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Rapid and Sensitive Method Using Multiplex Real-Time PCR for Diagnosis of Infections by Influenza A and Influenza B Viruses, Respiratory Syncytial Virus, and Parainfluenza Viruses 1, 2, 3, and 4

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Laboratory diagnosis of viral respiratory infections is generally performed by virus isolation in cell culture and immunofluorescent assays. Reverse transcriptase PCR is now recognized as a sensitive and specific alternative for detection of respiratory RNA viruses. A rapid real-time multiplex PCR assay was developed for the detection of influenza A and influenza B viruses, human respiratory syncytial virus (RSV), parainfluenza virus 1 (PIV1), PIV2, PIV3, and PIV4 in a two-tube multiplex reaction which used molecular beacons to discriminate the pathogens. A total of 358 respiratory samples taken over a 1-year period were analyzed by the multiplex assay. The incidence of respiratory viruses detected in these samples was 67 of 358 (19%) and 87 of 358 (24%) by culture and real-time PCR, respectively. Culture detected 3 influenza A virus, 2 influenza B virus, 57 RSV, 2 PIV1, and 2 PIV3 infections. All of these culture-positive samples and an additional 5 influenza A virus, 6 RSV, 2 PIV1, 1 PIV2, 1 PIV3, and 3 PIV4 infections were detected by the multiplex real-time PCR. The application of real-time PCR to clinical samples increases the sensitivity for respiratory viral diagnosis. In addition, results can be obtained within 6 h, which increases clinical relevance. Therefore, use of this real-time PCR assay would improve patient management and infection control.

Respiratory infections caused by influenza virus type A, influenza virus type B, respiratory syncytial virus (RSV), parainfluenza virus type 1 (PIV1), PIV2, and PIV3 are major causes of upper and lower respiratory tract diseases in infants and young children, causing croup, bronchiolitis, and pneumonia (12). Additionally, these viruses have all been identified as important causes of severe lower respiratory tract disease, with significant morbidity and mortality, in elderly and immunocompromised patients (10, 16, 26).

Currently, viral culture usually in combination with immunofluorescence (IF) is the "gold standard" for laboratory diagnosis. However, it is not a rapid diagnostic test, and therefore, its clinical value is limited. Rapid antigen detection tests are now widely used in routine laboratories, but these have been shown to be less sensitive and specific (6, 17, 18). PCR is found to be more sensitive, specific, and rapid for detection of respiratory viruses (14, 15, 22, 23, 24). In addition, it can be used for diagnosis of a wider range of pathogens, such as human metapneumovirus.

As these respiratory viral pathogens cause very similar clinical symptoms, differential diagnosis of the pathogens is required in one sample. Monospecific PCR assays require separate amplification of each target and are therefore expensive and resource intensive. Multiplex PCR for clinical diagnosis has a significant advantage, as it permits simultaneous amplification of several viruses in a single reaction mixture, facilitating cost-effective diagnosis (2, 4, 8, 11, 15). However, so far these multiplex PCR assays distinguish the target by PCR fragment size on electrophoresis or hybridization with probes post-PCR.

Real-time PCR with specific detection of the product by fluorescent probes improves the specificity of assays and significantly reduces hands-on time. Additionally, another feature of the real-time PCR method is the ability to perform multiplex amplification and detection. In some real-time PCR platforms, four different amplification products can be distinguished in a single tube. Published real-time PCR formats for detection of respiratory viruses to date has only included one or two organisms (13, 21, 25).

In this study, two multiplex RNA PCRs were designed for detection of the pathogenic respiratory RNA viruses influenza A and influenza B viruses, RSV, PIV1, PIV2, PIV3, and PIV4. The assays were compared to viral culture retrospectively, and the potential application in routine diagnosis was assessed.

