A Universal Influenza A and B Duplex Real-time RT-PCR Assay

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A high throughput universal influenza A and B duplex real-time RT-PCR was developed to meet effectively the heightened surveillance and diagnostic needs essential in managing influenza infections and outbreaks. Primers and probes, designed to target highly conserved regions of the matrix protein of influenza A and the nucleoprotein of influenza B, were optimized using the high-throughput LightCycler 480 II system. Analytical sensitivity and specificity were characterized using RNA transcripts diluted serially, archived non-influenza respiratory viruses, and proficiency test samples. Eighty-nine clinical samples were tested in parallel against existing influenza A and B monoplex assays. Once validated, the duplex assay was applied prospectively on 2,458 clinical specimens that were later subtyped. In April 2011, the emergence of an influenza B variant necessitated the inclusion of an additional modified probe for influenza B and revalidation of the revised protocol. The lower detection limits of the assay were 50 copies/PCR. There was no cross-reactivity against any non-influenza respiratory virus and all proficiency testing materials were identified correctly. The parallel testing revealed a 98.9% overall agreement. Routine application of the assay revealed high sensitivity and specificity for the detection of influenza A/H1N1/2009, A/H3N2 and influenza B. Assay C_q values correlated well between the pre- and post-revision protocols for influenza A ($r^2 = 0.998$) and B ($r^2 = 0.999$). The revised protocol detected three additional novel influenza B variant cases in 200 specimens reported previously as influenza B negative. This in-house assay offers a highly sensitive and specific option for laboratories seeking to expand their influenza testing capacity. *J. Med. Virol.* **84:1646–1651, 2012.** © 2012 Wiley Periodicals, Inc.

INTRODUCTION

In April 2009, a novel swine-origin influenza A (family *Orthomyxoviridae*, genus *Influenzavirus A*) emerging from Mexico was detected in the United States of America [Dawood et al., 2009]. By the end of May 2009, it had spread to over 40 countries worldwide [WHO, 2009a]. While the classification of this novel influenza virus as a pandemic strain has been debated [Cohen and Carter, 2010; Evans, 2010; Tang et al., 2010], it has prompted a reassessment of recent pandemic preparedness practices [Cordova-Villalobos et al., 2009; Del Rio and Hernandez-Avila, 2009; del Rio and Guarner, 2010; Rizzo et al., 2010; Tay et al., 2010]. An important lesson identified in many retrospective studies was the importance of collecting, analyzing and disseminating high quality surveillance

data for informing diagnostic, therapeutic and public health interventions [Cordova-Villalobos et al., 2009; Kumar et al., 2010; Rizzo et al., 2010; Sigmundsdottir et al., 2010; Tay et al., 2010].

Molecular testing is an essential surveillance tool, particularly when existing conventional viral culture and immunofluorescence testing is inadequate for detecting emerging, novel viruses [Lee et al., 2010; Tay et al., 2010]. Several real-time reverse transcription PCR (rRT-PCR) assays that have been developed for simultaneous detection of influenza A and B viruses in single-tube format, may fit this purpose [Selvaraju and Selvarangan, 2010; Chen et al., 2011; Huber et al., 2011; Papillard-Marechal et al., 2011; Shisong et al., 2011; Li et al., 2012; Salez et al., 2012]. However, the robustness of these assays has not been characterized extensively on routine clinical samples or proficiency testing materials. A sensitive and specific high-throughput duplex universal influenza A and B rRT-PCR method is described, which was validated on 2,458 clinical specimens and proficiency testing material from the Quality Control for Molecular Diagnostics and the Royal College of Pathologists of Australasia external quality assurance programs.

MATERIALS AND METHODS

Specimen Preparation

This study was approved by the local institutional ethics review board (National Healthcare Group Domain-Specific Review Board, reference numbers: B/09/360 and E/09/341). The workflow for respiratory sample collection and processing at the National University Hospital, Singapore had been described previously in detail [Lee et al., 2010]. The demographic details of the patients are summarized in Table I. Briefly, nasopharyngeal/nasal swabs collected into 3 ml of universal transport medium (Copan Diagnostics, Corona, CA) or dry/gel swabs pretreated with $1\times$-phosphate buffered saline (PBS), were kept at 4°C and processed within 24 hr. Viral RNA was extracted from 200 μl of the universal transport medium, or 200 μl of the $1\times$-PBS or from 200 μl of 1% N-acetylcysteine in $1\times$-PBS used to pre-treat sputum/endotracheal aspirates. The extracted viral RNA was eluted in 60 μl of the eluent.

