

Development of novel AllGlo-probe-based one-step multiplex qRT-PCR assay for rapid identification of avian influenza virus H7N9

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Abstract Recently, human deaths have resulted from infection with low-pathogenicity avian influenza virus H7N9 strains that have emerged recently in China. To strengthen H7N9 surveillance and outbreak control, rapid and reliable diagnostic methods are needed. To develop a sensitive quantitative real-time RT-PCR assay for rapid detection of H7N9 viral RNA, primers and AllGlo probes were designed to target the HA and NA genes of H7N9. Conserved sequences in the HA and NA genes were identified by phylogenetic analysis and used as targets for H7N9 virus detection. The similarities of the targeted HA and NA gene sequences from different H7 and N9 influenza virus strains were 93.2–99.9 % and 96.0–99.6 %, respectively. The specificity and sensitivity of the new multiplex real-time qRT-PCR was established. The test was used for the detection of

viral RNA in human pharyngeal swabs and environmental samples. The detection limit of the multiplex qRT-PCR was estimated to be about 10^{-1} TCID₅₀/reaction. Finally, the diagnostic sensitivities of the multiplex qRT-PCR, virus isolation and TaqMan qRT-PCR were compared using pharyngeal swabs and environmental samples. These analyses yielded positive results in 46.7 %, 43.3 % and 20.0 % of the samples, respectively. The novel multiplex AllGlo qRT-PCR is a rapid and sensitive method to identify H7N9 virus in clinical and environmental samples and can be used to facilitate studies on the epidemiology of H7N9 virus.

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Introduction

Recently, H7N9 influenza virus infection cases were found in many areas of China [1]. Clinical cases resulting from H7N9 virus infections have attracted great attention from scientists and public-health officials worldwide [2]. Enhanced surveillance was implemented, resulting in the identification of additional H7N9 clinical cases in Shanghai, Jiangsu, Anhui and Zhejiang provinces. Until now, more than 100 positive cases have been identified in these areas with an unusually high case fatality rate of nearly 30 % [3]. Though it does not appear that sustained spread of the H7N9 virus occurs between people, there is still concern that this virus may evolve to allow efficient human-to-human transmission. Perhaps of equal concern is the fact that the H7N9 virus does not seem to cause serious disease in birds. This greatly complicates the control of the virus in these species and could create reservoirs of the virus that could cause spillovers to the human population.

To strengthen surveillance and control of the novel avian influenza virus H7N9, a highly sensitive, specific,

and rapid method for H7N9 detection is needed. Such a test will also facilitate rapid antiviral treatment and supportive therapy. Methods to identify H7N9 infections include virus isolation and hemagglutination inhibition (HI) assays. These assays require 1-2 weeks for completion and therefore do not allow a rapid diagnosis of infection. In contrast, diagnosis of infection using genome-based detection methods can be performed within hours [4]. Reverse transcription loop-mediated isothermal amplification combined with a lateral-flow device has been developed for the rapid and sensitive detection of H7N9 [5]. There are also real-time RT-PCR kits for the detection of H7 and N9 influenza viruses, but these assays do not allow the simultaneous detection of both the HA and NA genes of the virus [6].

The qRT-PCR reported here makes use of the AllGlo probe, which is the latest generation of fluorescent quantitative probes developed by AlleLogic Biosciences Corporation (Hayward, California, USA). Because AllGlo probes do not employ a quencher and have two signal-generating reporter dyes per oligonucleotide, they are much simpler to manufacture and, in most of the cases, offer much higher sensitivity than traditional TaqMan probes. The excitation and emission wavelengths of conventional dyes used for the AllGlo assays, such as MAR and JUP, are similar to those of FAM and VIC respectively [7, 8]. In this study, AllGlo probes were designed and used to develop a rapid and reliable multiplex qRT-PCR method for the identification of the H7N9 virus by simultaneous detection of H7 and N9 genes.

