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A NASBA Method to Detect High- and Low-Pathogenicity H5 Avian Influenza Viruses

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SUMMARY. Nucleic acid sequence-based amplification (NASBA) 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 was 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 described NASBA technique is a specific, rapid, and sensitive method of detection of influenza A subtype H5 viruses. More importantly, it can be used to distinguish high- and low-pathogenicity strains of the H5 subtype.

RESUMEN. Un método de amplificación basada en la secuencia del ácido nucleico para detectar virus de influenza aviar de alta y baja patogenicidad del subtipo H5.

La amplificación basada en la secuencia del ácido nucleico (de las siglas en inglés NASBA) permite la amplificación rápida de regiones específicas de ácido nucleico obtenidos de diversas fuentes. Es especialmente adecuada para amplificar secuencias de ARN. Se desarrolló una técnica que permite la detección del virus de influenza aviar A subtipo H5 de fluido alantoideo cosechado de embriones de pollo inoculados con el virus. El ARN viral es detectado por electroluminiscencia. La técnica NASBA descrita es un método específico, rápido y sensible de detección de virus de influenza A subtipo H5. Lo más importante es que puede ser usada para distinguir cepas de patogenicidad alta o baja del subtipo H5.

Key words: NASBA, avian influenza, H5, hemagglutinin, pathogenic, electrochemiluminescence

Abbreviations: bp = base pair; DEPC = diethyl pyrocarbonate; ECL = electrochemiluminescence; ELISA = enzyme-linked immunosorbent assay; GuSCN = guanidine isothiocyanate; IC = internal control; NASBA = nucleic acid sequence-based amplification; PCR = polymerase chain reaction; RT/PCR = reverse transcription/polymerase chain reaction

In Hong Kong in 1997 a H5N1 avian influenza virus presumably spread from chickens to at least 18 humans and contributed to the deaths of six of them (7). Since this subtype was new to the human population, a major concern was the chance the virus might continue to spread in the human population causing a new influenza pandemic. Consequently, a program was implemented to depopulate all the poultry in Hong Kong, which resulted in the slaughter of over 1.4 million chickens and other poultry (5). When the poultry markets were reopened in 1998, an integrated H5 avian influenza monitoring and control program was introduced that included both serology and virus isolation. As a result of the large number of swab samples from waterfowl that were being cultured with only rare isolations of H5N1 viruses, there was considerable interest in developing tests for the rapid detection of H5

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influenza viruses. Additionally, the differentiation of these viruses into either the low- or high-pathogenicity phenotypes was desired. A rapid test with the required accuracy and ease of use would enhance the H5 avian influenza monitoring and control program.

Nucleic acid sequence-based amplification (NASBA) is an enzyme-based method for the amplification of nucleic acid (4). The technique is particularly suited for the amplification of single-stranded RNA and has been successfully used in the detection of numerous RNA and DNA viruses, bacteria, fungi, parasites, and cytokines.

The influenza hemagglutinin RNA is translated into a single precursor polypeptide, termed HA0, approximately 556 residues in length (8). To be infectious, HA0 must be cleaved into two peptides, HA1 and HA2, linked together by a disulphide bridge (6). The cleavage is performed by host proteases. The consensus sequence surrounding the cleavage site (*) in low-pathogenicity strains of H5 avian influenza was found to be PQRETR*GLF (1). In pathogenic strains, there are an increased number of basic residues at the cleavage site thought to arise from insertion or substitution. For example, the 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 (1). The increase in basic residues allows proteases present in tissues outside the gastrointestinal 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 avian influenza viruses (7).

Materials and Methods

Viruses. The viruses used in this study were isolated by intraallantoic cavity inoculation of 9–11-day-old chicken embryos (2) at the Castle Peak Veterinary Lab (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 ethylenediaminetetraacetic acid [EDTA], 1.3% Triton X-100) before delivery to the Department of Biology, Hong Kong University of Science and Technology, for further analysis.

Sequence alignment and primer selection. The nucleotide sequences of the hemagglutinin gene from about 50 avian influenza A subtype H5 isolates obtained from GenBank were aligned using the BioEdit software program (3). Conserved sequences within 100 nucleotides either side of the HA1/HA2 cleavage site were used for primer selection.

Nucleic acid isolation. Briefly, one volume of specimen was added to nine volumes of lysis buffer, and the sample was gently mixed with a vortexer. The lysis buffer both inactivated the virus and stabilized the viral RNA 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 were bound to the silica and collected in the solid phase. The silica and nucleic acid complex was pelleted by centrifugation for 30 sec at 10,000 × *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. Diethyl pyrocarbonate (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 × *g* to separate the silica from the water containing the eluted nucleic acid.

NASBA primers. Two pairs of DNA oligonucleotide primers were used 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. (New York, USA). For these assays, primers NASBA-P1 and NASBA-PP1 were identical in sequence.

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

Capture probe. The amplified products of the NASBA reaction (amplicons) were immobilized 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 sequence was determined after selection of the amplification primers by aligning the nucleotide sequences of the putative amplification products. The capture probe was 3′ end-labeled with biotin (Table 1). The capture probe was obtained from

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 GTC 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		

Gibco BRL. For these experiments, NASBA-CP was designed such that it was able to capture both generic and pathogenic H5 amplicons.

Detection of NASBA amplification products. The NASBA reaction amplicons were detected by hybridization analysis using an electrochemiluminescence (ECL)-based detection system following the manufacturer's instructions (Organon Teknika Inc., Boxtel, the Netherlands).

