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Comparison of the Eragen Multi-Code Respiratory Virus Panel with Conventional Viral Testing and Real-Time Multiplex PCR Assays for Detection of Respiratory Viruses▽†

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High-throughput multiplex assays for respiratory viruses are an important step forward in diagnostic virology. We compared one such assay, the PLx Multi-Code Respiratory Virus Panel (PLx-RVP), manufactured by Eragen Biosciences, Inc. (Madison, WI), with conventional virologic testing, consisting of fluorescent-antibody staining plus testing with the R-mix system and fibroblast tube cultures. The test set consisted of 410 archived respiratory specimens, mostly nasopharyngeal swabs, including 210 that had been positive by conventional testing for a balanced selection of common respiratory viruses. Specimens yielding discrepant results were evaluated using a panel of respiratory virus PCR assays developed, characterized, and validated with clinical specimens. PLx-RVP increased the total rate of detection of viruses by 35.8%, and there was a 25.7% increase in the rate of detection of positive specimens. Reference PCR assay results corroborated the PLx-RVP result for 54 (82%) of 66 discrepancies with conventional testing. Of the 12 specimens with discrepancies between PLx-RVp and the reference PCRs, 6 were positive for rhinovirus by PLx-RVP and the presence of rhinovirus was confirmed by nucleotide sequencing. The remaining six specimens included five in which the PLx-RVP failed to detect parainfluenza virus and one in which the detection of influenza A virus by PLx-RVP could not be confirmed by the reference PCR. Taking the results of the reference PCR assay results into account, the sensitivities of the PLx-RVP for individual viruses ranged from 94 to 100% and the specificities ranged from 99 to 100%. We conclude that PLx-RVP is a highly accurate system for the detection of respiratory viruses and significantly improves the rate of detection of these viruses compared to that by conventional virologic testing.

The rapid and accurate detection of respiratory viruses is clinically important. Potential advantages of specific viral detection include obtaining prognostic information, limiting additional diagnostic testing, instituting appropriate infection control precautions, and limiting unnecessary antibiotic usage. The use of respiratory specimens for this purpose is challenging because of the broad range of pathogens that may be present. This task is becoming even more complicated with the recent discoveries of several new viruses in respiratory tract samples, including human metapneumovirus (MPV) (19), coronaviruses (CoVs) NL63 (4, 20) and HKU1 (23), human bocavirus (3), and polyomaviruses KI (2) and WU (5).

Molecular methods offer the advantages of rapidity and the ability to detect viruses regardless of the growth requirements or the availability of reagents for rapid diagnostic testing. An exciting recent advance is the development of multiplex molecular assays that allow the simultaneous detection of multiple targets in the same reaction (12, 13, 15, 16). One such test is the xTAG Respiratory Virus Panel, produced by Luminex Molecular Diagnostics (Toronto, ON, Canada). This test, which uses the Luminex-100 or -200 flow cell instrument (12), has been cleared by the Food and Drug Administration for use in the United States for the simultaneous detection and identification of multiple respiratory viruses from nasopharyngeal swab specimens from individuals with suspected respiratory tract infection. In the present study, we have evaluated a different respiratory multiplex PCR test, the PLx MultiCode Respiratory Virus Panel (PLx-RVP), manufactured by Eragen Biosciences, Inc. (Madison, WI). Two important characteristics of the evaluation are, first, that we have included substantial numbers of specimens containing each of the common respiratory viruses detected by the test and, second, that we have designed and validated PCR assays to detect each of the respiratory viruses and used these assays to resolve discrepancies between the results of conventional testing (fluorescent-antibody [FA] staining and culture) and PLx-RVP.

MATERIALS AND METHODS

Specimens. The specimens used to evaluate the PLx-RVP were selected from samples submitted for respiratory virus detection to the Virology Laboratory (VL) at St. Louis Children's Hospital (SLCH). All types of respiratory specimens were included, with the majority being nasopharyngeal swabs. Specimens were mostly collected during the time period from October 2004 to May 2006, although in some cases it was necessary to collect samples outside this time period to achieve the desired number of specimens that had been positive for specific viruses (see below). Specimens were selected to include 30 that had been positive by conventional testing (FA staining, when applicable, and culture) in the VL for

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TABLE 1. Real-time multiplex assays for respiratory viruses[a]