Materials and methods

Clinical samples and viruses

This study was approved by the ethics committee of Zhejiang Provincial Center for Disease Control and Prevention (CDC), China. With permission of the patients, 15 pharyngeal swabs were collected from patients who were suspected to be infected with avian H7N9 influenza virus. Fifteen environmental samples, including feces and wastewater from cages, were collected at live-bird markets that had been visited by the H7N9-positive patients in the district of Huzhou. The samples were stored in 3-5 ml of Hanks' solution containing 100 U penicillin and 100 µg streptomycin per ml at -70 °C until tested. Avian influenza A virus strains (A/Zhejiang/1/2013(H7N9), A/environment/Zhejiang/S91/2013(H5N1), A/environment/Zhejiang/S52/2013(H9N2)) used in this study were all isolated from embryonated chicken egg culture. Seasonal influenza A virus (A/Zhejiang/1/2007(H1N1), A/Zhejiang/S16/2013(H3N2), A/Zhejiang/SWL5/2013(H1N1)) were all isolates from MDCK (Madin-Darby canine kidney) cell

culture. All strains used in this study were provided by Zhejiang Provincial CDC.

Sequence analysis and primer design

HA and NA gene sequences from H7N9 virus strains were downloaded from GenBank and used for phylogenetic analysis using MEGA 4.0 software (BioDesign Institute, AZ, USA). The Primer Express software package (Applied Biosystems, CA, USA) was used for primer and probe design. All of the primers and probes used in this study were synthesized by Invitrogen (Invitrogen, Shanghai, China). The targeted HA and NA gene sequences that were used for H7N9 virus identification were further used for conservation analysis using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Extraction of viral RNA

Viral RNA was extracted using an RNeasy Mini Kit (QIAGEN, CA, USA) according to the manufacturer's instructions. Briefly, 200 µl of the specimen was mixed with 600 µl of RLT buffer and 6 µl of β-mercaptoethanol and incubated for at least 1 min at room temperature. After the addition of 600 µl of cold 70 % ethanol, the mixture was vortexed and applied to a spin column. After the washing steps, RNA was eluted in 50 µl of RNase-free water.

T-A cloning and sequencing

A High Fidelity One Step RT-PCR Kit (TaKaRa, Shiga, Japan) was used to amplify the target gene segment. According to the protocol provided, the total volume per reaction was 50 µl, which included 10 pmol of each of the primers H7-FP, H7-RP, N9-FP and N9-RP (Table 1), 25 µl of 2× One Step RT-PCR Buffer III, 1 µl of TaKaRa Ex TagHS (5 U/µl), 1 µl of PrimeScript RT Enzyme Mix II, 4 µl of total RNA and 17 µl of RNase-free dH₂O. The reverse transcription reaction was performed by incubation at 42 °C for 5 min and terminated by incubation at 95 °C for 10 s. This was then followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. The products were detected in a 1.5 % ethidium-bromide-pre-stained agarose gel after electrophoresis. To confirm the identity of PCR products, the products were purified and cloned into plasmid pMD19-T using a T-A cloning kit (TaKaRa). The inserts of the recombinant plasmids (pMD19T-HA, pMD19T-NA) were then sequenced.

Multiplex real-time RT-PCR

Multiplex real-time RT-PCR was performed by using a One Step PrimeScript RT-PCR Kit (TaKaRa). Each

Table 1 Nucleotide sequences of the primers and probes used in this study

Primer/probe	Sequence (5' to 3')	Genomic position	PCR product size (bp)
H7-FP	CACTGCARAATAGAATACAGATWGAC		157
H7-RP	GTGCACYGCATGTTTCCATT	1514-1670	
H7-P	MAR-CTGGTTTAGCTTCGGGGCATC-MAR		
N9-FP	ACAATACACGATAGRTCCAGTA		107
N9-RP	CTAGTACTTGACCACCCAATGC	445-551	
N9-P	JUP-TRTCATCACCRCCACAGTAT-JUP		

reaction mixture consisted of 10 pmol of the primers H7-FP, H7-RP, N9-FP and N9-RP, 5 pmol of the probes H7-P and N9-P (Table 1), and 4 µl of template RNA in a final volume of 50 µl. RT-PCR reactions were performed according to the manufacturer's instructions, using the same conditions as for the conventional RT-PCR described above.

Sensitivity and specificity of real-time RT-PCR assays

Tenfold serial dilutions of H7N9 viral RNA templates were analyzed by real-time RT-PCR, and the results were used to generate a standard curve.

