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Detection of human novel influenza A (H1N1) viruses using multi-fluorescent real-time RT-PCR

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A B S T R A C T

The novel influenza A (H1N1) virus is now rapidly spreading across the world. Early detection is one of the most effective measures to prevent further transmission of the virus. 4 sets of proprietary primers and probes designed for detection of influenza A viruses (InfA), human and swine H1N1 viruses (SH1), the novel H1N1 viruses (NH1) and RNaseP gene (RP) respectively were pooled together in a single tube for a multi-fluorescent real-time RT-PCR assay. The detection limit was found to be one order more sensitive than that employing the WHO recommended protocol. The NH1 probe was negative for all control samples including human seasonal H1N1 virus, other subtypes of human influenza A viruses (H3, H5, H9), human influenza B virus and nasopharyngeal swabs from patients with noninfluenza respiratory diseases, indicating its high specificity, capable of discriminating the novel influenza A virus from the previously identified H1N1 viruses. For confirmation, the PCR amplified fragment of the hemagglutinin gene was sequenced which could provide enough information to identify the novel H1N1 virus as a distinct cluster among all viruses of subtype H1 through average distance clustering analysis. Although these assays should be useful in the current outbreak for rapid detection and discrimination of the novel H1N1 from swine H1N1 and other human seasonal H1N1 viruses, further design improvement is suggested to match possible sequence variations in the detected region along with the course of the epidemic.
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1. Introduction

The novel influenza A (H1N1) virus firstly detected in April 2009 is now rapidly spreading from human to human throughout the world. As the World Health Organization (WHO) announced on May 29, 2009, there were more than 15,000 of confirmed cases in 54 countries and regions including 99 deaths across the globe (http://www.who.int/csr/don/2009_05_29/en/index.html). More cases, more hospitalizations and more deaths related to this novel virus are expected to occur in the coming days, weeks, and months (Centers for Disease Control and Prevention, 2009; http://www.cdc.gov/h1n1flu/). Early epidemic findings indicated that the transmissibility of the novel H1N1 virus is higher than that of the seasonal H1N1, and the ratio of infected children with clinical symptom is twice higher than that of the adults (Fraser et al., 2009). Apparently, the novel H1N1 virus is antigenically distinct from human seasonal H1N1, and although vaccine against this virus has been developed, it takes time to realize the goal of global vaccination (Garten et al., 2009; Hébert and MacDonald,

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2009). Thus, early detection and separation of suspected patients are the most effective measures to prevent virus transmission. A protocol of real-time RT-PCR for influenza A (H1N1) detection was recommended by WHO (http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html). A conventional 1-step RT-PCR assay and a 1-step quantitative real-time RT-PCR assay have also been established for rapid detection of the novel H1N1 virus (Poon et al., 2009). Here we reported a multi-fluorescent real-time RT-PCR assay with proprietary primers and probes that seems more specific and sensitive than the recommended or conventional method under a "rapid-assay" condition. We also showed that, in combining with DNA sequencing, a confirmation assay could be established based on this technology.

2. Materials and methods

2.1. Virus RNA extracting

RNA from viral culture of 1 human novel H1N1 virus, 4 human seasonal H1N1 viruses, 4 human seasonal H3N2 viruses, 1 human H5N1 virus, 2 human H9N2 viruses, 1 swine H1N1 virus, 10 human influenza B viruses, and 43 nasopharyngeal swabs from patients with noninfluenza respiratory diseases was extracted using the QIAamp Virus RNA Mini Kit (Qiagen) according to the manufacture's protocol. RNA from nasopharyngeal swabs of 27 clinical patients infected by the novel H1N1 virus was also extracted according to this protocol.

