Simultaneous Detection of Influenza A, Influenza B, and Respiratory Syncytial Viruses and Subtyping of Influenza A H3N2 Virus and H1N1 (2009) Virus by Multiplex Real-Time PCR[▽]

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A multiplex real-time PCR assay was developed to simultaneously detect and discriminate influenza A virus subtypes, including novel H1N1 (2009) and seasonal H3N2 virus, influenza B virus, and respiratory syncytial virus (RSV) in a single test tube, with detection sensitivity and specificity of 99% and 100%, respectively, for the four pathogens.

Influenza virus and other respiratory viruses are common pathogens causing acute respiratory tract infection in humans. Among them, influenza virus A (Flu A), influenza virus B (Flu B), and respiratory syncytial virus (RSV) are the leading pathogens (2, 6, 10). Recently, several high-throughput multiplex molecular analytical techniques for simultaneous detection of multiple respiratory viruses were developed in other laboratories that provide promising solution for quick and definitive diagnosis of virus-infected respiratory tract diseases (4, 9, 11).

H1N1 (2009) virus, the new influenza A virus that emerged in Mexico in 2009, caused outbreaks of respiratory illness with high mortality in Mexico, resulting in a pandemic (3). Infections by the new H1N1 (2009) virus were diagnosed by viral genome amplification using PCR assay and virus isolation via tissue culture during the viral outbreak (1, 5, 7, 13, 15). Whereas the pandemic H1N1 (2009) virus became the most prevalent influenza A virus, infection with seasonal H1N1 virus was uncommon and infections by seasonal influenza A subtype H3N2 virus, Flu B, and RSV were restricted mainly to infants and children in 2009 (14). However, there is a need to develop a quick method to detect the common pathogens causing respiratory infection.

For this report, we developed a multiplex real-time PCR assay allowing the simultaneous detection and discrimination of Flu A, seasonal H3N2, new H1N1 (2009), Flu B, and RSV in a single test tube. Primers and probes designed for the multiplexing assay are listed in Table 1. In the listed sets of primers and probes, the RnaseP (RP) primer and probe set was designed specifically to target human nucleic acid as an

internal control (15). To evaluate the multiplex assay, we also designed sets of primers and probes used in the existing real-time PCR assays from previous reports (Table 1) (7, 11, 12, 15).

Samples of 1,017 nasopharyngeal swabs from patients with respiratory infections during April 2009 to April 2010 were collected and stored in viral transport medium at -80°C . For the multiplex PCR assay, total nucleic acids in individual specimens were extracted using a QIAamp RNeasy Minikit (Qiagen, Hilden, Germany) followed by cDNA synthesis using reverse transcriptase and random primers and a ReverTra Ace FSK-101 kit (Toyobo, Shanghai, China). The original copies of viral RNA transcripts were determined as described previously (13).

The real-time PCR was conducted using a 50- μl reaction solution containing 10 μl of cDNA and 40 μl of PCR mixture with a Premix Ex Taq Hot Start version (Takara, Shanghai, Japan) and thermal cycling parameters of denaturation at 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min on an ABI 7500 system (Applied Biosystems, Foster City, CA).

The assay sensitivities and specificities of the multiplex and simplex real-time PCR, as well as those of the existing real-time PCR method, were compared by assaying serially diluted samples of different types of virus and related RNA transcripts. In this study, the concentration of each of four types of viruses (seasonal H3N2 virus, new H1N1 [2009] virus, Flu B, and RSV) was 2×10^6 50% tissue culture infective doses (TCID₅₀)/ml, or 2×10^8 copies/ml. For each type of virus—seasonal H3N2 virus, new H1N1 (2009) virus, Flu B, or RSV—the ranges of detection sensitivity were from 2×10^{-1} to 2×10^0 TCID₅₀/ml, or 2×10^1 to 2×10^2 copies/ml.

