Appl Microbiol Biotechnol (2011) 90:1463–1470
DOI 10.1007/s00253-011-3192-8

METHODS AND PROTOCOLS

Simultaneous detection of influenza virus type B and influenza A virus subtypes H1N1, H3N2, and H5N1 using multiplex real-time RT-PCR

Fang Shisong · Li Jianxiong · Cheng Xiaowen · Zhao Cunyou · Wang Ting · Lv Xing · Wang Xin · Wu Chunli · Zhang Renli · Cheng Jinquan · Xue Hong · Yu Muhua

Received: 3 January 2011 / Revised: 25 February 2011 / Accepted: 27 February 2011 / Published online: 12 March 2011

Abstract Use of multiplex real-time reverse transcription polymerase chain reaction (RT-PCR) for the simultaneous detection of influenza type B virus and influenza A virus subtypes H5N1, H3N2, and H1N1 has been described. The method exhibited a high specificity and sensitivity of approximately 10^{1}–10^{2} copies per microliter or 10^{-3}–10^{-2} TCID50/L for each subtype, as well as a high reproducibility with coefficient of variation (CV) ranging from 0.27% to 4.20%. The assays can be performed commendably on various models of real-time PCR instruments; including ABI7500, ROCH 2.0, and Mx3005p. In an analysis of 436 clinical samples from patients during the year 2009, this detection method has successfully identified 261 positive samples, as compared to only 189 positive samples using the conventional cell culture systems, and at the same time further differentiated them as 35 type B, 21 subtype H1N1, and 205 subtype H3N2. The results indicate that the multiplex real-time RT-PCR method is a potential tool for rapid screening of influenza virus from a large pool of clinical samples during flu pandemics and facilitates early influenza virus identification in most public health laboratories around the world.

Keywords Influenza virus · Real-time RT-PCR · Differentiation

This study was supported by Science and Technology grant 2008B080702016 from Guangdong Provincial Department of Science and Technology.

Authors Fang Shisong and Li Jianxiong have contributed equally to this work.

F. Shisong · C. Xiaowen · L. Xing · W. Xin · W. Chunli · Z. Renli · C. Jinquan
Shenzhen Centre for disease control and prevention, Shenzhen, China

L. Jianxiong · W. Ting
School of Public Health, Sun Yat-Sen University, Guangzhou, China

Z. Cunyou · X. Hong
Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

Y. Muhua (✉)
Division of Microbiology Test, Shenzhen NanShan Centre for Disease Control and Prevention, No. 5, NanShang Rd., NanShan District, Shenzhen City, Guangdong Province, China
e-mail: yumuhua@vip.163.com

Introduction

Influenza is an acute infectious disease caused by a member of the family *Orthomyxoviridae*. There are three types of influenza viruses: type A, type B, and type C. Major outbreaks of influenza are always associated with influenza virus type A or B, especially influenza type A virus which has been accounted for four worldwide pandemics during the twentieth century (Luk et al. 2001; Oxford 2000). The most lethal flu pandemic, known as the "Spanish flu," happened during 1918–1919 and killed more than 20 million people. In 2009, a new strain of H1N1 influenza virus, often referred to as "novel influenza A (H1N1)," caused widespread infection to millions of people in a short time and deaths up to tens of thousands of people (Seth et al. 2010).

The influenza virus is an enveloped single negative strand RNA virus. Its genome includes eight gene segments except for type C, which has only seven gene segments. They encode ten proteins, abbreviated as PB1, PB2, PA, HA, NA, NP, M1, M2, NS1, and NS2. Influenza viruses are classified into A, B, and C types based on their antigenic differences in the NP and M proteins. And variations of the surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), determine the subtypes of influenza A virus responsible for an outbreak. Currently, 16 HA subtypes and 9 NA subtypes have been found (Fouchier et al. 2005). Human infections are mainly caused by influenza type B virus and three influenza A virus subtypes H1N1, H3N2, and the novel H1N1 (Xiyan et al. 2004). Cases of human infection with highly pathogenic avian influenza H5N1 virus have also been reported in several countries (Yu et al. 2006; WHO 2010).

