HHV-6 dual infections and triple infections with CMV, HHV-6, and HSV-1 were detected more commonly. A detailed study, including virus quantitation, is required to determine the clinical significance of these multiple viral infections.

ACKNOWLEDGMENTS

We thank the technical staff of the Clinical Virology Laboratory at UPMC for their assistance and support.

REFERENCES

Aberle JH, Aberle SW, Pracher E, Hutter HP, Kundi M, Popow-Kraupp T. 2005. Single versus dual respiratory virus infections in hospitalized infants: Impact on clinical course of disease and interferon-gamma response. Pediatr Infect Dis J 24:605–610.

Balasuriya UB, Leutenegger CM, Topol JB, McCollum WH, Timoney PJ, MacLachlan NJ. 2002. Detection of equine arteritis virus by real-time TaqMan reverse transcription-PCR assay. J Virol Methods 101:21–28.

Camps Serra M, Cervera C, Pumarola T, Moreno A, Perelló R, Torres A, Jiménez de Anta MT, Marcos MA. 2008. Virological diagnosis in community-acquired pneumonia in immunocompromised patients. Eur Respir J 31:618–624.

Dare R, Sanghavi S, Bullotta A, Keightley MC, St. George K, Wadowsky RM, Paterson DL, McCurry KR, Reinhart TA, Husain S, Rinaldo CR. 2007. Diagnosis of human metapneumovirus infection in immunosuppressed lung transplant recipients and children evaluated for pertussis. J Clin Microbiol 45:548–552.

Freymuth F, Quibriac M, Petitjean J, Daon F, Amiel ML. 1987. Viruses responsible for respiratory infections in pediatrics. Evaluation of 3,480 nasal aspirates performed in children over a 6-year period. Ann Pediatr (Paris) 34:493–501.

Gadsby NJ, Hardie A, Claas EC, Templeton KE. 2010. Comparison of the Luminex Respiratory Virus Panel fast assay with in-house real-time PCR for respiratory viral infection diagnosis. J Clin Microbiol 48:2213–2216.

Gordon SM. 2009. Update on 2009 pandemic ininfluenzaenza A (H1N1) virus. Cleve Clin J Med 76:577–582.

Guittet V, Brouard J, Vabret A, Lafay F, Guillois B, Duhamel JF, Freymuth F. 2003. Rhinovirus and acute respiratory infections in hospitalized children. Retrospective study1998–2000. Arch Pediatr 10:417–423.

Hindiyeh M, Levy V, Azar R, Varsano N, Regev L, Shalev Y, Grossman Z, Mendelson E. 2005. Evaluation of a multiplex real-time reverse transcriptase PCR assay for detection and differentiation of ininfluenzaenza viruses A and B during the 2001–2002 in influenzaenza season in Israel. J Clin Microbiol 43:589–595.

Karakelides H, Aubry MC, Ryu JH. 2003. Cytomegalovirus pneumonia mimicking lung cancer in an immunocompetent host. Mayo Clin Proc 78:488–490.

Kim SR, Ki CS, Lee NY. 2009. Rapid detection and identification of 12 respiratory viruses using a dual priming oligonucleotide system-based multiplex PCR assay. J Virol Methods 156:111–116.

Kuypers J, Wright N, Ferrenberg J, Huang ML, Cent A, Corey L, Morrow R. 2006. Comparison of real-time PCR assays with influenzaorescent-antibody assays for diagnosis of respiratory virus infections in children. J Clin Microbiol 44:2382–2388.

Leung AY, Chan M, Cheng VC, Yuen KY, Kwong YL. 2005. Quantification of adenovirus in the lower respiratory tract of patients without clinical adenovirus-related respiratory disease. Clin Infect Dis 40:1541–1544.

