ELSEVIER

Journal of Virological Methods 103 (2002) 213–225

Journal of Virological Methods

www.elsevier.com/locate/jviromet

Detection of highly pathogenic and low pathogenic avian influenza subtype H5 (Eurasian lineage) using NASBA

Richard A. Collins [a], Lung-Sang Ko [a], Ka-Lun So [b], Trevor Ellis [c], Lok-Ting Lau [a,b], Albert Cheung Hoi Yu [a,*]

[a] *Hong Kong DNA Chips Ltd, 1805-6, 18/F, Lu Plaza, 2 Wing Yip Street, Kowloon, Hong Kong SAR, China*
[b] *Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China*
[c] *Agriculture, Fisheries and Conservation Department, Castle Peak Veterinary Laboratory, San Fuk Road, Tuen Mun, New Territories, Hong Kong SAR, China*

Received 15 October 2001; received in revised form 4 February 2002; accepted 5 February 2002

Abstract

Nucleic acid sequence-based amplification (NASBA) is a technique that allows the rapid amplification of specific regions of nucleic acid obtained from a diverse range of sources. It is especially suitable for amplifying RNA sequences. A NASBA technique has been developed that allows the detection of avian influenza A subtype H5 from allantoic fluid harvested from inoculated chick embryos. The amplified viral RNA is detected by electrochemiluminescence. The NASBA technique described below is rapid and specific for the identification of influenza A subtype H5 viruses of the Eurasian lineage. More importantly, it can be used to distinguish highly pathogenic and low pathogenic strains of the H5 subtype.

Keywords: NASBA; Avian influenza; H5; Haemagglutinin; Pathogenic; Eurasian; Electrochemiluminescence

1. Introduction

Avian influenza virus type A subtype H5N1 contributed to the deaths of six people in Hong Kong in 1997 and was isolated from another 12 human cases (Yuen et al., 1998). A major concern was the possibility that the virus might spread to the larger population. Consequently, an eradication programme was implemented resulting in the slaughter of over 1.4 million chickens and other poultry (Shortridge et al., 2000). When the poultry markets were re-opened in 1998 an integrated H5 avian influenza monitoring and control programme was introduced. This involved veterinary inspection, quarantine and serological testing of poultry flocks in Hong Kong and Mainland China by the relevant government inspection and quarantine staff to ensure freedom from evidence of H5 influenza in flocks sending birds to the Hong Kong poultry markets. This was followed by audit testing of a random sample of serum

* Corresponding author. Tel./fax: +852-2111-2123.
E-mail address: dnachips@dnachip.com.hk (A.C.H. Yu).

0166-0934/02/$ - see front matter
PII: S0166-0934(02)00034-4

from every consignment of poultry for H5 antibodies and influenza virus culture of cloacal swabs from every consignment of ducks and geese at the point of entry to the wholesale poultry market. Control was enhanced further by removal of waterfowl from retail markets and complete segregation of land based poultry and waterfowl at all stages of production and marketing. A schedule of influenza virus culturing on random faecal swabs from retail market cages was also included as part of the monitoring programme. No incursions of H5 influenza virus occurred in the retail markets for over 3 years until February–May 2001 despite the detection of a number of H5N1 viruses in imported ducks and geese on occasions during 1999 and 2000. As a result of the large number of swab samples that were being cultured with only occasional detections of H5N1 viruses in waterfowl interest was generated in finding tests for rapid detection, or confirmation of H5 viruses and differentiating highly pathogenic H5 viruses from milder H5 viruses. A rapid test with the required accuracy and ease of use would enhance the H5 avian influenza monitoring and control programme.

Nucleic acid sequence-based amplification (NASBA) is a continuous, isothermal, enzyme-based method for the amplification of nucleic acid (Romano et al., 1996). The technique employs a mixture of reverse transcriptase, ribonuclease-H, RNA polymerase and two specially designed DNA oligonucleotide primers. The forward primer has a 5′ extension containing the promoter sequence for bacteriophage T7 DNA-dependent RNA polymerase. The reverse primer has a 5′ extension containing a complementary binding sequence for a DNA oligonucleotide detection probe labelled with a ruthenium-based electrochemiluminescent (ECL) tag. During the amplification process, the 5′ primer extensions are incorporated fully into the amplified sequence allowing both highly efficient production of complementary RNA template (directed by the RNA polymerase) and specific detection by the ECL-tagged probe during the detection stage. The technique is particularly suited for the amplification of single-stranded RNA and has been successfully used in the detection of numerous different RNA and DNA viruses, bacteria, fungi, parasites and cytokines. For example, NASBA protocols have been described for human immunodeficiency virus type 1 (Romano et al., 1996), simian immunodeficiency virus (Romano et al., 2000), cytomegalovirus (Blok et al., 1999), hepatitis C virus (Damen et al., 1999), Epstein-Barr virus (Hayes et al., 1999), measles (Chadwick et al., 1998), varicella-zoster (Mainka et al., 1998), human rhinovirus (Samuelson et al., 1998), human papillomavirus type 16 (Smits et al., 1995), potato leafroll virus (Leone et al., 1997), *Salmonella enterica* (Simpkins et al., 2000), *Chlamydia trachomatis* (Mahony et al., 2001), *Campylobacter jejuni* (Uyttendaele et al., 1997), *Mycobacterium leprae* (van der Vliet et al., 1996), *Listeria monocytogenes* (Uyttendaele et al., 1995), *Candida* spp. (Borst et al., 2001), *Aspergillus* spp. (Loeffler et al., 2001), *Plasmodium falciparum* (Schoone et al., 2000), macrophage-derived chemokine mRNA (Romano et al., 2001), tissue factor mRNA (van Deursen et al., 1999) and human TNF-alpha mRNA (Darke et al., 1998), among others.

Avian influenza viruses affecting chickens are described as either highly pathogenic or low pathogenic. To date, highly pathogenic avian influenza (HPAI) in chickens has been confined to certain strains of H5 and H7 subtypes only (Alexander, 2000). In general, HPAI viruses for chickens are low pathogenic for ducks but some can cause disease in geese via experimental or natural infections (Xu et al., 1999).

The type A influenza virus genome comprises eight single-stranded RNA gene segments that encode ten different proteins (Swayne and Suarez, 2000). The proteins can be divided into surface and internal proteins. The surface proteins include haemagglutinin (HA), neuraminidase (NA) and matrix two proteins. The HA and NA proteins provide the most important antigenic sites for the production of a protective immune response, primarily in the form of neutralising antibody. There is a great deal of antigenic variation among these proteins, with 15 HA and nine NA subtypes being recognised, based on haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests, respectively.

