JOURNAL OF CLINICAL MICROBIOLOGY, May 2007, p. 1535–1543 Vol. 45, No. 5
0095-1137/07/$08.00+0 doi:10.1128/JCM.02007-06

Design and Validation of an H5 TaqMan Real-Time One-Step Reverse Transcription-PCR and Confirmatory Assays for Diagnosis and Verification of Influenza A Virus H5 Infections in Humans[▽]

Joanna S. Ellis,* Joanne W. Smith, Sharleen Braham, Matthew Lock, Katrina Barlow, and Maria C. Zambon

Centre for Infections, Health Protection Agency, 61 Colindale Avenue, Colindale, London NW9 5HT, United Kingdom

Received 28 September 2006/Returned for modification 26 November 2006/Accepted 6 March 2007

Increasing diversity among influenza H5N1 viruses has resulted in the need for sensitive and specific diagnostic assays, fully validated for the detection of H5 viruses belonging to all hemagglutinin (HA) clades, particularly the recently circulating H5N1 viruses of clade 2. In this report, the development and validation of a real-time, one-step TaqMan reverse transcription-PCR (RT-PCR) assay specific for the detection of influenza A H5 viruses from clades 1, 1′, 2, and 3 is described. The real-time assay for H5 virus was shown to be highly sensitive, detecting H5 virus levels of <1 PFU from each of the HA clades. Specificity of the H5 RT-PCR for influenza A H5 viruses was demonstrated by using influenza A viruses of different subtypes, clinical samples containing influenza A viruses H1N1, H3N2, and H5N1, influenza B viruses, and other respiratory viruses. The usefulness of the inclusion of a distinguishable assay positive control and of confirmatory assays for the laboratory diagnosis and verification of H5 virus infections was demonstrated. A real-time RT-PCR pyrosequencing assay, a restriction enzyme digestion assay, and direct sequencing of the H5 real-time RT-PCR amplicon were validated for the confirmation of H5 detection by the diagnostic real-time assay. The H5 real-time assay was applied to diagnostic testing for suspected cases of influenza A virus H5 infection in the United Kingdom. Influenza A H5 viruses were not detected in the cases analyzed; however, influenza A H3N2 virus was detected in 57% of the suspected cases of H5. The H5 TaqMan real-time RT-PCR and confirmatory assays will be useful tools for the laboratory surveillance and rapid diagnosis of H5 infections in humans.

Since 1997, avian influenza A H5N1 viruses have been transmitted directly from birds to humans multiple times (http://www.who.int/csr/disease/avian_influenza/en/). Influenza H5N1 viruses have continued to evolve with increasing virulence and an expanding host range. In addition to terrestrial poultry and wild birds, H5 infections have been recorded for domestic cats, tigers, leopards, dogs, and viverrids as well as humans (15, 25, 27, 29). Furthermore, H5N1 viruses have spread geographically, with outbreaks reported in many countries. Phylogenetic analyses have indicated that the Z genotype has become dominant (17) and that the virus has evolved into at least two distinct clades, termed clades 1 and 2 (33). Human infections with highly pathogenic influenza H5N1 viruses from all the hemagglutinin (HA) clades have been associated with severe, often fatal disease. Clade 1 H5N1 viruses have been isolated from both humans and birds in Vietnam, Thailand, and Cambodia, but only from birds in Laos and Malaysia. Until July 2005, the more diverse clade 2 viruses were isolated only from birds in China, Indonesia, Japan, and South Korea. Subsequently, H5N1 clade 2 viruses have since been isolated from both birds and humans in Indonesia, China, Turkey, Egypt, Iraq, and Azerbaijan and from birds in many countries in Asia, Africa, and Europe, including Russia, Mongolia, Romania, Nigeria, Iran, Italy, Laos, and Malaysia (http://www.who.int/csr/disease/avian_influenza/timeline.pdf). Additionally, six subclades of H5 clade 2 viruses have been distinguished, three of which (subclades 1, 2, and 3) also differ in geographical distribution (http://www.who.int/csr/disease/avian_influenza/guidelines/recommendationvaccine.pdf). Human infections could occur outside of regions where H5N1 virus infections in birds have been detected, for example, in travelers returning from these areas. In such a situation, where the probability of H5N1 infection in humans is extremely low, it is imperative that further laboratory confirmation of H5N1 diagnosis, for instance by rapid nucleotide sequencing, is performed after a positive result. In this report, we describe the development and validation of a real-time, one-step reverse transcription-PCR (RT-PCR) assay specific for the detection of influenza A H5 viruses from clades 1, 1′, 2, and 3, together with the inclusion of a distinguishable assay positive control, and rapid confirmatory assays, which can be performed in various clinical and reference laboratories, for the verification of H5 virus infections. The H5 real-time diagnostic RT-PCR assay described here has been disseminated to laboratories participating in the United Kingdom National H5 Laboratory Network. National diagnostic testing is supported by the provision of quality assured, quantitated positive control material and national standard operating procedures, training programs, and proficiency testing to enable reliable surveillance and rapid diagnosis of H5 infections in humans.

* Corresponding author. Mailing address: Respiratory Virus Unit, Virus Reference Department, Centre for Infections, Health Protection Agency, 61 Colindale Avenue, Colindale, London NW9 5HT, United Kingdom. Phone: 44(0)20 8327 6239. Fax: 44(0)20 8327 8195. E-mail: joanna.ellis@hpa.org.uk.

▽ Published ahead of print on 14 March 2007.

MATERIALS AND METHODS

Virus stocks. The viruses analyzed in this study are shown in Table 1. The influenza viruses A/Turkey/Turkey/1/2005, A/Chicken/Turkiye/Av05-2006, and A/Q-CageBird/England/1219/2005 were provided by the Veterinary Laboratory

TABLE 1. Viruses analyzed in this study

Virus and panel	Influenza A subtype(s)	Clade(s) (subclade)[a]
Specificity panel A		
A/HongKong/483/1997	H5N1	3
A/HongKong/213/2003	H5N1	1′
A/Vietnam/1194/2003	H5N1	1
A/Vietnam/1203/2003	H5N1	1
A/Vietnam/1204/2003	H5N1	1
A/Indonesia/5/2005	H5N1	2 (1)
A/Indonesia/6/2005	H5N1	2 (1)
A/Turkey/Turkey/1/2005	H5N1	2 (2)
A/Chicken/TurkiyeAv05/2006	H5N1	2 (2)
A/Q-cage bird/England/1219/2005	H5N1	2 (3)
NIBRG12 (A/HongKong/213/2003)[b]	H5N1	1′
NIBRG14 (A/Vietnam/1194/2003)	H5N1	1
NIBRG23 (A/Turkey/Turkey/1/2005)	H5N1	2 (2)
Specificity panel B		
A/Taiwan/1/1986	H1N1	
A/Duck/Germany/1215/1973	H2N3	
A/Moscow/10/1999	H3N2	
A/Duck/Czechoslovakia/1956	H4N6	
A/Duck/Singapore-Q/F119-3/97	H5N3	3
A/Teal/HongKong/312/1997	H6N1	
A/AfricanStarling/Q-England/983/1979	H7N1	
A/Turkey/Ontario/6118/1968	H8N4	
A/HongKong/1073/1999	H9N2	
A/Turkey/Italy/1076/1967	H10N7	
A/Duck/England/1/1956	H11N6	
A/Duck/Albany/1976	H12N5	
A/Gull/Maryland//704/1077	H13N6	
A/Mallard/Gurjev/263/1982	H14N5	
A/Duck/Australia/341/1983	H15N8	
Specificity panel C		
Influenza B/Panama/45/1990		
Influenza C/Taylor/1/1947		
Adenoviruses 1, 2, 3, and 7		
Human coronaviruses 229E, NL63, and OC43		
Human metapneumovirus		
Rhinoviruses 16, 72, and 87		
Respiratory syncytial virus A (Long strain)		
Respiratory syncytial virus B (N2 strain)		
Specificity panel D		
N/T swab samples 2001–2006[c]	H1N1/H1N2/H3N2/B/Neg	
Simulated H5 clinical samples[d]	H5N1/H5N3	1 and 3

[a] Shown are results for the H5 HA clade; clade 2 subclades are shown in brackets.
[b] NIBRG indicates reverse genetics-derived virus.
[c] A total of 300 respiratory samples (mainly N/T swab samples) were taken from National Influenza Surveillance Program (14).
[d] Serum, sputum, combined N/T swab, and fecal samples (total 24) spiked with NIBRG14 (A/Vietnam/1194/2003) or A/Duck/Singapore-Q/F119-3/97 viruses.