Although virus isolation by cell culture has long served as the gold standard for the diagnosis of influenza virus, the approach alone is inefficient when worldwide outbreaks occur as it is less sensitive, time-consuming, and requires considerable technical expertise. Currently, more rapid nonculture methods with higher sensitivity and specificity have been developed, such as immunofluorescence, enzyme immunoassay, and molecular biology methods, which include one-step RT-PCR, real-time reverse transcription polymerase chain reaction (RT-PCR; Van et al. 2001), NASBA (Van et al. 2006), LAMP (Masahiro et al. 2006; Imai et al. 2006), TMA (Hill 2001), microarray (Dawson et al. 2007; Wang et al. 2006), and so on. Among them, real-time RT-PCR has become the main viral detection technique in the event of a pandemic outbreak due to its convenience, speed, and preciseness.

Materials and methods

Virus strains and clinical specimens

Reference strains of 10 H1N1 subtype, 11 H3N2 subtype, 1 H5N1 subtype, and 7 B type were obtained from the Center for Disease Control and Prevention (CDC) of Shenzhen. Reference strains of 20 novel H1N1, 2 adenovirus, 2 parainfluenza III and IV, and 2 respiratory syncytial virus were kindly provided by Guangdong CDC. Altogether, 436 throat swabs were collected from patients with respiratory disease symptoms in two Shenzhen hospitals during 2009.

Primer and probe design

Gene sequences of hemagglutinin (H1, H3, and H5) and neuraminidase (N1 and N2) of influenza A virus subtypes were obtained from GenBank database and analyzed with the MEGA software program (version 3.1; http://www.megasoftware.net). Based on multiple sequence alignments, five gene-specific primer pair/probe sets targeting consensus regions were derived using the Primer Premier (version 5.0; PREMIER Biosoft International, CA, http://www.premierbiosoft.com). The N1 primer pair/probe set was designed on the basis of the alignment outcome of over 100 influenza A virus sequences, including H1N1 and H5N1. The B primer pair/probe set targeting for the NS gene of influenza B virus was modified from those published in Jie et al. (2009). Sequence identity between primer pairs and probes was analyzed using BLASTN (version 2.2.1; NCBI, MD, http://www.ncbi.nlm.nih.gov). The primers and probes used in the present study are listed in Table 1.

Virus isolation and RNA extraction

All influenza reference viruses were cultured in 9- to 11-day-old embryonated chicken eggs as described previously (Wang 1999). The RNAs of reference viruses and clinical specimens were extracted with High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions and were stored at −80°C until use.

One-step multiplex real-time RT-PCR

In the present study, the two one-step multiplex real-time RT-PCR systems were developed using the One-step PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology Co., Dalian, China) and performed on ABI7500 (Applied Biosystems, USA). The duplex real-time RT-PCR system that was used to identify N1 and N2 subtypes was prepared in a 25-μl reaction mixture containing 12.5 ul of ×2 One-Step RT-PCR Buffer, 2.5 U TaKaRa Ex Taq™ HS, 0.5 μl PrimeScript™ RT Enzyme Mix II, 5 μl RNA sample, and two subtype-specific primer pair/probe sets, which comprised primer pairs specific for N1 and N2 genes at a final concentration of 0.6 μM and their detection probes (HEX-labeled N1 and ROX-labeled N2) at concentrations of 0.4 and 0.8 μM, respectively. The cycling parameters for RT-PCR included 5 min at 42°C for reverse transcription, 10 s at 95°C for Taq HS activation, followed by 40 cycles for amplification with 95°C for 5 s and 58°C for 40 s. Fluorescence signals were collected at the end of each amplification step. As for the quadruplex system used to detect H1, H3, H5, and NS genes, the RT-PCR conditions were similar to the duplex system, except different sets of primer pairs and probes were used. Four fluorogenic probes specific for H1, H3, H5, and NS genes, and marked differently with ROX, HEX, FAM, and CY5, were used at final concentrations of 0.2, 0.2, 0.16, and 0.24 μM, respectively. Final primer concentrations were 0.6 μM.