In order to evaluate the specificity of the real time RT-PCR established in this study, genomic nucleic acid extracts from seasonal influenza A virus (A/Zhejiang/1/2007(H1N1), A/Zhejiang/S16/2013(H3N2), A/Zhejiang/SWL5/2013(H1N1)) and avian influenza A virus A/environment/Zhejiang/S91/2013(H5N1), A/ environment/Zhejiang/S52/2013(H9N2) were prepared and used as templates. RNA templates of H7N9 virus were used as positive controls. Samples were analyzed in duplicate reactions on an ABI 7500 real-time PCR system.

Specimen detection by AllGlo multiplex RT-PCR

Genomic nucleic acid extracted from human pharyngeal swabs and environmental samples was used as template. Primers H7-FP, H7-RP, N9-FP and N9-RP, AllGlo probes H7-P and N9-P, and the multiplex real-time qRT-PCR developed in this study were applied for H7N9 virus detection. The amplification procedure was the same as described above.

Specimen detection by virus isolation and TaqMan RT-PCR

Embryonated chicken eggs were used for H7N9 virus isolation. Six 9-day-old embryonated chicken eggs were each inoculated with 200 µl of sample supplemented with antimicrobials in the allantoic fluid. The eggs were

incubated for 3 days and candled daily to check their viability. Chorioallantoic fluid was tested for hemagglutination of 0.5 % chicken red blood cells in phosphate-buffered saline. Hemagglutinin-inhibition tests with subtype-specific antisera were used to subtype all hemagglutination-positive samples. TaqMan qRT-PCR detection was performed as described before [2].

Results

Sequence and phylogenetic analysis

HA gene sequences of the H7 influenza A virus strains from Zhejiang (GenBank: JQ906576.1), Shiga (AB558257.1), Tsukuba (AB558263.1), Shimane (AB558255.1), Korea (FJ750852.1), Mongolia (AB473543.1), Hokkaido (AB243420.1), Akita (AB517639.1), Hadoree (FJ767721.1), Taiwan (AB269695.2), Thailand (JX307228.1) and Italy (CY099605.1) were used for conservation analysis. The NA gene sequences of N9 influenza A virus strains from Korea, Australia, Hokkaido, Tsukuba, Chiba, the Czech Republic, Vietnam, Spain, Mongolia, Siberia, Sweden, and Hunan were used for a similar comparison. The GenBank numbers of these strains are EU263337.1, CY025199.1, AB298284.1, AB472033.1, AB472037.1, JF789604.1, AB593473.1, HQ244409.1, AB481213.1, AB292784.1, GU052870.1, CY109684.1, respectively. As shown in Fig. 1, the similarities of the selected HA and NA gene nucleotide sequences from these different virus strains were 93.3-99.5 % and 96.5-99.6 %. Phylogenetic analysis showed that the average variability among these HA and NA sequences was 3.5 %.

After selection of target sequences, primers and probes were designed using Primer Express 5.0 software. The primer and probe sequences were generated, and the target segments were then applied for homology analysis using BLAST at NCBI. We found that the sequences of the target gene segments of HA and NA are conserved in H7 and N9 virus strains, respectively.

