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Switching gears for an influenza pandemic: validation of a duplex RT-PCR for simultaneous detection and confirmation of pandemic (H1N1) 2009.

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ABSTRACT

Rapid methods for detection and confirmation of pandemic influenza A [also known as pandemic (H1N1) 2009] are of utmost importance. In this study, a conventional reverse-transcriptase polymerase chain reaction (RT-PCR) was designed, optimized and validated for the detection of influenza A and the hemagglutinin of swine lineage H1 (swH1). Nucleic acids were extracted from 198 consecutive nasopharyngeal, nasal or throat swabs collected early in the outbreak [127 negatives, 66 pandemic (H1N1) 2009, 3 seasonal influenza A (H1N1) and 2 seasonal influenza A (H3N2)]. The performance characteristics of duplex RT-PCR were assessed compared to various detection methods: monoplex RT-PCR at the National Microbiology Laboratory (NML), a real-time RT-PCR using a Center for Disease Control and Prevention (CDC) protocol, a in-house multiplex RT-PCR [targeting influenza A, influenza B and respiratory syncytial virus (RSV)], and a rapid antigen test, the BinaxNOW Influenza A & B. For influenza A detection, the sensitivity of duplex RT-PCR was 97.2%, versus 74.6%, 71.8%, 47.8%, and 12.7% for the other assays, respectively. Duplex RT-PCR was also able to identify swH1 in the 94% of cases, thereby reducing the number of specimens forwarded to reference laboratories for confirmation. Only a limited number of influenza A-positive specimens fell below the limit of detection of the swH1 primers. Overall, duplex RT-PCR is a reliable method for simultaneous detection and confirmation of pandemic (H1N1) 2009 and would be particularly attractive to laboratories without real-time RT-PCR capability.

INTRODUCTION

Pigs and humans have many similarities with respect to influenza virus. Both have well-established, distinct and stable lineages of influenza A virus that cause periodic epidemics

associated with morbidity and mortality (21). Swine influenza A viruses currently circulating in North America are triple reassortants that have components of avian, human and swine origin (19). For pandemic (H1N1) 2009, the polymerase components PB2 and PA are derived from avian influenza lineages, PB1 is of human influenza origin, and the genes encoding hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein (M), and nonstructural protein (NS) are of swine lineages of influenza A (16). Sporadic cases of humans infection with triple reassortant swine influenza viruses had been previously documented; however, until recently human-to-human transmission was not sustained (6, 16, 19). In March 2009, Mexico reported clusters of respiratory disease that was subsequently identified as pandemic (H1N1) 2009. As of August 30, over 250,000 cases and 2837 deaths have been documented worldwide (22). This led the WHO to increase the pandemic alert to phase 6 and declare an international public health emergency.

Rapid diagnosis using molecular methods such as reverse-transcriptase polymerase chain reaction (RT-PCR) are the cornerstone to pandemic planning. As outlined in the Canadian Pandemic Influenza Plan (18), provincial Public Health or designate laboratories should have the capacity to identify and subtype influenza viruses using molecular methods. High sensitivity and specificity compared to conventional detection methods prompted many laboratories to implement RT-PCR for the detection of influenza viruses. With increasing rates of antiviral resistance in circulating seasonal human influenza A strains (H1N1 and H3N2), RT-PCR is now being used for influenza A subtyping in order to facilitate the clinical management of patients (8). Influenza A viruses that can not be subtyped must be forwarded to reference laboratories to rule out a novel influenza strain (3, 18). This was the scenario that played out in our and many other laboratories across North America.

On April 24, the Capital District Health Authority (CDHA) microbiology laboratory in Halifax, Nova Scotia received specimens from five symptomatic individuals with epidemiological links to Mexico (4). Three of five were identified as influenza A, but were non-typeable using primers targeting hemagglutinins H1 (huH1) and H3 (huH3) from seasonal influenza A viruses. These three and an additional case were confirmed as pandemic (H1N1) were confirmed by the NML using RT-PCR and sequencing of the M gene (4). While these methods enabled the detection of the first Canadian cases, more timely methods were necessary to help guide public health management. In fact, following confirmation of this novel influenza A virus in Nova Scotia, there was a dramatic increase in the number of respiratory specimens submitted for influenza RT-PCR. Initial strategies based on screening for influenza A followed by subtyping extended turn around times and put tremendous stress on both human resources and available reagents. As such, our traditional testing algorithm had to be quickly revised to accommodate this surge (Figure 1). With primers designed by the NML targeting the HA from H1-lineages of swine influenza A (swH1) and a second primer pair targeting influenza A (5), we validated a duplex RT-PCR for simultaneous detection and confirmation of pandemic (H1N1) 2009.

MATERIALS AND METHODS

Specimen collection

Nasal, nasopharangeal or throat swabs were collected from individuals during the recent outbreak in Nova Scotia (4). Swabs were placed in universal transport media (Copan Diagnostics, Corona, CA) and maintained at 4°C until testing or aliquoted and stored at -80°C for long-term storage. Duplex RT-PCR validation was performed using 198 consecutive

specimens collected between April 26 and April 28. An additional 50 positive and 50 negative specimens collected between April 23 and May 8 were used as part of the retrospective analysis, excluding specimens processed during the validation period. Analytical specificity was evaluated using a panel of archived viruses (Table 1). These included various human, avian, and swine influenza A viruses, influenza B virus, parainfluenza viruses (PIV)-1, -2, and -3, respiratory syncytial virus (RSV), herpes simplex virus (HSV) types 1 and 2, cytomegalovirus (CMV), enterovirus, mumps virus (genotype G), and adenovirus.

Influenza A rapid antigen testing

Rapid antigen testing was performed using a lateral flow assay, the Binax NOW Influenza A & B kit (Inverness Medical, Ottawa, ON). One hundred microlitres was processed in accordance with the manufacturer's instructions with visual inspection after 15 min.