The HA RNA is translated into a single precursor polypeptide, termed HA0, approximately 556 residues in length (Zambon, 1999). To be infectious, HA0 must be cleaved into two peptides, HA1 and HA2, linked together by a disulphide bridge (Webster and Rott, 1987). The cleavage is carried out by host proteases. The consensus sequence surrounding the cleavage site (*) in low pathogenic strains of H5 avian influenza was found to be PQRETR*GLF (Alexander, 2000). In highly pathogenic strains, there are an increased number of basic residues at the cleavage site thought to arise from insertion or substitution. For example, the consensus sequence surrounding the cleavage site in H5N1 isolates from the 1997 outbreak in Hong Kong and the H5N2 outbreak in Italy in the same year, were found to be RRRKKR*GLF (Alexander, 2000). The increase in basic residues allows proteases present in tissues outside the gastro-intestinal and respiratory tract to cleave and activate the precursor polypeptide and hence render the virus infectious to a greater number of tissues. This enhanced infectivity contributes to the increased systemic effects of viral infection and the multiple organ failure that is characteristic of infection by highly pathogenic forms of the virus (Yuen et al., 1998).

It is the purpose of this study to demonstrate the use of the NASBA detection system in amplifying and detecting nucleic acid derived from H5 isolates of the Eurasian lineage. Generic primers (for the amplification of both highly pathogenic and low pathogenic H5 HA sequences) and pathogenic primers (specific for pathogenic H5 HA sequences) were designed and tested. This preliminary study will act as the foundation for further studies into the potential application of this technique for monitoring a wider range of avian influenza subtypes.

2. Materials and Methods

2.1. Viruses

The viruses used in this study were isolated by intra-allantoic cavity inoculation of 9–11 day-old chicken embryos (Anon, 1992) at the Castle Peak Veterinary Lab (CPVL, Agriculture, Fisheries and Conservation Department, Hong Kong SAR, China). Viruses were inactivated in lysis buffer (5.25 M guanidine isothiocyanate (GuSCN), 50 mM Tris, pH 7.2, 20 mM EDTA, 1.3% Triton X-100) before delivery to the Department of Biology, Hong Kong University of Science and Technology for further analysis. Isolates used included A/Chicken/Hong Kong/258/97 (H5N1), A/Chicken/Hong Kong/1258-2/97 (H5N1), A/Chicken/Hong Kong/1258-3/97 (H5N1), A/Chicken/Hong Kong/1258-4/97 (H5N1), A/Chicken/Hong Kong/1258-5/97 (H5N1), A/Chicken/Hong Kong/1258-9/97 (H5N1), A/Chicken/Hong Kong/977-2/97 (H5N1), A/Chicken/Hong Kong/1000-2/97 (H5N1), A/Environment/Hong Kong/437-4/99 (H5N1), A/Environment/Hong Kong/437-6/99 (H5N1), A/Environment/Hong Kong/437-8/99 (H5N1), A/Environment/Hong Kong/437-10/99 (H5N1), A/Chicken/Hong Kong/3568-6/99 (H9N2), A/Chicken/Hong Kong/157-9/92 (H9N2), A/Porcine/Hong Kong/2662-2/99 (H1N1), A/Environment/Hong Kong/382-2/99 (H3N?), A/Quail/Hong Kong/665-8/2000 (H9N2), A/Chicken/Hong Kong/161-2/2000 (H9N2), A/Chicken/Hong Kong/161-4/2000 (H9N2), A/Chicken/Hong Kong/466-1/2000 (H9N2), A/Chicken/Hong Kong/466-3/2000 (H9N2), A/Chicken/Hong Kong/467-5/2000 (H9N2), A/Duck/Hong Kong/3461/99 (H6N?), A/Goose/Hong Kong/485-5/2000 (H5N1), A/Goose/Hong Kong/485.5/2000 (H5N1), A/Duck/Hong Kong/25/2000 (H5N1). Isolates were typed by HI tests at CPVL and NI tests and/or cDNA sequence analysis at Department of Microbiology, Hong Kong University using harvested allantoic fluid from inoculated chick embryos.

2.2. Sequence alignment and primer selection

The nucleotide sequences of the HA gene from about 50 avian influenza A subtype H5 isolates of the Eurasian lineage obtained from GenBank were aligned using the BioEdit software program (Hall, 1997). Conserved sequences within 100nt either side of the HA1/HA2 cleavage site were used for primer selection. Primer and probe se-

quences were compared with sequences submitted to the GenBank nucleotide database using a standard nucleotide–nucleotide comparison tool (BLASTN version 2.2.1, Altschul et al., 1997) using default search parameters. In addition, primer and probe sequences were aligned with specific sequences using a pairwise comparison tool (BLAST 2 sequences, Tatusova and Madden, 1999) using default comparison parameters.

2.3. Nucleic acid isolation

Briefly, one volume of inoculated egg allantoic fluid was added to nine volumes of lysis buffer. The sample was mixed gently by vortex mixing. This inactivated infectious virus and stabilised the nucleic acids by denaturing nucleases. Acid-treated silica (50 μl, 1 mg/ml) was added to the lysate. The sample was kept at room temperature for 10 min and vortexed vigorously every 2 min. The liberated influenza virus RNA segments bound to the silica and collected in the solid phase. The silica and nucleic acid complex was pelleted by centrifugation for 30 s at $10\,000 \times g$ and washed repeatedly (twice with 5.25 M GuSCN, 50 mM Tris, pH 6.4, 20 mM EDTA; twice with 70% ethanol, and once with acetone). The acetone was evaporated from the silica pellet by warming the sample in a 56 °C water bath for 10 min. DEPC-treated water (50 μl) was added to the dry pellet and incubated in a 56 °C water bath for 10 min. The tube was centrifuged for 1 min at $10\,000 \times g$ to separate the silica from the water containing the eluted nucleic acid.

2.4. NASBA primers

Two pairs of DNA oligonucleotide primers were utilised in this study. The primer pair used depended on whether generic H5 subtypes or pathogenic H5 subtypes were being amplified. The primers used for amplifying generic H5 subtypes were designated NASBA-P1 and NASBA-P2, respectively. The primers used to amplify H5 pathogenic strains were designated NASBA-PP1 and NASBA-PP2, respectively (Table 1). Primers were obtained from Gibco BRL, Life Technologies Inc., NY. For these assays, primers NASBA-P1 and NASBA-PP1 were identical in sequence.