TABLE I. Demographic Data of Clinical Samples Tested (n = 2,547) in the Entire Study

Demography	Frequency (%)
Gender	
Male	1,391 (54.6%)
Female	1,156 (45.4%)
Age group	
Paediatric (<18-year-old)	901 (35.4%)
Adult	1,538 (60.4%)
Unknown	8 (0.3%)
Ordering location	
Inpatient	1,092 (43.9%)
Outpatient	1,297 (50.9%)
Outreach	158 (6.2%)

Extraction was carried out on either the Bio Robot EZ1 or the QIAsymphony automated platforms (Qiagen, Valencia, CA), using the Qiagen EZ1 Virus mini kit v2.0 or the QIAsymphony Virus/Bacteria mini kit, respectively, according to the manufacturer's instructions.

Real-Time Reverse Transcription Polymerase Chain Reaction

The amplification and detection of influenza A and B viral RNA were performed in a single tube using the 5′ exonuclease probe rRT-PCR method. To ensure the detection of all influenza A and B subtypes, primers and probes with minimum secondary structures were designed, targeting the highly conserved regions of the matrix protein (A/California/07/2009(H1N1), GenBank accession: FJ969537) and nucleoprotein (B/North Carolina/WRAIR1582P/2009, GenBank accession: CY069566) genes, respectively.

The rRT-PCR was performed on the LightCycler 480 II System (Roche Molecular Diagnostics, Pleasanton, CA), using the SuperScript III Platinum One-step qRT-PCR reagent kit (Invitrogen, Carlsbad, CA). The 20-μl reaction volume is comprised of 5 μl of extracted RNA template, 0.5 μl of enzyme mix, 10 μl of $2\times$ reaction mix, 0.25 μmol/L influenza A forward primer, 0.2 μmol/L influenza A reverse primer, 0.25 μmol/L influenza A probe, 0.25 μmol/L influenza B forward primer, 0.2 μmol/L influenza B reverse primer, and 0.25 μmol/L influenza B original probe. All primers and probes were purchased from Eurogentec AIT (Seraing, Belgium) and listed in Table II.

The rRT-PCR was initiated by reverse transcription (55°C, 8 min) and initial denaturation (95°C, 2.5 min), followed by 45 amplification cycles at 95°C for 15 sec, 60°C for 20 sec, and 68°C for 15 sec. Fluorescence signals were collected at every 68°C stage. The influenza A and B amplification signal curves were analyzed at absorption wavelengths of 530 nm (FAM) and 560 nm (Yellow Yakima), respectively. Both fluorescence signals were color-compensated according to the manufacturer's recommendation. Appropriate positive control and non-template control were included in every test run.

Analytical Specificity

The specificity of the assay was evaluated using archived patient samples that were previously tested positive for human metapneumovirus, respiratory syncytial virus, parainfluenza virus types 1–4, and coronaviruses (229E and OC43). Proficiency testing materials for the influenza A and B panels, provided by the Quality Control for Molecular Diagnostics and the Royal College of Pathologiests of Australasia, were also used to assess the assay performance. Additionally, in silico analysis was performed to further characterize the specificity of the influenza A primers and probe by aligning the oligonucleotides against influenza A sequences published within the most

TABLE II. Primers and Probes Used in This Study

Primers/probe	Sequence 5′–3′	Gene target	Orientation	Nucleotide position
Influenza A				
Forward primer	GGAATGGCTAAAGACAAGACCAAT	Matrix protein	Forward	129–152[a]
Reverse primer	GGGCATTTTGGACAAAGCGTCTAC	Matrix protein	Reverse	250–227[a]
Probe	FAM-AGTCCTCGCTCACTGGGCACGGTG-BHQ1	Matrix protein	Reverse	221–198[a]
Influenza B				
Forward primer	CCAGGGATTGCAGACATTGA	Nucleoprotein	Forward	928–947[b]
Reverse primer	ACAGGTGTTGCCATATTGTAAAGAG	Nucleoprotein	Reverse	1,118–1,094[b]
Original probe	Yellow Yakima-TTGTTAGGCCCTCTGTGGCRAGCA-BHQ1	Nucleoprotein	Forward	980–1,003[b]
Modified probe	Yellow Yakima-TTGTTAGACCTTCTGTGGCRAGCA-BHQ1	Nucleoprotein	Forward	968–991[c]

