

Comparison of nucleic acid-based detection of avian influenza H5N1 with virus isolation

Songhua Shan,^a Lung-Sang Ko,^b Richard A. Collins,^b Zhongliang Wu,^a Jiahua Chen,^a Ka-Yun Chan,^b Jun Xing,^b Lok-Ting Lau,^{b,c} and Albert Cheung-Hoi Yu^{b,c,*}

^a Shanghai Entry-exit Inspection and Quarantine Bureau, Shanghai 200135, China

^b Hong Kong DNA Chips Limited, 1805-6, 18/F, Lu Plaza, 2 Wing Yip Street, Kowloon, Hong Kong SAR, China

^c Neuroscience Research Institute and Department of Neurobiology, Peking University, 38 Xue Yuan Road, Beijing 100083, China

Received 12 January 2003

Abstract

Nucleic acid sequence-based amplification with electrochemiluminescent detection (NASBA/ECL) of avian influenza virus was compared with viral culture in embryonated chicken eggs. Virus was isolated from blood or anal swabs of chickens artificially infected with highly pathogenic avian influenza A/Chicken/Hong Kong/1000/97 (H5N1). Viral nucleic acid was detected in blood samples by NASBA/ECL immediately prior to death, whilst nucleic acid extracted from anal swabs was detected from the day following artificial infection until death. Thus, blood and/or anal swabs are a suitable source of material for the detection of avian influenza in dead birds, but anal swabs are more suitable for detection of viral genetic material in live birds. Dilution of a known viral standard was used to determine the limit of sensitivity for both NASBA/ECL and egg culture detection methods. The NASBA/ECL method was equivalent in sensitivity to egg culture. The NASBA/ECL results agreed with egg culture data in 71/94 (75.5%) tissue samples obtained from artificially infected birds.

© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Highly pathogenic avian influenza; Nucleic acid sequence-based amplification; H5N1; Electrochemiluminescence; Virus isolation

The avian influenza virus was first recognised as the causative agent of fowl plague in 1955 [1]. Since then, many methods have been developed for the detection and identification of this economically important infectious agent. Simple and reliable methods of virus detection and identification would enable the characterisation of representatives of all circulating subtypes of influenza virus. Methods in routine use today for influenza detection and characterisation include haemagglutination, haemagglutination-inhibition (HI) [2], agar gel immunodiffusion [3], immunofluorescence, viral culture in embryonated eggs or Madin–Darby canine kidney cells [4], enzyme-linked immunosorbent assay (ELISA) for antibody or antigen [5,6], reverse transcriptase-polymerase chain reaction (RT-PCR) [7], Taqman-PCR [8], and nucleic acid sequence-based

amplification (NASBA) [9,10], among others. In order to limit the spread of highly pathogenic avian influenza (HPAI) rapid detection and identification of the infectious agent are required. The specificity, sensitivity, and speed of the available assays are important factors when considering which is the most appropriate to use in a particular situation. Genetic analysis with NASBA is one of the most sensitive and specific methods for typing and subtyping influenza viruses. HI and neuraminidase inhibition (NI) assays are commonly used to determine the subtype of influenza A viruses. However, the preparation of antisera used for HI or NI assays is time-consuming and the separation into different subtypes by these assays is sometimes error prone [11].

Hong Kong has been affected by outbreaks of HPAI on four separate occasions since 1997. In 1997, six people died and at least 12 others infected in the first documented incidence of an avian influenza being transmitted directly to humans without first undergoing reassortment in an intermediate host [12]. To prevent

* Corresponding author. Fax: +852-2111-9762.

