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Rapid and sensitive detection of avian influenza virus subtype H7 using NASBA

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Abstract

Nucleic acid sequence-based amplification with electrochemiluminescent detection (NASBA/ECL) is an isothermal technique allowing rapid amplification and detection of specific regions of nucleic acid from a diverse range of sources. It is especially suitable for amplifying RNA. A NASBA/ECL technique has been developed allowing the detection of RNA from avian influenza virus subtype H7 derived from allantoic fluid harvested from inoculated chick embryos and from cell cultures. Degenerate amplification primers and amplicon capture probes were designed enabling the detection of low and highly pathogenic avian influenza of the H7 subtype from the Eurasian and North American lineages and the Australian sub-lineage. The NASBA/ECL technique is specific for subtype H7 and does not cross-react with other influenza subtypes or with viruses containing haemagglutinin-like genes. The assay is 10- to 100-fold more sensitive than a commercially available antigen capture immunoassay system. The NASBA/ECL assay could be used in high throughput poultry screening programmes.

Keywords: NASBA; Avian influenza; H7; Haemagglutinin; Electrochemiluminescence

Since 1959, outbreaks of highly pathogenic avian influenza (HPAI) of the H7 subtype have occurred 11 times, affecting commercial poultry farms in Europe (United Kingdom and Germany, 1979; Italy, 1999–2000), Australia (1976–1997), and South America (Chile, 2002). Numerous outbreaks of low pathogenic avian influenza (LPAI) have occurred throughout the world, for example, H7N2 in the North-eastern United States in 2002. The economic consequences of H7 outbreaks are devastating. During a protracted LPAI/HPAI outbreak in Italy, over 13 million birds were affected [1] with about 5 million others infected and/or culled during a LPAI outbreak in Virginia/North Carolina (USA) during early 2002. Compensation for economic losses varies greatly depending on the location of the outbreak. The psychological and social impacts of disease on poultry workers have not been well studied.

There are also well-founded fears that HPAI may infect humans. Direct transfer of avian influenza to humans has been observed on at least three separate occasions; once during an outbreak of H5N1 in Hong Kong in 1997, where six people died and a further 12 confirmed cases of infection were reported and twice with H9N2 in China and Hong Kong in 1999 [2–4]. An epidemiological study in Italy immediately after the H7 HPAI 1999–2000 outbreak indicated that none of the tested poultry workers were sero-positive for H7 antibodies [1]. Similar studies following the 1997 H5N1 outbreak in Hong Kong indicated that 3.7% of health care workers exposed to patients infected with H5N1 had antibodies to the virus, compared with 0.7% of non-exposed health care workers [5]. In 1996, an avian influenza virus [A/England/268/96 (H7N7)] was isolated from a woman with conjunctivitis and an isolate of the same subtype was isolated from a man with infectious

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hepatitis [6,7]. Due to the risk of the rapid spread of disease afforded by modern means of transportation, sensitive, accurate, and rapid assays for H7 avian influenza are required.

Several methods for detecting avian influenza subtype H7 have been described previously. Reverse transcriptase polymerase chain reaction (RT-PCR) assays have been described with varying degrees of specificity and sensitivity [8–10]. One major aspect that must be overcome is the phylogenetic variation within the H7 subtypes in which distinct North American and Eurasian lineages, plus a major Australian sub-lineage, can be discerned [11].

NASBA (nucleic acid sequence-based amplification) is a continuous, isothermal, enzyme-based method for the amplification of nucleic acid [12]. 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 fully incorporated 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 avian influenza virus subtype H5 [13], foot-and-mouth disease virus [14], human, simian, and feline immunodeficiency viruses [12,15,16], dengue fever [17], West Nile encephalitis virus [18], hepatitis C virus [19], *Salmonella enterica* [20], *Chlamydia trachomatis* [21], *Candida* spp. [22], *Plasmodium falciparum* [23], macrophage-derived chemokine mRNA [24], and tissue factor mRNA [25], among many 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 [26].

The type A influenza virus genome comprises eight single-stranded RNA gene segments that encode ten different proteins [27]. The proteins can be divided into surface and internal proteins. The surface proteins include haemagglutinin (HA), neuraminidase (NA), and matrix 2 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 haemagglutinin RNA is translated into a single precursor polypeptide, termed HA0, approximately 556 residues in length [28]. To be infectious, HA0 must be cleaved into 2 peptides, HA1 and HA2, linked together by a disulphide bridge [29]. The cleavage is performed by host proteases. The consensus sequence surrounding the cleavage site (*) in low pathogenic strains of H7 avian influenza is commonly PKTR*G, PKPR*G, or PKGR*G [30]. 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 H7N1 isolates from the 1999 outbreak in Italy was found to be RVRR*G [11]. 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 [2].

