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Simultaneous typing and HA/NA subtyping of influenza A and B viruses including the pandemic influenza A/H1N1 2009 by multiplex real-time RT-PCR

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Abstract

Pandemic influenza A/H1N1 2009 and seasonal influenza viruses are currently co-circulating worldwide. A rapid, sensitive, and specific assay for distinguishing pandemic influenza A/H1N1 2009 from seasonal influenza viruses and for subtyping seasonal influenza A viruses could aid in the surveillance and control of these viral infections. Here, a multiplex real-time RT-PCR (rRT-PCR) assay for typing influenza A and B viruses and the pandemic influenza A/H1N1 2009 is developed. This assay can also subtype seasonal influenza A viruses simultaneously. The analytical sensitivity is 10–10^4 copies/reaction. The coefficients of variation of inter-assay and intra-assay are 0.04–0.45% and 0.08–0.97%, respectively. The new multiplex rRT-PCR assay is more sensitive in subtyping seasonal influenza viruses than the conventional PCR techniques. Results obtained with this assay for the detection of pandemic influenza A/H1N1 2009 are highly consistent (96.88%) with those achieved using the US CDC’s rRT-PCR protocol. A sample identified as “pandemic influenza A/H1N1 2009 positive” by the US CDC’s rRT-PCR was reclassified correctly as subtype H3N2 using this assay. Taken together, this new multiplex rRT-PCR protocol could be an important tool for improving diagnosis and management of the pandemic influenza A/H1N1 2009 and seasonal influenza viruses.

1. Introduction

In April 2009, outbreaks of influenza-like illnesses caused by a novel influenza A virus, pandemic influenza A/H1N1 2009 (also referred to as “swine-origin influenza virus, S-OIV”), were identified in Mexico and the United States (Centers for Disease Control and Prevention, 2009; Dawood et al., 2009). These outbreaks have led to a global influenza pandemic (Brownstein et al., 2009). Recent surveillance data show that the pandemic influenza A/H1N1 2009 is co-circulating with seasonal influenza viruses in many countries (http://www.cdc.gov/flu/weekly/pdf/External/F0939.pdf; http://www.who.int/csr/don/2009_10_23/en/index.html). Because pandemic influenza A/H1N1 2009 and seasonal influenza transmit in a similar manner and present almost identical symptoms (http://www.cdc.gov/h1n1flu/qa.htm), the control and prevention of this novel pandemic has been challenging. Critical for viral containment, surveillance, and treatment is the development of sensitive, accurate, and reliable diagnostic tests that can discriminate the H1N1 influenza virus from other strains.

Among the current laboratory diagnostic tests, real-time reverse transcriptase polymerase chain reaction (rRT-PCR), which determines virus copies by monitoring the fluorescence accumulations, has many advantages over other detection techniques. These include: ease of performance, great accuracy, high sensitivity and specificity, fast turn-around time, a high-throughput capacity, and minimal carry-over contamination (Mackay et al., 2002). However, rRT-PCR assays for subtyping seasonal influenza viruses (Daum et al., 2007) have not been suitable for the detection of pandemic influenza A/H1N1 2009 because of genetic variations.

Several rRT-PCR assays have been developed recently to detect the pandemic influenza A/H1N1 2009 (Carr et al., 2009; Hall et al., 2009; Jiang et al., 2010; Pabbaraju et al., 2009; Wang et al., 2009; Whiley et al., 2009). However, these assays are not able to type and subtype seasonal influenza viruses simultaneously. Another limitation of these rRT-PCR assays is that they only target the novel H1 gene of the pandemic influenza A/H1N1 2009 virus (Jiang et al., 2010).
A virus named Flu A M were synthesised as previously described (Whiley et al., 2009) and for the consensus influenza 2010; Whiley et al., 2009), resulting in a limited specificity that can result in false-negative diagnosis when sequence variations exist in the primer- or probe-targeted genes. In addition, there has been a report of some cross-reactivity between the pandemic influenza virus and the influenza A/H1N1 2009 virus and for seasonal influenza viruses such as human H1N1 and H3N2 were designed based on the sequence data obtained from the Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/)( Table 1). Primers/probes for typing of the viral strains were designed to target the conserved regions of the M gene segment for the influenza A virus and of the NP gene segment for the influenza B virus. Primers/probes for subtyping of viral strains were designed to target the HA gene segment for each of the three HA subtypes (H1-consensus, H1-pandemic A/H1N1 2009 and H3) and the NA gene segment for each of the three NA subtypes (N1-pandemic A/H1N1 2009 and N2).