Nucleic acid extraction

For monoplex influenza A or swH1 RT-PCR (performed at the NML), viral RNA was extracted from 265 µl of specimen with a BioRobot MDx Viral Kit (Qiagen Inc., Mississauga, ON) on the BioRobot MDx (Qiagen Inc., Mississauga, ON) and RNA was eluted in a final volume of 100 µl. All other nucleic acid extractions were performed (at the CDHA microbiology laboratory) using a MagNA Pure LC instrument (Roche Diagnostics, Branchburg, NJ) and a Total Nucleic Acid Isolation kit (Roche Diagnostics, Branchburg, NJ). One hundred and forty microlitres of specimen was extracted as recommended by the manufacturers' instructions and nucleic acids were eluted in a final volume of 60 µl. Five microlitres served as template in all RT-PCR reactions.

Conventional RT-PCR

Conventional RT-PCR assays (monoplex, duplex, triplex and HA subtyping) were performed using a Qiagen One-Step RT-PCR kit (Qiagen Inc., Mississauga, ON). Oligonucleotides (Table 2) were synthesized by Sigma Genosys (Oakville, ON) with the exception of the swH1 primer pair that was synthesized by the NML. RT-PCR amplifications were performed in 48- or 96-well plates on a DNA engine dyad thermocycler (Bio-Rad Laboratories Ltd., Mississauga, ON). Amplicons were resolved using 1% agarose gel electrophoresis with ethidium bromide staining.

Monoplex RT-PCR targeting influenza A or swH1

Viral RNA was amplified in a one-step RT-PCR reaction (Qiagen Inc., Mississauga, ON) following the manufacturer's recommendations. Briefly, 5 µl of RNA was added to 50 µl RT-PCR reactions containing: 2 µl One-Step RT-PCR enzyme mix, 1× One-Step RT-PCR buffer, 10 µl of Q-solution; 400 µM dNTPs (dATP, dCTP, dGTP, and dTTP), 0.6 µM of each primer. Primers for influenza A, FluA-M52C and FluA-M253R, have been previously described (5). Primers targeting the HA of swine lineage H1 (swH1), SwFluAH1F and SwFluAH1R, were developed by the NML based on HA sequence data from influenza A/California/04/2009 (H1N1)v (GenBank accession number FJ966082) obtained from the Global Initiative on Sharing Avian Influenza Data (GISAID). Thermocycling conditions were performed as recommended by the NML: reverse transcription at 50°C for 30 min; activation of the HotStart DNA polymerase at 95°C for 15 min; then 40 cycles of denaturation at 94°C for 30s, annealing at 59°C (influenza A) or 50°C (swH1) for 30s, and extension at 72°C for 1 min; followed by a final extension of 10 min at 72°C. Expected product sizes were 244 bp for influenza A and 517 bp for swH1.

Influenza A and swH1 duplex RT-PCR

For influenza A and swH1 duplex RT-PCR, 50 µl reactions contained: 5µl of template; 2 µl of One-Step RT-PCR enzyme mix; 1× One-Step RT-PCR buffer; 10 µl of Q-solution; 1 mM dNTPs; 5 units (U) of RNaseOUT recombinant ribonuclease inhibitor (Invitrogen Canada Inc., Burlington ON); and 1 µM of each primer pair: FluA-M52C and FluA-M253R for influenza A (5) and SwFluAH1F and SwFluAH1R for swH1. Thermocycling conditions were as follows: reverse transcription at 50°C for 30 min; activation of the HotStart DNA polymerase at 95°C for 15 min; then 40 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 1 min; followed by a final extension of 10 min at 72°C. During optimization, annealing temperatures of 50°C and 60°C were also evaluated. For optimization of the duplex assay, a gradient RT-PCR was performed on a DNA engine dyad thermocycler (Bio-Rad Laboratories Ltd., Mississauga, ON) using annealing temperatures ranging from 50 to 60°C.

For nested PCR reactions, 50 µl reactions contained 2 µl of amplicon, 1× buffer, 1.5 mM $MgCl_2$, 1 mM dNTPs, 2.5U of Taq DNA polymerase (Invitrogen Canada Inc., Burlington ON), and 1 µM of each primer. Thermocycling conditions were as follows: initial activation at 95°C for 5 min; then 25 cycles of denaturation at 95°C for 30s, annealing at 50 or 55°C for 30s, and extension 72°C for 60s; and a final elongation at 72°C for 7 min.

Influenza A, influenza B and RSV triplex RT-PCR

For influenza A, influenza B and RSV triplex RT-PCR (1), 50 µl reactions contained 5µl of template, 2 µl of One-Step RT-PCR enzyme mix; 1× One-Step RT-PCR buffer, 1 mM dNTPs, 20U RNaseOUT (Invitrogen Canada Inc., Burlington ON), and 1 µM of each primer pair: FluA-M52C and FluA-M253R for influenza A (5), FluB-B/MP and FluB/MP-1R for influenza B (13),

and HRSVMPFOR1 and HRSVMPRW2 for RSV (14). Thermocycling was performed as follows: reverse transcription at 50°C for 30 min; initial activation at 95°C for 15 min; then 40 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s and extension 72°C for 60s; and a final elongation at 72°C for 7 min. Expected amplicon sizes were 244 bp, 380 bp, and 525 bp for influenza A, influenza B, and RSV, respectively.