Table 1 Primer pairs and fluorogenic probes used in the two multiplex real-time RT-PCR systems

Target gene	Primer pairs/probe set	Sequence(5′-3′)	Location [a]
H1-HA	H1-F	AACTACTACTGGACTCTGCTGGAA	762–785
	H1-R	CCATTGGTGCATTTGAGGTGATG	897–875
	H1-probe	(ROX)-TGAYCCAAAGCCTCTACTCAGTGCGAAAGC-(BHQ2)	869–840
H3-HA	H3-F	AAGCATTCCYAATGACAAACC	872–892
	H3-R	ATTGCRCCRAATATGCCTCTAGT	1018–996
	H3-probe	(HEX)-CAGGATCACATATGGGSCCTGTCCCAG-(BHQ1)	908–934
H5-HA	H5-F	TGGAAAGTGTAARAAACGGAACGT	1481–1504
	H5-R	YGCTAGGGAACTCGCCACTG	1629–1610
	H5-probe	(FAM)-TGACTACCCGCAGTATTCAGAAGAAGCAAGACTAA	1506–1540
B-NS	Bns-R	TCATGTCAGCTATTATGGAGCTGTT	991–967
	Bns-F	ATGGCCATCGGATCCTCAACTCACTC	748–773
	Bns-probe	(CY5)-TATCCCAATTTGGTCAAGAGCACCGATTATCACCAG-(BHQ3)	838–873
N1-NA	N1-F	TGGACYAGTGGGAGCAGCAT	1332–1351
	N1-R	TGTCAATGGTRAAYGGCAACTC	1432–1407
	N1-probe	(HEX)-TGGTCTTGGCCAGACGGTGC-(BHQ1)	1386–1404
N2-NA	N2-F	CAGGAGTCRGAATGCGTT	650–667
	N2-R	AYATGCTGAGCACTYCCT	798–781
	N2-probe	(ROX)-TGTACAGTAGTAATGACKGATGGRAGTGC-(BHQ2)	683–711

[a] Primer and probe positions for subtype H1N1 influenza virus corresponding to the HA and NA genes of A/Guangzhou/483/2006 (H1N1, GenBank accession nos. EU382981 and EU382988), for subtype H3N2 corresponding to HA and NA genes of A/Shanghai/N20/2008 (H3N2, GenBank accession nos. GU068345 and GU064856), for subtype H5 corresponding to HA gene of A/Shenzhen/406H/2006 (H5N1, GenBank accession no. EF137706), for type B corresponding to those described previously (Jie et al. 2009)

The reaction was performed for 15 min at 42°C for reverse transcription, 2 min at 95°C for activating the Taq HS, followed by 40 cycles for amplification with 95°C for 10 s and 58°C for 40 s. The results were collected and analyzed using the ABI 7000 system SDS software (version 1.3, Applied Biosystems).

Specificity of the multiplex real-time RT-PCR system

Reference strains of human influenza A/B and strains from the genus *Respirovirus* that cause similar respiratory symptoms as influenza were used to evaluate the specificity of the two multiplex real-time RT-PCR systems. Positive controls of influenza A strains, H5N1, H3N2 and the novel H1N1, were previously identified by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays. Other *Respirovirus* strains that served as negative controls included adenovirus, parainfluenza III and IV, respiratory syncytial virus, and so on.

Sensitivity and reproducibility of the multiplex real-time RT-PCR system

The sensitivity of the multiplex amplification was evaluated by viral load testing in terms of RNA copy number. The target gene segments of H1, H3, N1, N2, and B were amplified from the influenza virus strains A/Jiangxi donghu/312/2006(H3N2), A/luohu/219/2006(H1N1) and B/shenzhen/155/2005. The cDNA of the H5 was obtained from the Center for Disease Control and Prevention of Shenzhen. The amplified products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and transformed into the DH5α (TaKaRa Biotechnology Co., Dalian, China). The recombinant plasmids were used as templates for RNA synthesis using RiboMax T7 Express In Vitro Transcription System (Promega, Madison, WI) following the manufacturer's instruction after linearization by SalI (TaKaRa Biotechnology Co., Dalian, China). The concentrations of the in vitro-transcribed RNAs were determined with BioPhotometer (Eppendorf, Hamburg, Germany), and tenfold serial dilutions of each transcribed RNA, ranging from 10^7 to 10^0 copies per microliter, were prepared. Both duplex and quadruplex real-time RT-PCR assays were then carried out using each dilution of target-transcribed RNAs according to the method described previously (Wu et al. 2008). The results were analyzed to determine the minimal detectable viral RNA copy number, mean threshold cycle (CT), standard deviation, and coefficient of variation.