E-mail addresses: achy@dnachip.com.hk, achy@bjmu.edu.cn (A.C.-H. Yu).

the spread of the virus, 1.4 million chickens and other poultry were destroyed [13] at a cost of HK\$ 90.4 million (USD 11.6 million). Despite the introduction of more stringent monitoring of domestically reared and imported poultry, another outbreak was detected in May 2001 and a further 1.4 million poultry were destroyed to prevent further dissemination of the virus. Government compensation to poultry retailers amounted to HK\$ 88.6 million (USD 11.4 million). In February 2002, a third outbreak resulted in 950,000 poultry being slaughtered. The Hong Kong SAR Government set aside HK\$ 25 million (USD 3.2 million) to compensate retailers for these losses. A fourth outbreak in December 2002/January 2003 had limited impact on the poultry industry. The isolate responsible for the 2001 and 2002 outbreaks did not infect humans. A rapid, highly specific, and sensitive assay for avian influenza is therefore of paramount importance to screen poultry prior to transport to market. This may help limit the spread of the disease by allowing rapid implementation of quarantine and/or eradication measures.

Nucleic acid sequence-based amplification with electrochemiluminescent detection (NASBA/ECL) is a continuous, isothermal, enzyme-based method for the amplification of nucleic acid [14]. The technique employs a mixture of reverse transcriptase, ribonuclease-H, DNA-dependent 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. 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 ruthenium-labelled ECL 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 [9,10,15].

We previously described an isothermal nucleic acid amplification and detection method for H5N1 viruses of the Eurasian lineage in which the speed and specificity of the test are described in detail [9]. Further studies on the

sensitivity of the method are described here with particular reference to performance relative to viral culture in embryonated chicken eggs.

Materials and methods

Reagents. Isothermal nucleic acid amplification of the avian influenza H5N1 haemagglutinin gene was performed with the Virus Detection System H5 (Pathogenic) obtained from Hong Kong DNA Chips Limited (Hong Kong SAR, China) and used according to the manufacturer's instructions. Electrochemiluminescence was detected with a NucliSens ECL Reader (bioMérieux bv, Boxtel, Netherlands).

Primers and probes. The Virus Detection System H5 (Pathogenic) contains a pair of DNA oligonucleotide primers (designated NASBA-PP1 and NASBA-PP2, respectively). The primers were used in a NASBA reaction to amplify highly pathogenic H5 strains. The amplified products of the NASBA reaction (amplicons) were immobilised by binding to paramagnetic silica beads to which had been attached a capture probe specific for the amplicons. The capture probe was 5' end-labelled with biotin. The primer and capture probe (NASBA-CP) sequences are shown in Table 1. The primers and capture probe were obtained from Gibco-BRL/Life Technologies (Tuen Mun, Hong Kong SAR, China).

Virus titration. The virus used in this study was A/Chicken/Hong Kong/1000/97 (H5N1). The 50% embryo lethal dose (ELD₅₀) was determined in specific-pathogen-free (SPF) 9–11-day embryonated chicken eggs by the method of Reed and Muench [16]. Virus was cultured in SPF 9–11-day white-shelled embryonated chicken eggs according to standard protocols [4]. Allantoic fluid from infected eggs was harvested, aliquoted, and stored at –70 °C.

Virus isolation. Cloacal and tracheal swabs were suspended in buffer containing antibiotics (isotonic PBS, pH 7.0–7.4, penicillin 2000 U/ml, and streptomycin 2 mg/ml) and inoculated via the allantoic sac into 9–11-day SPF chicken embryos according to the OIE Manual of Standards for Diagnostic Tests and Vaccines [4]. Five eggs were inoculated with each sample and allantoic fluid harvested from these embryos was checked by the haemagglutination test. Any haemagglutinin-positive samples would be further typed by HI tests as required.

Artificial infection. For artificial infection studies, A/Chicken/Hong Kong/1000/97 (H5N1) (ELD₅₀ 10^{7.5} infectious U/ml) was used to infect 5-week-old AA breed chickens. Each group comprising two birds was inoculated via the nasal or drinking water route with 0.1 ml of the bacterium-free virus stock. The birds were caged and groups were well separated. Food and water were provided ad libitum.

