Real-time RT-PCR for H5N1 avian influenza A virus detection

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The recent recurrence of highly pathogenic avian influenza virus A H5N1 was firstly reported in mid-December 2003 and continued through 2005. This study describes a sensitive and specific real-time RT-PCR method for the detection of influenza A subtype H5 and for monitoring virus loads. Using serial dilutions of influenza A H5N1 cultures, this assay reproducibly determined the lowest detection limit to be approximately 5×10^{-2} 50 % egg infective doses (EID₅₀). In contrast, the minimum detection limit was approximately 3 EID₅₀ in conventional RT-PCR with WHO primers and 10 EID₅₀ in antigen-capture ELISA. In tests of serial dilutions of *in vitro*-transcribed influenza A H5 gene RNA, there was linear amplification from 40 copies to 4×10^8 copies of target RNA per reaction and approximately six copies, and sometimes even as few as three copies, of target RNA tested positive in our assay. Thirty-five throat swabs from ill birds were tested: 33 samples tested positive using this assay. In comparison, 27, 13 and 19 samples tested positive using conventional RT-PCR, antigen-capture ELISA and virus isolation, respectively. To evaluate further the sensitivity of this real-time RT-PCR, a standard panel and 60 H5N1 isolates that contained different clades of influenza virus A/H5N1 were tested and all tested positive. To evaluate the specificity of the assay, 60 throat swabs from patients infected with influenza virus A H1 were tested; all were negative. Thirteen other viruses were also tested and all tested negative.

Received 20 October 2006

Accepted 11 January 2007

INTRODUCTION

The highly pathogenic avian influenza virus (HPAIV) H5N1 was first isolated from humans during the 1997 outbreak in Hong Kong. Eighteen patients were confirmed to have been infected with H5N1 after close contact with poultry and six patients died (CDC, 1997). The recent recurrence of influenza A H5N1, which was first reported in Southeast Asia in mid-December 2003, is severe and has involved increasing numbers of countries. It had infected 218 people and caused 124 deaths in ten countries by May 2006 (WHO, 2006). HPAIV has not only posed a continuing global human public health risk, but also endangered the poultry industry (CDC, 2004; FAO, 2005; Tran *et al.*, 2004; Viseshakul *et al.*, 2004). The recurrence of influenza A H5N1 has highlighted the need for a highly

sensitive, accurate and rapid diagnostic test for the infection. Such a test is important, not only for infection control, but also to facilitate early antiviral therapy. However, cell culture and subsequent haemagglutinin (HA) and neuraminidase subtyping by serological testing require 1–2 weeks for results; thus they are less useful in making therapeutic and infection-control decisions. On the other hand, commercially available rapid antigen tests for influenza A are rapid and simple, but subtyping (H1 and H5) of viruses shows cross-reactivity (WHO, 2005). Molecular diagnosis of influenza A H5 by real-time RT-PCR (RRT-PCR) can be a rapid assay: results, including subtyping, may be available in less than 1 day.

Standard RT-PCR has been applied previously to the detection of AIV and each of the 15 HA subtypes (Lee *et al.*, 2001; Munch *et al.*, 2001; Starick *et al.*, 2000). The World Health Organization (WHO) has also recommended a pair of primers for H5 subtype detection as a laboratory test (WHO, 2005). RRT-PCR with hydrolysis probes has been applied successfully to the detection of various RNA viruses (Chen *et al.*, 2004; Holland *et al.*, 1991; Livak *et al.*, 1995).

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Abbreviations: EID₅₀, 50 % egg infective dose; FAM, 6-carboxyfluorescein; HA, haemagglutinin; HPAIV, highly pathogenic avian influenza virus; RRT-PCR, real-time RT-PCR; TAMRA, 6-carboxytetramethylrhodamine; WHO, World Health Organization.

RRT-PCR offers the advantages of speed, viral load analysis, sensitivity and specificity compared with standard RT-PCR. In this study, we developed a rapid, highly sensitive and specific real-time, fluorescent, quantitative RT-PCR for direct detection of influenza A H5.