To further evaluate the sensitivity and detection efficiencies of the multiplex assays, the 50% tissue culture

Table 2 Reproducibility of the two multiplex assays

Quadruplex RT-PCR system								
Copy number	H1		H3		H5		B	
	Mean CT	CV	Mean CT	CV	Mean CT	CV	Mean CT	CV
10^7	21.57	1.23%	21.70	0.99%	16.15	0.92%	16.53	4.20%
10^6	25.06	1.00%	25.05	0.46%	18.62	9.13%	20.41	2.59%
10^5	28.42	1.18%	28.30	0.36%	22.37	0.61%	23.14	1.16%
10^4	31.86	1.11%	31.14	0.51%	25.47	0.46%	27.24	0.40%
10^3	34.79	0.78%	33.89	1.33%	28.30	0.27%	29.67	0.39%
10^2	37.19	0.98%	37.56	2.82%	31.19	1.09%	32.81	0.93%
10^1	NA	NA	NA	NA	35.65	3.07%	35.51	1.02%
Duplex RT-RCR system								
Copy number	N1				N2			
	Mean CT		CV		Mean CT		CV	
10^7	19.92		4.05%		21.57		1.23%	
10^6	22.99		1.06%		25.06		1.00%	
10^5	26.37		3.56%		28.42		1.18%	
10^4	29.71		0.39%		31.86		1.11%	
10^3	32.74		1.30%		34.79		0.78%	
10^2	36.85		2.86%		37.19		0.98%	
10^1	38.16		3.72%		NA		NA	

infectious doses ($TCID_{50}$) of H1N1, H3N2, type B strains were first determined as described in WHO (2002). Using the predetermined $TCID_{50}$ value, serially diluted virus strains ranging from 10^3 to 10^{-3} $TCID_{50}$ per milliliter were prepared and tested on the assays. At the same time, colloidal gold immunochromatographic assay (KaiBiLi, Hangzhou, China) was employed to determine the virus titer in the dilutions.

Applicability on different instruments and comparison with conventional method

In order to verify the assay applicability on different instruments, multiplex real-time RT-RCR was carried out using tenfold serial dilutions (from 10^7 to 10^0 copies per microliter) of each transcribed RNA on ABI7500 (Applied Biosystems, USA), ROCHE2.0 (Roche Diagnostics, France), and Stratagene Mx-3005p (Agilent Technologies Inc., USA). Four hundred thirty-six clinical specimens obtained from two Shenzhen hospitals were analyzed using the two multiplex assays, and the detection efficiencies were determined by comparison with the conventional culturing method.

Results

The two multiplex real-time RT-PCR systems

The six sets of primer pair/probe (Table 1) designed for the two multiplex real-time RT-PCR systems worked successfully under the cycling conditions as stated in "One-step multiplex real-time RT-PCR" Section. The results indicated that fluorescence could be detected for all strain-specific

Table 3 Reproducibility of the two multiplex assays on three different instruments

Copy number	Quadruplex system		Duplex system			
	H1	H3	H5	B	N1	N2
10^7	3.07%	1.98%	1.33%	4.90%	5.18%	1.87%
10^6	2.32%	2.39%	1.62%	3.64%	4.40%	1.53%
10^5	2.37%	2.33%	1.59%	2.23%	3.92%	2.04%
10^4	1.34%	2.11%	2.06%	2.00%	3.21%	1.33%
10^3	1.71%	1.69%	3.09%	2.94%	1.28%	1.59%
10^2	1.99%	1.08%	1.34%	2.18%	1.68%	2.12%
10^1	NA	NA	2.34%	1.76%	0.33%	1.63%

Table 4 Amplification efficiencies on different instruments

Instrument	H1	H3	H5	B	N1	N2
ABI7500	102.65%	116.00%	113.81%	111.19%	102.21%	101.78%
ROCH2.0	98.20%	113.49%	114.30%	99.66%	93.84%	109.37%
Mx3005p	99.40%	113.80%	104.50%	97.00%	121.30%	120.30%

probes in both duplex and quadruplex real-time RT-PCR systems, and the signal intensities specific for N1, N2, H1, H3, H5, and B genes were all comparable.

The specificity of the assays

Virus strains of influenza H1N1, H3N2, H5N1, novel influenza A (H1N1), influenza B, and other *Respirovirus* were subjected to the two multiplex assays to evaluate the assay specificity. Fluorescence signals for reference strains of 10 H1N1, 11 N3N2, 1 H5N1, and 7 B influenza viruses were strong, and none gave false-positive signals caused by non-specific PCR products. On the other hand, no positive fluorescent signal was yielded in the assays of other *Respirovirus*, including 20 novel H1N1 influenza virus, 2 adenovirus, 2 parainfluenza III and IV, and 2 respiratory syncytial virus. The results demonstrated that these systems be capable of detecting human influenza viruses with high specificity.