Table 1
Sequences of primers and probes in the multiplex rRT-PCR assay.

| Set | Primer | Sequence (5′ → 3′) | Gene target-nucleotide position | Amplicon (bp) | Origin |
|-----|--------|---------------------|---------------------------------|--------------|--------|
| Set 1 | Subtype H1-Consensus | H1-Consensus-F ATGCACAACTTCRACCGACAC* | H1 Hemagglutinin (Segment 4) | 187 | This study |
| | H1-Consensus-R AGTRATTTCRACCTTCTGGGTATG | 74 → 95 | This study |
| | H1-Consensus-P FAM-CANCCDCGGAYGTYRCANTTMCCCAAC-BHQ-1* | 230 → 205 | This study |
| Subtype H1-2009 | H1-2009-F TTATCATTTCAAGATACACACT | H1 Hemagglutinin (Segment 4) | 179 | This study |
| | H1-2009-R AATAGCAGGACCATCCT | 845 → 866 | This study |
| | H1-2009-P HEX-CACAGATGTTCAATACACT-BHQ-1 | 1023 → 1006 | This study |
| | | 1045 → 1067 | This study |
| Set 2 | Subtype N1-2009 | N1-2009-F CAGAGGCCCCACCCACACAAGA | N1 Neuraminidase (Segment 6) | 93 | (Whiley et al., 2009) |
| | N1-2009-R GCCAAGCAACCAACCA | 1281 → 1300 | This study |
| | N1-2009-P CY5-CAACATTTCCGACTCCGACGCT-BHQ-2 | 1373 → 1356 | (Whiley et al., 2009) |
| Subtype N2 | N2-F TGTATCTGACCAACACACCATAGA | N2 Neuraminidase (Segment 6) | 77 | (Whiley et al., 2009) |
| | N2-R TTGCGGCTTGACCAATTTC | N2 Neuraminidase (Segment 6) | 273 → 254 | This study |
| | N2-P HEX-AAGGAATATCGCCAAAATCTACGAGATAC-BHQ-1 | 223 → 252 | This study |
| Set 3 | Subtype H3 | H3-F ACCAGAGAACAAACACTAGAACATTAT | H3 Hemagglutinin (Segment 4) | 120 | This study |
| | H3-R TGTTCTGGCTCAGACATTTT | 1020 → 1046 | This study |
| | H3-P FAM-CCTGGTACCGGTTACATCGCAGGCA-BHQ-1 | 1119 → 1139 | This study |
| Subtype N1-Consensus | N1-F TATAAGACCTTGTCCTGGGTGA | N1 Neuraminidase (Segment 6) | 135 | This study |
| | N1-R GCCACTGTGCAAGAC | N1 Neuraminidase (Segment 6) | 1390 → 1407 | This study |
| | N1-P HEX-AATTTTGCACTGYGGACGACCTACATCTTTC-BHQ-1* | 1330 → 1362 | This study |
| Set 4 | Flu A M | Flu-M-F GACCRATCTCTCGCACTCTGAC* | Matrix (Segment 7) | 106 | WHO |
| | Flu-M-R AGGGCAATYTGGACAAAKGCTCA* | 91 → 112 | WHO |
| | Flu-M-P FAM-TGCAGTCTCTCGCACTCTGAC-BHQ-2 | 196 → 173 | WHO |
| | GAPDH FAM-GAPDH | GAPDH | 169 → 146 | WHO |
| | GAPDH-F CCAGGTGTTCCTGCTGGATCTC | GAPDH | 939 → 960 | This study |
| | GAPDH-R CACCCTGTCCTGGACAAAGA | GAPDH | 1074 → 1054 | This study |
| | GAPDH-P ROX-CTGCGATGCCGCTTCAAGCACCAC-BHQ-2 | GAPDH | 998 → 1020 | This study |
| | Flu B NP NP | Flu B-NP Flu-B-NP | Flu B-NP-R AGACTAGAGCTTGGTCTGGATCTC | Flu B-NP-F AGACTAGAGCTTGGTCTGGATCTC | N1-2009-P CY5-CACAATCTGGACTAGCGGGAGCAGCAT-BHQ-2* | 1246 → 1269 | This study |

