Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 167 (2010) 37–44

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

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

Yaowu Yang[a,1], Richard Gonzalez[a,b,1], Fang Huang[c,1], Wei Wang[a], Yongjun Li[a,b], Guy Vernet[b], Jianwei Wang[a,*], Qi Jin[a,*,2]

[a] Dr. Christophe Mérieux Laboratory, IPB, CAMS-Fondation Mérieux and State Key Laboratory of Molecular Virology and Genetic Engineering, Institute of Pathogen Biology (IPB), Chinese Academy of Medical Sciences (CAMS), Beijing 100730, China
[b] Fondation Mérieux, 69365 Lyon, France
[c] Beijing Centre for Disease Control and Prevention, Beijing 100013, China

Article history:
Received 13 November 2009
Received in revised form 9 March 2010
Accepted 10 March 2010
Available online 19 March 2010

Keywords:
Influenza virus
Pandemic influenza A/H1N1 2009
Seasonal influenza
Multiplex detection
Subtyping
Real-time RT-PCR

A B S T R A C T

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, such 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.,

* Corresponding author at: 9# Dong Dan San Tiao, Dongcheng District, Beijing 100730, China. Tel.: +86 10 65105188; fax: +86 10 65105188.
E-mail addresses: wangjw28@163.com (J. Wang), zdsys@vip.sina.com (Q. Jin).
[1] These authors contributed equally to this work.
[2] 9# Dong Dan San Tiao, Dongcheng District, Beijing 100730, China. Tel.: +86 10 65105161; fax: +86 10 65105160.

0166-0934/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2010.03.007

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 Hemagglutinin (Segment 4)	187	
	H1-Consensus-F	ATGCSAACAAYTCRACCGACAC[a]	74 → 95		This study
	H1-Consensus-R	AGTRATTCRCATTCTGGGTTT[a]	260 → 240		This study
	H1-Consensus-P	FAM-CANCCDGCAAYGYTRCANTTMCCCAA-BHQ-1[a]	230 → 205		This study
	Subtype H1-2009		H1 Hemagglutinin (Segment 4)	179	
	H1-2009-F	TTATCATTTCAGATACACCAGT	845 → 866		This study
	H1-2009-R	AATAGACGGGACATTCCT	1023 → 1006		This study
	H1-2009-P	HEX-CCACGATTGCAATACAACT-BHQ-1	1045 → 1067		This study
Set 2	Subtype N1-2009		N1 Neuraminidase (Segment 6)	93	
	N1-2009-F	CAGAGGGCGACCCAAAGAGA	1281 → 1300		(Whiley et al., 2009)
	N1-2009-R	GGCCAAGACCAACCCACA	1373 → 1356		(Whiley et al., 2009)
	N1-2009-P	Cy5-CACAATCTGGACTAGCGGGAGCAGCAT-BHQ-2	1302 → 1328		(Whiley et al., 2009)
	Subtype N2		N2 Neuraminidase (Segment 6)	77	
	N2-F	TGTATCTGACCAACACCACCATAGA	197 → 221		This study
	N2-R	TTGCGGCTTTGACCAATTTC	273 → 254		This study
	N2-P	HEX-AAGGAAATATGCCCCAAACTAGCAGAATAC-BHQ-1	223 → 252		This study
Set 3	Subtype H3		H3 Hemagglutinin (Segment 4)	120	
	H3-F	ACCAGAGAAACAAACTAGAGGCATATT	1020 → 1046		This study
	H3-R	TGTCCTGTGCCCTCAGAATTT	1119 → 1139		This study
	H3-P	FAM-CGGTTGGTACGGTTTCAGGCA-BHQ-1	1095 → 1115		This study
	Subtype N1-Consensus		N1 Neuraminidase (Segment 6)	135	
	N1-F	TATAAGACCTTGCTTCTGGGTTGA	1273 → 1296		This study
	N1-R	GCACCGTCTGGCCAAGAC	1390 → 1407		This study
	N1-P	HEX-AATYTGGACTAGYGGGAGCAGCATWTCYTTTTG-BHQ-1[a]	1330 → 1362		This study
Set 4	Flu A M		Matrix (Segment 7)	106	
	Flu-M-F	GACCRATCCTGTCACCTCTGAC[a]	91 → 112		WHO
	Flu-M-R	AGGGCATTYTGGACAAAKCGTCTA[a]	196 → 173		WHO
	Flu-M-P	FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ-2	169 → 146		WHO
	GAPDH		GAPDH	136	
	GAPDH-F	CCAGGTGGTCTCCTCTGACTTC	939 → 960		This study
	GAPDH-R	CACCCTGTTGCTGTAGCCAAA	1074 → 1054		This study
	GAPDH-P	ROX-CTGGCATTGCCCTCAACGACCAC-BHQ-2	998 → 1020		This study
	Flu B NP		NP (Segment 5)	121	
	Flu B-NP-F	AAGACCTRAGAGTTTTGTCTGCAYT[a]	1184 → 1208		This study
	Flu B-NP-R	ATCAGAGCTGCYCCCATTC	1304 → 1286		This study
	Flu B-NP-P	Cy5-TGCAAGGGTTTCCAYGTTCCAGCA-BHQ-2 [a]	1246 → 1269		This study

