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Evaluation of Three Influenza A and B Real-Time Reverse Transcription-PCR Assays and a New 2009 H1N1 Assay for Detection of Influenza Viruses[▽]

Suresh B. Selvaraju and Rangaraj Selvarangan*

Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, Missouri, and University of Missouri—Kansas City, School of Medicine, Kansas City, Missouri

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The performance characteristics of three real-time influenza A/B virus reverse transcription-PCR (RT-PCR) assays and two real-time 2009 H1N1 RT-PCR assays were evaluated using previously characterized clinical specimens. A total of 150 respiratory specimens from children (30 influenza A/H1 virus-, 30 influenza A/H3 virus-, 30 2009 H1N1-, and 30 influenza B virus-positive specimens and 30 influenza virus-negative specimens) were tested with the CDC influenza A/B PCR (CDC), ProFlu$^+$ multiplex real-time RT-PCR assay (ProFlu$^+$), and MGB Alert Influenza A/B & RSV RUO (MGB) assays. A second set of 157 respiratory specimens (100 2009 H1N1-, 22 seasonal influenza A/H1-, and 15 seasonal influenza A/H3-positive specimens and 20 influenza-negative specimens) were tested with a new laboratory-developed 2009 H1N1 RT-PCR and the CDC 2009 H1N1 assay. The overall sensitivities of the CDC, ProFlu$^+$, and MGB assays for detection of influenza A and B viruses were 100%, 98.3%, and 94%, respectively. The ProFlu$^+$ assay failed to detect one influenza A/H1 virus-positive specimen and yielded one unresolved result with another influenza A/H1 virus-positive specimen. The MGB assay detected 84/87 (96.5%) of influenza A and B viruses and 26/30 (86.6%) of 2009 H1N1 viruses. The new laboratory-developed 2009 H1N1 RT-PCR assay detected 100/100 (100%) 2009 H1N1 virus-positive specimens, while the CDC SW Inf A and SW H1 PCR assays failed to detect one and three low-positive 2009 H1N1-positive specimens, respectively. The CDC influenza A/B virus assay and the newly developed 2009 H1N1 RT-PCR assay with an internal control can be set up in two separate reactions in the same assay for routine clinical testing to detect influenza A and B viruses and to specifically identify the 2009 H1N1 influenza virus.

Influenza virus types A and B are two of the most important causes of human respiratory infection. Seasonal influenza causes substantial mortality and morbidity in the United States, with about 350,000 hospitalizations and 50,000 deaths occurring per year (14). A novel influenza A H1N1 virus strain emerged due to quadruple reassortment of human, swine, and bird influenza A viruses in April 2009 and has caused influenza-like illnesses (ILIs) worldwide. There have been 399,232 laboratory-confirmed cases of 2009 H1N1 influenza virus infections and over 4,735 deaths reported to the World Health Organization (17). In the United States, a total of 129 laboratory-confirmed 2009 H1N1-associated pediatric deaths have been reported (3). Rapid transmission of this 2009 influenza virus caused an unusually early start to the influenza season, with the ILI levels being elevated above the seasonal baseline (3).

Traditionally, rapid antigen test, direct fluorescent-antigen (DFA), and culture methods have been used for detection of influenza viruses. Recent reports have highlighted the poor sensitivities of rapid antigen-based tests (10% to 50%) in detecting 2009 H1N1 virus and thus are unsuitable for clinical testing (2, 5). Although the DFA method could provide a rapid turnaround time for immediate patient management decisions, the labor-intensive nature of the test, the need for highly trained technical staff to perform the test, and the subjectivity associated with result interpretation have reduced the utility of this test. Culture of influenza virus from respiratory specimens has been the "gold standard" for diagnosis; however, this procedure can take days to complete and some influenza virus subtypes grow poorly in cell culture. Most importantly, these traditional methods do not have the ability to differentiate seasonal influenza viruses from the 2009 H1N1 viruses. PCR testing is the most suitable method to detect influenza virus due to its high sensitivity and specificity, rapid turnaround time, and ability to detect and differentiate both seasonal and 2009 H1N1 virus strains. There have been a small number of laboratory-developed tests (LDTs) and commercially available influenza virus typing reverse transcription-PCR (RT-PCR) assays developed over the last few years (7, 9, 13). Most of these assays were developed to detect only seasonal influenza viruses and were not tested against recently emerged 2009 H1N1 strains. A few endpoint and real-time PCR assays have been specifically developed to detect 2009 H1N1 strains (8, 10, 16, 18). However, these assays either use more than one gene target for the detection of H1N1 strains or were developed when a limited number of H1N1 sequences were available in the GenBank database. Variants of the 2009 H1N1 strain have been noticed; hence, an updated assay capable of detecting currently circulating strains is important. Although the 2009 H1N1 strains accounted for more than 99% of circulating influenza viruses during outbreaks after April 2009, there is a probability that seasonal influenza A viruses will cocirculate during future respiratory viral seasons. Also, recent reports

* Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, 2401 Gillham Road, Kansas City, MO 64108. Phone: (816) 234-3031. Fax: (816) 234-1492. E-mail: rselvarangan@cmh.edu.

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TABLE 1. Sequences of primers and probes used in this study

Oligonucleotide	Sequence	Reference or source
CDC universal influenza A virus forward primer	5′-GAC CRA TCC TGT CAC CTC TGA C-3′	18
CDC universal influenza A virus reverse primer	5′-AGG GCA TTY TGG ACA AAK CGT CTA-3′	
CDC universal influenza A virus probe	5′-(FAM)-TGC AGT CCT CGC TCA CTG GGC ACG-(BHQ1)-3′	
CDC universal influenza B virus forward primer	5′-TCCTCAACTCACTCTTCGAGCG-3′	CDC
CDC universal influenza B virus reverse primer	5′-CGGTGCTCTTGACCAAATTGG-3′	
CDC universal influenza B virus probe	5′-(JOE)-CCAATTCGAGCAGCTGAAACTGCGGTG-(BHQ1)-3′	
H1N1 forward primer	5′-AAG CAA CAA AAA TGR AGG CAA TAC TA-3′	This study
H1N1 reverse primer	5′-TCT GTT GAA TTG TTC GCA TGA TAA-3′	
H1N1 probe	5′-(FAM)-TTR CAA CCG CAA ATG CAG ACA CAT TAT G-(BHQ1)-3′	
MS2 forward primer	5′-TGG CAC TAC CCC TCT CCG TAT TCA CG-3′	12
MS2 reverse primer	5′-GTA CGG GCG ACC CCA CGA TGA C-3′	
MS2 probe	5′-(JOE)-CAC ATC GAT AGA TCA AGG TGC CTA CAA GC-(BHQ1[a])-3′	

[a] BHQ1, Black Hole Quencher 1 dye.

have indicated that antiviral resistance patterns vary among various influenza A virus subtypes. Hence, it is important to develop an assay that detects both seasonal and 2009 H1N1 influenza viruses in a single PCR.

The aim of this study was to combine two real-time RT-PCR assays in a single test run: one to detect universal influenza A and B viruses, including 2009 H1N1 strains, and the second RT-PCR assay to detect and specifically identify 2009 H1N1 influenza virus only. To detect universal influenza A and B viruses, we compared the performance characteristics of three RT-PCR assays: the CDC influenza A/B virus (Flu A/B) PCR (CDC) assay, the ProFlu$^+$ multiplex real-time RT-PCR (ProFlu$^+$) assay, and the MGB Alert Influenza A/B & RSV RUO (MGB) assay. To specifically detect 2009 H1N1 influenza virus, we compared the performance characteristics of the CDC SW InfA and CDC SW H1 assays with a new laboratory-developed 2009 H1N1 RT-PCR assay. We describe the performance characteristics of these RT-PCR assays in this report, and we propose the use of the CDC Flu A/B assay combined with the new laboratory-developed 2009 H1N1 influenza virus/MS2 internal control in the same run to optimally detect universal influenza A and B viruses and specifically identify 2009 H1N1 influenza virus.