D = 33.3% mixture of A, T, G; H = 33.3% mixture of A, T and C; M = 50% mixture of A and C; N = 25% mixture of A, C, T and G; R = 50% mixture of A and G; S = 50% mixture of C and G; W = 50% mixture of A and T; Y = 50% mixture of C and T.

Sequence variations could aid in the surveillance, diagnosis, and management of the pandemic A/H1N1 2009 influenza and seasonal influenzas.

2. Materials and methods

2.1. Primers and probe design

The primers and TaqMan probes for the pandemic influenza A/H1N1 2009 virus and for seasonal influenza viruses such as human H1N1 and H3N2 were designed based on the sequence data obtained from the Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/)( Table 1). Primers/probes for typing of the viral strains were designed to target the conserved regions of the M gene segment for the influenza A virus and of the NP gene segment for the influenza B virus. Primers/probes for subtyping of viral strains were designed to target the HA gene segment for each of the three HA subtypes (H1-consensus, H1-pandemic A/H1N1 2009 and H3) and the NA gene segment for each of the three NA subtypes (N1-consensus, N1-pandemic A/H1N1 2009 and N2). In silico coverage of primers/probes was calculated using an in-house program based on work described previously (Bose et al., 2009), with some modifications. Briefly, a sequence was defined as a “hit” if there were two or less substitutions within the probe, or if there were no substitutions within five bases from the 3′ end; one or less substitutions within 10 bases from the 3′ end; or three or less substitutions within the primers. For degenerate primers or probes, an acceptable “hit” was defined by the presence of an accurate match between the sequence data consensus (made by BioEDIT 7.01 software) and its primers or probes.

All primers/probes were grouped into four sets based on the PCR product size, fluorochrome-labels, and the pilot tests (Table 1). The four sets were designed for the detection of different subtypes: set 1 for H1 of pandemic influenza A/H1N1 2009 and for the consensus H1 of influenza A viruses; set 2 for N1 of pandemic influenza A/H1N1 2009 virus and for seasonal influenza viruses such as human H1N1 and H3N2.
Table 2
Analysis of the specificity of the multiplex rRT-PCR assay.

| Virus strain      | Subtype       | TCID50/mL | Copies/rxn\(^b\) | H1-2009 | H1-Consensus | H3 | N1-2009 | N1-Consensus | N2 | Flu A M | Flu B NP |
|-------------------|---------------|-----------|-------------------|----------|---------------|----|----------|---------------|----|----------|----------|
| A/PR/8/1934       | H1N1          | \(1.8 \times 10^4\) | \(1 \times 10^8\) | –        | –              | +  | –        | –              | –  | +        | –        |
| A/Beijing/01/2009 | H1N1, H3N2    | \(0.7 \times 10^4\) | \(1 \times 10^8\) | +        | +              | +  | +        | +              | +  | +        | +        |
| A/Jiangxi/242/2004| H3N2          | \(1.5 \times 10^4\) | \(1 \times 10^8\) | –        | –              | +  | –        | –              | +  | +        | +        |
| A/Beijing/4046/2008| H1N1         | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| A/Beijing/4053/2008| H3N2          | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| A/Beijing/CY-30/2008| H3N2       | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| A/Beijing/3005/2008| H3N2          | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| A/Beijing/CY-29/2008| H3N2         | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| A/Beijing/4365/2008| H3N2         | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| A/Beijing/3022/2008| H3N2         | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| A/Beijing/3028/2008| H3N2         | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| A/Beijing/4368/2008| H3N2         | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| A/Beijing/3016/2008| H3N2         | \(1 \times 10^8\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| B/H1N1/361/2002    | Influenza B   | \(1.8 \times 10^4\) | –                  | –        | –              | +  | +        | +              | –  | +        | +        |
| OC43              |               | \(1.8 \times 10^1\) | –                  | –        | –              | –  | –        | –              | –  | –        | –        |
| Adenovirus Holden | Serotype 35   | \(1.8 \times 10^4\) | –                  | –        | –              | –  | –        | –              | +  | –        | –        |
| Influenza B (#426A)| Influenza B   | Sample     | –                  | –        | –              | –  | –        | –              | +  | –        | –        |
| Influenza C (#828A)| Influenza C   | Sample     | –                  | –        | –              | –  | –        | –              | +  | –        | –        |
| Adenovirus (#8313)| Unknown       | Sample     | –                  | –        | –              | –  | –        | –              | +  | –        | –        |
| hMPV (#1152A)     | Unknown       | Sample     | –                  | –        | –              | –  | –        | –              | +  | –        | –        |
| PIV (#2722)       | Serotype 3    | Sample     | –                  | –        | –              | –  | –        | –              | +  | –        | –        |

