



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.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



# One-step real-time reverse transcription-PCR assays for detecting and subtyping pandemic influenza A/H1N1 2009, seasonal influenza A/H1N1, and seasonal influenza A/H3N2 viruses

Mina Nakauchi<sup>a</sup>, Yoshihiro Yasui<sup>b</sup>, Tatsuya Miyoshi<sup>c</sup>, Hiroko Minagawa<sup>b</sup>, Tomoyuki Tanaka<sup>c</sup>, Masato Tashiro<sup>a</sup>, Tsutomu Kageyama<sup>a,\*</sup>

<sup>a</sup> Influenza Virus Research Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan

<sup>b</sup> Aichi Prefectural Institute of Public Health, 7-6 Nagare, Tsujimachi, Kita-ku, Nagoya, Aichi 462-8576, Japan

<sup>c</sup> Sakai City Institute of Public Health, 3-2-8 Kaichouhigashi, Sakai-ku, Sakai, Osaka 590-0953, Japan

## A B S T R A C T

Pandemic influenza A/H1N1 2009 (A/H1N1pdm) virus has caused significant outbreaks worldwide. A previous one-step real-time reverse transcription-PCR (rRT-PCR) assay for detecting A/H1N1pdm virus (H1pdm rRT-PCR assay) was improved since the former probe had a low melting temperature and low tolerance to viral mutation. To help with the screening of the A/H1N1pdm virus, rRT-PCR assays were also developed for detecting human seasonal A/H1N1 (H1 rRT-PCR assay) and A/H3N2 influenza viruses (H3 rRT-PCR assay). H1pdm, H1, and H3 rRT-PCR assays were evaluated using *in vitro*-transcribed control RNA, isolated viruses, and other respiratory pathogenic viruses, and were shown to have high sensitivity, good linearity ( $R^2 = 0.99$ ), and high specificity. In addition, the improved H1pdm rRT-PCR assay could detect two viral strains of A/H1N1pdm, namely, A/Aichi/472/2009 (H1N1)pdm and A/Sakai/89/2009 (H1N1)pdm, which have mutation(s) in the probe-binding region of the hemagglutinin gene, without loss of sensitivity. Using the three rRT-PCR assays developed, 90 clinical specimens collected between May and October 2009 were then tested. Of these, 26, 20, and 2 samples were identified as positive for A/H1pdm, A/H3, and A/H1, respectively, while 42 samples were negative for influenza A viruses. The present results suggest that these highly sensitive and specific H1pdm, H1, and H3 rRT-PCR assays are useful not only for diagnosing influenza viruses, but also for the surveillance of influenza viruses.

© 2010 Elsevier B.V. All rights reserved.

## Article history:

Received 29 July 2010

Received in revised form 12 October 2010

Accepted 19 October 2010

Available online 26 October 2010

## Keywords:

Diagnosis

Real-time RT-PCR

A/H1N1pdm

Influenza

## 1. Introduction

Since the identification of the novel H1N1 subtype of influenza A virus (pandemic influenza A/H1N1 2009 virus) in Mexico and the United States in March and April 2009, respectively, (WHO, 2009a,b), the virus has spread worldwide. According to WHO, as of February 7, 2010, more than 212 countries and overseas territories or communities have reported laboratory-confirmed cases of pandemic influenza, including at least 15,292 deaths (<http://www.who.int/csr/don/2010.02.12/en/index.html>).

To prepare for the entry of pandemic influenza A/H1N1 2009 (A/H1N1pdm) virus in Japan, the National Institute of Infectious Diseases (NIID) developed a one-step real-time reverse transcription-PCR (rRT-PCR) assay and conventional RT-PCR method for specifically detecting A/H1N1pdm virus by April 29,

2009 (Kageyama et al., *in press*). By sharing these laboratory diagnostic systems consisting of primers, probes, reagents, positive controls, and a manual with 75 prefectural and municipal public health institutes and 15 quarantine stations, preparations to detect A/H1N1pdm virus were completed by May 4, 2009 (Kageyama et al., *in press*). After the first cases of A/H1N1pdm virus infection were identified at Narita Airport Quarantine Station, Japan, on May 9, 2009 (Shimada et al., 2009), the virus spread and was confirmed (using these diagnostic assays) in all prefectures of Japan by July 16, 2009 (report by Infectious Disease Surveillance Center of NIID [[http://idsc.nih.gov/jp/disease/swine\\_influenza\\_e/index.html](http://idsc.nih.gov/jp/disease/swine_influenza_e/index.html)]).

In August 2009, a previous rRT-PCR assay was improved so as to detect A/H1N1pdm viruses more specifically and sensitively, since the probe that was used previously had a low melting temperature and low tolerance to viral mutation. Furthermore, to help with the screening of the A/H1N1pdm virus, rRT-PCR assays were developed also for detecting human seasonal A/H1N1 influenza (A/H1N1) and A/H3N2 influenza (A/H3N2) viruses. These highly sensitive and specific rRT-PCR assays for detecting and subtyping A/H1N1pdm,

\* Corresponding author. Tel.: +81 42 561 0771; fax: +81 42 561 0812.  
E-mail address: [tkage@nih.go.jp](mailto:tkage@nih.go.jp) (T. Kageyama).

seasonal A/H1N1, and seasonal A/H3N2 viruses will be useful not only for diagnosing influenza viruses, but also for the surveillance of influenza viruses.