[a] D = 33.3% mixture of A, T and 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.

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 A/H1N1 2009 virus and the influenza A virus H5N1 when using the US CDC's rRT-PCR assay, which is recommended by the World Health Organization (WHO) (Peacey et al., 2009). The development of an rRT-PCR assay with improved sensitivity, specificity, and performance, as well as the ability to distinguish pandemic influenza A/H1N1 2009 from seasonal influenza viruses would be most useful.

A TaqMan probe-based, multiplex rRT-PCR assay is described that can detect all currently circulating human seasonal influenza viruses, including the pandemic influenza A/H1N1 2009 virus. The assay can type simultaneously influenza viruses A and B, and the major human subtypes: human H1, H3, N1 and N2. This rRT-PCR protocol 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 named N1-2009 and for the consensus influenza A virus named Flu A M were synthesised as previously described (Whiley et al., 2009) and based on the WHO's recommendations (http://www.euro.who.int/Document/INF/CDC_realtime_RTPCR_H1N1.pdf). Other primers and 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

Table 2
Analysis of the specificity of the multiplex rRT-PCR assay[a].

Virus strain	Subtype	$TCID_{50}$/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	1.8×10^4	1×10^8	–	+	–	–	+	–	+	–
A/Beijing/01/2009	A/H1N1 2009	0.7×10^4	1×10^8	+	+	–	+	+	–	+	–
A/Jiangxi/242/2004	H3N2	1.5×10^4	1×10^8	–	–	+	–	–	+	+	–
A/Beijing/4046/2008	H1N1		1×10^8	–	+	–	–	+	–	+	–
A/Beijing/4053/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
A/Beijing/3012/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
A/Beijing/CY-30/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
A/Beijing/3005/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
A/Beijing/CY-29/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
A/Beijing/4365/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
A/Beijing/3032/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
A/Beijing/3028/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
A/Beijing/4368/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
A/Beijing/3016/2008	H3N2		1×10^8	–	–	+	–	–	+	+	–
B/SH/361/2002	Influenza B	1.8×10^4		–	–	–	–	–	–	–	+
OC43		1.8×10^3		–	–	–	–	–	–	–	–
Adenovirus Holden	Serotype 35	1.8×10^4		–	–	–	–	–	–	–	–
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_Ts were all at 15.3–22.7, Std. Dev. C_Ts 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

Two 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_H1N1.pdf).

2.4. Viral nucleic acid extraction

To extract viral nucleic acids, 200 μL 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 according to the manufacturer's protocol. The viral nucleic acids were stored at −80 °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 μL of nucleic acid solution was added to 23 μL of master mix containing the following components at the indicated final concentrations: 1× reaction buffer, 1 μL 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 the baseline fluorescence was defined as negative.

Table 3
In silico coverage of the primers and probes in the multiplex rRT-PCR assay.