MATERIALS AND METHODS

Clinical samples and standard materials. For the influenza A/B virus RT-PCR assays, a total of 150 respiratory specimens (30 influenza A/H1 virus-, 30 influenza A/H3 virus-, 30 influenza A/2009 H1N1 virus-, and 30 influenza B virus-positive specimens and 30 influenza virus-negative) previously characterized as being influenza virus positive or negative by rapid antigen test were used for analysis. Among the 150 specimens, 100 were subsequently confirmed to be positive by R-Mix shell vial culture results, and the remaining 50 specimens were characterized as being positive by three influenza A/B virus RT-PCR assays. A specimen was considered true positive if influenza A or B virus was isolated by culture and/or was detected by all three Flu A/B RT-PCR assays tested. A specimen was considered true negative if influenza A or B virus was not isolated by culture or not detected by any of the three Flu A/B RT-PCR assays tested. The three influenza A/B RT-PCR assays used in this study were the CDC influenza A/B PCR assay, the ProFlu$^+$ multiplex real-time RT-PCR assay (Prodessa, Inc., Waukesha, WI), and the MGB Alert Influenza A/B & RSV RUO assay (Epoch Biosciences, Inc., Bothell, WA). The sequences of CDC Flu A were published previously (18), while the CDC Flu B primer and probe sequences were obtained by material transfer agreement with CDC (Table 1). The identification of influenza A virus H1/H3 in these clinical specimens was performed by the CDC H1/H3-specific real-time PCR. The primers and probe sequences for H1/H3 subtyping were obtained from CDC through a material transfer agreement. Discrepant analysis was performed with specimens whose results between any of the three RT-PCRs differed by repeating the run with same aliquot or using fresh nucleic acid extract from the specimen.

For 2009 H1N1 RT PCR assays, a total of 157 respiratory specimens (100 characterized by the Luminex respiratory viral panel [RVP] assay as influenza A virus nonsubtypeable, 22 seasonal influenza A/H1 virus- and 15 influenza A/H3 virus-positive specimens, and 20 influenza virus-negative specimens) were tested with the laboratory-developed 2009 H1N1 RT-PCR, CDC SW Inf A, and CDC SW H1 assays. These 2009 H1N1-positive specimens were obtained from children seen at Children's Mercy Hospitals and Clinics, Kansas City, MO, during the 2009 H1N1 outbreak from April 2009 through September 2009. This study was approved by Institutional Review Board of Children's Mercy Hospitals and Clinics.

Influenza A/H1 virus [Solomon Islands/03/06 (H1N1), stock of $10^{6.7}$ 50% tissue culture infective doses ($TCID_{50}$)/ml], influenza A/H3 virus [Brisbane/10/07 (H3N2), stock of $10^{5.6}$ $TCID_{50}$/ml], and influenza B virus (Florida/04/06, stock of $10^{6.7}$ $TCID_{50}$/ml stock) were kindly provided by ZeptoMetrix Corporation (Buffalo, NY) and used for limit-of-detection (LOD) and reproducibility analyses of the influenza A/B RT-PCR assays. A 2009 H1N1 virus-positive patient specimen was used as the standard for 2009 H1N1 virus due to a lack of commercial standard material. Bacteriophage MS2 was obtained from ATCC (15597-B1) and used as an internal control (for both extraction and amplification) for the laboratory-developed 2009 H1N1 influenza virus real-time PCR. The primer and probe sequences for the MS2, CDC influenza A and B, and laboratory-developed 2009 H1N1 virus, PCRs are given in Table 1. An MS2 lyophilized stock was resuspended with 1 ml of viral transport medium (VTM) and serially diluted 10-fold using VTM to a level that would yield a threshold cycle (C_T) value of ~33. The internal controls supplied for the ProFlu$^+$ assay were used as recommended by the manufacturer.

2009 H1N1 RT-PCR primer and probe design. The primers and probes specific for 2009 H1N1 strains were designed by aligning 272 hemagglutinin (HA) gene sequences of U.S. H1N1 strains. The HA sequences were obtained from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (1). The 1,746-bp consensus sequence was used to design the H1N1-specific primers and probes with Primer Express software (version 3.0; Applied Biosystems, Foster City, CA) (Table 1).

Nucleic acid extraction and amplification. For all RT-PCR assays, nucleic acid was extracted from 200 μl of specimen (180 μl of clinical specimen plus 20 μl of internal control) using a NucliSENS easyMAG automated extraction system and eluted in 55 μl of elution buffer (bioMerieux Inc., Durham, NC).

The CDC Flu A/B assay was performed to detect universal influenza A and B viruses in a single tube. The CDC Flu A- and B-specific probes were labeled with 6-carboxyfluorescein (6-FAM) and 4′,5′-dichloro-2′,7-dimethoxyfluorescein (JOE) dyes, respectively. PCR assay was performed using a SuperScript III Platinum one-step quantitative RT-PCR system with carboxy-X-rhodamine (ROX; Invitrogen Corporation, Carlsbad, CA) on a model 7500 fast real-time