## 2. Materials and methods

### 2.1. Primer and probe design

The probe for detecting the A/H1N1pdm virus was redesigned by comparing the former probe sequence and the corresponding sequences of hemagglutinin (HA) genes from A/H1N1pdm viruses posted on the Global Initiative on Sharing Avian Influenza Data (GISAID) database between August and October 2009. The nucleotide sequences were aligned using Clustal W software (Larkin et al., 2007). The sequence of the probe was modified slightly to match the HA gene of the recently circulating virus, and the redesigned probe was elongated (seven nucleotides longer than the former probe) to increase the melting temperature and thus improve the sensitivity and specificity of the assay. Subtyping primers and probes for detecting human seasonal A/H1N1 and A/H3N2 viruses were designed to correspond to each HA gene by comparing sequences posted over the past five years on the GISAID database. The type A typing primers and probe for detecting influenza A virus were designed previously to correspond to conserved regions of the matrix gene segment (Kageyama et al., in press). The rRT-PCR assay for detecting influenza A virus (Type A rRT-PCR assay) was used as a control for evaluating the new and improved rRT-PCR assays. The sequences and position of the primers and probes are listed in Table 1. The sequences of the primers and probes for Type A and H1pdm rRT-PCR assays are disclosed on the Global Influenza Programme website ([http://www.who.int/csr/resources/publications/swineflu/WHO\\_Diagnostic.RecommendationsH1N1\\_20090521.pdf](http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic.RecommendationsH1N1_20090521.pdf)).

### 2.2. Viruses

All influenza viruses included in this study were isolated in Madin-Darby Canine Kidney (MDCK) cells. In addition to A/H1N1pdm, several subtypes/types of seasonal influenza viruses used for validating the specificity of the assay were as follows: A/Narita/1/2009 (H1N1)pdm, A/Hiroshima/310/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, A/Sakai/89/2009 (H1N1)pdm, A/Brisbane/59/2007 (H1N1), A/Shiga/8/2008

(H1N1), A/Ehime/38/2008 (H1N1), A/Yokohama/95/2008 (H1N1), A/Kanagawa/69/2008 (H1N1), A/Fukui/91/2008 (H1N1), A/Akita/10/2008 (H1N1), A/Fukui/77/2008 (H1N1), A/Sapporo/16/2008 (H1N1), A/Uruguay/716/2007 (H3N2), A/Toyama/123/2008 (H3N2), A/Yamanashi/135/2008 (H3N2), A/Hiroshima-C/41/2008 (H3N2), B/Sakai/41/2008, and B/Mie/1/2009.

The viral respiratory pathogens used for validating the specificity of the assay were as follows: respiratory syncytial virus A, respiratory syncytial virus B, human parainfluenza viruses types 1–4, Human rhinoviruses types 1B, 2, 14, 36, and 89, human metapneumovirus, and human coronaviruses OC43, 229E, and NL63. Human metapneumovirus and human parainfluenza viruses 2 and 4 were obtained from Sendai Medical Center. Human rhinoviruses types 1B, 2, 14, 36, and 89 were obtained from Nagasaki Prefectural Institute for Environmental Research and Public Health. Respiratory syncytial viruses A and B, human parainfluenza viruses 1 and 3, and human coronaviruses were stored at NIID.

### 2.3. Preparation of RNA transcript controls

To construct an RNA-positive control for each rRT-PCR assay, each target gene segment was amplified by RT-PCR, and the resulting PCR product containing T7 promoter was then transcribed *in vitro*. The detailed procedure is described below.

The primer Uni12 (5'-AGCAAAAGCAGG-3') (Hoffmann et al., 2001) was used for RT using a SuperScript<sup>®</sup> III Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's instructions. The matrix gene of the A/Narita/1/2009 (H1N1)pdm virus was amplified using the following paired primers: Narita\_MP.F (5'-ATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTTCTATCATC-3') and T7\_Narita\_MP.R (5'-TAATACGACTACTATAGGGTTACTCTAGCTCTATGTTGACAAAATGACCATCGTC-3'). The HA gene segments of A/Narita/1/2009 (H1N1)pdm, A/Brisbane/59/2007 (H1N1), and A/Uruguay/716/2007 (H3N2) viruses were amplified using the following paired primers: Narita\_HA.F (5'-AGCAAAAGCAGGGGAAAA-CAAAAGCAACAAAATGAAGCAATACTAGTAGTCTG-3') and T7\_Narita\_HA.R (5'-TAATACGACTACTATAGGGAGTAGAAACAAGGGT-GTTTTTCTCATGCTTCTGAAATCCTAATGTTAAATAC-3'), Brisbane\_HA.F (5'-AGCAAAAGCAGGGGATAATAAAAAACAACCAGAATGAAAG-TAAAACACTGCTGCTTAT-3') and T7\_Brisbane\_HA.R (5'-TAATACGACTACTATAGGGTAGTAGAAACAAGGGTGTTCCTTATATTC-TGAAATCTGGTCTTAGATG-3'), and Uruguay\_HA.F (5'-AGCAAAA-