MATERIALS AND METHODS

Viruses. Respiratory viral strains (RSV subtype A [RSV-A-2, ATCC VR-1302], RSV subtype B [RSV-B-9320, ATCC VR-955], PIV1 [HA-2, ATCC VR-94], PIV2 [ATCC VR-92], PIV4a [M25, ATCC VR1378], PIV4b [CH19503, ATCC VR1377], and influenza B virus [B/Maryland/1/59, ATCC VR-296]) were obtained from the American Type Culture Collection. PIV3 (MK9, NCPV 00019) was obtained from the United Kingdom National Collection of Pathogenic Viruses. Influenza virus isolates were obtained from the Dutch National Influenza Center (Erasmus MC, Rotterdam, The Netherlands). Influenza A virus isolates included A/HK/1/68 (H3N2), A/Johannesburg/33/94 (H3N2), 10 typed influenza A virus isolates from The Netherlands in 2001, 5 influenza A virus H1N1 isolates, 5 influenza A virus H3N2 isolates, and influenza B isolates

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TABLE 1. Primers and probes for real-time PCR assays[a]

Primer	Sequence	Labels (5′, 3′)
Influenza As	AAAGCGAATTTCAGTGTGAT	
Influenza Aas	GAAGGCAATGGTGAGATTT	
Influenza A FAM	GCTGCCAGGGCTTTCACCGAAGAGGGGGCAGC	FAM, Dabcyl
Influenza Bs	GTCCATCAAGCTCCAGTTTT	
Influenza Bas	TCTTCTTACAGCTTGCTTGC	
Influenza B TXR	GCTGCCAACGAAGTAGGTGGAGACGGAGGGGCAGC	Texas Red, Dabcyl
RSVs	TTTCCACAATATYTAAGTGTCAA	
RSVas	TCATCWCCATACTTTTCTGTTA	
RSVHEX	GCGAGCCCATGTGAATTCCCTGCATCAATGCTCGC	HEX, BHQ-1
PIV1s	ACCTACAAGGCAACAACATC	
PIV1as	CTTCCTGCTGGTGTGTTAAT	
PIV1HEX	GCTGCC CAAACGATGGCTGAAAAAGGGA GGCAGC	HEX, BHQ-1
PIV2s	CCATTTACCTAAGTGATGGAA	
PIV2as	CGTGGCATAATCTTCTTTTT	
PIV2TXR	GCTGCCAATCGCAAAAGCTGTTCAGTCACGGCAGC	Texas Red, Dabcyl
PIV3s	GGAGCATTGTGTCATCTGTC	
PIV3as	TAGTGTGTAATGCAGCTCGT	
PIV3FAM	CGCGCTACCCAGTCATAACTTACTCAACAGCAACAGCGCG	FAM, Dabcyl
PIV4s	CCTGGAGTCCCATCAAAAGT	
PIV4as	GCATCTATACGAACACCTGCT	
PIV4Cy-5	GCTGCCGTCTCAAAATTTGTTGATCAAGACAATACAATTGGCAGC	Cy-5, BHQ-2

[a] Abbreviations: s, sense; as, antisense; TXR, Texas Red; Cy-5, indodicarbocyanine; BHQ, black hole quencher; Dabcyl, 4(4′-dimethylaminophenylazo)-benzoic acid. Nucleotides forming the stem of the molecular beacon are underlined.

including B/Netherlands/22/95 and B/Netherlands/138/95. From the repository of viral isolates in our department, 26 influenza A virus isolates, 11 influenza B virus isolates, 94 RSV isolates, 8 PIV1 isolates, 6 PIV2 isolates, 15 PIV3 isolates, and 1 PIV4 isolate were included for evaluation of the multiplex PCR.

Virus stocks. RSV type A and B isolates were cultured on HEp-2 cells, and PIV and influenza A and influenza B virus isolates was cultured on LLC-MK_2 cells. About 5 ml of each virus was prepared, and the stocks were stored in 0.5-ml aliquots. The limit of sensitivity of the multiplex RNA PCR was determined by testing 10-fold dilutions of the 50% tissue culture infectious dose ($TCID_{50}$) for each virus type. Each viral dilution series was inoculated in duplicate onto 24-well plates, centrifuged for 30 min at 1,500 × *g*, and incubated in CO_2 for 5 days. The cells were stained with monoclonal antibody (Dako, Glostrup, Denmark) to determine the $TCID_{50}$, except for PIV4, where cytopathic effect was used, as no suitable monoclonal antibody was available. Viral RNA was extracted from 0.2 ml of each dilution and tested by the multiplex RNA PCR under the conditions described below. The detection limit of the assay was determined as the highest dilution that resulted in a positive reaction.