TABLE 2. Analytical sensitivities of influenza A/B and 2009 H1N1 RT-PCR assays

Specimen	No. of specimens positive/total no. of specimens tested (%)					
	Influenza A/B assays (n = 150)			2009 H1N1 assays (n = 157)		
	CDC	ProFlu$^+$	MGB	New H1N1	CDC SW Inf A	CDC SW H1
Influenza A/H1 virus[a]	29/29 (100)[b]	27/29 (93.1)	27/29 (93.1)	0/22	0/22	0/22
Influenza A/H3 virus[a]	28/28 (100)[c]	28/28 (100)	28/28 (100)	0/15	0/15	0/15
Influenza A/2009 H1N1 virus[d]	30/30 (100)	30/30 (100)	26/30 (86.6)	100/100 (100)	99/100 (99)	97/100 (97)
Influenza B virus	30/30 (100)	30/30 (100)	29/30 (95)			
Influenza virus negative	0/33	0/33	0/33	0/20	0/20	0/20

[a] Influenza A/H1 and A/H3 subtypes were determined by CDC H1/H3-specific real-time PCR.
[b] One rapid-antigen-positive influenza A/H1 specimen was negative by the R-Mix shell vial culture gold standard.
[c] Two rapid-antigen-positive influenza A/H3 specimens were negative by the R-Mix shell vial culture gold standard.
[d] Influenza A/H1N1 2009 strains used in this study were identified by the Luminex RVP assay as nonsubtypeable influenza A virus. The Luminex RVP assay was considered the gold standard for the 2009 H1N1 assays.

PCR system (Applied Biosystems) with a 25-μl reaction volume containing 12.5 μl of 2× reaction mixture with ROX, 0.5 μl of influenza A and B virus forward and reverse primers (50 μM stock) and probe (10 μM stock), 0.5 μl of SuperScript III reverse transcriptase-Platinum *Taq* mix, 4.0 μl of nuclease-free water, and 5 μl of template RNA. The following thermal cycling protocol was used: 50°C for 30 min (reverse transcription), 95°C for 2 min (reverse transcriptase enzyme inactivation), and 45 cycles of 95°C for 15 s (denaturation) and 55°C for 30 s (annealing and signal acquisition).

The ProFlu$^+$ assay was performed on a SmartCycler II apparatus (Cepheid, Sunnyvale, CA) by adding 19.45 μl of an influenza A virus-influenza B virus-respiratory syncytial virus (RSV) mixture, 0.30 μl of Moloney murine leukemia virus reverse transcriptase, 0.25 μl of RNase inhibitor, and 5 μl of template RNA with the following thermal cycling parameters: reverse transcription at 42°C for 30 min, enzyme inactivation at 95°C for 10 min, 5 cycles of 95°C for 30 s and 55°C for 60 s with optics on for fluorescence detection, and 45 cycles of 95°C for 10 s and 55°C for 60 s with optics on for fluorescence detection. Results were interpreted according to the manufacturer's instructions.

The MGB assay was carried out on ABI 7500 fast real-time PCR system with the following reagents per sample: 1.25 μl of MGB Alert Influenza A/B & RSV RUO detection reagent, 1.25 μl of RNase inhibitor (20 U/μl), 2.5 μl of 10× PCR enhancer, 12 μl of 2× QuantiTect probe RT-PCR master mixture, 0.25 μl of QuantiTect reverse transcriptase mixture, 2.75 μl of nuclease-free water, and 5 μl of template RNA. The thermal cycling parameters followed were 50°C for 30 min for reverse transcription, 95°C for 15 min for reverse transcriptase inactivation, 50 cycles of 95°C for 15 s and 56°C for 30 s with signal acquisition, and 76°C for 30 s, followed by melting curve analysis at 95°C for 15 s, 35°C for 15 s, and 95°C for 15 s. The MGB assay differentiates the influenza virus subtypes by melting curve analysis.

For the 2009 H1N1 RT-PCR assay, nucleic acid was extracted from 180 μl of clinical specimen spiked with 20 μl of an appropriate dilution of MS2 (the concentration that yielded a C_T value of ~33 cycles) using the easyMAG automated extraction system. The PCR reagents and cycling conditions were similar to those described for the CDC Flu A/B RT-PCR except for the substitution of the H1N1 and MS2 primer and probe set: 0.75 μl (50 μM stock) of H1N1 forward and reverse primers and H1N1 probe (10 μM stock) and 0.25 μl (10 μM stock) of MS2 forward and reverse primers and probe.