Nucleic acid extraction. The nucleic acid extraction process has been described previously [9,10,15]. Briefly, one volume of blood or material from anal swabs was added to nine volumes of lysis buffer. The sample was mixed gently by vortex mixing. This inactivated infectious virus and stabilised the nucleic acids by denaturing nucleases. Acid-treated silica (50 µl, 1 mg/ml) was added to the lysate. The sample was kept at room temperature for 10 min and vortex mixed 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

Table 1
Primer sequences used in this study

Name	Sequence (5'–3')
NASBA-PP1	AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GTC CCC TGC TCA TTG CTA TGG TGG TA^a
NASBA-PP2	<u>GAT GCA AGG TCG CAT ATG AGG AGA GAA GAA GAA AAA AGA GAG GAC^b</u>
NASBA-CP	Biotin-CTA TTT GGA GCT ATA GCA GGT T

^a Bold type indicates the sequence of the bacteriophage T7 DNA-dependent RNA polymerase binding site.

^b Underscore indicates the region complementary to the sequence of the ECL detection oligonucleotide.

complex was pelleted by centrifugation (30 s × 10,000g) and washed repeatedly (twice with 5.25 M guanidine thiocyanate, 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. 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 (1 min × 10,000g) to separate the silica from the water containing the eluted nucleic acid.

Nucleic acid amplification and detection. The NASBA method has been described previously [9,10,15]. To 5 µl of nucleic acid extract, 10 µl of a mixture containing 80 mM Tris, pH 8.3, 24 mM MgCl₂, 140 mM KCl, 10 mM DTT, 2 mM each dNTP, 4 mM each NTP, 30% DMSO, and 0.4 µM each primer was added. This mixture was heated to 65 °C for 5 min in a water bath and then cooled to 41 °C for 5 min. Once cool, 5 µl enzyme mix (6.4 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 90 min in a water bath. The final volume was 20 µl. 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 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 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 (NucleiSens ECL Reader, bioMérieux bv, Boxtel, Netherlands).

Sensitivity assay. A solution of A/chicken/Hong Kong/1000/97 (H5N1) with an ELD₅₀ of 10^{7.5} infectious U/ml was serially diluted in sterile phosphate-buffered saline (PBS) and processed for NASBA/ECL detection as described above. At the same time, diluted samples were cultured in embryonated chicken eggs as described above.

Cut-off value. The cut-off value for the qualitative determination of positive and negative values in NASBA/ECL was defined as 0.015 × the Instrument Reference Solution (IRS), after five consecutive known negative control samples were assayed in order to determine the average background signal.

Results

Cut-off value

The ECL signal obtained during the experiments was 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 25,000–35,000 arbitrary luminescence units. While ECL emission is proportional to the amount of amplified nucleic acid products, in the absence of standardisation with appropriate internal amplification controls, no information on the initial amount of infectious agent, target RNA or degree of amplification can be obtained. The cut-off value for differentiating positive and negative samples is critical to the appropriate interpretation of data. In this study, cut-off values are defined as 0.015 × IRS. We have reported a similar method for calculating cut-off values [9,10,15]. The use of appropriate internal controls for quantifying HIV viral load using the NASBA technique has been described [14] and the technique is applicable to other targets.

Dynamic range of ECL detector

The dynamic range of the ECL detector was determined using a serial dilution of a known positive sample of avian influenza H5N1. A sample of A/chicken/Hong Kong/1000/97 (H5N1) was diluted 1/5, 1/25, 1/125, 1/625, and 1/3125 in duplicate and analysed using the standard NASBA/ECL protocol. The results are shown in Table 2. Even at a dilution of 1/3125 (equivalent to about 10⁴ infectious U/ml), the ECL signal was almost 50 times higher than the cut-off value for the qualitative determination of positive samples. The theoretical limit of detection under these conditions would be obtained after a 15,500-fold dilution of the original stock solution (equivalent to about 10^{2.3} infectious U/ml).

Artificial infection studies

Two AA breed chickens were artificially infected with 0.1 ml (ELD₅₀ 10^{7.5} U/ml) A/Chicken/Hong Kong/1000/97 (H5N1) via the nasal or oral routes. The birds were housed in separate cages and examined daily until death. Blood (1 ml from the wing vein) and anal swabs were obtained daily, beginning 24 h after infection, for nucleic acid extraction and analysis by NASBA/ECL. Anal swabs were taken only from a single bird that subsequently died 6 days post-infection. A blood sample prior to infection was taken as a control. A positive control (A/chicken/Hong Kong/1000/97 (H5N1)) and negative control were also analysed. The results of the blood and anal swab analyses are shown in Tables 3 and 4, respectively. In the blood analysis of both chickens, viraemia is apparent only immediately before death. However, the presence of virus in the lower intestinal tract is apparent 24 h after infection, continues until death, and can be detected post-mortem.

