A Sensitive, Specific, and Cost-Effective Multiplex Reverse Transcriptase-PCR Assay for the Detection of Seven Common Respiratory Viruses in Respiratory Samples

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Cell culture and direct fluorescent antibody (DFA) assays have been traditionally used for the laboratory diagnosis of respiratory viral infections. Multiplex reverse transcriptase polymerase chain reaction (m-RT-PCR) is a sensitive, specific, and rapid method for detecting several DNA and RNA viruses in a single specimen. We developed a m-RT-PCR assay that utilizes multiple virus-specific primer pairs in a single reaction mix combined with an enzyme-linked amplicon hybridization assay (ELAHA) using virus-specific probes targeting unique gene sequences for each virus. Using this m-RT-PCR-ELAHA, we examined the presence of seven respiratory viruses in 598 nasopharyngeal aspirate (NPA) samples from patients with suspected respiratory infection. The specificity of each assay was 100%. The sensitivity of the DFA was 79.7% and the combined DFA/culture amplified-DFA (CA-DFA) was 88.6% when compared to the m-RT-PCR-ELAHA. Of the 598 NPA specimens screened by m-RT-PCR-ELAHA, 3% were positive for adenovirus (ADV), 2% for influenza A (Flu A) virus, 0.3% for influenza B (Flu B) virus, 1% for parainfluenza type 1 virus (PIV1), 1% for parainfluenza type 2 virus (PIV2), 5.5% for parainfluenza type 3 virus (PIV3), and 21% for respiratory syncytial virus (RSV). The enhanced sensitivity, specificity, rapid result turnaround time and reduced expense of the m-RT-PCR-ELAHA compared to DFA and CA-DFA, suggests that this assay would be a significant improvement over traditional assays for the detection of respiratory viruses in a clinical laboratory. (*J Mol Diagn* 2004, 6:125–131)

Common etiological viral agents of respiratory infections include adenoviruses (ADV), influenza types A and B (Flu A and B), parainfluenza types 1, 2, and 3 (PIV1, 2, 3), and respiratory syncytial virus (RSV).^{1–4} These viruses are responsible for a spectrum of acute upper and lower respiratory tract disease. In children, the elderly and other immunocompromised groups, respiratory viruses can cause more serious clinical complications, such as croup, bronchiolitis, and pneumonia, which often require hospitalization.^{5,6} Virus isolation by cell culture and direct immunofluorescent antibody assay (DFA) staining with monoclonal antibodies are two of the most commonly used laboratory techniques for detecting respiratory viruses. Both these methods have significant limitations in sensitivity and specificity. DFA detection is more rapid but less sensitive than viral culture, and results may be affected by specimen quality (ie, presence of intact, infected cells), virus type, and interpretation of a positive result, which is subjective and requires a great deal of technical skill.^{2,7–10} DFA is also unable to detect minor variations in amino acid sequence on envelope or capsid proteins.¹¹ Viral culture is still considered the “gold standard” for respiratory virus detection, but is limited by a prolonged result turnaround time (ie, 2 days to 1 week) and is dependent on stringent specimen transport and storage conditions to preserve the infectivity of the virus.^{9,12–15} Although the combination of both these techniques can provide an increase in the number of positive results, a significant proportion of specimens still remain negative, despite clinical suspicion of viral infection.⁸

Several studies have shown that polymerase chain reaction (PCR) amplification can resolve the intrinsic limitations associated with traditional diagnostic techniques by combining increased sensitivity, specificity, and rapid result turnaround time.^{16,17} Also, PCR results are not dependent on infectious virus or viable cells. However,

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PCR may be affected by the presence of sequence variation that can be overcome by designing the assay to target highly conserved nucleic acid sequences.¹⁸ Also, using conventional PCR technology to detect several viruses individually is labor-intensive and expensive.^{18,19} These limitations can be overcome by using a multiplex PCR assay. The multiplex format is a significant improvement over conventional PCR protocols, achieved by incorporating multiple primers that amplify RNA or DNA from several viruses simultaneously in a single reaction.^{9,20–25}