**Table 1**  
Primers and probes.

Primer and probe names	Primer and probe sequences (5'–3')	Orientation	Target gene	Position <sup>a</sup>
Primers and probe set for the <b>H1pdm</b> rRT-PCR assay				
NIID-swH1 TaqMan Primer-F1	AGAAAAGAATGTAACAGTAACACTCTGT	+	HA	111–140
NIID-swH1 TaqMan Primer-R1	TGTTCCACAATGTARGACCAT	–	HA	276–297
NIID-swH1 Probe2	(FAM) <u>CAGCCAGCAATRTTCATTACC</u> (MGB) <sup>b</sup>	–	HA	208–230
Primers and probe set for the <b>H1</b> rRT-PCR assay				
NIID-H1 TaqMan Primer-F1	CCCAGGGYATTTTCGCGACTATGAG	+	HA	324–348
NIID-H1 TaqMan Primer-R1	CATGATGCTGAYACTCCGGTTACG	–	HA	432–455
NIID-H1 Probe1	(FAM)TCTCAAAYGAAGATACTGAACT(MGB)	–	HA	367–387
Primers and probe set for the <b>H3</b> rRT-PCR assay				
NIID-H3 TaqMan Primer-F1	CTATTGGACAATAGTAAAACCGGGRGA	+	HA	744–770
NIID-H3 TaqMan Primer-R1	GTCAATGGGRATGCTCCATTG	–	HA	898–921
NIID-H3 Probe1	(FAM)AAGTAACCCCKAGGAGCAATTAG(MGB)	–	HA	799–821
Primers and probe set for the <b>Type A</b> rRT-PCR assay				
MP-39-67For	CCMAGGTCGAAACGTAYGTTCTCTCATC	+	M	14–42
MP-183-153Rev	TGACAGRATYGGTCTTGTCTTAGCCAYTCCA	–	M	128–159
MP-96-75ProbeAs	(FAM)ATYTCGGCTTTGAGGGGCGCTG(MGB)	–	M	50–71

The NIID-swH1 Probe2 is seven nucleotides longer than the former probe, NIID-swH1 Probe. The seven nucleotides are underlined.

<sup>a</sup> The nucleotide positions of HA and M genes are based on cRNA sequences obtained from GenBank. Accession numbers for HA genes of A/Narita/1/2009 (H1N1)pdm, A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2) viruses, and the M gene of the A/Narita/1/2009 (H1N1)pdm virus are GQ165815, CY030230, EU716428, and GQ169302, respectively.

<sup>b</sup> Probes are labeled with FAM at the 5' end and MGB at the 3' end.

GCAGGGGATAATCTATTAACCATGAAGACTATCATTGCTTTGAGCT-ACATTCTATG-3') and T7.Uruguay\_HA\_R (5'-TAATACGACTC-ACTATAGGGCAGTAGAAACAAGGGTGTTTTAAATTAATGCACTCAAA-TGCAAATGTTGC-3'). PCR was performed using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). The PCR products were purified using NucleoSpin® (Macherey-Nagel GmbH & Co. KG, Duren, Germany) and transcribed using the MegaScript® T7 In Vitro Transcription Kit (Ambion) according to each manufacturer's instructions. After DNase digestion to remove residues of RT-PCR products, the transcribed RNA was purified twice using an RNA Isolation Kit (Agilent Technologies, Santa Clara, CA, USA) and quantified by spectrophotometric analysis.

#### 2.4. Sequencing of the partial HA gene of pandemic influenza A/H1N1 2009

To confirm the sequence of target region for primers and probe of H1pdm rRT-PCR assay, partial HA genes of A/Narita/1/2009 (H1N1)pdm, A/Hiroshima/310/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, and A/Sakai/89/2009 (H1N1)pdm were sequenced. To amplify the partial HA gene between -32 and +366 (from the start codon) using extracted RNA, RT-PCR was carried out with the paired primers H1HA1-BEGIN (5'-AGCAAAAGCAGGGGAAAATAA-3') and swine H1-385-366R

(5'-CAAATGATGACACTGAGCTC-3') using the OneStep RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RT-PCR conditions were as follows: 30 min at 50 °C to activate RT followed by initial denaturation for 15 min at 95 °C, with a subsequent 45 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 40 s). The PCR products were purified and then sequenced using swine H1-56-76F primer (5'-CATTATGTATAGGTTATCATG-3'). Total RNA was prepared as described in Section 2.6.

#### 2.5. Clinical specimens

Nasal and/or pharyngeal swabs collected from suspected and contact cases of influenza and suspended in virus transport medium were obtained from prefectural and municipal public health institutes and quarantine stations in Japan. These specimens were collected between May and October 2009.

#### 2.6. RNA preparation

Supernatants of cultured MDCK cells were centrifuged at 10,000 × g for 10 min. Viral RNA was prepared from 140 µl of the supernatant using the QIAamp® Viral RNA kit (Qiagen) according to the manufacturer's instructions with a slight modification in that the viral RNA was eluted in 70 µl of AVE (Qiagen).

Total RNA was prepared from clinical specimens using the QIAamp® Viral RNA kit (Qiagen) (using 140 µl of clinical specimen) or MagMAX™ 96 Viral Isolation Kit (Ambion, Austin, TX, USA) (using 50 µl of clinical specimen) with KingFisher Flex (Thermo Fisher Scientific, Waltham, MA, USA) according to each manufacturer's instructions. Total RNA from the 140 µl clinical specimen was eluted with 60 µl of AVE (Qiagen), whereas total RNA from the 50 µl clinical specimen was eluted with 30 µl of elution buffer (Ambion).

#### 2.7. One-step real-time RT-PCR assay

The reaction was performed using a QuantiTect® Probe RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Briefly, the 25 µl assay contained 12.5 µl of 2× QuantiTect Probe PCR Master Mix, 0.25 µl of QuantiTect RT Mix, 0.1 µl of RNase Inhibitor

(Applied Biosystems, Foster City, CA, USA), 1.5 µl of 10 µM forward primer, 1.5 µl of 10 µM reverse primer, 0.5 µl of 5 µM probe, 3.65 µl of water, and 5 µl of RNA template. Cycling was performed as follows: 30 min at 50 °C to activate RT, followed by initial denaturation for 15 min at 95 °C with a subsequent 45 cycles of amplification (denaturation at 95 °C for 15 s and annealing as well as extension at 56 °C for 75 s) using LightCycler® 480 (Roche Molecular Biochemicals). Fluorescent signals were collected during annealing and extension steps, and amplification data were analyzed using the LightCycler® 480 SW1.5 software according to the manufacturer's instructions.