Multiplex	Virus assay (reference)[b]	Primer/probe name	Primer/probe target	Sequence (5′–3′)	Analytical sensitivity (no. of copies per reaction)
1	InfA (22)	Pkz/Glxo FluAfor	Matrix gene	AAGACCAATCCTGTCACCTCTGA	5
		Inf A R/A probe		VIC-TTCACGCTCACCGT-MGB	
		Pkz/Glxo FluA Rev		CAAAGCGTCTACGCTGCAGTCC	
	InfB (22)	GSK FluB for	Matrix gene	GAGACACAATTGCCTACCTGCTT	1
		GSK FluB Pro MGB		FAM-GCAAAGCAGAACTAGC-MGB	
		GSK FluB Rev4		TCTTTCCCACCGAACCAACA	
		GSK FluB Rev5		TCTTTCCCACCAAACCAACA	
2	CoV OC43	BC OC43 N-gene For	N gene	GTTGTACAGGATGTGGGA	<10
		BC OC43 N-gene probe		FAM-CTGGATACCAGGAGTT-MGB	
		BC OC43 N-gene Rev		CGAACTTAGTCGTCATGT	
	CoV 229E	BC 229E N-gene For	N gene	TGCATTTTTATTATCTTGGC	<10
		BC 229E N-gene probe		FAM-CTGTTGATGGTGCTAAA-MGB	
		BC 229E N-gene Rev		TGAATTCTTGCGCCTAAC	
	CoV NL63 (4)	Fouchier et al. (4), set 1 For	N gene	TGTCAACGAGGTTTTGCATTAAAT	<10
		Fouchier et al. (4), set 1 probe		FAM-ATGCGTTTAGCGCATGA-MGB	
		Fouchier et al. (4), set 1 Rev		ACTGGCCTACCATTGTGTGTAAGA	
	CoV HKU1	HKU1 N gene564 F	N gene	GGCTCAGGAAGGTCTGCTTCTA	<10
		HKU1 N Gene588 Tpr		FAM-AGTCGACCAGGTTCACGTT-MGB	
		HKU1 N Gene 631 R		CGATTATTGGGTCCACGTGAT	
3	RSV group A (21)	RSV van Elden RSA-1	N gene	AGATCAACTTCTGTCATCCAGCAA	ND
		RSV van Elden RSA Probe		FAM-CACCATCCAACGGAGCACAGGA GAT-MGB	
		RSV van Elden RSA-2		TTCTGCACATCATAATTAGGAGTATCAAT	
	RSV group B (21)	RSV van Elden RSB-1	N gene	AAGATGCAAATCATAAATTCACAGGA	ND
		RSV van Elden RSB Probe		FAM-ATATGCTATGTCCAGGTTAG-MGB	
		RSV van Elden RSB-2		TGATATCCAGCATCTTTAAGTATCTTTATAGTG	
4	PIV types 1 and 3	HPIV 1+3 For3	L gene	AGTGCAATMCATCTWGCRGCTGTTA	PIV type 1 = 16, PIV type 3 = 258
		HPIV3MQAFor4		ATTATAAGTAGCAAGATGTTCATATATAGCAAA	
		HPIV 1+3 Probe3		FAM-CAAGGAGAYAATCAAGCYATA-MGB	
		HPIV3MQAPro4		FAM-GATGGGAGAATTCTTC-MGB	
		HPIV1+3 Rev7		GTGATRTCTTCATAGACGTGAGTCTTT	
		HPIV3MQARev8		ACCARAAGACACATCTAGATAATGCTTT	
	PIV type 2	HPIV2 MQA For2	Phosphoprotein gene	CCCAGCATCTGCGGAGAAT	1,455
		HPIV2 MQA Pro2		FAM-AAGGAGATCATTGAGCTCT-MGB	
		Para2MQAPro2b		FAM-AGGGAGATCATTGAGCTCT-MGB	
		HPIV2 MQA Rev2		AAGGCGAAGATCAAGTCCCTTT	
		HPIV2MARev2a		CTACAGTCTGAAGGCGAAGATCAAG	
		Para2MQARev2b		AAGGCGAAGATCAAGCCCCTTT	
	PIV types 4a and 4b	HPIV4MQAPhosFor4	Phosphoprotein gene	CACTCAACAAATYAAAGGTTCACTTGC	356
		HPIV4MQAPhosPro4		FAM-ATTGAGGGCCTAATCAC-MGB	
		HPVI4MQAPhosRev4		GACTCCAGGGTCCATTATTTTCA	
5	AdV	Ad Hex MQA5 For	Hexon gene	CGCAGTGGKCTTACATGCACATC	Species A = 34, species B = 11, species C = 9, species D = 12, species E = 5
		Ad Hex MQA1 probe	Hexon gene	FAM-CCGGGCTGGTGCAGTT-MGB	
		Ad Hex MQA2 probe	Hexon gene	FAM-CGGGTCTGGTGCAGTT-MGB	
		Ad Hex 31 probe	Hexon gene	FAM-CCGGTCTGGTGCAATT-MGB	
		Ad Hex B/E probe	Hexon gene	FAM-GCTTCGGAGTACCT-MGB	
		Ad Hex A/C/D probe	Hexon gene	FAM-GCCTCGGAGTACCT-MGB	
		Ad Hex MQA6 Rev	Hexon gene	CCACTGTGGGGTTTCTAAATTTGTT	
		Ad Hex MQA7 Rev	Hexon gene	ACCGTGGGGTTTCTAAACTTGTT	
		Ad Hex 31 Rev	Hexon gene	CCACAGTGGGATTCCTAAACTTGTT	
		Ad Pent PE15841 For	Penton gene	AAACGTTCCTGCTCTCACAGATC	
		Ad Pent PE15939 RProbe	Penton gene	FAM-CAGGTGCGGCGTCT-MGB	
		Ad Pent PE15963 Rev	Penton gene	CCCAGGGCCTTGTARACGTA	