The sensitivity and reproducibility of the assays

The detection limits of N1 and N2 genes in the duplex real-time RT-PCR system were as low as 10^1 and 10^2 RNA copies per microliter, respectively, whereas H1, H3, H5, and B genes in the quadruplex system have their respective detection limits of 10^2, 10^2, 10^1, and 10^1 RNA copies per microliter. The results showed that these systems have high sensitivity to be explored as potential diagnostic tools.

Efficiency and reproducibility of the assay

The standard curves of various target genes were plotted with the threshold cycle numbers (CT) against the dilution factors of their transcribed RNAs. The correlation coefficient between CT values and dilution factors exceeded 99% for all detected genes in the two multiplex amplification assays. Calculating from the slope of the linear regression line, the amplification efficiencies of N1 and N2 genes in the duplex real-time RT-PCR assay were estimated to be 103.98% and 107.23%, respectively. And in the quadruplex real-time RT-PCR assay, the amplification efficiencies of H1, H3, H5, and B-NS genes were estimated to be 107.23%, 110.17%, 108.20%, and 107.71%, respectively.

As shown in Table 2, the coefficient of variations (CV) of all target genes, except that of B type, were between 0.27% and 4.20%, indicating that the amplification of each target gene in the two multiplex systems was highly reproducible.

In another study using serial diluted virus strains, the standard curves were plotted with the threshold cycle numbers (CT) against the dilution factors based on previously determined $TCID_{50}$ of each virus strain. The two multiplex assays could detect influenza virus concentrations as low as 10^{-2} $TCID_{50}$ per milliliter, whereas the lowest detectable virus concentration using colloidal gold-based immunochromatographic assay was 10^1–10^2 $TCID_{50}$ per milliliter. The amplification efficiencies of viral RNAs of H1N1, H3N2, and B were found to be 106.76%, 164.73%, 116.56% for H1, H3, B genes in the quadruplex assay and 112.75% and 124.32% for N1 and N2 genes in the duplex real-time RT-PCR assay. The results showed that the multiplex systems had high amplification efficiency and reproducibility.

Applicability of the assays

Multiplex assays using ABI7500, ROCHE2.0, and Stratagene Mx-3005p were carried out on each dilution of the in vitro-transcribed RNAs. The results are shown in Table 3. Except N1, all target genes exhibited high reproducibility in both systems, with comparable CVs around 1–2%. Similarly, the amplification efficiencies (Table 4) of each gene were between 90% and 120%. Even though some of them exhibited significant discrepancies between different instruments, there was no significant statistical difference ($P>0.05$)

Table 5 Comparison of the diagnostic efficiency between multiplex real-time RT-PCR and conventional culturing method

Type/subtype	No. of positive samples by multiplex real-time RT-PCR	No. of positive samples by conventional culturing
H1	21	11
H3	205	160
B	35	18
Total	261	189