Agency, Weybridge, United Kingdom. Human H5N1 isolates were kindly provided by the World Health Organization Collaborating Centre for Reference and Research on Influenza, Mill Hill, London, United Kingdom, and the National Institute for Biological Standards and Controls, Potters Bar, Hertfordshire, United Kingdom. The genetically modified H5N1 viruses (NIBRG12, NIBRG14, and NIBRG23) generated by reverse genetics (23) were also provided by the National Institute for Biological Standards and Controls. The H5N3 isolate A/Duck/Singapore-Q/F119-3/1997 was kindly provided by the Director of Primary Production, Veterinary Laboratory Branch, Central Veterinary Laboratory, Singapore. A/Duck/Singapore-Q/F119-3/1997 is also known as A/Duck/Malaysia/F119-3/1997 (19).

Virus culture. All influenza viruses in Table 1 were grown in the allantoic cavities of embryonated hen's eggs for 1 to 3 days at 35 to 39°C. High-pathogenicity influenza H5N1 viruses were cultivated in a DEFRA containment level 4 (CL4) facility. Genetically modified influenza H5N1 viruses were cultivated in a CL3 laboratory. Influenza A viruses H1 to H4, H5N3, and H6 to H15 and influenza B and C viruses were propagated in CL2 laboratories. Infected allantoic fluids were harvested, and viral titers were determined by HA with turkey erythrocytes by using standard methods. Virus containing allantoic fluids were then stored at −80°C until required. Other respiratory viruses in Table 1 were obtained from the archive of the Respiratory Virus Unit, Health Protection Agency (HPA), Colindale, London, United Kingdom.

Specificity panel preparation. Specificity panels were prepared to determine the cross-reactivity of the H5 RT-PCR assay with other influenza A viruses (Table 1). Specificity panel A contained cultured avian and human influenza A H5N1 viruses diluted in viral transport medium (VTM). Specificity panel B contained egg-grown influenza A viruses of different subtypes (H1 to H15) diluted in VTM. Specificity panel C was prepared by spiking pooled respiratory clinical samples (combined nose and throat [N/T] swabs) that had previously tested negative for influenza A viruses H1 and H3, influenza B viruses, and respiratory syncytial virus A (RSVA) and RSVB by RT-PCR (28) with human influenza B and C viruses and other respiratory viruses listed in Table 1. For

panel D, 300 combined N/T swab samples from patients presenting with influenza and influenza-like illness were analyzed. The samples were collected by a subset of the practitioners in the Birmingham Unit of the Royal College of General Practitioners Network during the influenza seasons of 2000 through 2006 (12).

Determination of sensitivity of H5 RT-PCR assays. (i) Titration of virus infectivity. Infectivity assays were performed as previously described by using Madin-Darby canine kidney cells (11). Tenfold dilutions (10^{-1} to 10^{-12}) of influenza A viruses representative of H5 HA clades (clade 1, NIBRG14 [A/Vietnam/1194/2004]; clade 2, NIBRG23 [A/Turkey/Turkey/1/2005]; and clade 3, A/Duck/Singapore-Q/F119-3/1997) were prepared in VTM. Aliquots of each virus dilution (100 μl) were analyzed by the infectivity assay.

(ii) RT-PCR. Freshly prepared aliquots of each virus dilution were subjected to nucleic acid extraction, and RT-PCR was performed with the H5 TaqMan real-time assay to determine the sensitivity of the assay and the amplification efficiency of the RT-PCR.

(iii) Spiked clinical material. Tenfold dilution series (10^{-1} to 10^{-12}) of NIBRG14 and A/Duck/Singapore-Q/F119-3/1997 H5 viruses were also spiked in clinical material as previously described. Viral RNA was purified from aliquots of each dilution and amplified with the H5 real-time RT-PCR to determine the end point of detection. The NIBRG14 and A/Duck/Singapore-Q/F119-3/1997 viruses were also spiked at three different concentrations (10^{-2}, 10^{-4}, and 10^{-6}) into N/T swab, sputum, and human serum samples that previously tested negative for influenza and RSV viruses by multiplex RT-PCR (28). Nucleic acid was extracted from these spiked samples, and the detection of H5 viral RNA was performed with the H5 TaqMan real-time RT-PCR assay.

H5 positive control material. Aliquots of 600 μl of influenza A H5N3 A/Duck/Singapore-Q/F119-3/1997 virus (2.5×10^8 PFU/ml) mixed with 200 μl guanidinium isothiocyanate lysis buffer (Severn Biotech Ltd., Worcester, United Kingdom) were prepared and stored at −80°C until use. The control material was validated with the H5 TaqMan real-time RT-PCR assay and quality assured (Conformité Européenne marked) following standard stability and stress testing (http://www.hpa.org.uk/srmd/div_esl_qcu/products.htm).

Clinical samples. Respiratory samples (N/T swabs, sputum, and nasopharyngeal aspirates) from returning travelers who were suspected to have avian H5 influenza infections according to the HPA case definition (http://www.hpa.org.uk/infections/topics_az/influenza/avian/case_definition.htm) presenting to hospitals in England with influenza-like illnesses were sent directly to HPA for primary H5 diagnostic testing. Patient samples (150 μl) were aliquoted into 840 μl guanidinium thiocyanate (GSCN) and Triton X-100 containing lysis buffer (Roche Diagnostics, Mannheim, Germany) in a CL3 laboratory prior to viral RNA extraction at CL2. Viral RNA or cDNA was analyzed in parallel, following a diagnostic algorithm for analyzing samples from patients suspected of H5 infection, by using the H5 TaqMan real-time RT-PCR assay, a multiplex RT-PCR for the detection of influenza A H1 and H3, influenza B, and RSVA and RSVB viruses (28), and a pan-influenza A RT-PCR targeting the matrix gene of all influenza A viruses (11). The H5 positive control virus was processed in the same way as the clinical specimens.

In addition, five respiratory samples (nasopharyngeal and lung) collected during an H5N1 human outbreak in Europe during 2006 were kindly provided by the WHO Collaborating Centre for Reference and Research on Influenza, Mill Hill, London, United Kingdom. Patient samples (100 μl) were aliquoted into lysis buffer, as described above, in a CL4 laboratory prior to viral RNA extraction at CL2. Viral RNA was then analyzed using the H5 TaqMan real-time RT-PCR assay.

Nucleic acid extraction and RT. The choice of extraction technique depended on the location of analysis (CL2, CL3, or CL4 laboratory), urgency of sample processing, and logistic factors, e.g., the number of samples to be analyzed.

The automated MagNAPure nucleic acid isolation system with a total nucleic acid isolation kit (Roche Diagnostics) was used to purify viral RNA from aliquots (150 μl) of samples in specificity panels B, C, and D. MagNAPure-purified nucleic acids were eluted in a final volume of 100 μl. Viral RNA was extracted from samples (150 μl) in specificity panel A and from respiratory secretions taken from suspected H5 patients by using 840 μl GSCN lysis buffer and 10 μl silica suspension (Severn Biotech Ltd.) as previously described and eluted in 30 μl nuclease-free water (4, 28).