Liolios L, Jenney A, Spelman D, Kotsimbos T, Catton M, Wesselingh S. 2001. Comparison of a multiplex reverse transcription-PCR-enzyme hybridization assay with conventional viral culture and immunoinfluenzaorescence techniques for the detection of seven viral respiratory pathogens. J. Clin. Microbiol 39:2779–2783.

Locatelli G, Santoro F, Veglia F, Gobbi A, Lusso P, Malnati MS. 2000. Real-time quantitative PCR for human herpesvirus 6 DNA. J Clin Microbiol 38:4042–4048.

Lu X, Chittaganpitch M, Olsen SJ, Mackay IM, Sloots TP, Fry AM, Erdman DD. 2006. Real-time PCR assays for detection of bocavirus in human specimens. J Clin Microbiol 44:3231–3235.

Machado CM, Vilas Boas LS, Mendes AVA, Santos MFM, da Rocha IF, Sturaro D, Dulley FL, Pannuti CS. 2003. Low mortality rates related to respiratory virus infections after bone marrow transplantation. Bone Marrow Transplant 31:695–700.

Merk J, Schmid FX, Fleck M, Schwarz S, Lehane C, Boehm S, Salzberger B, Birnbaum DE. 2005. Fatal pulmonary failure attributable to viral pneumonia with human herpes virus 6 (HHV-6) in a young immunocompetent woman. J Intensive Care Med 20:302–306.

Muir D, Pillay D. 1998. Respiratory virus infections in immunocompromised patients. J Med Microbiol 47:561–562.

Murali S, Langston AA, Nolte FS, Banks G, Martin R, Caliendo AM. 2009. Detection of respiratory viruses with a multiplex polymerase chain reaction assay (MultiCode-PLx Respiratory Virus Panel) in patients with hematologic malignancies. Leuk Lymphoma 50:619–624.

Nichols WG, Gooley T, Boeckh M. 2001. Community-acquired respiratory syncytial virus and parainfluenzaenza virus infections after hematopoietic stem cell transplantation: The Fred Hutchinson Cancer Research Center experience. Biol Blood Marrow Transplant 7:11S–15S.

Rafailidis PI, Mourtzoukou EG, Varbobitis IC, Falagas ME. 2008. Severe cytomegalovirus infection in apparently immunocompetent patients: A systematic review. Virol J 5:47–53.

Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. Am J Hyg 27:493–497.

Sanghavi SK, Abu-Elmagd K, Keightley MC, St. George K, Lewandowski K, Boes SS, Bullotta A, Dare R, Lassak M, Husain S, Kwak EJ, Paterson DL, Rinaldo CR. 2008. Relationship of cytomegalovirus load assessed by real-time PCR to pp65 antigenemia in organ transplant recipients. J Clin Virol 42:335–342.

Scalera NM, Mossad SB. 2009. The first pandemic of the 21st century: A review of the 2009 pandemic variant ininfluenzaenza A (H1N1) virus. Postgrad Med 121:43–47.

St. George K, Rinaldo CR. 1994. Effects of enhancing agents on detection of cytomegalovirus in clinical specimens. J Clin Microbiol 32:2024–2027.

St. George K, Patel NM, Hartwig RA, Scholl DR, Jollick JA, Jr, Kauffmann LM, Evans MR, Rinaldo CR, Jr. 2002. Rapid and sensitive detection of respiratory virus infections for directed antiviral treatment using R-Mix cultures. J Clin Virol 24:107–115.

Templeton KE, Scheltinga SA, Beersma MRC, Kroes ACM, Claas ECJ. 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by ininfluenzaenza A and ininfluenzaenza B viruses, respiratory syncytial virus, and parainfluenzaenza viruses 1, 2 3, and 4. J Clin Microbiol 42:1564–1569.

van Elden LJ, van Loon AM, van Alphen F, Hendriksen KA, Hoepelman AI, van Kraaij MG, Oosterheert JJ, Schipper P, Schuurman R, Nijhuis M. 2004. Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reverse-transcriptase polymerase chain reaction. J Infect Dis 189:652–657.