In this study we combine the multiplex RT-PCR with an enzyme-linked amplicon hybridization assay (ELAHA) developed in our laboratory to detect amplification products using a colorimetric detection method.²⁶ The ELAHA method can increase the sensitivity and specificity of PCR assays by detecting amplicon with a sequence-specific biotinylated probe.^{26,27} We also incorporated an internal control PCR reaction. One of the major limitations of PCR detection is false-negative results as a consequence of PCR inhibitors present in the clinical sample that are not removed by the extraction process. The internal control PCR reaction incorporated in this multiplex RT-PCR, targeted sequences of the human endogenous retrovirus (ERV-3).²⁸ To date, no published multiplex-PCR assay has been able to simultaneously detect adenovirus, flu A and B, RSV A and B, and Para 1, 2, and 3.

We evaluated the suitability of the m-RT-PCR-ELAHA as an alternative laboratory method to the traditional DFA or viral culture. In all, 598 nasopharyngeal aspirates from patients with suspected respiratory infection were tested for the presence of seven respiratory viruses. The results of the m-RT-PCR-ELAHA were compared to those obtained by a testing algorithm used in our hospital's laboratory combining DFA and a culture augmented DFA (CA-DFA) method. Testing of a panel of DNA and RNA extracted from 41 unrelated organisms confirmed the specificity of the primers and probes used in the m-RT-PCR-ELAHA.

Materials and Methods

Patient Specimens

Between December 2000 and September 2001, nasopharyngeal aspirate (NPA) specimens were collected from 598 pediatric patients who presented to Royal Children's hospital with suspected acute respiratory infection. Age ranges for the patients were 10 days to 15 years. All NPAs submitted to the Queensland Health Pathology Service (QHPS) for the routine investigation of respiratory viruses and bacteria, were used in this study. NPA samples collected from a patient were immediately added to 2.0 ml of viral transport medium (VTM) containing minimal essential medium with 2% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml), amphotericin B (20 µg/ml), neomycin (40 µg/ml) and NaHCO₃ buffer. A 1.0-ml volume of each sample was used to prepare cells for DFA and to inoculate cell monolayers for

CA-DFA. Residual specimens in VTM were stored at –70°C and were thawed at –4°C before PCR testing.

Extraction of Viral Nucleic Acids

Viral nucleic acids were extracted from 0.2 ml of each specimen in VTM using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Castle Hill, NSW, Australia), following the manufacturer's instructions. Purified specimen nucleic acid was eluted from the column in 50 µl of kit elution buffer (Roche Diagnostics). Nucleic acid extracts were stored at –70°C until analysis and were used in the multiplex RT-PCR-ELAHA and the monospecific RT-PCR assays.

Multiplex Reverse Transcriptase PCR (m-RT-PCR)

The m-RT-PCR was performed using 0.2 ml thin-walled PCR tubes in a Perkin Elmer 9600 thermal cycler (Applied Biosystems, Foster City, CA, USA). The RT-PCR mixture used in the assay was the Superscript One-Step RT-PCR kit (with Platinum *Taq*) (Invitrogen, USA). Each reaction contained the following kit reagents: 1 µl of RT/Platinum *Taq* mix and 25 µl of 2X Reaction Mix (containing 0.4 mmol/L of each dNTP and 2.4 mmol/L MgSO₄). To this were added four units of extra Platinum *Taq* (Invitrogen, Carlsbad, CA, USA) 10 pmol of each primer (including the ERV primers to act as internal control; Table 1), 0.1 nmoles of digoxigenin-11-dUTP (Roche Diagnostics) and 5 µl of specimen DNA or RNA extract or control. The final reaction volume was adjusted to 50 µl with PCR-grade water and RT-PCR amplification was performed using the following conditions: initial incubation for 30 minutes at 50°C for reverse transcription, followed by a denaturation step at 95°C for 2 minutes; followed by 45 cycles of denaturation at 95°C for 20 seconds; primer annealing at 58°C for 20 seconds; extension at 72°C for 20 seconds; and 1 cycle of further extension at 72°C for 7 minutes.

