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A One-Step, Real-Time PCR Assay for Rapid Detection of Rhinovirus

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One-step, real-time PCR assays for rhinovirus have been developed for a limited number of PCR amplification platforms and chemistries, and some exhibit cross-reactivity with genetically similar enteroviruses. We developed a one-step, real-time PCR assay for rhinovirus by using a sequence detection system (Applied Biosystems; Foster City, CA). The primers were designed to amplify a 120-base target in the noncoding region of picornavirus RNA, and a TaqMan (Applied Biosystems) degenerate probe was designed for the specific detection of rhinovirus amplicons. The PCR assay had no cross-reactivity with a panel of 76 nontarget nucleic acids, which included RNAs from 43 enterovirus strains. Excellent lower limits of detection relative to viral culture were observed for the PCR assay by using 38 of 40 rhinovirus reference strains representing different serotypes, which could reproducibly detect rhinovirus serotype 2 in viral transport medium containing 10 to 10,000 $TCID_{50}$ (50% tissue culture infectious dose endpoint) units/ml of the virus. However, for rhinovirus serotypes 59 and 69, the PCR assay was less sensitive than culture. Testing of 48 clinical specimens from children with cold-like illnesses for rhinovirus by the PCR and culture assays yielded detection rates of 16.7% and 6.3%, respectively. For a batch of 10 specimens, the entire assay was completed in 4.5 hours. This real-time PCR assay enables detection of many rhinovirus serotypes with the Applied Biosystems reagent-instrument platform. ***(J Mol Diagn 2010, 12:102–108; DOI: 10.2353/jmoldx.2010.090071)***

Rhinoviruses are the most common cause of viral upper respiratory tract infections and have been associated with more severe lower tract infections in compromised patients.[1–3] Several real-time, RT-PCR assays have been developed; these have improved the diagnosis of rhinovirus infection over traditional culture methods, which are slow and insensitive.[4–7] However, only a few published PCR assays have combined reverse transcription and PCR in the same real-time reaction (ie, one-step assay).[8,9] The advantages of the one-step assay over the two-step assay include improved workflow, reduction in assay preparation time, and elimination of cross contamination from the transfer of cDNA from the reverse transcription reaction into the PCR reaction. One-step assays have been developed for a limited number of PCR amplification platforms and chemistries and may not necessarily perform optimally with other platforms.[8] In addition, cross-reactivity with genetically similar enteroviruses has been reported with some assays.[8,9]

A one-step, real-time PCR assay has not been described for the ABI Prism Sequence Detection System (Applied Biosystems; Foster City, CA) though this platform is widely used in clinical and research laboratories. High PCR efficiency with this platform generally requires the use of primers and a TaqMan probe (Applied Biosystems) with melting temperatures of 58°C to 60°C and 68°C to 70°C, respectively, and an amplicon size of 50 to 150 bp. The 5′ noncoding region of the rhinovirus genome is most commonly targeted in PCR assays. It consists of six subregions (designated A through F) of approximately 20 bases that are highly conserved in picornaviruses and separated by longer, intervening variable sequences.[10] These characteristics complicate the design of efficient one-step, real-time assays. We have observed that a 16-base sequence immediately downstream from subregion E is reasonably well-conserved in rhinoviruses but not in enteroviruses, suggesting that this sequence could serve as a target for a TaqMan probe in a one-step, real-time PCR assay for rhinovirus.

The aim of this study was to develop a rapid, sensitive, and specific one-step, real-time PCR assay for rhinovirus for use with the ABI Prism Sequence Detection System.

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Table 1. Detection Limits of the One-step PCR Assay for Various Rhinovirus Serotypes