Sensitivity studies

The NASBA/ECL method and a standard viral culture technique were compared for the ability to detect subtype H5N1. The isolate A/chicken/Hong Kong/1000/

Table 2
Dynamic range of ECL detection

Sample ^a	ECL signal (mean ± range)
1/5 dilution	10,000,001 ^b
1/25 dilution	10,000,001 ^b
1/125 dilution	715,879 ± 6987
1/625 dilution	120,133 ± 278
1/3125 dilution	22,800 ± 1600
Instrument reference solution (IRS)	30,570
Cut-off value (0.015 × IRS)	459

^a A/chicken/Hong Kong/1000/97 (H5N1) ELD₅₀ 10^{7.5} infectious U/ml diluted in PBS and tested in duplicate.

^b Upper limit of detection.

Table 3
Detection of H5N1 virus in blood

Sample	Chicken 1 ^a	Chicken 2 ^b
Pre-infection control	96	181
Day 1	89	153
Day 2	93	360
Day 3	233	1,134,776
Day 4	215	894,243
Day 5	76	N/A
Day 6	1,727,214	N/A
Instrument reference solution (IRS)	27,142	34,599
Cut-off value (0.015 × IRS)	407	519

NA, not applicable.

^a Died on Day 6.

^b Died on Day 4.

Table 4
Detection of H5N1 virus in anal swabs

Sample ^a	Analysis 1 ^b	Analysis 2 ^b
Day 1	440,395	631,031
Day 2	460,252	1213
Day 3	534,916	864,889
Day 4	4399	974,965
Day 5	375,573	1,059,623
Positive control	340,811	1,304,349
Negative control	131	52
Instrument reference solution (IRS)	32,161	33,490
Cut-off value (0.015 × IRS)	482	502

^a Anal swabs were taken from the chicken in Table 3 that died 6 days post-infection.

^b Analyses 1 and 2 are replicate analyses performed on different days using the same RNA extracted from the anal swab.

97 (H5N1), with an ELD₅₀ of 10^{7.5} infectious U/ml, was used as the stock virus sample in both assays. A series of 10-fold dilutions of the stock virus sample in sterile PBS were prepared. Replicate analyses of the NASBA/ECL samples were performed. The death of the embryo and the haemagglutination activity of the allantoic fluid were used as the end-points to indicate qualitatively the

Table 5
Comparison of NASBA/ECL and viral culture in the detection of avian influenza H5N1

Sample	NASBA/ECL (1) ^a	NASBA/ECL (2) ^a	Viral culture
Undiluted	984,489	ND	ND
10 ⁻¹ dilution	674,817	726,886	Positive
10 ⁻² dilution	731,321	986,486	Positive
10 ⁻³ dilution	696,142	1,742,771	Positive
10 ⁻⁴ dilution	661,271	1,002,002	Positive
10 ⁻⁵ dilution	1,906,473	1,284,430	Positive
10 ⁻⁶ dilution	318	1,079,156	Positive
Negative control	195	96	Negative
IRS	32,486	31,093	NA
Cut-off value (0.015 × IRS)	487	466	NA

NA, not applicable; ND, not done.

^a Replicate analyses performed on RNA extracted from A/chicken/Hong Kong/1000/97 (H5N1) with ELD₅₀ of 10^{7.5} infectious U/ml.

Table 6
Comparison of NASBA/ECL and virus isolation by egg culture

Virus isolation	NASBA/ECL	Number
Positive	Positive	30
Negative	Negative	41
Positive	Negative	9
Negative	Positive	14
Total		94

presence or absence of avian influenza in the viral cultures. The results are shown in Table 5.