Dilution series of A/Duck/Singapore-Q/F119-3/1997 H5 virus prepared in duplicate in N/T swab clinical material or VTM were also extracted in parallel by the GSCN-silica method (150 μl) and with a QIAGEN viral RNA extraction kit (140 μl) (QIAGEN Ltd., West Sussex, United Kingdom). Viral RNA purified with a QIAGEN kit was eluted in a final volume of 60 μl.

RT of viral RNA to cDNA for use in two-step RT-PCR assays was performed as previously described (11, 28).

H5 TaqMan real-time one-step RT-PCR assay. Conserved target regions of the HA of influenza A H5 viruses were identified following alignment of all human A H5N1 and representative avian H5 virus HA nucleotide sequences publicly available from the Los Alamos influenza sequence database (19). HA-specific influenza virus H5 primers and a minor groove binder probe were designed for the H5 TaqMan real-time assay by using Primer Express software (Applied Biosystems, Cheshire, United Kingdom). Optimal primer and probe concentration and amplification conditions were determined. Final conditions for a 50-μl reaction mixture used 25 μl 2× QuantiTect probe RT-PCR master mix containing 6-carboxy-X-rhodamine as a passive reference (QIAGEN QuantiTect probe RT-PCR kit), 900 nM each primer (H5Forward [5′ GCCGAATG ATGCMATMAAYT 3′] and H5Reverse [5′ CGCACCCATTGGAGTTTGAC 3′]), 200 nM TaqMan minor groove binder probe (H5probe [5′ 6-carboxyfluorescein-CATTGCTCCAGAAWAT-MGBNFQ 3′]), 0.5 μl QuantiTect RT mix (QIAGEN QuantiTect probe RT-PCR kit), and 2 μl viral RNA template. The determination of the sensitivity of the H5 real-time RT-PCR assay was performed using the above protocol and also using the following 50-μl reaction mixture: 25 μl 2× RT-PCR mix (SuperScript III Platinum one-step quantitative RT-PCR kit; Invitrogen, Paisley, Scotland), 900 nM each primer (H5Forward and H5Reverse), 200 nM TaqMan minor groove binder probe (H5probe), 8 μl SuperScript III RT/Platinum *Taq* mix, 1 μl 6-carboxy-X-rhodamine reference dye, and 2 μl viral RNA template. All samples and controls were tested in duplicate. The PCR thermal profile consisted of an initial RT step of 50°C for 30 min, followed by 15 min at 95°C, and 40 cycles of 15 s at 94°C and 60 s at 60°C. Amplification, detection, and data analysis were performed on an ABI Prism 7000 real-time thermal cycler, with "FAM no quencher" selected as the detector. The baseline was set to where no fluorescence emissions were detectable and five cycles before the start of amplification was detected. Since cycle threshold (C_T) values greater than 38.00 were observed to approach the sensitivity limits of the real-time detection system, C_T values of less than 38.00 were considered positive detections by amplification of the target template.

Confirmatory strategies. The strategies for the diagnosis and confirmation of H5 virus infection were shown in Fig. 1. Confirmatory strategies were based on nucleotide differences between H5 clade 1, 2, and 3 viruses identified following the alignment of HA sequences. The phylogenetic tree was constructed using publicly available H5 HA sequences, representing clades 1, 1′, 2, and 3, with sequences (nucleotide positions 181 to 1273) aligned by ClustalW (16).

(i) H5 real-time RT-PCR restriction enzyme digestion confirmation analysis. For confirmation of the amplification of wild-type (WT) H5 viral RNA and differentiation from H5 positive control amplicons, the digestion of real-time RT-PCR products with two restriction enzymes was performed. The enzyme MseI recognizes and cuts the sequence TTAA present at nucleotide positions 792 to 795 in the HA of the positive control virus A/Duck/Singapore-Q/F119-3/1997. The sequence TCGA (nucleotides 840 to 844) observed in HA sequences of clade 1 and 2 H5 viruses, but absent in A/Duck/Singapore-Q/F119-3/1997, is recognized and cleaved by the restriction enzyme Taq$^{\alpha}$I. Fifteen microliters of the H5 TaqMan real-time RT-PCR mix containing the 151-bp PCR product amplified from the HA1 portion of the influenza A H5 HA was added to 0.5 μl of the restriction enzymes MseI (5 U) or Taq$^{\alpha}$I (10 U) (New England Biolabs, Beverly, MA) for a minimum of 60 min at 37°C or 60°C, respectively, in 50 mM NaCl–10 mM Tris-HCl–10 mM $MgCl_2$–1 mM dithiothreitol–100 μg/ml bovine serum albumin [pH 7.9] for MseI digestion or 100 mM NaCl–50 mM Tris-HCl–10 mM $MgCl_2$–1 mM dithiothreitol–100 μg/ml bovine serum albumin [pH 7.9] for Taq$^{\alpha}$I digestion. The products of enzyme digestion were visualized by ethidium bromide staining following electrophoresis on a 3% molecular screening agarose gel (Roche Diagnostics).

(ii) Pyrosequencing confirmation analysis. For confirmation of the detection of WT H5 viral RNA by differentiation from H5 positive control amplicons, and analysis of H5 WT sequence, a pyrosequencing assay was performed. By using PSQ assay design software (Biotage, Uppsala, Sweden), PCR primers (H5pyroF1 [5′ biotin-ACA GAG CAG GTT GAC ACA ATA AT-3′] and H5pyroR2 [5′-CGT GTT TCA CTT CTT CAT AGT C-3′]) were designed to amplify a 274-bp region (113 to 386 bp) of the HA1 portion of the influenza H5 virus HA molecule and sequence a 5-bp region (232 to 236 bp) within the PCR amplicon by using the sequencing primer H5pyroS1 (5′-TCC AGC TAC ACT ACA AT-3′). RT of viral RNA to cDNA for use in the pyrosequencing PCR was performed as previously described (11, 28).

Optimal primer concentrations were determined for a 50-μl real-time PCR containing 25 μl 2× QuantiTect SYBR green master mix (QIAGEN), 200 nM biotinylated forward primer, 200 nM of reverse primer, and 5 μl cDNA template. Cycling conditions used were 50°C for 2 min, followed by 10 min at 95°C and 40 cycles of 15 s at 94°C and 60 s at 60°C. Amplification and detection were performed using an ABI Prism 7000 real-time thermocycler.