Rhinovirus serotype	Rhinovirus species*	Source	$TCID_{50}$ equivalents per reaction,† mean (SD)	Negative log_{10} titer for assay,‡ mean (SD)	
				PCR	Culture
1A	A	ATCC	0.00001 (0.00000)	—	—
1B	A	NCH	—	5.5 (0.7)	5.5 (0.7)
2	A	ATCC	0.001 (0.000)	—	—
3	B	ATCC	0.0055 (0.0064)	—	—
4	B	NCH	—	5.0 (0.0)	4.0 (0.0)
5	B	NCH	—	4.0 (0.0)	2.5 (0.7)
6	B	NCH	—	4.5 (0.7)	4.0 (0.0)
7	A	ATCC	0.001 (0.000)	—	—
8	A	NCH	—	4.0 (1.4)	4.5 (0.7)
9	A	NCH	—	6.0 (0.0)	4.0 (0.0)
13	A	NCH	—	4.0 (0.0)	5.0 (1.4)
15	A	NCH	—	5.0 (0.0)	4.0 (0.0)
17	B	ATCC	0.055 (0.064)	—	—
21	A	ATCC	0.01 (0.00)	—	—
23	A	NCH	—	5.0 (0.0)	4.0 (0.0)
29	A	ATCC	0.0001 (0.0000)	—	—
31	A	NCH	—	6.0 (0.0)	4.5 (0.7)
35	B	NCH	—	4.0 (0.0)	4.5 (0.7)
37	B	ATCC	0.055 (0.064)	—	—
38	A	NCH	—	5.0 (0.0)	4.5 (0.7)
39	A	ATCC	0.01 (0.00)	—	—
40	A	ATCC	0.001 (0.000)	—	—
41	A	NCH	—	3.0 (0.0)	−1.0 (0.0)§
44	A	NCH	—	4.5 (0.7)	3.0 (0.0)
49	A	NCH	—	4.0 (0.0)	2.0 (0.0)
50	A	NCH	—	5.0 (0.0)	4.0 (0.0)
53	A	NCH	—	4.0 (0.0)	5.0 (0.0)
55	A	NCH	—	4.5 (0.7)	1.5 (0.7)
58	A	ATCC	0.0055 (0.0064)	—	—
59	A	NHC	—	1.5 (0.7)	5.0 (0.0)
62	A	ATCC	0.0001 (0.0000)	—	—
64	A	NCH	—	3.0 (0.0)	3.5 (0.7)
66	A	ATCC	0.001 (0.000)	—	—
69	B	NCH	—	2.5 (0.7)	4.5 (0.7)
72	B	ATCC	0.01 (0.00)	—	—
74	A	NCH	—	4.0 (0.0)	5.0 (0.0)
86	B	NCH	—	5.0 (0.0)	5.0 (0.0)
92	B	NCH	—	4.0 (0.0)	4.0 (0.0)
97	B	NCH	—	3.0 (0.0)	0.0 (0.0)¶
99	B	NCH	—	4.0 (0.0)	4.0 (0.0)

*Ledford et al.[11]
†Determined from testing dilution series of rhinovirus stock extract in duplicate PCR reactions in each of two separate experiments.
‡Determined from testing dilutions series of rhinovirus stock suspension in duplicate PCR reactions (following extraction) and in duplicate WI-38 cell culture tubes in each of two separate experiments.
§Value signifies that no cytopathic effect was observed from the undiluted and diluted suspensions.
¶Value signifies that cyptopathic effect was observed only from undiluted suspension.

Materials and Methods

Source and Cultivation of Rhinovirus Reference Strains

Reference strains of rhinoviruses were obtained as frozen suspensions from the American Type Culture Collection (ATCC; Manassas, VA) and the Clinical Virology Laboratory at Nationwide Children's Hospital (NCH; Columbus, OH) (Table 1).[11] WI-38 human embryonic lung fibroblast cell monolayers in 16 × 125-mm cell culture tubes (Viromed Laboratories; Minnetonka, MN) were used in preparing replicate frozen aliquots for limit of detection studies and for measuring the concentration of virus in stock suspensions. Frozen virus suspensions were thawed in a 37°C water bath and, if necessary for the experiment, diluted serially in 10-fold steps by using Eagle's minimal essential medium containing 2% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 10 μg/ml gentamicin, 2.5 μg/ml Fungizone, and 20 mmol/L HEPES buffer (Viromed Laboratories). After removing the medium from the cell culture tubes, 0.2-ml portions from the virus suspensions were inoculated onto duplicate monolayers. The cell culture tubes were then incubated stationary at 33°C for 1 hour to promote adsorption of virus to the cells. After adsorption, 1 ml of the medium was added to the tubes. To enhance infection of the cells by the virus, the tubes were incubated on a roller drum (0.5 to 0.75 rpm) for 10 days at 33°C. The tubes were examined microscopically for the appearance and progression of a cytopathic effect. Virus concentration in

stock suspensions prepared from the ATCC strains was determined by the method of Reed and Muench.[12]

