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Duplex Real-Time Reverse Transcriptase PCR Assays for Rapid Detection and Identification of Pandemic (H1N1) 2009 and Seasonal Influenza A/H1, A/H3, and B Viruses▽

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Reports of a novel influenza virus type A (H1N1), now designated by the World Health Organization as pandemic (H1N1) 2009, emerged from the United States and Mexico in April 2009. The management of the pandemic in Australia required rapid and reliable testing of large numbers of specimens for the novel influenza strain and differentiation from seasonal influenza strains. A real-time reverse transcriptase PCR (RT-PCR) assay for the detection of pandemic (H1N1) 2009 was designed and used with existing real-time RT-PCR assays for seasonal influenza viruses A and B. MS2 coliphage was added to all samples and amplified as a quality control. Three duplex RT-PCR assays, each containing two primer pairs and corresponding 5′ nuclease probes, were initially evaluated on control material and stored samples and showed high sensitivity and specificity. More than 11,000 clinical samples were then tested for influenza A and B matrix gene targets and specific hemagglutinin gene targets for seasonal influenza A/H1, A/H3, and pandemic A (H1N1) 2009. Minimum sensitivities and specificities were 98.8% and 100%, respectively, for pandemic (H1N1) 2009, 81.5% and 98.9% for seasonal A/H1, and 96.3% and 99.6% for A/H3. Automated sample extraction facilitated the rapid processing of samples so that the assays allowed accurate, rapid, and cost-effective screening of large numbers of clinical samples.

The first reports of human infection with a novel swine origin influenza A/H1 virus appeared in April 2009 (1), and the virus has since been named by the World Health Organization (WHO) as pandemic (H1N1) 2009. The first cases were identified in California and were retrospectively linked to contact with infected people in Mexico. Between that time and 5 December 2009, it was reported to the WHO from over 200 countries, with 9,596 deaths due to the virus (http://www.who.int/csr/disease/swineflu/updates/en/index.html). It was first reported in Australia on 12 May and in Western Australia on 25 May, with over 37,000 cases and 191 deaths during the 2009 season (http://www.health.gov.au/internet/healthemergency/publishing.nsf/Content/ozflucurrent.htm). Since the first appearance of the virus, it has been imperative to deliver rapid, accurate test results for large numbers of samples and to detect and differentiate seasonal and pandemic influenza. To meet that demand, we developed and introduced three duplex real-time RT-PCR assays to detect and type influenza A/H1 (seasonal), A/H3, pandemic (H1N1) 2009, and influenza B viruses and evaluated the performance of these assays.

MATERIALS AND METHODS

Primer and probe design. Primers and probe sequences listed in Table 1 were designed in-house using Primer Express software (Applied Biosystems), with the exception of those for the influenza A matrix gene (9). Fluorophore-labeled oligonucleotide probes were synthesized by Applied Biosystems (MGB probes) or Biosearch Technologies, Inc. (Table 1). Sequence information for pandemic (H1N1) 2009 was available in late April 2009 from the Global Initiative on Sharing Avian Influenza Data (GISAID) Internet site (http://platform.gisaid.org/), and regions of the hemagglutinin (HA) gene that differentiated the pandemic (H1N1) 2009 from seasonal H1N1 strains were targeted for primer and probe design. Regular examination of the updated sequence information ensured that the oligonucleotide primers and probes remained appropriate for circulating pandemic (H1N1) 2009 strains.

Sample preparation. The majority of specimens consisted of deep nasal swabs from both nostrils plus a throat swab from patients presenting with influenza-like illness at hospital emergency departments or general medical practices. These were either collected into viral transport medium (VTM) or left as dry swabs, which were kept cool and promptly transported to PathWest Laboratory Medicine in Perth, Western Australia. The in-house-manufactured VTM contained Hanks balanced salt solution (BSS) with 1% bovine serum albumin, sodium bicarbonate, phenol red, and gentamicin (5). Nasopharyngeal aspirates in normal saline, when collected, were also tested. Samples were held at 4°C, and most were tested within 24 h of collection, except those collected from patients in rural and remote locations. From 27 April to 19 July 2009, 11,092 samples were received for influenza testing. A portion of each of the VTM samples was reserved for virus cultivation and stored over liquid nitrogen, while dry swabs were vortex mixed in VTM for testing and storage. For all samples, nucleic acid was then extracted from a 200-μl volume of VTM using a magnetic bead viral RNA isolation kit on an automated nucleic acid extraction instrument (MagMAX Express-96; Applied Biosystems). Liquid-handling robots were used to dispense all extraction reagents and for the addition of samples to lysis buffer (CAS-1200, CAS-1820; Corbett, Australia). In-house modifications to the manufacturer's protocol included a single wash buffer 1 step and the elution of purified RNA with 90 μl nuclease-free water preheated to 80°C. A standardized amount of MS2 RNA coliphage (MS2) was added to the lysis buffer supplied with the kit to

