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Development of a diagnostic system for novel influenza A(H7N9) virus using real-time RT-PCR assay in Japan

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Keywords

Influenza A(H7N9) virus; one-step real-time RT-PCR assay; diagnosis

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Summary

The first human cases of infection with avian influenza A(H7N9) virus were reported in March 2013 in China. The number of confirmed cases continues to increase, although almost all the cases are limited to China. In this study, a one-step real-time RT-PCR assay was developed for detecting the novel A(H7N9) virus. This assay was shown to have high specificity, good linearity, and high sensitivity to a broad range of Eurasian H7 viruses. The assay is useful both for diagnostic purposes in cases of suspected human infection with the influenza A(H7N9) virus and in the surveillance of both avian and human influenza viruses. A diagnostic system using this assay was prepared at 74 prefectural and municipal public health institutes and 16 quarantine stations in Japan early into the human H7N9 infection outbreaks, enabling potential diagnoses of H7N9 infection across Japan.

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Introduction

The first human cases of infection with avian influenza A(H7N9) virus were reported by the National Health and Family Planning Commission, China on March 31, 2013. A total of 135 cases of H7N9 human infection, including 44 deaths, were confirmed by 13 August 2013, and more than 200 additional human cases of infection with H7N9 were reported in China between October 2013 and February 2014 (1, 2). The H7N9 viruses are reassortants that acquired the H7 hemagglutinin (HA) and N9 neuraminidase (NA) genes from Eurasian avian influenza viruses and the remaining 6 genes from the recent influenza A(H9N2) virus spreading in China. In addition, human H7N9 viruses have several characteristic amino acid changes in the HA gene and the PB2 RNA polymerase subunit that probably facilitate binding to human-type receptors and efficient replication in mammals, respectively, and which highlight the pandemic potential of the novel viruses (3, 4). Indeed, imported human H7N9 infections from China have been confirmed in Taiwan and Malaysia (2, 5, 6).

A laboratory-based diagnostic system for the H7N9 virus using a highly sensitive and specific one-step real-time RT-PCR assay for detecting Eurasian lineage

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H7 influenza viruses (H7 rRT-PCR assay), along with conventional RT-PCR methods, was developed by the National Institute of Infectious Diseases (NIID) in Japan to monitor and diagnose human H7N9 infections. These methods have been shown to perform well as a result of using the human H7N9 isolate received from the Chinese Center for Disease Control and Prevention (CCDC) on April 12, 2013. Screening of individuals with suspected H7N9 infection, defined as fever $\geq$38 °C, acute respiratory symptoms and a history of residence or travel in China within 10 days, continues across Japan at bases in 74 prefectural and municipal public health institutes and 16 quarantine stations using the H7N9 diagnostic kit distributed on April 16, 2013. As of March 14, 2014, no cases of imported human H7N9 infection have been confirmed in Japan.

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Materials and Methods

Primer and probe design: The primers and probe for the H7 rRT-PCR assay for specific detection of Eurasian lineage H7 subtype influenza A viruses were designed based on multiple alignments of Eurasian H7 HA gene sequences from the Global Initiative on Sharing All Influenza Data (GISAID) database. Nucleotide sequences of

primers and the minor groove binder (MGB) TaqMan® probe (Life Technologies Japan, Tokyo, Japan) for this assay are shown in Table 1 and were designed in the highly conserved HA2 regions.

Viruses and viral RNA extraction for evaluation: The novel human H7N9 virus, A/Anhui/1/2013 (H7N9), and 7 Eurasian H7 avian influenza A viruses—A/duck/Fukui/1/2004 (H7N7), A/quail/Aichi/1/2009 (H7N6), A/mallard/Netherlands/12/2000 (H7N3), A/Netherlands/33/2003 (H7N7), A/duck/Gunma/466/2011 (H7N9), A/duck/Hong Kong/301/1978 (H7N2), and A/duck/Hong Kong/293/1978 (H7N2)—were used to evaluate the novel H7 rRT-PCR assay. Viral RNA was extracted from 60–140 μL of each virus propagated in embryonated chicken egg using a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

In vitro-transcribed RNA: In vitro-transcribed RNAs were used as standards for the H7 rRT-PCR assay. RNA transcripts of the full-length H7 HA and matrix (M) genes were synthesized from RNA extracted from A/Anhui/1/2013 (GISAID accession no. EPI439507) and A/Narita/1/2009 (H1N1pdm09) (EPI179437), respectively, by the