### 3. Results

#### 3.1. Development of real-time RT-PCR assays

Three rRT-PCR assays were developed (Table 1): H1pdm rRT-PCR assay for detecting pandemic influenza A/H1N1 2009 (A/H1N1pdm) virus, H1 rRT-PCR assay for detecting human seasonal A/H1N1 influenza (A/H1N1) virus, and H3 rRT-PCR assay for detecting A/H3N2 influenza (A/H3N2) virus.

The specificity of each rRT-PCR assay was evaluated using A/H1N1, A/H3N2, and A/H1N1pdm viruses, influenza B viruses, and other viral respiratory pathogens. The improved H1pdm rRT-PCR assay reacted specifically to A/H1N1pdm viruses, but did not cross-react with A/H1N1 and A/H3N2 viruses. The new H1 rRT-PCR assay reacted specifically to A/H1N1 viruses, but did not cross-react with A/H3N2 and A/H1N1pdm viruses, and the H3 rRT-PCR assay reacted specifically with the A/H3N2 viruses, but did not cross-react with A/H1N1 and A/H1N1pdm viruses. The Type A rRT-PCR assay could detect only influenza A viruses. All four assays showed no cross-reactivity against influenza B viruses and other viral respiratory pathogens.

The detection limit of each assay was determined by performing serial dilutions of *in vitro*-transcribed control viral RNA for six replicates in each assay. The detection limit of each assay was determined by calculating the concentration where there is 95% positivity using the results shown in Table 2. The detection limits of Type A, H1pdm, H1, and H3 rRT-PCR were determined to be 7.5, 6.8, 7.3, and 7.1 copies/reaction, respectively.

The sensitivity of improved H1pdm, H1, and H3 rRT-PCR assays was compared with that of the Type A rRT-PCR assay using serial dilutions of A/Narita/1/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, A/Sakai/89/2009 (H1N1)pdm, A/Brisbane/59/2007 (H1N1), or A/Uruguay/716/2007 (H3N2) viruses in six replicates for each assay (Tables 3 and 4). A/Aichi/472/2009 (H1N1)pdm was shown to have an HA gene in which the adenine nucleotide was substituted with a cytosine nucleotide at position 218 from the start codon located in the target region of the former probe (NIID-swH1 Probe1) (Kageyama et al., *in press*). A/Sakai/89/2009 (H1N1)pdm showed an HA gene in which the guanine nucleotide and the thymine nucleotide were substituted with adenine nucleotides at positions 208 and 210, respectively, located in the target region of the NIID-swH1 Probe 1. Since both viruses had mutation(s) in the probe-binding region of the HA gene, the sensitivity of the former H1pdm rRT-PCR assay decreased for these viruses (Table 4). However, the improved H1pdm rRT-PCR assay could detect these viruses with high sensitivity (Table 4).

As shown in Tables 3 and 4, the minimum viral titers for 100% detection of A/Narita/1/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, and A/Sakai/89/2009 (H1N1)pdm using the Type A and H1pdm rRT-PCR assays were  $3.16 \times 10^{-2}$ ,  $5.00 \times 10^{-3}$ , and  $1.07 \times 10^{-3}$  TCID<sub>50</sub>/reaction, respectively. The minimum viral titer for 100% detection of A/Brisbane/59/2007 (H1N1) using Type A and H1 rRT-PCR assays was  $2.19 \times 10^{-3}$  TCID<sub>50</sub>/reaction, and that of

**Table 2**Detection limits of each assay using serial dilutions of *in vitro*-transcribed control viral RNA.

Template RNA concentrations (copies/reaction)	Number of positive replicates/number of tests for each assay (positive %)			
	Type A <sup>a</sup>	H1pdm <sup>b</sup>	H1 <sup>c</sup>	H3 <sup>d</sup>
10	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)
5	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)
1	2/6 (33.3)	5/6 (83.3)	3/6 (50)	4/6 (66.7)
0.1	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)

<sup>a</sup> Template RNA for each assay is the matrix gene segment of A/Narita/1/2009 (H1N1)pdm.<sup>b</sup> Template RNA for each assay is the HA gene segment of A/Narita/1/2009 (H1N1)pdm.<sup>c</sup> Template RNA for each assay is the HA gene segment of A/Brisbane/59/2007 (H1N1).<sup>d</sup> Template RNA for each assay is the HA gene segment of A/Uruguay/716/2007 (H3N2).**Table 3**

Detection limits of each real-time RT-PCR assay using serial dilutions of viral RNA.