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TABLE 1. Primers and probes included in the three duplex real-time RT-PCR assays

Duplex mix	Target	Primer[a]/probe	Sequence 5′–3′[c]	Gene target	Product (bp)
1	Influenza A matrix[b]	FLUA-MAT-F	CTTCTAACCGAGGTCGAAACGTA	Matrix protein	155
		FLUA-MAT-R	GGTGACAGGATTGGTCTTGTCTTTA		
		FA-MAT-PR	CALO-TCAGGCCCCCTCAAAGCCGAG-BHQ1		
1	Influenza B matrix	FBMAT-24	TGCCTACCTGCTTTMMYTRACA	Matrix protein	75
		FBMAT-98	CCRAACCAACARTGTAATTTTTCTG		
		FBMAT-51	FAM-TGCTTTGCCTTCTCCA-MGBNFQ		
2	Influenza A H1	H1N1 HA-435	AAGCTCATGGCCCAACCA	Hemagglutinin	57
		H1N1 HA-491	CCATTATGGGAGCATGATGCT		
		H1HA-454	VIC-ATACTCCGGTCACGGT-MGBNFQ		
		H1HA-454mod	VIC-ACACTCCGGTTACGGT-MGBNFQ		
2	Influenza A H3	H3N2 HA-857	ACGAAGTGGGAAAAGCTCAATAAT	Hemagglutinin	72
		H3N2 HA-928	GGAGTGATGCATTCAGAATTGC		
		H3HA-889	FAM-ATGCACCCATTGGC-MGBNFQ		
3	Pandemic (H1N1) 2009	SWHA-440	AAGGTGTAACGGCAGCATGTC	Hemagglutinin	106
		SWHA-545	TAGGATTTGCTGAGCTTTGGGTAT		
		SWHA-465	FAM-AGAAGCTTTTTGCTCCAGCA-MGBNFQ		
3	MS2 RNA coliphage	MS2-105	GTCGACAATGGCGGAACTG	Coat protein	66
		MS2-170	TTCAGCGACCCCGTTAGC		
		MS2-127	CALO-ACGTGACTGTCGCCCCAAGCAACTT-BHQ1		

[a] Forward primer listed first.
[b] Primers and probe sequences were designed in-house using Primer Express software, with the exception of those for the influenza A matrix gene (9).
[c] FAM, 6-carboxyfluorescein.

monitor the efficiency of sample extraction, the removal of reverse transcription and PCR inhibitors, and the cDNA production process (2). Negative controls were included in the extraction process between every 5 clinical samples and treated as samples for the completion of the assays.

Real-time RT-PCR. The 20-μl one-step RT-PCR mix contained 1× reaction buffer (Invitrogen), 0.3 μl Superscript III RT/Platinum *Taq* mix (Invitrogen), 0.5 units iStar *Taq* DNA polymerase (Intron Biotechnology), 10 units RNaseOUT (Invitrogen), 0.3 μM primers, 0.1 μM TaqMan probes, and 8 μl RNA extracted from sample or control material. The PCR mixes, minus target RNA, were prepared in large volumes and stored in single-use aliquots at −20°C. All batches were quality checked with negative-control material and 10-fold dilutions of positive-control material and were accepted when titration endpoint cycle threshold (C_T) values were within 2 standard deviations (SD) of the determined mean value. Liquid-handling robots (CAS-1200; Corbett, Australia) were used to dispense the reaction mixes (12 μl) into 100-tube rings (Rotor-Disc 100; Qiagen, Australia). A similar robot located in a separate laboratory was used to add the sample or positive-control RNA (8 μl) to the reaction mix, and the rings were sealed with a heat-treated film. Three reaction mixes were required for each sample. Mix 1 contained primers and probes for the detection of influenza A and B matrix genes, mix 2 contained primers and probes for the circulating human seasonal H1 and H3 influenza A hemagglutinin genes, and mix 3 contained primers and probes for the pandemic (H1N1) 2009 hemagglutinin gene and MS2 RNA coliphage coat protein gene (Table 1). Appropriate positive-control materials for each gene segment targeted were included on each 100-tube ring. Valid positive-, negative-, and extraction/inhibitor control results were required for assay batches to be accepted. Samples producing C_T values of ≤40 were considered positive for that gene segment.