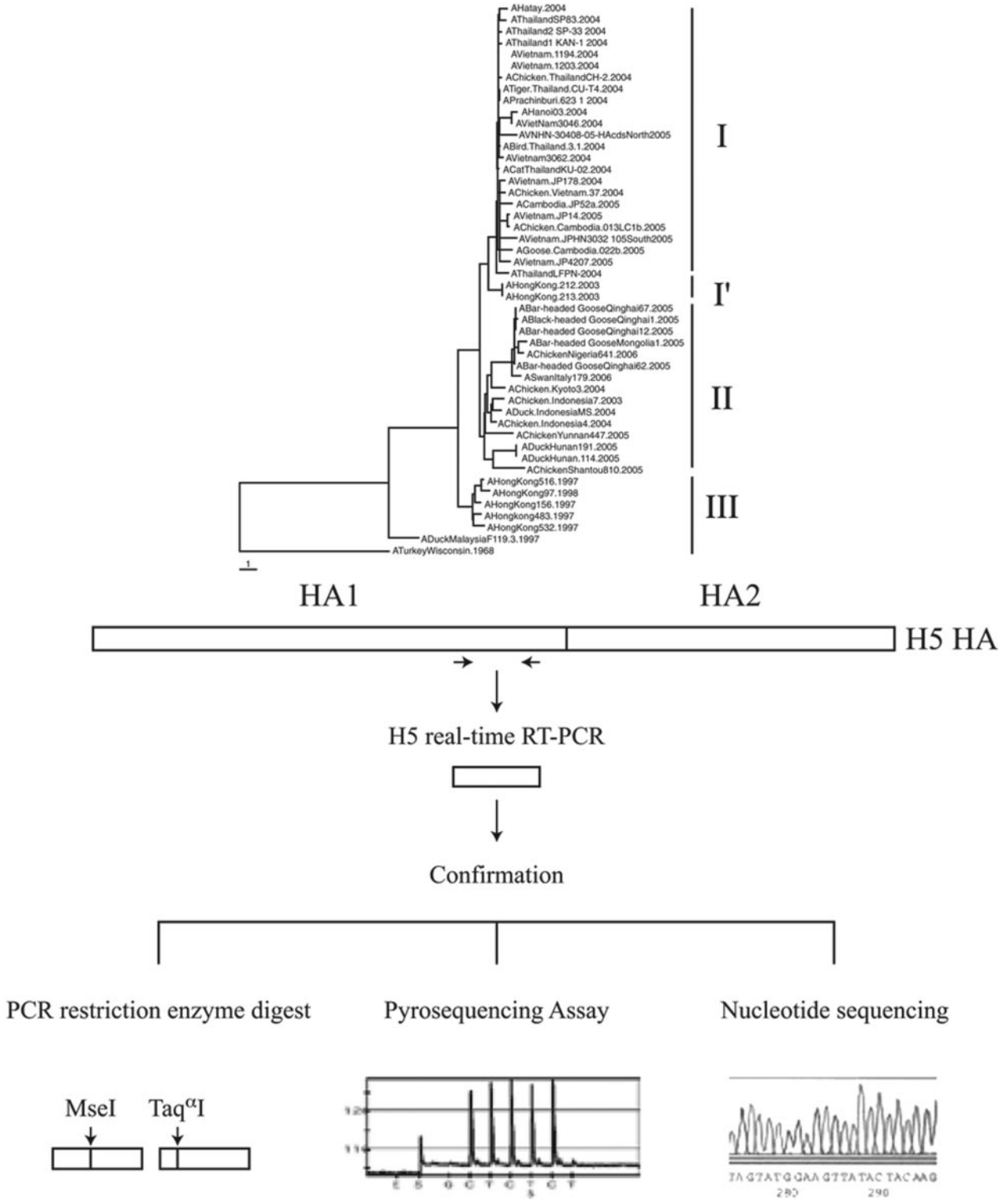

FIG. 1. Diagram of H5 RT-PCR strategy and confirmatory assays for diagnosis and verification of H5N1 infection as described in this report. The lengths of the horizontal lines are proportional to the nucleotide differences (%) between sequences as indicated by the scale. The detection of H5 viral RNA by the TaqMan H5 real-time RT-PCR assay is confirmed by PCR restriction enzyme digest or direct sequencing of the real-time amplicon or by pyrosequencing RT-PCR of a 5-nucleotide discriminatory region of the H5 HA gene.

Pyrosequencing was performed according to the manufacturer's protocol (Biotage). In brief, 40 μl of biotinylated PCR product was immobilized onto streptavidin Sepharose High Performance (Amersham Biosciences, Buckinghamshire, United Kingdom) in binding buffer (10 mM Tris-HCl [pH 7.6], 2 M NaCl, 1 mM EDTA, and 0.1% Tween 20) and vigorously shaken for 10 min at room temperature. Sequencing primer (440 nM) in annealing buffer (20 mM Tris-acetate [pH 7.6] and 2 mM $MgAc_2$) was incubated at 80°C for 2 min and then allowed to cool slowly to RT. Single-stranded DNA template was obtained by using the PyroMark vacuum prep workstation (Biotage). Immobilized PCR product was initially washed in 70% ethanol and then incubated in 0.2 M NaOH. A final wash in 10 mM Tris-acetate, pH 7.6, was carried out before annealing single-stranded DNA to the sequencing primer.

Sequencing was performed using an automated PSQ PyroMark ID instrument (Biotage). Substrate and enzyme were dispensed, followed by the sequential addition of nucleotides, in the order CTCTC. Sequence results were obtained in the form of pyrograms and analyzed using IdentiFire software (Biotage).

(ii) Cycle sequencing confirmation analysis. PCR products (amplified by the H5 TaqMan real-time RT-PCR assay from reference H5 viruses) were sequenced by automated nucleotide cycle sequencing. PCR products were first purified using a QIAquick PCR purification kit (QIAGEN Ltd.) and then sequenced using a Beckman Coulter CEQ 2000 capillary sequencer and CEQ 2000 dye terminator cycle sequencing quick-start kit (Beckman Coulter, Fullerton, CA). Nucleotide sequences were compared by BLAST analysis using the influenza sequence database (19) and GenBank sequence database (2).

RESULTS

H5 TaqMan real-time RT-PCR assay. The amplification efficiency of the real-time H5 RT-PCR assay was determined by using a 10-fold dilution series of NIBRG14, NIBRG23, and A/Duck/Singapore-Q/F119-3/1997 viruses. Standard curves were generated with duplicate samples over a template dilution range of 5 to 6 logs by using obtained C_T values of less than or equal to 36.00, with a difference between replicates of <0.5 C_T values. By using the C_T slope method (3, 18) and slope values of -3.36 with NIBRG14 viral RNA, -3.35 with NIBRG23, and -3.28 for A/Duck/Singapore-Q/F119-3/1997, the amplification efficiency values of the RT-PCRs were calculated to be 98, 99, and 101%, respectively.

Sensitivity. The sensitivity of the H5-specific TaqMan real-time RT-PCR for the detection of influenza H5 viruses was determined on at least five separate occasions by using 10-fold dilutions (10^{-1} to 10^{-12}) of three influenza H5 viruses representative of clade 1 (NIBRG14), clade 2 (NIBRG23), and clade 3 (A/Duck/Singapore-Q/F119-3/1997). An analysis of 1 PFU of virus reliably and reproducibly generated C_T values of 34.00 ± 0.28.

By using the QuantiTect probe RT-PCR kit, the real-time H5 RT-PCR assay detected <1 PFU of NIBRG14 and A/Duck/Singapore-Q/F119-3/1997 viruses belonging to HA clade 1 and clade 3, respectively, and 1 to 10 PFU of the clade 2 H5 virus NIBRG23. When SuperScript III Platinum one-step RT-PCR master mix was used, the H5 assay detected <1 PFU of the H5 viruses analyzed from clade 1, 2, or 3.

The sensitivity of the real-time H5 assay using cultured H5 viruses spiked into clinical material was also determined on five separate occasions. When NIBRG14 or A/Duck/Singapore-Q/F119-3/1997 viruses were diluted in either VTM or clinical N/T material, the end point of detection observed was <1 PFU of each virus. NIBRG14 and A/Duck/Singapore-Q/F119-3/1997 viruses were also spiked at three different concentrations into influenza virus- and RSV-negative N/T swab, sputum, and human serum samples with 1.6×10^4, 1.6×10^2, and 1.6 PFU of virus analyzed, respectively. Influenza A H5 viral RNA was detected ($C_T < 38.00$) with the H5 TaqMan real-time RT-PCR assay in all of the N/T swab, serum, and sputum samples spiked with the A/Duck/Singapore-Q/F119-3/1997 or NIBRG14 H5 viruses, at all of the virus concentrations analyzed, with the C_T values obtained with H5 viruses spiked at the same concentration in different simulated clinical sample types differing by C_T values of 0.08 to 1.10.