Reverse transcription/polymerase chain reaction (RT/PCR). 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, USA), 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 RNase 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 performed 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 cycles: 94°C for 1 min, 55°C for 40 sec, and 72°C for 1 min. Further extension at 72°C for an additional 10 min completed the reaction. PCR products were analyzed by electrophoresis on 2% agarose gels. The PCR products were subsequently sequenced to confirm their identity (data not shown).

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). DNA sequences were analyzed by sequence similarity searches with EMBL and GenBank DNA databases.

RESULTS

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

Table 2 shows the ECL signal detected following amplification of nucleic acid isolated from 12 different H5N1 allantoic fluid samples with the generic and pathogenic H5 primers. This indicates that the generic and pathogenic primers amplify H5 nucleic acid efficiently.

Table 3 shows the ECL signal detected when various influenza type A subtypes were amplified using the generic H5 primers. Two different studies were conducted. The studies 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. These indicate that the generic and pathogenic primers are specific for H5.

DISCUSSION

The NASBA-based technique described in this paper provides a rapid genome-based detection system for H5 influenza virus, which has advantages for confirmation of H5 virus isolation over antigenic

Table 2. Efficacy of the generic and pathogenic NASBA primers for the amplification of avian influenza H5 hemagglutinin sequences.[A]

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

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

[B]Cutoff value = negative control mean + 3 SD.

(enzyme-linked immunosorbent assay [ELISA], immunoperoxidase, immunofluorescence) or other 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 hr. The NASBA-based H5 detection system is very convenient to use, requiring a minimum of staff training. The ECL reader generates the data automatically, eliminating the need for gel electrophoresis.

The NASBA-based H5 test using the pathogenic primers (NASBA-PP1 and NASBA-PP2) provides a useful tool to rapidly determine whether an H5 influenza isolate has characteristics of a high-pathogenicity avian influenza (HPAI) virus. The standard intravenous pathogenicity index test is the definitive test to classify HPAI viruses but takes 10 days to complete (2). However, the presence of a polybasic amino acid sequence at the cleavage site of HA0 correlates with increased virulence and is a corequirement for avian influenza viruses to be classed as HPAI (2). 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 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 (1997–2000), and by species (e.g., goose, duck, and chicken). The influenza hemagglutinin constantly mutates by the well-described processes of antigenic drift. From time to time it may be necessary to replace one or more of the primers 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. However, the regions covered by the generic primers appear to be 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 signal strength of the generic primers was consistently lower than that produced by the pathogenic primers (Table 2). The ECL signal produced by the pathogenic primers exceeded that of the generic primer by an average of over 13,000-fold. One of the primers is common to both the generic and pathogenic amplification systems. The

Table 3. Cross-reactivity of the generic H5 primers with other avian influenza hemagglutinin subtypes.[A]

Sample No./case No.	ECL signal	Result[B]
Reference standard	39,933	
H1 feces	131	Negative
H1 swab	223	Negative
H1 (2662.2/99)	181	Negative
H3 swab	161	Negative
H3 (3821.2)	102	Negative
H6 + H1 (3461/99)	130	Negative
H9 (3568.6/99)	110	Negative
H5 positive sample	1,753,013	Positive
Negative control 1	125	Negative
Negative control 2	110	Negative
Reference standard	40,478	
H1 (485.5) + H5	187,817	Positive
H1 (485.3) + H5	128,305	Positive
H5 (437.10)	2595	Positive
H5 (25)	30,366	Positive
H5 (437.4)	1584	Positive
H5 (437.6)	102,516	Positive
H5 (437.8)	17,268	Positive
H9 (665.8)	151	Negative
H9 (161.2)	263	Negative
H9 (161.4)	99	Negative
H9 (157.9)	110	Negative
H9 (466.10)	146	Negative
H9 (467.5)	139	Negative
H9 (466.3)	144	Negative

[A]Two separate experiments were conducted. All samples tested were allantoic fluid harvests of 9–11-day-old chicken embryos inoculated from swabs from the indicated cases.

[B]Cutoff value = mean blank value + 3 SD.

pathogenic amplicon is considerably shorter than the generic amplicon (165 base pairs [bp] *vs.* 269 bp) and allows a more rapid turnover and increased level of amplification than the generic amplicon. The reaction blank and negative controls all gave extremely low ECL signals. 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 hundredfold (Table 2). 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.

Table 3 demonstrates the specificity of the generic primers for avian influenza type A H5 subtypes. The generic primer was chosen since 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 two studies conducted, H5 was accurately and specifically detected compared with other influenza A subtypes, including H1, H3, H6, and H9. The H5 signal was at least tenfold higher than the negative control. In addition, the H5 could be accurately detected even in the presence of other contaminating subtypes, such as H1 (Table 3).

The H5 assay described here proved to be 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 here 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 programs 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 highly pathogenic H5 avian influenza isolates and in establishing a method for quantification of viral load. In addition, the applicability of this system to detect viral RNA directly in fecal and swab samples must be assessed, since 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 efficiently generated directly from swab samples without prior amplification in allantoic fluid.

REFERENCES

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

2. Anon. Highly pathogenic avian influenza (Fowl plague). In: OIE manual of standards for diagnostic tests and vaccines, 2nd ed. OIE, Paris, France. A15, pp.123–129. 1992.

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

4. Romano, J. W., B. van Gemen, and T. Kievits. NASBA: a novel, isothermal detection technology for

qualitative and quantitative HIV- RNA measurements. Clin. Lab. Med. 16:89–103. 1996.

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

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

7. Yuen, K. Y., 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, and A. F. B. Cheng, and members of the H5N1 study group. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet 351:467–471. 1998.

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

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