Set	Primer	Coverage years[a]	Total sequences[b]	Hits	Coverage (%)
Set 1	H1-Consensus-F	1918–2009	1402	1390[c]	99.1
	H1-Consensus-R	1918–2009	1402	1311[c]	93.5
	H1-Consensus-P	1918–2009	1402	1402[d]	100
	H1-2009-F	2009	270	269	99.6
	H1-2009-R	2009	270	270	100
	H1-2009-P	2009	270	269	99.6
Set 2	N1-2009-F	2009	188	188	100
	N1-2009-R	2009	188	188	100
	N1-2009-P	2009	188	188[c]	100
	N2-F	1957–2009	2274	2104	92.5
	N2-R	1957–2009	2274	2226	97.9
	N2-P	1957–2009	2274	2230	98.1
Set 3	H3-F	1968–2009	1389	1322	95.2
	H3-R	1968–2009	1389	1251	90.1
	H3-P	1968–2009	1389	1382	99.5
	N1-F	1918–2009	1495	1470	98.3
	N1-R	1918–2009	1495	1445	96.7
	N1-P	1918–2009	1495	1457	97.5
Set 4	Flu-M-F	1918–2009	1326	1314[c]	99.1
	Flu-M-R	1918–2009	1326	1300[c]	98.0
	Flu-M-P	1918–2009	1326	1324	99.8
	Flu B-NP-F	1940–2008	234	234	100
	Flu B-NP-R	1940–2008	234	232	99.1
	Flu B-NP-P	1940–2008	234	233	99.6

[a] The host parameter from the Influenza Virus Resource database was "Human".
[b] The parameters selected from the Influenza Virus Resource database were "Coding region", "Full-length sequences only", "Remove identical sequences" and "Include Lab strains".
[c] "Hits" were calculated using an in-house program (S base matching C or G base, Y base matching C or T base and R base matching A or G base) considering degenerate bases.
[d] "Hits" were determined by the accurate match between the sequence data consensus and its probe.

2.6. Sensitivity, reproducibility, and specificity of the multiplex rRT-PCR assay

The titres of the three known influenza A virus strains (seasonal H1N1, H3N2 and pandemic A/H1N1 2009), influenza B virus, OC43 and Ad35 were measured by 50% tissue culture infective dose ($TCID_{50}$) and calculated by the Reed–Muench method (Cunningham, 1973). Copies of RNA extracts of the three known and 11 unknown subtypes of influenza A virus strains were quantified by SYBR green rRT-PCR. Quantification was done using a standard curve made by serial dilutions of pGEM-NP, a plasmid containing a partial NP gene of influenza A virus (data not shown). The limits of detection (LODs) were determined using 10-fold serial dilutions (10^8–1 copies/reaction or 1.8×10^4 to 1.8×10^{-6} $TCID_{50}$/mL) of the quantified viral RNA extracts and of the plasmids cloned with 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 $TCID_{50}$s (Table 2). The analytical reproducibility assay was calculated at a degree of dilution of 10^5 copies/reaction (3.6×10^{-2} $TCID_{50}$/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-assay and intra-assay variability (coefficient of variation, CV), both measures of the reproducibility of the rRT-PCR assays, was determined by the following formula:

$$\mathrm{CV}(\%) = \frac{\mathrm{SD}\ C_T\mathrm{s}}{\mathrm{Mean}\ C_T\mathrm{s}} \times 100\%$$

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 CNIC. The sequences and nucleotide positions of the primers were H1-F768: 5′-ACTACTGGACTCTGCTGGAAC-3′ (768-788nt), H1-R1094: 5′-CAATGAAACCGGCAATGGCTCC-3′ (1094-1073nt) for the H1 subtype; N1-F1059: 5′-AAGGGGTTTTCATACAGGGTATGGT-3′ (1059-1083nt), N1-R1165: 5′-TCTGTCCATCCATTAGGATCC-3′ (1165-1185nt) for the N1 subtype; H3-F671: 5′-ATCAGGGAGAGTCACAGTCTC-3′ (671-691nt), H3-R940: 5′-ATGCTTCCATTTGGAGTGATGC-3′ (940-919nt) for the H3 subtype and N2-F779: 5′-GGAAATCGTTCATATTAGCCCATTG-3′ (779-803nt), N2-R955: 5′-AGCACACATAACTGGAAACAATGC-3′ (955-932nt) for the N2 subtype. Conventional RT-PCR assays were performed and optimised using the SuperScript™ 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 SuperScript™ III RT/Platinum® 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
Analysis of the limits of detection (LODs) of the multiplex rRT-PCR assay[a].