\(^a\) The mean C\(_T\)s were all at 15.3–22.7, Std. Dev. C\(_T\)s were all at 0.0–0.4, and CVs were all less than 5%.

\(^b\) rxn refers to ‘reaction’.

A/H1N1 2009 and for the consensus N2 of influenza A viruses; set 3 for the consensus N1 and H3 of influenza A viruses; and set 4 for conserved regions of the M gene of influenza A viruses, the NP gene for influenza B viruses and the GAPDH internal marker gene for *homo sapiens*. The four sets of primers and probes were aliquoted into four different tubes for the multiplex rRT-PCR assay.

2.2. Virus stocks

Tw0 known subtypes of influenza A virus strains, A/PR/8/34 (H1N1) and A/Jiangxi/242/2004 (H3N2), together with an influenza B virus B/Shanghai/361/2002 strain were provided by the Chinese National Influenza Centre (CNIC). An isolate of pandemic influenza A/H1N1 2009 [A/Beijing/01/2009 (GenBank accession number: GQ183617–GQ183624)] and 11 seasonal influenza A virus isolates of unknown subtypes (named A/Beijing/4046, 4053, 3012, CY-30, 3005, CY-29, 4365, 3032, 3028, 4368 and 3016/2008) were isolated from Beijing, China (Table 2). Human coronavirus OC43 (OC43) was provided by the Peking Union Medical College Hospital. The adenovirus serotype 35 (Ad35; Holden strain) was purchased from American Type Culture Collection (ATCC, Manassas, VA). All of the known viruses were grown on continuous cell lines (influenza A virus strains on MDCK cells, influenza B virus strain and OC43 on LLC-MK2 cells, and Ad35 on Hela cells).

2.3. Clinical specimens

Nasopharyngeal swab specimens were obtained from Peking Union Medical College Hospital and from Beijing Children’s Hospital between January 2007 and December 2008. Samples were preliminarily screened for influenza viruses and other respiratory viruses using PCR assays, as described elsewhere (Ren et al., 2009). Among the 119 randomly selected samples, 105 samples tested positive for influenza A virus, two positive for influenza B virus, two positive for coronavirus OC43, two positive for adenovirus, and eight negative for all known respiratory viruses.

In addition to these samples, 32 nasopharyngeal swab specimens that were positive for pandemic influenza A/H1N1 2009 were collected by the Beijing CDC between May and July 2009. These latter samples were screened using the US CDC’s real-time RT-PCR protocol for pandemic influenza A/H1N1 2009 as recommended by WHO (http://www.euro.who.int/Document/INF/CDC_realtime_RTPCR_A/H1N1.pdf).

2.4. Viral nucleic acid extraction

To extract viral nucleic acids, 200 \(\mu\)L of each nasopharyngeal swab, clinical sample or viral culture were added into 2 mL of lysis buffer and incubated at room temperature for 10 min. After lysis, the samples were loaded onto the NucliSENS easyMAG (bioMérieux, Marcy L’Etoile, France) for total nucleic acid extraction. The viral nucleic acids were stored at \(-80^\circ\)C prior to detection.

