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Nucleic acid sequence-based amplification methods to detect avian influenza virus

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Abstract

Infection of poultry with highly pathogenic avian influenza virus (AIV) can be devastating in terms of flock morbidity and mortality, economic loss, and social disruption. The causative agent is confined to certain isolates of influenza A virus subtypes H5 and H7. Due to the potential of direct transfer of avian influenza to humans, continued research into rapid diagnostic tests for influenza is therefore necessary. A nucleic acid sequence-based amplification (NASBA) method was developed to detect a portion of the haemagglutinin gene of avian influenza A virus subtypes H5 and H7 irrespective of lineage. A further NASBA assay, based on the matrix gene, was able to detect examples of all known subtypes (H1–H15) of avian influenza virus. The entire nucleic acid isolation, amplification, and detection procedure was completed within 6 h. The dynamic range of the three AIV assays was five to seven orders of magnitude. The assays were sensitive and highly specific, with no cross-reactivity to phylogenetically or clinically relevant viruses. The results of the three AIV NASBA assays correlated with those obtained by viral culture in embryonated fowl's eggs.

Keywords: Avian influenza; Haemagglutinin; Nucleic acid sequence-based amplification; NASBA

Influenza A viruses have a segmented genome of single-stranded negative-sense RNA and belong to the family *Orthomyxoviridae* [1]. They have been isolated from a variety of animals, including humans, pigs, horses, sea mammals, and birds [2]. In humans influenza viruses cause a highly contagious acute respiratory disease that has probably been responsible for epidemic and pandemic disease in humans for centuries [3]. In avian species most influenza virus infections cause mild localized infections of the respiratory and intestinal tract, and the disease is termed low pathogenicity avian influenza (LPAI). Some extremely virulent viruses cause highly pathogenic avian influenza (HPAI) in poultry, a systemic infection in which mortality may be as high as 100%.

All AIVs are type A and can be further subtyped according to the antigenicity of two envelope glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Fifteen subtypes of HA (H1–15) and nine subtypes of NA (N1–9) have been identified to date. Only viruses of H5 and H7 subtypes cause HPAI, although not all viruses of these two subtypes actually cause HPAI [4]. There have been 13 reported outbreaks of HPAI due to subtype H7 and 12 outbreaks due to subtype H5 (counting the four incidents in Hong Kong since 1997 as separate outbreaks) since 1959. In addition, many outbreaks of LPAI infection have occurred from these two subtypes. In the northeast United States, an ongoing outbreak of H7 LPAI has been observed since 1994, primarily in live-bird markets [5]. There is

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evidence that HPAI viruses arise from LPAI strains after they cross from the aquatic bird reservoir into domestic poultry [6,7]. In northern Italy, outbreaks of avian influenza due to an H7N1 virus of low pathogenicity occurred in domestic poultry from March to December 1999 when a HPAI virus emerged. In the following months, 13 million birds were affected [8,9]. In early 2003, an outbreak of highly pathogenic H7N7 in Netherlands (spreading to Belgium and Germany) resulted in the death or culling of about 30 million birds.

In recent years there have been examples of AIVs directly crossing the species barrier to infect humans. In 1996, an avian influenza virus [A/England/268/96 (H7N7)] was isolated from a woman with conjunctivitis [10], and a virus of the same subtype was isolated from a man with infectious hepatitis [11]. The highly pathogenic H7N7 outbreak in the Netherlands of 2003 resulted in one human fatality and approximately 100 other confirmed human AIV infections. The most significant transmission took place in Hong Kong where an outbreak of HPAI subtype H5N1 occurred in chickens resulting in high mortality for infected birds in 1997 [12,13]. The same virus was then isolated from 18 individuals in Hong Kong, six of whom died. This was the first reported instance of an avian influenza virus H5N1 directly crossing the species barrier and infecting humans. Epidemiological studies indicated that 3.7% of healthcare workers who came into contact with the infected patients had antibodies against H5N1 compared with 0.7% of healthcare workers who had no documented contact [14]. In addition, 3% of government workers instructed to carry out the poultry culling and cleanup operation were subsequently found to have antibodies to H5N1 [15], and a further 10% of poultry workers were also found to be seropositive with respect to H5N1 [15]. HPAI infections of the H5N1 subtype subsequently recurred in Hong Kong in May 2001, February and April 2002, and December 2002/January 2003. In February 2003, an H5N1 virus genetically distinct from that isolated from humans in 1997 was isolated from two people who had visited Fujian province in Mainland China. Of these confirmed infections one person died. Thus, H5N1 viruses in Hong Kong and China represent a continued economic and public health risk with the potential for a pandemic strain to arise in this area [16].