It is the purpose of this study to demonstrate the utility of the NASBA detection system in amplifying and detecting nucleic acid derived from H7 isolates of the Eurasian and North American lineages and Australian sub-lineage. Generic primers, able to amplify both highly pathogenic and low pathogenic H7 haemagglutinin 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.

Materials and methods

Viruses and viral RNA. The viruses and viral RNA used in this study were obtained from a number of sources. A/common iora /Singapore/F89/95(H7N1), A/fairy bluebird/Singapore/F92/94(H7N1), A/teal/Taiwan/WB2-37-2TPFE2/98(H7N1), A/turkey/Northern Ireland/VF-1545 C5/98 (H7N7), and A/chicken/Victoria/1/85 (H7N7) were provided by the Veterinary Laboratories Agency, New Haw, Addlestone, Weybridge, Surrey, UK. Isolates 160/V00 (HPAI from chicken) and 223/V01 (LPAI from turkey) obtained during an H7N1 outbreak in Italy during 1999–2000 were provided by the Department of Virology, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy. A/swine/Belgium/1/98 (H1N1) and A/swine/Gent/80/01 (H3N2) were provided by the Laboratory of Virology, Faculty of Veterinary Medicine, Universiteit Gent, Merelbeke, Belgium. A/chicken/Victoria/85 (H7N7), A/chicken/Victoria/76 (H7N7), A/emu/New South Wales/1742/97 (H7N4), A/chicken/Victoria/224/92 (H7N3), and A/chicken/Queensland/447/94 (H7N3) were provided by CSIRO-Livestock Industries, Australian Animal Health Laboratory, East Geelong,

Victoria, Australia. A/swine/Ontario/01911-1/99 (H4N6) was generously provided by the School of Veterinary Medicine, University of Wisconsin-Madison, WI, USA. Parainfluenza-3 virus (strain SF-4), avian paramyxovirus type 2 (P/chicken/CA/Yucaipa/56), and avian paramyxovirus type 3 (P/turkey/Wisconsin/68) were purchased from the National Veterinary Services Laboratory, US Department of Agriculture, Animal and Plant Health Inspection Service, Ames, IA, USA. A/quail/Hong Kong/2929-8/2000 (H6N?), A/chicken/Hong Kong/3130-2/2000 (H9N?), A/chicken/Hong Kong/1000/97 (H5N1), and a local isolate of Newcastle disease virus were provided by the Agriculture, Fisheries and Conservation Department, Hong Kong SAR, China.

Amplification primer and capture probe design. The nucleotide sequences of the haemagglutinin gene from 126 avian influenza A subtype H7 isolates of the Eurasian and North American lineages and the Australian sub-lineage obtained from GenBank were aligned using the BioEdit software program [31]. Conserved sequences within the HA1 segment were used for NASBA amplification primer selection. Selected degenerate primer sequences were compared with sequences submitted to the GenBank nucleotide database using a standard nucleotide-nucleotide comparison tool (BLASTN version 2.2.1, [32]) using default search parameters. In addition, the degenerate primer sequences were aligned with specific sequences using a pairwise comparison tool (BLAST 2 sequences, [33]) using default comparison parameters. The degenerate primer pair used for amplifying H7 subtypes was designated H7-P1 and H7-P2, respectively (Table 1). Primers were obtained from BioAsia, Shanghai, China. The degenerate capture probe sequences (H7-CP-1 and H7-CP-2) were determined after selection of the amplification primers by aligning the nucleotide sequences of the putative amplification products. The capture probes were 5′ end-labelled with biotin (Table 1). The capture probes were obtained from BioAsia.

Nucleic acid isolation. Briefly, 0.1 ml inoculated egg allantoic fluid was added to 0.9 ml 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 (10,000*g*, 30 s) and washed repeatedly (twice with 5.25 M guanidine isothiocyanate, 50 mM Tris, pH 6.4, and 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. Diethyl pyrocarbonate-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 (10,000*g*, 1 min) to separate the silica from the water containing the eluted nucleic acid.

Amplification by NASBA. To 5 μl nucleic acid extract, 10 μl of a mixture containing 80 mM Tris, pH 8.3, 24 mM $MgCl_2$, 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 U/μl T7 RNA polymerase, 1.3 U/μl avian myeloblastosis virus reverse transcriptase, 0.02 U/μl ribonuclease-H, and 0.42 μg/μl BSA) was added and the reaction was incubated at 41 °C for 150 min in a water bath. The potassium chloride concentration was adjusted to 90 mM. The final volume was 20 μl.

Detection of NASBA amplification products. The NASBA reaction amplicons were detected by hybridisation analysis using an electrochemiluminescent (ECL) detection system following the manufacturer's instructions. Briefly, the amplicons were immobilised by hybridisation to a single-stranded DNA 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 was measured by the detector (Nuclisens ECL Reader, bioMérieux, Lyon, France).