Specificity of multiplex PCR. A panel of respiratory viruses and bacteria commonly found in the respiratory tract was used to determine the specificity of the multiplex RNA PCR. Nucleic acids were extracted from adenovirus (type 5), coronavirus (229E), metapneumovirus, rhinovirus (type 1b and 16), echovirus type 7, mumps, measles, *Bordetella bronchiseptica*, *Bordetella parapertussis*, *Bordetella pertussis*, *Burkholderia cepacia*, *Chlamydia pneumoniae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* and tested by the multiplex PCR.

Clinical samples. From December 2001 to December 2002, 358 respiratory samples (178 nasopharyngeal aspirates, 128 throat swabs, 23 throat wash samples, 13 bronchoalveolar lavage samples, 13 sputum samples, and 3 tracheal aspirates) were received in the laboratory for routine culture of respiratory viruses. From each sample, an aliquot was stored at −70°C for PCR analysis.

Respiratory viral culture. Cultures were performed by inoculating HEp-2, human embryo lung (HEL), and LLC-MK_2 cells with each clinical sample for the detection of respiratory viruses. Approximately 100 μl of each sample was inoculated onto each cell line in shell vials. Each shell vial was centrifuged at 1,500 × *g* for 30 min. One HEL shell vial was incubated at 32°C, and the other was incubated at 37°C. The LLC-MK_2 and HEp2 cells were incubated at 35°C. All shell vials were incubated for 14 days and examined daily for cytopathic effect. Immunofluorescence with commercial monoclonal antibodies for influenza A and influenza B viruses, RSV, PIV1, PIV2, PIV3, and adenoviruses confirmed positive cytopathic effects (Dako Diagnostics). Rhinoviruses were distinguished from enteroviruses by means of acid lability testing.

RNA extraction. Two methods were used for RNA extraction. The QIAamp Viral RNA Mini spin protocol (Qiagen, Hilden, Germany) was used for extraction in the development of the assay. All samples were extracted according to the manufacturer's instructions. Briefly, 140 μl of sample was extracted and the nucleic acids were eluted in 60 μl of buffer and stored at −70°C. In the negative control, sterile distilled water was added instead of specimen.

The second method was the automated MagNA Pure nucleic acid isolation system (Roche Diagnostics, Almere, The Netherlands), and this was used for testing the clinical samples to assess suitability for the assay in routine diagnosis. Nucleic acids were extracted from the original clinical material. Briefly, 200 μl of material was isolated with the Total Nucleic Acid isolation kit (Roche Diagnostics), as described by the manufacturer. The purified nucleic acid was eluted in a final volume of 100 μl. The MagNA Pure automatically performed all the steps of the procedure. Within the tips, nucleic acids were bound to magnetic beads, washed free of impurities, and finally eluted from the magnetic beads into a sealed cartridge. A total of 32 samples could be extracted in a single run, and this process took 2 h. Negative template controls were included in each MagNA Pure run. In the negative control, sterile distilled water was added instead of specimen.

Primer and probe design for real-time PCR. Primer and molecular beacon sequences were selected from an alignment of nucleotide sequences of the influenza A and influenza B viruses, RSV, PIV1, PIV2, PIV3, and PIV4 from GenBank. The alignment was performed to select a highly conserved region for each virus. The PCR primers and molecular beacons were optimized with Beacon Designer, version 2.0, to perform with an annealing temperature of 55°C (Premier Biosoft International, Palo Alto, Calif.). Each primer and molecular beacon selected was checked against the alignment to ensure that each primer and probe was targeted to a conserved region in the respective alignments. Additionally, the program checked interactions between primers and probes. The reference sequences used in Beacon Designer, version 2.0, were as follows: influenza A virus, accession no. AF348204; influenza B virus, accession no. AB036876; RSV, accession no. M75730; PIV1, accession no. U70948; PIV2, accession no. AF213352; PIV3, accession no. M18760; PIV4, accession no. M55976. Molecular beacons and primers were prepared by Biolegio, Malden, The Netherlands. Selected primers and probes are shown in Table 1.