METHODS

Samples. Throat swab samples were collected from 35 birds presenting with abnormal neurological signs and diarrhoea in Qinghai Lake, China, during the HPAIV (H5N1) outbreak in 2005 (Liu *et al.*, 2005). Throat swab samples were also collected from 60 patients who were confirmed to be infected with influenza A H1 by RT-PCR assay (Wright *et al.*, 1995) at Beijing 301 Hospital, China. The swabs were kept at 4 °C and transported to the laboratory within 48 h in PBS at 4 °C supplemented with 200 mg streptomycin ml⁻¹, 100 U penicillin ml⁻¹ and 10 µg amphotericin B ml⁻¹. On receipt of the specimen, prior to any manipulation of the specimen, some aliquots were removed in a type II biological safety cabinet for molecular analysis and virus isolation.

Viruses. Sixty isolates of AIV/H5N1 that originated from different species from 1997 to 2005 were provided by the HPAIV study team. The genomes of these isolates were sequenced and they divided into different clades (Table 1 and unpublished data). The other 14 viruses were kindly provided by China Agriculture University, the Academy of Military Medicine Science and the National Institute for the Control of Pharmaceutical and Biological Products: influenza virus A/PR/8/34 (H1N1); influenza virus A/Beijing/30/95 (H3N2); influenza virus A/duck/Taiwan/4201/99 (H7N7); influenza virus A/Swine/Shandong/nb/2003 (H9N2); influenza virus B (Hongkong/5/72); parainfluenza viruses 1, 2 and 3; severe acute respiratory syndrome-associated coronavirus (TJF); respiratory syncytial virus; human immunodeficiency virus 1; cytomegalovirus; Epstein-Barr virus; and adenovirus type 2. The titres of all influenza viruses were determined by 50% egg infective dose (EID₅₀), whilst the titres of the other viruses were determined by quantitative PCR (Chen *et al.*, 2004; Kubar *et al.*, 2005; Kuypers *et al.*, 2006; Palmer *et al.*, 2003; Perkins *et al.*, 2005); they were determined to be approximately 10⁴ EID₅₀ ml⁻¹ or 10⁶ copies ml⁻¹, respectively.

Primers and probe. In view of the divergence of the H5 gene, we aligned all H5 gene sequences in the NCBI GenBank database

(*n*=252) and our unpublished H5N1 genome sequences (*n*=60) and chose a conservative region from which to design the primers and probe using PRIMER EXPRESS software v2.0 (Table 2). Another H5 subtype primer set was synthesized according to data published by WHO (WHO, 2005). The probe was labelled at the 5' end with 6-carboxyfluorescein (FAM) reporter dye and at the 3' end with 6-carboxytetramethylrhodamine (TAMRA) quencher dye. All primers and the probe were synthesized by Shanghai Sangon Company.

Transcription of AIV H5 RNA *in vitro*. We designed a pair of universal primers to amplify the complete HA gene (forward: 5'-TATTGGTCTCAGGAGCGAAAGCAGGGG-3'; reverse: 5'-ATA-TGGTCTCGTATTAGTAGAAACAAGGGTGT-3'). The influenza A virus [A/Swine/Anhui/cb/2004 (H5N1)] H5 gene was amplified by RT-PCR and the product was cloned into the pGEM-T Easy vector (Promega). The recombinant plasmid was linearized with *Pst*I, purified with a PCR purification kit (Shanghai Biotech) and transcribed with T7 RNA polymerase using a RiboMax Express large-scale RNA production system (Promega). The template DNA was degraded with 5 U RNase-free DNase I and the RNA transcripts were purified twice with an RNeasy kit (Qiagen). The RNA was quantified spectrophotometrically at 260 nm, divided into aliquots and stored at -80 °C. Diluted AIV H5 transcripts (4 × 10⁸-4 × 10⁻² copies µl⁻¹ at tenfold dilutions; 4 × 10¹-7.8 × 10⁻² copies µl⁻¹ at twofold dilutions) were used for the determination of detection limits and the amplification efficiency of the assay.