Influenza A enzyme immunosorbent assay. Influenza A subtype H7N7 (A/turkey/Northern Ireland/VF1545 C5/94) was serially diluted 10-fold in phosphate-buffered saline. An aliquot (125 μl) of each dilution was used in a commercially available enzyme immunosorbent assay (EIA, Directigen Flu A; Becton–Dickinson, NJ, USA) according to the protocol included in the kit.

Results

Several different experiments were conducted to examine the efficacy of the NASBA primers and capture probes in amplifying and capturing sequences derived from avian influenza subtype H7.

Use of electrochemiluminescence

The ECL signals obtained during the experiments were normalised relative to an instrument reference solution (IRS) supplied by the manufacturer of the ECL reader. The IRS solution produces an ECL signal of about 45,000–55,000 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

Table 1
Primer sequences used in this study

Name	Sequence (5′–3′)	GC content (%)	Product size (nucleotides)
H7-P1	**AAT TCT AAT ACG ACT CAC TAT AGG G**AG AAG GTI ACT GTG TCA TT(AG) G[a,c]	17–18/46 (37–39)	313 (H7-P1 + H7-P2)
H7-P2	GAT GCA AGG TCG CAT ATG AGA GA(TA) CAG GAT CTT CAT TCT ATG CAG AG(AC) TGA A[b,c]	22–23/52 (42–44)	
H7-CP-1	Biotin-G(AG)C CAC AAG TGA ATG G(ACT)C AAT	10–11/21 (48–52)	NA
H7-CP-2	Biotin-GAC CAC (AC)AG TAA ATG GTC AGT	9–10/21 (43–48)	NA

I, inosine; NA, not applicable.
[a] Bold type indicates the sequence of the bacteriophage T7 DNA-dependent RNA polymerase promoter.
[b] Underscore indicates the region complementary to the sequence of the ECL detection oligonucleotide.
[c] Degenerate nucleotides are given in parentheses.

are defined as $0.025 \times$ IRS. We have reported similar methods for calculating the cut-off value previously [13,14]. The use of appropriate internal controls for quantifying HIV viral load using the NASBA/ECL technique has been described [12] 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).

Sequence alignment and primer specificity

The NASBA primers and probes used in this study were designed by aligning the haemagglutinin nucleotide sequences of a relatively large number of Eurasian, North American, and Australian avian influenza subtype H7 isolates that were deposited in public sequence databases. Primers were designed to amplify segments of the HA1 region that were conserved in both highly pathogenic and low pathogenic isolates. Due to phylogenetic variation between different H7 lineages, it was not possible to design a single pair of primers for use with the NASBA/ECL system. Consequently, the forward primer, H7-P1, contained two degenerate nucleotides (inosine was used to represent A, C, G, or T at one location and A or G was used at the second location), resulting in a mixture of two different primers. The reverse primer, H7-P2, also contained two degenerate nucleotides (A/T and A/C), resulting in a mixture of four different primers. Excluding the 5′ extensions required for the NASBA process, the forward and reverse degenerate H7 NASBA primers were 21 and 32 nucleotides in length, respectively. The primer mixture had a high degree of sequence identity to H7 isolates obtained from various species, such as chicken, turkey, goose, quail, and guinea fowl, isolated at various times and locations, including North America, Europe, Australia, and Africa (data not shown). To test whether the selected primers would amplify regions from other haemagglutinin subtypes, a standard nucleotide–nucleotide search (BLASTN) was conducted. The forward primer displayed similarity only to other H7 avian influenza isolates, with the exception of a single H5 isolate (A/chicken/Jalisco/1994), which was identical to one of the degenerate H7-P1 primers by 20 out of 21 nucleotides (data not shown). Using a pairwise nucleotide comparison (BLAST 2 sequences), the forward primer was found to have no significant sequence similarity to haemagglutinin genes from other avian influenza viruses (subtypes H1–H15 inclusive) or avian paramyxoviruses, such as Newcastle disease virus submitted to GenBank. A similar analysis was conducted for the reverse primer (H7-P2). Again, the only other sequences with which the reverse primers exhibited similarity were H7 haemagglutinin genes, with the exception of the same H5 isolate as noted previously, which was identical to one of the degenerate H7-P2 primers by 30 out of 32 nucleotides (data not shown). Using a pairwise nucleotide comparison, the reverse primers were found to have no significant sequence similarity to haemagglutinin genes from other avian influenza viruses (subtypes H1–H15 inclusive) or avian paramyxoviruses (data not shown). Thus, it is predicted that the H7 NASBA primers are specific for H7 strains within the Eurasian and North American lineages and the Australian sub-lineage.