Real-time PCR conditions. The assays were first optimized in a monospecific PCR; subsequently, two multiplex PCRs were performed. One multiplex PCR was performed with the influenza A and influenza B viruses and RSV, and the other multiplex PCR was performed with PIV1, PIV2, PIV3, and PIV4. Real-time PCR was performed in 50 μl of reaction mixture consisting of 10 μl of 5× one-step reverse transcription (RT)-PCR buffer (Qiagen one-step RT-PCR kit), 10 mM deoxynucleoside triphosphates (dNTPs), 4.5 mM $MgCl_2$, 1.25 U of RNAsin (Invitrogen, Paisley, Scotland), 0.6 μM concentrations of each primer,

0.34 μM (for influenza A virus, RSV, PIV2, PIV3, and PIV4) or 0.5 μM (for influenza B virus and PIV1) concentrations of molecular beacon, and 5 μl of template. The PCR thermal profile consisted of an initial cDNA step of 30 min at 50°C followed by 15 min at 95°C and 50 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Amplification, detection, and data analysis were performed with the iCycler IQ real-time detection system (Bio-Rad, Veenendaal, The Netherlands).

Influenza A virus conventional PCR. All samples found to be positive for influenza A virus by the real-time PCR were tested by a second influenza A virus PCR to confirm the results. PCR amplification was performed with primers described by Claas et al. (3), which target the same gene as the real-time PCR primers. Briefly, 5 μl of isolated RNA was converted to cDNA and amplified for 40 cycles with influenza A virus primers, designed to the nonstructural gene segment sequence. Cycling conditions were 3 min at 95°C, followed by 40 cycles of 60 s at 92°C, 120 s at 50°C, and 120 s at 74°C, followed by a 10-min hold at 72°C. The PCR product was detected by an enzymatic reaction after hybridization to a digoxigenin-labeled probe in an enzyme immunoassay system.

Real-time RSV PCR to the nucleocapsid. All samples found to be positive for RSV by the real-time PCR were confirmed by a second real-time PCR for RSV designed to the nucleocapsid, a different gene than that used for the multiplex real-time PCR. This was performed in 50 μl of reaction mixture consisting of 10 μl of 5× one-step RT-PCR buffer (Qiagen one-step RT-PCR kit), 10 mM dNTPs, 6.5 mM $MgCl_2$, 1.25 U of RNAsin (Invitrogen), 0.6 μM of concentrations of the sense and antisense primers (380RSVs, AATGGAAAAGAAATG AAATT, and 381as, GGAGAATCATGCCTGTATTC, respectively), 0.34 μM molecular beacon (Hex-GCTGCC-CAAYATTGAGATAGAATCTAGAAAA TCCTAC-GGCAGC-BHQ1), and 5 μl of template. The PCR thermal profile consisted of an initial cDNA step of 30 min at 50°C, followed by 15 min at 95°C and 50 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Amplification, detection, and data analysis were performed with the iCycler IQ real-time detection system (Bio-Rad).

PIV conventional PCR. All samples found to be positive for PIV by the real-time PCR were tested by a second PCR to confirm the results. All of these secondary PCRs targeted a different region of the same gene as the real-time PCR. This was performed in 50 μl of reaction mixture consisting of 10 μl of 5× one-step RT-PCR buffer (Qiagen one-step RT-PCR kit), 10 mM dNTPs, 4.5 mM $MgCl_2$, 1.25 U of RNAsin (Invitrogen), 0.6 μM concentrations of the sense and antisense primers, and 5 μl of template. The primers used for PIV1 were 229PIV1s (GATATAACAACCCCATGAC) and 230PIV1as (TGTAGCAACA TTGACTGCAT). For PIV2, the primers used were as described by Echevarria et al. (5). For PIV3, the primers used were as described by Swierkosz et al. (19), and for PIV4, the primers used were as described by Aguilar et al. (2). The PCR thermal profile consisted of an initial cDNA step of 30 min at 50°C, followed by 15 min at 95°C and 50 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The product was detected by agarose gel electrophoresis.