Standard influenza virus A/H5N1 panel. The standard avian influenza A virus/H5N1 panel was supplied by the Influenza virus A/H5N1 Quality Assurance Laboratory, National Institute for the Control of Pharmaceutical and Biological Products. It contained eight volumes of different isolates of influenza virus A/H5N1: Hong Kong/213/03 (clade 1), black-headed Goose/QH/1/05 (clade 2), Anhui/1/2005 (clade 2) and Hong Kong/156/97 (clade 3) (two volumes of each). The concentration of each volume was approximately 10⁴ EID₅₀ ml⁻¹.

RNA extraction. Total RNA was extracted using a QIAamp RNA extraction kit (Qiagen) according to the manufacturer's instructions. Briefly, 140 µl of each sample was used for the extraction of viral genomic RNA. The RNA was eluted from the columns with 50 µl DEPC-treated water and used in the following experiments immediately or stored at -80 °C. All of the above methods were performed in a Biosafety level 3 facility.

DNA extraction. DNA was extracted using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. Briefly, 100 µl of each virus culture was used for the extraction of viral genomic

Table 1. Species of origin and clades of 60 H5N1 isolates

Species	Clade				Total
	1	2	3	Others	
Chicken		5	17	16	38
Duck	1	2	2	2	7
Goose			2	1	3
White swan		1			1
Mallard		1			1
Black-headed goose		2			2
Great black-headed gull		1			1
Black-headed gull		1			1
Human			2		2
Swine		2	1	1	4
Total	1	15	24	20	60

Table 2. PCR primers and probe used for AIV H5 subtype detection

Primer/probe	Primer sequences (5'→3')	PCR product (bp)
H5f-53*	ACATGCCCAAGACATACTGGAA	130
H5r-182	GAATTCGTCACACATTGGGTTTC	
H5 Probe-79	FAM-CACACAACGGGAAGCTCT-GCGATCT-TAMRA	
WHOf-839	GCCATTCCACAACATACACCC	219
WHOOr-1057	CTCCCTGCTCATTGCTATG	

*Reference strain: influenza A virus [A/great black-headed gull/Qinghai/1/2005 (H5N1)].

DNA. The DNA was eluted from the columns with 50 μ l double-distilled water. DNA was used in the following experiments immediately or stored at -80°C .

Conventional RT-PCR. The Qiagen one-step RT-PCR kit was used for RT-PCR with a 30 μ l reaction mixture containing 10 μ l RNA, 6 μ l $5\times$ buffer, 1.2 μ l dNTP mix (25 mM), 1.2 μ l enzyme mix ($25\times$), 0.3 μ l RNase inhibitor (20 U μl^{-1}), 0.6 μ M WHO forward primer and 0.6 μ M WHO reverse primer. The reverse transcriptase step was carried out at 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of amplification (94°C for 30 s; 55°C for 30 s; 72°C for 30 s). Amplified products were detected by agarose gel electrophoresis and sequence identification. Sequencing was carried out using an ABI PRISM 3730 DNA sequencer.

RRT-PCR. RRT-PCR was carried out in a 30 μ l mixture containing 10 μ l RNA, 15 μ l $2\times$ Taqman one-step RT-PCR master mix (ABI), 0.75 μ l $40\times$ MultiScribe and RNase inhibitor mixture, 0.25 μ M forward primer, 0.25 μ M reverse primer and 0.125 μ M probe in a fluorometric PCR thermocycler (ABI 7300). The reaction was carried out for 30 min at 48°C , followed by 10 min at 95°C , with a subsequent 40 cycles of amplification (95°C for 15 s; 60°C for 1 min; fluorescence was recorded at 60°C).