Viral titer (TCID <sub>50</sub> /reaction)	No. of positive replicates/No. of tests for each assay (positive %)		Viral titer (TCID <sub>50</sub> /reaction)	No. of positive replicates/No. of tests for each assay (positive %)		Viral titer (TCID <sub>50</sub> /reaction)	No. of positive replicates/No. of tests for each assay (positive %)	
	Type A	H1pdm		Type A	H1		Type A	H3
A/Narita/1/2009 (H1N1)pdm			A/Brisbane/59/2007 (H1N1)			A/Uruguay/716/2007 (H3N2)		
3.16 × 10 <sup>-1</sup>	6/6 (100)	6/6 (100)	2.19 × 10 <sup>-2</sup>	6/6 (100)	6/6 (100)	4.68 × 10 <sup>-2</sup>	6/6 (100)	6/6 (100)
3.16 × 10 <sup>-2</sup>	6/6 (100)	6/6 (100)	2.19 × 10 <sup>-3</sup>	6/6 (100)	6/6 (100)	4.68 × 10 <sup>-3</sup>	6/6 (100)	6/6 (100)
3.16 × 10 <sup>-3</sup>	3/6 (50)	2/6 (33.3)	2.19 × 10 <sup>-4</sup>	4/6 (66.7)	3/6 (50)	4.68 × 10 <sup>-4</sup>	1/6 (16.7)	1/6 (16.7)
3.16 × 10 <sup>-4</sup>	0/6 (0)	0/6 (0)	2.19 × 10 <sup>-5</sup>	0/6 (0)	0/6 (0)	4.68 × 10 <sup>-5</sup>	0/6 (0)	0/6 (0)

A/Uruguay/716/2007 (H3N2) using Type A and H3 rRT-PCR assays was 4.68 × 10<sup>-3</sup> TCID<sub>50</sub>/reaction (Table 3). As described above, the minimum viral titer for 100% detection of each virus using H1pdm, H1, or H3 rRT-PCR assays was identical to that for 100% detection of each virus using the Type A rRT-PCR assay (Tables 3 and 4). Detection of further dilutions of each virus was not 100% over six replicates of each assay (Tables 3 and 4).

A standard curve of each assay was also generated (Fig. 1). The standard curve showed a linear relationship between the log of the viral titer and the crossing point (Cp) value for all assays (Fig. 1). The correlation coefficient of the standard curve was 0.99 for all assays, indicating a precise log-linear relationship between the viral titer and Cp value (Fig. 1).

### 3.2. Validation of real-time RT-PCR assays performed using clinical specimens

From May 2009 to October 2009 clinical specimens (nasal and/or pharyngeal swabs) from suspected cases of pandemic (H1N1) 2009 virus infection (*n* = 90) were obtained from prefectural and municipal public health institutes and quarantine stations in Japan. Type A rRT-PCR, improved H1pdm rRT-PCR, H1 rRT-PCR, and H3 rRT-PCR assays were performed for all clinical specimens. As a result, 48 samples were identified as positive for Type A, whereas 42 samples were negative for Type A. All 48 Type A-positive samples were subtyped, revealing that 26, 20, and 2 samples were positive for H1pdm, H3, and H1, respectively.

**Table 4**

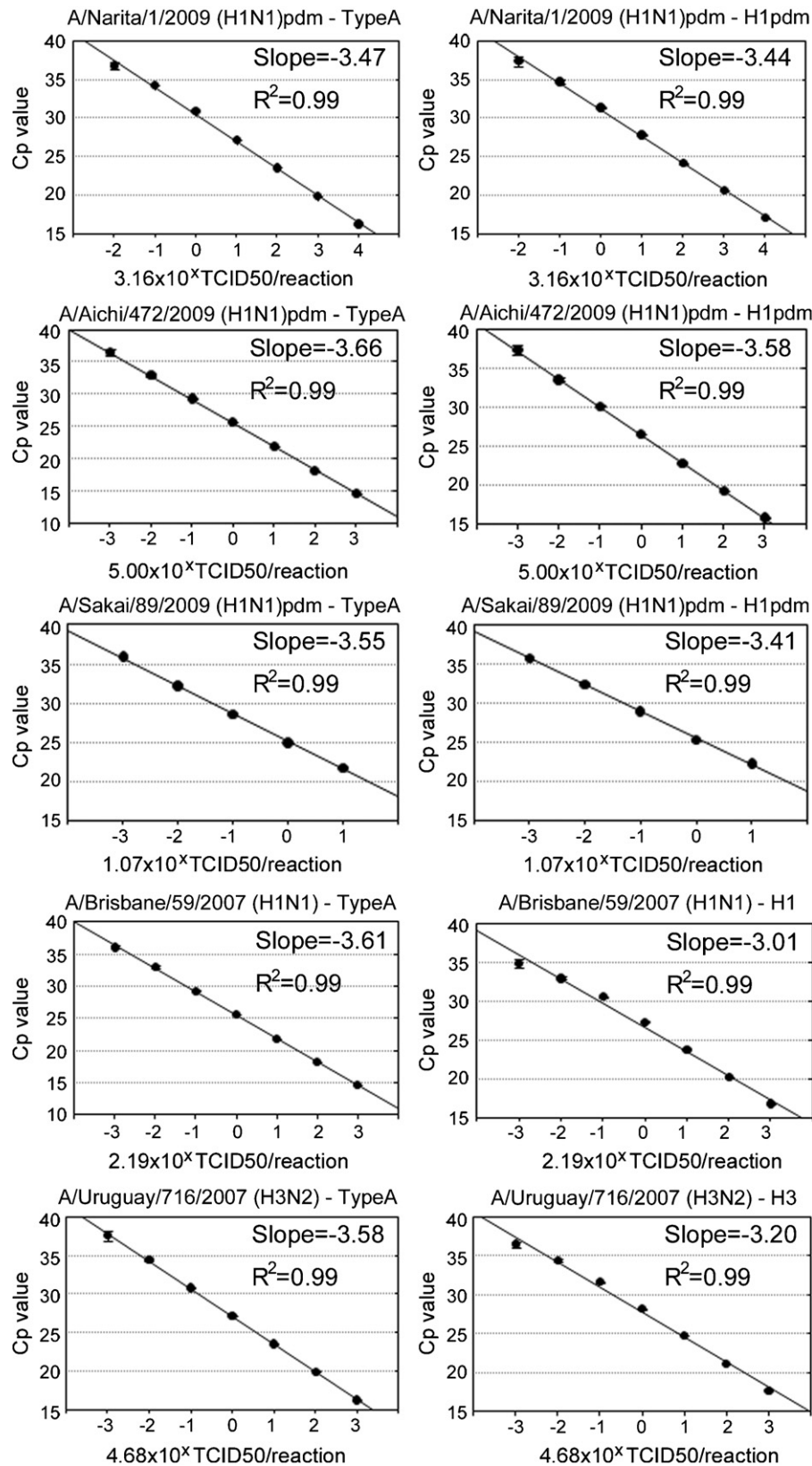
Comparison of detection limits of previous and modified H1pdm rRT-PCR assays using serial dilutions of viral RNA.