2.5. Amplification by NASBA

To 5 μl of nucleic acid extract, 10 μl of a mixture containing 80 mM Tris, pH 8.3, 24 mM $MgCl_2$, 140 mM KCl, 10 mM DTT, 2 mM each dNTP, 4 mM each NTP, 30% DMSO, and 0.4 μM each primer was added. This mixture was heated to 65 °C for 5 min in a water bath, and then cooled to 41 °C for 5 min. Once cool, 5 μl enzyme mix (6.4 units/μl T7 RNA polymerase, 1.3

Table 1
Primer sequences used in this study

Name	Sequence from 5′ to 3′	GC content	Product size (bp)
NASBA-P1/ NASBA-PP1	**AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG G**TC CCC TGC TCA TTG CTA TGG TGG TA	25/56 = 45%	
NASBA-P2	GAT GCA AGG TCG CAT ATG AGT GCC ATT CCA CAA CAT ACA CCC CCT CA	24/47 = 51%	165 (NASBA-P1 + NASBA-P2)
NASBA-PP2	GAT GCA AGG TCG CAT ATG AGG AGA GAA GAA GAA AAA AGA GAG GAC	20/45 = 44%	269 (NASBA-PP1 + NASBA-PP2)
NASBA-CP	Biotin-CTA TTT GGA GCT ATA GCA GGT T		
IC-forward	TCC CCT GCT CAT TGC TAT GGT GGT A		
IC-reverse	TGC CAT TCC ACA ACA TAC ACC CCC TCA		

Bold type indicates the sequence of the bacteriophage T7 DNA-dependent RNA polymerase promoter.Underscore indicates the region complementary to the sequence of the ECL detection oligonucleotide.

units/μl AMV-RT, 0.02 units/μl RNase H, and 0.42 μg/μl BSA) was added and the reaction incubated at 41 °C for 90 min in a water bath. The final volume was 20 μl.

2.6. Capture probe

The amplified products of the NASBA reaction (amplicons) were immobilised by binding to paramagnetic silica beads to which had been attached a capture probe specific for both the generic and pathogenic amplicons. The capture probe (NASBA-CP) sequence was determined after selection of the amplification primers by aligning the nucleotide sequences of the putative amplification products. The capture probe was 5′ end-labelled with biotin (Table 1). The capture probe was obtained from Gibco BRL. For these experiments, NASBA-CP was designed such that it was able to capture both generic and pathogenic H5 amplicons.

2.7. Detection of NASBA amplification products

The NASBA reaction amplicons were detected by hybridisation analysis using an ECL detection system following the manufacturer's instructions. Briefly, the amplicons were immobilised by hybridisation to a capture probe that had been attached to paramagnetic silica beads through a streptavidin:biotin interaction. The amplicon solution (5 μl) was added to 20 μl of a 1:1 mixture of the ECL generic probe solution and capture probe solution and incubated at 41 °C for 30 min. Assay buffer (0.3 ml) was added to this mixture and the emitted light intensity at 620 nm measured by the detector (NucliSens ECL Reader, Organon Teknika Inc., Boxtel, Netherlands).

2.8. Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was used to generate a DNA copy of the RNA amplicons to facilitate sequencing. The reaction mixture contained 5 μM random hexamer (Gibco BRL) and 1 μg RNA from the nucleic acid extraction procedure in RNase-free water. The mixture was incubated at 70 °C for 10 min. Then, 1 U/μl RNasin inhibitor (Promega, WI), 1 × first strand buffer (Gibco BRL), 10 mM DTT (Gibco BRL) and 1 mM dNTPs (Pharmacia Biotech, Sweden) were added and the mixture kept at room temperature for 10 min followed by incubation at 42 °C for 2 min. Finally, 10 U/μl SUPERSCRIPT™ II RNase H reverse transcriptase (Gibco BRL) was added into the reaction mixture and incubated at 42 °C for 50 min followed by 70 °C for 15 min in order to stop the reaction. After the RT reaction, 0.15 U/μl Ribonuclease-H (Gibco BRL) was added to the reaction mixture and incubated for 20 min at 37 °C to degrade the RNA in the DNA–RNA hybrid.

PCR was carried out in a thermocycler (Robocycler, Stratagene, Amsterdam, Holland). The reaction mixture contained 1 × PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, 200 μM dNTPs (Pharmacia Biotech, Sweden), internal control primers (IC, Table 1), and 0.04 U/μl *Taq* DNA polymerase (Gibco BRL). The PCR cycle began with 2 min at 94 °C, followed by 40 amplification cycle: 94 °C for 1 min, 55 °C for 40 s, and 72 °C for 1 min. Further extension at 72 °C for an additional 10 min completed the reaction. PCR products were analysed by electrophoresis on 2% agarose gels. The PCR products were sequenced subsequently to confirm their identity (data not shown).

2.9. DNA sequencing and analysis

The products of the amplification reaction were sequenced to ensure that the amplicons corresponded to the intended target sequence (data not shown). DNA sequencing was performed with an AutoRead 100 Sequencing Kit (Pharmacia Biotech, Sweden) using an automated fluorescent sequencer (Pharmacia LKB. A.L.F. DNA Sequencer, Pharmacia Biotech, Sweden). In brief, 8 μg template DNA was denatured with 400 mM NaOH and incubated at room temperature for 10 min. The DNA was precipitated with 100% ethanol and 3 M potassium acetate (pH 4.8) and then washed with 70% ethanol. The DNA pellet was

dried and resuspended in distilled water (10 μl). Annealing primer was added to the DNA template to 0.15 nM. The mixture was heated at 65 °C for 5 min and immediately incubated at 37 °C for another 10 min. They were incubated at room temperature for at least 10 min following which extension buffer, DMSO and T7 DNA polymerase were added. The reaction mixture was divided equally into four different tubes containing adenine-, cytosine-, guanine- and thymine-dideoxynucleotides, respectively. The reaction mixture was further incubated at 37 °C for 5 min, followed by the addition of stop solution. They were heated at 90 °C for 3 min before being loaded into a sequencing gel. DNA sequences were analysed by sequence similarity searches with EMBL and GenBank DNA databases.