Source and Cultivation of Clinical Specimens

Nasal aspirate specimens ($N = 48$) were obtained from 28 school-aged children with symptomatic cold-like illnesses. Serial nasal wash specimens were obtained from one of the study investigators with a cold-like illness. Approval for use of these materials was obtained from the Institutional Review Board of the Children's Hospital of Pittsburgh. Specimens were stabilized by either addition of bovine serum albumin at a final concentration of 0.025% or by suspension in M4 transport medium before storage at −80°C until testing. Because human fibroblast cells can vary greatly in sensitivity to rhinovirus infection,[13] clinical specimens were inoculated onto WI-38, MRC-5, and secondary human foreskin fibroblast cells. Except for the two additional types of cell culture monolayers, the clinical specimens were cultured in the same way as described above for the reference virus strains. A positive rhinovirus culture result required the development of a cytopathic effect in a cell culture tube and a positive rhinovirus PCR result from the testing of the supernatant from that tube, as described below. All of the supernatants evaluated from tubes exhibiting a cytopathic effect were positive by the rhinovirus PCR assay.

PCR Specificity Panel

The specificity of the one-step PCR assay was assessed with a panel of 76 total nucleic acids extracts derived from high density suspensions of coxsackievirus types A6, A7, A11, A12, A13, A15, A18, A20, A22, B1 (two strains), B2, B3 (two strains), B4, B5, and B6, echovirus types 1, 2, 3 (two strains), 4 (two strains), 5, 6 (two strains), 7 (two strains), 8, 9, 11, 13, 14, 15, 21, 24, 25, 30, 31, and 33, enterovirus types 68 (two strains) and 70, adenovirus, cytomegalovirus, herpes simplex virus types 1 and 2, human metapneumovirus, influenza A virus, parainfluenza virus types 1, 2, and 3, respiratory syncytial virus, varicella-zoster virus, *Acinetobacter anitratus*, *Bordetella pertussis*, *Candida albicans*, *Corynebacterium* species, *Enterobacter cloacae*, *Escherichia coli*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Neisseria meningitidis*, *Neisseria mucosa*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus* species, coagulase negative, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus* species, β-hemolytic group C, *Streptococcus* species, viridans group, and human lymphoblastoid CEM cells.

RNA Isolation

RNA was isolated from reference strains, clinical specimens, and cell culture supernatants by using the QIAamp Viral RNA kit (Qiagen; Valencia, CA) according to the manufacturer's instructions. A total of 140 μl of specimen or sample was extracted, the entire extract was loaded onto the spin column, and the final elution volume was 60 μl. Each extraction run included a suspension of rhinovirus serotype 2 (positive extraction control) and viral transport medium (negative extraction control).

Primers and Degenerate TaqMan Probe

The primers and probe were designed by analyzing the nucleotide sequence of the 5′ noncoding region between positions 377 and 496 of rhinovirus strain 9503031 (GenBank accession no. AF108174) by using Primer Express version 2.0 (Applied Biosystems) software.[10] The primers were selected from subregions E and F and were expected to produce an amplicon of 120 bp.[10] The probe was selected from the region immediately downstream of subregion E. To provide a suitable melting temperature property and to detect rhinovirus strains with some sequence variation in the probe target region, the probe was designed with a minor groove binder moiety at the 3′ end and with degeneracies at positions 1, 10, and 11 relative to the 5′ end. The 5′ to 3′ sequences of the oligonucleotides designed for the assay were as follows: primer Pic-1 (positions 377 to 393), 5′-TCCTCCGGCCCCTGAAT-3′ (melting temperature of 59.9°C); primer Pic-3 (positions 496 to 474), 5′-GAAACACGGACACCCAAAGTAGT-3′ (melting temperature of 58.1°C); and probe Pic-5 (positions 395 to 410), 5′-YGGCTAACCYWAACCC-3′ (melting temperature of 68.8°C to 76.8°C). In the preceding sequence, Y represents either C or T and W represents either A or T.