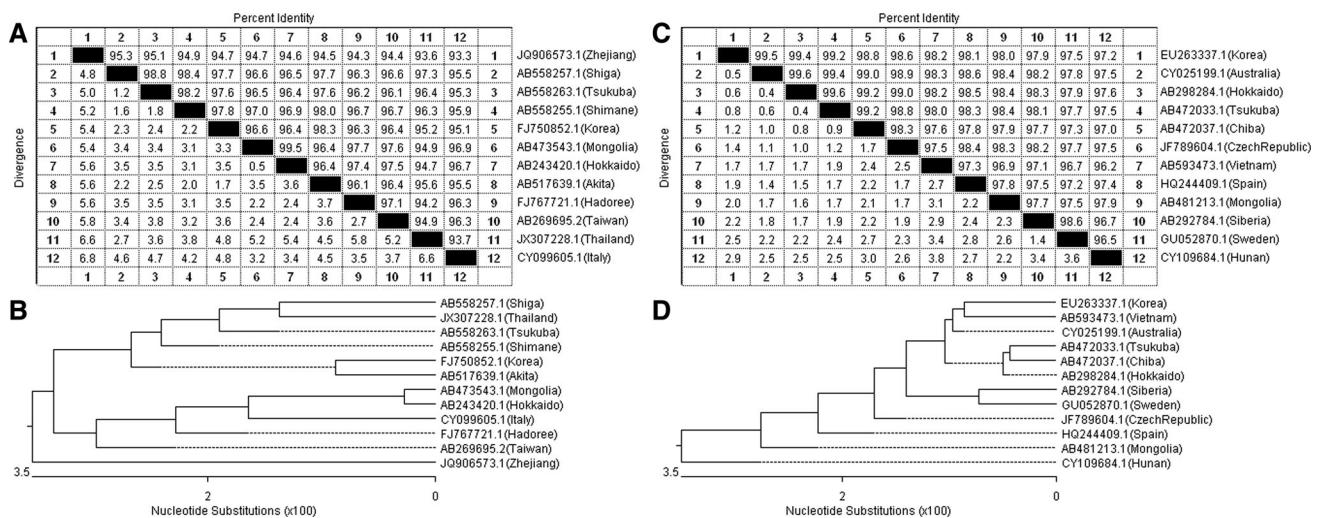


Fig. 1 Homology comparison and phylogenetic analysis of the HA and NA gene sequences from different H7 and N9 influenza A virus strains. **A**. The nucleotide sequence identities of the HA genes of the H7 influenza virus strains from Zhejiang, Taiwan, Hokkaido, Mongolia, Akita, Shimane, Shiga, Tsukuba, Italy, Korea, Hadoree and Thailand are between 93.3–99.5 %. **B**. Phylogenetic analysis

PCR amplification and TA cloning

After PCR amplification using genomic nucleic acid from the A/Zhejiang/1/2013(H7N9) virus as template, products were visualized on a 1.5 % agarose gel. The sizes of the amplification products of the HA gene (157 bp) and NA gene (107 bp) were found to be correct. The results also demonstrated the high specificity of the primers. The PCR products were purified and cloned into the plasmid pMD19-T. The inserts in the resulting recombinant plasmids pMD19-T-HA and pMD19-T-NA were further confirmed by sequencing. After comparison with reference sequences from H7 and N9 influenza A viruses, we observed that the similarities between them were all above 95 %. In order to improve the detection sensitivity of the qRT-PCR, degenerate oligonucleotides were used when appropriate (Table 1).

Sensitivity and specificity of the real-time RT-PCR assay

Using AllGlo technology, a multiplex qRT-PCR method was established for the detection of H7N9 virus in pharyngeal swabs. In order to evaluate the sensitivity of the established detection method, serial dilutions of H7N9 viral RNA templates generated previously were used to establish standard curves in triplicate. As shown in Fig. 2A and C, the lower detection limit of the real-time qRT-PCR method is 10^{-1} TCID₅₀/reaction. Data were then subjected to log-linear analysis to generate a standard curve for calculation of unknowns ($R^2 > 0.99$) (Fig. 2B and D).

shows that the average variability among these sequences is 3.5 %. **C**. The nucleotide sequence identities of the NA genes of the N9 influenza virus strains from Korea, Siberia, Hokkaido, Tsukuba, Chiba, Mongolia, Vietnam, Australia, Hunan, Sweden, Spain and the Czech Republic are between 96.5–99.6 %. **D**. Phylogenetic analysis shows that the average variability among these sequences is 3.5 %

GenBank was explored to evaluate if the primer sequences could pair with sequences other than those targeted in the HA and NA genes of the H7 and N9 influenza A viruses. BLAST analyses did not reveal such sequences. Then, the specificity of the established multiplex real-time qRT-PCR was tested by using the genomic nucleic acid from other viruses of the same genus or viruses that may cause similar symptoms in humans. We used all of the influenza A viruses available at our institute for specificity evaluation, and we found that only the H7N9 influenza A virus gave CT values above the detection limit after real-time qRT-PCR (Fig. 3). Specificity studies included H1, H3, H5, and H9 influenza viruses, human respiratory syncytial virus, and human adenovirus, which can cause similar clinical symptoms [6].