Viral titer (TCID <sub>50</sub> /reaction)	No. of positive replicates/No. of tests for each assay (positive %)			Viral titer (TCID <sub>50</sub> /reaction)	No. of positive replicates/No. of tests for each assay (positive %)		
	Type A	H1pdm	Former H1pdm		Type A	H1pdm	Former H1pdm
A/Aichi/472/2009 (H1N1)pdm				A/Sakai/89/2009 (H1N1)pdm			
5.00 × 10 <sup>1</sup>	6/6 (100)	6/6 (100)	0/6 (0)	1.07 × 10 <sup>0</sup>	6/6 (100)	6/6 (100)	0/6 (0)
5.00 × 10 <sup>0</sup>	6/6 (100)	6/6 (100)	0/6 (0)	1.07 × 10 <sup>-1</sup>	6/6 (100)	6/6 (100)	0/6 (0)
5.00 × 10 <sup>-1</sup>	6/6 (100)	6/6 (100)	0/6 (0)	1.07 × 10 <sup>-2</sup>	6/6 (100)	6/6 (100)	0/6 (0)
5.00 × 10 <sup>-2</sup>	6/6 (100)	6/6 (100)	0/6 (0)	1.07 × 10 <sup>-3</sup>	6/6 (100)	6/6 (100)	0/6 (0)
5.00 × 10 <sup>-3</sup>	6/6 (100)	6/6 (100)	0/6 (0)	1.07 × 10 <sup>-4</sup>	5/6 (83.3)	5/6 (83.3)	0/6 (0)
5.00 × 10 <sup>-4</sup>	5/6 (83.3)	2/6 (33.3)	0/6 (0)	1.07 × 10 <sup>-5</sup>	3/6 (50)	0/6 (0)	0/6 (0)
5.00 × 10 <sup>-5</sup>	0/6 (0)	0/6 (0)	0/6 (0)	1.07 × 10 <sup>-6</sup>	0/6 (0)	0/6 (0)	0/6 (0)

## 4. Discussion

After the worldwide outbreak of pandemic influenza A/H1N1 2009 (A/H1N1pdm), many diagnostic methods for detecting the causative virus, including the rRT-PCR assay, were established by many groups (Beck et al., 2010; Bose et al., 2009; Carr et al., 2009; Chidlow et al., 2010; Dong et al., 2010; Ge et al., 2009; Gunson et al., 2010; Hall et al., 2009; He et al., 2009; Jiang et al., 2010; Kubo et al., 2010; Lau et al., 2009; LeBlanc et al., 2009; Liu et al., 2009; Pabbaraju et al., 2009; Poon et al., 2009; Wang et al., 2009; Wenzel et al., 2009; Whaley et al., 2009; Wu et al., 2010; Yang et al., 2009). Type A rRT-PCR and improved H1pdm rRT-PCR assays, as well as the newly developed H1 rRT-PCR and H3 rRT-PCR assays used for subtyping human seasonal influenza A viruses, were shown to have good linearity (*R*<sup>2</sup> = 0.99) and high sensitivity (Tables 2–4 and Fig. 1). No cross-reactivity or nonspecific reactions were observed in any of the assays performed using isolated viruses and clinical specimens. In the subtyping assay of clinical specimens, all 48 Type A-positive samples were subtyped by H1pdm, H1, and H3 rRT-PCR assays developed in this study, and there were no untyped Type A-positive samples. These results suggest that H1pdm, H1, H3, and Type A rRT-PCR assays are highly sensitive and specific for each target gene and thus should be of great use for detecting A/H1N1pdm virus and subtyping seasonal influenza A viruses.

The A/Aichi/472/2009 (H1N1)pdm and A/Sakai/89/2009 (H1N1)pdm viruses with mutation(s) in the sequence corresponding to the former probe sequence could not be detected using the



**Fig. 1.** Standard curves of each assay. Ten-fold serial dilutions of viral RNA were used for each rRT-PCR assay performed in six replicates. The standard curves were generated using average crossing point (Cp) values obtained from the assays performed in six replicates. The correlation coefficient and slope of the standard curve are represented in the graphs. Standard curves were made based on Type A and H1pdm rRT-PCR assays performed using A/Narita/1/2009 (H1N1)pdm, A/Aichi/472/2009 (H1N1)pdm, and A/Sakai/89/2009 (H1N1)pdm (upper 6 graphs), those based on Type A and H1 rRT-PCR assays performed using A/Brisbane/59/2007 (H1N1) (second 2 graphs from the bottom), and those developed based on Type A and H3 rRT-PCR assays performed using A/Uruguay/716/2007 (H3N2) (bottom 2 graphs).