Human influenza A subtyping

For huH1 and huH3 subtyping, 50 µl reactions contained 5µl of template, 2 µl of One-Step RT-PCR enzyme mix, 1× One-Step RT-PCR buffer, 1 mM dNTPs, 20U RNaseOUT, and 1 µM of each primer pair (Table 2): HA1-230-F and HA1-757-R for huH1 and H3ha100f and H3ha415r for huH3. Thermocycling was performed as follows: reverse transcription at 50°C for 30 min; initial activation at 95°C for 15 min; 40 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s and extension 72°C for 60s; and a final elongation at 72°C for 7 min. Expected amplicon sizes were 611 bp and 976 bp, respectively. An alternative primer pair was also used (H1-1 and H1-2 for huH1 and H3-1 and H3-2 for huH3) when subtyping failed by the conventional assay (25). Expected product sizes were 529 bp and 316 bp, respectively.

Real-time RT-PCR

Detection of influenza A or swH1 by real-time RT-PCR was performed independently on a LightCycler 2.0 (Roche Diagnostics, Branchburg, NJ) using thermocycling and reaction conditions described by the CDC (2). One-step RT-PCR was performed using a Qiagen QuantiTect Multiplex NoROX RT-PCR kit (Qiagen Inc., Mississauga, ON) in 20 µl reactions consisting of: 1× Master Mix; 0.2 µl QuantiTect Multiplex NR enzyme mix; 20U RNaseOUT,

400 nM of primers (InfA Forward and InfA Reverse for influenza A; SW H1 Forward and SW H1 Reverse for swH1), and 200 nM of FluA probe (InfA Probe for influenza A and SW H1 Probe for swH1) (Table 2).

Viral copy number was estimated in relation to a standard curve generated using linearized plasmid harboring the M gene of influenza A/Wisconsin/67/2005. Briefly, amplicon generated from PCR amplication using primer pair FWISM and FWISM was subcloned into the XhoI and BamHI restriction sites of similarily digested pBlueScript II KS- (Stratagene, La Jolla, CA). Following electroporation into *Escherichia coli* XL10 Gold (Stratagene, La Jolla, CA), ampicillin-resistant transformants were confirmed by PCR. Plasmid was extracted using a QIAprep Spin miniprep kit (Qiagen Inc., Mississauga, ON), digested with BamHI and subjected to agarose gel electrophoresis. Following purification using a QIAquick gel extraction kit (Qiagen Inc., Mississauga, ON), linearized plasmid was quantified by spectrophotometry. Ten-fold serial dilutions of linearized plasmid were used as template for real-time RT-PCR. An inverse linear relationship ($y = -3.10\times + 38.95$; $R^2 = 0.9979$) generated by plotting crossing points (Cp) values against plasmid concentration.

DNA sequencing

Matrix gene amplicon from influenza A-positive RT-PCR reactions in the duplex RT-PCR were purified using QIAquick PCR purification kit (Qiagen Inc., Mississauga, ON) and subjected to sequence analysis using primers FluA-M52C and FluA-M253R (5). To ensure discrepant results were not due to amplicon contamination, additional positive detected by duplex RT-PCR were subjected to a second RT-PCR and sequencing reaction using primer FluA-M52C and a second primer (swH1M351R) located downstream of the original targeted

region (Table 2). Sequencing was conducted on an ABI 3100 sequencer (Applied BioSystems, Streetsville, ON) at the DNA Core Facility at the NML or using BigDye Terminator chemistry on an ABI 3130xL DNA Sequencer (Applied BioSystems, Streetsville, ON) at York University (Toronto, ON). Sequence analysis was performed using Lasergene 7.1 Sequence Analysis Software (DNAStar, Madison, WI) and consensus sequences (from forward and reverse sequencing reactions) were compared to reference data available in the GenBank database by using BLAST analysis.

Statistics

Since the optimal RT-PCR assay for pandemic (H1N1) 2009 is unknown, a modified gold standard was used to assess the clinical performance of all RT-PCR assays where a positive case was defined by concordant results between at least two RT-PCR reactions targeting different genomic regions and subsequent sequence analysis to ensure specificity of the primers. The performance of each method was compared to this modified gold standard to determine sensitivity (Sn), specificity (Sp), positive predictive values (PPV), and negative predictive values (NPV). 95% confidence intervals were calculated for each value. Chi-square and two-tailed Fisher's exact tests were used and a probability (*P*) value of <0.05 was considered statistically significant.

RESULTS

Monoplex versus duplex RT-PCR

Ten-fold serial dilutions of pandemic (H1N1) 2009 RNA was performed to compare the analytical sensitivity between monoplex (influenza A or swH1) and duplex (influenza A and

swH1) RT-PCR reactions. In three independent experiments, both assays demonstrated similar limits of detection (LOD). For influenza A, the LOD was estimated at 2 to 20 copies per reaction (10^{-6} or 10^{-7} dilution) whereas for swH1, the LOD was approximately 20 to 200 copies per reaction (10^{-5} and 10^{-6} dilutions) (Figure 2). The increased sensitivity of influenza A versus swH1 suggests that confirmation would be required with specimens containing low viral loads (Figure 1B). No additional benefit was afforded by nested PCR (except increased amplicon quantity). On the other hand, gradient RT-PCR which varied the annealing temperature to values spanning 50°C to 60°C (Figure 3) demonstrated that at 50°C, amplification of swH1 may not be optimal. When 10-fold serial dilutions pandemic (H1N1) 2009 RNA was performed using annealing temperatures of 50°C, 55°C, and 60°C, detection of swH1 was optimal at 55°C (Figure 3). At this temperature, the detection of swH1 was approximately 10-fold more sensitive than the recommended 50°C. Whatever the annealing temperature used, no differences were observed for influenza A.

Analytical specificity

Identical primers targeting a highly conserved region of the influenza A matrix gene are present in monoplex and duplex RT-PCR assays (5). While specificity of the influenza A primers has been well documented (1, 5), influence of the swH1-specific primers was unknown. The specificity of duplex RT-PCR was assessed using a panel of archived viruses (Table 1). Influenza A amplicon was observed in all influenza A strains, whereas swH1 amplicon was only observed with pandemic (H1N1) 2009 viral isolates or A/Swine/Minnesota/3068ZT/98 (H1N1). No amplification was observed using nucleic acids extracted from others viruses: RSV, HSV-1, HSV-2, CMV, PIV-1, PIV-2, PIV -3, enterovirus, mumps virus, adenovirus and influenza B.