The amplification process was performed in RotorgeneQ real-time thermocyclers (Qiagen, Australia) as follows: 50°C for 30 min (reverse transcription), 95°C for 5 min (DNA polymerase activation), followed by 45 cycles of 94°C for 12 s (denaturation), 51°C for 11 s (Rotor-Disc temperature correction step), 55°C for 60 s (annealing), 68°C for 0 s (Rotor-Disc temperature correction step), and 72°C for 15 s (extension). The Rotor-Disc temperature correction steps were inserted according to advice from the Rotorgene and Rotor-Disc design engineers (Corbett Life Sciences, Australia). Probe emission signals were acquired during the extension step of the cycling program.

DNA sequencing of pandemic (H1N1) 2009. A nested RT-PCR was used to amplify a 180-bp segment of the HA gene of pandemic (H1N1) 2009, and the PCR products were sequenced using the AB BigDye Terminator version 3.1 sequencing kit on the AB 3130xl genetic analyzer (Applied Biosystems) and identified by GenBank database searches.

Control material. Positive-control material stocks were stored at −80°C as extracted RNA (QIAmp viral RNA minikit, Qiagen, Australia). The RNA was extracted from clarified cell culture fluids of local strains of influenza A/New Caledonia/20/99-like (H1N1), A/Sydney/5/97-like (H3N2), B/Beijing/184/93-like, and A/California/7/2009-like (H1N1) viruses propagated in MDCK cells. Ten replicates of 10-fold dilutions of the RNA were previously tested to determine the endpoint titers of the viral RNA, and that material at 10-fold above the endpoint titer was stored in single-use aliquots for use as positive-control material in each batch of sample tests. C_T values for each positive control in each PCR assay were recorded on Shewhart plots, and trends were monitored. After 100 batches of tests had been performed, SD were calculated, and assay batches with control values deviating by 2 SD were rejected.

Large batches of MS2 to monitor the extraction, reverse transcription, and amplification processes were harvested from broth cultures of *Escherichia coli* and stored in single-use aliquots at −20°C. The original MS2 coliphage material was a gift from Simon Toze (CSIRO, Australia), and its identity was confirmed by DNA sequencing and subsequent GenBank database search.

The MS2 phage concentration was adjusted to produce C_T values of 24 to 26 in the MS2 PCR assay, which corresponded to approximately 6.4×10^3 copies per sample. Evaluation of the diagnostic yields in relation to MS2 phage C_T values had demonstrated that these were not affected until C_T values exceeded 37 (data not shown). Samples producing negative influenza results with MS2 C_T values of >37 were retested, and those with persistent C_T values of >37 were reported as invalid and excluded from this study. In practice, <1% of samples had C_T values of >37 following the automated extraction procedure.

Assay efficiency. The comparative efficiency of individual and duplex PCR assays was determined on real-time PCR data collected from at least four replicates of 10-fold dilutions of target RNA (influenza A/H1N1 and A/H3N2, pandemic H1N1 2009, and influenza B control material), using the Rotorgene Q software (Qiagen, Australia).

Analytical sensitivity. The limit of detection (LOD) of cell culture and real-time RT-PCR was determined using six replicates of 10-fold dilutions of pandemic (H1N1) 2009 virus (A/Perth/29/2009). The cell culture assay utilized trypsin-enhanced MDCK shell vial cultures and monoclonal antibody detection of infection to calculate the 50% tissue culture infective dose ($TCID_{50}$) value.