The applicability of two different widely used extraction procedures for the purification of viral RNA for use in the H5 TaqMan real-time assay was then determined. Serial dilutions of A/Duck/Singapore-Q/F119-3/1997 virus prepared in VTM or negative N/T swab clinical material were extracted in parallel by a GSCN-silica method (4, 28) and a commercial viral RNA purification kit (QIAGEN Ltd.), and purified RNA was analyzed in duplicate with the H5 real-time assay on 10 separate occasions. Using either method, <1 PFU of A/Duck/Singapore-Q/F119-3/1997 virus was reliably detected in the dilutions prepared in VTM (data not shown). Moreover, no difference was observed in the sensitivity of detection of viral RNA purified by either extraction method from A/Duck/Singapore-Q/F119-3/1997 virus spiked in N/T swab clinical material (data not shown).

Specificity. The H5 TaqMan real-time RT-PCR assay was tested for the ability to detect human and avian influenza H5 viruses from different H5 HA clades and subclades (specificity panel A) (Table 1). Amplification was observed with the H5 RT-PCR assay when influenza H5 viruses of avian or human origin (from the H5 HA clades 1, 1′, 2, or 3 [33]) were analyzed. The H5 real-time RT-PCR assay was also tested for cross-reactivity with RNA prepared from influenza A virus isolates of subtypes H1 to H15 (specificity panel B), influenza B and C viruses, and other cultured respiratory viruses (specificity panel C) and clinical samples (combined N/T swabs) containing human influenza virus H1 or H3 or influenza B viruses (specificity panel D). No detectable amplification ($C_T < 38.00$) was observed with viral RNA purified from influenza A virus subtypes H1 to H15, with the exception of the H5N3 virus A/Duck/Singapore-Q/F119-3/1997. No detectable amplification ($C_T < 38.00$) was observed following analysis of the respiratory viruses in specificity panel C or the clinical samples containing human influenza H1 or H3 or influenza B viruses (specificity panel D) by the H5 real-time RT-PCR assay, indicating the specificity of the H5 RT-PCR assay for influenza H5 viruses (data not shown).

Confirmatory strategies. (i) H5 real-time RT-PCR restriction enzyme digestion confirmation analysis. For confirmation of the amplification of WT H5 viral RNA and differentiation of WT from H5 positive control amplicons, a restriction enzyme digestion assay was developed based on nucleotide differences at two sites in the sequence of the respective amplicons. Positive control amplicons were cut following digestion with the enzyme MseI to yield two fragments of 69 and 82 bp (Fig. 2a). Since the MseI recognition site is absent in the DNA sequence of WT-derived PCR products, MseI did not digest these amplicons. The restriction enzyme $Taq^{\alpha}I$ cuts PCR products amplified by the H5 real-time assay from WT H5 HA clade 1 and 2 strains at one site, yielding two fragments of 21 and 130 bp, whereas positive control amplicons were not digested by this enzyme since the $Taq^{\alpha}I$ restriction recognition site is absent (Fig. 2a and b). The avian H5 HA clade 2 virus A/Turkey/

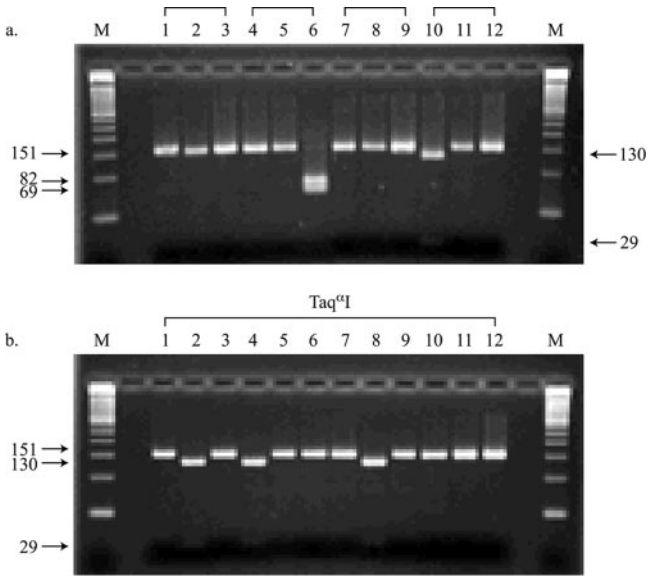

FIG. 2. Restriction enzyme analysis of influenza A H5 HA real-time RT-PCR amplicons. (a) MseI and Taq$^{\alpha}$I digestion of H5 WT (clade 1 and 2) and positive control (clade 3) HA amplicons. In the lane descriptions below, numbers in brackets after strain names refer to H5 HA clades. Lanes 1 and 7, undigested A/Vietnam/1194/2004 (1); lanes 2 and 8, undigested NIBRG23 (2); 3 and 9, undigested A/Duck/Singapore-Q/F119-3/1997 (3); lane 4, MseI-digested A/Vietnam/1194/2004 (1); lane 5, MseI-digested NIBRG23 (2); lane 6, MseI-digested A/Duck/Singapore-Q/F119-3/1997 (3); lane 10; Taq$^{\alpha}$I-digested A/Vietnam/1194/2004 (1); lane 11, Taq$^{\alpha}$I-digested NIBRG23 (2); lane 12, Taq$^{\alpha}$I-digested A/Duck/Singapore-Q/F119-3/1997 (3); lane M, DNA molecular weight markers. (b) Taq$^{\alpha}$I digestion of HA RT-PCR amplicons from WT H5 (clade 2) and positive control (clade 3) viruses. Lane 1, undigested A/Indonesia/5/2005 (2); lane 2, Taq$^{\alpha}$I-digested A/Indonesia/5/2005 (2); lane 3, undigested A/Indonesia/6/2005 (2); lane 4, Taq$^{\alpha}$I-digested A/Indonesia/6/2005 (2); lane 5, undigested A/Turkey/Turkey/1/2005 (2); lane 6, Taq$^{\alpha}$I-digested A/Turkey/Turkey/1/2005 (2); lane 7, undigested A/Q-CageBird/England/1219/2005 (2); lane 8, Taq$^{\alpha}$I-digested A/Q-CageBird/England/1219/2005 (2); lane 9, undigested NIBRG23 (2); lane 10, Taq$^{\alpha}$I-digested NIBRG23 (2); lane 11, undigested A/Duck/Singapore-Q/F119-3/1997 (3); lane 12, Taq$^{\alpha}$I-digested A/Duck/Singapore-Q/F119-3/1997 (3); lane M, DNA molecular weight markers.

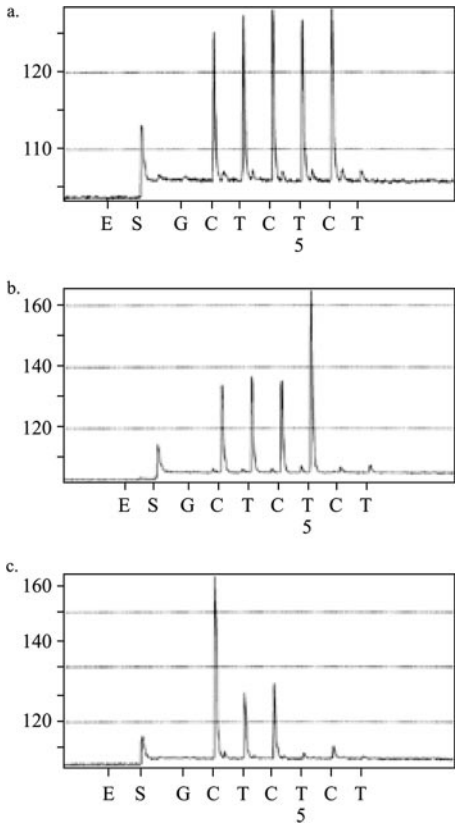

FIG. 3. Pyrograms of raw data obtained from pyrosequencing a 5-nucleotide region of HA1 of the HA gene of H5 viruses. The *y* axis determines the luminescence intensity, with peak heights relative to the number of each nucleotide. The order of enzyme (E), substrate (S), and sequential nucleotide addition is indicated on the *x* axis. (a) A/Vietnam/1194/2004 (clade 1) sequence CTCTC. (b) NIBRG23 (clade 2) sequence CTCTT. (c) A/Duck/Singapore-Q/F119-3/1997 (clade 3) sequence CCCTC.