[a]Based on influenza A/California/07/2009(H1N1), GenBank accession: FJ969537.
[b]Based on influenza B/North Carolina/WRAIR1582P/2009, GenBank accession: CY069566.
[c]Based on influenza B/Singapore/1/2011, GenBank accession: CY093580.

recent 5 years in GenBank (n = 200) that included H1N1/2009, seasonal H1N1, seasonal H3N2, and H5N1subtypes.

In addition, in order to assess the capability of this assay to detect the recent novel reassortant influenza A/H3N2/v, all the sequences available for this virus [Lindstrom et al., 2012] were downloaded for an in silico comparison, that is, JN638729, JQ070795, JN655534, JQ070780, and JQ290167.

Clinical Sensitivity and Specificity

The agreement between the universal influenza A/B duplex assay and the existing modified influenza A and influenza B monoplex assays [Lee et al., 2010] was determined by parallel testing of 89 consecutive clinical samples received for routine testing between March 31 and April 10, 2010. The 89 samples were further subjected to H1N1/2009 and seasonal H1N1/H3N2 subtyping assays for identification [Lee et al., 2010]. Upon completion of the initial evaluation, the duplex assay was implemented as the influenza-screening assay at the National University Hospital, Singapore. Specimens testing positive for influenza A using the duplex assay were further subtyped with an in-house developed H1N1/2009 dual-gene rRT-PCR [Lee et al., 2010] and published seasonal H1N1 and H3N2 subtyping assays [HKCHP, 2009].

Discovery of Influenza B Variant Strain and Addition of Influenza B Probe

On April 12, 2011, after the routine application of this laboratory-developed assay, a novel influenza B variant (B/Singapore/1/2011, GenBank accession number: CY093580) that was detected by direct immunofluorescence staining and virus culture, tested negative on this assay. The cause of failure to detect the variant virus by this assay was the presence of two single nucleotide substitutions at the probe region of the assay. This incidence has been described in detail [Lee et al., 2011b].

To overcome this, an additional probe that fully complements the influenza B variant was designed (Table II), and included in equimolar amounts with

the original probe to a final concentration of 0.25 μmol/L. The revised protocol was tested in parallel with the original method that contained only the original probe, on each of 20 randomly selected influenza A/H1N1/2009, A/H3N2, and B positive samples with C_q values between 15.8 and 37.3. Furthermore, 200 clinical specimens that had been reported as negative for influenza A/B during April 2011 using the original method was retested with this revised protocol. Any positive sample arising from this additional testing was subjected to Sanger sequencing to confirm the identity of the pathogen.

Lower Detection Limit of Revised Protocol

The amplicons of the matrix protein of influenza A and the nucleoprotein of influenza B were cloned into the pCR2.1-TOPO cloning vector (Invitrogen) and transcribed in vitro into RNA transcripts using Ribo-MAX Large Scale RNA Production System-T7 (Promega, Madison, WI), according to manufacturers' instructions. The sequences of the cloned plasmids were confirmed by direct sequencing prior to transcription. The synthesized transcripts were quantified using the NanoDrop ND-1000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), using absorption wavelength of 260 nm. The lower detection limit and linearity studies of the revised universal influenza A and B rRT-PCR assay were performed in triplicates, using transcripts diluted serially in 10-fold (1×10^8 copies/μl).

RESULTS

Analytical Specificity

The duplex assay did not exhibit any cross reactivity towards any non-influenza respiratory viruses in the archived specimens. The universal influenza A/B assay was also able to identify correctly all the proficiency testing materials provided by the Quality Control for Molecular Diagnostics and Royal College of Pathologiests of Australasia.