6	MPV (11)	Forward		CATATAAGCATGCTATATTAAAAGAGTCTC	MPV-B = 9
		NLN1MGB		FAM-TGCAATGATGAGGGTGTCA-MGB	
		NLN2MGB		FAM-TAATGATGAGGGTGTCACTG-MGB	
		Reverse		CCTATTTCTGCAGCATATTTGTAATCAG	
7	RV (10)	CDC Rhino F	5′ NCR	Cy+[d]AGCC+[d]TGCGTGGC	Clade A = 2-25,[c] clade B = 13, clade C = 13
		CDC Rhino P		FAM-TCCTCCGGCCCCTGAATGYGGC-3BHQ_1	
		CDC Rhino R		GAAACACGGACACCCAAAGTA	
8	*BCR*/ABL	BCRABLfor		CCGCTGACCATCAATAAGGAA	ND
		BCRABLprobe		NED-AGCCCTTCAGCGGC-MGB	
		BCRABLrev		TGAGGCTCAAAGTCAGATGCTACT	

[a] ND, not determined; NCR, noncoding region.
[b] The source of the PCR assay is shown by the reference citation. In some instances, the primer and probe sequences listed represent modifications of the published sequences. If no reference citation is provided, the assay was developed for this study. The *BCR/ABL* primers and probe are from Asuragen, Inc.
[c] Two clade A viruses were evaluated. The LOD for rhinovirus type 2, the sequence of which matches the sequences of the primers and probes, was two copies. The LOD for rhinovirus type 33, the sequence of which has one mismatch in the forward primer, was 25 copies.
[d] Locked nucleic acid.

the following viruses: influenza A virus (InfA), influenza B virus (InfB), respiratory syncytial virus (RSV), adenovirus (AdV), and rhinovirus (RV). Because parainfluenza viruses (PIVs) are not routinely typed in the VL, 60 specimens positive for PIV were selected to try to achieve representation of each PIV type. In addition, a total of 198 specimens that had been negative for respiratory viruses in the VL were included. Nucleic acid extracts were prepared using a Bio-Robot M48 apparatus with the MagAttract virus mini M48 kit (Qiagen Inc., Valencia, CA) from 200-μl aliquots of supernatant prepared from specimen transport medium, eluted in 100 μl, and stored at −70°C. These extracts were used in the PLx-RVP and the reference PCR assays developed in our laboratory (see below).

In addition to the respiratory specimens used to evaluate the PLx-RVP, other respiratory specimens submitted to the VL were used to validate the respiratory virus PCR assays developed in our research and development laboratory at Washington University to serve as reference assays (see below). For those viruses that are included in the panel of fluorescent-antibody stains used in the VL (InfA and -B, RSV, PIV types 1 to 3, AdV), validation specimens were selected to include some that were positive by direct FA staining of the specimen and some that were negative by FA staining but positive by culture. For influenza virus, negative specimens included some that had been obtained during the winter and others that had been obtained during summer months, when no influenza viruses were detected in the VL. For RV, the specimens selected included some that had been found to be positive for RV by other molecular assays being evaluated in the laboratory. The results of previous molecular tests were not known by personnel performing the validation testing. The human MPV assay was validated by comparing its results to those obtained with different PCR assays that had been developed by one of the authors (1). The targets for these assays were a segment of the N gene of genotype A and a segment of the P gene of genotype B. The MPV assay was also validated by comparing its results to those of FA staining performed in the VL using a commercially available fluorescein-labeled antibody (Diagnostic Hybrids, Athens, OH).

Virology Laboratory procedures. Respiratory specimens submitted to the VL were processed by low-speed centrifugation. The cell pellet was spotted on glass microscope slides that were stained using Simulfluor reagents (Diagnostic Hybrids) and visualized under a fluorescence microscope to detect InfA and -B, RSV, PIV types 1 to 3, and AdV. Virus culture was performed using the R-mix system (Diagnostic Hybrids). The cells were stained approximately 16 and 40 h after inoculation using the same Simulfluor reagents. In addition, a roller tube of MRC-5 human fibroblast cells was inoculated and incubated for 10 to 14 days to detect rhinovirus by the appearance of the characteristic cytopathic effect.

PLx-RVP. The MultiCode-PLx platform, manufactured by Eragen Biosciences, Inc., utilizes multiplex PCR to amplify target sequences, followed by interrogation of the resulting amplicons via target-specific extension and, finally, solid-phase hybridization and detection (7). The assay is driven by a proprietary novel base pair that is used for both site-specific labeling and room temperature capture of the extension products. The assay is read on the Luminex-100 instrument, and the raw data are analyzed using proprietary software. A positive signal is typically defined by reporter values that are greater than 6 standard deviations above the background signals observed when target nucleic acid is absent (see Table S1 in the supplemental material).