Evaluation of the qRT-PCR assay with different sample types

Pharyngeal swab samples were obtained from patients, and environmental swabs were obtained from the live-bird market that they had recently visited. Clinical samples were collected from patients with pneumonia and abnormal neurological symptoms not normally observed in patients suffering from seasonal H1N1 or H3N2 influenza A virus infection. Fourteen samples from patients (4/15) and the associated environment (10/15) were positive for H7N9 using AllGlo-probe-based multiplex real-time qRT-PCR (Fig. 3B). The PCR products were also visualized on an agarose gel to confirm their correct size and sequenced to confirm their identity. However, 13 samples from patients

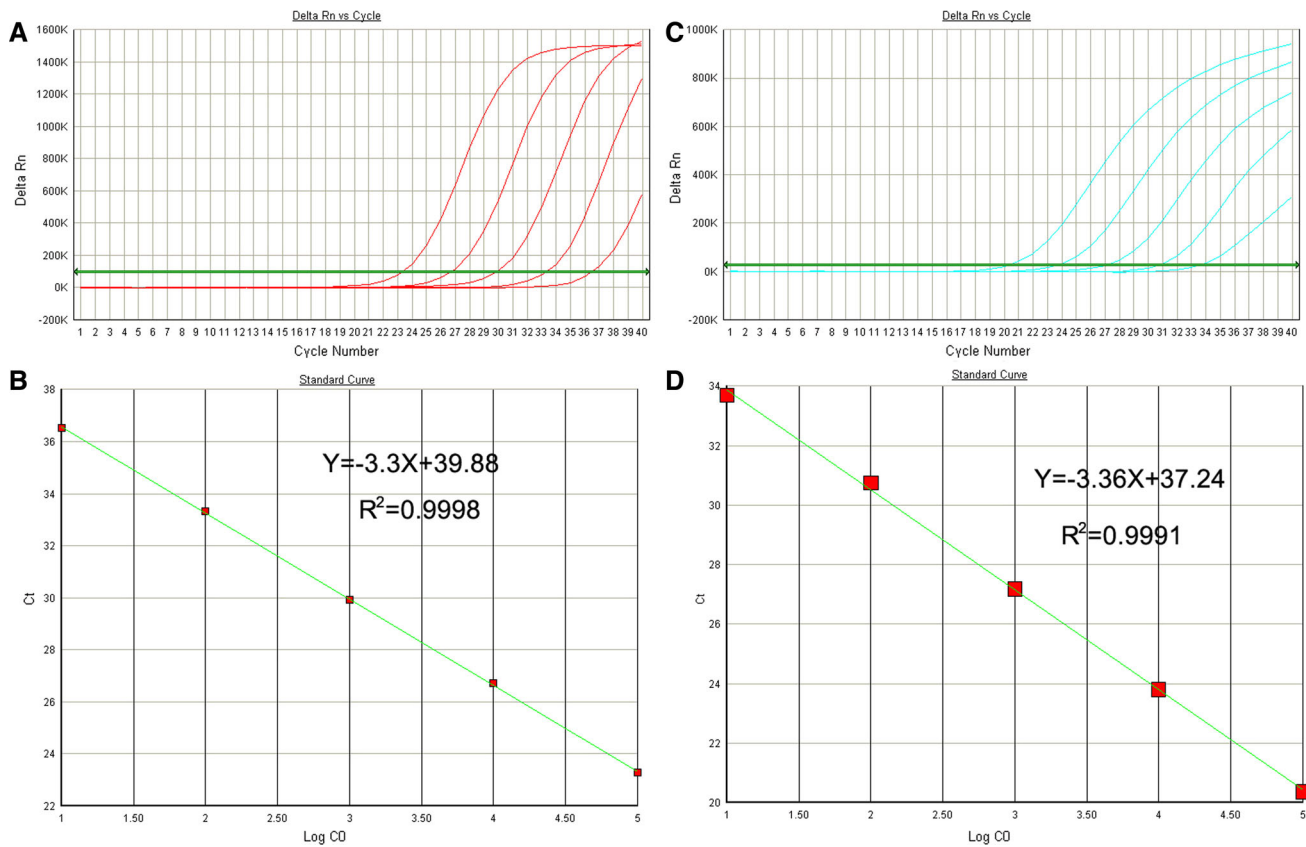


Fig. 2 Sensitivity of the multiplex real-time qRT-PCR. **A.** Real-time RT-PCR amplification plot of the HA viral gene segment. A PCR baseline subtractive curve fit view of the data is shown with relative fluorescence units (RFU) plotted against cycle number. The default setting of 10 times the standard deviation of fluorescence in all wells over the baseline cycles was used to calculate the threshold cycle or Ct value for a positive reaction (horizontal line). **B.** Standard curve

analysis of the amplification plots with Ct values plotted against starting copy numbers. A typical amplification plot derived from serial tenfold dilutions of templates ranged from 10^3 to 10^{-1} TCID₅₀/reaction ($R^2 = 0.9998$). **C.** Real-time RT-PCR amplification plot of the NA viral gene segment from serial tenfold dilutions of templates. **D.** Standard curve analysis of the amplification plots with Ct values plotted against starting copy numbers ($R^2 = 0.9991$)