former H1pdm rRT-PCR assay (Table 4). These two viruses were isolated from clinical specimens that failed to be detected by the former H1pdm rRT-PCR assay. The redesigned probe in this study was seven nucleotides longer than the former probe (Table 1). This was done to increase the melting temperature and thus to improve the sensitivity and specificity of the assay. The results of the improved H1pdm rRT-PCR assay performed using serial dilutions of the A/Aichi/472/2009 (H1N1)pdm and A/Sakai/89/2009 (H1N1)pdm viruses demonstrated that the sensitivity of this assay was identical to that of the Type A rRT-PCR assay (Table 4 and Fig. 1), suggesting that the improved H1pdm rRT-PCR assay can detect mutated A/H1N1pdm viruses without losing sensitivity. Thus far, additional mutations of the target sequence of the redesigned probe used in H1pdm rRT-PCR assays have not been observed in recent circulating A/H1N1pdm viruses in Japan.

Using the four rRT-PCR assays, a diagnostic scheme was developed in which H1pdm, H1, and H3 rRT-PCR assays for subtyping and Type A rRT-PCR assay for evaluating the subtyping assays were all performed simultaneously. To prepare for viruses with other possible mutations in the HA gene and emerging influenza viruses of other subtypes, as well as to decrease the risk of false negatives, subtype-specific rRT-PCR assays must always be performed in combination with the Type A rRT-PCR assay.

The highly sensitive and specific H1pdm, H1, and H3 rRT-PCR assays developed in this study and Type A rRT-PCR assay were performed as laboratory diagnostic tests for pandemic influenza A/H1N1 2009 virus at most prefectural and municipal public health institutes and quarantine stations in Japan, and have been confirmed to be very useful not only for laboratory diagnostic tests but also for the surveillance of the spread of influenza viruses.

## Acknowledgments

The authors declare no potential conflict of interest relevant to this article. None of the primers and probes listed in this article are patented.

We would like to thank all the members of the Influenza Virus Research Center for their helpful technical advice. We also thank Dr. Komei Shirabe of Yamaguchi Prefectural Institute of Public Health and Environment for his informative input. We are also grateful to all the members of the Japan Association of Prefectural and Municipal Public Health Institutes and quarantine stations for their cooperation in the laboratory diagnosis of pandemic (H1N1) 2009 virus and for providing us with clinical specimens. We especially thank Shinichi Takao (Hiroshima Prefectural Technology Research Institute), Yoshimi Ohuchi (Shiga Prefectural Institute of Public Health), Yuka Ootsuka (Ehime Prefectural Institute of Public Health and Environmental Science), Chiharu Kawakami (Yokohama City Institute of Health), Sumi Watanabe (Kanagawa Prefectural Institute of Public Health), Eiko Hirano (Fukui Prefectural Institute of Public Health and Environmental Science), Hiroyuki Saito (Akita Prefectural Research Center for Public Health and Environment), Masayuki Kikuchi (Sapporo City Institute of Public Health), Eiji Horimoto (Toyama Institute of Health), Hiroyoshi Asakawa (Yamanashi Institute for Public Health), Katsuhiko Abe (Hiroshima City Institute of Public Health), Takuya Yano (Mie Prefecture Health and Environment Research Institute), and Narita Airport Quarantine Station for providing us with isolated influenza viruses. We are greatly obliged to Dr. Hidekazu Nishimura (Sendai Medical Center), Dr. Masanobu Agoh and Dr. Akinori Yamaguchi (Nagasaki Prefectural Institute for Environmental Research and Public Health), and Dr. Fumihiro Taguchi (Department of Virology III, NIID and Nippon Veterinary and Life Science University) for providing us with the respiratory pathogens. We are grateful to Dr. Shigeru Morikawa, Dr. Tetsuya Mizutani, and Dr. Masayuki Saijo for their helpful advice.