Comparison of NASBA/ECL with egg culture

Ninety-four tissue samples obtained from chickens artificially infected with A/chicken/Hong Kong/1000/97 (H5N1) were inoculated into embryonated hen's eggs. The same samples were also analysed by NASBA/ECL for the presence of avian influenza nucleic acid. The results are shown in Table 6. The NASBA/ECL data agreed with the egg culture data in 71/94 (75.5%) of cases and disagreed in 23/94 (24.5%) of cases.

Discussion

This paper further characterises the sensitivity of a nucleic acid sequence-based amplification and electrochemiluminescent detection (NASBA/ECL) system for avian influenza virus subtype H5N1 first described by Collins et al. [9]. The NASBA/ECL method has advantages for confirming H5 virus identification over antigenic (ELISA, immunoperoxidase, and immunofluorescence) and genomic (RT-PCR/DNA sequencing) methods. NASBA/ECL is highly appropriate for RNA analytes, such as avian influenza genetic material, due to the direct incorporation of the reverse transcription process into the amplification reaction. Under standard conditions, the reaction can be completed in about 4 h. The assay can be performed in the presence of

contaminants typically encountered in field samples, such as cloacal, pharyngeal and anal swabs, faecal samples, cage sweepings, and blood, which may cause problems in other assays, such as ELISA and RT-PCR.

The NASBA/ECL method using the primers and capture probe described here provides a useful tool to determine rapidly if H5 influenza isolates have the characteristics of a highly pathogenic avian influenza (HPAI) virus. The standard intravenous pathogenicity index test is the definitive test for classifying HPAI viruses but takes 10 days to complete [4]. The presence of a polybasic amino acid sequence at the cleavage site of the HA0 haemagglutinin precursor polypeptide correlates with increased virulence and is a co-requirement for avian influenza viruses to be classified as HPAI [4]. The NASBA-based pathogenic H5 test, which has one primer located at the cleavage site of the HA0 gene, can give a rapid confirmation that a newly isolated H5 influenza virus is likely to be a HPAI.

The ECL detector allows highly automated analysis of the products of the NASBA reaction. The reduction in manual handling and the highly sensitive signal detection increases the sensitivity of the assay. Dilution of a stock virus solution 3125-fold yielded an ECL signal almost 50-fold higher than the cut-off value used to establish positive samples (Table 2). In addition, the reproducibility of the assay in this limited study was high with ECL signal duplicates varying by 7% or less.

Blood tests are commonly used in veterinary studies of viral infection, especially those where antibody responses are being examined. In the studies presented here of chickens artificially infected with A/chicken/Hong Kong/1000/97 (H5N1), significant viraemia as evidenced by the ability to amplify specific regions of the haemagglutinin gene by the NASBA/ECL method was detected only on the day of death or the day preceding death. Closer inspection of the data appears to indicate a rising NASBA/ECL signal in the days following infection, but it did not exceed the cut-off limit for determining positive infection. These data indicate that analysing blood samples in live birds by NASBA/ECL for the presence of virus may not be suitable in a mass screening programme due to the low virus titre. However, for the examination of dead or dying birds, blood samples are an excellent source of viral nucleic acid and may be very satisfactory for confirming the presence or otherwise of a HPAI. The routine examination of dead birds collected during monitoring of live poultry markets in Hong Kong has been implemented as a feature of enhanced surveillance techniques for H5N1 viruses.

Anal swabs proved to be an excellent source of viral nucleic acid immediately following artificial infection of chickens with A/chicken/Hong Kong/1000/97 (H5N1). Waterfowl are primarily infected with avian influenza through the gastrointestinal tract via faecal contamina-

tion of drinking water. Consequently, viral titres are expected to be high in samples of the upper and lower alimentary tract. The wide distribution of avian influenza in wild fowl and domesticated poultry has been demonstrated [17,18]. In replicate analyses of anal swabs taken from the artificially infected bird that died six days after infection, a consistently high ECL signal is obtained. Excluding the obvious outliers in each sample set, the positive signal was at least 700-fold higher than the cut-off value (Table 4). The outliers were not due to errors in obtaining the anal swab, as the counterpart of each outlier sample in the other data set is normal. The error is most likely due to failure of the amplification reaction. Even so, the reduced signal from each of the partially successful NASBA reactions is also clearly positive and exceeds the cut-off by 9-fold and 2.4-fold, respectively. This demonstrates the robustness and sensitivity of the NASBA method as positive data can still be extracted under less than optimal conditions.