Turkey/1/2005 and the genetically modified A/Turkey/Turkey/1/2005 virus (NIBRG23) real-time RT-PCR products were not digested by the MseI or Taq$^{\alpha}$I restriction enzyme (Fig. 2a and b). An analysis of the H5 HA sequence of A/Turkey/Turkey/1/2005 indicated that a nucleotide substitution (C to T) at position 799, in the region amplified by the H5 real-time RT-PCR assay, resulted in the loss of the Taq$^{\alpha}$I restriction site. However, amplicons from the other clade 2 H5 viruses analyzed retained the Taq$^{\alpha}$I restriction site and gave restriction patterns characteristic of clade 2 viruses (Fig. 2b).

(ii) Pyrosequencing RT-PCR and sequence analysis. For confirmation of the detection of WT H5 viral RNA by differentiation from H5 positive control amplicons and analysis of H5 WT sequence, a pyrosequencing assay, which differentiates between sequences from viruses belonging to H5 HA clades 1, 2, and 3, was developed based on a 5-bp region (236 to 232 bp) of HA1. Following the amplification of viral RNA by pyrosequencing real-time RT-PCR of the 274-bp fragment encompassing this region, pyrosequencing was performed and the resulting pyrograms were analyzed (Fig. 3). In the 5-nucleotide region sequenced, clade 1 and 1′ viruses were shown to have the nucleotide sequence CTCTC (Fig. 3a), whereas clade 2 viruses had the sequence CTCTT (Fig. 3b). The H5 positive control A/Duck/Singapore-Q/F119-3/1997, representative of clade 3 viruses, had the sequence CCCTC in this region (Fig. 3c).

(iii) Nucleotide sequencing analysis. The specificity of the H5 real-time RT-PCR assay was confirmed by direct sequencing of the H5 HA gene PCR amplicons of the WT (A/Vietnam/1194/2004) and positive control (A/Duck/Singapore-Q/F119-3/1997) viruses. BLAST analysis of the HA nucleotide sequences of the PCR products confirmed that the amplicons were derived from the WT or positive control viruses analyzed (data not shown).

(iv) Diagnostic testing for influenza H5 in returning travelers. Returning travelers presenting to hospitals in England with influenza-like illness, who were suspected to be harboring avian H5 virus according to the HPA avian H5 virus infection case definition, were managed according to the HPA H5 algorithm (http://www.hpa.org.uk/infections/topics_az/influenza/avian/algorithm.htm). A total of 15 clinical specimens (nose swabs, throat swabs, sputum samples, and nasopharyngeal aspirates) from seven suspected cases of avian H5 virus infection

that precisely matched the HPA H5 influenza virus infection case definition were analyzed between October 2004 and April 2006. Nucleic acid was analyzed directly with the H5 TaqMan real-time RT-PCR in parallel with a two-step multiplex RT-PCR for influenza A H1 and H3 viruses, influenza B viruses, RSVA and RSVB and a pan-influenza A virus RT-PCR (11, 28). Influenza H5 viral RNA was not detected in any of the samples analyzed from the suspected H5 cases. However, influenza AH3 viral RNA was detected in samples from four patients by RT-PCR. Where serum was also collected from these patients, serological testing confirmed that there was no evidence of H5 infection (data not shown).

Respiratory samples collected during an H5N1 human outbreak in Europe in 2006 were also analyzed. Of these five samples, three had previously been demonstrated to contain influenza A H5N1 viral RNA when tested by the WHO Collaborating Centre for Reference and Research on Influenza (A. Hay, personal communication). Following analysis, a lung sample and one of four nasopharyngeal specimens examined were shown to contain influenza A H5 viral RNA by the H5 TaqMan real-time RT-PCR.

DISCUSSION

RT-PCR methods have been shown to be useful and the diagnostic method of choice during outbreaks of H5N1 infections in Hong Kong and South East Asia (8, 30, 34). However, a disadvantage of RT-PCR methodology is the inherent sensitivity, with the potential for false-positive results due to carryover contamination. In addition to following precautionary anticontamination measures in the laboratory (5, 7, 31), the reporting of false-positive results can be avoided by the use of confirmatory tests, along with a distinguishable assay positive control. By using the H5 TaqMan real-time RT-PCR described in this report, a reliable subtype-specific diagnostic result can be generated within a few hours following specimen collection, including verification of positive results.

In countries such as the United Kingdom, where H5N1 infections in birds are not endemic, diagnostic testing for H5 avian influenza is most likely to be performed on travelers returning from H5N1-affected regions or on potentially exposed poultry workers. In both instances, there is a necessity for the use of algorithms to provide robust differential diagnosis. For poultry workers, exposure to avian influenza A viruses of subtypes other than H5 should also be considered.

The H5 real-time RT-PCR described in this report was designed as a one-step, real-time assay to improve efficient processing and reduce the risk of carryover contamination. The H5 assay was designed for use on a TaqMan sequence detection platform (Applied Biosystems); however, the test will be useful in a range of laboratories since the assay is transferable to other real-time platforms, such as the Roche LightCycler, Corbett Rotor-Gene, or Cepheid Smart Cycler, with little reoptimization. Primers that fit the criteria for suitable real-time PCR primers were selected from highly conserved regions of the HA gene of H5 influenza viruses, with the range limited by the high rate of variation in the gene. In addition, the probe sequence selection from a highly conserved region was aided by the use of the minor groove binder at the 3′ end of the probe, which increased the melting temperature of the probe, allowing the use of shorter probes. Despite the selection of conserved regions, in order to ensure the amplification of target viral RNA from the HA gene of influenza viruses belonging to all three H5 HA clades, three degenerate bases were incorporated into the forward primer and one was incorporated into the probe sequence. The influenza A H5N3 virus A/Duck/Singapore-Q/F119-3/1997 was selected as the positive control material for the real-time RT-PCR assay, since this strain is representative of H5 clade 3 viruses, which have not been detected since 1997, and can easily be distinguished genetically from H5 circulating viruses of clade 1 and clade 2. False-positive results arising from the contamination of test samples by the positive control can therefore be confirmed by sequence-dependent confirmatory assays. In addition, A/Duck/Singapore-Q/F119-3/1997 virus has the advantage of being apathogenic and can be easily cultured at CL2. While this is a useful strategy, it does not discriminate from cross-contamination of WT from other specimens, so in the event of a positive signal, it is important to reconfirm the result by reextraction at a time of low prevalence.

The H5 TaqMan real-time RT-PCR assay was shown to be extremely sensitive for the detection of influenza A H5 viruses, detecting clade 1, 2, and 3 H5 virus levels of 1 PFU or less by using a QuantiTect Probe RT-PCR master mix (QIAGEN Ltd.). Furthermore, when a different commercially available one-step RT-PCR master mix was used (Superscript III Platinum RT-PCR kit; Invitrogen), it was observed that the H5 real-time assay was 1 log more sensitive for the detection of NIBRG23 (A/Turkey/Turkey/1/2005) clade 2 virus, detecting <1 PFU of this virus. This was also seen with the A/Chicken/Turkiye/Av05/2006 clade 2 virus, but not with the other clade 2 viruses analyzed from Table 1 (data not shown). An examination of the sequence of the H5 assay target region of A/Turkey/Turkey/1/2005 and A/Chicken/Turkiye/Av05/2006 identified three mismatches relative to the primer and probe sequences that were not present in the HA sequences of the other clade 2 viruses analyzed or in those of clade 1, 1′, or clade 3 H5 viruses: nucleotide 16 in the forward primer (C to A, degenerate in the primer), nucleotide 7 in the reverse primer (C to T), and nucleotide 14 in the probe (T to A, degenerate in the probe). Differences in the composition of the two RT-PCR master mixes, including magnesium ion concentration, reverse transcriptase, and polymerase enzymes, could have resulted in a greater tolerance of the mismatches between the target sequence and primers/probe. It is therefore essential to use the Superscript III Platinum real-time master mix in this H5 RT-PCR assay to ensure optimum sensitivity of detection of H5 viruses belonging to all H5 HA clades and subclades.