Performance specifications of RT-PCRs. The performance specifications of the RT-PCR assays (influenza A/B and 2009 H1N1 assays) were analyzed according to Clinical and Laboratory Standards Institute guidelines (4). The LOD of the test was determined by testing serial 10-fold dilutions of seasonal influenza A/H1, influenza A/H3, and influenza B viruses obtained from ZeptoMetrix and a clinical specimen of 2009 H1N1 for the influenza A/B virus PCRs. The clinical specimen positive for 2009 H1N1 was used for the 2009 H1N1 RT-PCR. PCR runs of both influenza A/B virus and 2009 H1N1 assays were performed with appropriate positive controls extracted along with test specimens. The specificity of the influenza A/B virus PCR reagents was tested against the following organisms, obtained from American Type Culture Collection (ATCC), at the given concentration: adenovirus (ATCC VR-3; 4.1 $TCID_{50}$/ml), respiratory syncytial virus types A (ATCC VR-26; 3.3 $TCID_{50}$/ml) and B (ATCC VR-955; 1.8 $TCID_{50}$/ml), parainfluenza virus type 1 (ATCC VR-94; 1.9 $TCID_{50}$/ml), parainfluenza virus type 2 (ATCC VR-92; 2.9 $TCID_{50}$/ml), parainfluenza virus type 3 (ATCC VR-93; 3.8 $TCID_{50}$/ml), echovirus type 9 (ATCC VR-39; 3.3 $TCID_{50}$/ml), rhinovirus type 39 (ATCC VR-340; 2.9 $TCID_{50}$/ml), *Streptococcus pneumoniae* (ATCC 49619; 0.5 McFarland standard, or ~1.5×10^8 cells/ml), and *Haemophilus influenzae* (ATCC 10211; ~1.5×10^8 cells/ml). For the 2009 H1N1 RT-PCR, 22 seasonal influenza A/H1 virus-, 15 seasonal influenza A/H3 virus-, and 20 influenza B virus-positive clinical specimens and 1 H5N1 avian influenza virus-positive specimen (nucleic acid extract kindly provided by John Lednicky, Midwest Research Institute, Kansas City, MO) were tested, in addition to the organisms mentioned above. Further, primers and probe sequences of the new laboratory-developed RT-PCR for 2009 H1N1 were analyzed against full-length HA gene sequences of 20 swine influenza virus sequences (H1N1, H1N2, H3N1, H3N2) and 20 avian influenza virus sequences (H1N1, H1N2, H2N2, H3N8, H4N8, H5N1, H5N2, H5N8, H6N2, H7N2, H7N3, H9N2, H10N7, H11N2, H11N3, H11N9, H12N5, H13N6) available in GenBank for sequence similarity using Lasergene (version 8.0) software. The accuracy of detection of influenza viruses was determined on the basis of the number of true-positive and -negative patient specimens accurately detected by each RT-PCR assay. The reproducibilities of the assays were tested by using culture fluids obtained from ZeptoMetrix at $10^{0.7}$ $TCID_{50}$/ml of influenza A/H1 virus, $10^{0.6}$ $TCID_{50}$/ml of influenza A/H3 virus, $10^{0.7}$ $TCID_{50}$/ml of influenza B virus, and a 10^{-4} dilution of an H1N1-positive clinical specimen. The nucleic acid amplification reactions were performed in triplicate on three different runs with fresh nucleic acid extracted for each run.

RESULTS

Analytical sensitivity. The results of the analytical sensitivity study of three influenza A/B virus RT-PCRs are given in Table 2. Of the 150 respiratory specimens tested, 117 were determined to be true positive for either influenza A or B virus. The CDC assay demonstrated good overall sensitivity of 100% in detecting both influenza A virus (H1, H3, and 2009 H1N1 subtypes) and influenza B virus. The FDA-approved ProFlu$^+$ assay demonstrated an overall sensitivity of 98.2% in detecting both influenza A and B viruses. It failed to detect one seasonal influenza A/H1 virus and yielded another unresolved result due to an internal control failure with an influenza A/H1 virus-positive specimen (27/29). The MGB assay had an overall sensitivity of 94% for detection of both influenza A and B viruses. Although it detected all influenza A/H3 viruses (28/28), the assay failed to detect 1 influenza B virus (29/30), 2 influenza A/H1 viruses (27/29), and 4 2009 H1N1 influenza viruses (26/30).

The LODs of the influenza A/B virus and 2009 H1N1 RT-PCR assays are given in Table 3. The LOD of the CDC assay is comparable to that of the FDA-approved ProFlu$^+$ assay except with influenza A/H3 virus, where the ProFlu$^+$ assay showed a 1-log decreased detection. The MGB assay showed a 1-log decreased detection with influenza A/H3 and influenza B viruses and a 2-log (10^{-4} dilution) decreased detection with the 2009 H1N1 strain.