Antigen-capture ELISA. All samples were also tested by antigen-capture ELISA employing biotin-labelled monoclonal antibodies (Wang *et al.*, 2004). The monoclonal antibodies (H5) were a gift from Professor Qin, Institute of Microbiology and Epidemiology, Academy of Military Medical Science. Briefly, 96-well plates (Shenzhen Jinchuanhua International) were coated overnight at 4°C with purified monoclonal antibodies (3B2B9C5, 3B5C11A10) to AIV H5 diluted 1000-fold in 50 mM NaHCO_3 buffer (pH 9.6). Each well was rinsed and blocked with PBS containing 0.05 % Tween 20 and 3 % BSA. Samples were centrifuged (3000 r.p.m., 5 min) and the supernatant was tested. After incubation at 37°C for 1 h, the plates were washed five times with PBS/0.5 % Tween 20. The other monoclonal antibody (3D1D11B2, diluted 1 : 3000) labelled with biotin was added to the plates. The plates were incubated at 37°C for 30 min and washed five times with PBS/0.5 % Tween 20. Diluted HRP-streptavidin (diluted 1 : 1000 in PBS supplemented with 0.5 % Tween 20 and 1.5 % BSA) was added to each well, followed by incubation at 37°C for 30 min. The plates were washed five times with PBS/0.5 % Tween 20 before the addition of tetramethylbenzidine substrate. The reaction was stopped by the addition of 2 M H_2SO_4 . The $A_{450/630}$ value was measured in triplicate. A blank control, a

negative control and a positive control were included on each plate. All reagents were used in a standard volume of 100 μ l.

Virus isolation. Samples were diluted in PBS containing 2 mg streptomycin ml^{-1} , 1000 IU penicillin ml^{-1} and 20 μg amphotericin B ml^{-1} . Nine-day-old embryonated chicken eggs were inoculated with 100 μ l sample by the chorioallantoic sac route. Five eggs were inoculated per dilution. At 4 days post-inoculation, chorioallantoic fluid was collected and tested by RT-PCR combined with sequencing and by RRT-PCR. RNA extraction was carried out using a QIAamp RNA extraction kit (Qiagen) as described previously. Virus isolation was undertaken in a Biosafety level 3 facility.

RESULTS AND DISCUSSION

Sensitivity and specificity of the RRT-PCR assay

Serial dilutions of *in vitro*-transcribed AIV H5 gene RNA were tested. A wide linear range (from 40 copies per reaction to 4×10^8 copies per reaction of target RNA, $R^2=0.997$) was detected (Fig. 1). To determine the minimum copy number of H5 gene RNA that could be detected, each low-concentration dilution was repeated 48 times. Approximately six copies of *in vitro*-transcribed RNA per reaction could be detected reproducibly (95 % confidence lower limits $P=94.5\%$), and sometimes even as few as three copies of target RNA tested positive in our assay (Table 3). In view of the divergence of the H5 gene, a standard panel containing different clades of influenza virus A/H5N1 was tested. All isolates tested positive. For further evaluation of the sensitivity of the system, 60 H5N1 isolates originating from different species were tested and all tested positive.

To determine the specificity, throat swabs from 60 patients previously shown to have H1N1 infection were tested and all samples tested negative. To test cross-reactivity with other viruses, nuclear acids (RNA or DNA) extracted from clinical isolates of 14 other viruses (listed in Methods) were evaluated. None of these viruses reacted positively in our assay.

Fig. 1. Sensitivity and dynamic range of RRT-PCR in detection of AIV H5 RNA. Serial dilutions of *in vitro*-transcribed AIV H5 gene RNA were tested. A wide linear range (from 40 copies to 4×10^8 copies of the control RNA per reaction) was determined in this assay. The intercept of the magnitude of the fluorescence signal (PCR baseline minus curve fit relative fluorescence units) with the horizontal threshold line represents the C_t value for a given sample.

Table 3. Detection of different concentrations of *in vitro*-transcribed AIV H5 gene RNA tested by RRT-PCR

RRT-PCR*	Concentration of <i>in vitro</i> -transcribed AIV H5 gene RNA (copies μl^{-1})					
	2.5	1.25	0.625	0.313	0.155	0.078
Positive	48	48	47	15	0	0
Negative	0	0	1	33	48	48

*Each reaction mix contained 10 μl RNA.