Analysis of results. To compare cycle threshold (C_t) values in culture-positive and -negative samples, a Student *t* test was performed by using SSPS (Munich, Germany).

RESULTS

Multiplex real-time PCR. Each assay was primarily set up as a monospecific assay which used a 10-fold dilution series of the target viruses. A standard curve was generated with the C_t values obtained, and this was used to optimize the PCR efficiency. The monospecific assays were then combined in two multiplex reactions and optimized further. One was comprised of the influenza A and influenza B viruses and RSV, and the other one was comprised of PIV1, PIV2, PIV3, and PIV4, with both assays having the same PCR protocol so they could be run in the same plate. The influenza A and influenza B virus and RSV probes were labeled with the fluorophores 6-carboxyfluorescein (FAM), Texas Red, and hexachlorofluorescein (HEX), respectively. The PIV probes were also labeled with these three fluorophores (PIV1, HEX; PIV2, Texas Red; PIV3, FAM), and in addition, the PIV4 probe was labeled with Cy5. Therefore, differentiation of the target was possible without post-PCR analysis.

TABLE 2. Comparison of C_t values after amplification of a dilution series of titrated virus in monospecifc and multiplex real-time PCR[a]

Virus and dilution ($TCID_{50}$)	C_t value for:	
	Monospecific PCR	Multiplex PCR
Influenza A		
10	36.63	37.11
1	39.52	40.10
0.1	42.51	43.25
Influenza B		
0.1	32.39	32.54
0.01	36.25	36.12
0.001	40.27	39.67
RSV		
1	32.36	33.27
0.1	35.87	36.12
0.01	41.56	41.75
PIV-1		
1	36.83	35.11
0.1	39.45	39.63
0.01	43.42	42.82
PIV-2		
0.1	33.25	33.45
0.01	37.23	37.42
0.001	40.13	40.03
PIV-3		
10	32.45	33.02
1	36.25	36.54
0.1	41.12	42.03
PIV-4		
0.01	33.0	34.12
0.001	38.25	40.15
0.0001	42.0	NEG

[a] For each virus, a 10-fold dilution series is shown with the mean relative C_t values. For each virus, the lowest level of detection is shown as well as the two 10-fold dilutions immediately prior to the lowest detection limit. Each dilution series was tested in triplicate to determine sensitivity. NEG, negative.

Specificity and sensitivity. To assess the integrities of the primers and probes, the two multiplex reactions were tested for cross-reactivity among the 7 viruses included and a panel of closely related and common human respiratory pathogens, as listed in Materials and Methods. No nonspecific reactions or any interassay cross-amplification were observed, and only the correct virus was amplified by the two multiplex reactions. In addition, several virus strains and clinical isolates were assayed, and all were correctly identified by the multiplex PCR.

The assay sensitivity was determined by amplification of RNA extracted from dilutions of a $TCID_{50}$ titrated stock of each virus. The fluorescent signal in the monospecific assay observed at various dilutions for each virus type corresponded to a calculated minimal amount of detectable RNA of 0.1 of the $TCID_{50}$ for influenza A virus, 0.001 of the $TCID_{50}$ for influenza B virus, 0.01 of the $TCID_{50}$ for RSV, 0.01 of the $TCID_{50}$ for PIV1, 0.001 of the $TCID_{50}$ for PIV2, 0.1 of the $TCID_{50}$ for PIV3, and 0.0001 of the $TCID_{50}$ for PIV4. As shown in Table 2 the C_t values of the 10-fold dilution series of the target viruses in the multiplex assay were within ±1 cycle of those of the monospecific assays. Only the PIV4 assay showed decreased sensitivity of 1 log, but the assay was still very sensitive in comparison to cell culture. There was no difference in sensitivity of the monospecific and the multiplex assays for

influenza A and influenza B viruses, RSV, PIV1, PIV2, and PIV3.