The PLx-RVP detects the presence of nucleic acids from the following viruses: InfA and -B; RSV types A and B; PIV types 1, 2, 3, 4a, and 4b; RV; human CoVs OC43, 229E, and NL63; MPV; and AdV species B, C, and E. The specific procedures for performing the PLx-RVP have been described previously (13, 15). Testing with the PLx-RVP was performed at the Eragen Biosciences laboratory in Madison, WI, using nucleic acid extracts prepared from specimens at Washington University, as described above. All Eragen Biosciences personnel involved in this testing were blinded to the results obtained in the VL. The results were sent to Washington University for analysis. The PLx-RVP was classified as being for research use only at the time that testing was performed.

Respiratory virus PCR assays. Because it was anticipated that there might be discrepancies between the results of PLx-RVP and conventional testing, real-time PCR assays for each respiratory virus were established to serve as reference assays for the purpose of adjudicating discrepancies. The primers and probes used for some of these assays were derived from the literature, and others were based on analysis of sequences available in public databases. The primer and probe sequences are shown in Table 1. Most probes included minor groove binder (MGB) moieties to take advantage of relatively short conserved target sequences. Primers and probes were ordered from Applied Biosystems (ABI; Foster City, CA). The samples were initially evaluated in singleplex assays, but as development progressed, primer/probe sets from the singleplex assays were combined with other primer/probe sets and the samples were tested in multiplex assays, as shown in Table 1. Validation was performed by use of the same assay

TABLE 2. Validation of reference real-time respiratory virus PCR assays[a]

Multiplex assay	Virus target(s)	No. of specimens with pos PCR VL result/no. tested (% PCR pos)		
		FA staining pos	FA staining neg, culture pos	FA staining neg, culture neg
1[b]	Influenza A virus	30/30 (100)	30/30 (100)	1/152 (0.7)
1[b]	Influenza B virus	31/31 (100)	27/30 (90)	0/152
3	Respiratory syncytial virus	30/30 (100)	9/9 (100)	6/39 (15)
4	Parainfluenza virus	16/16 (100)	8/11 (73)	0/10
5	Adenovirus	3/3 (100)	26/26 (100)	0/33
6	Human metapneumovirus	16/16 (100)	NA[c]	7/104 (7)[c]
6	Rhinovirus	NA[d]	27/27 (100)[d]	26/66 (39)[d]

[a] NA, not applicable; pos, positive; neg, negative.
[b] The influenza virus multiplex assay detects both influenza A and B viruses and can distinguish between them with different probe labels.
[c] No cultures were performed for human metapneumovirus. Each of the seven specimens that were positive by reference PCR was also positive by other PCR assays (1).
[d] No FA staining was performed.

configuration that was used to test specimens for comparison with the PLx-RVP results. All assays except the RV and MPV assays included an amplification control in each reaction. For RNA viruses, this consisted of 300 copies per reaction mixture of *BCR/ABL* RNA obtained from Asuragen, Inc. (Austin, TX), plus *BCR/ABL* primers and probes, as shown in Table 1. The *BCR/ABL* probe was labeled with NED (Applied Biosystems) or VIC (Applera) to allow its signal to be distinguished from that of the virus probes, which were labeled with 6-carboxyfluorescein (FAM) for all probes except the InfA probe, which was labeled with VIC. For the adenovirus assays, the amplification control consisted of a proprietary internal positive control purchased from Applied Biosystems. The probe used to detect the DNA amplification control was labeled with VIC. All assays except the adenovirus assay were set up using the Qiagen QuantiTect multiplex reverse transcription-PCR (RT-PCR) mix with a reaction volume of 50 μl. The adenovirus assay used the ABI universal master mix. All assays were run on an ABI 7500 thermal cycler with the following parameters: 50°C for 20 min, followed by 95°C for 15 min and then 45 cycles of 94°C for 45 s and 60°C for 1 min. Before being used to resolve discrepancies, the Washington University assays were validated using the validation panels described above.

Rhinovirus typing. Typing of RV was done by analyzing a 260-bp variable sequence of the 5′ noncoding region of the RV genome (9). Briefly, the target sequences were amplified from total cDNA by seminested PCR. The PCR fragment was purified and then cloned into a plasmid vector. For each sample, three or more plasmids were then retrieved, purified, and sequenced. The resultant sequences were compared with the homologous sequences of 101 classical serotypes and the recently identified new strains (prefixed with W) using phylogenetic tree reconstruction and bootstrap analysis with ClustalX software. The RV in each specimen was assigned the serotype or strain to which it clustered within the phylogenetic tree with a significant bootstrap value (>50%). All assigned serotypes/strains in this study had 96 to 100% sequence identities with the respective reference serotypes/strains. In contrast, if a new sequence did not cluster with one of the reference sequences in the phylogenetic tree and had >8% pairwise nucleotide divergence from the nearest reference serotype/strain, it was designated a new strain.