3. Results

Several different experiments were conducted to examine the efficacy of the NASBA primers and capture probes for amplifying and capturing generic and pathogenic strains of avian influenza H5.

3.1. Use of electrochemiluminescence

The ECL signal obtained during the experiments was normalised relative to an internal reference standard (IRS) solution supplied by the manufacturer of the ECL reader. The IRS solution produces an ECL signal of about 30–40 000 arbitrary luminescence units. While ECL emission is proportional to the amount of amplified nucleic acid products, in the absence of standardisation with appropriate internal amplification controls, no information on the initial amount of infectious agent, target RNA or degree of amplification can be obtained. The cut-off value for differentiating positive and negative samples is critical to the appropriate interpretation of data. In this study, cut-off values are defined as the mean of the negative controls plus three standard deviations. Other users of NASBA systems have reported different methods for calculating the cut-off value, including $0.01–0.025 \times$ IRS (Greene, 2001; Rahman et al., 2000; Mahony et al., 2001) and 200 ECL units (Witt et al., 2000). The use of appropriate internal controls for quantitating HIV viral load using the NASBA technique has been described (Romano et al., 1996) and the technique is applicable to other targets. Analysing the same sample multiple times assessed reproducibility. Replicate analyses of the same sample conducted over a period of several months by different operators, produced the same qualitative result as the original determination (data not shown).

3.2. Sequence alignment and primer specificity

The NASBA primers and probes used in this study were designed by aligning the nucleotide sequences of a limited number of Eurasian avian influenza subtypes, comprising H5N1 sequences derived from the Hong Kong and Southern China outbreak of 1997. Primers were designed to amplify regions conserved in both highly pathogenic and low pathogenic isolates. Excluding the 5′ extensions required for the NASBA process, the forward and reverse generic H5 NASBA primers were 25 and 27 nucleotides in length, respectively. The forward primer (NASBA-P1) showed 100% sequence identity to multiple H5N1 isolates derived from various species (i.e. chicken, duck and goose), isolated at various times and locations, e.g. Guangdong 1996 and Hong Kong 1997 (data not shown). To test whether the selected primer would amplify regions from other HA subtypes, a standard nucleotide–nucleotide search (BLASTN) was conducted. Apart from the expected Hong Kong and Southern China isolates, the forward primer displayed similarity only to other H5 avian influenza isolates (Table 2). The greatest similarity was to isolates of the Eurasian lineage. However, significant similarity to certain isolates of the North American lineage was observed, for example A/Gull/Pennsylvania/4175/83, A/Mallard/Wisconsin/169/75 and A/Emu/Texas/39442/93 (22 out of 23 consecutive nucleotides, 88% overall). Using a pairwise nucleotide comparison (BLAST 2 sequences), the forward primer was found to have no significant sequence similarity with HA genes from other avian influenza viruses (subtypes H1 to H15 inclusive) or avian

Table 2
Sequence similarity between NASBA primers and probes and sequences from GenBank

GenBank sequence	Subtype	Sequence identity			
		NASBA-P1/NASBA-PP1	NASBA-P2	NASBA-PP2	NASBA-CP
		(25 nt)	(27 nt)	(25 nt)	(22 nt)
A/Duck/Hong Kong/205/77	H5N3	24/25	25/27		20/20
A/Duck/Ireland/113/83	H5N8	24/25		19/20	20/20
A/Turkey/Ireland/1378/83	H5N8	24/25		19/20	19/20
A/Duck/Potsdam/2216-4/84	H5N6	24/25	24/27		19/20
A/Duck/Potsdam/1402-6/86	H5N2	24/25	25/27		20/20
A/Turkey/England/50-92/91	H5N1	24/25			19/20
A/Duck/Malaysia/F119-3/97	H5N3	24/25	25/27		19/20
A/Chicken/Italy/9097/97	H5N9	24/25			
A/Duck/Ho Chi Minh/014/78	H5N3	23/25	25/27		20/20
A/Chicken/Italy/312/97	H5N2	23/25	26/27	21/22	19/20
A/Chicken/Italy/367/97	H5N2	23/25	26/27	21/22	19/20
A/Guinea Fowl/Italy/330/97	H5N2	23/25	26/27	21/22	19/20
A/Chicken/Italy/8/98	H5N2	23/25	26/27	21/22	19/20
A/Mallard/Wisconsin/169/75	H5N3	22/23			
A/Gull/Pennsylvania/4175/83	H5N1	22/23			
A/Mallard/Ohio/556/87	H5N9	22/23			19/20
A/Emu/TX/39442/93	H5N2	22/23			19/20
A/Chicken/Puebla/8623-607/94	H5N2	22/23			
A/Duck/Hong Kong/698/79	H5N3	21/22	19/19		22/22
A/Duck/Michigan/80	H5N2	17/17			
A/Turkey/Minnesota/3689-1551/81	H5N2	17/17			19/20
A/Mallard/WI/944/82	H5N2	17/17			
A/Mallard/Pensylvania/10218/84	H5N2	17/17			
A/Chicken/Queretaro/7653-20/95	H5N2	17/17			
A/Chicken/Queretaro/14588-19/95	H5N2	17/17			
A/Tern/South Africa/61	H5N3				21/22
A/Duck/Hong Kong/342/78	H5N2		25/27		

The nucleotide sequences were used to perform a nucleotide–nucleotide search of the entries in GenBank using the BLASTN search tool with default search parameters. Avian influenza sequences not corresponding to isolates derived from the 1997 Hong Kong or Southern China H5N1 outbreak and located within the first 80–250 search descriptions are shown. Blank cells indicate no significant sequence similarity.

paramyxoviruses, such as Newcastle disease virus submitted to GenBank. A similar analysis was conducted for the reverse generic primer (NASBA-P2) and the reverse pathogenic primer (NASBA-PP2, Table 2). Again, apart from the expected high similarity with H5N1 isolates from Hong Kong and Southern China (data not shown), the only other sequences with which the primers exhibited similarity were H5 HA genes of primarily Asian origin (Table 2). Using a pairwise nucleotide comparison, the primers were found to have no significant sequence similarity with HA genes from other avian influenza viruses (subtypes H1 to H15 inclusive) or avian paramyxoviruses (data not shown). In this study, the sequence of the forward pathogenic primer, NASBA-PP1, is identical to NASBA-P1, whose predicted specificity has been described. Thus, it is predicted that both the generic H5 and pathogenic H5 NASBA primers are specific for H5 strains of the Eurasian lineage only.