The assay sensitivity was also assessed in the presence of common bacterial pathogens. The C_t values observed in either multiplex assay were not affected in the presence of *P. aeruginosa*, *S. aureus*, and *S. pneumoniae*.

Automated nucleic acid extraction by MagNA Pure. To enable further automation of the procedure, 30 clinical samples (10 nasopharyngeal aspirates and 20 throat swab samples), all positive by cell culture for RSV, were extracted by the Qiagen RNA extraction method and the MagNA Pure method. Thereafter, the two RNA extracts were run in the multiplex PCR in the same run to compare the efficacy of the MagNA Pure extraction method. No significant difference in C_t values was observed (results not shown), and therefore, nucleic acids were extracted from the clinical samples with the MagNA Pure system.

Clinical evaluation. A total of 358 clinical samples collected from 279 patients were used in this study. Viral culture and multiplex PCR were performed on each sample. Viral culture with confirmation by IF revealed 67 positive (influenza A and influenza B viruses, RSV, PIV1, and PIV3) samples of the total 358 tested (19%). The same specimens were screened for the seven viruses with the two multiplex PCR assays, and the result was 87 positive samples (24%). All different targets were detected (Table 3). Results for 32 samples (25 samples and 7 controls) could be obtained in 6 h with only 1 h of hands-on time.

The multiplex PCR-positive specimens included all the samples that were positive by cell culture and 20 additional ones. The C_t values were also recorded for all the real-time PCR results. In culture-positive samples, the mean C_t value was 26 (range, 15.3 to 37.4), whereas in culture-negative samples, the mean C_t value was 37.5 (range, 25.8 to 45.8) ($P < 0.001$). Table 3 shows the results of the comparison of viral culture and the multiplex PCR in respiratory samples.

The 20 samples that were positive by the real-time PCR and were culture negative were tested by a second RNA PCR for confirmation of the results. Seventeen were found to be positive by this additional testing, supporting the findings of the multiplex real-time PCR (Table 3). The three additional positive samples by PCR were from patients in whom previous or subsequent samples were culture positive, suggesting that the samples can be considered true positives.

TABLE 3. PCR and viral culture results for 358 clinical samples

Virus	No. of samples with positive result by:		
	Multiplex real-time PCR	Viral culture	Secondary PCR after discrepant result
Influenza A	8	3	5
Influenza B	2	2	
RSV	66	58	8
PIV1	4	2	1
PIV2	1	0	1
PIV3	3	2	0
PIV4	3	0	2
Total (%)	87 (24)	67 (19)	

DISCUSSION

A two-tube real-time multiplex PCR assay was developed to detect and distinguish RSV, influenza A and influenza B viruses, PIV1, PIV2, PIV3, and PIV4. These viruses cause the majority of viral lower respiratory tract infections in children (12) and are a significant cause of disease in immunocompromised patients (10). The assay was found to be sensitive and specific. Previously, multiplex assays using hybridization have been shown to reduce sensitivity in comparison to a single target PCR (11). However, the real-time PCR method enables controlled optimization of PCR efficiency. Except for the PIV4 reaction, which lost some sensitivity, a multiplex PCR assay could be developed to detect six important viral respiratory pathogens with the same sensitivity as the single-target assays.

Multiplex real-time PCR was found to be more sensitive than cell culture on a range of different respiratory samples. These findings are consistent with those of other studies, which employed RT-PCR for the detection of viral infections (3, 7, 9, 15, 22, 23, 24). Conventional respiratory viral cell culture is limited by a lack of speed and therefore has little impact on patient care (1, 27). Rapid immunological tests partly overcome this problem, but the low sensitivity requires cell culture to be performed on negative specimens (6, 14, 17, 18). Using automated nucleic acid extraction procedures and real-time PCR, 32 samples can be processed in 6 h with 1 h of hands-on time. In comparison, conventional PCR assays use gel electrophoresis, blotting, or hybridization to detect the amplified targets and take significantly more hands-on time (15). The multiplex real-time PCR generates a diagnostic result within one working day and within a few hours of the rapid antigen detection tests. Elimination of post-PCR processing not only increases the speed but also reduces the risk of cross-contamination. Real-time PCR applications on respiratory viruses have been described previously (13, 21, 25), either on the LightCycler or on the ABI 7700 platform. These systems can detect only two different fluorophores in a single tube, thus reducing the number of targets that can be challenged. In this study, the iCycler IQ real-time PCR detection platform was used, which is able to detect four different fluorophores in a single well.