(4/15) and the associated environment (9/15) were positive by TaqMan-probe-based single-plex qRT-PCR. Six H7N9 virus isolates were generated from samples from patients (2/4) and the associated environment 4/10 (Table 2). Experiments were done to compare the detection efficiency of H7N9 by AllGlo-probe-based multiplex qRT-PCR and the established TaqMan-probe-based qRT-PCR. The results confirmed that the AllGlo-probe-based multiplex qRT-PCR method was sensitive and reliable for H7N9 virus detection (S1).

Discussion

When different subtypes of influenza A viruses co-infect a cell, they can exchange their gene segments and produce novel viruses with new genotypes, a process known as reassortment [9]. Preliminary studies have suggested that the new virus H7N9 stems from a reassortment of three virus strains that were known to infect only birds. It was

found that the gene encoding the N protein is similar to that of avian H11N9 viruses that were found in South Korea in 2011. The gene for the HA protein seems to belong to a Eurasian group of H7 avian influenza viruses.

It is striking that the HA sequence of the novel H7N9 virus is similar to that of viruses that do not cause severe sickness in birds. As a result, H7N9 viruses could be spreading in poultry undetected and thereby creating a reservoir of the virus that could periodically lead to spillover to the human population. At this moment, much effort is being put into the identification of birds or other animals that could function as hosts of the H7N9 virus. The multiplex qRT-PCR developed in the current work could facilitate these studies. It could also be used to facilitate rapid identification of new human cases. Early diagnosis of infection with H7N9 virus is not only critical for the treatment of infected patients but also for the control of the spread of the disease.

To strengthen H7N9 surveillance and outbreak control, detection tools with optimal sensitivity and specificity