Comparison of RRT-PCR with conventional RT-PCR

The WHO primers are a reliable identification method and are used for H5 gene detection in many countries as a laboratory test. To compare the sensitivity of RRT-PCR with that of conventional RT-PCR using the WHO primers, throat swabs from 35 birds taken during an HPAIV (H5N1) outbreak and serial dilutions of AIV H5N1 cultures were tested. Thirty-three samples tested positive in our assay, but only 27 samples tested positive by conventional RT-PCR (Table 4). Six of the amplified products that tested positive only in the RRT-PCR were cloned into the pGEM-T Easy vector and sequence identification showed that they were true positive results. The detection limits determined for each test may explain the discordance among the tests, as conventional RT-PCR could only detect 3 EID₅₀ whilst our assay detected down to approximately 5×10^{-2} EID₅₀ (Ct value=37.5).

Comparison of RRT-PCR with antigen-capture ELISA

To compare the sensitivity of RRT-PCR with that of antigen-capture ELISA, the same 35 avian samples and serial dilutions of AIV H5N1 cultures were analysed. Two samples were negative by both assays and 13 samples were positive by both assays; i.e. 42.9% of the samples were in agreement (Table 4). The 20 samples for which the results differed were positive by RRT-PCR but negative by ELISA; thus ELISA detected 39.4% of the samples that were positive by RRT-PCR. In tests of serial dilutions of AIV H5N1 cultures, the minimum dilution of AIV H5N1 cultures was approximately 10 EID₅₀ for antigen-capture ELISA, showing that it has lower sensitivity than RRT-PCR. This coincides with previously reported data (Ng *et al.*, 2005).

Comparison of RRT-PCR with virus isolation

The sensitivity of the RRT-PCR assay was compared with that of virus isolation in embryonating eggs from clinical swab samples. One sample was negative by both assays and 18 were positive by both assays (Table 4). The serum sample from the bird whose throat swab was negative in both assays was positive by an HA inhibition assay. Overall, the results of the two assays agreed for 19 samples (54.3%) and disagreed for 16 samples (45.7%). Of the 16 samples for which results differed, one sample was positive by virus isolation and negative by RRT-PCR, and 15 samples were positive by RRT-PCR but negative by virus isolation. Thus virus isolation detected 54.5% of the samples that were positive by RRT-PCR, but 45.5% (15/33) of the samples that were positive by RRT-PCR were negative by virus isolation. This can partly be explained by the fact that virus isolation detects only live virus; thus virus that may have been inactivated during long-term shipping or storage from Qinghai to Beijing would not be detected, whereas it potentially can be detected by RRT-PCR. On the other hand, one of the 35 samples (2.9%) that was negative by RRT-PCR was positive by virus isolation. The cultured material was tested further by RRT-PCR and tested positive. Factors that may adversely affect the sensitivity of the RRT-PCR assay include RT-PCR inhibitory substances in the samples, inefficient RNA extraction procedures and rapid degradation of RNA before testing.

Conclusions

We have developed a sensitive and specific RRT-PCR assay for the detection of influenza virus A H5 subtype. Compared with conventional RT-PCR, antigen-capture ELISA and virus isolation, RRT-PCR is a more sensitive and convenient method.

Table 4. Comparison of RRT-PCR results with conventional RT-PCR, antigen-capture ELISA and virus isolation results for H5 subtype influenza virus

RRT-PCR	Conventional RT-PCR		Antigen-capture ELISA		Virus isolation	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	27	6	13	20	18	15
Negative	0	2	0	2	1	1

ACKNOWLEDGEMENTS

We thank the HPAI pathogens study team for virus culture and titre determination in this work. This study was supported by grants from the National High Technology Research and Development Program of China (863 Program) from the Ministry of Science and Technology, People's Republic of China.

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