2.5. Multiplex rRT-PCR amplification

One-step reverse transcription-PCR (RT-PCR) was performed and optimised using SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, 2 \(\mu\)L of nucleic acid solution was added to 23 \(\mu\)L of master mix containing the following components at the indicated final concentrations: 1 \(\times\) reaction buffer, 1 \(\mu\)L of SuperScript III RT/Platinum Taq Mix, 500 nM of each primer, and 300 nM of the probe labelled at the 5’ end with a reporter dye and at the 3’ end with a non-fluorescent quencher based on the four sets of multiplex rRT-PCR assays (Table 1). Thermocycling was carried out as follows: 30 min at 50°C and 3 min at 95°C for reverse transcription (RT) and denaturation; five pre-cycles at 95°C for 15 s, at 50°C for 30 s, and at 72°C for 1 min; followed by 40 cycles at 95°C for 10 s, and at 55°C for 40 s. The multiplex rRT-PCR assay was performed on a CFX 96 (Bio-Rad Laboratories Inc., Hercules, CA). The baseline fluorescence threshold for each analysis was manually adjusted based on the background fluorescence of the ‘no template’ control reaction using the Baseline Subtracted Curve Fit analysis mode in Bio-Rad CFX Manager Software. For each analysis, a sample amplification curve exceeding baseline fluorescence with a corresponding \(C_T\) (Cycle threshold) value (not exceeding 36 in a 40-cycle run) was considered as positive. In addition, a \(C_T\) value between 36 and 40 was considered as weakly positive. An amplification curve lower than 36.5 exceeded baseline fluorescence was defined as negative.
tested at high TCID50s (Table 2). The analytical reproducibility including influenza B virus, coronavirus OC43 and adenovirus were of the rRT-PCR assay. Other respiratory viral nucleic acid extracts were tested at high concentrations (10^8 copies/reaction #2722) were recruited for the cross-reaction assay. Viral RNA movirus (hMPV; #1152A) and parainfluenza virus type 3 (PIV3; influenza C virus (#828A), adenovirus (#8313), human metapneumovirus (hMPV; #1152A) and parainfluenza virus type 3 (PIV3; #2722) were tested at high TCID50/mL) of the quantified viral RNA extracts and of the plasmid cloning of genes of influenza A viruses including H1–2009, N1–2009, H1, N1, H3, N2, and M. In addition, another five clinical respiratory specimens positive for influenza B virus (#426A), influenza C virus (#828A), adenovirus (#8313), human metapneumovirus (hMPV; #1152A) and parainfluenza virus type 3 (PIV3; #2722) were recruited for the cross-reaction assay. Viral RNA extracts were tested at high concentrations (10^8 copies/reaction above the LOD) (Table 2) to determine the analytical specificity of the rRT-PCR assay. Other respiratory viral nucleic acid extracts including influenza B virus, coronavirus OC43 and adenovirus were tested at high TCID50s (Table 2). The analytical reproducibility assay was calculated at a degree of dilution of 10^8 copies/reaction (3.6 × 10^-2 TCID50/reaction for influenza B virus). All of the analytical assays were run at least three times and each run was performed on different days. Inter-assy and intra-assy variability (coefficient of variation, CV), both measures of the reproducibility of the rRT-PCR assays, was determined by the following formula:

\[
CV(\%) = \frac{SD_{C_T} - \text{Mean}_{C_T}}{100} 
\]

SD C_Ts: standard deviation of C_T values; Mean C_Ts: mean C_T values.