Enzyme-Linked Amplicon Hybridization Assay (ELAHA)

The ELAHA method used seven biotinylated probes for the detection of ADV, Flu A and B, PIV1, 2, 3, and RSV (Table 1) using the protocol previously described.²⁶ Briefly, following amplification, 5 µl of reaction mix was added to each of eight reaction tubes (200 µl) containing 10 ng of probe, specific for each of the seven respiratory viruses and the ERV, diluted in 5 µl of 1X sodium chloride/sodium citrate buffer (SSC) (3 mol/L sodium chloride, 0.3 mol/L sodium citrate). A 40-µl volume of 1X SSC was added to each tube giving a final reaction volume of 50 µl. The tubes were then placed into a thermocycler and held at 94°C for 5 minutes to denature the amplicon followed by rapid cooling to 4°C. The contents of the tubes were transferred to streptavidin-coated wells of a 96-well microtitre plate (Thermo-Labsystems, Ryolalme, NSW, Australia) and incubated for 20 minutes at 37°C. The

Table 1. Primers and Probes Used in this Study

Designation	Sequence (5' to 3')	Target gene	Reference
Primers			
FluAs	GGA CCT CCA CTT ACT CCA AAA CAG AAA C	NS	This study
FluAas	GTA AGG CTT GCA TGA ATG TTA TTT GCT C	NS	This study
FluBs	GAT ATA CGT AAT GTG TTG TCC TTG AG	NS	This study
FluBas	GAC CAG TCT AAT TGT CTC CCT CTT C	NS	This study
PIV1s	ATG CTC CTT GCC CAC TGT GAA TG	HN	This study
PIVas	AAT CTT TAT CCC ACT TCC TAC ACT TG	HN	This study
PIV2s	GAC ATT CCT GAT ACC CTT AAT CAC CAA A	N	This study
PIV2as	CTT ACT ACC ATT GAC TTG CGG ATG	N	This study
PIV3s	ACC AGG AAA CTA TGC TGC AGA ACG GC	N	35
PIV3as	GAT CCA CTG TGT CAC CGC TCA ATA CC	N	35
RSVs	GCC AAA AAA TTG TTT CCA CAA TA	L	This study
RSVas	TCT TCA TCA CCA TAC TTT TCT GTT A	L	This study
ADVs	ACT ACA ACA TTG GCT ACC AGG GC	H	This study
ADVas	GCC GAG AAG GGC GTG CGC AGG TA	H	33
PHP10-s	CATGGGAAGCAAGGGAACCTAATG	Human ERV-3	28
PHP10-as	CCCAGCGAGCAATACAGAATTT	Human ERV-3	28
Probes (all labeled with biotin at 5' end)			
FluA	TTG ACC TAG TTG TTC TCG CCA	NS	This study
FluB	CTC CCA CCG CAG TTT CAG CTG	NS	This study
PIV1	CAG ATT ACT CGA GTG AAG GTA TAG A	HN	This study
PIV2	CTG CTC CTG ATC AAC CAC CAG TAT C	N	This study
PIV3	GGT ATC CAT CAT GTT TAG GAG CTC T	N	35
RSV	GGA ATT CAC ATG GTC TAC TAC TGA CTG T	L	This study
ADV	CAC CGC GGC GTC ATC GAG AC	H	This study
PHP-P505	TCTTCCTCGAACCTGCACCATCAAGTCA	Human ERV-3	28

NS, non-structural protein; HN, haemagglutinin neuraminidase; N, nucleocapsid protein; L, L protein; H, hexon protein; ERV, endogenous retrovirus.