The potential for other subtypes of AIV to infect humans must always be considered and this has been demonstrated by the direct transfer of AIV H9N2 to humans on at least two separate occasions [17,18], emphasizing the need for continued research into rapid diagnostic tests for influenza. Several techniques are used to isolate, detect, and identify influenza viruses. Viral culture in embryonated fowl's eggs is the currently accepted and the most widely used standard for the laboratory diagnosis of influenza [3]. This method is very sensitive but also labor-intensive and time-consuming (2–10 days). By comparison, viral antigen detection techniques (enzyme-linked immunoassay and immunofluorescence) are relatively rapid and straightforward [19,20]. However, they are significantly less sensitive and specific than isolation in embryonated eggs. Molecular techniques, such as PCR [21,22], are highly sensitive and allow rapid diagnosis of influenza infection; however, these techniques may require additional time for result confirmation by viral culture or ELISA. Real-time PCR assays for influenza A and B [23] and subtypes H1, H3, H5, and H7 [24,25] have been reported.

Nucleic acid sequence-based amplification (NASBA) methods based on the amplification of the nucleoprotein gene for influenza A viruses and the HA gene of the H5 Eurasian lineage have been described [26–28]. A preliminary study of a NASBA assay to detect avian influenza subtype H7 has also been made [29]. NASBA is a transcription-based amplification system specifically designed for the detection of RNA targets [30] and allows the continuous amplification of nucleic acids in a single mixture at a single temperature [31].

In this paper, we describe a rapid detection method for all subtypes of influenza A viruses (NASBA-AIV), in addition to H5-specific (NASBA-H5) and H7-specific (NASBA-H7) detection methods using NASBA technology. The NASBA-AIV assay is able to detect influenza A virus H1–H15 subtypes, while NASBA-H7 can detect the Eurasian and North American lineages (also the Australian sub-lineage) and NASBA-H5 detects most lineages. The sensitivity of the different NASBA assays with viral culture in embryonated fowl's eggs is also compared.

Materials and methods

Viruses. In this study, all viruses were derived from the virus repository of the EU/OIE/FAO international reference laboratory for AI at the Veterinary Laboratories Agency, New Haw, Addlestone, Weybridge, Woking, Surrey, UK (Tables 1–3). Representative strains were derived principally from avian hosts to include H subtypes 1–15 but also included some viruses from mammalian hosts. Several other clinically relevant viral pathogens were used for specificity testing (listed in Tables 4–6).

Embryo titration. Serial 10-fold dilutions (10^{-2}–10^{-9}) of virus were prepared in virus dilution media. For each dilution, 0.1 ml virus-containing medium was inoculated into the allantoic cavity of embryonated fowls' eggs ($n = 4$–6). After 72 h of incubation at 37 °C, the eggs were chilled and harvested individually and the undiluted allantoic fluids were tested for HA activity [32]. The ELD_{50} per ml was calculated using the method of Karber [33]. The egg infectious dose is a measure of viral infectivity where the EID_{50} is the smallest amount of virus capable of initiating infection in 50% of the embryonated fowls' eggs [34].

Design of primers and probes. The influenza A subtype H1–H15 amplification primers and capture probe were derived after comparison of the conserved sequences of the matrix protein (M) gene obtained from viruses of avian, human, swine, and equine origin. The