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procedure described as follows. Uni12 primer (5′-AGCAAAAGCAGG-3′) (7) was used for reverse transcription using a SuperScript ® III Reverse Transcriptase Kit (Invitrogen, Carlsbad, Cal., USA), according to the manufacturer's instructions. The H7 HA and M genes were amplified by PCR using Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, Mas., USA) with the paired primers Anhui-HA-F (5′-CAGGGAGCAAAAGCAGGGGATACAAAATGAACACTCAAATCCTG-3′) and Anhui-H7-R+T7 (5′-TAATACGACTCACTATAGGGAGTAGAAACAAGGGTG TTTTTTTC-3′), and M-1F (5′-ATGAGTCTTCTAACCGAGGTCGAAA-3′) and M-958R+T7 (5′-TAATACGACTCACTATAGGGTTACTCTAGCTCTATGTTGACA AAA-3′), respectively. (Underline indicates the T7 promoter sequence.) RNAs were transcribed using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Madison, Wis., USA) and treated with TURBO® DNase (Ambion, Austin, Tex., USA) to degrade the template DNA. The dNTPs and NTPs were removed using MicroSpin G-25 Columns (GE Healthcare, Piscataway, NJ, USA) following the manufacturer's instructions. Transcribed RNAs were quantified using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, Mas., USA), and the

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absorbance value was used to calculate the copy number. The integrity of the transcribed RNAs was assessed with a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, Cal., USA). Transcript dilutions were carried out in nuclease-free water containing 10 ng/μL of carrier RNA (Qiagen).

One-step real-time RT-PCR assay: The rRT-PCR assay was performed in a 25 μL PCR reaction comprised of 600 nM of each forward and reverse primer, 100 nM of TaqManMGB probe, 12.5 μL of 2×QuantiTect Probe RT-PCR Master Mix, 0.25 μL of QuantiTect RT Mix, with the QuantiTect Probe RT-PCR kit (Qiagen), 2 U RNase Inhibitor (Applied Biosystems, Foster City, Cal., USA), and 5 μL of RNA as the template. The rRT-PCR assays were performed with LightCycler® 480 II (Roche, Basel, Switzerland) under the following conditions: 50 °C for 30 min, 95 °C for 15 min, 45 cycles of 15 s at 94 °C, and 75 s at 56 °C. Amplification data were collected and analyzed using the Second Derivative Maximum Method in Light Cycler® 480 SW1.5 software. In this study, the copy number of M gene in the 8 H7 viruses was determined quantitatively by the influenza A (Type A) rRT-PCR assay targeted for the universal M gene of all influenza A viruses (8) using known concentrations of in vitro-transcribed

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A/Narita/1/2009 M gene RNA as the standard.

Validation and evaluation of the one-step real-time RT-PCR assay: The analytical sensitivity of the assay was assessed by serial dilutions of quantified in vitro-transcribed RNA in 6 replicates at each concentration. The limit of detection (LOD) was calculated by probit regression analysis (9) with a 95% probability endpoint.

For evaluation of the H7 rRT-PCR assay, both the H7 and Type A rRT-PCR assays were performed at the same time using three 10-fold serial dilutions of the 8 Eurasian H7 influenza A viruses in triplicate; the number of positive results obtained by each assay was compared and the average crossing point (Cp) values defined.

The specificity of the H7 rRT-PCR assay was validated using 23 representative subtypes of influenza A and influenza B viruses, and 18 viral respiratory pathogens (Table 2A and B).

Phylogenetic analysis of HA gene: Phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis software (MEGA, version 5.0) (10). The evolutionary history was inferred using the neighbor-joining method (11). The evolutionary distances were computed using Kimura's two-parameter method (12). The

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bootstrap values of the HA genes were calculated from 1000 replicates (13).

Results

Sensitivity and specificity of the one-step real-time RT-PCR assay: The LOD of the H7 rRT-PCR assay was determined by testing serial dilutions of quantified in vitro-transcribed HA gene RNA of A/Anhui/1/2013 in 6 replicates, resulting in 5.2 copies per reaction. The efficiency of this assay was 99.2% and the R^2 values, which compare the log of the template concentrations with Cp values, was 1.0 at between 5.0 to 5.0×10^7 copies per reaction (Fig. 1). A precise log-linear relationship between the template concentration and Cp value was observed over a broad dynamic range.

Evaluation of the H7 rRT-PCR assay was performed using three 10-fold serial dilutions of 8 Eurasian H7 virus RNAs. The M gene copy number of each virus was quantitated by the Type A rRT-PCR assay (Table 3). All viruses were detectable by the H7 rRT-PCR assay. The 7 viruses except for A/quail/Aichi/1/2009 could be identified as H7 HA subtypes using the H7 rRT-PCR assay with a low concentration of templates; the difference in Cp values between the Type A rRT-PCR and the H7 rRT-PCR assay

(ΔCp) was less than 1.4 and the standard deviation (SD) of the triplicate Cp values was less than 0.85. These results indicated that the H7 rRT-PCR assay has highly sensitivity for these 7 viruses just like the Type A rRT-PCR assay. When the assays were performed using A/quail/Aichi/1/2009, the ΔCp of the 10^{-5} dilution was high and the HA gene could not be detected by the H7 rRT-PCR assay at lower concentrations.