The use of a biotin-labelled capture probe (H7-CP) introduced an added level of specificity to the NASBA/ECL assay, as only appropriately amplified molecules are carried over to the detection stage. Non-specific or partially cross-reactive molecules generated during the NASBA reaction fail to hybridise with the capture probe and are washed away prior to detection. H7-CP was designed in a similar manner as the H7 NASBA primers. Due to phylogenetic differences between H7 lineages it was necessary to use a mixture of two degenerate capture probes. H7-CP-1 contained two degenerate nucleotides (A/G and A/C/T), resulting in a mixture of six different capture probes. Capture probe H7-CP-2 contained a single degenerate nucleotide (A/C), resulting in a mixture of two different capture probes. Between them, the H7-CP probes had a high degree of sequence similarity to a wide range of H7 isolates from North America, Europe, Australia, and Africa submitted to GenBank (data not shown). The potential for the capture probe to detect haemagglutinin genes other than those specified by the NASBA primers was examined by conducting a BLASTN search using the 21-nucleotide capture probe sequence as query. H7-CP-1 and -2 had no significant sequence similarity with any other haemagglutinin genes from either avian influenza viruses or avian paramyxoviruses, with the exception of A/chicken/Jalisco/1994 (H5), which shared 100% sequence similarity with one of the degenerate capture probe sequences. Thus, H7-CP-1 and -2 are predicted to be highly specific for H7 haemagglutinin sequences.

Assay for influenza A subtype H7

After obtaining consensus sequences for primer design based on alignments of multiple Eurasian and North American H7 isolates, the ability of the selected primers to function efficiently in the amplification of extracted nucleic acid was examined. RNA extracts from LPAI and HPAI H7 viruses derived from multiple geographic locations and host species were obtained. Table 2 shows the ECL signal detected following amplification of isolated nucleic acid with the H7 primers. These data indicate that the primers amplify H7-derived nucleic acid efficiently. The products of amplification

Table 2
Efficacy of avian influenza H7 detection reagents

Sample[a]	Subtype and pathogenicity	ECL signal	Sensitivity[b]	Result
A/chicken/Victoria/76	H7N7 HPAI	2486	2.11	Positive
A/chicken/Victoria/85	H7N7 HPAI	9064	7.70	Positive
A/chicken/Victoria/1/85	H7N7 HPAI	156,714	133	Positive
A/chicken/Victoria/224/92	H7N3 HPAI	109,937	93.4	Positive
A/chicken/Queensland/447/94	H7N3 HPAI	290,678	247	Positive
A/fairy bluebird/Singapore/F92/94	H7N1 LPAI	257,367	219	Positive
A/common iora/Singapore/F89/95	H7N1 LPAI	360,581	306	Positive
A/emu/New South Wales/1742/97	H7N4 HPAI	107,883	91.7	Positive
A/teal/Taiwan/WB2-37-2TPFE2/98	H7N1 LPAI	261,086	222	Positive
A/turkey/N. Ireland/VF-1545 C5/98	H7N7 LPAI	218,953	186	Positive
223/V00 (A/turkey/Italy)	H7N1 LPAI	388,258	330	Positive
160/V00 (A/chicken/Italy)	H7N1 HPAI	283,592	241	Positive
Negative control	NA	140	0.119	Negative
Instrument Reference Solution (IRS)	NA	47,091	NA	NA
Cut-off limit ($0.025 \times$ IRS)	NA	1177	NA	NA

NA, not applicable.
[a] All samples comprise RNA extracts obtained from infected allantoic fluid of embryonated eggs or tissue culture, except A/turkey/N. Ireland/VF-1545 C5/98, which was obtained as a whole virus preparation.
[b] Defined as ECL signal/cut-off limit.

were also examined by agarose gel electrophoresis in the presence of ethidium bromide where bands of the appropriate size (~300 nucleotides) were visualised (data not shown). The ECL signal generated from each isolate varied widely. This may be due to variation in virus titre in egg/cell culture and the amount of nucleic acid isolated subsequently, or individual variation 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 provided for analysis.

Effect of potassium chloride

The efficiency of amplification was examined at various potassium chloride (KCl) concentrations using RNA prepared from A/common iora/Singapore/F98/95 (H7N1). The results are shown in Table 3. As the amplified RNA is extracted from the amplification medium prior to detection, there is no carryover of KCl into the detection stage. Thus, the ECL signal generated reflects the effect of KCl on nucleic acid amplification and not on other components of the detection process. As KCl increased to 90 mM, the ECL signal became greater and the variation between duplicates decreased. During these studies, experiments were performed at 90 mM KCl.