		$TCID_{50}$/mL	$TCID_{50}$/rxn[b]	Copies/rxn	$TCID_{50}$/mL	$TCID_{50}$/rxn	Copies /rxn	$TCID_{50}$/mL	$TCID_{50}$/rxn	Copies /rxn	$TCID_{50}$/mL	$TCID_{50}$/rxn	Copies/rxn
Virus strain	Subtype	Set 1 (H1-2009/H1-Consensus)			Set 2 (N1-2009/N2)			Set 3 (N1-Consensus/H3)			Set 4 (Flu A M/GAPDH/Flu B NP)		
A/PR8	H1N1	N[c]/1.8	N/3.6×10^{-3}	N/10^4	N/N	N/N	N/N/N	1.8×10^{-1}/N	3.6×10^{-4}/N	10^3/N	1.8×10^{-3}/1.8/N	3.6×10^{-6}/3.6×10^{-3}/N	$10/10^4$/N
A/Beijing/01/2009	A/H1N1 2009	6.7×10^{-1}/667	1.3×10^{-3}/1.334	$10^4/10^7$	6.7×10^{-3}/N	1.3×10^{-5}/N	10^2/N	6.7×10^{-2}/N	1.3×10^{-4}/N	10^3/N	6.7×10^{-2}/6.7×10^{-1}/N	1.3×10^{-4}/1.3×10^{-3}/N	$10^3/10^4$/N
A/Jiangxi/242/2004	H3N2	N/N	N/N	N/N	N/1.5×10^{-1}	N/3×10^{-4}	N/10^3	N/1.5×10^{-3}	N/3×10^{-6}	N/10	1.5×10^{-3}/1.5×10^{-3}/N	3×10^{-6}/3×10^{-6}/N	10/10/N
B/SH/361/2002		N/N	N/N	/[d]	N/N	N/N	/	N/N	N/N	/	N/N/1.8×10^{-6}	N/N/3.6×10^{-9}	/
pGEM T-H1-2009		/	/	1/N	/	/	N/N	/	/	N/N	/	/	N/N/N
pGEM T-N1-2009		/	/	N/N	/	/	1/N	/	/	N/N	/	/	N/N/N
pGEM T-H1		/	/	N/1	/	/	N/N	/	/	N/N	/	/	N/N/N
pGEM T-N1		/	/	N/N	/	/	N/N	/	/	1/N	/	/	N/N/N
pGEM T-H3		/	/	N/N	/	/	N/N	/	/	N/10	/	/	N/N/N
pGEM T-N2		/	/	N/N	/	/	N/1	/	/	N/N	/	/	N/N/N
pGEM T-Flu-A-M		/	/	N/N	/	/	N/N	/	/	N/N	/	/	1/N/N

[a] The amplification curves of LODs are all exceeding the baseline fluorescence (Section 2.5) in a 40-cycle run, and the CVs of the C_T values of LODs are all less than 4% for intra-assays and inter-assays (data not shown).
[b] rxn refers to "reaction".
[c] N refers to "nondetectable".
[d] No item.

35 cycles at 94 °C for 30 s, at 52 °C for 30 s, and at 72 °C for 1 min, followed by 10 min at 72 °C. Amplified products were analysed using electrophoresis on a 1% agarose gel stained with ethidium bromide.

2.8. Nucleotide sequencing

Purified PCR amplicons were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced using an ABI3730 DNA Analyzer (Applied Biosystems, Norwalk, CT).

2.9. Statistical analysis

Statistical significance for the multiplex rRT-PCR assay and other standard detection methods such as for conventional subtyping PCR and the US CDC's real-time PCR was determined via McNemar's chi-square test for matched pairs. A P-value ≤ 0.05 was considered significant.