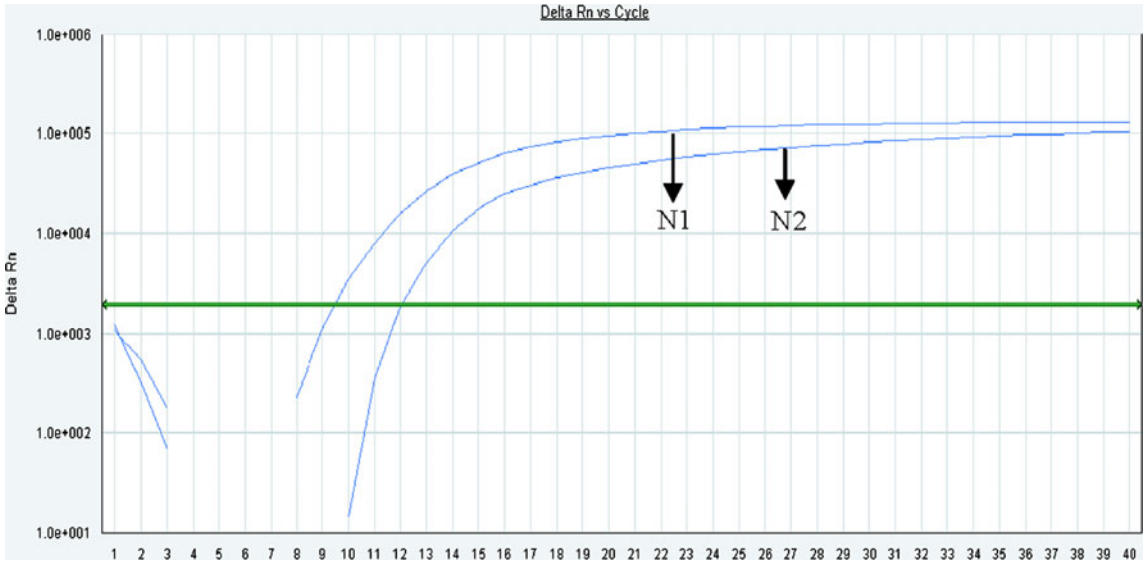

Fig. 1 Amplification plot for detection of N1 and N2 genes in a duplex real-time RT-PCR

between their CT values. The study indicated that the multiplex systems can be performed on different instruments.

Comparison between the two multiplex real-time RT-PCR and conventional culture methods

Four hundred thirty-six clinical samples (throat swabs) collected from two Shenzhen hospitals during 2009 were tested using the multiplex real-time RT-PCR assays and the cell culture methods. Influenza virus type and subtyping of positive sample cultures were identified by the aerosol and HI tests as described in WHO (2002).

As seen in Table 5, 261 (35 positive for type B, 21 positive for subtype for H1N1, and 205 positive for subtype H3N2) out of 436 samples (59.86%) were confirmed positive by the multiplex real-time RT-PCR assays, while by conventional culturing method, only 189 samples (43.35%) were identified as H1, H3, and B influenza virus, and they could all be typed or subtyped by the multiplex real-time RT-PCR assays. The multiplex RT-PCR was therefore more sensitive than the conventional culturing method.

Discussion

A rapid and accurate diagnostic tool for influenza infection is very important for patient management and prevention of influenza pandemics, especially during an outbreak, where traditional methods like cell culture, embryonated egg culture, and immunoassay were unable to accomplish.

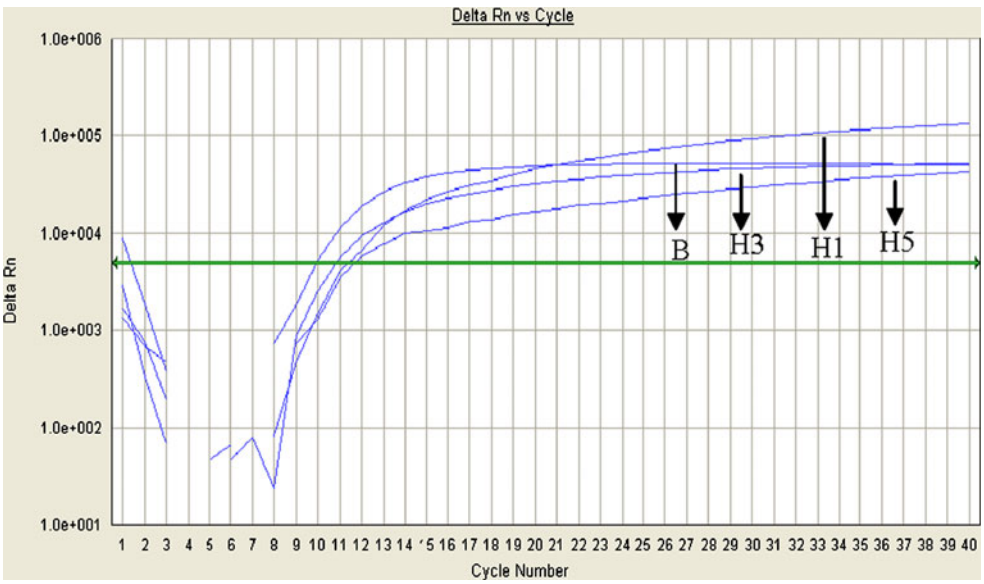

Fig. 2 Amplification plot for detection of H1, H3, H5, and B genes in a quadruplex real-time RT-PCR

Nowadays, more and more laboratories have started to research and employ molecular methods in the detection of influenza viruses, and lots of molecular assays for diagnosis of influenza have been developed.