One-Step, Real-Time PCR Assay

In this one-step, real-time PCR assay, reverse transcription and PCR amplification were performed in the same reaction tube. The total reaction volume was 50 μl, and the reaction mixture contained 0.9 μmol/L (each) of the primers, 0.15 μmol/L of the probe, TaqMan One Step PCR Master Mix Reagents kit (Applied Biosystems) components, and 5 μl of extracted sample. All sample lysates and controls, unless otherwise noted, were tested in duplicate reactions. The thermal cycling program consisted of 48°C for 30 minutes, 95°C for 10 minutes, and 45 two-step cycles of 95°C for 15 seconds and 60°C for 1 minute, and was conducted by using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each run included the testing of the positive and negative extraction control lysates, Tris-EDTA buffer in four reactions (no template controls), and diethylpyrocarbonate-treated water (Ambion; Austin, TX) in duplicate reactions for each set of five specimen lysates (negative reagent controls). The no-template controls and negative reagent controls were used to detect any nonspecific fluorescent signal or carry-over contamination. Run acceptability required obtaining the expected results from each control. The total run time for this protocol was 2.5 hours. Samples were considered positive if the amplification plots (ie, change in normalized reporter signal [delta Rn] versus PCR cycle number) from duplicate reactions showed definite exponential increase in fluorescent signal.

An internal positive control was not included in this one-step assay. However, specimen lysates yielding a negative result in the assay were evaluated for inhibition of reverse transcription and/or PCR amplification by testing the lysates in one-step reactions seeded with RNA from rhinovirus serotype 2 (100 $TCID_{50}$ [50% tissue culture infectious dose endpoint] equivalents per reaction).

Reproducibility of the Assay

The reproducibility of the entire assay (ie, extraction through PCR amplification) was assessed by using a cell-culture titrated stock suspension of rhinovirus 2 to prepare replicate samples. The samples were prepared by thawing an aliquot of the stock suspension in a 37°C water bath and serially diluting the suspension in 10-fold steps in the M4 transport medium to provide concentrations ranging from 1 to 10,000 $TCID_{50}$ units/ml. A separate dilution series was prepared for the intra- and interassay assessments. Replicate aliquots of each dilution were stored at −80°C. For the intra-assay assessment, six replicate aliquots from each dilution were tested in the same run. For the interassay assessment, aliquots from each dilution were tested in six independent runs. Reproducibility was measured by determining the proportion of samples tested yielding a positive result (ie, positive amplification plots from both duplicate reactions) and by determining cycle threshold (C_T) values from each reaction. The C_T value is the fractional PCR cycle number corresponding to the intersection of a threshold line and the amplification plot. For these experiments, the threshold line was manually set at a delta Rn value of 0.10, which corresponds to the mid-exponential phase of the PCR amplification plots.

Limits of PCR and Viral Culture Detection for Reference Rhinovirus Serotypes

Detection limits for the rhinovirus strains were determined in two separate trials. For the ATCC strains, RNA was extracted from the stocks, serially diluted in 10-fold steps in diethlpyrocarbonate-treated water (Ambion) containing 30 ng/ml of yeast tRNA (Ambion), and the dilutions were tested by the PCR assay; the endpoint was defined as the highest dilution yielding positive results in duplicate reactions and was expressed as $TCID_{50}$ equivalents/reaction. For the NCH strains, the stocks were serially diluted 10-fold in the Eagle's minimal essential medium. The diluted suspensions were extracted by the RNA isolation procedure, and the extracts were tested by the PCR assay. The diluted suspensions were also tested for rhinovirus by the cell culture assay. Endpoint-detection titers were defined as the highest dilutions yielding positive results in duplicate PCR reactions or cell culture tubes, respectively.

Pyrosequencing of Amplicons

Pyrosequencing studies were conducted in selected cases from the set of 48 clinical specimens to determine whether amplicons from PCR-positive, culture-negative cases contained sequences matching with rhinovirus sequences deposited in GenBank, to confirm the identification of rhinoviruses isolated by culture, and to assess the genetic diversity of rhinoviruses detected by the PCR assay. Pyrosequencing (Biotage; Uppsala, Sweden) was performed as previously described by using the above primers as sequencing primers.[14] The PSQ 96 SQA software (Biotage) determined the base sequences and assessed the quality of the pyrograms. The sequences generated were compared with known sequences within the National Center for Biotechnology Information GenBank by using the Basic Local Alignment Search Tool algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Confirmation of a rhinovirus sequence required a pyrosequence length of at least 29 bases, a 100% match with a rhinovirus sequence deposited in GenBank, and no more than a 65% match with other relevant targets.