2.2. Multi-fluorescent real-time RT-PCR

The multi-fluorescent real-time 1-step RT-PCR assays were carried out using Quant One Step qRT-PCR (Probe) Kit (Qiagen). Briefly, 5 μl of RNA, 25 μl of 2× RT-PCR Master mix, 1.5 μl of 20 μM InfA, SH1, NH1 and RP primers and probes, 0.5 μl of 25 mM dNTPs, 0.8 μl of Quant RTase and 2 μl of Hotmaster Taq Polymerase were added to a total volume of 50 μl. The thermal cycles were performed on ABI 7500 (Applied Biosystems) following the conditions: 30 min at 50 °C for reverse transcription; 3 min at 92 °C, then 45 cycles at 92 °C for 15 s, 60 °C for 30 s and 68 °C for 40 s for PCR amplification. Chi-square test was applied in the statistical analysis of the real-time RT-PCR data.

2.3. SYBR Green real-time PCR and sequencing

RNA of the samples obtained from the above procedure was reverse transcribed with SuperScript III RNase H^- Reverse Transcriptase (Invitrogen), generating the first strand cDNA to be used as PCR template in the following PCR reaction. A real-time PCR assay was performed using the SYBR Green Real-time PCR Kit (Toyobo). A reaction system with its final volume of 25 μl was made with 12.5 μl of 2× Master mix, 1 μl of 20 μM H1F722, 1 μl of 20 μM H1R1062 and 2.5 μl of the first strand cDNA added. Reactions were first incubated on ABI 7500 (Applied Biosystems) at 95 °C for 60 s, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s. The products of SYBR Green real-time PCR with ideal amplification and melting curves were sequenced directly without running the gel electrophoresis for observing the amplicons. Sequencing reaction was carried out using BigDye Terminator Cycle Sequencing Kits and ABI 3730 DNA Sequencers (Applied Biosystems).

3. Results

3.1. Designing specific primers and probes for the novel type A (H1N1) virus

Compared with sequences of the novel H1N1 virus (A/California/06/2009, GenBank accession no. FJ966960), two and one mismatched nucleotides were found respectively in the reverse primer and the probe sequences provided by the WHO protocol (SW H1, refer to Table 1) (http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html). These mismatched nucleotides between the primers/probes and the viral cDNA templates may result in decreased sensitivity of the PCR assay. Therefore, we designed a new set of primers and probes for the hemagglutinin gene (HA) (NH1, refer to Table 1) to specifically detect the novel H1N1 virus, but not seasonal H1N1 viruses.

Due to the limits of biosafety facilities, we were not able to handle the novel H1N1 virus in our lab. Only the cDNA from a cultured human novel type A (H1N1) virus sample with known titer of 1×10^3 $TCID_{50}$ (median tissue culture infective dose) per microlitre was available. Using serial dilutions of this cDNA preparation, the detection limit of the NH1 primer and probe set was found to be 1×10^{-7} of diluted cDNA per reaction in the single fluorescent PCR assay. While employing the SW H1 primer and probe set recommended by the WHO protocol showed lower sensitivity (1×10^{-6} of diluted cDNA per reaction) in the same assay (Fig. 1).

3.2. Multi-fluorescent real-time RT-PCR assay for detecting the novel H1N1 virus

Instead of performing RT-PCR detection of the novel H1N1 virus in 4 tubes individually as described in the WHO protocol (http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html), we developed a multi-fluorescent real-time RT-PCR assay in which 4 sets of primers and probes including InfA, SH1, NH1 and RP (Table 1) were pooled together in a single tube to discriminate the novel H1N1 virus from swine virus and other seasonal H1N1 viruses. As recommended by the WHO protocol, the InfA primer and probe set was used for universal detection of influenza A viruses, and the RP primer and probe set was designed as an internal control to monitor the quality of clinical specimen. The SH1 set was designed by ourselves within a certain region of the HA gene that is relatively conserved among all H1 subtypes, so that it could be used to detect all human and swine H1N1 viruses. The sensitivity of NH1 primer and probe set remained to be 1×10^{-7} of diluted cDNA per reaction in the multi-fluorescent PCR assay (data not shown) as its performance for a single RT-PCR reaction shown above. Thus, the NH1 primer and probe set designed in this study was sensitive enough to be used in the multi-fluorescent PCR assay.