Table 1
Sequence of primers and probes used in this study

Virus specificity	Name	Sequence (5′–3′)	Sequence coordinates
H5	H5-ECL	**GAT GCA AGG TCG CAT ATG AG** GT GA(C/T) AAT GAA TG(C/T) ATG GAA	1457–1476[a]
	H5-T7	AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG G CCA IAA AGA (C/T)AG ACC AGC TA	1653–1634[a]
	H5-CP	Biotin-GC(A/G) AGT TC(C/T) CTA GCA CTG GCA AT	1606–1628[a]
H7	H7-ECL	**GAT GCA AGG TCG CAT ATG AG** AGA (A/T)CA GGA TCT TCA TTC TAT GCA GAG (A/C)TG AA	445–476[b]
	H7-T7	AAT TCT AAT ACG ACT CAC TAT AGG G AGA AGG TIA CTG TGT CAT T(A/G)G	752–743[b]
	H7-CP-1	Biotin-G(A/G)C CAC AAG TGA ATG G(A/C/T)C AAT	
	H7-CP-2	Biotin-GAC CAC (A/C)AG TAA ATG GTC AGT	686–706[b]
A	AIV-ECL	**GAT GCA AGG TCG CAT ATG AG** CTT CTA ACC GAG GTC GAA ACG TA	25–47[c]
	AIV-T7	AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG G A(A/G)G GCA TT(C/T) TGG ACA AA(G/T) CGT CTA	269–246[c]
	AIV-CP	Biotin-CCG TCA GGC CCC CTC AAA GCC GA	64–86[c]

I, inosine.
Underscore indicates the T7 DNA-dependent RNA polymerase binding sequence.
Bold type indicates the sequence complementary to the ECL detection probe.
[a] A/Mallard duck/Pennsylvania/10218/84 (H5N2) haemagglutinin gene (AF100180).
[b] A/turkey/Italy/5074/99 (H7N1) haemagglutinin gene (AF364171).
[c] A/Duck/Hong Kong/552/79 (H9N2) matrix gene (AF523495).

Table 2
Sensitivity of NASBA-AIV and NASBA-H7 assays in detecting avian influenza H7N1 virus

Dilution	A/chicken/Italy/1081/99 (H7N1)[a]			
	NASBA-AIV		NASBA-H7	
	ECL signal	Result	ECL signal	Result
10^{-1}	1,328,502	Positive	335,510	Positive
10^{-2}	1,206,822	Positive	295,700	Positive
10^{-3}	1,486,492	Positive	288,692	Positive
10^{-4}	1,409,444	Positive	289,765	Positive
10^{-5}	1,216,368	Positive	58,448	Positive
10^{-6}	1,181,705	Positive	89,067	Positive
10^{-7}	538	Negative	122	Negative
10^{-8}	342	Negative	52	Negative
10^{-9}	352	Negative	−115	Negative
10^{-10}	288	Negative	69	Negative
IRS	54,715	NA	49,415	NA
Cut-off value ($0.025 \times$ IRS)	1367	NA	1235	NA

IRS, instrument reference solution.
NA, not applicable.
[a] $10^{8.92}$ EID_{50}/ml.

Table 3
Sensitivity of NASBA-AIV and NASBA-H5 assays in detecting avian influenza virus H5N3

Dilution	A/pekin duck/Singapore-Q/F119/3/97 (H5N3)[a]			
	NASBA-AIV		NASBA-H5	
	ECL signal	Result	ECL signal	Result
10^{-1}	715,693	Positive	1,323,942	Positive
10^{-2}	726,276	Positive	1,283,673	Positive
10^{-3}	666,961	Positive	1,339,277	Positive
10^{-4}	782,306	Positive	1,085,974	Positive
10^{-5}	800,196	Positive	1,437,995	Positive
10^{-6}	226	Negative	1,965,036	Positive
10^{-7}	202	Negative	10,000,001[b]	Positive
10^{-8}	277	Negative	65	Negative
10^{-9}	180	Negative	162	Negative
10^{-10}	232	Negative	269	Negative
IRS	45,712	NA	44,787	NA
Cut-off value ($0.025 \times$ IRS)	1142	NA	1120	NA

IRS, instrument reference solution
NA, not applicable.
[a] $10^{8.92}$ EID_{50}/ml.
[b] Upper limit of detection.