The use of a biotin-labelled capture probe (NASBA-CP) introduces an added level of specificity to the NASBA assay, as only appropriately

amplified molecules will be carried over to the detection stage. Non-specific or partially cross-reactive molecules generated during the NASBA reaction will fail to hybridise with the capture probe and be washed away prior to detection. NASBA-CP was designed in a similar manner as the generic H5 and pathogenic H5 NASBA primers. The NASBA-CP probe had 100% sequence identity with H5N1 HA sequences derived from Hong Kong and Southern China sources between 1997 and 1999 submitted to GenBank (data not shown). The potential for the capture probe to detect HA genes other than those specified by the NASBA primers was examined by conducting a BLASTN search using the 22-nucleotide capture probe sequence as query. Apart from the expected H5N1 strains from Hong Kong and Southern China (data not shown), NASBA-CP displayed partial sequence identity (between 86 and 100%) only with other H5 HA molecules (Table 2) and had no significant sequence similarity with any other HA genes from either avian influenza viruses or avian paramyxoviruses. Partial similarity (18 out of 19 consecutive nucleotides, 82% overall) was observed between NASBA-CP and A/black-headed gull/Astrachan/227/84 (H13N6). However, the failure of the NASBA primers to amplify this isolate means that this relatively uncommon subtype is highly unlikely to contaminate assays for H5 using this system. Thus, NASBA-CP is predicted to be highly specific for H5 HA sequences.

3.3. Assay for H5

After obtaining consensus sequences for primer design based on alignments of multiple Eurasian H5N1 isolates, the ability of the selected primers to function efficiently in the amplification of extracted nucleic acid was examined. Twelve pathogenic H5N1 viruses obtained from Hong Kong and Southern China were inoculated into embryonated hen's eggs. Table 3 shows the ECL signal detected following amplification of isolated nucleic acid with the generic and pathogenic H5 primers, respectively. These data indicate that the generic and pathogenic primers amplify H5 nucleic acid efficiently. The ECL signal generated from each isolate varied widely. This may be due to variation in the amount of virus propagated in eggs and the amount of nucleic acid subsequently isolated from allantoic fluids or individual varia-

Table 3
Efficacy of the generic and pathogenic NASBA primers for the amplification of Eurasian avian influenza H5 HA sequences

Case no./Sample no.	Generic H5 primers		Pathogenic H5 primers	
	ECL signal	Result[a]	ECL signal ($\times 10^{-6}$)	Result[a]
Internal reference standard	35 368		0.038	
A/Ck/HK/258/97 (H5N1)	4229	Positive	11.8	Positive
A/Ck/HK/977-2/97 (H5N1)	6961	Positive	5.3	Positive
A/Ck/HK/1000/97 (H5N1)	33 835	Positive	23.1	Positive
A/Ck/HK/1258-2/97 (H5N1)	2500	Positive	61.8	Positive
A/Ck/HK/1258-3/97 (H5N1)	2400	Positive	85.1	Positive
A/Ck/HK/1258-4/97 (H5N1)	10 494	Positive	68.4	Positive
A/Ck/HK/1258-5/97 (H5N1)	3089	Positive	158.4	Positive
A/Ck/HK/1258-9/97 (H5N1)	4883	Positive	5.4	Positive
A/Env/HK/437-4/99 (H5N1)	5165	Positive	48.4	Positive
A/Env/HK/437-6/99 (H5N1)	22 200	Positive	27.1	Positive
A/Env/HK/437-8/99 (H5N1)	5142	Positive	21.1	Positive
A/Env/HK/437-10/99 (H5N1)	511	Positive	11.6	Positive
Negative control 1	1	Negative		
Negative control 2	1	Negative		

All samples tested were allantoic fluid harvests of 9–11 day-old chicken embryos inoculated from swabs of the indicated cases.
[a] Cut-off value = negative control mean + 3 S.D.

Table 4
Cross-reactivity of the generic H5 primers with other Eurasian avian influenza HA subtypes

Sample no./Case no.	ECL signal	Result[a]
Internal reference standard	39 933	
Internal reference standard	40 478	
A/Porcine/Hong Kong/2662-2/99 (H1N1) mock infected faeces	131	Negative
A/Porcine/Hong Kong/2662-2/99 (H1N1) mock infected cloacal swab	223	Negative
A/Porcine/Hong Kong/2662-2/99 (H1N1)	181	Negative
A/Environment/Hong Kong/3821-2 (H3N?) mock infected cloacal swab	161	Negative
A/Environment/Hong Kong/3821-2 (H3N?) cage swab	102	Negative
A/Duck/Hong Kong/3461/99 (H6N?)+A/Porcine/Hong Kong/2662-2/99 (H1N1) (1:1)	130	Negative
A/Chicken/Hong Kong/3568-6/99 (H9N2)	110	Negative
A/Chicken/Hong Kong/977-2/97 (H5N1)	1 753 013	Positive
A/Porcine/Hong Kong/2662-2/99 (H1N1)+A/Goose/Hong Kong/485-5/2000 (H5N1) (1:1)	187 817	Positive
A/Porcine/HongKong/2662-2/99 (H1N1)+A/Goose/Hong Kong/485-3/2000 (H5N1) (1:1)	128 305	Positive
A/Duck/Hong Kong/25/2000 (H5N1)	30 366	Positive
A/Environment/Hong Kong/437-4/99 (H5N1)	1584	Positive
A/Environment/Hong Kong/437-6/99 (H5N1)	102 516	Positive
A/Environment/Hong Kong/437-8/99 (H5N1)	17 268	Positive
A/Environment/Hong Kong/437-10/99 (H5N1)	2595	Positive
A/Quail/Hong Kong/665-8/2000 (H9N2)	151	Negative
A/Chicken/Hong Kong/157-9/2000 (H9N2)	110	Negative
A/Chicken/Hong Kong/161-2/2000 (H9N2)	263	Negative
A/Chicken/Hong Kong/161-4/2000 (H9N2)	99	Negative
A/Chicken/Hong Kong/466-3/2000 (H9N2)	144	Negative
A/Chicken/Hong Kong/466-1/2000 (H9N2)	146	Negative
A/Chicken/Hong Kong/467-5/2000 (H9N2)	139	Negative
Negative control 1	125	Negative
Negative control 2	110	Negative

All samples tested were allantoic fluid harvests of 9–11 day-old chicken embryos inoculated from swabs from the indicated cases.