The specificity of the multi-fluorescent real-time PCR assay was examined in cDNA samples from 1 human novel H1N1 virus, 4 human seasonal H1N1 viruses, 4 human seasonal H3N2 viruses, 1 human H5N1 virus, 2 human H9N2 viruses, 1 swine H1N1 virus, 10 human influenza B viruses and 43 nasopharyngeal swabs from patients with noninfluenza respiratory diseases. As shown in Fig. 2, the InfA primer and probe set was able to detect all influenza A infected samples, but not influenza B (Fig. 2A); the SH1 primer and probe set was only positive in human novel H1N1, human seasonal H1N1 and swine H1N1 samples, but not in other subtypes of influenza A viruses (Fig. 2B). The NH1 primer and probe set specifically detected the human novel H1N1 virus, but not human seasonal H1N1 and swine H1N1 viruses (Fig. 2C). All the 43 nasopharyngeal swabs from patients with noninfluenza respiratory diseases and 10 influenza B viruses showed negative results with the InfA,

Table 1
Primers and probes used for this study.

Set	Primers and probes	Position (bp)	Sequences (5′ > 3′)	Targeted genes	GenBank accession no.
InfA	InfA forward	146–167	GACCRATCYTGTCACCTCTGAC	Matrix protein	FJ969513
	InfA reverse	251–228	AGGGCATTYTGGACAAAKCGTCTA		
	InfA probe	224–201	ROX-TGCAGTCCTCGCTCACTGGGCACG-BHQ2		
RP	RP forward	50–68	AGATTTGGACCTGCGAGCG	Ribonuclease P	NM_001104546
	RP reverse	114–95	GAGCGGCTGTCTCCACAAGT		
	RP probe	71–93	CY5-TTCTGACCTGAAGGCTCTGCGCG-BHQ3		
SH1	SH1 forward	1585–1607	CAGATYYTGGCGATCTATTCAAC	Hemagglutinin	FJ966960
	SH1 reverse	1674–1654	CCCATTRGARCACATCCAGAA		
	SH1 probe	1634–1650	HEX-TCTCCCTGGGGGCAATC-BHQ1		
NH1	NH1 forward	393–416	TGAGATATTCCCCAAGACAAGTTC	Hemagglutinin	FJ966960
	NH1 reverse	489–467	TTTGTAGAAGCTTTTTGCTCCAG		
	NH1 probe	426–451	FAM-TCATGACTCGAACAAAGGTGTAACGG-BHQ1		
SW H1[a]	SW H1 forward	902–924	GTGCTATAAACACCAGCCTYCCA	Hemagglutinin	FJ966960
	SW H1 reverse	1017–994	CGGGAT[1]ATTCCTT[2]AATCCTGTRGC		
	SW H1 probe	928–957	FAM–CAGAATATACAT(BHQ1)CCRG[3]TCACAATTGGARAA-PO4		
	H1F 722	722–744	AAGGGAGAATGAACTATTACTGG	Hemagglutinin	FJ966960
	H1R1062	1062–1046	AATGAAACCGGCAATGG		

[a] The mismatched nucleotides against the sequences of the novel human type A (H1N1) virus (A/California/06/2009, FJ966960) were shaded and the details were marked as: [1]T → C, [2]T → C, [3]G → A.

SH1 and NH1 probes, although they were all positive for the RP primer and probe set (Fig. 2D). These results indicated the multi-fluorescent real-time PCR assay could specifically discriminate the human novel H1N1 virus from swine H1N1 virus and other human seasonal H1N1 viruses.

3.3. Confirming the presence of novel H1N1 virus by sequencing

After being screened by the multi-fluorescent real-time RT-PCR assay, samples positive for influenza A H1N1 were subjected to an additional SYBR Green real-time PCR assay followed by direct sequencing. Primers specific for subtype H1 (H1F722/H1R1062, refer to Table 1) recently reported by our group were used in this assay (Yan et al., 2009). We chose the region between primers H1F722/H1R1062 for sequencing because the sequence information it provided could clearly identify the novel H1N1 virus as a distinct cluster among all viruses of subtype H1 through average distance clustering analysis (Yan et al., 2009). The sensitivity of primer H1F722/H1R1062 was also found to be 1×10^{-7} of diluted cDNA per reaction, which was examined by either gel electrophoresis or SYBR Green real-time PCR assay (Fig. 3).