For four mixed virus samples, the final concentration of each virus in 200 μl of the mixed virus samples was 2×10^4 TCID₅₀/ml, and total nucleic acids of the mixed viruses were extracted to produce the corresponding RNA transcript mixture at the final concentration of 2×10^6 copies/ml for each type of transcript. The detection sensitivities of the multiplex assay for

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TABLE 1. Real-time PCR primers and probes designed and used in this study

Primer and probe set and oligonucleotide category	Sequence ^a	Gene target	Location (bp)	GenBank accession no.
Designed and used in this study				
Flu A universal forward primer	5'-GACCRATCCTGTACCTCTGAC-3'	M	156–177	HM590431
Flu A universal reverse primer	5'-GGGCATTYTGACAAAKCGTCTACG-3'	M	226–250	HM590431
Flu A probe	5'-HEX-TGCAGTCCTCGCTCACTGGGCACG-BHQ1-3'	M	201–224	HM590431
Flu B forward primer	5'-TCCTCAACTCACTCTTCGAGCG-3'	NP	733–754	CY040733
Flu B reverse primer	5'-CGGTGCTCTTGACCAAATTGG-3'	NP	815–835	CY040733
Flu B probe	5'-ROX-CCAATTCGAGCAGCTGAAACTGCGGTG-BHQ2-3'	NP	777–803	CY040733
Novel H1N1 2009 forward primer	5'-TGAGATATCCCCAAGACAAGTTC-3'	HA	393–416	CY065770
Novel H1N1 2009 reverse primer	5'-TTTGTAGAAGCTTTTTGCTCCAG-3'	HA	480–502	CY065770
Novel H1N1 2009 probe	5'-FAM-TCATGACTCGAACAAGGTGTAACGG-BHQ1-3'	HA	439–464	CY065770
Seasonal H3 forward primer	5'-ACCCTCAGTGTGATGGCTTCCAAA-3'	HA	280–303	CY064879
Seasonal H3 reverse primer	5'-TAAGGGAGGCATAATCCGGCACAT-3'	HA	364–387	CY064879
Seasonal H3 probe	5'-CY5-ACGCAGCAAAGCCTACAGCAACTGT-BHQ3-3'	HA	327–351	CY064879
RSV universal forward primer	5'-GGAYTGTTTATGAATGCCTATGGT-3'	L	733–756	DQ780569
RSV universal reverse primer	5'-GGRTTRTTCAATATATGGTAGAATCC-3'	L	895–920	DQ780569
RSV probe	5'-LC-Red 705-TCCACAACCTGYTCCATTCTGC-BHQ3-3'	L	835–857	DQ780569
Designed in previous study and used in this study ^b				
Flu A universal forward primer	GACCRATCCTGTACCTCTGAC			
Flu A universal reverse primer	AGGGACTTYTGACAAAKCGTCTA			
Flu A probe	FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ1			
Flu B forward primer	AAATACGGTGGATTAAATAAAGCAA			
Flu B reverse primer	CCAGCAATAGCTCCGAAGAAA			
Flu B probe	FAM-CACCCATATTGGGCAATTTCTATGGC-TAMRA			
Novel H1N1 2009 forward primer	GTGCTATAAACACCAGCCTYCCA			
Novel H1N1 2009 forward primer	CGGGATATTCCTTAATCCTGTRGC			
Novel H1N1 2009 probe	FAM-CAGAAATATACA"TT"CCRGTCACAATTGGARAA-BHQ1			
Seasonal H3N2 forward primer	CATGCAGTACCAACGGAAC			
Seasonal H3N2 reverse primer	CATCACACTGAGGGTCTCCCAA			
Seasonal H3N2 probe	FAM-ATGACCAAATTGAAGTIACTAATGCTACTGAGC-BHQ1			
RSV universal forward primer	AACAGATGTAAGCAGCTCCGTTATC			
RSV universal reverse primer	CGATTTTATTGGATGCTGTACATTT			
RSV universal probe	FAM-TGCCATAGCATGACACAATGGCTCCT-TAMRA			

^a HEX, hexachlorofluorescein phosphoramidite; BHQ1, BHQ2, and BHQ3, Black Hole Quencher 1, 2, and 3, respectively; ROX, carboxy-X-rhodamine; FAM, a proprietary fluorophore similar to 6-carboxyfluorescein fluorophore; CY5, indodicarbocyanine (blue reporter dye); LC-Red 705, LightCycler Red 705 dye; TAMRA, 6-carboxytetramethylrhodamine; bp, base pairs. All the probes contained oligonucleotides with the 5' reporter dye and the 3' quencher dye.