conserved sequences were selected by sequence alignment of more than 300 M gene sequences retrieved from the GenBank database (National Center of Biotechnology Information, NCBI). Sequence alignments were performed using CLUSTALX software [35]. To further subtype avian influenza viruses using NASBA, we designed H5- and H7-specific amplification primers and capture probes, each based on the conserved sequences of the haemagglutinin genes of these subtypes. Over 100 haemagglutinin gene sequences were retrieved from GenBank for each subtype of avian, human, swine, and equine origin. The H5-ECL and H7-ECL amplification primers each contain two degenerate nucleotides resulting in a mixture of four possible oligonucleotide sequences (Table 1). The H5-T7 and H7-T7 amplification primers each contain two degenerate nucleotides, including inosine, resulting in a mixture of two possible oligonucleotide sequences. The AIV-T7 amplification primer contains three degenerate nucleotides resulting in a mixture of eight possible sequences. The H5-CP capture probe contains two degenerate nucleotides resulting in a mixture of four possible sequences. Two different H7-CP capture probes were designed. H7-CP-1 contains two degenerate nucleotides, producing six possible sequences, while H7-CP-2 contains a single degenerate nucleotide resulting in two possible H7-CP-2 sequences, respectively. The amplification primer and capture probe sequences for each subtype assay are shown in Table 1.

RNA extraction and amplification. RNA was extracted using the RNeasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Briefly, 0.4 ml of allantoic fluid was mixed with 0.4 ml RLT buffer. To this, 0.4 ml of 70% ethanol was added and the entire sample was applied to an RNeasy spin column. The spin column was washed once with 0.7 ml RW1 buffer and twice with 0.5 ml RPE buffer. RNA was eluted in 50 μl nuclease-free water and subjected to amplification reaction using the NASBA method. Briefly, extracted

Table 4
Specificity of NASBA-AIV assay in detecting subtypes of avian influenza virus and other clinically relevant pathogens

Sample	HA subtype	NASBA	
		ECL signal	Result
A/Bayern/7/95	H1	637,166	Positive
A/swine/England/195852/92	H1	744,731	Positive
A/Singapore/1/57	H2	1,056,667	Positive
A/duck/Taiwan/17.2-35-2/98	H2	828,145	Positive
A/Sydney/5/97	H3	834,293	Positive
A/budgerigar/China/2873/V00	H3	843,635	Positive
A/duck broiler/Malaysia/F1110702	H3	979,793	Positive
A/duck/Taiwan/22/98	H4	1,427,384	Positive
A/mallard/England/B871/9/96	H4	1,430,347	Positive
A/chicken/South Africa/1050/94	H5	993,027	Positive
A/goose/Italy/1/92	H5	1,308,032	Positive
A/pekin duck/Singapore-Q/F119/3/97	H5	1,310,023	Positive
A/chicken/RSA/01	H6	950,113	Positive
A/turkey/Canada/Ontario/98	H6	1,177,148	Positive
A/shearwater/E.Australia/1/72	H6	997,015	Positive
A/turkey/Germany/R81/99	H6	901,868	Positive
A/ostrich/SA/1609/91	H7	1,309,856	Positive
A/psittacine/Italy/2/91	H7	755,780	Positive
A/equine/Prague/1/56	H7	1,204,701	Positive
A/turkey/Ontario/6118/68	H8	992,758	Positive
A/quail/UAE/369-1642/02	H9	1,343,281	Positive
A/chicken/Pakistan/5/99	H9	1,155,994	Positive
A/shell duck/RSA/145/2/98	H10	886,497	Positive
A/mandarin duck/Singapore/F64/7/93	H10	10,000,001[a]	Positive
A/duck/MEM546/74	H11	88,616	Positive
A/white fronted goose faeces/England/01	H11	10,000,001[a]	Positive
A/duck/Alberta/60/76	H12	1,027,528	Positive
A/gull/Medina/204/77	H13	689,134	Positive
A/mallard/Gurjev/263/82	H14	822,846	Positive
A/shearwater/Australia/79	H15	729,580	Positive
Herpes (11T) 1994	NA	77	Negative
Canary Pox 1997	NA	68	Negative
Parvovirus (GPV)	NA	-22	Negative
Picornavirus (IAE) 1995	NA	4	Negative
Pneumonia (APV F83) Subtype A	NA	74	Negative
Reovirus (S1133)	NA	-239	Negative
Rotavirus (353/87)	NA	21	Negative
Coronavirus (793/B) 1991	NA	50	Negative
Adenovirus (CELO)	NA	86	Negative
Influenza B Hawaii 10/01	NA	146	Negative
Influenza C 67	NA	121	Negative
Negative control (water)	NA	215	Negative
Instrument Reference Solution	NA	49,436	NA
Cut-off limit (0.025 × IRS)	NA	1235	NA

NA, not applicable.
[a] Upper limit of detection.