3.4. Detecting the novel H1N1 virus in clinical specimens

The multi-fluorescent real-time RT-PCR assay developed in this study was applied in detecting human novel H1N1 virus in 27 clinical specimens from infected patients followed by sequencing for confirmation. Starting from RNA extracted from nasopharyngeal swab of the patients (performed in a biosafety-ensured CDC laboratory), we carried out the multi-fluorescent real-time RT-PCR assay in our laboratory as described above. The InfA, SH1, NH1 and RP probes were all positive in assays for the 27 clinical specimens (Fig. 4). The SW H1 primer and probe set provided by the WHO protocol was also employed in examining the same specimens in parallel, while only 15 specimens (56%) were positive for the SW H1 probe (Supplementary Table 1). The presence of human novel H1N1 virus in these clinical specimens was finally confirmed by sequencing the SYBR Green real-time PCR products; i.e. the sequences of the corresponding HA segment showed 100% identity to that of A/California/06/2009. These results indicated that specifically designed NH1 primer and probe set was significantly more sensitive than the SW H1 set ($p < 0.01$).

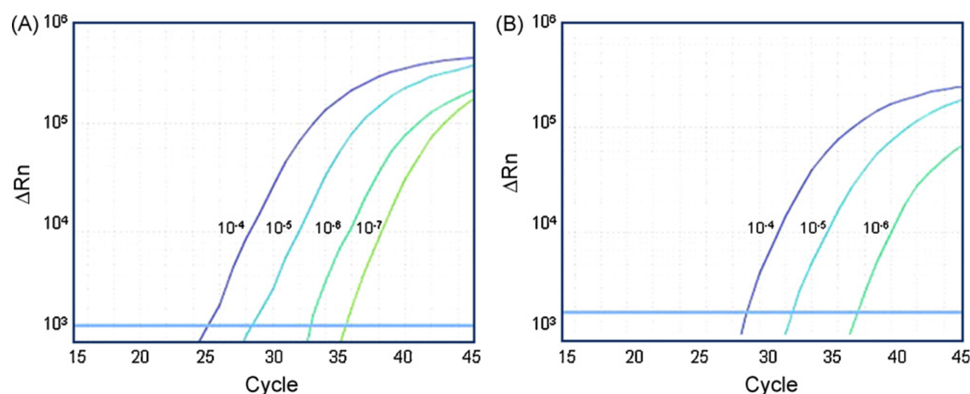

Fig. 1. RT-PCR detection limits of the NH1 primer and probe set specific for the novel type A (H1N1) virus. Real-time PCR amplification curves using serially diluted cDNA (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}, from left to right) prepared from a cultured human novel type A (H1N1) virus sample with known titer of 1×10^3 $TCID_{50}$ as templates with the NH1 primers and probe are shown in panel A. Similar kind of amplification curves using the same serially diluted cDNA templates (ranging in order from left to right: 1×10^{-4}, 1×10^{-5}, and 1×10^{-6}) with the SW H1 primer and probe set are shown in panel B.