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_Ts were in the range of 15.3–22.7, the standard deviations (SDs) of the C_T values ranged between 0.0–0.4, and the coefficients of variation (CVs) were all less than 5%. These data demonstrate the high specificity of the primers/probes designed for this novel multiplex rRT-PCR assay.

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 (6.67×10^2 $TCID_{50}$/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_T values of the LODs were all less than 4% based on statistical analysis, suggesting that these results were reproducible.

Table 5
Reproducibility of the multiplex rRT-PCR assay[a].

	Virus	Subtype	$TCID_{50}$/rxn	Copies/rxn[b]	Set 1			Set 2			Set 3			Set 4		
					Mean C_T	SD C_T	CV (%)	Mean C_T	SD C_T	CV (%)	Mean C_T	SD C_T	CV (%)	Mean C_T	SD C_T	CV (%)
Intra-assays	A/PR8	H1N1	3.6×10^{-2}	10^5	26.68	0.16	0.62	N	N	N	27.09	0.02	0.08	23.80	0.14	0.57
	A/Beijing/01/2009	A/H1N1 2009		10^5	25.72	0.14	0.54	27.22	0.15	0.54	29.13	0.13	0.43	26.06	0.08	0.31
	A/Jiangxi/242/2004	H3N2		10^5	N[c]	N	N	30.01	0.29	0.97	26.30	0.13	0.49	26.88	0.14	0.52
	B/SH/361/2002				N	N	N	N	N	N	N	N	N	27.28	0.08	0.31
Inter-assays	A/PR8	H1N1		10^5	26.62	0.12	0.44	N	N	N	27.05	0.05	0.17	23.72	0.11	0.45
	A/Beijing/01/2009	A/H1N1 2009		10^5	25.77	0.04	0.16	27.24	0.02	0.09	29.06	0.11	0.37	26.13	0.18	0.67
	A/Jiangxi/242/2004	H3N2		10^5	N	N	N	29.93	0.11	0.36	26.27	0.05	0.18	26.92	0.05	0.20
	B/SH/361/2002				N	N	N	N	N	N	N	N	N	27.29	0.01	0.04

[a] The amplification curves of all the reactions exceeded the baseline fluorescence (Section 2.5) in a 40-cycle run.
[b] rxn refers to "reaction".
[c] N refers to "nondetectable".

3.4. Reproducibility of multiplex rRT-PCR assay

To assess the reproducibility of the multiplex rRT-PCR assay, mean C_T values and SD C_Ts were calculated at 10^5 copies/reaction for influenza A viruses and at 3.6×10^{-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

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[a]			H3N2			Influenza B			A/H1N1 2009		
	+	–	Total	+	–	Total	+	–	Total	+	–	Total	+	–	Total	+	–	Total
+	119	18	137	0	31	31	52	13	65	68	4	72	0	2	2	31	0	31
–	0	14	14	0	120	120	0	86	86	0	79	79	0	149	149	1	119	120
Total	119	32	151	0	151	151	52	99	151	68	83	151	0	151	151	32	119	151
$\chi 2$	16.06			29.03			11.08			2.25			0.50			0.00		
P[b]	6.15×10^{-5}			7.12×10^{-8}			8.74×10^{-4}			0.13			0.48			1.00		