In silico analysis of the sequences of the universal influenza A primers and probes against the A/H1N1/

2009, seasonal A/H3N2, and A/H3N2/v virus sequences showed 100% complementation. Single base-pair mismatches were found near the 5' end of the forward primer against seasonal H1N1, and the 5' ends of the forward and reverse primers against the H5N1 strain, but these were not expected to affect significantly the performance of the assay.

Clinical Sensitivity and Specificity

Of the 89 clinical specimens tested in parallel, 26 were positive for influenza A using the duplex assay, one more than the 25 detected by the existing monoplex influenza A assay. All influenza A positive cases were confirmed by the H1N1/2009 and seasonal H1N1/H3N2 subtyping assays. There were 25 H1N1/2009 and 1 H3N2 positives detected. The discrepant specimen was identified as H1N1/2009 by the subtyping assay. The overall percent agreement was 98.9% (96.1% positive agreement; 100% negative agreement).

For influenza B detection, the duplex screening assay detected an additional influenza B positive case (n = 3) compared to the two positives detected by the existing monoplex influenza B method.

Between April 10, 2010 and January 5, 2011, the universal influenza A/B duplex assay was tested on 2,458 samples. During this period, there were 640 influenza A, 146 influenza B, and 2 influenza A/H1N1/2009 and B co-infections detected. Of the 640 influenza A positive cases, 355 were confirmed as H1N1/2009, 270 as seasonal H3N2, and 2 as seasonal H1N1 subtypes (98.0% positive agreement). The 13 cases that were not detected by the subtyping methods had C_q values between 36.2 and 40 on the duplex assay.

Although this assay should, from the in silico analysis, be able to detect the A/H3N2/v reassortant virus, unfortunately, no positive sample was available for experimental testing, so the potential for this assay to detect this strain remains undetermined.

Comparison Between Pre- and Post-revision Protocols

Comparison between the pre- and post-revision universal influenza A/B protocols showed good correlation of the C_q values, with $r^2 = 0.998$ and 0.999 for influenza A and B detection, respectively (Fig. 1A and B). Of the 200 previously influenza negative samples tested by the revised protocol, 3 additional influenza B/Singapore/1/2011 samples were identified and confirmed by Sanger sequencing [Lee et al., 2011b].

Lower Limit of Detection

The estimated lower limits of detection of the revised universal influenza A/B duplex assay were similar for influenza A/H1N1, A/H3N2, and B at 50 copies/PCR. A summary of the C_q values at concentrations of $5-5 \times 10^8$ copies/PCR is provided in Table III. The
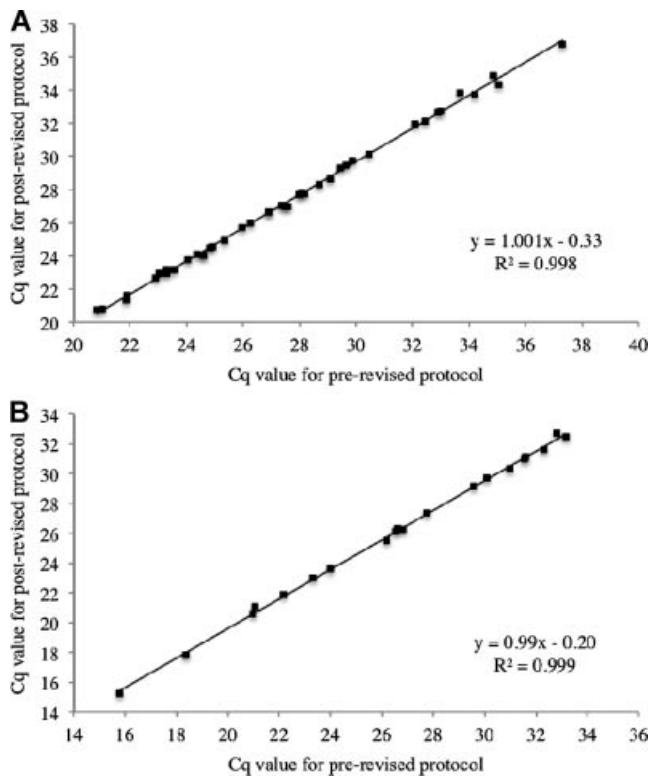

Fig. 1. C_q-value plots for pre- and post-revision protocols on (**A**) 20 clinical samples for each of influenza A/H1N1/2009 and A/H3N2, showing a gradient of 1.00 and r^2 of 0.998. **B**: 20 clinical samples for influenza B, showing a gradient of 0.99 and r^2 of 0.999.