Positive controls and LODs. Formal limits of detection (LODs) were determined for each of the reference PCR assays except the RSV and MPV type A (MPV-A) assays. For the InfA and -B assays, the positive controls for the assays, which were also used to determine the LODs, were purchased from Advanced Biotechnologies, Inc. (Columbia, MD), and consisted of purified virus preparations (InfA/PR/8/34/H1N1 and InfB/Lee/40) that had been quantified by determination of particle counts. Viral RNA was extracted from a known amount of virus with a MagNa Pure compact apparatus (Roche Diagnostics, Indianapolis, IN) using the MagNa Pure LC total nucleic acid isolation kit. For the remaining viruses, a segment of approximately 1,000 bp surrounding the PCR product was cloned into plasmid pCR II-TOPO (Invitrogen Corp., Carlsbad, CA). For rhinovirus, four different rhinovirues were selected to determine the LODs for each clade, including rhinoviruses 2 and 33 for clade A, rhinovirus 17 for clade B, and rhinovirus QPM for clade C. All these rhinoviruses except for QPM were obtained from ATCC; QPM was an isolate from our laboratory identified by nucleotide sequencing of the 5′ noncoding region of the genome. As determined by sequencing of the cloned segment in our laboratory, rhinoviruses 17 and 33 each have one mismatch with the forward primer and rhinoviruses 2 and QPM each match the primer and probe sequences completely. RNA transcripts were created using SP6 or T7 RNA polymerase and were quantitated using a spectrophotometer (NanoDrop Technologies, Wilmington, DE) or a Cubitt fluorometer (Invitrogen). The transcripts were stored at −70°C and diluted in 10-fold dilutions in RNase-free water immediately before use. For the adenovirus assays, plasmid DNA was purified and the copy number was calculated using a Nanodrop spectrophotometer and the known molecular weight of the plasmid and insert. The AdVs used were AdV A (type 31), AdV B (type 3), AdV C (type 5), AdV D (type 19), and AdV E (type 4). The type 31 strain was provided by Gregory Gray, University of Iowa Center for Emerging Infectious Diseases; the other strains were isolates from the SLCH VL that were typed in Gregory Gray's laboratory by sequencing a segment of the hexon gene (6). The LODs for each assay were determined by testing six replicates of a series of dilutions and calculating by probit analysis the number of copies that could be detected with 95% confidence (SPSS software, version 16; SPSS Inc. Chicago, IL). The LODs are shown in Table 1.

RESULTS

Validation of the reference PCR assays. The reference PCR assays were validated by testing total nucleic acid extracts from patient specimens that had been analyzed in the VL. The results of the validation testing are shown in Table 2. The reference PCR assays gave positive results for all specimens that were positive by FA staining. They were also positive for all specimens that were positive by culture with a negative FA staining result except three specimens that were culture positive for InfB and three specimens that were culture positive for PIV. For RV, no FA staining was performed in the VL, but the reference RV PCR was positive for all specimens that had been culture positive for RV. For InfA and -B, PIV, and AdV, the reference PCRs were negative for all specimens that had been negative by conventional testing, with the exception of one specimen that was positive for InfA. For RSV, the reference PCR was positive for 15% of specimens that were negative by FA staining and culture, and for RV, the reference PCR was positive for 39% of specimens that had been negative for rhinovirus by culture. For MPV, the reference PCR was positive for all 16 specimens that were positive by FA staining and also for 7 (7%) of the 104 specimens that were negative by FA staining. The MPV specimens were also tested by M- and P-gene PCRs. The reference PCR was positive for 23 of 24 specimens that were positive by the M- and P-gene PCRs and was negative for all 96 specimens that were negative by the M- and P-gene PCR assays. The 23 specimens that were positive

TABLE 3. PLx-RVP compared to conventional virus testing using fluorescent-antibody staining and culture

Virus	PLx-RVP result	Conventional testing result (no. of specimens)[a]		Sensitivity[b] (%)	Specificity[c] (%)	PPV[d] (%)	NPV[e] (%)
		Positive	Negative				
Influenza A virus	Positive	29	12	91	97	71	99
	Negative	3	365				
Influenza B virus	Positive	29	0	97	100	100	99.7
	Negative	1	379				
Respiratory syncytial virus	Positive	33	5	100	99	87	100
	Negative	0	370				
	Equivocal	0	1				
Parainfluenza virus	Positive	50	3	83	99	94	98
	Negative	6	343				
	Equivocal	4	3				
Rhinovirus	Positive	30	33	100	91	48	100
	Negative	0	345				
	Equivocal	0	1				
Adenovirus	Positive	30	6	100	98	83	100
	Negative	0	371				
	Equivocal	0	2				

[a] Positive by either FA staining or culture. No FA staining was performed for rhinovirus.
[b] In calculations of sensitivity, a result of "equivocal" was included in the calculation and was counted as a false-negative result.
[c] In calculations of specificity, results of "equivocal" were not considered false positive and therefore were not included in the calculation.
[d] PPV, positive predictive value.
[e] NPV, negative predictive value.

by the reference PCR also included all 16 that were positive by FA staining.