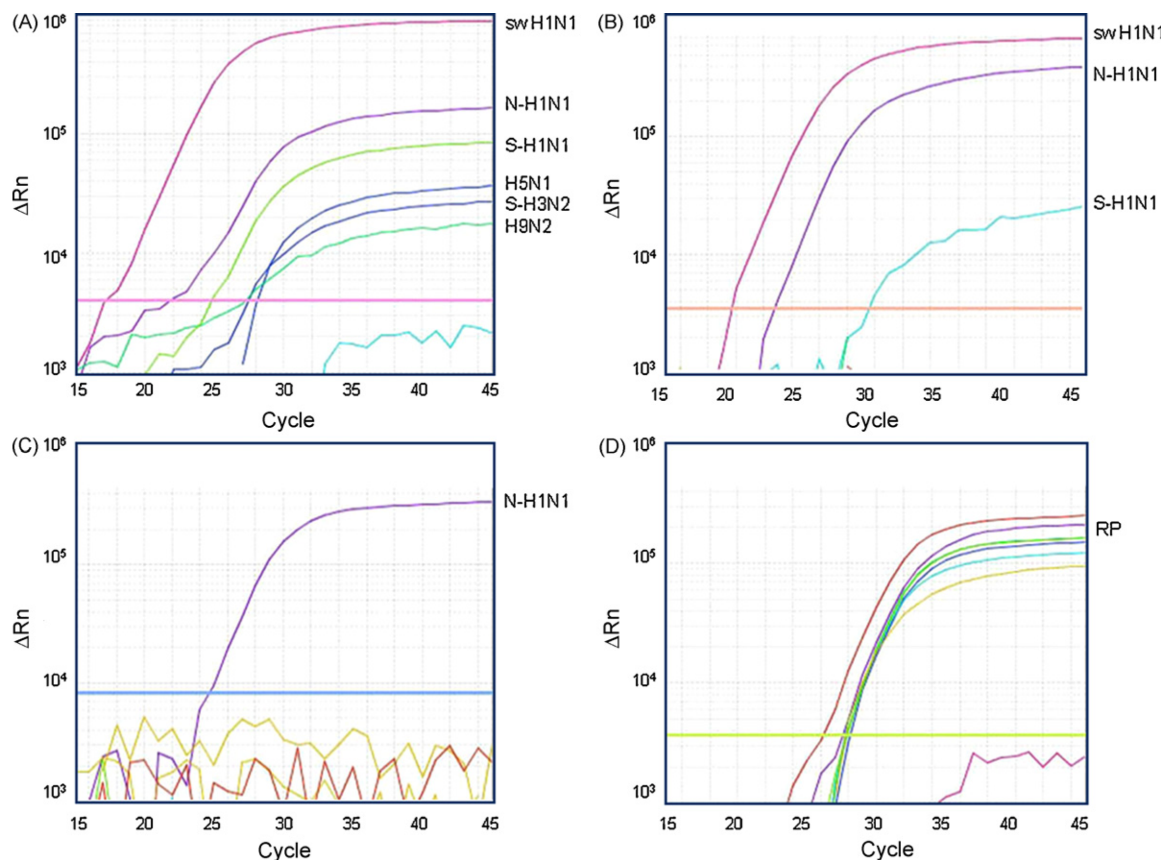

Fig. 2. Detection of the novel type A (H1N1) virus by multi-fluorescent real-time RT-PCR assay. The real-time PCR amplification curves for templates of the cDNA prepared from 1 swine H1N1 (SW H1N1), 1 human novel H1N1 (N-H1N1), 4 human seasonal H1N1 (S-H1N1), 1 human H5N1 (H5N1), 4 human seasonal H3N2 (S-H3N2), and 2 human H9N2 (H9N2) viruses, as well as 10 human influenza B viruses and 43 nasopharyngeal swabs from patients with noninfluenza respiratory diseases, employing primer and probe sets of InfA (A), SH1 (B), NH1 (C) and RP (D). For internal control, 20 ng of human total RNA was added to the RT-PCR reaction and the human RNaseP message should be detected by its corresponding primers and probe RP. ΔRn indicates the magnitude of the PCR signal.

4. Discussion

Increasing number of total infected cases of the novel H1N1 virus has been observed day by day since the first case was reported in April 2009. Developing rapid and specific molecular tests for early detection and confirmation of this novel virus is a matter of great urgency. However, in the real-time RT-PCR protocol recommended by WHO, the primers and the probe were designed for detection of swine H1N1 influenza viruses, of which, the HA sequence was slightly different from that found in all of the human infection cases. Thus, the SW H1 primer and probe set in the WHO protocol did not match exactly the sequence of the novel H1N1 virus (Table 1). The 6th and 13th bases of SW H1 reverse primer altered from "T" to "C", and the 16th base of SW H1 probe altered from "G" to "A" in novel H1N1 sequences. The mutation frequency of these 3 nucleotides in the swine H1N1 viruses was 19%, 4%, and 29% respectively. However, alignment of the 325 novel H1N1 sequences submitted to NCBI database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) before June 8, 2009 showed that, the frequency of "T → C" (6th base), "T → C" (13th base) and "G → A" mutations occurred in novel H1N1 viruses was as high as 98%, 100% and 100% respectively (Supplementary Table 2). Therefore, when 27 clinical specimens were tested, the SW H1 primer and probe were only able to detect 15 out of them, and the lower annealing affinity caused by 3 mismatched base pairs in addition to the probable lower virus titers of the other 12 samples is likely to account for the negative results (Supplementary Table 1). Nonetheless, the sensitivity of employing the SW H1 primer/probe set was one order lower than that employing the proprietary NH1 primers/probe set, which was designed solely based on the specific sequences of the novel H1N1 virus. The advantage of multi-fluorescent RT-PCR assay applied in our study is its ability to detect human influenza A, seasonal H1N1 and novel H1N1 viruses simultaneously in a single tube. It thus significantly simplified the experimental procedures and rendered it suitable for rapid detection and discrimination of seasonal H1N1 versus the novel H1N1 viruses.