wells were washed four times with 1X SSC. Anti-digoxigenin peroxidase conjugate (Roche Diagnostics) was prepared by diluting the conjugate in phosphate-buffered saline (PBS, pH 7.2) to a final concentration of 150 mU/ml. A 100- μ l volume was added to each well and incubated for 20 minutes at 37°C. Wells were again washed four times with 1X SSC. Finally, 100 μ l of tetramethylbenzidine substrate (TMB, Elisa Systems, Windsor, Queensland, Australia) was added to each well and incubated at room temperature for 10 minutes. The reaction was stopped by the addition of 100 μ l of 1 mol/L HCl. The optical density for each well was recorded using a plate spectrophotometer (Murex Diagnostics, Norcross, GA, USA) at a wavelength of 450 nm using 690 nm as a reference. An optical density of 0.2 or greater was indicative of a positive result.

Sensitivity of the Multiplex Reverse Transcriptase PCR

The sensitivity of the m-RT-PCR assay was determined for each of the targets using virus suspensions in cell culture media that had been quantified in terms of TCID₅₀. The Tissue Culture Infectious Dose 50 (TCID₅₀) is the quantity of virus in a specified suspension volume that will infect 50% of cell cultures. This was calculated for each virus suspension using the Karber method²⁹ by inoculating a 10-fold dilution series of the original suspension onto cell monolayers cultured in the wells of a 96-well tissue culture plate (Nunc A/S, Kamstrup, Roskilde, Denmark). Each dilution was inoculated onto eight separate wells (cul-

tures), and the number of ensuing infected monolayers was determined by direct fluorescent staining for each dilution.

To determine the sensitivity of the m-RT-PCR for each of the seven respiratory viruses (ADV, Flu A and B, PIV1, 2, 3, and RSV), six 10-fold dilutions in VTM were made of virus suspensions for which a TCID₅₀ had previously been determined using standard virological technique.^{29–32} Dilutions ranged from 5×10^4 to 0 TCID₅₀ for each virus. Viral RNA or DNA was extracted from 0.2 ml of each dilution and tested by the m-RT-PCR assay using the conditions described above. Viral targets, for which the assay returned a sensitivity between 5 to 50 TCID₅₀ per milliliter, were repeated using serially twofold dilutions ranging from 5 to 80 TCID₅₀ per milliliter. This was done to determine a more accurate estimation of the analytical sensitivity in this range of viral concentrations. The highest dilution returning a positive RT-PCR result was considered to be representative of the assay's analytical sensitivity.

Specificity of the m-RT-PCR

Specificity testing between the seven viral targets was performed using RNA and DNA extracted as detailed above from clinical isolates of Flu A, Flu B, PIV1, PIV2, PIV3, RSV types A and B, and Adenovirus. These isolates were subsequently used as the positive controls in each run. In addition, a panel of respiratory organisms found in the respiratory tract was used to determine the inter-specificity of the m-RT-PCR. This consisted of reference strains and clinical isolates shown in Table 2. Nucleic