In our study, two rapid and sensitive real-time RT-PCR assays able to accurately differentiate major influenza type/subtypes in a short time have been designed. The sensitivity of the assays can attain a detection limit of approximately between 10^2 and 10^1 copies per microliter, which are more sensitive than normal RT-PCR assays Boonsuk et al. (2008); Wei et al. 2006; Jie et al. 2009) and are similar to that of signal real-time RT-PCR and multiplex real-time RT-PCR previously reported by other researchers (Joanna et al. 2007; Suwannakarn et al. 2008; Van et al. 2001). The sensitivity difference among various subtypes might be attributed to a number of factors. For instance, the designed primer pairs/probes or the reaction conditions are more favorable for amplifications of H5, B, and N1 genes. Moreover, the sensitivity of all tested virus strains based on $TCID_{50}$ can be as low as 10^{-2} $TCID_{50}$ per milliliter, and even down to 10^{-3} $TCID_{50}$ per milliliter for type B. In comparison with the conventional methods, such as culture methods, immunoassays, or the colloidal gold immunochromatographic assay (Chana et al. 2007), the multiplex real-time RT-PCR assays in the present study have proved to have high specificity and sensitivity almost approaching 100% as they successfully recognized all positives, as well as subtypes, from a large number of clinical samples.

The PCR amplification efficiency is an important indication to evaluate the performance of real-time PCR systems. In the present study, two methods were used to determine the PCR amplification efficiency. One was by measuring the linear regression slope of the amplification plot with CT versus each serial dilution of the in vitro-transcribed RNAs, while another was the linear regression slopes of the reflected multiplex amplification CT values versus each serial dilution based on $TCID_{50}$ of the virus strains. PCR amplification efficiency using the in vitro-transcribed RNAs was between 103% and 110%, which is slightly higher than previously reported (Wu et al. 2008; Figs. 1 and 2) . The results indicated that non-specific interactions of these primers and probes used in the two multiplex systems were weak. However, in another testing using virus strains, H1 subtype gave an inconsistent efficiency/reproducibility with its serial dilutions possibly caused by non-specific amplifications. The complexity of viral extracts isolated from the embryonated egg culturing, for example, the cellular nucleic acids, protein, and other genes of influenza, has an inhibitory effect on the PCR amplification. Another factor reducing amplification efficiency is the type of specimens for detection of respiratory virus—throat swabs may have reduced sensitivity for detection of human influenza when compared to nasopharyngeal swabs (Robinson et al. 2008).

To evaluate the assay performance on various real-time PCR instruments, tenfold serial dilutions of the in vitro-transcribed RNA were amplified on three different instruments, ABI7500, ROCHE2.0, and Stratagene Mx-3005P. The multiplex assays on all target genes gave very stable amplification performances on each of the three instruments. The CV difference from the results of three instruments was found to be higher than that from the six repetitive experiments on ABI7500. The CT values and amplification efficiencies are also varied for each subtype and on different instruments. That was probably due to the differences in the instrument's detection technique and sensitivity, yet the differences were not statistically significant, and the relative relationships between different subtypes were alike.

Large numbers of reference influenza virus strains and clinical samples isolated by cultivation were employed to study the assay specificity. The primers and probes used in the present study were designed to target the NS gene specific for Type B, and the HA genes of subtypes H1, H3, H5, as well as the NA genes of N1, N2 influenza virus. In addition, other influenza subtypes, such as the novel influenza A (H1N1), and *Respirovirus* were also used as the clinical symptoms of these virus infections were the same as influenza. Fluorescence signal could only be detected when B, H1N1, H3N2, and H5N1 viruses were tested on the quadruplex multiplex assay and also when the H3N2, H1N1, and H5N1 virus samples were assayed on the duplex multiplex system. However, marked differences in nucleotide sequence between human seasonal H1N1 and novel H1N1 virus make one weakness that these two multiplex RT-PCR systems cannot differentiate novel H1N1 virus from seasonal H1N1.

In conclusion, the two one-step multiplex real-time RT-PCR systems offer a rapid, sensitive, specific, stable, and widely applicable method for the identification and subtyping of human infectious influenza virus, including H1N1, H3N2, H5N1, and B. The method can be further explored to become a useful molecular diagnostic tool for influenza virus detection when dealing with lots of clinical samples in daily surveillance and during influenza virus outbreaks.

Acknowledgments We thank Guangdong CDC for provision of reference strains of novel H1N1, adenovirus, parainfluenza III and IV, and respiratory syncytial virus. We also thank China National Influenza Centre for their technical support and confirmation to these two real-time RT-PCR systems.

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