Up to now, WHO has been strongly recommending that the nucleic acid of all samples containing influenza A non-typable virus should be sequenced to confirm the presence of the novel H1N1 virus (Chinese Center for Disease Control and Prevention, 2009; http://www.chinacdc.net.cn/n272442/n272530/n3479265/n347-9284/appendix/WHO_shiyanshizhinan_english.pdf). Therefore, after the rapid detection through multi-fluorescent RT-PCR assay, sequencing was used for final confirmation of the presence of this novel H1N1 virus. Instead of performing conventional PCR in which the products should be observed by gel electrophoresis before being sequenced, SYBR Green real-time PCR was carried out in this study. The products of SYBR Green real-time PCR with ideal amplification and melting curves could be sequenced directly after PCR reaction without agarose gel electrophoresis for amplicon verification, which not only eliminated amplicon carryover contamination, but also avoided the potential chemical hazard of electrophoresis to both the environment and the laboratory operators.

Our method could clearly discriminate the human novel H1N1 virus from swine H1N1 and other human seasonal H1N1 viruses, which has been validated through successfully detecting the

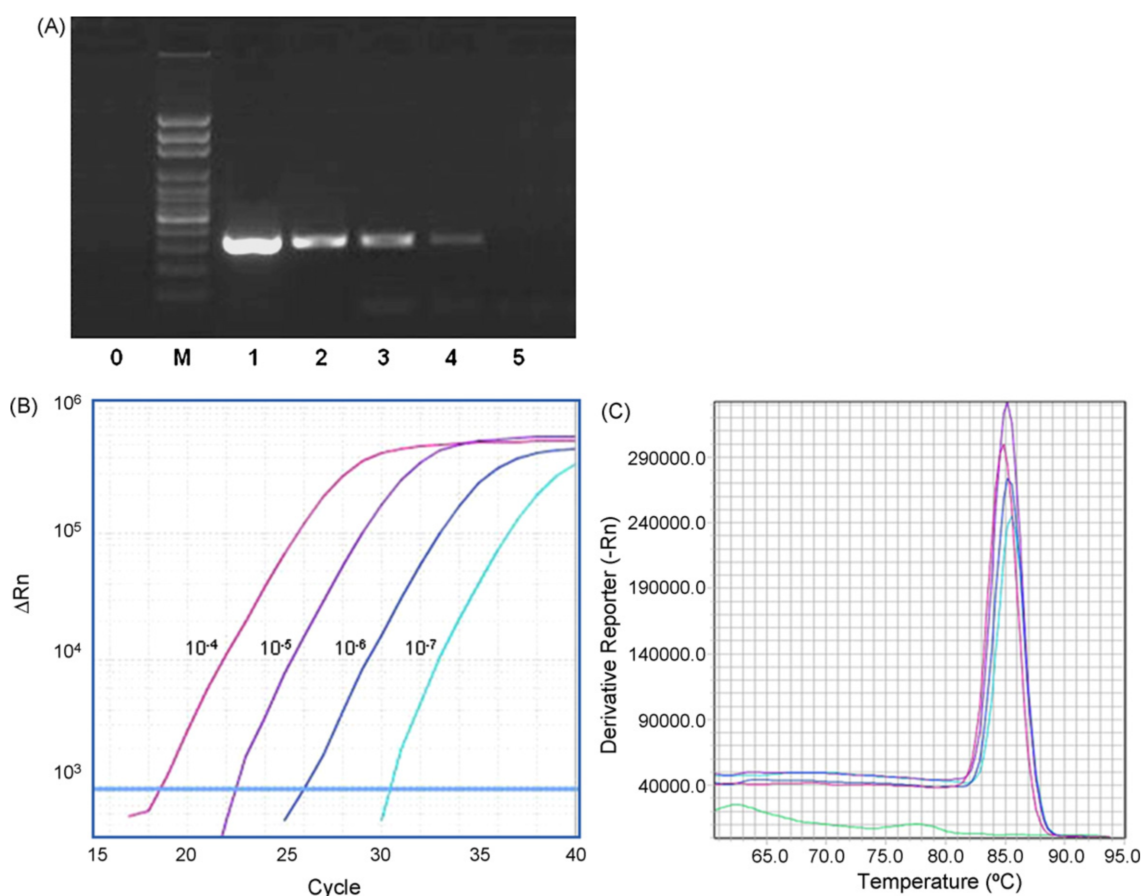

Fig. 3. Detection limits of primer H1F722/H1R1062. Conventional and SYBR Green real-time PCR amplification using serially diluted cDNA described in Fig. 1 as templates with the H1F722/H1R1062 primers. (A) Conventional PCR products analyzed by gel electrophoresis. 