### 2.7. Conventional RT-PCR for subtyping of seasonal influenza viruses

The primer sequences of conventional RT-PCR for subtyping of seasonal influenza A viruses were provided by the NCBI. The sequences and nucleotide positions of the primers were H1-F678: 5’-ACTACTGGACTCTGCTGGGAAC-3’ (768–788nt), H1-R1094: 5’-CAATGAAACCGCCATGGCTCC-3’ (1094–1073nt) for the H1 subtype; N1-F1059: 5’-AAGGGTTTTCATACAGGGTATGGT-3’ (1402nt) for the N1 subtype; H3 subtypes; N1-2009-P: 5’-AACTGTTCATTTGGAGTGATG-3’ (1059–1083nt), N1-2009-R: 5’-GGAAATCGTTCATATTAGCCCATTG-3’ (940–919nt) for the N1 subtype; H3-P: 5’-AAGGGTTTTCATACAGGGTATGGT-3’ (1326nt) for the H3 subtype; N2-F779: 5’-ACTACTGGACTCTGCTGGGAAC-3’ (768–788nt), N2-R955: 5’-CAATGAAACCGCCATGGCTCC-3’ (1326nt) for the N2 subtype. Conventional RT-PCR assays were performed and optimised using the SuperScriptIII™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen) according to the manufacturer's instructions. Briefly, 5 μL of each nucleic acid sample were added to a 20 μL master mix containing the following components at the indicated final concentrations: 1 × reaction buffer, 1 μL of SuperScriptIII™ III RT/Taq Mix and 500 nM of each primer. Thermocycling was carried out as follows: 1 min at 60 °C, 10 min at 42 °C, 30 min at 50 °C and 15 min at 95 °C for reverse transcription (RT) and denaturation;
Table 4

| Subtype | LOD1 (H1-2009)/H1-Consensus | LOD2 (N1-2009)/N2 | LOD3 (N1-Consensus/H3) | LOD4 (Flu A M/GAPDH/Flu B NP) |
|---------|-----------------------------|-------------------|-------------------------|-------------------------------|
| A/PR8   | N/1.3                       | 10^3 N/1.3        | 10^3 N/1.3              | 10^3 N/1.3                   |
|           |                              |                   |                         |                              |
| A/Beijing/01/2009 | N/1.8                      | 10^3 N/1.3        | 10^3 N/1.3              | 10^3 N/1.3                   |
| A/Jiangxi/242/2004 | N/3.3                      | 10^3 N/1.3        | 10^3 N/1.3              | 10^3 N/1.3                   |
| B/SH/361/2002   | N/1.5                       | 10^3 N/1.3        | 10^3 N/1.3              | 10^3 N/1.3                   |

3. Results

3.1. In silico coverage of the primer/probe sets

To evaluate the coverage of all the primers/probes designed for the new multiplex rRT-PCR, an in silico analysis using an in-house program was performed (see Section 2.1). The results showed that the coverage of these primers/probes – including sequences acquired from the literature (Whiley et al., 2009) and from the WHO – was 90.1–100% (Table 3).

3.2. Specificity of singleplex rRT-PCR

To assess the specificity of the multiplex rRT-PCR, the sequences of primers/probes were analysed by Blast analysis. No cross-reactivity with other respiratory viruses was found (data not shown). Subsequently, the cross-reactivity of the singleplex primers/probe was examined using a panel of virus strains and clinical specimens (Table 2). No obvious cross-reactivity was observed within influenza A virus type and its subtypes nor with influenza B virus type or any other respiratory viruses. In addition, one (A/Beijing/4046/2008) of the 11 influenza A virus isolates with unknown subtypes was identified as the H1N1 subtype, and the other 10 strains as the H3N2 subtype (Table 2). These results were confirmed by subsequent sequencing of the PCR products. All of the specific reactions had high positive fluorescence signals. The mean C\textsubscript{T}s were in the range of 15.3–22.7, the standard deviations (SDs) of the C\textsubscript{T}s values of LODs were all less than 4% for intra-assays and inter-assays (data not shown).

3.3. Sensitivity of multiplex rRT-PCR assay

The limits of detection (LODs) for each influenza virus in the multiplex reaction ranged from 10 to 10\(^4\) copies/reaction (Table 4). The LOD for pandemic influenza A/H1N1 2009 detected by H1-consensus primers/probe in Set 1 was 10\(^7\) copies/reaction (6.67 \times 10\(^5\) TCID\(_{50}\)/mL). However, as the LODs of the H1-2009 in Set 1 and the N1-2009 in Set 2 were 10\(^4\) and 10\(^2\) copies/reaction, respectively, the detection sensitivity of pandemic influenza A/H1N1 2009 was not affected. The CVs of the C\textsubscript{T} values of the LODs were all less than 4% based on statistical analysis, suggesting that these results were reproducible.
3.4. Reproducibility of multiplex rRT-PCR assay

To assess the reproducibility of the multiplex rRT-PCR assay, mean Ct values and SD Crs were calculated at $10^5$ copies/reaction for influenza A viruses and at $3.6 \times 10^{-2}$ TCID$_{50}$/reaction for influenza B viruses. The results showed that the CVs were 0.08–0.97% for intra-assays and 0.04–0.45% for inter-assays, suggesting that the multiplex rRT-PCR assay is highly reproducible (Table 5).