TABLE 3. Limit of detection of influenza A/B and 2009 H1N1 RT-PCR assays

Organism	Limit of detection[a]					
	Influenza A/B assays			2009 H1N1 assays		
	CDC-Influenza A/B	ProFlu$^+$	MGB Alert Flu A/B & RSV	New H1N1	CDC-SW Inf A	CDC-SW H1
Influenza A/H1 virus	$10^{0.07}$	$10^{0.07}$	$10^{0.07}$			
Influenza A/H3 virus	$10^{0.006}$	$10^{0.06}$	$10^{0.06}$			
Influenza B virus	$10^{0.007}$	$10^{0.007}$	$10^{0.07}$			
Influenza A/2009 H1N1 virus	10^{-6}	10^{-6}	10^{-4}	10^{-6}	10^{-6}	10^{-5}

[a] Influenza A/H1 virus [Solomon Islands/03/06 (H1N1); $10^{6.7}$-$TCID_{50}$/ml stock], influenza A/H3 virus [Brisbane/10/07 (H3N2); $10^{5.6}$-$TCID_{50}$/ml stock], and influenza B virus [Florida/04/06; $10^{6.7}$-$TCID_{50}$/ml stock], obtained from ZeptoMetrix Corporation, were used to determine the LODs of the influenza A/B virus RT-PCR assays. A patient isolate of 2009 H1N1 was used to determine the LODs of the 2009 H1N1 RT-PCR assays. The values for the influenza A/B virus assays for influenza A/H1 virus, influenza A/H3 virus, and influenza B virus are in $TCID_{50}$/ml, whereas the values for the influenza A/B virus assays and the 2009 H1N1 assays for influenza A/2009 H1N1 virus are in dilutions.

The Luminex RVP assay was used as a gold standard to determine the analytical sensitivity of the laboratory-developed 2009 H1N1 RT-PCR and the CDC SW Inf A and SW H1 assays (Table 2). The laboratory-developed 2009 H1N1 RT-PCR assay was able to detect all 100 positive specimens with an analytical sensitivity of 100% (Table 2). This assay did not detect 2009 H1N1 RNA in any of the 20 negative specimens and did not show any cross-reactivity with seasonal influenza A/H1 and A/H3 virus-positive specimens. The analytical sensitivities of the CDC SW Inf A and CDC SW H1 assays in detecting 2009 H1N1 influenza virus were 99% and 97%, respectively. The LODs of the RT-PCR assays for 2009 H1N1 influenza virus were determined by testing 10-fold serial dilutions of a 2009 H1N1-positive clinical specimen. The LDT for 2009 H1N1 influenza virus and the CDC SW Inf A assay were able to detect virus present at up to a 10^{-6} dilution in the sample, while the CDC SW H1 assay demonstrated 1-log decreased detection (10^{-5} dilution).

Analytical specificity. No cross-reactivity was observed with any of the viruses or bacteria used to test for the specificity of the influenza A/B virus and the RT-PCR assays for 2009 H1N1 influenza virus. The three influenza virus A/B RT-PCR assays and the three 2009 H1N1 RT-PCR assays did not detect any influenza virus signal in 33 respiratory specimens determined to be true negative. The LDT for 2009 H1N1 influenza virus did not show cross-reactivity with any of the seasonal influenza A/H1 or A/H3 viruses, influenza B viruses, or H5N1 avian influenza virus tested. Sequence analysis with swine influenza viral sequences showed several missing bases in the probe binding region ($n = 2$ to 6) and base mismatches in the forward primer ($n = 1$ to 22), probe ($n = 3$ to 18), and reverse primer ($n = 4$ to 15). The avian influenza virus sequences analyzed also showed missing bases in the probe binding region ($n = 4$ to 21) and base mismatches in the forward primer ($n = 7$ to 21), probe ($n = 7$ to 23), and reverse primer ($n = 1$ to 18). The sequence analyses further indicate the high specificity of the new LDT for 2009 H1N1 influenza virus.

Accuracy. Accuracy estimation showed that the CDC assay had 100% agreement with influenza specimens defined as true positive or true negative. The ProFlu$^+$ and MGB assays yielded 99% and 94% agreements, respectively. The newly developed 2009 H1N1 RT-PCR assay demonstrated 100% concordance with 2009 H1N1 influenza virus-positive and -negative specimens, while the SW Inf A and SW H1 assays demonstrated 99% and 97% agreements, respectively.