0, water control; M, 100 bp DNA ladder; 1, 1×10^{-4} diluted cDNAs; 2, 1×10^{-5} diluted cDNAs; 3, 1×10^{-6} diluted cDNAs; 4, 1×10^{-7} diluted cDNAs; and 5, 1×10^{-8} diluted cDNAs. (B) SYBR Green real-time PCR amplification curves. The dilution factors are indicated. (C) Melting curves of the amplicons in the SYBR Green real-time PCR assay.

novel H1N1 virus in 27 clinical specimens from infected patients. Although this method apparently is both specific and sensitive in detection as well as confirmation of the novel H1N1 infection in the current outbreak, due to the hyper-variable property of the nucleotide sequences in this detected region, the NH1 primers and probe may not be able to detect all the novel H1N1 viruses along with the lapse of time. Analyzing 701 novel H1N1 virus sequences submitted to NCBI database after June 8, 2009, we found that additional emerging variations in the detection region have made the NH1 primers and probe failed to match all the novel H1N1 sequences currently available (Supplementary Table 2). Therefore, the nucleotide sequences of the detection primers and probes should be modified in due course according to the variation of influenza virus sequences, and final confirmation by sequencing should be regarded as a golden standard.

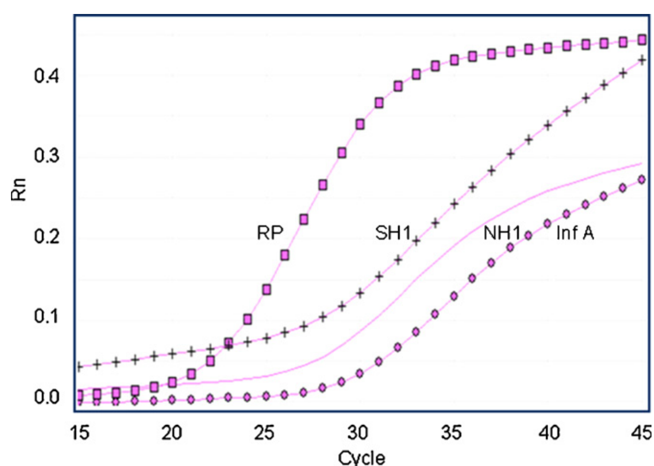

Fig. 4. Detection of the novel H1N1 virus from a clinical specimen by multi-fluorescent real-time RT-PCR assay. Amplification curves marked with squares are for the RP probe, crosses for the SH1 probe, circles for the InfA probe, while the one with no marker is for the NH1 probe.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2009.10.011.

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