3.5. Validation of multiplex rRT-PCR assay

To validate the performance of the new multiplex rRT-PCR assay, parallel detections were performed using conventional subtyping PCR protocols recommended by the CNIC and the US CDC’s protocol for pandemic influenza A/H1N1 2009 with a panel of 151 clinical samples. The results of these detections and those for influenza A virus subtyping are summarised in Table 6.

The multiplex rRT-PCR assay subtyped the 105 seasonal influenza A viruses into 34 H1N1 and 71 H3N2. Furthermore, the multiplex rRT-PCR assay identified four new H1N1 and four new H3N2 strains, which had not been identified by conventional PCR previously. These new strains were confirmed through sequencing of the PCR products. Statistical analysis (Table 6) suggested that there were significant differences between the new multiplex rRT-PCR and the conventional subtyping PCR in the detection of seasonal influenza A virus ($P = 6.15 \times 10^{-5}$) and in H1N1 subtyping ($P = 8.74 \times 10^{-4}$). However, there was no significant difference between the two methods in H3N2 subtyping ($P = 0.13$). Based on these observations, the new multiplex rRT-PCR assay is more sensitive in subtyping influenza A viruses than the conventional PCR techniques.

The newly developed multiplex rRT-PCR showed high consistency (96.88%) in detecting pandemic influenza A/H1N1 2009. This is consistent with US CDC’s rRT-PCR protocol, as no significant differences were found between the two assays ($P = 1.00$) (Table 6). Interestingly, sample #LS1125-4, which was identified as “pandemic influenza A/H1N1 2009 positive” by the US CDC’s rRT-PCR, was subtyped as H3N2 with the multiplex rRT-PCR assay. The identification of H3N2 was later confirmed by sequencing the PCR product.

4. Discussion

In this report, a new and rapid four-tube multiplex rRT-PCR system for influenza virus diagnosis was developed. This system can simultaneously detect pandemic influenza A/H1N1 2009 virus, distinguish seasonal influenza A and B viruses, and subtype influenza A viruses into three HA and three NA subtypes (pandemic influenza A/H1N1 2009, seasonal H1N1, and H3N2) (Fig. 1). The assay improves on current standard assays and may aid in minimizing the transmission of pandemic influenza A/H1N1 2009 by identifying and distinguishing new cases of pandemic influenza A/H1N1 2009 from seasonal influenza.

The newly developed multiplex rRT-PCR system has several advantages over other assays for detection of influenza viruses. First, it is more sensitive than conventional PCR assays in subtyping influenza A viruses ($P = 6.15 \times 10^{-5}$ compared with seasonal influenza A virus detection; and $P = 8.74 \times 10^{-4}$ for H1N1 subtyping). The analysis of the LOD for pandemic influenza A/H1N1 2009 virus indicates that the strategy of multiple-targeting detection ensures the sensitivity of the assay system and effectively lowers the false-negative rate. Second, owing to the de-novo design of primers/probes for two target genes (H1-2009 and N1-2009 of the pandemic influenza A/H1N1 2009 virus, and H3 and N2 subtypes of seasonal influenza A virus)
Table 6
Performance of the multiplex rRT-PCR compared to other molecular tests for influenza subtyping and for the detection of pandemic influenza A/H1N1 2009.

| Multiplex rRT-PCR | Conventional subtyping PCR | US CDC's rRT-PCR |
|-------------------|-----------------------------|------------------|
|                   | Influenza A                  | A/H1N1 2009      | H1N1*          | H3N2        | Influenza B         | A/H1N1 2009      |
| +                 | 119                         | 18               | 137            | 0           | 31               | 31               | 52               | 65           |
| −                 | 0                           | 14               | 14             | 0           | 120              | 120              | 0                | 86           |
| Total             | 119                         | 32               | 151            | 0           | 151              | 151              | 52               | 99           |
| $\chi^2$         | 6.15 × 10⁻⁵                 | 7.12 × 10⁻⁸      | 8.74 × 10⁻⁴    | 0.13        | 0.48             | 1.00             |