TABLE 2. Preliminary evaluation of the influenza duplex real-time RT-PCR assays using stored respiratory specimens and known pathogens

Specimen or known pathogen	Duplex RT-PCR assay result		
	Influenza A matrix	Influenza A HA (H1/H3/pH 1)	Influenza B matrix
Specimens (no.)			
Seasonal influenza A/H1N1 (26)	+	H1	−
Influenza A/H3N2 (100)	+	H3	−
Influenza B (11)	−	−	+
Pandemic A (H1N1) 2009 (3)	+	pH 1[a]	−
Pandemic A (H1N1) 2009 RNA (1)	+	pH 1	−
Noninfluenza pathogen (64)[b]	−	−	−
No respiratory pathogen (14)	−	−	−
Known pathogens			
Influenza A viruses			
A/Auckland/1/2009 p(H1N1)	+	pH 1	−
A/California/7/2009 p(H1N1)	+	pH 1	−
A/Solomon Islands/3/2006 (H1N1)	+	H1	−
A/Brisbane/59/2007 (H1N1)	+	H1	−
A/Brisbane/10/2007 (H3N2)	+	H3	−
A/H2N2	+	−	−
A/H5N1/clade 2.1	+	−	−
A/H5N1/clade 2.2	+	−	−
A/H5N1/clade 2.3.4	+	−	−
A/H7N4	+	−	−
A/mallard/NL/1/05 (H9)	+	−	−
Influenza B viruses			
B/Brisbane/60/2008	−	−	+
B/Florida/4/2006	−	−	+
B/Malaysia/2506/2004	−	−	+
Influenza C virus	−	−	−
Noninfluenza respiratory pathogen[c]	−	−	−

[a] Pandemic (H1N1) 2009 HA gene.

[b] The specimens contained parainfluenza virus ($n = 18$), human metapneumovirus ($n = 13$), rhinovirus ($n = 8$), respiratory syncytial virus ($n = 17$), *Bordetella pertussis* ($n = 8$), adenovirus ($n = 3$), cytomegalovirus ($n = 1$), herpes simplex virus ($n = 1$), and *Haemophilus influenzae* ($n = 1$).

[c] One each of adenovirus, *Aspergillus* species, human bocavirus, *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Bordetella holmesii*, *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, *Chlamydia trachomatis*, human coronaviruses (229E, HKU1, NL63, and OC43), cytomegalovirus, untyped enterovirus, enterovirus 68, *Haemophilus influenzae*, human metapneumovirus, *Klebsiella pneumoniae*, *Legionella longbeachae*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, parainfluenzavirus (types 1, 2, 3, 4a, and 4b), *Pneumocystis jirovecii*, *Pseudomonas aeruginosa*, human rhinovirus, respiratory syncytial virus (types A and B), KI and WU polyomaviruses, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*.

Probit regression analysis was then used to determine the comparative sensitivity of individual and duplex PCR assays in relation to the measured limit of detection (3, 4, 8). Twenty replicates of twofold dilutions of target RNA material covering the range of 0% to 100% positive results were analyzed, and RT efficacy was assumed to be 100%. A P value of <0.05 was accepted as significant, and the sensitivity of the assays at a probability of detection of 95% was calculated (StatsDirect statistical package version 2.7.2).

Preliminary evaluation of the clinical performance of assays (Table 2). A collection of 137 seasonal influenza-positive pernasal aspirates or combined nose/throat swabs was utilized to perform a preliminary rapid evaluation of the automated extraction and duplex RT-PCR assays. The previous results were obtained using in-house assays that had been validated according to national standards (*NPACC Standards for Validation of In-House Assays*; http://www.health.gov.au/npaac) and approved by the National Association of Testing Authorities (http://www.nata.asn.au/). These comprised manual RNA extraction techniques (QIAmp viral RNA minikit; Qiagen, Australia) and individual real-time RT-PCR assays. Influenza viruses cultured from samples collected into VTM were independently confirmed by hemagglutination inhibition typing (WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia). Combined nose/throat swabs from the first three WHO-confirmed pandemic (H1N1) 2009 cases in Western Australia and inactivated A/Auckland/1/2009 cell culture material (WHOCC, Melbourne, Australia) were also included in the preliminary evaluation.

Nucleic acids extracted from a stored isolate of influenza A (H2N2) virus (identity confirmed by matrix gene sequencing); from H5N1 (clades 2.1, 2.2, and 2.3.4) and H7N4 viruses supplied by the RCPA Quality Assurance Programs, Australia; and from H9 virus (A/Mallard/NL/1/05) supplied by the QCMD Quality Assurance Program, Scotland, were tested in the duplex RT-PCR assays.