Table 2. Organisms Used to Test the Specificity of the m-RT-PCR-ELAHA

Reference strains	Clinical isolates
<i>Acinetobacter baumannii</i> ACM 686	<i>Acinetobacter calcoaceticus</i> sub. <i>Anitratus</i>
<i>Bacteroides distasonis</i> ATCC 8503	<i>Aspergillus flavus</i>
<i>Bordetella pertussis</i> ATCC 12742	<i>Aspergillus fumigatus</i>
<i>Burkholderia cepacia</i> ATCC 17765	<i>Aspergillus niger</i>
<i>Candida albicans</i> ATCC 14053	<i>Chlamydia pneumoniae</i> ,
<i>Corynebacterium diphtheriae</i> ATCC 13812	cytomegalovirus,
<i>Escherichia coli</i> ATCC 35218	coxsackie B6 virus
<i>Haemophilus influenzae</i> ATCC 10211	Epstein-Barr virus
<i>Klebsiella pneumoniae</i> ATCC 13883	<i>Enterobacter cloacae</i>
<i>Neisseria lactamica</i> ATCC 23970	enteroviruses
<i>Neisseria meningitidis</i> ATCC 13102	human metapneumovirus
<i>Porphyromonas gingivalis</i> ATCC 33277	herpes simplex virus type 1
<i>Proteus vulgaris</i> ATCC 6380	<i>Klebsiella oxytoca</i>
<i>Pseudomonas aeruginosa</i> ATCC 27853	<i>Morganella morganii</i>
<i>Staphylococcus haemolyticus</i> ATCC 29970	<i>Mycoplasma pneumoniae</i>
<i>Streptococcus agalactiae</i> ATCC 12386	rhinovirus
<i>Streptococcus mutans</i> ATCC 35668	<i>Serratia marcescens</i>
<i>Streptococcus pneumoniae</i> ATCC 27336	<i>Staphylococcus aureus</i>
<i>Streptococcus pyogenes</i> ATCC 19615	<i>Staphylococcus epidermidis</i>
<i>Streptococcus salivarius</i> ATCC 13419	<i>Stenotrophomonas maltophilia</i>
	<i>Ureaplasma urealyticum</i>
	varicella zoster virus

acid from these organisms was purified and tested in the m-RT-PCR assay using the conditions described above. The amount of non-specific nucleic acid ranged from 0.4 to 0.9 µg per reaction.

Direct Fluorescent Antibody (DFA) Assay

The DFA and CA-DFA testing algorithm was used in the diagnostic virology laboratory of the QHPS to test clinical specimens for the presence of respiratory viruses. This involved screening the specimens initially by DFA, and specimens that produced a negative result were then further tested by CA-DFA. The DFA was performed using the Bartel's Viral Respiratory Screening and Identification kit (Intracel Corporation, Frederick, MD, USA) according to manufacturer's instructions. Briefly, glass substrate slides were prepared from epithelial cells present in the NPA specimen. These were air-dried and fixed in acetone for 10 minutes. Each well of the slide was covered with 30 µl of monoclonal antibody specific for each of the seven respiratory viruses. Slides were incubated in a humid chamber for 30 minutes at 37°C, rinsed with distilled water, followed by three washes in PBS. Thirty µl of anti-mouse FITC conjugate (Intracel) was used to cover each well, and slides were incubated again for 30 minutes at 37°C. Wells were rinsed and washed as above. After a final rinse in distilled water the slides were dried before mounting with a coverslip. Cells in each well were examined for fluorescence under UV illumination at ×400 magnification using an epifluorescent microscope (Olympus BX60; Olympus Optical Co., Tokyo, Japan).

Culture Augmented-DFA

The culture augmented-DFA (CA-DFA) method was developed in our laboratory to replace viral isolation by cell culture. This method adopted the principles of a standard