Reproducibility. The reproducibility analysis showed that each influenza A/B virus RT-PCR assay demonstrated very little variation in C_T values for the two test strains. The interrun and intrarun C_T variabilities for influenza A/H1 virus were as follows: 0.5 and 0.3 cycles, respectively, for the CDC Flu A/B assay; 0.9 and 0.5 cycles, respectively, for the ProFlu$^+$ assay; and 0.7 and 0.3 cycles, respectively, for the MGB assay. The interrun and intrarun C_T variabilities for influenza A/H3 virus were as follows: 0.6 and 0.3 cycles, respectively, for the CDC Flu A/B assay; 0.4 and 0.2 cycles, respectively, for the ProFlu$^+$ assay; and 1.2 and 0.2 cycles, respectively, for the MGB assay. For influenza B virus, the C_T variabilities were 0.7 and 0.6 cycles, respectively, for the CDC Flu A/B assay; 0.1 and 0.2 cycles, respectively, for the ProFlu$^+$ assay; and 0.3 cycles each (for both interrun and intrarun variability) for the MGB assay. The respective interrun and intrarun C_T variabilities for the 2009 H1N1 strain were as follows: 0.59 and 0.18 cycles, respectively, for the CDC Flu A/B assay; 0.56 and 0.02 cycles, respectively, for the ProFlu$^+$ assay; and 0.07 and 0.33 cycles, respectively, for the MGB assay.

With the 2009 H1N1 virus control, the new LDT yielded highly reproducible results, with interrun and intrarun C_T variabilities of 1.2 and 1 cycles, respectively. The interrun and intrarun variabilities of the CDC SW Inf A and SW H1 assays were at an acceptable level of reproducibility, with the C_T variabilities being in the range of 0.5 to 1.6 cycles.

Clinical sample testing. Of the 150 respiratory specimens tested, 117 were considered true positive for Flu A or B and 33 were considered true negative for both viruses. The performance characteristics of all three Flu A/B RT-PCRs were comparable, although minor deficiencies in the sensitivities of the ProFlu$^+$ and MGB assays in detecting influenza A viruses were noted. The C_T values obtained for the CDC and ProFlu$^+$ assays were comparable, with the respective median C_Ts being 22.9 and 23.2, respectively, for Flu A-positive specimens and 21.3 and 20, respectively, for Flu B-positive specimens. The C_T values for the MGB assay were delayed compared to those for the CDC assay, with the median C_T values being 30 for influenza A virus (range, 1.1 and 12.6 cycles) and 27.9 for influenza B virus (range, 1.4 and 12 cycles). The MGB assay C_T values were delayed compared to those for ProFlu$^+$ assay: 0.6 and 6.7

cycles, respectively, for Flu A and 3.7 and 10 cycles, respectively, for Flu B. The melting temperatures (T_ms) for influenza A virus (including 2009 H1N1) ranged from 57.1 to 58.9°C, with the median T_m being 57.5°C. For influenza B virus, the T_ms were in the range of 62.6 to 64.5°C, with the median T_ms being 63.8°C.

The newly developed 2009 H1N1 RT-PCR assay and the CDC SW Inf A assay detected all H1N1-positive specimens and had comparable C_T values. The CDC SW H1 PCR was comparatively less sensitive, as it failed to detect three 2009 H1N1 virus-positive specimens and yielded delayed C_T values in the range of 0.1 to 6.0, with the median C_T difference being 3.1 cycles compared with C_T values obtained by the new 2009 H1N1 RT-PCR developed in this study. The C_T value for the MS2 internal control ranged from 32.0 to 33.8 cycles, with the median C_T being 33.2 cycles. The internal control yielded negative results when high numbers of copies of 2009 H1N1 RNA were present in the specimen and produced a positive signal only when there was a low level of 2009 H1N1 RNA ($C_T \geq 32.0$ cycles).