Specificity studies

After the primers were demonstrated to work effectively in the amplification of diverse haemagglutinin genes from avian influenza subtype H7, their specificity against a limited panel of influenza isolates (H1, H3, H4, H5, H6, and H9) was examined. The ECL signals did not exceed the cut-off value for the assay (Table 4). The H7 primers were also tested against distantly related viruses that also contain haemagglutinin molecules, such

Table 3
Effect of KCl on amplification

KCl (mM)	ECL signal[a] (mean ± range, $n = 2$)	Sensitivity[b]	Amplification enhancement (w.r.t. 70 mM KCl)
70	209,187 ± 31,731	156	1.00
80	278,233 ± 116,898	207	1.33
90	334,805 ± 33,826	249	1.60
Negative control	105	0.078	NA
Instrument reference solution (IRS)	53,737	NA	NA
Cut-off limit ($0.025 \times$ IRS)	1343	NA	NA

NA, not applicable.
[a] Sample used was A/common iora/Singapore/F98/95 (H7N1).
[b] Defined as ECL signal/cut-off limit.

Table 4
Specificity of avian influenza subtype H7 detection reagents

Sample[a]	Subtype or strain	ECL signal	Result
A/swine/Belgium/1/98	H1N1	578	Negative
A/swine/Gent/80/01	H3N2	209	Negative
A/swine/Ontario/01911-1/99	H4N6	143	Negative
A/chicken/Hong Kong/1000/97	H5N1	285	Negative
A/quail/Hong Kong/2929-8/2000	H6N?	227	Negative
A/chicken/Hong Kong/3130-2/2000	H9N?	339	Negative
Parainfluenza-3 virus	SF4	187	Negative
Avian paramyxovirus type 2	P/chicken/CA/Yucaipa/56	269	Negative
Avian paramyxovirus type 3	P/turkey/Wisconsin/68	381	Negative
Newcastle disease virus	Hong Kong isolate	242	Negative
Chicken muscle	NA	507	Negative
Pig muscle	NA	69	Negative
Sheep muscle	NA	146	Negative
Cattle muscle	NA	0	Negative
Avian influenza immunodiffusion negative reference serum	NA	243	Negative
Negative control	NA	188	Negative
Positive control: A/common iora/Singapore/F89/95	H7N1	105,299	Positive
Instrument Reference Solution (IRS)	NA	47,258	NA
Cut-off limit ($0.025 \times$ IRS)	NA	1181	NA

NA, not applicable.
[a] All samples comprise RNA extracts obtained from infected allantoic fluid of embryonated eggs or tissue culture.

as Newcastle disease virus, parainfluenza virus type 3, and avian paramyxovirus type 2 and type 3. No amplification of these molecules was observed (Table 4). Non-specific amplification of genomic DNA extracted from animals susceptible to influenza (chicken and pig) and other mammalian species (cattle and sheep) did not occur (Table 4). The data indicate that among the limited sample of subtypes tested the degenerate amplification primers and capture probes were specific for H7.

Limit of detection

The limit of detection of the NASBA/ECL method was examined by determining the ability of the H7 primers to amplify a serially diluted RNA template derived from A/turkey/Northern Ireland/VF-1545 C5/98 (H7N7). The amount of nucleic acid in the initial RNA preparation could not be correlated with virus concentration. Thus, the results of the serial dilution assay can be used only to give an estimate of the minimum number of virus particles necessary to yield a positive signal. The RNA template was serially diluted in nuclease-free water prior to amplification. The ECL signal for each dilution of template is shown in Table 5. Dilution of the template by 10^5-fold yielded positive ECL signals. At the same time, the whole virus preparation was serially diluted in phosphate-buffered saline and aliquots were applied to a commercially available antigen capture system for influenza A. Dilution of the stock virus 10^4-fold produced a weak positive signal. The virus could not be detected upon further dilution (Table 5). Thus, the NASBA/ECL assay for H7 is extremely sensitive,

Table 5
Limit of detection

Sample dilution[a]	NASBA/ECL			EIA
	ECL signal	Sensitivity[b]	Result	Result
Undiluted	190,568	158	Positive	ND
10^{-1}	254,833	211	Positive	Positive
10^{-2}	157,897	131	Positive	Positive
10^{-3}	163,259	135	Positive	Positive
10^{-4}	177,978	147	Positive	Weak positive
10^{-5}	82,327	68.2	Positive	Negative
10^{-6}	282	0.233	Negative	Negative
10^{-7}	271	0.224	Negative	ND
IRS	48,334	NA	NA	NA
Cut-off limit ($0.025 \times$ IRS)	1208	NA	NA	NA

NA, not applicable; ND, not done.
[a] A/turkey/Northern Ireland/VF-1545 C5/98 (H7N7).
[b] Defined as ECL signal/cut-off limit.

able to detect viral RNA from very small samples, and is 10- to 100-fold more sensitive than current antigen capture detection systems.