[a] Cut-off value = mean blank value + 3 S.D.

tion in the level of RNA amplification. It is possible to quantify the amount of virus present in a sample by appropriate use of internal controls. In this preliminary study, which focused on the feasibility of the general detection method, such experiments were not performed and no comment can be made as to the relative amounts of virus present in each sample of allantoic fluid.

3.4. Specificity studies

After the generic and pathogenic primers were demonstrated to work effectively, the specificity of the generic primers toward H5 was tested against a limited panel of influenza isolates. Table 4 shows the ECL signal detected when various influenza type A subtypes were amplified using the generic H5 primers. The experiment compared the ECL signals obtained from a small number of chick embryo allantoic fluid harvests of influenza type A subtypes H1, H3, H6 and H9 viruses. The H1 samples were used to mock infect pooled faeces and cloacal swabs, respectively, and were also mixed with H5 and H6 samples to determine if H5 sequences could be detected against a background of similar genetic material. The H3 samples were derived from a cage swab of pet birds at a pet shop. The data indicate that among the limited sample of subtypes tested, the generic and pathogenic primers are specific for H5.

3.5. Sequencing

The amplified target RNA molecules were se-

quenced to confirm their identity. No differences between the expected sequence and that obtained were observed (data not shown).

4. Discussion

The NASBA-based technique described in this communication provides a rapid genome-based detection system for H5 influenza virus, which has advantages for confirmation of H5 virus isolation over antigenic (ELISA, immunoperoxidase, immunofluorescence) or genomic (RT-PCR/DNA sequencing) methods. Amplification by NASBA is most appropriate for RNA analytes, such as the genetic material from influenza virus, due to the direct incorporation of the reverse transcription process into the amplification reaction. Under standard conditions, the test can be completed in about 4 h. The assay can be performed in the presence of contaminants, e.g. heparin, EDTA, citrate, haemoglobin, albumin and lipids (Romano et al., 1996). The presence of PCR inhibitors in allantoic fluids is an important factor affecting assay reproducibility and requires the use of appropriate internal control primers.

The product of the NASBA reaction is single-stranded RNA, which enables the user to select their preferred hybridisation-based detection system, enhancing the sensitivity and specificity of the technique. The amplification by AMV-RT and T7 RNA polymerase has a low combined error rate (Sooknanen et al., 1994), which, combined with the milder incubation conditions and reduced incubation time compared with PCR, increases the fidelity of the transcripts. This may be important for the phylogenetic analysis of viral isolates. In addition, the NASBA-based H5 detection system is highly automated, resulting in fewer handling errors and contributing to an increase in sensitivity and specificity. The ECL reader eliminates the need for gel electrophoresis, is convenient to use and requires minimum staff training.

The NASBA-based H5 test using the pathogenic primers (NASBA-PP1 and NASBA-PP2) provides a useful tool to rapidly determine if an H5 influenza isolate has characteristics of a HPAI virus. The standard intravenous pathogenicity index test is the definitive test to classify HPAI viruses but takes ten days to complete (Anon, 1992). However, the presence of a polybasic amino acid sequence at the cleavage site of HA0 correlates with increased virulence and is a co-requirement for avian influenza viruses to be classed as HPAI (Anon, 1992). The NASBA-based pathogenic H5 test, which has one primer located at the cleavage site of the HA0 gene, can give a rapid confirmation that a newly isolated H5 influenza virus is likely to be a HPAI.

To enhance the specificity of the NASBA-based avian influenza detection system two H5 sequence-specific primers and an additional capture probe are included to confirm the presence of amplified RNA. The key to the success of the NASBA technique lies in the initial selection of primers for the amplification reaction. The primers used in this study were compatible with a wide range of H5 isolates separated geographically (e.g. Hong Kong, Guangdong (China) and Italy), temporally (1996–2000), and by species (e.g. goose, duck and chicken). A BLASTN search of the GenBank database using the generic and pathogenic H5 forward primer sequences as query revealed that the only partially cross-reactive sequences were derived from other avian influenza H5 HA molecules (Table 2). There was a distinct difference in the degree of cross-reactivity between the primer sequences and the HA nucleotide sequences of isolates of the Eurasian lineage (88–96%) and isolates of the North American lineage (68–88%), clearly reflecting phylogenetic differences. While some strains of the North American lineage are likely to be amplified by the forward generic and pathogenic primers, the reverse primers displayed little sequence similarity with such isolates. It is highly likely that different primers will be required to adequately detect North American H5 subtypes using the NASBA system described in this paper. In addition, as the influenza HA constantly mutates by the well-described processes of antigenic drift and shift it may be necessary to replace one or more of the primers from time to time to accommodate the evolution of the H5 virus. This is particularly true of the primer NASBA-PP2, which detects pathogenicity by spanning the HA1/HA2 cleavage site

and which was the least conserved of the primers used in this study (Table 2). Highly pathogenic isolates of avian influenza are also observed in certain H7 strains. A BLASTN search and pairwise nucleotide comparison of the GenBank database using the NASBA-PP2 sequence as query did not reveal significant sequence similarity with any highly pathogenic strains of H7 avian influenza. Thus, it seems that the NASBA-PP2 primer is specific for H5 strains currently in circulation in Southeast Asia. Furthermore, genes for HA-like molecules from Newcastle disease virus or other avian paramyxoviruses revealed no significant sequence similarity to any of the NASBA amplification primers or capture probe, emphasising the wide genetic differences between these viruses. In the absence of comprehensive specificity studies using a diverse range of avian influenza HA and HA-like molecules from related viruses, these data indicate specificity of the NASBA test system for H5.

The regions covered by the generic primers are highly conserved and are likely to mutate less frequently than the cleavage site region and may be used to enable rapid amplification and sequencing of novel strains permitting new pathogenic primers to be made in a timely manner should future outbreaks occur.

The added specificity provided by the capture probe increases greatly the accuracy of the NASBA assay system. The capture probe displays significant cross-reactivity (between 86 and 100%) only for other H5 HA sequences (Table 2). In addition to the haemagglutin genes from the H5N1 isolates of Hong Kong and Southern China, the generic H5 primers and the capture probe displayed greatest cross-reactivity with the HA sequences from A/Duck/Hong Kong/205/77, A/Duck/Ho Chi Minh/014/78, A/Duck/Potsdam/1402/86, and A/Duck/Malaysia/F119-3/97. The fact that the majority of these sequences were isolated from South-east Asia may indicate the high degree of conservation of particular domains within the HA gene of viruses from this region. The cross-reactivity of the other primers to HA genes from other South-east Asian isolates, e.g. NASBA-P2 with A/Duck/Hong Kong/342/78 (96% sequence identity) and NASBA-CP with A/Duck/Hong Kong/698/79 (100% sequence identity), supports this idea.