Analytical sensitivity

The analytical sensitivity of duplex, triplex, and real-time RT-PCR assays were evaluated using nucleic acids extracted from 10-fold dilutions of a pandemic (H1N1) 2009 influenza A isolate, A/Canada-NS/RV1535/2009(H1N1)v (Figure 4). As seen in Figure 2, the LOD of influenza A in the duplex assay was approximately two copies per reaction (10^{-5} dilution) whereas the LOD for swH1 was approximately 20 to 200 copies per reaction (10^{-3} or 10^{-4} dilution) (Figure 4A). The sensitivity of real-time RT-PCR for influenza A was approximately two copies per reaction (10^{-5} dilution, Figure 4C); however, at this concentration, a positive signal was only obtained in 2 of 5 reactions. All five reactions were detected by duplex RT-PCR, suggesting real-time RT-PCR may be less sensitive. As for triplex RT-PCR, a lower LOD was observed at approximately 200 copies per reaction (10^{-3} dilution) (Figure 4B). The lack of sensitivity of triplex RT-PCR was not unique to pandemic (H1N1) 2009. Using nucleic acids extracted from 10-fold serial dilutions of seasonal influenza A viruses (H1N1 and H3N2), triplex RT-PCR was 10- to 100-fold less sensitive than duplex RT-PCR (data not shown).

Clinical performance

RT-PCR assays and rapid antigen testing were assessed using 198 consecutive specimens collected early in the Nova Scotia outbreak. Of 71 sequence-confirmed influenza A viruses, 66 were identified as pandemic (H1N1) 2009, three as seasonal H1N1 and two as seasonal H3N2. The sensitivity of duplex RT-PCR for influenza A detection was 97.2% (69/71) whereas all other assays were significantly ($P<0.001$) less sensitive: 74.6% (53/71) for influenza A monoplex RT-PCR; 71.8% (51/71) for real-time RT-PCR; 47.8% (34/71) for triplex RT-PCR; and 12.7%

(9/71) for rapid antigen testing (Table 3). With a prevalence of 36% (71/198), the negative predictive values were 98.4% for duplex RT-PCR, 87.6% for influenza A monoplex RT-PCR, 86.4% for real-time RT-PCR, 77.4% for triplex RT-PCR and 67.2% for rapid antigen testing (Table 3). No false positives were observed, giving all assays positive predictive values of 100%.

Of 71 influenza A viruses identified in this study, 66 were confirmed as pandemic (H1N1) 2009. Of the RT-PCR assays able to identify swH1 (monoplex, duplex and real-time RT-PCR), sensitivities differed (Table 3). Duplex RT-PCR identified swH1 in the most cases, with a sensitivity of 94% (62/66). However, this high sensitivity was only observed if the annealing temperature was set at 55°C. Using an annealing temperature of 50°C, the sensitivity for swH1 detection fell to 77% (51/66). Interestingly, a similar sensitivity [80% (51/66)] was observed using swH1 monoplex RT-PCR, which is performed using an annealing temperature of 50°C. This data, along with Figure 3, suggests that the duplex RT-PCR should be performed using an annealing temperature of 55°C.

As described in the Material and Methods, a positive pandemic (H1N1) 2009 case was defined by concordant results between at least two RT-PCR reactions targeting different genomic regions and subsequent sequence analysis to ensure specificity of the primers. However, duplex RT-PCR identified 18 additional influenza A that were considered negative by real-time influenza A RT-PCR. To ensure the additional influenza A cases detected by duplex RT-PCR were not attributed to amplicon contamination, several strategies were undertaken. First, re-extraction and repeat duplex RT-PCR reaction generated identical results with two exceptions. The influenza A monoplex RT-PCR previously had identified two cases of pandemic (H1N1) 2009 that were considered negative by all other assays, including duplex RT-PCR; however, upon repeat of the duplex RT-PCR, these two cases were influenza A positive (but swH1

negative). Secondly, the real-time swH1 RT-PCR confirmed 91% (60/66) of the pandemic (H1N1) cases (Table 3). All cases that were positive by real-time RT-PCR were also positive by duplex RT-PCR. Of note, 10 of the 60 swH1 positive specimens detected by real-time RT-PCR had Cp values between 35 and 40, suggesting these specimens had a low viral load. Finally, a second conventional RT-PCR was used which incorporated both a higher concentration of template RNA (obtained using larger amount of specimen eluted in the same volume) and primers designed outside the region targeted by the duplex RT-PCR. Amplicon was present in all discrepant results analyzed, including those confirmed by real-time swH1 RT-PCR. These amplicon were purified and subjected to sequencing of the M gene which revealed 15 pandemic (H1N1) 2009 and three seasonal influenza A viruses (two H1N1 and one H3N2).

Retrospective analysis

To ensure adequate performance of duplex RT-PCR following its implementation, 50 negative and 50 positive influenza A specimens were subjected to both duplex and triplex RT-PCR. Of 50 positive specimens, 29 were detected as influenza A by both duplex and triplex RT-PCR. Using sequencing and huH1 and huH3 subtyping, these isolates were identified as: 16 pandemic (H1N1) 2009; 7 seasonal H1N1; and 6 seasonal H3N2 isolates. All 16 pandemic (H1N1) 2009 viruses were swH1-positive by duplex RT-PCR. Secondly, 21 pandemic (H1N1) 2009 viruses were only detected by duplex RT-PCR, suggesting triplex RT-PCR would have missed 42% of cases. While 18 of these 21 cases were detected as swH1 positive by the duplex RT-PCR, the remaining three influenza A-positive specimens could not be resolved by subtyping (swH1, huH1 or huH3) or nested PCR. Using sequence analysis following RT-PCR with

primers designed outside the original targeted region and real-time swH1 RT-PCR, these three were confirmed as pandemic (H1N1) 2009.