Bioinformatics

The Entrez and Basic Local Alignment Search tools from the National Center for Biotechnology Information were used for retrieving rhinovirus 5′ noncoding region sequences from GenBank and for interrogating the primer and probe sequences against enterovirus sequences deposited in GenBank, respectively.

Statistical Analysis

Differences in limits of detection between the PCR and viral culture assays were analyzed by the Wilcoxon test by using SPSS version 16.0 software (SPSS, Inc; Chicago, IL). The difference in PCR and culture positivity rates was analyzed by a test of two proportions in relation to the z statistic.

Results

Bioinformatics

A total of 126 rhinovirus strains, representing 60 of the 100 known serotypes, with nucleotide sequences between the primer annealing sites were identified in GenBank (date of accession: October 28, 2008). The primers and probe had no more than a one-base mismatch with 1.6% and 9.5% of these targets, respectively. Although over 1000 enterovirus strains with sequences identical to the primers were identified in GenBank (date of accession: October 28, 2008), the closest matches to the probe included two-base mismatches with an echovirus 11 strain and a coxsackievirus B1 strain and five-base mismatches with several enteroviruses.

Analytical Specificity

All 76 nonrhinoviral nucleic acid samples in the specificity panel yielded negative results by the PCR assay, dem-

Table 2. Intra- and Inter-run Reproducibility of the One-step, Real-time Rhinovirus PCR Assay*

Concentration of rhinovirus type 2 in suspensions ($TCID_{50}$ units/ml)	Intra-run reproducibility			Inter-run reproducibility		
	Proportion of positive samples†	C_T		Proportion of positive samples†	C_T	
		Mean‡	CV, %		Mean‡	CV, %
1	0.50§	43.9	2.2	0.83¶	43.3	1.8
10	1.00	39.1	1.7	1.00	40.3	0.9
100	1.00	35.0	1.1	1.00	36.8	0.6
1000	1.00	29.9	0.7	1.00	32.7	1.4
10,000	1.00	26.2	1.1	1.00	28.9	1.9

*The intra-run experiment was conducted by testing six replicates from each of the suspensions in the same run. The inter-run experiment was conducted by testing one replicate from each suspension in six independent runs. All samples underwent extraction, and the lysates were tested in duplicate PCR reactions (ie, 12 reactions per each condition). Samples were scored as positive if definite PCR amplification was observed in both of the duplicate one-step PCR reactions. The C_T values were determined by setting the baseline threshold value at 0.1 delta Rn units.

†Proportion was calculated by dividing the number of positive samples by the number of samples tested ($N = 6$).

‡In calculating means, reactions yielding a negative result were assigned a C_T value of 45.0 corresponding to the total number of programmed PCR cycles.

§PCR amplification occurred in 8 of the 12 PCR reactions.

¶PCR amplification occurred in 11 of the 12 PCR reactions.

onstrating a high degree of specificity for the primers and probe used here.

Reproducibility

To evaluate the reproducibility of the assay, samples containing concentrations of rhinovirus serotype 2 ranging from 1 to 10,000 $TCID_{50}$ units/ml were tested in replicates of six in the same assay (ie, intrarun assessment) and individually in six independent assays (ie, interrun assessment) (Table 2). In both assessments, all of the samples containing 10 to 10,000 $TCID_{50}$ units/ml yielded a positive result. For the samples containing the lowest concentration of virus tested (ie, 1 $TCID_{50}$ units/ml), the proportion of samples yielding a positive result was 0.50 in the intrarun experiment and 0.83 in the interrun experiment. This concentration of virus corresponds to 0.01 $TCID_{50}$ equivalents/PCR reaction, which is only 10-fold higher than the limit of detection for this virus (Table 1). The coefficients of variation for the C_T values determined for each virus concentration varied from 0.7 to 2.2% in the intrarun assessment and 0.6 to 1.9% in the interrun assessment. The minor differences in mean C_T values observed between the intrarun and interrun assessments are likely attributed to using separate dilution series in preparing the samples for the two experiments.