RNA (5 μl) was added to 10 μl amplification mixture according to the protocol of the Basic Kit Amplification Module (bioMérieux bv, Boxtel, Netherlands), followed by incubation at 65 °C for 5 min, and cooling to 41 °C for 5 min. Once cool, 5 μl enzyme mix (containing RNase-H, T7 RNA polymerase, AMV-RT, BSA) was added and the reaction mixture was incubated for 90–120 min at 41 °C for isothermal amplification of RNA.

Signal detection. Detection reagents were prepared by vortex mixing a suspension comprising biotinylated influenza A virus oligonucleotide capture probes bound to streptavidin-coated paramagnetic beads until an opaque solution was formed, as described in the manufacturer's protocol (bioMérieux). The bead-oligo suspension (10 μl) and 10 μl ruthenium-labeled electrochemiluminescent (ECL) probe were mixed and this mixture (20 μl) was added to 5 μl of the NASBA product and incubated for 30 min at 41 °C. Finally, ECL signal detection was performed in the NucliSens ECL Reader (bioMérieux) according to the manufacturer's protocol.

Table 5
Specificity of NASBA-H7 assay in detecting distinct lineages of influenza virus and clinically relevant pathogens

Sample	HA subtype	NASBA	
		ECL signal	Result
A/ostrich/SA/1609/91	H7N1	488,880	Positive
A/psittacine/Italy/2/91	H7N2	606,191	Positive
A/turkey/N. Ireland/VF-1545 C5/98	H7N7	270,511	Positive
A/turkey/England/262/79	H7N3	99,776	Positive
A/ostrich/RSA/W-4/96	H7N7	370,427	Positive
A/blackbird/Singapore/F92/9/94	H7N1	256,342	Positive
A/bird/Pakistan/447/95	H7N3	372,163	Positive
A/equine/Prague/1/56[a]	H7N7	188	Negative
A/chicken/Bendigo/Victoria/85	H7N7	86,424	Positive
A/chicken/Pakistan/5/99	H9	140	Negative
A/chicken/Pakistan/3/99	H9	129	Negative
A/chicken/Korea/25232-006/96	H9	161	Negative
A/Bayern/7/95	H1	121	Negative
A/Singapore/1/57	H2	118	Negative
A/chicken/RSA/01	H6	193	Negative
A/duck/Singapore-Q/F119-3/97 (H5N3)	H5	121	Negative
Herpes (11T) 1994	NA	−81	Negative
Canary Pox 1997	NA	114	Negative
Parvovirus (GPV)	NA	66	Negative
Picornavirus (IAE) 1995	NA	81	Negative
Pneumonia (APV F83) subtype A	NA	124	Negative
Reovirus (S1133)	NA	89	Negative
Rotavirus (353/87)	NA	155	Negative
Coronavirus (793/B) 1991	NA	100	Negative
Adenovirus (CELO)	NA	110	Negative
Instrument Reference Solution	NA	51,764	NA
Cut-off limit (0.025 × IRS)	NA	1294	NA

NA, not applicable.
[a] This reference strain was not detected by the NASBA-H7 assay.

Results

Sensitivity of NASBA compared with culture in embryonated fowl's eggs

The detection limit of the NASBA/ECL and embryonated egg culture methods was examined using 10-fold serial dilutions of H7N1 (for NASBA-AIV and NASBA-H7) and H5N3 virus preparations (for NASBA-AIV and NASBA-H5). The results are summarized in Tables 2 and 3, respectively. Both the NASBA-AIV and NASBA-H7 methods were able to detect the H7N7 subtype at a level of 10^6-fold dilution (i.e., $10^{2.92}$ EID_{50}/ml). The NASBA-AIV method could detect the H5N3 subtype at a level of 10^5-fold dilution (i.e., $10^{3.92}$ EID_{50}/ml),

Table 6
Specificity of NASBA-H5 assay in detecting distinct lineages of influenza virus and clinically relevant pathogens