It is vital that diagnostic assays are highly sensitive for the diagnosis of H5N1 infection using different clinical sample types. The pattern of replication of influenza H5N1 viruses in the human respiratory tract is unclear, and it may be appropriate to analyze various clinical samples. Efficient replication of H5N1 viruses has been demonstrated in cells in the lower region of the respiratory tract, where the avian virus receptor (SAα2,3Gal) is prevalent (26, 32), and more recently, the infection of nasopharyngeal and oropharyngeal epithelial cells as well as lung tissue has been demonstrated (22).The sensitivity of the H5 real-time assay for different clinical sample types, such as secretions from the upper respiratory tract and lower

respiratory tract, was analyzed in this study first by using simulated respiratory clinical H5 samples due to the limited availability of H5 clinical samples from human cases and also by analysis of a small number of respiratory samples from suspect H5 cases during an H5N1 human outbreak in Europe in 2006. The study demonstrated that the H5 real-time RT-PCR assay is suitable for analyzing both upper respiratory and lower respiratory tract samples.

Serum samples spiked with H5 viruses were also analyzed since invasive H5N1 infection has been documented for mammals and humans (9, 13, 14, 35). Previously reported RT-PCR assays for the detection of H5 viruses have been designed to detect human and avian clade 3 viruses, human clade 1′ viruses, or clade 1 human, avian, and felid viruses from 1997, 2003, and 2004, respectively (20, 21, 24), and have not included validation data on the detection of H5 clade 2 viruses. It is particularly important that the sensitivity and specificity for clade 2 viruses are determined for any potentially useful diagnostic H5 assay, since clade 2 viruses are becoming geographically more widespread and the predominant H5 strain isolated from humans and birds. The H5 real-time RT-PCR described here was shown to be specific for the detection of influenza A H5 viruses, amplifying viral RNA from viruses belonging to all of the H5 HA clades and subclades identified since 1997. Nonreactivity of the H5 real-time RT-PCR assay with other influenza A virus subtypes, influenza B and C viruses, and noninfluenza respiratory viruses was demonstrated, confirming the specificity of the assay for H5 viruses.

The majority of published assays for the detection of H5 viruses have relied on detection in a single assay (20, 24). In contrast, in this report, the incorporation of a distinguishable H5-positive assay control and confirmatory assay options enable the verification of the integrity of positive results.

For diagnostic laboratories that do not have access to sequencing facilities, a simple restriction enzyme digest assay was developed. The selection of the two restriction sites chosen was based on differences in the HA sequence of the H5 real-time assay amplicons between clade 1, 1′, and 2 viruses, compared to the sequence from the clade 3 positive control PCR product. Positive control amplicons have the MseI restriction site, which is absent in amplicons from WT viruses. In contrast, the enzyme Taq$^{\alpha}$I digests PCR products from H5 clade 1 and 2 viruses, whereas positive control-derived amplicons are not digested since the Taq$^{\alpha}$I site is absent. During the study, it was observed that the Taq$^{\alpha}$I restriction site has been lost from a subclade of H5 clade 2 viruses, demonstrating the importance of using at least two different enzymes for the confirmatory analysis. Confirmation of the amplification of a clade 2 subclade 2 virus requires sequence analysis of the real-time RT-PCR amplicon by traditional nucleotide sequencing or confirmation with the RT-PCR pyrosequencing assay. The restriction digest assay is easy to perform, and the confirmatory assay can be performed in 3.5 h. A confirmation of H5 diagnosis with the H5 real-time RT-PCR can be also be achieved by direct sequencing of the PCR amplicon, since the sequence of the H5 amplicon generated by amplification from WT viruses is distinguishable from that of the H5N3-positive control virus. Sequence confirmation can be achieved in 5 to 6 h following diagnosis with the H5 real-time RT-PCR assay.

Pyrosequencing of RT-PCR amplicons has previously been used to genotype viruses (1, 10), and although pyrosequencing has been utilized to assess adamantane resistance among influenza A strains (6), there have been no reports to date of the applicability of pyrosequencing to genotype influenza viruses. The pyrosequencing RT-PCR was designed to amplify a region of the HA gene that is distinguishable between H5 viruses belonging to the different H5 HA clades. The results demonstrated that the pyrosequencing assay was reproducible and easy to interpret and that confirmation was achieved more rapidly than with standard nucleotide cycle sequencing, with results available 2.25 h after H5 diagnosis with the real-time RT-PCR assay.

The application of the H5 real-time RT-PCR to the diagnosis of H5 infection in upper respiratory tract and lower respiratory tract secretions from suspected cases presenting in the United Kingdom was analyzed. Although no positive cases were detected, the diagnosis of influenza A H3N2 virus infection in 57% of cases analyzed highlights the importance of differential diagnosis for suspected avian influenza virus infection patients, particularly for travelers returning from regions where seasonal influenza viruses may be circulating.

In the design of this H5 real-time RT-PCR, an alignment of conserved regions of influenza A H5 viruses was made with publicly available sequences. The restriction PCR digest and pyrosequencing confirmatory assays were also designed based on the viral HA gene sequences. Due to the high mutation frequency of influenza viruses and the heterogeneity of the circulating strains, it is theoretically possible that mutations in the primer or probe regions or the regions used for confirmation may occur and continual analysis of sequences from recently circulating strains must be performed. The addition of an internal control would further improve the assay as a diagnostic tool, as each sample would be checked for the quality of the nucleic acid extraction.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance provided by Carol Sadler, Adriana Alvarez-Aguero, and Vicky Goulding. We also thank Alison Bermingham and Ruth Reith for preparation of the virus panels, Joe Vincini for undertaking the Conformité Européenne marking of the H5 positive control material, and Alan Hay and YiPu Lin for kindly providing virus isolates and clinical material.

REFERENCES

1. **Adelson, M. E., M. Feola, J. Trama, R. C. Tilton, and E. Mordechai.** 2005. Simultaneous detection of herpes simplex virus types 1 and 2 by real-time PCR and pyrosequencing. J. Clin. Virol. **33:**25–34.
2. **Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and D. L. Wheeler.** 2005. GenBank. Nucleic Acids Res. **33:**D34–D38.
3. **Bernard, P. S., and C. T. Wittwer.** 2002. Real-time PCR technology for cancer diagnostics. Clin. Chem. **48:**1178–1185.
4. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
5. **Borst, A., A. T. Box, and A. C. Fluit.** 2004. False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy. Eur. J. Clin. Microbiol. Infect. Dis. **23:**289–299.
6. **Bright, R. A., M. J. Medina, X. Xu, G. Perez-Oronoz, T. R. Wallis, X. M. Davis, L. Povinelli, N. J. Cox, and A. I. Klimov.** 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet **366:**1175–1181.
7. **Burkardt, H. J.** 2000. Standardization and quality control of PCR analyses. Clin. Chem. Lab. Med. **38:**87–91.
8. **Chotpitayasunondh, T., K. Ungchusak, W. Hanshaoworakul, S. Chunsuthiwat, P. Sawanpanyalert, R. Kijphati, S. Lochindarat, P. Srisan, P. Suwan, Y. Osotthanakorn, T. Anantasetagoon, S. Kanjanawasri, S. Tanupattarachai, J. Weerakul, R. Chaiwirattana, M. Maneerattanaporn, R. Poolsavathitikool, K.**