Of interest, the original five nasal specimens submitted to the CDHA microbiology laboratory (4) were evaluated within the retrospective data. Three were identified as influenza A using triplex RT-PCR, four by monoplex RT-PCR, and all five were identified using duplex RT-PCR. In addition, two of five were swH1-positive by duplex RT-PCR. The additional cases diagnosed by monoplex and duplex RT-PCR assays required sequence analysis suggesting these specimens contained low viral loads. All five were confirmed as pandemic (H1N1) 2009 by sequence analysis.

DISCUSSION

Early detection of infected patients, implementation of isolation measures, and contact tracing is imperative for the management of influenza virus. Rapid antigen tests can generate a result in 30 minutes or less (18, 22), unfortunately, these methods lack sensitivity (Table 3) compared to RT-PCR (7, 8, 10, 23) and should not be used to exclude influenza infection. Early in the outbreak, the only available guidelines suggested novel influenza viruses should be confirmed using viral culture and at least partial sequencing of the viral genome (3, 23). While sequencing is considered the gold standard for confirmation of novel influenza viruses, this approach is impractical for most laboratories and poses problems for the routine detection of influenza by RT-PCR (discussed later). Using sequence data from pandemic (H1N1) 2009, we designed primers targeting swH1 and validated a conventional RT-PCR reaction that is capable of simultaneously detection and confirming this novel influenza A virus.

Duplex RT-PCR was significantly more sensitive than all other assays for the detection of influenza A (Table 3), including the conventional triplex RT-PCR previously used in our laboratory (Figure 4). This prompted a rapid modification of our testing algorithm during the Nova Scotia outbreak (Figure 1). Interestingly, duplex RT-PCR was also more sensitive than a real-time influenza A RT-PCR designed by the CDC (2). The lower sensitivity of real-time RT-PCR could be partly attributed to the protocol which had not yet been optimized for the LightCycler platform. However, Poon et al. (17) recently found that a real-time RT-PCR assay also was less sensitive than a conventional RT-PCR in the detection of A/Swine/Hong Kong/PHK1578/03. It remains to be determined whether sensitivity of real-time influenza A RT-PCR could be enhanced using other platforms or a modified protocol.

Several conclusions could be derived by comparing monoplex and duplex RT-PCR. Despite identical primers being used in both assays, duplex RT-PCR was significantly ($P<0.001$) more sensitive (Figure 2 and Table 3). Several possibilities could explain these discrepant results. First, the two assays differed in respect to the extraction methods, RT-PCR reaction conditions and thermocycling conditions (annealing temperatures). For example, the higher annealing temperature in duplex RT-PCR (55°C) compared to the swH1 monoplex RT-PCR (50°C) contributed to increased sensitivity (Figure 3 and Table 3). Some of the discrepant results may simply reflect a Poisson distribution due to sampling error with low concentrations of template (11, 20). The phenomenon is almost impossible to control, and is most pronounced at low target concentrations where a small changes in nucleic acid template in a PCR reaction could generate a relatively large difference in the number of amplicon produced. A large number of replicates would be necessary to overcome this limitation. Similarly, PCR inhibitors are known to affect PCR amplification and could lead to considerable variations in the PCR efficiency. This

hypothesis is highly plausible since monoplex and duplex RT-PCR assays were performed using different extraction methods; however, the lack of an exogenous internal control prevents assessment of the contribution of PCR inhibitors. Further studies are being undertaken to combine the duplex RT-PCR to detection of an internal control such as MS2 bacteriophage.

In addition to identifying influenza A, duplex RT-PCR was highly sensitive for identifying swH1 (Table 3). During the validation period and retrospective analysis, 94% (62/66) and 92% (34/37) of cases of pandemic (H1N1) were identified as swH1-positive by duplex RT-PCR. It should be noted that the annealing temperature of the duplex RT-PCR greatly influenced the ability to detect swH1 (Figure 3 and Table 3). Using an annealing temperature of 55°C compared to 50°C increased the number of swH1 targets detected (Table 3). Using this strategy, duplex RT-PCR identified swH1 in the majority of cases of pandemic (H1N1) 2009, thereby considerably reducing the number of specimens forwarded to reference laboratories for confirmation. However, we recognize there will be circumstances where influenza A-positive specimens will fall below the limit of detection of the swH1 primer pair. For the few cases that fit this criterion, low viral loads were observed by real-time RT-PCR. Since the sensitivity of RT-PCR assays targeting influenza A are not equivalent (Table 3), confirmation of pandemic (H1N1) 2009 could be problematic if specimens containing low viral loads are forwarded to reference laboratories using a less sensitive RT-PCR. Sequencing has been proposed by some to confirm the detection of pandemic (H1N1) 2009 in clinical specimens; however, this methodology can be problematic. Sequencing reactions use amplicon generated from RT-PCR as template; therefore, amplicon contamination could result in false positives sequence data. Recognizing that sequencing will always play an important role in the

confirmation of novel influenza viruses, other methods of confirmation should be sought for confirmation of low positive RT-PCR results (discussed below).