Limits of Detection

The PCR assay detection limits for the 14 ATCC strains were <0.1 $TCID_{50}$ equivalents/reaction (Table 1). For the 26 NCH rhinovirus strains, the PCR endpoint-detection titers were somewhat higher than the culture endpoint-detection titers ($P = 0.087$). PCR endpoint-detection titers were 100 to 1000-fold higher than culture endpoint-detection titers for rhinovirus serotypes 9, 41, 49, 55, and 97, but culture endpoint-detection titers were 100 to 3000-fold higher than PCR endpoint-detection titers for rhinovirus serotypes 59 and 69.

Detection of Rhinovirus in Clinical Specimens

Testing of 48 nasal aspirate specimens from 28 children by the PCR and viral culture assays yielded three PCR-positive, culture-positive results, five PCR-positive, culture-negative results, and 40 PCR-negative, culture-negative results. Use of three cell lines did not improve the isolation of rhinovirus; each isolate grew in each cell type. The PCR and culture positivity rates were 16.7% and 6.3%, respectively ($P = 0.05$). No child had more than one episode of rhinovirus infection identified. Material was available for pyrosequencing from three of the five PCR-positive, culture-negative cases and the three culture-positive cases. In each case, rhinovirus-specific sequences were confirmed (data not shown). Sequencing did not identify the rhinovirus serotype, but in five of the six cases, the rhinoviruses were clearly genotypically different indicating that the PCR assay can detect multiple strains of rhinovirus circulating in the community. Lysates from the 40 specimens with the negative PCR results were tested for inhibitors of the one-step PCR assay in reactions containing 100 $TCID_{50}$ equivalents of rhinovirus serotype 2 RNA; no inhibition was observed.

A cold-like illness in a study investigator who was symptomatic for 5 days was evaluated by collecting daily nasal wash specimens ($N = 13$) between the 2nd and 14th day after onset of symptoms. The PCR assay was positive for the specimens ($N = 8$) collected between the second and ninth days after onset with a corresponding increase in mean C_T values from 29.8 to 44.6 and was negative for the remaining samples. Pyrosequencing confirmed a rhinovirus-specific sequence from a representative sample. In contrast, all of the samples were negative by culture even after blind subculture.

Discussion

This new one-step, real-time PCR assay facilitates rapid detection of rhinoviruses. Because the primers and the

TaqMan MGB probe (Applied Biosystems) are fully compatible with the Applied Biosystems TaqMan One step PCR Master Mix Reagents kit components and ABI Prism Sequence Detection System, the assay may be welcomed by many of the laboratories using this platform. In contrast, the two previously described one-step, real-time PCR assays have been designed for the iCycler real-time detection system (Bio-Rad; Hercules, CA).[8,9] The authors of one of these studies,[8] observed a reduction in amplification efficiency when the Applied Biosystems TaqMan One Step PCR Master Mix reagents kit was used with the iCycler real-time detection system underscoring the importance of establishing compatibility of all components of the PCR assay platform. Furthermore, most real-time assays for rhinovirus detection are two-step assays requiring a manual transfer of cDNA into PCR reactions and are consequently more cumbersome and more susceptible to contamination than a one-step PCR assay.[4–7] For the testing of a batch of 10 nasal aspirate or wash specimens including controls, a moderate run-size for our laboratory, the one-step assay design saves approximately 30 minutes in assay preparation time over the two-step assay design. The entire protocol for this number of specimens can easily be completed in 4.5 hours.

The one-step PCR assay has high analytical specificity. Importantly, no false-positive results occurred from the testing of relatively high concentrations of enterovirus RNA, as has been described with other one-step PCR assays.[8,9] Despite the existence of only a two-base mismatch between the probe and echovirus 11 and coxsackievirus B1 sequences deposited in GenBank, our stocks of these viruses showed no cross-reactivity in the PCR assay.