The NASBA/ECL method was compared against standard viral culture in SPF chicken eggs. Serial dilutions of stock HPAI virus ($ELD_{50} 10^{7.5}$ infectious U/ml) were assayed by each method. The NASBA/ECL method and the standard viral culture method detected the presence of the virus in all samples, even at the lowest dilution tested (10^{-6} -fold). The viral culture method required 3 days to confirm the presence of the virus in the most diluted sample. The NASBA/ECL analysis was completed in 4 h. Thus, in the analysis of A/chicken/Hong Kong/1000/97 (H5N1), the NASBA/ECL method was shown to have equivalent sensitivity to the “gold standard” viral culture method.

Serological tests on a flock basis are considered a useful tool for determining whether a flock has been infected with influenza. However, serological tests may miss recent infections, as a measurable antibody titre does not develop until after approximately one week [2,19]. Genetic tests have the potential to be far more sensitive and specific than serological assays. Genetic analysis methods that are comparable to currently accepted “gold standard” methods yet are much quicker and easier to perform are highly applicable to frontline screening programmes, such as at customs and import control checkpoints and live bird markets, where the rapid isolation and confirmation of HPAI are required. In addition, the ability of methods such as NASBA/ECL to be used readily on environmental samples such as faecal samples, cage sweepings, butcher slabs, utensils, and clothing makes it an appropriate technology to use for confirming the completeness of cleansing and sterilisation operations following HPAI outbreaks or during routine market cleaning programmes.

Vaccines against H5N1 have been developed [20] and may be an effective method for controlling future outbreaks of H5N1 in live bird markets. However, vaccination will disrupt current antibody-based detection

methods, as the vaccine-induced immune response will be indistinguishable from naturally induced immunity following infection. The NASBA/ECL method is not affected by the presence of antibodies and is able to identify birds actively infected with H5N1 in a vaccinated population.

Tissue samples obtained from birds artificially infected with HPAI H5N1 were compared by NASBA/ECL and inoculation into embryonated hen's eggs. The results agreed in 71/94 (75.5%) of cases, with 30/39 (76.9%) of egg culture positive cases and 41/55 (74.5%) of egg culture negative cases being confirmed by NASBA/ECL. Some 23/94 (24.5%) of cases were in disagreement. Further studies on larger sample sizes are required to provide more accurate data on the concordance between egg culture and NASBA/ECL results. In a recent study of a comparison between real-time PCR and virus isolation by egg culture for avian influenza detection, concordance between methods was 89%, although with a much larger data set (1550 samples) [21]. Differences in the detection of avian influenza between the assays can be explained in part by the nature of the assay itself. Virus isolation by egg culture detects only live virus. Viruses that have been inactivated during transport or by the addition of disinfectants or other treatments will not be detected, whereas such samples will be detected by NASBA/ECL. Factors that adversely affect the sensitivity of the NASBA/ECL with respect to virus isolation by egg culture include NASBA-inhibitory substances in the samples, inefficient RNA extraction, and the ubiquitous presence of RNA degrading enzymes.

The rapid identification of HPAI is a critical factor in ensuring that the disease does not spread. Rapid implementation of quarantine and eradication measures is required following confirmation of HPAI outbreaks. Antibody-based detection methods lack sensitivity while viral culture methods may take several days. Other techniques, such as RT-PCR, are prone to contamination, especially when used to analyse samples such as allantoic fluids or faeces. NASBA/ECL offers a rapid, specific, and sensitive method for the detection of HPAI and is a highly appropriate technique for use in a centralised front-line mass-screening programme of poultry prior to delivery to market.