## References

- Beck, E.T., Jurgens, L.A., Kehl, S.C., Bose, M.E., Patitucci, T., LaGue, E., Darga, P., Wilkinson, K., Witt, L.M., Fan, J., He, J., Kumar, S., Henrickson, K.J., 2010. Development of a rapid automated influenza A, influenza B, and respiratory syncytial virus A/B multiplex real-time RT-PCR assay and its use during the 2009 H1N1 swine-origin influenza virus epidemic in Milwaukee, Wisconsin. *J. Mol. Diagn.* 12, 74–81.
- Bose, M.E., Beck, E.T., Ledebor, 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 virus species during the 2009 swine origin influenza A H1N1 virus epidemic in Milwaukee, Wisconsin. *J. Clin. Microbiol.* 47, 2779–2786.
- 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.
- Chidlow, G., Harnett, G., Williams, S., Levy, A., Speers, D., Smith, W.D., 2010. Duplex real-time RT-PCR assays for the rapid detection and identification of pandemic (H1N1) 2009 and seasonal influenza viruses A/H1, A/H3 and B. *J. Clin. Microbiol.* 48, 862–866.
- Dong, H., Zhang, Y., Xiong, H., Yan, A., Ding, G., Chen, Y., Xie, L., Chen, J., Zhang, G., Hao, P., Cong, L., Lu, Y., Che, X., Wang, X., Li, Y., Yuen, K.Y., Zhao, G., Jin, W., 2010. Detection of human novel influenza A (H1N1) viruses using multi-fluorescent real-time RT-PCR. *Virus Res.* 147, 85–90.
- Ge, Y., Cui, L., Qi, X., Shan, J., Shan, Y., Qi, Y., Wu, B., Wang, H., Shi, Z., 2009. Detection of novel swine origin influenza A virus (H1N1) by real-time nucleic acid sequence-based amplification. *J. Virol. Methods*.
- Gunson, R., Maclean, A., Davies, E., Bennett, S., Miller, R., Carman, W.F., 2010. Development of a multiplex real-time RT-PCR that allows universal detection of influenza A viruses and simultaneous typing of influenza A/H1N1/2009 virus. *J. Virol. Methods* 163, 258–261.
- 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.
- He, J., Bose, M.E., Beck, E.T., Fan, J., Tiwari, S., Metallo, J., Jurgens, L.A., Kehl, S.C., Ledebor, N., Kumar, S., Weisburg, W., Henrickson, K.J., 2009. Rapid multiplex reverse transcription-PCR typing of influenza A and B virus, and subtyping of influenza A virus into H1, 2, 3, 5, 7, 9, N1 (human), N1 (animal), N2, and N7, including typing of novel swine origin influenza A (H1N1) virus, during the 2009 outbreak in Milwaukee, Wisconsin. *J. Clin. Microbiol.* 47, 2772–2778.
- Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol.* 146, 2275–2289.
- 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.
- Kageyama, T., Shirakura, M., Kishida, N., Nakauchi, M., Obuchi, M., Ujiike, M., Itamura, S., Odagiri, T., Tashiro, M., in press. Establishment of pandemic influenza A 2009 (H1N1) virus diagnosis system in Japan using conventional RT-PCR assay and real-time RT-PCR assay. *Jpn. J. Infect. Dis.*
- Kubo, T., Agoh, M., Mai, I., Q., Fukushima, K., Nishimura, H., Yamaguchi, A., Hirano, M., Yoshikawa, A., Hasebe, F., Kohno, S., Morita, K., 2010. Development of a reverse transcription-loop-mediated isothermal amplification assay for detection of pandemic (H1N1) 2009 virus as a novel molecular method for diagnosis of pandemic influenza in resource-limited settings. *J. Clin. Microbiol.* 48, 728–735.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948.
- Lau, S.K., Chan, K.H., Yip, C.C., Ng, T.K., Tsang, O.T., Woo, P.C., Yuen, K.Y., 2009. Confirmation of the first Hong Kong case of human infection by novel swine origin influenza A (H1N1) virus diagnosed using ultrarapid, real-time reverse transcriptase PCR. *J. Clin. Microbiol.* 47, 2344–2346.
- LeBlanc, J.J., Li, Y., Bastien, N., Forward, K.R., Davidson, R.J., Hachette, T.F., 2009. Switching gears for an influenza pandemic: validation of a duplex reverse transcriptase PCR assay for simultaneous detection and confirmatory identification of pandemic (H1N1) 2009 influenza virus. *J. Clin. Microbiol.* 47, 3805–3813.
- Liu, S., Hou, G., Zhuang, Q., Shu, Y., Chen, J., Jiang, W., Li, J., 2009. A SYBR Green I real-time RT-PCR assay for detection and differentiation of influenza A (H1N1) virus in swine populations. *J. Virol. Methods* 162, 184–187.
- 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.
- Poon, L.L., Chan, K.H., Smith, G.J., Leung, C.S., Guan, Y., Yuen, K.Y., Peiris, J.S., 2009. Molecular detection of a novel human influenza (H1N1) of pandemic potential by conventional and real-time quantitative RT-PCR assays. *Clin. Chem.* 55, 1555–1558.
- Shimada, T., Gu, Y., Kamiya, H., Komiya, N., Odaira, F., Sunagawa, T., Takahashi, H., Toyokawa, T., Tsuchihashi, Y., Yasui, Y., Tada, Y., Okabe, N., 2009. Epidemiology of influenza A (H1N1)v virus infection in Japan, May–June 2009. *Euro Surveill.* 14.
- Wang, R., Sheng, Z.M., Taubenberger, J.K., 2009. Detection of novel (swine origin) H1N1 influenza A virus by quantitative real-time reverse transcription-PCR. *J. Clin. Microbiol.* 47, 2675–2677.

- Wenzel, J.J., Walch, H., Bollwein, M., Niller, H.H., Ankenbauer, W., Mauritz, R., Holtke, H.J., Zepeda, H.M., Wolf, H., Jilg, W., Reischl, U., 2009. Library of prefabricated locked nucleic acid hydrolysis probes facilitates rapid development of reverse-transcription quantitative real-time PCR assays for detection of novel influenza A/H1N1/09 virus. *Clin. Chem.* 55, 2218–2222.
- 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.
- WHO, 2009a. Global influenza surveillance network: laboratory surveillance and response to pandemic H1N1. *Wkly. Epidemiol. Rec.* 84, 361–365.
- WHO, 2009b. New influenza A(H1N1) virus infections: global surveillance summary, May 2009. *Wkly. Epidemiol. Rec.* 84, 173–179.
- Wu, W., Kang, X., Bai, Z., Liu, L., Li, J., Wu, X., Sun, H., Hu, T., Yang, M., Wang, P., Yang, Y., Di, B., Chen, W., 2010. Detection of pandemic influenza A/H1N1/2009 virus by real-time reverse transcription polymerase chain reaction. *J. Virol. Methods* 165, 294–296.
- Yang, J.R., Lo, J., Liu, J.L., Lin, C.H., Ho, Y.L., Chen, C.J., Wu, H.S., Liu, M.T., 2009. Rapid SYBR green I and modified probe real-time reverse transcription-PCR assays identify influenza H1N1 viruses and distinguish between pandemic and seasonal strains. *J. Clin. Microbiol.* 47, 3714–3716.