[a] 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

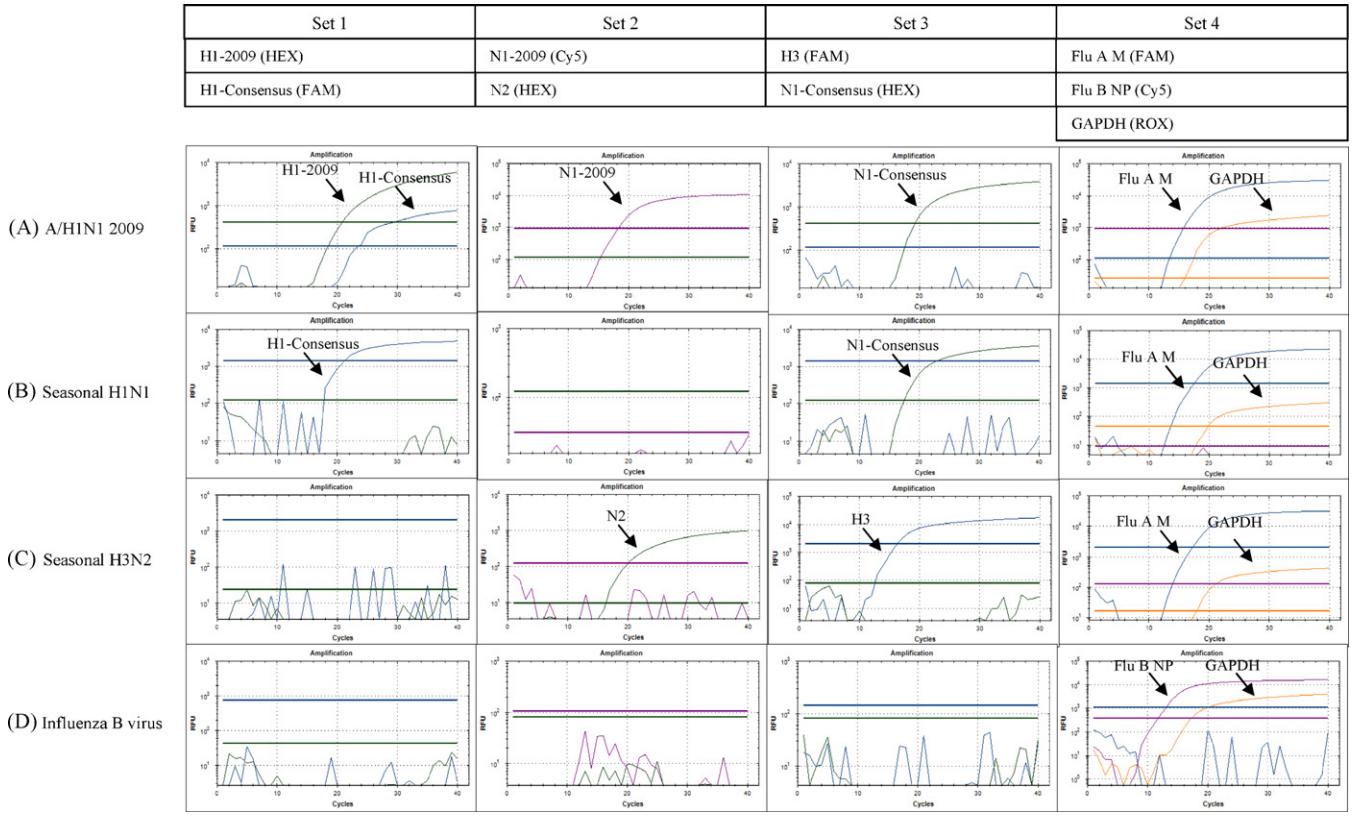

Fig. 1. Representative amplification curve showing typical patterns obtained from the multiplex rRT-PCR assay. (A) Pandemic influenza A/H1N1 2009 virus detected by H1-2009 (HEX channel, green curve) and H1-Consensus (FAM channel, blue curve) in Set 1, N1-2009 (Cy5 channel, violet curve) in Set 2, N1-Consensus (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 2, and H3 (FAM channel, blue curve) in Set 3, and Flu A M (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.)