DISCUSSION

Nucleic acid amplification tests are the preferred methods for the identification of respiratory viral infections, including influenza. PCR-based methods provide rapid and sensitive detection and, most importantly, help with identifying different subtypes of influenza viruses. The current study describes the performance characteristics of three real-time RT-PCR assays for detection of all influenza viruses and a newly developed 2009 H1N1 RT-PCR assay for specific detection of 2009 H1N1 viruses. We designed the assay to test specimens in two separate master mixtures: in a first reaction for detection of influenza A and B viruses and in a second reaction for detection of 2009 H1N1 virus and the MS2 internal control. This diagnostic algorithm allows both detection of seasonal influenza A and B viruses and specific identification of 2009 H1N1 viruses. A recent report from Germany indicates that the seasonal influenza viruses A and B cocirculated during the pandemic 2009 H1N1 viral season (15). Therefore, despite increased interest in the 2009 H1N1 virus, seasonal influenza cannot be neglected in diagnosis of the illness in patients with flu-like symptoms. The Luminex xTAG RVP and ProFlu$^+$ multiplex real-time RT-PCR assays are both FDA-approved *in vitro* diagnostic assays available to detect influenza viruses. The 8- to 12-h turnaround time and labor-intensive nature of the Luminex RVP assay does not lend itself to rapid detection of influenza viruses. The ProFlu$^+$ assay is currently approved for analysis on a SmartCycler instrument but has a limited throughput of only 16 samples per instrument. Hence, two other influenza A/B virus assays, namely, the CDC and MGB assays, were evaluated on a high-throughput platform with the 96-well ABI 7500 real-time instrument, and their performance characteristics were compared with those of the ProFlu$^+$ assay. The CDC protocol for influenza virus testing suggests the use of separate reaction wells for each analyte; we modified the protocol in an attempt to detect influenza A and B viruses in a single tube by labeling the influenza A virus probe with 6-FAM and the influenza B virus probe with JOE dye. The results of the CDC assay were comparable to those of the FDA-approved ProFlu$^+$ assay. Interestingly, the CDC assay detected two additional influenza A virus-positive samples that were negative or that yielded an unresolved result with the ProFlu$^+$ assay due to internal control failure. We have observed that up to 3% (13/431) of respiratory specimens produced an unresolved result with the ProFlu$^+$ assay during routine clinical testing in our facility (data not shown). According to the manufacturer, either PCR inhibition or reagent failure could be the reason for such a result. However, the CDC assay did not show any such PCR inhibition with the sample that demonstrated an unresolved result with the ProFlu$^+$ assay. The MGB assay did not generate an amplification curve or a C_T for 4 of the 2009 H1N1-positive specimens tested; however, a characteristic melting profile (T_m = 58°C) in the acceptable range was detected for each of the specimens. This may be due to base mismatches in the probe binding region.

A new 2009 H1N1 PCR assay was developed with an internal control and designed to be tested with the CDC influenza A/B virus PCR in the same run. The 2009 H1N1-positive specimens were identified by Luminex RVP assay as nonsubtypeable influenza A viruses. The nonsubtypeable influenza A virus-positive specimens were further confirmed to be 2009 H1N1 strains by the CDC 2009 H1N1 real-time PCR assay (18). Other investigators have used a similar approach to identify 2009 H1N1 strains and differentiate them from seasonal influenza viruses using Luminex RVP assay results (6, 10). Even though the CDC-developed real-time PCR protocol was used for detection of 2009 H1N1 strains, both the SW Inf A and SW H1 assays have been reported to have some drawbacks. The SW Inf A assay was reported to cross-react with H5N1 viruses (11), and the sequence mismatches in the SW H1 assay reverse primer binding region may potentially reduce the sensitivity of this assay (16). With the CDC SW H1 assay, we observed a 1-log lower sensitivity with 2009 H1N1-positive clinical specimens. Also, three of the 2009 H1N1 low-positive specimens were not detected by the CDC SW H1 assay. A similar report from an earlier study indicated that 5 out of 39 of the 2009 H1N1 strains were not detected (10). A few 2009 H1N1 PCR assays have been developed since the first report of the 2009 H1N1 outbreak; however, these assays were developed much earlier, when the outbreak was reported and the number of sequences in the GenBank database was limited (10, 16). Constant addition of new sequences into the GenBank database revealed mismatches in the primer and probe regions of these previously developed PCR methods that could potentially reduce the sensitivities of these assays. As of October 2009, there were about 280 hemagglutinin sequences of the United States-associated 2009 H1N1 strains available in NCBI's Influenza Virus Source, and the primers and probe of this newly developed H1N1 RT-PCR assay have good matches with all available sequences.

In summary, this newly developed and validated 2009 H1N1 RT-PCR assay is a recently updated assay capable of detecting the currently circulating 2009 H1N1 viruses with high sensitivity. The CDC influenza A/B virus assay and this new 2009 H1N1 RT-PCR with an internal control can be used in combination for diagnosis and surveillance of universal influenza A and B viruses and 2009 H1N1 viruses.

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