Acknowledgments

This work was supported by a University–Industry Collaboration Programme Grant (UIM44) from the Innovation and Technology Fund administered by the Innovation and Technology Commission, Commerce and Industry Bureau of the Hong Kong SAR Government. Any opinions, findings, conclusions or recommendations expressed in this material (or by members of the project team) do not reflect the views of the Government of the Hong Kong SAR or the Industry Department. The authors thank Dr. Wendy Wen Luan Hsiao

(Department of Biology, Hong Kong University of Science and Technology), and Marco Mou (bioMérieux China Ltd) for technical support.

References

- [1] W. Schäfer, Vergleichende sero-immunologische Untersuchungen über die Viren der Influenza und klassischen Geflügelpest, *Z. Naturforsch. C (Biosci.)* 10B (1955) 81–91.
- [2] D.E. Swayne, D.A. Senne, C.W. Beard, Influenza, in: D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson, W.M. Reed (Eds.), *Isolation and Identification of Avian Pathogens*, American Association of Avian Pathologists, Pennsylvania, 1998, pp. 150–155.
- [3] C.W. Beard, Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion, *Bull. World Health Organ.* 42 (1970) 779–785.
- [4] Anon., Highly pathogenic avian influenza (fowl plague), in: *OIE Manual of Standards for Diagnostic Tests and Vaccines*, fourth ed., OIE, Paris, 2000.
- [5] E.M. Zhou, M. Chan, R.A. Heckert, J. Riva, M.F. Cantin, Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein, *Avian Dis.* 43 (1998) 517–522.
- [6] S. Davison, A.F. Ziegler, R.J. Eckroade, Comparison of an antigen-capture enzyme immunoassay with virus isolation for avian influenza from field samples, *Avian Dis.* 42 (1998) 791–795.
- [7] E. Starick, A. Romer-Oberdorfer, O. Werner, Type- and subtype-specific RT-PCR assays for avian influenza A viruses (AIV), *J. Vet. Med. B* 47 (2000) 295–301.
- [8] B. Schweiger, I. Zadow, R. Heckler, H. Timm, G. Pauli, Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples, *J. Clin. Microbiol.* 38 (2000) 1552–1558.
- [9] 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* 103 (2002) 213–225.
- [10] R.A. Collins, L.-S. Ko, K.-Y. Fung, K.-Y. Chan, J. Xing, L.-T. Lau, A.C.H. Yu, Rapid and sensitive detection of avian influenza virus subtype H7 using NASBA, *Biochem. Biophys. Res. Commun.* 300 (2003) 507–515.
- [11] E. Hoffmann, J. Stech, Y. Guan, R.G. Webster, D.R. Perez, Universal primer set for the full-length amplification of all influenza A viruses, *Arch. Virol.* 146 (2001) 2275–2289.
- [12] K.F. Shortridge, P. Gao, Y. Guan, T. Ito, Y. Kawaoka, D. Markwell, A. Takada, R.G. Webster, Interspecies transmission of influenza viruses: H5N1 virus and a Hong Kong SAR perspective, *Vet. Microbiol.* 74 (2000) 141–147.
- [13] M.L. Perdue, D.L. Suarez, D.E. Swayne, Avian influenza in the 1990s, *Poult. Avian Biol. Rev.* 11 (2000) 1–20.
- [14] 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.
- [15] 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.
- [16] L.J. Reed, H. Muench, A simple method of estimating fifty per cent endpoints, *Am. J. Hyg.* 27 (1938) 493–497.
- [17] D.J. Alexander, A review of avian influenza in different bird species, *Vet. Microbiol.* 74 (2000) 3–13.
- [18] K.F. Shortridge, Avian influenza A viruses of southern China and Hong Kong: ecological aspects and implications for man, *Bull. World Health Organ.* 60 (1982) 129–135.

- [19] D.E. Swayne, D.L. Suarez, Highly pathogenic avian influenza, *Rev. Sci. Tech. Off. Int. Epiz.* 19 (2000) 463–482.
- [20] D.E. Swayne, J.R. Beck, M.L. Perdue, C.W. Beard, Efficacy of vaccines in chickens against highly pathogenic Hong Kong H5N1 avian influenza, *Avian Dis.* 45 (2001) 355–365.
- [21] 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.