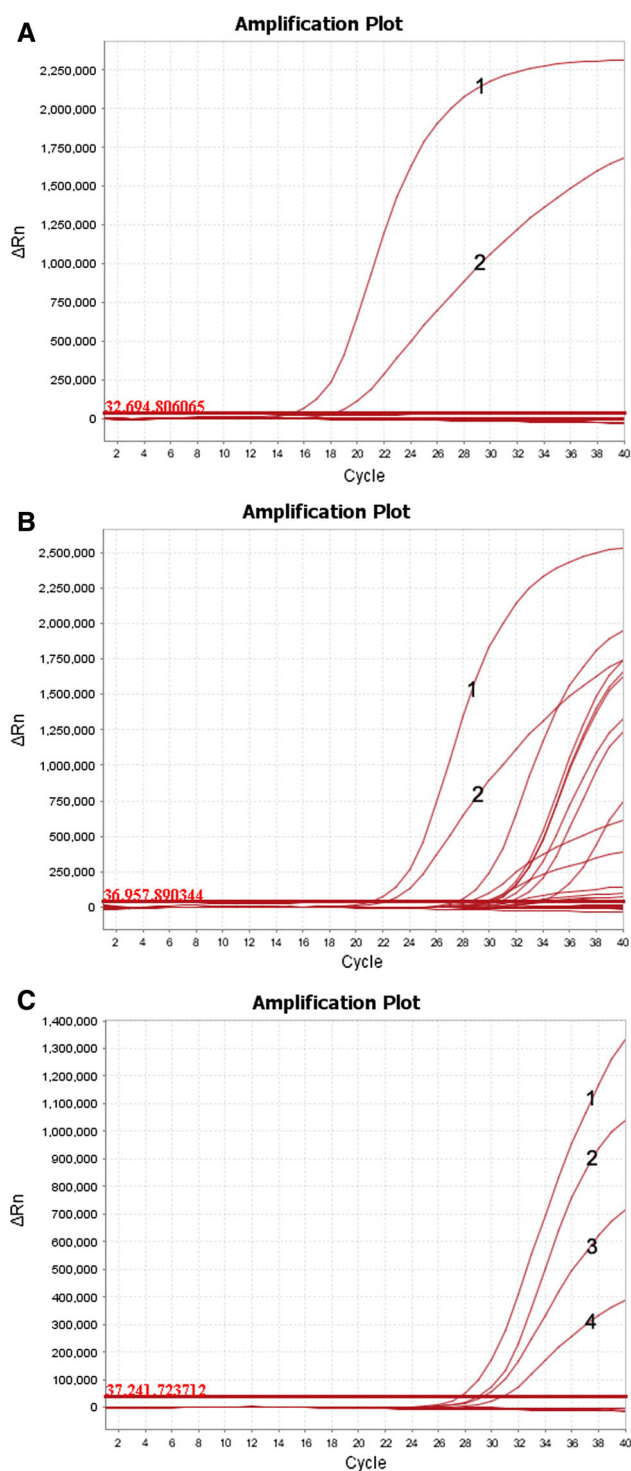


Fig. 3 Specificity analysis of the multiplex real-time qRT-PCR and sensitivity comparison by specimen detection. **A.** Specificity analysis of the real time RT-PCR was performed using genomic nucleic acid from seasonal influenza A virus H1N1, swine influenza A virus H1N1, influenza A virus H3N2, avian influenza virus H5N1, avian influenza virus H9N2 strains as controls. For the primer-probe sets, no positive results were obtained except with the RNA transcripts of H7N9 virus. (1 and 2, positive control). **B.** Clinical and environmental specimens identified by the real-time RT-PCR. Using the RNA of H7N9 virus as positive control, RNA transcripts from positive samples gave a typical amplification plot. (1 and 2, positive control). **C.** Sensitivity comparison of the AllGlo multiplex qRT-PCR and TaqMan qRT-PCR. 1 and 3, AllGlo multiplex qRT-PCR amplification plot of the HA and NA gene segments from H7N9 virus RNA; 2 and 4, TaqMan qRT-PCR amplification plot of the HA and NA gene segments from H7N9 virus RNA

Table 2 Comparison of detection of H7N9 virus from clinical and environmental specimens by different methods

Result	Number of samples with the indicated result		
	Virus isolation and hemagglutination assays	TaqMan qRT-PCR	AllGlo qRT-PCR
Positive	6	13	14
Negative	24	17	16
Total	30	30	30

is the latest generation of fluorescent quantitative probes, which has the advantages of good reproducibility, high sensitivity, and high specificity, and it can facilitate high-throughput diagnostics. We set out to design an AllGlo-probe-based multi-quantitative real-time RT-PCR assay to identify and quantify H7N9 virus present in different samples.

The multiplex real-time qRT-PCR assay for the identification of the H7N9 virus has many advantages over conventional methods with respect to the detection limit of the assay as well as specificity [10]. Standard curves show that the amplification efficiency of templates with different concentrations of RNA is similar, and the maximum sensitivity achieved was 10^{-1} TCID₅₀/reaction. Multiple negative controls as well as positive controls have been included in diagnostic multiplex qRT-PCR in order to achieve an acceptable level of confidence in the absence of false-positive and/or false-negative results. An investigation of the specificity of the multiplex real-time qRT-PCR assay revealed that only the templates of the H7N9 RNA showed positive detection results.

Finally, the multiplex real-time qRT-PCR established in this study was further applied to identify H7N9 virus in different samples. After preliminary analysis and diagnosis by the monitoring hospitals of our province, pharyngeal swab samples from patients with suspected H7N9 infection were collected and analyzed in our laboratory. Using the

should become available to clinical laboratories and public-health departments. Methods used to identify influenza viruses in birds should allow differentiation between antigenically and genetically distinct influenza virus subtypes. qRT-PCR is particularly suitable for this purpose, allowing rapid detection of influenza viruses in animal and human specimens. The AllGlo technology used in the current work

established multiplex qRT-PCR, the positive rate of H7N9 infection in patients was 4/15. In addition, the environmental samples collected from the poultry flocks to which the positive patients had been exposed were also used for H7N9 virus identification and showed a positive rate of 10/15. For H7N9 detection, the TaqMan qRT-PCR-positive samples and virus-isolation-positive samples all gave positive results in the AllGlo qRT-PCR. Although this study showed some relationship between the poultry flocks and H7N9 infection disease in humans, we still do not know where the virus comes from. In order to identify the birds or animals from which the affected humans acquired the virus, the method established here would be very useful for identification of the host animal.

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Conflict of interest The authors declare that they have no competing interests.

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