* Including pandemic influenza A/H1N1 2009 strains because the H1 and N1 consensus primers/probes of this current multiplex rRT-PCR and the H1 and N1 primers of the conventional subtyping PCR method detected pandemic influenza A/H1N1 2009 as H1N1.

b Statistical significance was determined via McNemar's chi-square test for matched pairs. A P-value of ≤0.05 was considered significant.

of the H3N2 subtype, etc.), the multiplex rRT-PCR assay is specific for the detection of the pandemic influenza A/H1N1 2009 virus (Fig. 1A) and that of the H3N2 subtype (Fig. 1C and Fig. 2A). As shown here, one sample identified as pandemic influenza A/H1N1 2009 by the US CDC's rRT-PCR assay was confirmed to be the H3N2 subtype by the new assay. Based on sequence alignment, the H1-2009 primers/probes had very low homologies with that for H3N2 (Fig. 2B). However, there were high homologies between the SW H1 primers/probes of the US CDC's rRT-PCR and some H3N2 strains (such as A/Hong Kong/1-9-MA21-3/1968, a strain that is highly homologous to the sequencing product of this test). In particular, the SW H1 reverse primer has eight continuous matches with HA sequences found at the 3' end in H3N2, an important terminal site for primer specificity (Kwok et al., 1990; Peacey et al., 2009) (Fig. 2C). Third, in the new multiplex rRT-PCR system, the consensus primers/probes cover the majority of HA and NA sequences of each human influenza A subtype (H1, H3, N1 and N2) found in the Influenza Virus Resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/) (Table 3). This allows the new assay to identify a wide range of influenza subtypes, as seen in Tables 6 and 2, which present data for subtyping 105 randomly selected influenza A virus positive samples collected between January 2007 and December 2008, and 11 influenza A virus isolates of unknown subtypes. Fourth, the new multiplex rRT-PCR assay is highly flexible, as primers/probes can be easily adapted for the detection of some other or newly occurring

Fig. 1. Representative amplification curve showing typical patterns obtained from the multiplex rRT-PCR assay. (A) Pandemic influenza A/H1N1 2009 virus detected by H1-2009 (HEX channel, green curve) and H1-Consensus (FAM channel, blue curve) in Set 1, N1-2009 (Cy5 channel, violet curve) in Set 2, N1-Consensus (HEX channel, green curve) in Set 3, and Flu A M (FAM channel, blue curve) in Set 4. (B) Seasonal H1N1 virus detected by H1-Consensus (FAM channel, blue curve) in Set 1, N1-Consensus (HEX channel, green curve) in Set 3, and Flu A M (FAM channel, blue curve) in Set 4. (C) Seasonal H3N2 virus detected by N2 (HEX channel, green curve) in Set 3, and H3 (FAM channel, blue curve) in Set 4. (D) Influenza B virus detected by Flu B NP (Cy5 channel, violet curve) in Set 4. All the nucleic acid extractions of human clinical specimens and mammalian cell lines were detected by GAPDH (ROX channel, brown curve) in Set 4 (see A, B, C and D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
subtypes of influenza viruses. This adaptation would make the multiplex rRT-PCR a useful platform for the simultaneous detection and surveillance of multiple influenza viruses and/or possibly other pathogens.

In summary, a rapid multiplex rRT-PCR assay with high analytical sensitivity, specificity, and reproducibility has been developed. The assay will be useful for the surveillance, control, and prevention of pandemic influenza A/H1N1 2009 and seasonal influenza.

Acknowledgments

This work was supported in part by the National Basic Research Program (973 Program) of China (2010CB534003), the Scientific Research Development Program from the Science and Technology Commission of Shanghai Municipality (09DZ1906603), China, and an intramural grant from the Institute of Pathogen Biology, Chinese Academy of Medical Sciences (2006IPB05).

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