A collection of 64 pernasal aspirates or nasal and throat swabs previously positive for common respiratory pathogens were retested in the automated extraction/influenza duplex PCR system and individual PCR assays to ensure that the previously reported pathogens were still detectable with a different extraction method and following prolonged storage at −20°C and to check for cross-reaction of the duplex assays with any of these organisms. A further 14 samples from patients with symptoms of acute respiratory infection but for which no respiratory pathogen was identified were also included to control for nonspecific amplification in the duplex assays.

Archived DNA and RNA extracts from a range of respiratory pathogens were also checked for nonspecific reactivity in the three duplex influenza PCR assays. These materials, stored at −80°C, were tested at 100-fold above the endpoint titer. Where practical, the material was originally from cell culture or bacterial culture material, but for noncultivable agents, sample extracts were utilized. The identities of the organisms were confirmed by DNA sequencing of PCR products, as described for the pandemic (H1N1) 2009 virus sequencing.

RESULTS

Assay efficiency. The reaction efficiencies for the real-time RT-PCR assays were satisfactory (4), ranging from 83% to 99% for both the individual and duplex assays, with no significant difference between the two types of assays.

Analytical sensitivity. Pandemic (H1N1) 2009 culture material at $10^{5.4}$ $TCID_{50}$/0.1 ml tested in the influenza matrix duplex assay, the pandemic (H1N1) 2009/MS2 duplex assay, and the individual pandemic (H1N1) 2009 real-time RT-PCR assay was detectable at a dilution of $1/10^6$ (95% confidence interval [CI]) in all three assays, equivalent to an LOD of 0.23 $TCID_{50}$.

Multiple replicates of positive-control material of influenza A/H1N1, A/H3N2, pandemic (H1N1) 2009, and influenza B viruses at suitable limiting dilutions tested in the real-time RT-PCR assays produced a Poisson distribution of positive and negative results that reflected the original concentration of target RNA. Probit regression analysis estimated that the analytical sensitivities of the assays for the detection of pandemic (H1N1) 2009 virus, at a 95% confidence level, ranged from 223 to 294 copies/ml (equivalent to 2.3 $TCID_{50}$/ml). Similar analyses for the influenza A/H1 (seasonal), A/H3, and B virus assays estimated analytical sensitivities of 275 to 495 copies/ml, but no comparison to the $TCID_{50}$ was made for these agents. There was no significant difference in sensitivity between the duplex and individual assays (data not shown).

Preliminary evaluation of the clinical performance of assays (Table 2). The expected results were obtained for the 137 known positive samples from seasonal influenza A and B cases, the first three local pandemic (H1N1) 2009 virus samples in Western Australia, and the inactivated pandemic (H1N1) 2009 cell culture material A/Auckland/1/2009. All produced equivalent results with comparable C_T values in the automated extraction/duplex RT-PCR and manual extraction/individual RT-PCR assays, with no evidence of nonspecific cross-reactivity among the different influenza types and subtypes. Hemagglutination inhibition typing results confirmed that the assay had been able to detect influenza virus strains A/Solomon

Islands/3/2006 (H1N1), A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), B/Brisbane/60/2008, B/Florida/4/2006, B/Malaysia/2506/2004, and A/California/7/2009 (H1N1).

Nucleic acid extracts of influenza A virus subtypes H2N2, H5N1 (clades 2.1, 2.2, and 2.3.4), H7N4, and H9 (A/Mallard/NL/1/05) were reactive in the duplex assay directed against the influenza A matrix gene target but showed no reactivity in the other assays, while the 64 samples previously positive for non-influenza respiratory pathogens and a collection of 14 specimens from patients with acute respiratory illness for which no pathogen was identified were all negative in the matrix and HA gene assays. The 64 stored samples known to contain noninfluenza respiratory pathogens were retested to ensure that there was no deterioration of the nucleic acid. Similarly, nucleic acid extracted from a comprehensive range of common respiratory pathogens showed no evidence of false-positive reactivity in the three duplex assays.

Prospective evaluation of the assays. More than 11,000 individual patient samples were tested during the containment and protect phases of the 2009 influenza pandemic in Western Australia (http://www.health.gov.au/internet/healthemergency/publishing.nsf/Content/resources#phases). Seasonal influenza A virus was detected in 614 samples (H1, 88 samples; H3, 526 samples), influenza B was detected in 12 samples, and pandemic (H1N1) 2009 was found in 1,647 samples. Of these, three samples were positive, as determined by the HA gene PCR (one H1 and two H3 samples) but not the influenza A matrix gene by PCR. In addition, there were 20 samples that were positive for the influenza A matrix gene by PCR but could not be subtyped, and these were designated false-negative results for each of the HA gene PCRs for the sensitivity calculations. Therefore, the HA gene PCR assays had minimum sensitivities, compared with the influenza A matrix gene PCR, of 1,647/1,667 (98.8%) for pandemic (H1N1) 2009, 526/546 (96.3%) for H3, and 88/108 (81.5%) for seasonal H1 virus.