Discussion

The NASBA/ECL technique described in this paper provides a rapid genome-based detection system for H7 influenza virus, which has advantages for confirmation of H7 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 [12].

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 avian myeloblastosis virus reverse transcriptase and bacteriophage T7 RNA polymerase has a low combined error rate [34], which, combined with the milder incubation conditions and reduced incubation time compared with PCR, increases the fidelity of the transcripts. This may be important in the phylogenetic analysis of viral isolates. In addition, the NASBA-based H7 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.

It may be possible to modify the H7 assay by using specific pathogenic primers to determine whether a H7 influenza isolate has characteristics of a highly pathogenic avian influenza (HPAI) virus. The standard intravenous pathogenicity index test is the definitive test to classify HPAI viruses but takes 10 days to complete [35]. 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 [35]. A NASBA-based pathogenic H7 test, having one primer located at the cleavage site of the HA0 gene, would give a rapid confirmation that a newly isolated H7 influenza virus is likely to be a HPAI. Such an assay has been developed to discriminate LPAI and HPAI H5 strains [13].

To enhance the specificity of the NASBA-based avian influenza detection system, two degenerate H7 sequence-specific primers and a mixture of two degenerate capture probes were included to confirm the presence of amplified RNA. The key to the success of the NASBA/ECL technique lies in the initial selection of primers for the amplification reaction. The combination of six degenerate forward and reverse primers used in this study allowed the detection of a wide range of H7 isolates separated geographically (e.g., Taiwan, Singapore, Australia, Northern Ireland, and Italy), temporally (1976–2001), and by species (e.g., chicken, turkey, emu, teal, common iora, and fairy bluebird). A BLASTN search of the GenBank database using the H7 forward primer sequences as query revealed that the only partially cross-reactive sequences were derived from other avian influenza H7 haemagglutinin molecules, with the exception of A/chicken/Jalisco/94 (H5). As the influenza haemagglutinin constantly mutates by the well-described processes of antigenic drift and antigenic shift it may be necessary to replace one or more of the primers from time to time to accommodate the evolution of the H7 virus. Genes for haemagglutinin-like molecules from Newcastle disease virus, other avian paramyxoviruses, or parainfluenza virus 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 haemagglutinin and haemagglutinin-like molecules from related viruses, these data indicate the specificity of the NASBA/ECL test system for H7.

The added specificity provided by the degenerate capture probes greatly increases the accuracy of the NASBA/ECL assay system. The capture probe displays significant cross-reactivity only for other H7 haemagglutinin sequences and for a single H5 isolate as noted previously.

The negative controls all gave very low ECL signals (<200 arbitrary ECL units) relative to the instrument reference solution (IRS) (~50,000 ECL units). All positive ECL signals were in excess of the cut-off limit ($0.025 \times$ IRS) for differentiating positive and negative samples by an average of 173-fold (Table 2). Two samples (A/chicken/Victoria/76 and A/chicken/Victoria/1/85) gave positive ECL signals approximately 2- and 8-fold higher than the cut-off limit (Table 2). This may reflect a poor amplification, inhibition of the NASBA reaction due to the presence of unknown contaminants in the template solution, or mutation of the template during multiple passages in culture. Another sample of A/chicken/Victoria/85 obtained from a separate source was amplified to a very high degree (Table 2). These data indicate that the H7 primers are capable of efficiently amplifying the nucleic acid isolated from several different avian influenza A subtype H7 samples.

The effect of KCl on the efficiency of nucleic acid amplification is well known [36]. Generally, primer pairs with long amplification products work more efficiently at low salt concentrations, whereas primer pairs with

short amplification products work more efficiently at high salt concentrations, where longer products become harder to denature [36]. Standard PCR buffers contain about 50 mM KCl, but this can vary depending upon the application. The optimum KCl concentration may help overcome non-optimal parameters such as product size and GC content. The products of the NASBA reaction described in this work are about 313 nucleotides in length and so are expected to be amplified more efficiently at relatively high KCl concentrations. This was demonstrated for the H7 primers, which produced their maximum amplification and least variability between replicates at 90 mM (Table 3). High concentrations of salt are not the only factor affecting amplification efficiency. Most data relating to the effects of salt concentration have been derived from standard PCR and RT-PCR applications. Little data are available for NASBA applications, which contain two additional enzymes. For this reason, it was decided to limit the KCl concentration used in these studies to 90 mM.