This study assessed the reproducibility of the entire assay from RNA extraction through PCR amplification by testing a range of rhinovirus serotype 2 concentrations in M4 specimen transport medium, which is commonly used for transporting and storing specimens for virus detection by either culture or PCR-based assays. The assay was found to be highly reproducible for detecting this virus in concentrations ranging from 10 to 10,000 $TCID_{50}$ units/ml and was also positive in 50 to 83% of the replicates containing 1 $TCID_{50}$ units/ml (ie, near the limit of detection for the assay). Furthermore, the intra- and interrun variations in measuring C_T values over a wide range of concentrations of this virus, is quite low, overall ranging from 0.6 to 2.2%. This degree of variation is similar to the variation observed with a real-time rhinovirus PCR assay for the iCycler platform.[8]

Ideally, PCR assays should be evaluated by testing a wide variety of rhinovirus strains at low concentration of virus. In this study, we demonstrated that the one-step PCR assay not only detected 40 different stock serotypes of rhinovirus but also detected most of them at a relatively low concentration (Table 1). The exceptions occurred with rhinovirus serotypes 59 and 69 that were detected at lower concentrations by culture than by the PCR assay. DNA sequencing studies could help in determining if this difference is attributed to partial mismatches in sequences between the oligonucleotides in the assay and the 5′ noncoding region targets and also identify additional primers or probe that could be incorporated into the one-step assay to improve the detection of these serotypes. However, using the primers described for the one-step assay, we have been unsuccessful in generating sufficient quantities of amplicons for the sequencing studies. Our present resources did not allow us to explore the use of primers that could amplify a larger portion of the 5′ noncoding region, but one that contains the primer and probe target sites of the assay, a strategy that may generate sufficient quantity of amplicon for sequencing studies. The epidemiological importance of serotypes 59 and 69 is not well understood partly because of a lack of recent prevalence studies. However, during the period of 1962 to 1998, serotypes 59 and 69 represented 2.1% and 0.3%, respectively, of all of the serotyped isolates from 11 studies.[15] Although, the prevalent serotypes can change from year to year, typically multiple serotypes circulate simultaneously in a given population.[15] Thus, even during periods when serotypes 59 and 69 may be more prevalent, the one-step PCR assay should be helpful in detecting other circulating strains. In addition to the 40 serotypes evaluated in this study, our PCR assay should, in theory, be able to detect at least 19 additional rhinovirus serotypes that have sequences, deposited in GenBank, with perfect matches to the primer and probe sequences. In contrast to our study, the authors of a recent study of another one-step PCR assay evaluated all 100 rhinovirus serotypes, but only at a high concentration of virus, and found that all serotypes were detected.[8]

The results from our small clinical evaluation suggest that the one-step PCR assay may be particularly good for the detection of rhinovirus in nasal specimens collected from a pediatric population. The PCR assay had a higher detection rate than culture (16.7% vs. 6.3%, respectively) for the testing of 48 nasal aspirate specimens. Pyrosequencing studies supported the view that the PCR-positive, culture-negative cases may represent true positive results for the PCR assay and false-negative results for the culture assay. Because cell culture is insensitive for the detection of rhinovirus,[4–7] it should not be used as a gold standard reference method for determining the diagnostic sensitivity and specificity of rhinovirus PCR assays. Accordingly, it is not surprising that all of the culture-positive cases were also PCR positive nor that several PCR-positive, culture negative cases were identified. Evaluation of a cold-like illness in one of the study investigators also showed that PCR results can be dramatically positive with corresponding negative culture results. The diagnostic sensitivity and specificity of PCR assays for rhinovirus could be determined in larger prospective studies including careful serological assessment of rhinovirus infection. However, such studies would be very difficult to conduct because of the numerous rhinovirus serotypes that would need to be covered serologically.

Our assay system is limited in not having an internal positive control for validating the RNA extraction, reverse transcription, and PCR amplification steps of every clinical specimen tested. As done in our study, specimen lysates that yield a negative result by the assay can be evaluated for inhibitors of reverse transcription or PCR

amplification by testing them in one-step reactions seeded with rhinovirus RNA. To ensure that the assay is capable of extracting RNA from negative specimens, in addition to removing inhibitors of reverse transcription or PCR amplification, laboratories could add a known quantity of a rhinovirus strain into a portion of the specimen and process this sample through the entire assay from extraction through PCR amplification. Additional studies are needed to determine the importance of including these types of positive controls in the assay system.

In conclusion, this one-step, real-time PCR assay enables sensitive detection of many serotypes of rhinovirus and is highly specific. It can detect rhinovirus when viral cultures are negative. The assay is easy to perform, reproducible, compatible with the Applied Biosystems TaqMan One Step PCR Master Mix Reagents Kit and ABI Prism 7000 Sequence Detection System, and can provide results from respiratory specimens within 4.5 hours of receipt.

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