^b Flu A and novel H1N1 2009 primers and probes are from reference 8. Flu B primers and probe are from reference 14. Seasonal H3N2 primers and probe are from reference 9. RSV universal primers and probe are from reference 6.

mixed viruses ranged from 2×10^1 to 2×10^0 TCID₅₀/ml or 2×10^2 to 5×10^2 copies/ml.

The results indicated that the detection sensitivity of the multiplex assay for mixed virus samples was slightly lower than that for a single type of virus. We also found that the detection sensitivity of the simplex assay shown in this study was similar to the existing assay sensitivity reported in other laboratories (7, 11, 12, 15). The lower detection sensitivity in the multiplex assay format may have resulted from the amplification competition and interference occurring during simultaneous amplification of two or more types of virus when using multiplex primers and probes in one reaction. With respect to the assay specificity, we found no cross-reactions among four types of viruses (seasonal H3N2 virus, new H1N1 [2009] virus, Flu B, and RSV) or among 27 other common respiratory pathogens and commensal organisms (seasonal H1N1 virus, enterovirus type 71, human parainfluenza virus type 3, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Neisseria meningitidis*, and so on) at high concentrations ($>10^4$ TCID₅₀, copies, or cells/ml) in the tests.

For definitive diagnosis of viral infection, tissue culture has

been considered the "gold standard" (10). From the 1,017 nasopharyngeal swab samples, we selected the first 182 specimens collected from the patients with respiratory infections for virus isolation by inoculating the samples into Madin-Darby canine kidney (MDCK) cells or human epidermoid carcinoma (HEp-2) cells. Among 182 clinical samples, 76 samples were positive for viral isolation: 51 Flu A-positive samples, including 32 novel H1N1 (2009) virus samples, 15 seasonal H3N2 virus samples, and 4 other samples of subtypes of Flu A, 13 Flu B-positive samples, and 12 RSV-positive samples. We also identified two samples with H1N1 (2009) virus-RSV coinfection and one sample with H3N2 virus-RSV coinfection.

In comparison with the gold standard tissue culture, the multiplex real-time PCR assay for the 182 samples showed 100% sensitivity (95% confidence interval [CI], 95% to 100%) and 96% specificity (95% CI, 98% to 100%) for these viruses. For example, the sensitivities and specificities for H1N1 (2009) virus were 100% (95% CI, 89% to 100%) and 99% (95% CI, 99% to 100%), respectively; for seasonal H3N2 virus, 100% (95% CI, 68% to 100%) and 99% (95% CI, 99% to 100%), respectively; for Flu B, 100% (95% CI, 75% to 100%) and 100% (95% CI, 99% to 100%), respectively; and for RSV,

TABLE 2. Sensitivity and specificity of multiplex real-time PCR in comparison with simplex assay format for detecting respiratory viruses in 1,017 clinical specimens

Virus	No. of samples with indicated result				Sensitivity (%)	Specificity (%)
	Multiplex assay (6 indeterminates) ^a		Simplex assay (7 indeterminates) ^b			
	+	−	+	−		
Flu A	299	718	303	714	99	100
Novel H1N1 (2009)	187 ^{c,e}	830	191 ^{c,e}	826	98	100
Seasonal H3N2	70 ^{d,e}	947	70 ^{d,e}	947	100	100
Flu B	57	960	57	960	100	100
RSV	49 ^{c,d}	968	51 ^{c,d}	966	96	100
Total (<i>n</i> = 1,017)	405	612	411	606	99	100

^a Six indeterminate samples consisted of three positives for H1N1(2009) virus, two positives for H3N2 virus, and one positives for RSV by the viral genomic sequence analysis.