shell vial technique.¹⁵ Four continuous cell lines, MRC-5, A549, HEP-2, and LLC-MK2 (Biowhittaker, Walkersville, MD, USA), susceptible to respiratory virus infection were cultured in 96-well sterile tissue-culture plates (Nunc, Australia). An 80-µl volume of each NPA specimen was centrifuged onto the cell monolayers at 4000 rpm for 30 minutes at 35°C using a Hettich Rotanta 96R microtiter plate centrifuge (Hettich-Zentrifugen, Tuttlingen, Germany). Supernatants from each well were then aspirated and replaced with 200 µl of maintenance medium consisting of 194 µl of Eagle's minimum essential medium (Biowhittaker) containing 2% (v/v) fetal bovine serum (Trace Biosciences, Sydney, NSW, Australia), 9.75 units of penicillin G (5000U/ml; CSL Biosciences, Australia), 10 µg of streptomycin sulfate (5000 µg/ml; CSL Biosciences, Melbourne, VIC, Australia), and 0.25 µg of fungizone (5 mg/ml; Apoteco, Princeton, NJ, USA). After 40 hours of incubation at 37°C in a 5% CO₂ atmosphere, wells were aspirated and cell monolayers were fixed with an acetone/methanol mixture (1:1 v/v) for 10 minutes at -20°C. Cell monolayers in the wells were dried and then overlaid with 30 µl of appropriate monoclonal antibody (Intracel) specific for the target respiratory virus as described in the DFA method above. After incubation with anti-mouse FITC conjugate (Intracel), the presence of a respiratory virus was indicated by the detection of at least one single fluorescent cell under UV illumination using an inverted fluorescent microscope (Eclipse TE200; Nikon-Kawasaki, Kanagawa, Japan) at ×100 magnification.

Discrepant Analysis

Results that were discrepant between the m-RT-PCR-ELAHA and the DFA/CA-DFA algorithm were resolved by discrepant analysis using monospecific PCR assays for each virus. Adenovirus-positive specimens were con-

Table 3. Detection of Each of the Seven Respiratory Viruses by DFA, DFA/CA-DFA, and m-RT-PCR-ELAHA (number of confirmed positive samples, 202)

	DFA positive no.	(%)*	DFA/CA-DFA positive no.	(%)	m-RT-PCR-ELAHA positive† no.
ADV	11	(64.7)	14	(82.3)	17
Flu A	5	(35.7)	9	(64.2)	14
Flu B	2	(100)	2	(100)	2
PIV 1	1	(16.6)	5	(83.3)	6
PIV 2	5	(71.4)	6	(85.7)	7
PIV 3	29	(87.8)	29	(87.8)	33
RSV	108	(87.8)	114	(92.6)	123
TOTAL	161	(79.7)	179	(88.6)	202

*Percentage of total number of proven positive samples.

†All m-RT-PCR-ELAHA positive samples were confirmed by monospecific RT-PCR for each virus.

firmed using an ADV nested PCR assay targeting a sequence of the hexon gene³³ 2050-bp upstream from the m-RT-PCR target for this virus. Specimens positive for Flu A were confirmed using a LightCycler (Roche) assay, which targeted the matrix gene.³⁴ The single specimen positive for PIV1 was confirmed by a monospecific RT-PCR assay using primers, which targeted the hemagglutinin neuraminidase gene 161-bp upstream from the m-RT-PCR target for this virus.⁷ The PIV2 specimen was confirmed using an RT-PCR assay targeting a sequence of the gene coding for the nucleocapsid protein, 46-bp upstream from the sense primer of the multiplex target.³⁵ The additional specimens positive for PIV3 were confirmed using a monospecific RT-PCR assay, targeting the hemagglutinin-neuraminidase gene (unpublished data). RSV-positive specimens were confirmed using a PCR-ELAHA assay that targets the RSV nucleocapsid gene.³⁵ Previous evaluations of these confirmatory tests in our laboratory have shown that each assay is highly suitable for the detection of its respective target (unpublished data).

Results

A total of 598 nasopharyngeal aspirate (NPA) samples from patients with suspected respiratory infection were tested by m-RT-PCR and a combination of the DFA and CA-DFA. In total, 202 (33.7%) specimens were positive by m-RT-PCR, 179 (29.9%) were positive by the DFA and CA-DFA combination, and 396 (66.2%) specimens were negative by all three methods. All 179 specimens that tested positive by the DFA and CA-DFA combination also tested positive by the m-RT-PCR-ELAHA and 161 (89.9%) of these were detected by DFA alone (see Table 3). The m-RT-PCR-ELAHA detected 23 additional positive specimens (see Table 3).