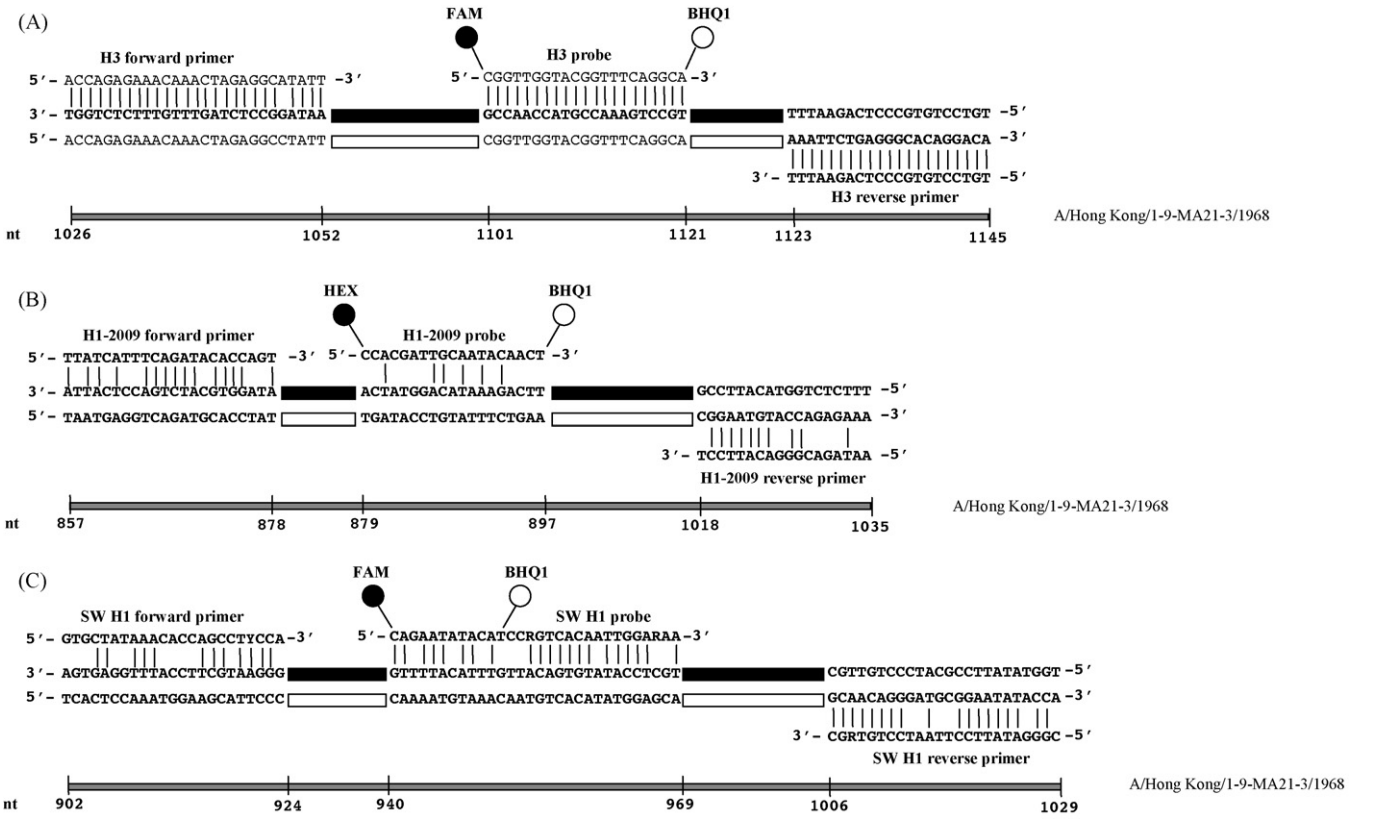

Fig. 2. Sequence alignment of the primers/probes with A/Hong Kong/1-9-MA21-3/1968, a H3N2 strain highly homologous to the sequencing product of sample #LS1125-4. (A) H3 primers/probe designed in this study. (B) H1-2009 primers/probe designed in this study. (C) SW H1 primers/probe of the US CDC's real-time PCR.

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 influenzas.

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).

References

Bose, M.E., Beck, E.T., Ledeboer, N., Kehl, S.C., Jurgens, L.A., Patitucci, T., Witt, L., Lague, E., Darga, P., He, J., Fan, J., Kumar, S., Henrickson, K.J., 2009. Rapid semi-automated Subtyping of influenza during the 2009 swine-origin influenza A H1N1 virus epidemic in Milwaukee, Wisconsin. J. Clin. Microbiol. 47, 2779–2786.

Brownstein, J.S., Freifeld, C.C., Madoff, L.C., 2009. Influenza A (H1N1) virus, 2009 – online monitoring. N. Engl. J. Med. 360, 2156.