The specificity of the assay was validated using representative non-H7 influenza A and influenza B viruses and other viral respiratory pathogens (Table 2A and B). The assay showed no cross-reactivity with any of the other subtypes of influenza A and B viruses or respiratory pathogens (data not shown).

Phylogenetic analysis: The phylogenetic tree of 69 representative H7 HA coding sequences from GISAID database, including the 8 Eurasian H7 viruses, is shown in Fig. 2. Among the 8 Eurasian H7 viruses used for evaluation of the H7 rRT-PCR assay, the HA gene of A/duck/Fukui/1/2004 had the shortest distance and A/quail/Aichi/1/2009 the longest from A/Anhui/1/2013 (nucleotide identity 94.7% vs. 84.7%, respectively).

Discussion

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The HA gene of A/duck/Fukui/1/2004 (H7N7) (EPI447361) shares the highest nucleotide identity (94.7%) with A/Anhui/1/2013 in our influenza virus library (Fig. 2), and this virus was therefore used for developing the H7 rRT-PCR assay until the arrival of H7N9 virus from the CCDC on April 12, 2013. The newly established rRT-PCR assay for detecting novel H7N9 showed good linearity and high sensitivity using in vitro-transcribed RNA of A/Anhui/1/2013 HA gene (Fig. 1). The LOD of the H7 rRT-PCR assay was comparable to that of our previously developed rRT-PCR assay for the universal detection of the M gene of all influenza A viruses (8). Since the sequences of both primers and probe were designed for the highly conserved region of HA gene of Eurasian H7 viruses, the H7 rRT-PCR assay has shown high sensitivity to a broad range of Eurasian H7 avian influenza viruses (Table 3). No cross-reactivity or nonspecific reactions were observed in this assay performed using other subtypes of influenza A and influenza B viruses (Table 2A) or other respiratory viruses (Table 2B).

A/quail/Aichi/1/2009 has 4 mismatches in the forward primer, 2 in the reverse primer, and 2 in the probe region; however, A/duck/Fukui/1/2004 has no mismatches in either of these regions. These nucleic acid discrepancies are believed to lower the

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sensitivity of the H7 rRT-PCR assay when using A/quail/Aichi/1/2009. A/Netherlands/33/2003, A/duck/Gunma/466/2011, A/duck/Hong Kong/301/1978, and A/duck/Hong Kong/293/1978 have only 1 mismatch in the probe region. As the endpoint fluorescence intensities of the H7 rRT-PCR assays using these 4 viruses were lower than those using the other viruses due to 1 mismatch (data not shown), the sensitivity of the H7 rRT-PCR assay remained unchanged (Table 3).

The viral RNA of A/duck/Fukui/1/2004 was distributed to 74 prefectural and municipal public health institutes and 16 quarantine stations in Japan as a positive control for the assay along with the protocol, primers, probe, and reagents as a diagnostic kit for H7N9 on April 16, 2013. In Japan, these aforementioned facilities are involved in screening for human H7N9 infections. No human H7N9 infection cases have been confirmed to date, despite the many diagnostic procedures performed across Japan.

At present, the animal reservoirs and the specific mode of transmission of the virus to humans are still being investigated and the virus may be more widespread among poultry (14). The risk of H7N9 exposure may be limited to China; however,

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continuous surveillance activities are still needed to monitor avian, animal, and human viral infections in other areas. The newly developed H7 rRT-PCR assay was shown to be highly sensitive with the ability to detect both novel H7N9 and other avian Eurasian H7 influenza A viruses. This H7 rRT-PCR assay is useful not only for diagnostic purposes in cases of suspected human H7N9 infection but also for the surveillance of avian and animal influenza infections.

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Conflict of interest

None to declare.

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Figure legends

Fig. 1. Dynamic range of the H7 rRT-PCR assay.

Standard curve (Cp value vs. $\log_{10}$ concentration) for serial dilutions of in vitro-transcribed RNA of the HA gene of A/Anhui/1/2013. The standard curve was generated using the average Cp values obtained from 6 replicates. The correlation coefficient and slope of the standard curve are shown in the graph.

Fig. 2. Phylogenetic relationships of the HA gene of H7 influenza A viruses.

The tree was constructed using the neighbor-joining method. The evolutionary distances were computed using Kimura's two-parameter method. Bootstrap values were calculated from 1000 replicates and values higher than 50% are shown next to the branches. The sequences of the viruses used in this study are shown in bold.

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Table 1. Primer and probe sequences used in the H7 rRT-PCR assay.

Primer or probe	Sequence (5'-3')	Position *	Product size (bp)
NIID-H7 TMPrimer-F1	TGTGATGAYGAYTGYATGGCCAG	1447-1469	156
NIID-H7 TMPrimer-R1	ACATGATGCCCCGAAGCTAAAC	1581-1602	
NIID-H7 Probe1	(FAM)ATCTGTATTCTATTTTGCATTGCYTC(MGB)	1510-1535	

* Nucleotide numbering is based on the HA gene of A/Anhui/1/2013(H7N9).

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