Limit of detection studies were performed using the H7 primers and capture probe mixture and nucleic acid prepared from A/turkey/Northern Ireland/VF-1545 C5/98 (Table 5). The RNA template was serially diluted and amplified. Even after 10^5-fold dilution, a detectable ECL signal greater than the cut-off limit was obtained for the subtype tested. Commercially available antigen capture immunoassay detection systems were 10–100 times less sensitive than the NASBA/ECL assay described here. The absolute ECL signal in terms of luminescence units and the sensitivity (ECL signal/cut-off limit) increased significantly when the sample was diluted 10-fold from the original undiluted RNA template preparation. The degree of signal enhancement due to this limited dilution was about 33%. This enhancement may be due to decreased inhibition of amplification caused by the presence of unidentified inhibitory substances in the RNA template preparation. This phenomenon has been observed during NASBA/ECL with other RNA templates, such as foot-and-mouth disease virus [14].

Table 4 demonstrates the specificity of the NASBA primers for avian influenza type A H7 subtypes. In the studies conducted H7 was accurately and specifically detected compared with other influenza A subtypes, including H1, H3, H4, H5, H6, and H9.

The novel molecular detection system described here is a rapid, sensitive, accurate, robust, and reproducible assay for the efficient and convenient qualitative identification of avian influenza virus type A subtype H7. It has the potential to make a useful contribution to the confirmation of H7 influenza virus infection in monitoring programmes for H7 influenza in the poultry industry. It is important that further studies be conducted to determine the sensitivity and specificity of the primers in detecting other historical H7 isolates and in establishing a method for quantification of viral load. In addition, the applicability of this system to detect viral RNA directly in tracheal and cloacal 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 H7 amplicons can be efficiently generated directly from swab samples without prior amplification in allantoic fluid.

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References

[1] I. Capua, F. Mutinelli, M.D. Pozza, I. Donatelli, S. Puzelli, F.M. Cancellotti, The 1999–2000 avian influenza (H7N1) epidemic in Italy: veterinary and human health implications, Acta Trop. 83 (2002) 7–11.

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

[3] Y.J. Guo, J.W. Li, I. Cheng, Discovery of humans infected by avian influenza A (H9N2) virus, Chin. J. Exp. Clin. Virol. 15 (1999) 105–108.

[4] M. Peiris, K.Y. Yuen, C.W. Leung, K.H. Chan, P.L. Ip, R.W. Lai, W.K. Orr, K.F. Shortridge, Human infection with influenza H9N2, Lancet 354 (1999) 916–917.

[5] C. Buxton Bridges, J.M. Katz, W.H. Seto, P.K. Chan, D. Tsang, W. Ho, K.H. Mak, W. Lim, J.S. Tam, M. Clarke, S.G. Williams, A.W. Mounts, J.S. Bresee, L.A. Conn, T. Rowe, J. Hu-Primmer, R.A. Abernathy, X. Lu, N.J. Cox, K. Fukuda, Risk of influenza A (H5N1) infection among health care workers exposed to patients with influenza A (H5N1), Hong Kong, J. Infect. Dis. 181 (2000) 344–348.

[6] J. Kurtz, R.J. Manvell, J. Banks, Avian influenza virus isolated from a woman with conjunctivitis, Lancet 348 (1996) 901–902.

[7] C.H. Campbell, R.G. Webster, S.S. Breese Jr., Fowl plague virus from man, J. Infect. Dis. 122 (1970) 513–516.

[8] E. Starick, A. Romer-Oberdorfer, O. Werner, Type- and subtype-specific RT-PCR assays for avian influenza A viruses (AIV), J. Vet. Med. B. Infect. Dis. Vet. Public Health 47 (2000) 295–301.

[9] M. Munch, L.P. Nielsen, K.J. Handberg, P.H. Jorgensen, Detection and subtyping (H5 and H7) of avian type A influenza