Comparison of Multicode-PLx Respiratory Virus Panel with Virology Laboratory results. A total of 410 specimens that had been submitted to the VL were tested using the PLx-RVP. One sample that was negative in the VL had a failed internal control in the PLx-RVP, despite repeat testing, and was excluded from analysis. In all, the PLx-RVP detected 292 viruses in 264 specimens, whereas conventional testing detected 215 viruses in 210 specimens. This represents a 35.8% increase in yield in viruses and a 25.7% increase in positive specimens. The increased yield was accounted for primarily by the greatly enhanced detection of RV plus the detection of MPV and CoVs, which were not detected by conventional testing. The comparison of the PLx-RVP results with those of conventional testing are summarized in Table 3 for all viruses except CoV and MPV, which are not included because these viruses could not be detected by conventional testing. The sensitivity of the PLx-RVP for specific viruses ranged from 83% (PIV) to 100% (RSV, AdV, and RV). The specificity ranged from 91% (RV) to 100% (InfB). Of the 38 RSVs detected by the PLx-RVP, 25 were identified as group A and 13 as group B. Of the 53 PIVs identified by the PLx-RVP, 28 were identified as type 1, 13 as type 2, 9 as type 3, and 3 as type 4b. Of the total of 10 PIVs that were detected by conventional testing but not by the PLx-RVP, 4 were PIV type 1, 2 were PIV type 2, 2 were PIV type 3, 1 was PIV type 4, and 1 was not typed. Of the 36 AdVs detected, 30 were group C and 6 were group B. In addition, the PLx-RVP detected MPV in 17 specimens and CoV in 15 specimens (for NL63, $n = 8$; for OC43, $n = 7$). There were a total of 27 mixed virus infections detected by the PLx-RVP (Table 4). Two viruses were detected in 26 specimens, and 3 viruses were detected in 1 specimen.

Reference PCR assays. The reference PCR assays were performed with all specimens that were positive either by the PLx-RVP or by conventional testing in the VL and also with a sample of approximately 25% of the specimens that were negative for any viruses by both the PLx-RVP and by FA staining and culture in the VL. The results of this testing are shown in Table 5. A total of 637 reference PCR tests were performed. Excluding nine specimens for which the PLx-RVP result was equivocal and one specimen for which the reference PCR

TABLE 4. Multiple virus infections detected by the PLx-RVP

Virus	No. (%) of multiple virus infections detected[a]	
	Total detected	Detected as component of mixed infection
Influenza A virus	41	4 (10)
Influenza B virus	29	0
Respiratory syncytial virus	38	9 (24)
Parainfluenza virus	53	8 (15)
Rhinovirus	63	13 (21)
Adenovirus	36	9 (25)
Metapneumovirus	17	4 (24)
Coronavirus	15	8 (57)

[a] The numbers of mixed infections were as follows: PIV and RV, 6; AdV and RV, 4; RSV and OC43, 4; RSV and AdV, 2; AdV and MPV, 2; InfA and AdV, 1; InfA and RSV, 1; InfA and OC43, 1; RSV and PIV, 1; RSV and NL63, 1; RV and MPV, 1; RV and NL63, 1; MPV and NL63, 1; InfA, PIV, and RV, 1.

TABLE 5. Results of reference PCR assays compared to results of PLx-RVP and conventional viral testing

Virus	Result of PLx-RVP/result of conventional testing[a]	No. of specimens		
		Evaluated by reference PCR	With the indicated result by reference PCR	
			Positive	Negative
Influenza A virus	Pos/Pos	29	29	0
	Pos/Neg	11[b]	10	1
	Neg/Pos	3	0	3
	Neg/Neg	64	0	64
Influenza B virus	Pos/Pos	29	29	0
	Pos/Neg	0	0	0
	Neg/Pos	1	0	1
	Neg/Neg	73	1	72
Respiratory syncytial virus	Pos/Pos	33	33	0
	Pos/Neg	5	5	0
	Neg/Pos	0	0	0
	Neg/Neg	30	0	30
Parainfluenza virus	Pos/Pos	49[b]	49	0
	Pos/Neg	3	3	0
	Neg/Pos	6	5	1
	Neg/Neg	32[c]	0	32
	Eq/Pos	4	4	0
	Eq/Neg	3	0	3
Rhinovirus	Pos/Pos	29[b]	29	0
	Pos/Neg	32[b]	26	6
	Neg/Pos	0	0	0
	Neg/Neg	33	0	33
Adenovirus	Pos/Pos	29[b]	29	0
	Pos/Neg	5[b]	5	0
	Neg/Pos	0	0	0
	Neg/Neg	33	0	33
	Eq/Neg	2	2	0
Coronavirus	Pos/ND	15	14[c]	0
	Neg/ND	33	0	33
Metapneumovirus	Pos/ND	17	17	0
	Neg/ND	34	0	34

[a] Pos, positive; Neg, negative; ND, not determined; Eq, equivocal.
[b] One specimen was not available for reference PCR testing.
[c] The reference PCR result for one specimen was equivocal.

result was equivocal, the results of the reference PCR assays were in agreement with the PLx-RVP result for 614 (97.9%) of the 627 tests performed. There were 66 discrepancies between PLx-RVP and conventional testing. Of these, the reference PCR results were in agreement with the PLx-RVP result for 54 (82%). The 12 discrepancies were the following: 1 specimen that was positive for InfA by the PLx-RVP, negative in the VL, and negative by the reference InfA PCR; 5 specimens that were negative for PIV by the PLx-RVP, positive in the VL, and positive by the reference PCR; and 6 specimens that were positive for RV by the PLx-RVP, negative in the VL, and negative by the reference RV PCR.