In the design of these real-time PCRs, an alignment of conserved regions of the target viruses was made with publicly available GenBank sequences. However, new strains of viruses will continue to emerge. Because of the high mutation frequency of RNA viruses and the heterogeneity of the circulating strains, it is theoretically possible that mutations in the primer and probe regions may evolve (25). However, so far the selection of the target sequences in the multiplex PCR has not contributed to any known false negatives in our studies. Inhibition of the PCR can also lead to false-negative results. The majority of the samples tested in this study were throat swabs, which were previously shown to be rarely inhibitory by PCR (20). Inhibition of sputum and bronchoalveolar lavage samples has been assessed by spiking experiments, and no inhibition was seen (results not shown). The addition of an internal control reaction would further improve the assay as a routine diagnostic assay, as each sample would be checked for the quality of the nucleic acid extraction and for inhibition.

In the clinical evaluation, the real-time multiplex PCR detected all viral culture-positive clinical samples and resulted in additional positives, which would otherwise have been missed by routine methods. Twenty PCR-positive samples (23%) were missed by viral culture, which most likely was due to the amount and viability of the viruses present in the clinical samples. This was reflected by the C_t values obtained. In culture-positive samples, the mean C_t value was 26, whereas in culture-negative samples, the mean C_t value was 38 ($P < 0.001$), indicating that less virus was present in the culture-negative samples. In addition, there is no need for replication-competent viruses in the clinical samples, making the PCR method less dependent on specimen quality. Dual infections occur as well (11, 14), and two have been detected by the multiplex PCR. Both dual infections were RSV in combination with influenza A virus.

Although of limited value when considering cell culture, with its limited sensitivity, as the gold standard, the specificity of the real-time PCR was 93% and the sensitivity was 100%. However, all of the additional 20 positives were patients with respiratory symptoms and are therefore unlikely to represent false positives. Seventeen of those tested by secondary PCR assays were all confirmed, and no other respiratory pathogen was found by conventional bacteriological and virological techniques. In some cases, the secondary PCR assays are probably less sensitive than the real-time PCR assays, as shown by the 3 samples missed by the secondary PCRs. Currently, many cases of respiratory infection go undiagnosed, owing to the sensitivity of the diagnostic assays and the range of respiratory pathogens. Therefore, a more-sensitive technique helps to elucidate more respiratory infections.

The multiplex PCR assay described in this study detects seven of the possible agents that might cause clinically significant respiratory tract infections. It is possible to include other targets, such as rhinoviruses, coronaviruses (OC43, 229E, and severe acute respiratory syndrome coronavirus), and human metapneumovirus, in an additional multiplex PCR. Diagnosis of the adenovirus with its DNA genome might be best employed in a multiplex assay with bacterial respiratory pathogens.

Cost-effective implementation of molecular testing in routine diagnostics requires further attention. Although reagents for PCR are more expensive than those for cell culture, automation of the extraction process and the use of real-time PCR reduce the hands-on time in the laboratory. Additional cost benefits may result from the more-rapid diagnosis in reduced time of hospitalization, decreased nosocomial spread, and decreased use of antibiotics (1, 27). Obviously, strategies for application of these real-time PCR assays will have to be designed. During an influenza epidemic, not all multiplex assays would be applied simultaneously to all samples. Dependent on the season and viral etiology, specific algorithms or innovative strategies may optimize the implementation of these tests.

In conclusion, the multiplex real-time PCR assay constitutes a specific and sensitive alternative to conventional culture and IF methods, and use of this assay would aid in the diagnosis of respiratory disease. Specific and sensitive results within 6 h are important in a clinical setting, and therefore, this assay could improve patient management by appropriate therapy following rapid diagnosis of a viral infection.

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