virus by reverse transcription-PCR and PCR-ELISA, Arch. Virol. 146 (2001) 87–97.
[10] E. Spackman, D.A. Senne, T.J. Myers, L.L. Bulaga, L.P. Garber, M.L. Perdue, K. Lohman, L.T. Daum, D.L. Suarez, Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes, J. Clin. Microbiol. 40 (2002) 3256–3260.
[11] J. Banks, E.C. Speidel, J.W. McCauley, D.J. Alexander, Phylogenetic analysis of H7 haemagglutinin subtype influenza A viruses, Arch. Virol. 145 (2000) 1047–1058.
[12] J.W. Romano, B. van Gemen, T. Kievits, NASBA: a novel, isothermal detection technology for qualitative and quantitative HIV—RNA measurements, Clin. Lab. Med. 16 (1996) 89–103.
[13] R.A. Collins, L.-S. Ko, K.-L. So, T. Ellis, L.-T. Lau, A.C.H. Yu, Detection of highly pathogenic and low pathogenic avian influenza subtype H5 (Eurasian lineage) using NASBA, J. Virol. Methods 102 (2002) 213–225.
[14] R.A. Collins, L.-S. Ko, K.Y. Fung, L.-T. Lau, J. Xing, A.C.H. Yu, A method to detect major serotypes of foot-and-mouth disease virus, Biochem. Biophys. Res. Commun. 297 (2002) 267–274.
[15] J.W. Romano, R.N. Shurtliff, E. Dobratz, A. Gibson, K. Hickman, P.D. Markham, R. Pal, Quantitative evaluation of simian immunodeficiency virus infection using NASBA technology, J. Virol. Methods 86 (2000) 61–70.
[16] H.L. Jordan, L.A. Scappino, M. Moscardini, M. Pistello, Detection of feline immunodeficiency virus RNA by two nucleic acid sequence based amplification (NASBA) formats, J. Virol. Methods 103 (2002) 1–13.
[17] A.J. Baeumner, N.A. Schlesinger, N.S. Slutzki, J. Romano, E.M. Lee, R.A. Montagna, Biosensor for dengue virus detection: sensitive, rapid, and serotype specific, Anal. Chem. 15 (2002) 1442–1448.
[18] R.S. Lanciotti, A.J. Kerst, Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses, J. Clin. Microbiol. 39 (2001) 4506–4513.
[19] M. Damen, P. Sillekens, T.H.M. Cuypers, I. Frantzen, R. Melsert, Characterization of the quantitative HCV NASBA assay, J. Virol. Methods 82 (1999) 45–54.
[20] S.A. Simpkins, A.B. Chan, J. Hays, B. Popping, N. Cook, An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*, Lett. Appl. Microbiol. 30 (2000) 75–79.
[21] J.B. Mahony, X. Song, S. Chong, M. Faught, T. Salonga, J. Kapala, 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 (2001) 1429–1435.
[22] A. Borst, M.A. Leverstein-Van Hall, J. Verhoef, A.C. Fluit, Detection of *Candida* spp. in blood cultures using nucleic acid sequence-based amplification (NASBA), Diagn. Microbiol. Infect. Dis. 39 (2001) 155–160.
[23] G.J. Schoone, L. Oskam, N.C. Kroon, H.D. Schallig, S.A. Omar, Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification, J. Clin. Microbiol. 38 (2000) 4072–4075.
[24] J.W. Romano, R.N. Shurtliff, M. Grace, E.M. Lee, C. Ginocchio, M. Kaplan, R. Pal, Macrophage-derived chemokine gene expression in human and macaque cells: mRNA quantification using NASBA technology, Cytokine 13 (2001) 325–333.
[25] P.B. van Deursen, A.W. Gunther, C.C. van Riel, M.M. van der Eijnden, H.L. Vos, B. van Gemen, D.A. van Strijp, N.M. Tacken, R.M. Bertina, A novel quantitative multiplex NASBA method: application to measuring tissue factor and CD14 mRNA levels in human monocytes, Nucleic Acids Res. 27 (1999) e15.
[26] D.J. Alexander, A review of avian influenza in different bird species, Vet. Microbiol. 74 (2000) 3–13.
[27] D.E. Swayne, D.L. Suarez, Highly pathogenic avian influenza, Rev. Sci. Technol. 19 (2000) 463–482.
[28] M.C. Zambon, Epidemiology and pathogenesis of influenza, J. Antimicrob. Chemother. 44 (1999) 3–9.
[29] R.G. Webster, R. Rott, Influenza virus A pathogenicity: the pivotal role of the hemagglutinin, Cell 50 (1987) 665–666.
[30] D.L. Suarez, M. Garcia, J. Latimer, D. Senne, M. Perdue, Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States, J. Virol. 73 (1999) 3567–3573.
[31] T. Hall, BioEdit biological sequence alignment editor for Windows 95/98/NT. v5.0.9., 1997. Available from <http://www.mbio.ncsu.edu/BioEdit/bioedit.html> [accessed June 22, 2001].
[32] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[33] T.A. Tatusova, T.L. Madden, Blast 2 sequences—a new tool for comparing protein and nucleotide sequences, FEMS Microbiol. Lett. 174 (1999) 247–250.
[34] R. Sooknanen, M. Howes, L. Read, L.T. Malek, Fidelity of nucleic acid amplification with avian myeloblastosis virus reverse transcriptase and T7 RNA polymerase, Biotechniques 17 (1994) 1077–1080, 1083–1085.
[35] Anon., Highly pathogenic avian influenza (Fowl plague), Manual of Standards for Diagnostic Tests and Vaccines, second ed., Office International des Épizooties, Paris, 1992.
[36] O. Heregariu, N.A. Heerema, S.R. Dlouhy, G.H. Vance, P.H. Vogt, Multiplex PCR: critical parameters and step-by-step protocol, BioTechniques 23 (1997) 504–511.