Rhinovirus typing. Rhinovirus molecular typing was performed with a total of 32 specimens that had been positive by the PLx-RVP, including all 6 that were negative by the reference PCR. RV RNA was detected and the 260-base 5′ noncoding region sequence was determined in each of these specimens. The RV types of the six discrepant specimens were 51 ($n = 2$), 30, 78, W12, and W23. The RV types in the remaining specimens were 30 ($n = 1$), 49 ($n = 2$), 51 ($n = 6$), 53 ($n = 1$), 57 ($n = 1$), 78 ($n = 1$), 80 ($n = 1$), 96 ($n = 1$), W10 ($n = 3$), W17 ($n = 1$), W23 ($n = 2$), W28 ($n = 1$), W32 ($n = 1$), W38 ($n = 1$), W39 ($n = 1$), W41 ($n = 1$), and W42 ($n = 1$). The strains with the prefix "W" are new strains that are different from the 101 classical serotypes. All eight classical serotypes (serotypes 30, 49, 51, 53, 57, 78, 80, and 96) are in clade A. Analysis of the sequences of the 420-base VP4/VP2 region of the new strains showed that types W10, W12, W17, W23, W32, W38, W39, W41, and W42 are in clade C and that type W28 is in clade A. Strains W10, W12, W17, W23, W28, W32, and W38 have previously been reported to be detected in infants with cold symptoms in Madison, WI (9). Strains W39 and W41 resemble the new RV strains described by Tapparel et al. in association with respiratory infection in Switzerland (18).

TABLE 6. Comparisons of PLx-RVP and conventional viral detection to reference PCR assay detection

Virus	Test system	No. of specimens positive or negative/total no. tested (%)			
		Sensitivity[a]	Specificity[a]	Positive predictive value	Negative predictive value
Influenza A virus	PLx-RVP	39/39 (100)	67/68 (99)	39/40 (98)	67/67 (100)
Influenza A virus	Conventional	29/39 (74)	65/68 (96)	29/32 (91)	64/75 (87)
Influenza B virus	PLx-RVP	29/30 (97)	73/73 (100)	29/29 (100)	73/74 (99)
Influenza B virus	Conventional	29/30 (97)	72/73 (99)	29/30 (97)	72/73 (99)
Respiratory syncytial virus	PLx-RVP	38/38 (100)	30/30 (100)	38/38 (100)	30/30 (100)
Respiratory syncytial virus	Conventional	33/38 (87)	30/30 (100)	33/33 (100)	30/35 (86)
Parainfluenza virus	PLx-RVP	52/61 (95)	33/33 (100)	52/52 (100)	33/38 (87)
Parainfluenza virus	Conventional	58/61 (95)	35/36 (97)	58/59 (98)	35/38 (92)
Rhinovirus	PLx-RVP	55/55 (100)	33/39 (85)	55/61 (90)	33/33 (100)
Rhinovirus	Conventional	29/55 (53)	39/39 (100)	29/29 (100)	39/65 (60)
Adenovirus	PLx-RVP	34/36 (94)	33/33 (100)	34/34 (100)	33/33 (100)
Adenovirus	Conventional	29/34 (85)	33/33 (100)	29/29 (100)	33/38 (87)
Human metapneumovirus	PLx-RVP	17/17 (100)	34/34 (100)	17/17 (100)	34/34 (100)
Coronaviruses	PLx-RVP	14/14 (100)	33/33 (100)	14/14 (100)	33/33 (100)

[a] Equivocal results are included for calculations of sensitivity but excluded for calculations of specificity.

Strain W42 matches isolate Resp_3898 reported in GenBank (accession number GQ476669).

Recalculated test parameters. In order to calculate more meaningful test parameters, the sensitivity, specificity, and positive and negative predictive values were recalculated for the PLx-RVP and for conventional virus testing using the results of the reference PCR assays as the standard for comparison. The recalculated test parameters are shown in Table 6. The PLx-RVP was more sensitive than conventional testing for all viruses except InfB (equal sensitivity) and PIV. The increased sensitivity of the PLx-RVP for rhinoviruses was especially notable. The specificity of the PLx-RVP was between 99 and 100% for all viruses except rhinovirus, for which it was 85%. If the six specimens that were negative for RV in the reference PCR assay but that were shown to contain RV sequences by the molecular typing assay are considered to have been truly positive for RV rather than negative, the adjusted specificity increases to 100%.