Since the optimal RT-PCR assay for pandemic (H1N1) 2009 is unknown, a modified gold standard was used in this study to assess the clinical performance of all RT-PCR assays. A positive case was defined by concordant results between at least two RT-PCR reactions targeting different genomic regions and subsequent sequence analysis to ensure specificity of the primers. As some discordant results were observed between the various RT-PCR assays, other experiments were required to ensure the additional influenza A cases detected were not attributed to amplicon contamination. Specimens displaying discordant results were subjected to: re-extraction and repeat RT-PCR using the same duplex assay; a real-time swH1 RT-PCR; and re-extraction using a larger volume of specimen (1 ml versus 140 μl) and a second RT-PCR using primers designed outside the original targeted region followed by sequencing of the M gene. Using these strategies, all discordant results could be resolved. In light of all results, duplex RT-PCR was deemed to be highly sensitive for the detection of influenza A and confirmation of pandemic (H1N1) 2009.

Until now, most infections attributed to pandemic (H1N1) 2009 have been mild and self-limited. There is growing concern that this virus will evolve and lead to subsequent outbreaks of severe disease. Rapid detection of this novel influenza virus is paramount for implementation of control measures. We have shown that duplex RT-PCR is a highly sensitive, accurate, and reliable method for detection and confirmation of pandemic (H1N1) 2009. While real-time RT-PCR could permit a more timely diagnosis, not all laboratories have the infrastructure to offer such testing. Duplex RT-PCR is undoubtedly an attractive option for laboratories without this capability. Since the fate of this novel influenza virus unclear, a conventional RT-PCR using

generic reagents (without probes or specialized kits) is ideal for any laboratory transitioning from low- to high-throughput screening of pandemic (H1N1) 2009. Reagents used here are of a more generic nature, supplies may be more readily accessible as global demand for testing peaks.

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FIGURE LEGENDS

FIG. 1. Testing algorithms for detection of influenza A viruses. A) Prior to pandemic (H1N1) 2009, routine testing for influenza A (Flu A), influenza B (Flu B) and respiratory syncytial virus (RSV) was performed in Nova Scotia. Followed detection of influenza A, hemagglutinin subtyping (huH1 and hu H3) was performed. Specimens that could not be subtyped were forwarded to the NML for sequence analysis. B) Following confirmed cases of pandemic (H1N1) 2009, testing strategies focused on screening for influenza A and swH1. Influenza A specimens that were weakly positive or failed subtyping were referred to the NML.

FIG. 2. Monoplex and duplex RT-PCR have equivalent limits of detection. Ten-fold serial dilutions of pandemic (H1N1) 2009 RNA were subjected to monoplex (influenza A or swH1) or duplex (influenza A and swH1) reactions. Lane 1: 100 bp ladder; lanes 2-10: dilutions ranging from 10^{0}-10^{-8}; lane 11: reagent control.

FIG. 3. Optimization of the duplex RT-PCR. A) Using RNA concentrations of 10-fold less than the limit of detect of swH1, a gradient RT-PCR was performed using annealing temperature spanning 50°C to 60°C. An estimation of achieved temperatures is as follows: 50.0°C (lane 2); 50.3°C (lane 3); 50.9°C (lane 4); 51.7°C (lane 5); 52.8°C (lane 6); 54.3°C (lane 7); 56.0°C (lane 8); 57.4°C (lane 9); 58.5°C (lane 10); 59.3°C (lane 11); 59.8°C (lane 12); 60.0°C (lane 13). A 100 bp ladder are found in lanes 1 and 14. B) Duplex RT-PCR was performed using annealing temperatures of 50°C, 55°C and 60°C.

FIG. 4. End-point analysis of three RT-PCR assays targeting influenza A. Nucleic acids extracted form 10-fold serial dilutions of pandemic (H1N1) 2009 were subjected to: A) influenza A and swH1 duplex RT-PCR; B) influenza A, influenza B, and RSV triplex RT-PCR; and C) real-time RT-PCR targeting influenza A. Lane 1: 100 bp ladder; lanes 2-7: dilutions ranging from 10^{0} to 10^{-5}; lane 8: reagent control.

TABLE 1. Virus isolates used for the specificity panel.

Virus	Description	Duplex RT-PCR Result	
		Influenza A	SwH1
Influenza A	A/Canada-NS/RV 1535/2009 (H1N1)v	+	+
	A/Canada-NS/RV 1536/2009 (H1N1)v	+	+
	A/Canada-NS/RV 1538/2009 (H1N1)v	+	+
	A/Swine/Minnesota/3068ZT/98 (H1N1)	+	+
	A/Solomon Islands/03/06 (H1N1)	+	-

	A/New Caledonia/20/99 (H1N1)	+	-
	A/Wisconsin/67/05 (H3N2)	+	-
	A/Swine/Texas/4199-2/98 (H3N2)	+	-
	A/Duck/Czech/56 (H4N6)	+	-
	A/Turkey/Wisconsin/68 (H5N9)	+	-
	A/Turkey/Massachusetts/3740/65 (H6N2)	+	-
	A/Shearwater/Australia/72 (H6N5)	+	-
	A/Turkey/Oregon/71 (H7N3)	+	-
	A/Turkey/Ontario/3778/68 (H8N4)	+	-
	A/Turkey/Wisconsin/1/66 (H9N2)	+	-
	A/Quail/Italy/1117/65 (H10N8)	+	-
	A/Duck/England/56 (H11N6)	+	-
	A/Duck/Wisconsin/480/79 (H12N6)	+	-
	A/Gull/Maryland/704/77 (H13N6)	+	-
Influenza B	B/Malaysia/2506/04	-	-
	B/Florida/07/07	-	-
RSV	Clinical isolate	-	-
PIV-1	Clinical isolate	-	-
PIV-2	Clinical isolate	-	-
PIV-3	Clinical isolate	-	-
Enterovirus	Clinical isolate	-	-
Mumps virus	Clinical isolate	-	-
Adenovirus	Clinical isolate	-	-