^b Seven indeterminate samples consisted of four positive for H1N1(2009) virus, one positive for H3N2 virus, and two positives for RSV by the viral genomic sequence analysis. Indeterminate results by the genomic sequence analysis were considered to represent definitive positive diagnoses, and the numbers were included in the sensitivity and specificity calculations.

^c H1N1(2009) virus-RSV coinfection were identified in three samples.

^d H3N2 virus-RSV coinfection was identified in one sample.

^e H1N1(2009) virus-seasonal H3N2 virus coinfections were identified in two samples.

100% (95% CI, 74% to 100%) and 99% (95% CI, 99% to 100%), respectively; and the positive predictive values (PPV) were 94% (Flu A), 94% [H1N1 (2009)], 94% (H3N2), 100% (FluB), and 92% (RSV). The negative predictive value (NPV) was 100%. In this test, a discrepancy was found in 6 samples for which the PCR result was positive at a high threshold cycle (C_T) value ($35 < C_T \leq 40$) whereas the tissue culture result for virus isolation was negative. The discrepancy may have resulted from a very low concentration of virus in the samples, leading to the difficulty of viral isolation, or from the presence of possible inhibitory components in the samples that might have inhibited the virus replication in tissue cultures.

Analyses of 1,017 specimens by the use of multiplex and simplex real-time PCR assays demonstrated that the two assay systems we developed had comparable sensitivities and specificities (Table 2). The multiplex PCR results showed that 299 samples were positive for Flu A, including 187 samples with H1N1 (2009) virus, 70 samples with seasonal H3N2 virus, and 42 samples with other Flu A subtypes, that 57 samples were positive for Flu B, and that 49 samples were positive for RSV. There were 612 samples with negative results. Using the simplex PCR assay, we detected 303 Flu A-positive samples, including 191 samples with H1N1 (2009) virus, 70 samples with seasonal H3N2 virus, and 42 samples with other subtypes, whereas 57 samples were positive for Flu B, 51 samples were positive for RSV, and 606 samples gave negative results. Notably, two novel samples showing H1N1 (2009) virus-H3N2 virus coinfection, three samples showing H1N1 (2009) virus-RSV coinfection (including two positive samples from the viral isolation), and one sample showing H3N2 virus-RSV coinfection (including one positive sample from the viral culture) were also confirmed by multiplex PCR assay.

Furthermore, in comparison with the sensitivities and specificities of existing assays, we found that the sensitivity for Flu A, H1N1 (2009) virus, seasonal H3N2 virus, Flu B, and RSV detection was 100% and that the specificities were 99%, 98%, 100%, 100%, and 96%, respectively, which strongly suggests that the multiplex real-time PCR assay described in this report

is fully comparable with assay systems previously reported (1, 5, 7, 8, 12, 13, 15).

Of six results that were indeterminate in comparisons of the multiplex assay and tissue culture, three represented novel H1N1 (2009) virus, two represented seasonal H3N2 virus, and one represented RSV, for which a positive result was also given by the simplex PCR assay. Of seven results that were indeterminate in comparisons of the simplex and multiplex assays, four represented novel H1N1 (2009) virus, one represented H3N2 virus, and two represented RSV by simplex PCR; in contrast, the multiplex assay result was negative. We thought that interference among the multiplex primers and probes may have led to the discrepancy and that the sensitivity would therefore have decreased when the viral concentration was extremely low. To confirm the positive result from the weakly positive samples, we amplified DNA products in the samples and then cloned them in pMD18T vector (Takara, Dalian, China). The genomic sequences of the plasmids with positive results were verified by Sangon Biotech (Shanghai) Co., Ltd. Therefore, these indeterminate results were considered positive and were included in the sensitivity and specificity calculations.

In summary, a sensitive and specific multiplex PCR assay was developed to detect the most common pathogens causing respiratory tract diseases, including Flu A, Flu B, and RSV. This assay may provide a quick and easy method for clinical diagnostics and viral infection surveillance.

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