The sensitivity of m-RT-PCR-ELAHA was consistently better than DFA alone for all viruses. This finding was most noted in influenza A and PIV type 1 where the DFA detected only 5 of 14 (36%) and 1 of 6 (17%) positive samples respectively. For the remaining respiratory viruses, the sensitivity of DFA detection ranged from 11 of 17 (64%) for ADV to 108 of 123 (88%) for RSV (Table 3). All influenza B-positive specimens were detected by DFA, but considering these comprised only two speci-

mens, the validity of this result was difficult to interpret. Comparing the conventional methods to m-RT-PCR shows a sensitivity of 79.7% for the DFA and a sensitivity of 88.6% for the DFA/CD-DFA testing algorithm. Of the 396 specimens that were negative by all assays, only five were found to be negative for the internal control sequence (human ERV). All specimens that were positive by m-RT-PCR-ELAHA for respiratory viruses were positive for human ERV.

The analytical sensitivity of the m-RT-PCR-ELAHA was greatest for RSV with a value of 5 TCID₅₀ per milliliter of culture. Similarly, the assay detected 10 TCID₅₀ per milliliter of ADV, Flu A and PIV1, 15 TCID₅₀ per milliliter of Flu B and PIV3, with the detection of PIV2 the least sensitive at 40 TCID₅₀ per milliliter.

The specificity of all three methods was found to be 100%. The 23 specimens that were positive by m-RT-PCR-ELAHA alone were also positive by conventional PCR assays targeting different regions of each viral genome. The specificity of the m-RT-PCR-ELAHA was further confirmed by testing RNA and DNA that had been extracted from 41 unrelated viruses, bacteria, yeast, and fungi. No positive reactions were observed. In addition, the specificity of the ELAHA probes was established by testing for cross-reactions of amplification products obtained from the seven target respiratory viruses with unrelated probes. We found there were no cross-reactions in the ELAHA detection step.

The turnaround time for conventional virus detection methods ranged from approximately 2 to 3 hours for DFA to 2 to 3 days for CA-DFA and up to 1 to 2 weeks for virus isolation. The m-RT-PCR-ELAHA assay, on the other hand, had a turnaround time for results that was less than 5 hours. The cost of reagents alone for DFA and CA-DFA in our laboratory was calculated as \$USD 16.23, and \$USD 19.92, respectively, whereas the m-RT-PCR-ELAHA was \$USD 11.07.

Discussion

Traditionally, detection of respiratory viruses including ADV, Flu A and B, PIV types 1, 2, and 3, and RSV, has relied on the direct visualization of viral antigens by DFA. However, most laboratories supplement rapid antigen testing with some method of virus culture and indirect

antigen detection, to increase the overall level of sensitivity. Doing so may prolong the diagnostic procedure from approximately 3 hours to 7 days. Recently, molecular methods have been developed that have increased sensitivity over traditional methods, but these are often limited to the detection of a single respiratory virus per reaction.^{7,36} The aim of this study was to develop a rapid multiplex PCR assay that identified seven respiratory viruses in a single reaction and to evaluate the utility of this method in our clinical setting.

An inherent limitation in designing a multiplex PCR assay is the loss of sensitivity that results when combining a large number of primer sets in a single reaction. In this study, we carefully optimized the reaction conditions to obtain maximum sensitivity (data not shown). As a result, despite the presence of eight primer sets in the PCR reaction mixture, the m-RT-PCR-ELAHA was able to specifically detect all virus types tested at a high level of sensitivity. This sensitivity was enhanced by the incorporation of the ELAHA detection step, which was approximately 100-fold more sensitive than conventional agarose gel detection methods.²⁶ As a result, our findings show that PCR assays were more sensitive than traditional diagnostic methods, which is consistent with previous reports.^{7,12,13,16}