	HSV-1	Clinical isolate	-	-
	HSV-2	Clinical isolate	-	-
	CMV	Clinical isolate	-	-

1 TABLE 2. Oligonucleotides and probes used in this study

Virus	Location	Name	Sequence (5'-3')	References
Influenza A (M gene)	17-37[a]	FluA-M52C	CTTCTAACCGAGGTCGAAACG	5
	238-261[a]	FluA-M253R	AGGGCATTTTGGACAAAKCGTCTA	5
	331-351[a]	swH1M351R	TCCTTGGCCCCATGGAAYGTT	This study
	151-177[a]	InfA Forward[f]	GACCRATCCTGTCACCTCTGAC	2
	211-234[a]	InfA Reverse[f]	AGGGCATTYTGGACAAAKCGTCTA	2
	238-261[a]	InfA Probe[f]	TGCAGTCCTCGCTCACTGGGCACG	2
	11-31[a]	FWISM	GGCGGATCCATGAGCCTTCTAACCGAGGTC	This study
	972-992[a]	RWISM	GGCCTCGAGTTACTCCAACTCTATGCTGAC	This study
Influenza A (HA gene)	107-127[b]	SwH1F	CAGACACTGTAGACACAGTAC	This study
	602-623[b]	SwH1R	CTAGTAGATGGATGGTGAATGC	This study
	920-942[b]	SW H1 Forward[f]	GTGCTATAAACACCAGCCTYCCA	2
	1010-1035[b]	SW H1 Reverse[f]	CGGGATATTCCTTAATCCTGTRGC	2
	946-975[b]	SW H1 Probe[f]	CAGAATATACATCCRGTCACAATTGGARAA	2
	63-86[b]	H1-1	GATGCAGACACAATATGTATAGG	25
	635-658[b]	H1-2	CICTACAGAGACATAAGCATTT	25
	248-269[b]	HA1-230-F	GGATCTTAGGAAACCCAGAATG	This study
	756-775[b]	HA1 757-R	GTTCCAGCAGAGTCCACTAG	This study
	100-120[c]	H3ha100f	CATGCAGTACCAAACGGAACG	This study
	394-415[c]	H3ha415r	CATTGTTAAACTCCAGTGTGCC	This study
	144-165[c]	H3-1	TCAGATTGAAGTGACTAATGCT	25
	1100-1120[c]	H3-2	AATTTTGATGCCTGAAACCGT	25

Influenza B (M gene)	90-109[d]	FluB-B/MP	TTACACTGTTGGTTCGGTGG	13
	594-613[d]	FluB/MP-1R	GGCAGTTTTTGGACGTCTTC	13
RSV (F gene)	1111-1133[e]	HRSVMPFO2	AACAGTTTAACATTACCAAGTGA	14
	1468-1490[e]	HRSVMPRW2	TCATTGACTTGAGATATTGATGC	14

[a-e]Oligonucleotides are numbered as aligned to GenBank accession numbers: FJ998210 for sequences encoding the matrix protein[a] and FJ998207 for the hemagglutinin H1[b] of influenza A/Canada-NS/RV1535/2009 (H1N1); EU399751 for the hemagglutinin H3[c] of influenza A/Ontario/1252/2007 (H3N2); CY018486 for the M gene[d] of influenza B/Canada/1688/2000; and AF013254 for the fusion gene[e] human RSV.

[f]Primers and probes sequences as well as the protocol for the real-time RT-PCR were provided by the CDC (2). The Taqman probe was labeled at the 5'end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3'end with the quencher, BlackHole Quencher 1 (BHQ1) (BioSearch Technologies, Inc., Novato, CA).

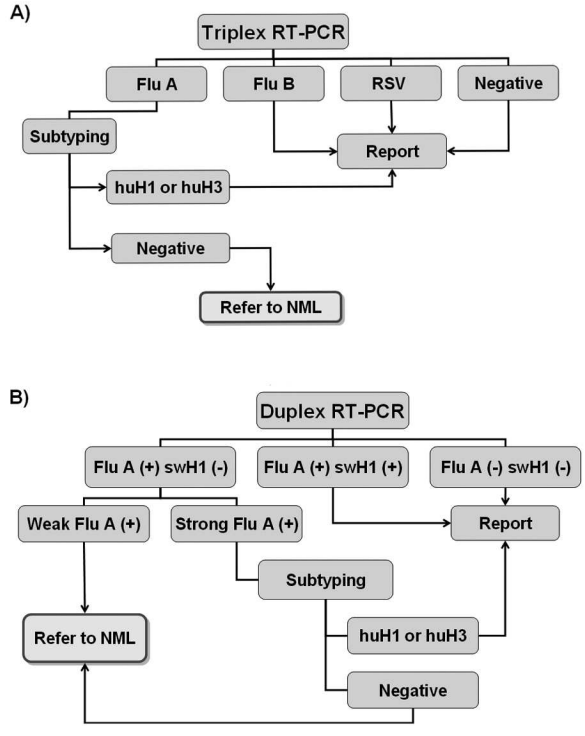
TABLE 3. Performance characteristics of the various assays.