Chokephaibulkit, A. Apisarnthanarak, and S. F. Dowell. 2005. Human disease from influenza A (H5N1), Thailand, 2004. Emerg. Infect. Dis. **11:**201–209.
9. **de Jong, M. D., C. P. Simmons, T. T. Thanh, V. M. Hien, G. J. Smith, T. N. Chau, D. M. Hoang, N. Van Vinh Chau, T. H. Khanh, V. C. Dong, P. T. Qui, B. Van Cam, D. Q. Ha, Y. Guan, J. S. Peiris, N. T. Chinh, T. T. Hien, and J. Farrar.** 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat. Med. **12:**1203–1207.
10. **Elahi, E., N. Pourmand, R. Chaung, A. Rofoogaran, J. Boisver, K. Samimi-Rad, R. W. Davis, and M. Ronaghi.** 2003. Determination of hepatitis C virus genotype by pyrosequencing. J. Virol. Methods **109:**171–176.
11. **Ellis, J. S., and M. C. Zambon.** 2001. Combined PCR-heteroduplex mobility assay for detection and differentiation of influenza A viruses from different animal species. J. Clin. Microbiol. **39:**4097–4102.
12. **Fleming, D. M., P. Chakraverty, C. Sadler, and P. Litton.** 1995. Combined clinical and virological surveillance of influenza in winters of 1992 and 1993-4. BMJ **311:**290–291.
13. **Govorkova, E. A., J. E. Rehg, S. Krauss, H. L. Yen, Y. Guan, M. Peiris, T. D. Nguyen, T. H. Hanh, P. Puthavathana, H. T. Long, C. Buranathai, W. Lim, R. G. Webster, and E. Hoffmann.** 2005. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J. Virol. **79:**2191–2198.
14. **Hatta, M., P. Gao, P. Halfmann, and Y. Kawaoka.** 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:**1840–1842.
15. **Keawcharoen, J., K. Oraveerakul, T. Kuiken, R. A. Fouchier, A. Amonsin, S. Payungporn, S. Noppornpanth, S. Wattanodorn, A. Theambooniers, R. Tantilertcharoen, R. Pattanarangsan, N. Arya, P. Ratanakorn, D. M. Osterhaus, and Y. Poovorawan.** 2004. Avian influenza H5N1 in tigers and leopards. Emerg. Infect. Dis. **10:**2189–2191.
16. **Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei.** 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics **17:**1244–1245.
17. **Li, K. S., Y. Guan, J. Wang, G. J. Smith, K. M. Xu, L. Duan, A. P. Rahardjo, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. T. Estoepangestie, A. Chaisingh, P. Auewarakul, H. T. Long, N. T. Hanh, R. J. Webby, L. L. Poon, H. Chen, K. F. Shortridge, K. Y. Yuen, R. G. Webster, and J. S. Peiris.** 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature **430:**209–213.
18. **Liu, W., and D. A. Saint.** 2002. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal. Biochem. **302:**52–59.
19. **Macken, C., H. Lu, J. Goodman, and L. Boykin.** 2001. The value of a database in surveillance and vaccine selection, p. 103–106. *In* A. D. M. E. Osterhaus, N. Cox, and A. W. Hampson (ed.), Options for the control of influenza IV. Elsevier Science, Amsterdam, The Netherlands.
20. **Ng, E. K., P. K. Cheng, A. Y. Ng, T. L. Hoang, and W. W. Lim.** 2005. Influenza A H5N1 detection. Emerg. Infect. Dis. **11:**1303–1305.
21. **Ng, L. F., I. Barr, T. Nguyen, S. M. Noor, R. S. Tan, L. V. Agathe, S. Gupta, H. Khalil, T. L. To, S. S. Hassan, and E. C. Ren.** 2006. Specific detection of H5N1 avian influenza A virus in field specimens by a one-step RT-PCR assay. BMC Infect. Dis. **6:**40.
22. **Nicholls, J. M., M. C. Chan, W. Y. Chan, H. K. Wong, C. Y. Cheung, D. L. Kwong, M. P. Wong, W. H. Chui, L. L. Poon, S. W. Tsao, Y. Guan, and J. S. Peiris.** 2007. Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nat. Med. **13:**147–149.
23. **Nicolson, C., D. Major, J. M. Wood, and J. S. Robertson.** 2005. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine **23:**2943–2952.
24. **Payungporn, S., S. Chutinimitkul, A. Chaisingh, S. Damrongwantanapokin, C. Buranathai, A. Amonsin, A. Theamboonlers, and Y. Poovorawan.** 2006. Single step multiplex real-time RT-PCR for H5N1 influenza A virus detection. J. Virol. Methods **131:**143–147.
25. **Roberton, S. I., D. J. Bell, G. J. Smith, J. M. Nicholls, K. H. Chan, D. T. Nguyen, P. Q. Tran, U. Streicher, L. L. Poon, H. Chen, P. Horby, M. Guardo, Y. Guan, and J. S. Peiris.** 2006. Avian influenza H5N1 in viverrids: implications for wildlife health and conservation. Proc. Biol. Sci. **273:**1729–1732.
26. **Shinya, K., M. Ebina, S. Yamada, M. Ono, N. Kasai, and Y. Kawaoka.** 2006. Avian flu: influenza virus receptors in the human airway. Nature **440:**435–436.
27. **Songsermn, T., A. Amonsin, R. Jam-on, N. Sae-Heng, N. Meemak, N. Pariyothorn, S. Payungporn, A. Theamboonlers, and Y. Poovorawan.** 2006. Avian influenza H5N1 in naturally infected domestic cat. Emerg. Infect. Dis. **12:**681–683.
28. **Stockton, J., J. S. Ellis, M. Saville, J. P. Clewley, and M. C. Zambon.** 1998. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. J. Clin. Microbiol. **36:**2990–2995.
29. **Thanawongnuwech, R., A. Amonsin, R. Tantilertcharoen, S. Damrongwatanapokin, A. Theamboonlers, S. Payungporn, K. Nanthapornphiphat, S. Ratanamungklanon, E. Tunak, T. Songserm, V. Vivatthanavanich, T. Lekdumrongsak, S. Kesdangsakonwut, S. Tunhikorn, and Y. Poovorawan.** 2005. Probable tiger-to-tiger transmission of avian influenza H5N1. Emerg. Infect. Dis. **11:**699–701.
30. **Tran, T. H., T. L. Nguyen, T. D. Nguyen, T. S. Luong, P. M. Pham, V. C. Nguyen, T. S. Pham, C. D. Vo, T. Q. Le, T. T. Ngo, B. K. Dao, P. P. Le, T. T. Nguyen, T. L. Hoang, V. T. Cao, T. G. Le, D. T. Nguyen, H. N. Le, K. T. Nguyen, H. S. Le, V. T. Le, D. Christiane, T. T. Tran, M. de Jong, C. Schultsz, P. Cheng, W. Lim, P. Horby, and J. Farrar.** 2004. Avian influenza A (H5N1) in 10 patients in Vietnam. N. Engl. J. Med. **350:**1179–1188.
31. **Valentine-Thon, E.** 2002. Quality control in nucleic acid testing—where do we stand? J. Clin. Virol. **25**(Suppl. 3)**:**S13–S21.
32. **van Riel, D., V. J. Munster, E. de Wit, G. F. Rimmelzwaan, R. A. Fouchier, A. D. Osterhaus, and T. Kuiken.** 2006. H5N1 virus attachment to lower respiratory tract. Science **312:**399.
33. **World Health Organization Global Influenza Program Surveillance Network.** 2005. Evolution of H5N1 avian influenza viruses in Asia. Emerg. Infect. Dis. **11:**1515–1521.
34. **Yuen, K. Y., P. K. Chan, M. Peiris, D. N. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. Ho, R. Sung, and A. F. Cheng.** 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet **351:**467–471.
35. **Zitzow, L. A., T. Rowe, T. Morken, W. J. Shieh, S. Zaki, and J. M. Katz.** 2002. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J. Virol. **76:**4420–4429.