The signal strength of the generic primers was consistently lower than that produced by the pathogenic primers (Table 3). The ECL signal produced by the pathogenic primers exceeded that of the generic primer by an average of over 13 000-fold (range 680–51 000-fold). The reason for this is unclear. One of the pair of primers is common to both the generic and pathogenic amplification systems. The other generic primer has a higher GC content than its corresponding pathogenic primer (51 vs. 44% GC) and should lead to a more stable polymerisation initiation complex allowing more efficient amplification. However, the pathogenic amplicon is considerably shorter than the generic amplicon (165 vs. 269 bp) and allows a more rapid turnover and increased level of amplification than the generic amplicon. It may be that the increased turnover of the pathogenic amplicon compensates for the less efficient binding. The reaction blank and negative controls all gave extremely low ECL signals (1–125 arbitrary ECL units) relative to the IRS ($>35\,000$ ECL units). All positive ECL signals were greatly in excess of the cut-off limit ($+3$ standard deviations of the mean negative control) for differentiating positive and negative samples by a minimum of several 100-fold (Table 3). These data indicate that the generic and pathogenic H5 primers are capable of efficiently amplifying the nucleic acid isolated from several different avian influenza A subtype H5 samples, including isolates obtained from the 1997 H5N1 outbreak in Hong Kong, in addition to isolates obtained from avian influenza surveillance studies conducted throughout 1999 and 2000.

Table 4 demonstrates the specificity of the generic primers for avian influenza type A H5 subtypes. The generic primer was chosen as it gave a significantly lower ECL signal than the pathogenic primer allowing a more stringent analysis of the efficiency of the amplification. In addition, it is likely that the pathogenic primer would be used to determine the pathogenicity of samples demonstrated to be H5 positive following use of the generic primers. In the studies conducted H5 was detected accurately and specifically compared

with other influenza A subtypes, including H1, H3, H6 and H9. The H5 signal was at least 10-fold higher than the negative control. In addition, the H5 could be accurately detected even in the presence of equal amounts of other contaminating subtypes, such as H1 (Table 3). Analysis of mock infected faeces and cloacal swabs in addition to allantoic fluids was attempted. Faecal and cloacal residues did not inhibit the NASBA reaction.

The H5 assay described above proved to be very robust given the limited number of samples tested. During the course of the study there were no confirmed instances of false negative or false positive results. The novel molecular detection system described above is a rapid, sensitive, accurate, robust and reproducible assay for the efficient and convenient qualitative identification of avian influenza virus type A subtype H5. It has the potential to make a useful contribution to the confirmation of H5 influenza virus infection in monitoring programmes for H5 influenza in the poultry industry generally. It is important that further studies be conducted to determine the sensitivity and specificity of the primers in detecting other historical H5 isolates and in establishing a method for quantification of viral load. In addition, the applicability of this system to detect viral RNA directly in faecal and swab samples must be assessed, as these are important parameters contributing to the wider utility of this detection system. These studies are ongoing, although preliminary experiments indicate that H5 amplicons can be generated efficiently directly from swab samples without prior amplification in allantoic fluid. Furthermore, the primers and probes used in this study also displayed 100% sequence identity with the H5N1 sequences isolated from the human victims of the 1997 H5N1 outbreak in Hong Kong. An investigation into the use of a NASBA-based technique to identify influenza in human clinical samples is therefore warranted.

Acknowledgements

The authors would like to thank Pamela Li Chui Har and Clara Li Suk Man at the AFCD laboratories for their excellent technical assistance; Prof. Kennedy Shortridge, Dr Malik Peiris and Dr Yi Guan from the University of Hong Kong for helpful discussions; and Gary T.F. Lee and John Ford from Organon Teknika China Ltd for technical support.

References

Alexander, D.J., 2000. A review of avian influenza in different bird species. Vet. Microbiol. 74 (1–2), 3–13.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Anon, 1992. Highly Pathogenic Avian Influenza (Fowl Plague) in OIE Manual of Standards for Diagnostic Tests and Vaccines, vol. A15, second ed, pp. 123–129.

Blok, M.J., Lautenschlager, I., Christiaans, M.H.L., van Hooff, J.P., Goossens, V.J., Middeldorp, J.M., Sillekens, P., Ramon, A., Hockerstedt, K., Bruggeman, C.A., 1999. Nucleic acid sequence-based amplification: a new technique for monitoring cytomegalovirus infection in transplant recipients. Transplant. Proc. 31 (1–2), 308–309.

Borst, A., Leverstein-Van Hall, M.A., Verhoef, J., Fluit, A.C., 2001. Detection of *Candida* spp. in blood cultures using nucleic acid sequence-based amplification (NASBA). Diagn. Microbiol. Infect. Dis. 39 (3), 155–160.

Chadwick, N., Bruce, I., Davies, M., van Gemen, B., Schukkink, R., Khan, K., Pounder, R., Wakefield, A., 1998. A sensitive and robust method for measles RNA detection. J. Virol. Meth. 70 (1), 59–70.

Damen, M., Sillekens, P., Cuypers, T.H.M., Frantzen, I., Melsert, R., 1999. Characterization of the quantitative HCV NASBA assay. J. Virol. Meth. 82 (1), 45–54.

Darke, B.M., Jackson, S.K., Hanna, S.M., Fox, J.D., 1998. Detection of human TNF-alpha mRNA by NASBA. J. Immunol. Meth. 212 (1), 19–28.

Greene S.R., 2001. Norwalk-like viruses (G1&G2). Application list for NucliSens Basic Kit. Application #2 (Available from): http://www.teknika.nl/BasicKit/ [accessed January 22, 2002].

Hall T., 1997. BioEdit biological sequence alignment editor for Windows 95/98/NT. v5.0.9. Available from http://www.mbio.ncsu.edu/BioEdit/bioedit.html [accessed June 22, 2001].

Hayes, D.P., Brink, A.A.T.P., Vervoort, M.B.H.J., Middeldorp, J.M., Meijer, C.J.L.M., van den Brule, A.J.C., 1999. Expression of Epstein-Barr virus (EBV) transcripts encoding homologues to important human proteins in diverse EBV associated diseases. Mol. Pathol. 52 (2), 97–103.