DISCUSSION

The newly developed multiplex molecular assays to detect respiratory viruses (12, 13, 15, 16) are a major step forward in diagnostic virology. In the present study, we evaluated one such assay, the PLx-RVP, by comparing it to conventional virologic testing, consisting of FA staining and culture as performed in a routine diagnostic virology laboratory. We used laboratory-developed PCR assays as the reference method to resolve discrepancies between the two approaches. We found that the multiplex method achieved a 35.3% increase in the detection of respiratory viruses. The increased yield was largely accounted for by a very substantial increase in the rate of detection of RV by the PLx-RVP. The other large contribution was the detection of MPV and CoV, viruses that were not detected by the conventional methods in use in the VL at the time that the study was performed.

An alpha prototype version of the PLx-RVP was evaluated in a clinical laboratory setting by Nolte et al. (15). In that evaluation, the comparative test methods were direct fluorescent-antibody staining and shell vial culture. Singleplex PCR assays were used to confirm the presence of viruses (RV, MPV, CoV, and PIV types 4a and 4b) that were not detected by the methods used in the clinical laboratory. The PLx-RVP detected 53% more viruses than the comparative methods, with the results for 90% of the discrepancies being confirmed by singleplex PCRs. A limitation of the study is that aside from influenza A virus, which was detected in 78 samples by one or both methods, the number of other viruses detected was relatively small, ranging from 1 to 16. Reports by Lee et al. (8) and Marshall et al. (13) used earlier versions of the assay and evaluated relatively small numbers of individual respiratory viruses. The present evaluation extends the results of the previous studies by using an evaluation panel that included a substantially larger number of specimens containing each of the viruses that can be detected by the PLx-RVP. Specifically, the evaluation panel included at least 30 of each of the viruses that could be detected in the clinical VL. Although they were not specifically selected, there were also 17 samples containing MPV and 15 containing human CoVs. The results indicate that the PLx-RVP is at least as sensitive as conventional methods for the detection of each of these viruses.

Because we anticipated that the PLx-RVP would be positive for some specimens that were negative by conventional testing, we placed a high priority on establishing comparative molecular assays that could be used to adjudicate discrepancies. The molecular assays used for adjudication were, in some cases, based on previously published assays or, in other cases, were developed using publically available sequence data. With the exception of the RV and MPV assays, each assay had an internal amplification control, and all assays with the exception of the RSV and MPV-A assays had a defined analytic limit of detection, defined as the minimum number of nucleic acid copies that could be detected by the assay with 95% confidence on the basis of probit analysis. In addition, each assay (except that for CoV) was validated using a separate panel of specimens that had been shown to contain the target analyte by conventional testing. For each of the viruses for which direct FA staining was being performed in the Virology Laboratory,

where the specimens were originally tested, some of the validation samples were selected because they had been negative by FA staining and positive by culture, suggesting the presence of a low level of virus. The reference assays corroborated the findings of the PLx-RVP in almost 98% of the samples which had yielded discrepancies between the PLx-RVP and conventional testing.

The two viruses which were associated with the largest numbers of discrepancies were RV and PIV. As was seen in the other evaluations (8, 13, 15), the PLx-RVP detected many more RVs than conventional testing. The presence of RV was confirmed in the large majority of the discrepant specimens by the reference PCR assay, supporting the enhanced sensitivity of PLx-RVP for RV. Of the six specimens that were positive by PLx-RVP and negative by the reference PCR, further molecular testing based on amplification and sequencing of a segment of the 5′ noncoding region of the RV genome revealed RV in all six. Thus, although the adjusted specificity of the PLx-RVP for rhinovirus, on the basis of the results of the reference PCR, was calculated to be 85%, it should be considered to be 100%, since all six of the discrepant samples were shown by an independent molecular method to have RV RNA. It is notable that the sensitivity of the PLx-RVP appears to exceed that of the reference PCR, even though the LODs determined for the reference PCR were very low, ranging from 2 to 25 copies per reaction mixture, depending on whether the sequence of the serotype used to evaluate the LOD matched or had a mismatch with the primers and probes of that assay. For PIV, the PLx-RVP was either negative or equivocal for 10 of the 60 samples in which PIV had been detected by conventional testing. The reference PCR assay confirmed the detection of PIV in 9 of the 10 samples, suggesting that the PLx-RVP was truly less sensitive than conventional testing for the detection of PIV. PIV types 1 to 4 were represented among the samples in which PIV was missed by PLx-RVP, suggesting that the decreased sensitivity of PLx-RVP extends across all PIV types.

In summary, with the exception of PIV, the PLx-RVP showed excellent sensitivity and specificity across the full range of viruses that it has the ability to detect, measured against conventional virologic testing, with discrepancies being adjudicated with highly sensitivity reference PCR assays. The excellent sensitivity of the assay for rhinovirus is a notable strength of the assay, particularly in the face of mounting evidence for the clinical significance of rhinoviruses (14, 17). This study adds to the body of evidence that respiratory virus multiplex PCR assays can enhance the detection of respiratory viruses. Further studies are now required to determine the clinical utility and cost-effectiveness of such tests in the clinical arena.

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