Our results illustrated the limitations of virus culture and DFA-based techniques for the detection of viruses in clinical specimens.^{8,12,14,36} The failure to detect virus by these techniques (DFA/CA-DFA) might be due to low viral load of or the presence of non-infectious virions in the specimen, which resulted from insufficient specimen or inappropriate transport or storage conditions (ie, specimens were kept for extended period at room temperature or higher).¹³ RT-PCR technology is not affected by these limitations because it is dependent on the presence of viral nucleic acid rather than infectious or intact virions.¹⁸

The incorporation of an internal control in a PCR reaction is highly desirable, and we included human ERV to monitor inhibition of the PCR. However, our results showed that the majority of respiratory specimens do not contain inhibitory substances. This low level of inhibition may be attributed to the fact that NPA specimens were placed in 2 ml of VTM, which may have diluted the inhibitors and limited their effect on the PCR. Alternatively, it may reflect the efficiency of the extraction procedure to remove inhibitors that are commonly present in respiratory secretions. Whatever the case, the low incidence of inhibitory substances in our specimens suggests that it may not be cost-effective to incorporate the internal control into the assay. On the other hand, the addition of the internal control would be beneficial for laboratories that use other extraction protocols and experience higher levels of inhibition in their specimens.

The specificity of the m-RT-PCR-ELAHA, the DFA and the CA-DFA assays was 100%. The specificity of the m-RT-PCR-ELAHA was enhanced by the use of the ELAHA detection method, which used oligonucleotide probes to specifically detect the amplification product for each virus. This meant that both the amplification and the detection steps in this assay were virus specific. This gave a greater degree of confidence in the results when

compared to conventional amplicon detection by gel electrophoresis, where non-specific or spurious bands may affect result interpretation.²⁶ Also, the ELAHA detection method is a safer alternative to agarose gel detection that uses ethidium bromide, a reported mutagen and suspected carcinogenic agent.²⁶ The specificity of the m-RT-PCR-ELAHA was further demonstrated by the lack of cross-reactions in both the RT-PCR and ELAHA steps when testing RNA or DNA from unrelated viruses, bacteria, yeast, or fungi.

In addition to improved sensitivity and high specificity, we found that the m-RT-PCR-ELAHA offered other significant advantages, including improved result turnaround times and greater cost-effectiveness. However, an experienced operator can perform the DFA faster (3 hours) than the m-RT-PCR-ELAHA (5 hours), but our results showed that testing by DFA alone would result in failure to detect the respiratory pathogen in approximately 20% of PCR-positive specimens. Thus, as an alternative, the m-RT-PCR-ELAHA offers increased sensitivity with a same day turnaround time for results, and in laboratories where DFA might remain the test of first choice, m-RT-PCR ELAHA could be used as a substitute for cell culture-based assays, resulting in improved clinical service.

Our evaluation showed that the m-RT-PCR-ELAHA was more cost-effective than DFA and the CA-DFA. Considering reagent costs alone, the cost per test of the m-RT-PCR-ELAHA was \$USD 5.16 to \$USD 7.37 cheaper than DFA and the CA-DFA respectively. In addition, labor costs were less for the multiplex PCR, as the training of personnel in this technology and result interpretation were relatively generic, whereas performance and interpretation of cell culture and DFA-based assays is more labor intensive and requires special training. Therefore, to our knowledge, this assay is the only one described thus far that combines maximum sensitivity with high specificity and cost-effective use of reagents and operator time.

Clinically, the rapid and accurate diagnosis of respiratory viruses is important to improve patient management and direct therapy following a specific diagnosis. Identification of the pathogen will limit unnecessary antibiotic usage, and prevent nosocomial spread of viruses such as RSV to hospitalized infants and immunocompromised patients.^{3,5,37} In addition, the m-RT-PCR-ELAHA may be used as a tool to evaluate the effectiveness of, or to justify the development of, a new viral vaccine. In this regard, we suggest that m-RT-PCR ELAHA described here is an effective clinical laboratory technique for the detection of seven common respiratory viruses in samples from patients with respiratory infections.

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