C_q-value change per logarithmic concentration, represented by the slopes of the graphs, were -3.37, -3.26, and -3.44 for influenza A/H1N1, A/H3N2, and B, respectively. The PCR efficiencies, derived from the formula $E = 10^{-1/\text{slope}}$, where a value of 2 represents 100% efficiency, were 1.98 (influenza A/H1N1), 2.03 (influenza A/H3N2), and 1.95 (influenza B).

DISCUSSION

The use of universal influenza assays are important to identify influenza patients, not just for seasonal but now for pandemic A/H1N1 and avian A/H5N1 influenza viruses, which are now known to be less detected readily by more traditional diagnostic virology methods, such as cell culture and immunofluorescence assays [WHO, 2005a, 2009b]. The targeting of highly conserved regions of the continually evolving influenza virus more reliably ensures the detection of all strains, including novel strains. Subsequent failure to subtype a patient into H1N1/2009, seasonal H3N2 or H1N1 should raise the suspicion of a novel variant or, more rarely, a novel subtype, or perhaps an avian A/H5N1 infection [WHO, 2005a, 2009b].

This universal influenza A/B assay was able to achieve better specificity and sensitivity compared to the existing monoplex assays. In addition, optimizing this assay for a high-throughput platform, the

TABLE III. Summary of the C_q Values (Triplicate) of In Vitro Synthesized RNA Transcripts of Influenza A/H1N1/2009, A/H3N2, and B, at Concentrations of 5–5 × 10^8 Copies/PCR

Log (copies/PCR)	H3N2		H1N1/2009		Flu B	
	Mean	SD	Mean	SD	Mean	SD
0.7	40.00	—	40.00	—	—	—
1.7	37.92	1.44	37.52	0.59	38.89	0.97
2.7	35.39	0.35	34.37	0.30	35.53	0.29
3.7	31.79	0.08	30.64	0.10	32.13	0.10
4.7	28.44	0.09	27.34	0.10	28.65	0.06
5.7	24.94	0.02	23.85	0.04	25.20	0.03
6.7	21.53	0.06	20.40	0.10	21.76	0.10
7.7	18.00	0.08	16.91	0.02	18.23	0.06
8.7	14.64	0.06	13.57	0.04	14.91	0.07

LightCycler® 480 II, may make it more applicable for diagnostic laboratories with high workloads. The 13 discrepant cases that were undetected by the subtyping assays were likely due to stochastic variations at low viral loads approaching the lower limit of detection of the assays. The quantitative capability of this universal influenza A assay has been demonstrated in a viral load comparison study between influenza A/H1N1/2009, seasonal influenza A/H1N1, and A/H3N2 in Singapore [Lee et al., 2011a].

Within the month of April 2011, four out of five influenza B positive samples detected during routine diagnostic testing turned out to be a new variant, later named as influenza B/Singapore/1/2011. This potential drift variant was missed by the primers and probes of the earlier version of this influenza A/B duplex screening assay and a redesign was undertaken [Lee et al., 2011b]. The good correlation between the C_q values of the pre- and post-revision protocols demonstrated that the performance of the redesigned assay had not been affected adversely by the inclusion of the additional probe.

In conclusion, this duplex universal influenza A/B assay offers a useful option for laboratories requiring a high-throughput testing capacity, with the simultaneous detection of influenza A and B viruses saving time and cost. This maybe particularly useful in the post-pandemic influenza era when heightened awareness and continued surveillance by hospital clinical teams and community general practitioners may increase the diagnostic laboratory workload. This assay also fulfills the ongoing surveillance needs in all phases of pandemic preparedness planning, as recommended by the World Health Organization [WHO, 2005b], and in addition, has demonstrated the potential, at least in silico, to detect the recent reassortant virus, A/H3N2/v.

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