Carr, M.J., Gunson, R., Maclean, A., Coughlan, S., Fitzgerald, M., Scully, M., O'Herlihy, B., Ryan, J., O'Flanagan, D., Connell, J., Carman, W.F., Hall, W.W., 2009. Development of a real-time RT-PCR for the detection of swine-lineage influenza A (H1N1) virus infections. J. Clin. Virol. 45, 196–199.

Centers for disease control and prevention (CDC), 2009. Swine influenza A (H1N1) infection in two children – Southern California, March–April 2009. MMWR Morb. Mort. Wkly. Rep. 58, 400–402.

Cunningham, C.H., 1973. Quantal and enumerative titration of virus in cell cultures. In: Kruse Jr., P.F., Patterson Jr., M.K. (Eds.), The Tissue Culture: Methods and Application. Academic Press, Inc., New York, pp. 527–532.

Daum, L.T., Canas, L.C., Arulanandam, B.P., Niemeyer, D., Valdes, J.J., Chambers, J.P., 2007. Real-time RT-PCR assays for type and subtype detection of influenza A and B viruses. Influenza Other Respi. Viruses 1, 167–175.

Dawood, F.S., Jain, S., Finelli, L., Shaw, M.W., Lindstrom, S., Garten, R.J., Gubareva, L.V., Xu, X., Bridges, C.B., Uyeki, T.M., 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N. Engl. J. Med. 360, 2605–2615.

Hall, R.J., Peacey, M., Huang, Q.S., Carter, P.E., 2009. Rapid method to support diagnosis of swine origin influenza virus infection by sequencing of real-time PCR amplicons from diagnostic assays. J. Clin. Microbiol. 47, 3053–3054.

Jiang, T., Kang, X., Deng, Y., Zhao, H., Li, X., Yu, X., Yu, M., Qin, E., Zhu, Q., Yang, Y., Qin, C., 2010. Development of a real-time RT-PCR assay for a novel influenza A (H1N1) virus. J. Virol. Methods 163, 470–473.

Kwok, S., Kellogg, D.E., McKinney, N., Spasic, D., Goda, L., Levenson, C., Sninsky, J.J., 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. 18, 999–1005.

Mackay, I.M., Arden, K.E., Nitsche, A., 2002. Real-time PCR in virology. Nucleic Acids Res. 30, 1292–1305.

Pabbaraju, K., Wong, S., Wong, A.A., Appleyard, G.D., Chui, L., Pang, X.L., Yanow, S.K., Fonseca, K., Lee, B.E., Fox, J.D., Preiksaitis, J.K., 2009. Design and validation of real-time reverse transcription-PCR assays for detection of pandemic (H1N1) 2009 virus. J. Clin. Microbiol. 47, 3454–3460.

Peacey, M., Hall, R.J., Bocacao, J., Huang, Q.S., 2009. Diagnostic assay recommended by the world health organization for swine origin influenza a (h1n1) virus cross-reacts with H5N1 influenza virus. J. Clin. Microbiol. 47, 3789–3790.

Ren, L., Gonzalez, R., Wang, Z., Xiang, Z., Wang, Y., Zhou, H., Li, J., Xiao, Y., Yang, Q., Zhang, J., Chen, L., Wang, W., Li, Y., Li, T., Meng, X., Zhang, Y., Vernet, G., Paranhos-Baccalà, G., Chen, J., Jin, Q., Wang, J., 2009. Prevalence of human respiratory viruses in adults with acute respiratory tract infections in Beijing, 2005–2007. Clin. Microbiol. Infect. 15, 1146–1153.

Wang, R., Sheng, Z.M., Taubenberger, J.K., 2009. Detection of novel (swine origin) H1N1 influenza A virus by quantitative real-time RT-PCR. J. Clin. Microbiol. 47, 2675–2677.

Whiley, D.M., Bialasiewicz, S., Bletchly, C., Faux, C.E., Harrower, B., Gould, A.R., Lambert, S.B., Nimmo, G.R., Nissen, M.D., Sloots, T.P., 2009. Detection of novel influenza A(H1N1) virus by real-time RT-PCR. J. Clin. Virol. 45, 203–204.