Sample	HA subtype	NASBA	
		ECL signal	Result
A/ostrich/Zimbabwe/4066/95	H5N2	1,418,518	Positive
A/chicken/South Africa/1050/94	H5N9	1,959,125	Positive
A/tern/South Africa/61	H5N3	1,389,904	Positive
A/pekin duck/Malaysia/F59/4/98	H5N2	1,322,112	Positive
A/chicken/Italy/367/97	H5N2	1,313,721	Positive
A/chicken/South Africa/1050/94	H5N9	1,931,494	Positive
A/chicken/Italy/RA9097/98	H5N2	10,000,001[a]	Positive
A/turkey/England/50-92/91	H5N1	949,321	Positive
A/ostrich/Denmark/72420/96 pool 2	H5N2	1,044,893	Positive
A/goose/Italy/1/92	H5N2	1,733,398	Positive
A/duck/Singapore-Q/F119-3/97	H5N3	710,641	Positive
A/Bayern/7/95	H1	−166	Negative
A/duck/Taiwan/17.2-35-2/98	H2	105	Negative
A/duck broiler/Malaysia/F1110702	H3	139	Negative
A/shearwater/E.Australia/1/72	H6	152	Negative
A/bird/Pakistan/447/95	H7	129	Negative
A/chicken/Pakistan/5/99	H9	563	Negative
Herpes (11T) 1994	NA	149	Negative
Canary Pox 1997	NA	161	Negative
Parvovirus (GPV)	NA	109	Negative
Picornavirus (IAE) 1995	NA	121	Negative
Pneumonia (APV F83) Subtype A	NA	88	Negative
Reovirus (S1133)	NA	146	Negative
Rotavirus (353/87)	NA	122	Negative
Coronavirus (793/B) 1991	NA	101	Negative
Adenovirus (CELO)	NA	135	Negative
Instrument Reference Solution	NA	43,316	NA
Cut-off limit (IRS × 0.025)	NA	1082	NA

NA, not applicable.
[a] Upper limit of detection

whilst the NASBA-H5 method detected the H5N3 subtype after 10^7-fold dilution ($10^{1.92}$ EID_{50}/ml). For both H7N7 and H5N3, the viral culture method was able to routinely detect virus after 10^7-fold dilution (data not shown).

Specificity of NASBA

The specificity of the NASBA-AIV, NASBA-H7, and NASBA-H5 assays was examined with influenza A virus subtypes H1–15 obtained from widely varying geographic locations and times. Other phylogenetically related viruses containing haemagglutinin-like molecules and clinically relevant pathogens were also examined (Table 4). The NASBA-AIV assay was able to detect representative strains of all 15 subtypes of influenza A virus, and no false positive signals were observed with other viral pathogens. The NASBA-H7 assay detected all the H7 reference strains with the exception of A/equine/Prague/1/56 (Table 5). No false positive signals were observed with other viral pathogens or influenza A viruses of subtypes other than H7. Similarly, the NASBA-H5 assay detected all H5 reference strains (Table 6). Again, no false positive signals were observed with other viral pathogens or influenza A viruses of subtypes other than H5.

Discussion

The AIV assays described here are the first NASBA-based systems capable of rapidly detecting avian influenza H1–H15, H5, and H7 subtypes, irrespective of lineage. It is not intended to compare these NASBA-based methods with other technologies, for example real-time PCR, which have been described elsewhere [24–26]. The economic consequences of avian influenza, as evidenced in the 2003 outbreak of HPAI H7N7 in the Netherlands, demand a rapid, sensitive, and accurate diagnostic test be readily available. The NASBA-H5 assay described here was previously evaluated using tissue samples (viscera and blood) from chickens experimentally infected with highly pathogenic A/chicken/Hong Kong/1000/97 (H5N1) [28]. The samples gave positive results with the NASBA H5-specific primers [28]. In other studies conducted in our laboratories, cloacal and tracheal samples collected from a dead chicken infected with HPAI subtype H5N1 during an outbreak in Hong Kong in 2002 were examined using the NASBA-H5 assay. Freshly extracted RNA from the cloacal and tracheal samples generated positive results (data not shown). In addition, the NASBA-H5 assay has also utilized avian influenza strains propagated in embryonated fowl's eggs. Therefore, the NASBA-H5 assay is an efficient method for the direct amplification of viral nucleic acid from both tissue and cultured samples.