When primary samples had been cultured, all culture-positive samples were also appropriately positive in the duplex real-time RT-PCR assays. For the samples that were pandemic (H1N1) 2009 virus PCR positive but not culture confirmed, i.e., culture was either not performed or was negative, all were positive in the influenza A matrix PCR assay and negative in the A/H1- and A/H3-specific PCRs. Furthermore, 42 samples were randomly selected for sequencing, and all were confirmed as pandemic (H1N1) 2009 virus. One of the 88 seasonal H1-PCR positive samples and two of the 526 H3 PCR-positive samples were negative in the influenza A matrix gene PCR. Therefore, the minimum specificity for the pandemic (H1N1) 2009 and seasonal H1- and H3-specific PCRs were 100%, 98.9%, and 99.6%, respectively.

DISCUSSION

The duplex assays were developed in response to the dramatic increase in demand for influenza testing as a result of the emergence of pandemic influenza (H1N1) 2009 in April 2009. Traditional PCR tests to detect influenza A and B viruses and to further subtype the A strains as H1, H3, or pandemic (H1N1) 2009 with the inclusion of a control for the RNA extraction and subsequent PCR process would have required 6 individual PCR assays. The duplex format halved the number of PCR assays required, significantly decreasing the cost of the assay and reducing the burden on thermocycling equipment and staff, while retaining high sensitivity and specificity. The consumable cost for the three duplex assays, including the positive and negative controls included in each batch, was $10 per sample. If necessary, further economies could be made by eliminating the seasonal H1/H3 duplex assay, particularly in the absence of community seasonal influenza activity. Infections with those viruses would still be detected in the influenza A matrix assay, and further H1 and H3 testing could then be completed.

The automated sample extraction instrument extracted nucleic acid from 80 samples and 16 water controls in less than 30 min, and the thermocycling protocol took 2.75 h to complete. Extra time was required for liquid-handling robots to dispense PCR mix, samples, and wash buffers.

Recently, other real-time RT-PCR assays have been evaluated for the detection of pandemic (H1N1) 2009 virus by targeting the HA gene using either specific probes (6) or SYBR green detection (10) or, in a novel approach, amplifying an apparently specific pandemic (H1N1) 2009 sequence in the matrix gene (7). These were evaluated with small numbers of clinical samples and varied in their capacities to identify non-pandemic influenza. Clearly, a number of different assays will be developed that can be adapted to a range of testing platforms and work environments. However, in contrast to the evaluation of other published assays, we have evaluated ours on very large numbers of clinical samples, including many positive samples. Our assays provide primary typing and subtyping and have shown excellent performance and cost efficiency in a high-throughput automated testing protocol.

The data do suggest that our assay had a lower sensitivity for the detection of seasonal A/H1 virus in clinical samples (81.5%) than that for A/H3 (96.3%) and pandemic (H1N1) 2009 (98.8%) viruses. We believe that was because samples that were matrix gene PCR positive only were called false-negative results for all three HA gene assays. These were mainly weak positives (C_T values of >35) in the matrix gene assay and so were presumably below the detection limit for the HA gene PCRs. Since pandemic (H1N1) 2009 was the predominant influenza virus detected with our clinical specimens, it is probable that most of those samples contained the pandemic virus and that the negative results for the A/H1 and A/H3 PCR assays were correct. Therefore, it is likely that our assay has a sensitivity for seasonal A/H1 virus similar to that for the other subtypes, as was suggested by the analytical sensitivity estimates. The lack of seasonal A/H1 activity has meant that we have not been able to assess this further.

Our paper describes an automated sample extraction technique coupled with three duplex real-time RT-PCR assays to facilitate accurate and rapid screening of large numbers of samples for influenza viruses. The technique proved invaluable in the containment phase of the current influenza pandemic, when contact tracing, patient quarantining, and the management of antiviral therapy required rapid turnaround times for the reporting of influenza virus results.

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