Leone, G., van Schijndel, H.B., van Gemen, B., Schoen, C.D., 1997. Direct detection of potato leafroll virus in potato

tubers by immunocapture and the isothermal nucleic acid amplification method NASBA. J. Virol. Meth. 66 (1), 19–27.

Loeffler, J., Hebart, H., Cox, P., Flues, N., Schumacher, U., Einsele, H., 2001. Nucleic acid sequence-based amplification of *Aspergillus* RNA in blood samples. J. Clin. Microbiol. 39 (4), 1626–1629.

Mahony, J.B., Song, X., Chong, S., Faught, M., Salonga, T., Kapala, J., 2001. Evaluation of the NucliSens Basic Kit for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genital tract specimens using nucleic acid sequence-based amplification of 16S rRNA. J. Clin. Microbiol. 39 (4), 1429–1435.

Mainka, C., Fuss, B., Geiger, H., Hofelmeyr, H., Wolff, M.H., 1998. Characterization of viremia at different stages of varicella-zoster virus infection. J. Med. Virol. 56 (1), 91–98.

Rahman, A., Fox, J.D., Westmoreland, D., 2000. Development and evaluation of NASBA and real-time RT-PCR for diagnosis of viral respiratory tract infections. J. Clin.Virol. 18, 174 (P200).

Romano, J.W., van Gemen, B., Kievits, T., 1996. NASBA: a novel, isothermal detection technology for qualitative and quantitative HIV–RNA measurements. Clin. Lab. Med. 16 (1), 89–103.

Romano, J.W., Shurtliff, R.N., Dobratz, E., Gibson, A., Hickman, K., Markham, P.D., Pal, R., 2000. Quantitative evaluation of simian immunodeficiency virus infection using NASBA technology. J. Virol. Meth. 86 (1), 61–70.

Romano, J.W., Shurtliff, R.N., Grace, M., Lee, E.M., Ginocchio, C., Kaplan, M., Pal, R., 2001. Macrophage-derived chemokine gene expression in human and macaque cells: mRNA quantification using NASBA technology. Cytokine 13 (6), 325–333.

Samuelson, A., Westmoreland, D., Eccles, R., Fox, J.D., 1998. Development and application of a new method for amplification and detection of human rhinovirus RNA. J. Virol. Meth. 71 (2), 197–209.

Schoone, G.J., Oskam, L., Kroon, N.C., Schallig, H.D., Omar, S.A., 2000. Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. J. Clin. Microbiol. 38 (11), 4072–4075.

Shortridge, K.F., Gao, P., Guan, Y., Ito, T., Kawaoka, Y., Markwell, D., Takada, A., Webster, R.G., 2000. Interspecies transmission of influenza viruses: H5N1 virus and a Hong Kong SAR perspective. Vet. Microbiol. 74 (1–2), 141–147.

Simpkins, S.A., Chan, A.B., Hays, J., Popping, B., Cook, N., 2000. An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. Lett. Appl. Microbiol. 30 (1), 75–79.

Smits, H.L., van Gemen, B., Schukkink, R., van der Velden, J., Tjong-A-Hung, S.P., Jebbink, M.F., ter Schegget, J., 1995. Application of the NASBA nucleic acid amplification method in the detection of human papillomavirus type 16 E6-E7 transcripts. J. Virol. Meth. 54 (1), 75–81.

Sooknanen, R., Howes, M., Read, L., Malek, L.T., 1994. Fidelity of nucleic acid amplification with avian myeloblastosis virus reverse transcriptase and T7 RNA polymerase. Biotechniques 17 (6), 1077–1080, 1083–1085.

Swayne, D.E., Suarez, D.L., 2000. Highly pathogenic avian influenza. Rev. Sci. Tech. 19 (2), 463–482.

Tatusova, T.A., Madden, T.L., 1999. Blast 2 sequences—a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. 174, 247–250.

Uyttendaele, M., Schukkink, R., van Gemen, B., Debevere, J., 1995. Development of NASBA, a nucleic acid amplification system, for identification of *Listeria monocytogenes* and comparison to ELISA and a modified FDA method. Int. J. Food Microbiol. 27 (1), 77–89.

Uyttendaele, M., Bastiaansen, A., Debevere, J., 1997. Evaluation of the NASBA nucleic acid amplification system for assessment of the viability of *Campylobacter jejuni*. Int. J. Food Microbiol. 37 (1), 13–20.

van der Vliet, G., Cho, S.-N., Kampirapap, K., van Leeuwen, J., Skukkink, R., van Gemen, B., Das, P.K., Faber, W.R., Walsh, G.P., Klatser, P.R., 1996. Use of NASBA RNA amplification for detection of *Mycobacterium leprae* in skin biopsies from untreated and treated leprosy patients. Int. J. Lepr. Other Mycobact. Dis. 64 (4), 396–403.

van Deursen, P.B., Gunther, A.W., van Riel, C.C., van der Eijnden, M.M., Vos, H.L., van Gemen, B., van Strijp, D.A., Tacken, N.M., Bertina, R.M., 1999. A novel quantitative multiplex NASBA method: application to measuring tissue factor and CD14 mRNA levels in human monocytes. Nucleic Acids Res. 27 (17), e15.

Webster, R.G., Rott, R., 1987. Influenza virus A pathogenicity: the pivotal role of the hemagglutinin. Cell 50 (5), 665–666.

Witt, D.J., Kemper, M., Stead, A., Sillekens, P., Ginocchio, C.C., Espy, M.J., Paya, C.V., Smith, T.F., Roeles, F., Caliendo, A.M., 2000. Analytical performance and clinical utility of a nucleic acid sequence-based amplification assay for detection of cytomegalovirus infection. J. Clin. Microbiol. 38 (11), 3994–3999.

Yuen, K.Y., Chan, P.K.S., Peiris, M., Tsang, D.N.C., Que, T.L., Shortridge, K.F., Cheung, P.T., To, W.K., Ho, E.T.F., Sung, R., Cheng, A.F.B. and members of the H5N1 Study Group, 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet 351 (9101) 467–471.

Xu, X., Subbarao, K., Cox, N.J., Guo, Y., 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 261 (1), 15–19.

Zambon, M.C., 1999. Epidemiology and pathogenesis of influenza. J. Antimicrob. Chemother. 44 (Suppl. B), 3–9.