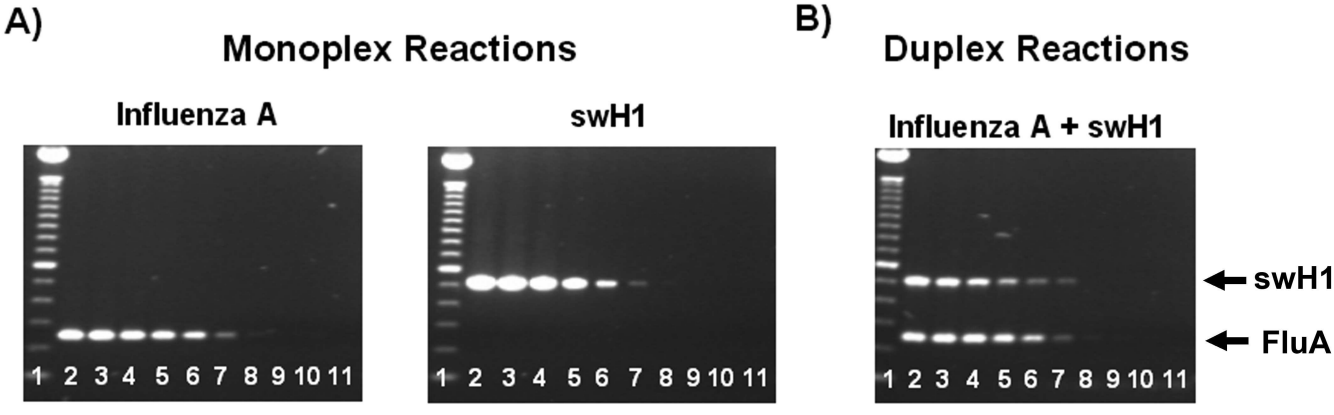
	Influenza A				swH1			
	Sn[a] % (CI)	Sp[a] % (CI)	PPV[a] % (CI)	NPV[a] % (CI)	Sn[a] % (CI)	Sp[a] % (CI)	PPV[a] % (CI)	NPV[a] % (CI)
Duplex RT-PCR (55°C)[b]	97.2 (93.8-97.2)	100 (98.1-100)	100 (96.5-100)	98.4 (93.1-98.4)	93.9 (90.0-93.9)	100 (98.0-100)	100 (95.8-100)	97.1 (95.1-97.1)
Duplex RT-PCR (50°C)[b]	97.2 (93.8-97.2)	100 (98.1-100)	100 (96.5-100)	98.4 (93.1-98.4)	77.3 (72.6-77.3)	100 (97.7-100)	100 (94.0-100)	89.8 (87.7-89.8)
Monoplex RT-PCR[c]	74.6 (70.3-74.6)	100 (97.6-100)	100 (94.1-100)	87.6 (85.4-87.6)	80.3 (75.7-80.3)	100 (97.7-100)	100 (94.3-100)	91.0 (89.0-91.0)
Real-time RT-PCR[c]	71.8 (67.4-71.8)	100 (97.5-100)	100 (93.9-100)	86.4 (84.3-86.4)	90.9 (86.7-90.9)	100 (97.9-100)	100 (95.4-100)	95.7 (93.6-100)
Triplex RT-PCR	47.9 (43.4-47.9)	100 (97.5-100)	100 (90.6-100)	77.4 (75.5-77.4)	-	-	-	-
Rapid antigen testing	12.7 (9.0-12.7)	100 (97.9-100)	100 (71.0-100)	67.2 (65.8-67.2)	-	-	-	-

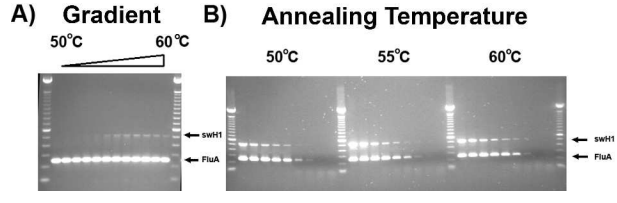
[a]Sensitivity (Sn), specificity (Sp), positive predictive values (PPV), and negative predictive values (NPV) were calculated compared to a modified gold standard (see Material and Methods) with 95% confidence intervals (CI) indicated in parentheses. Results for influenza A are based on 71 confirmed cases [66 pandemic (H1N1) 2009, 3 seasonal influenza A H1N1 and 2 seasonal influenza H3N2]. Results for swH1 were calculated based on the 66 confirmed cases of pandemic (H1N1) 2009. A probability value (P)<0.05 considered statistically significant.

[b]The duplex RT-PCR was evaluated using annealing temperatures of 50°C and 55°C.

[c]For monoplex and real-time RT-PCR reactions, detection of influenza A and swH1 were performed in independent reactions.

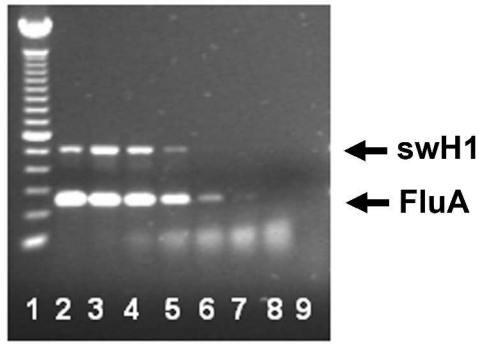

A)
Triplex RT-PCR
Flu A
Flu B
RSV
Negative
Subtyping
Report
huH1 or huH3
Negative
Refer to NML
B)
Duplex RT-PCR
Flu A (+) swH1 (-)
Flu A (+) swH1 (+)
Flu A (-) swH1 (-)
Weak Flu A (+)
Strong Flu A (+)
Report
Subtyping
Refer to NML
huH1 or huH3
Negative


A)
Monoplex Reactions
Influenza A
swH1
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
B)
Duplex Reactions
Influenza A + swH1
swH1
FluA
1 2 3 4 5 6 7 8 9 10 11


A) Gradient
50℃
60℃
swH1
FluA
B) Annealing Temperature
50℃
55℃
60℃
swH1
FluA

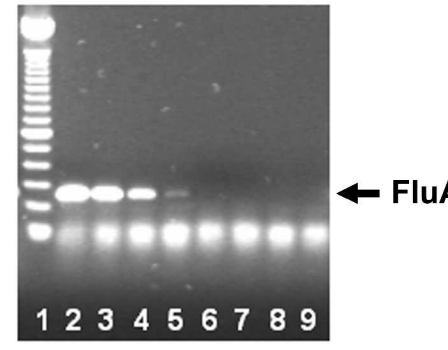
A)

Influenza A + swH1 duplex



B)

Influenza A, B and RSV triplex



C)