NASBA is a continuous isothermal reaction in which a thermal cycler is not required. The NASBA assays described here are rapid molecular techniques for the detection of influenza A virus (NASBA-AIV), subtype H7 (NASBA-H7), and subtype H5 (NASBA-H5) in veterinary samples. Using these assays, an accurate identification of influenza A and subtype H7 or H5 can be made within 6 h. The amplification primers and capture probes are specific to AIV, H7 or H5, and do not cross-react with other haemagglutinin subtypes, phylogenetically related viruses containing haemagglutinin-like molecules, or other clinically relevant pathogens (Tables 4–6).

Without the need for thermal denaturation for DNA strand separation, as required by conventional PCR, the NASBA assay has the advantage of being able to amplify specific single-stranded RNA target sequences in the presence of genomic DNA contaminants [30]. NASBA/ECL is especially suitable for the detection of RNA viruses, such as influenza, foot-and-mouth disease virus, dengue fever virus, human immunodeficiency virus, and cytomegalovirus, among many others [27,29, 36–39]. As the end-product of the NASBA reaction is

RNA, which tends to be unstable under normal environmental conditions, the possibility of carryover contamination of equipment from previous experiments is minimized. The NASBA assay, like other nucleic acid-based technologies, has the ability to detect whole infectious particles in addition to partially degraded, non-infectious or mis-packaged virions [25].

The dynamic range of the NASBA/ECL assays was at least five orders of magnitude for NASBA-AIV and at least seven orders of magnitude for NASBA-H5 and NASBA-H7. The NASBA/ECL assays were 10- to 1000-fold more sensitive than commercially available antigen-capture immunoassay and more sensitive than conventional polymerase chain reaction (PCR) methods (data not shown). PCR can amplify DNA greater than 1×10^7-fold while NASBA can amplify RNA 1×10^9-fold [31]. Using H7N1 or H5N3 RNA extracts as targets for amplification, the NASBA-AIV, H7, and H5 were comparable in sensitivity with egg culture, which is the currently accepted "gold standard" for virus detection. The NASBA/ECL detection system is highly automated, resulting in fewer handling errors and contributing to an increase in sensitivity and specificity. The target-specific capture probes and generic ECL detection probes hybridize with the complementary amplified target molecules, thus increasing assay specificity during the detection stage. NASBA/ECL has a relatively high throughput, as many as 50 samples can be processed in the ECL reader at the same time. Therefore, it may be a suitable assay to use for routine screening methods, such as in cross-border or poultry market surveillance operations involving analysis of multiple samples simultaneously. The requirements for sample format are extremely robust and swabs taken from cages, work surfaces, clothing, utensils, and litter are suitable for analysis as are tissue samples taken from suspect animals or products from in vitro amplification. This extends the use of the NASBA/ECL method to monitor decontaminated premises or equipment prior to restocking with livestock

The NASBA-AIV, NASBA-H5, and NASBA-H7 assays could readily give expected results for certain avian influenza virus subtypes, except in one case that the NASBA-H7 assay failed to detect one particular H7 subtype sample, A/equine/Prague/1/56 (H7N7) (Table 5). The same sample was analysed using the NASBA-AIV assay and yielded a positive result (Table 4). Thus, the failure of the NASBA-H7 assay with respect to this sample was not due to sample degradation. Further analysis of the A/equine/Prague/1/56 H gene sequence (GenBank, X62552) indicated that the H7 amplification primers and capture probe contain several mismatches, probably resulting in a low-efficiency amplification and signal detection for this particular subtype.

The Instrument Reference Solution (IRS), used to monitor the stability of the ECL reader, typically produced ~30,000–50,000 arbitrary ECL reader units. The negative controls generated very low ECL signals (less than a few hundred ECL units). The cut-off limit was defined as IRS $\times$ 0.025 and was further calibrated before each experiment using at least five known negative samples. All the positive reference strains used in this study generated ECL signals greatly exceeding the cut-off limit.

In summary, NASBA/ECL assays for influenza A virus, subtype H7, and subtype H5 have been developed. These assays are comparable in sensitivity to embryonated fowl's eggs for culture. The rapid assay can generate results within a few hours and may be a suitable alternative to isolation in embryonated eggs (which can take weeks) for the routine screening of poultry or other birds for the presence of influenza A virus.

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