

Development and Evaluation of a Real-Time Nucleic Acid Sequence Based Amplification Assay for Rapid Detection of Influenza A

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The development and introduction of effective treatment for influenza A in the form of neuraminidase inhibitors have made the rapid diagnosis of infection important especially in high-risk populations. The aim of this study was to develop a real-time nucleic acid sequenced based amplification (NASBA) using a molecular beacon that could detect a wide range of influenza A subtypes and strains in a single reaction by targeting a conserved region of the influenza genome, and to evaluate its sensitivity and specificity against traditional laboratory techniques on a range of clinical samples usefulness during the 2003/2004 influenza season. The results demonstrated the assay to be highly sensitive and specific, detecting <0.1 TCID₅₀ of virus stock. Three hundred eighty-nine clinical samples were tested in total from two patient groups. Overall, the real-time NASBA assay detected 64% (66/103) more influenza positive samples than cell culture and direct immunofluorescence (IF) and, therefore, was shown to be more sensitive in detecting influenza A in a wide range of respiratory samples than traditional methods. In conclusion, the real-time influenza A assay demonstrated clinical usefulness in both hospital and community populations. **J. Med. Virol. 74:619–628, 2004.**

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INTRODUCTION

Influenza A virus is an important respiratory pathogen globally. It is subject to regular antigenic changes

brought about by either point mutation in the genes coding for hemagglutinin or neuraminidase (genetic drift) or by re-assortment of genes from two distinct types of influenza (genetic shift). In both situations, prior immunity to influenza might not prevent infection with the new type, leading to localized epidemics or, in the case of genetic shift, a global pandemic of influenza [Cox and Subbarao, 1999; Horimoto and Kawaoka, 2001; Kilbourne et al., 2002; Nicholson et al., 2003; Treanor, 2004].

Infection with influenza A can lead to a wide spectrum of clinical disease from asymptomatic infection to the acute, self-limiting influenza syndrome to severe sometimes fatal complications. The severity of disease depends generally on the age and health of the patient with most influenza associated fatalities seen in the elderly or those who have underlying pulmonary or cardiac disease [Chan et al., 2002; Hayden and Palese, 2002; Nicholson et al., 2003]. The WHO estimates that inter-pandemic influenza epidemics cause in excess of three to five million cases of severe illness and up to 500,000 deaths each year in industrialized countries alone. In addition, influenza epidemics are associated with high economic loss through lost working days and increased burden to the health service [Nicholson et al., 2000; Stohr, 2003].

Annual immunization remains the best way to prevent infection in 'at risk' populations. Each year the

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influenza strains circulating are monitored by global surveillance schemes to determine the vaccine composition for that year. Minor genetic drift occurring in the circulating virus can reduce the effectiveness of the vaccine in preventing illness but, even then, partial immunity afforded by the vaccine will often attenuate the infection, reducing the occurrence of severe illness and complications [Kilbourne et al., 2002; Palese and Garcia-Sastre, 2002; Nicholson et al., 2003; Treanor, 2004].

Treatment and prophylaxis are available against influenza. Presently, the antiviral of choice in both situations is oseltamivir (Tamiflu™), due primarily to its ease of administration [Stohr, 2003]. Belonging to a group of compounds called the neuraminidase inhibitors, it effectively blocks the ability of the virus to cleave itself from the host cell preventing further infection of neighboring cells [Gubareva et al., 2000; Stiver, 2003]. UK guidelines limit its use to 'at risk' individuals who have not been vaccinated or in outbreak situations in closed communities and it must be used within 48 hr of symptom onset or contact, thus preventing its use in the community at large [National Institute for Clinical Excellence, 2003]. As vaccination is also not offered to otherwise healthy individuals, the impact of influenza on lost working days through illness is not reduced by either measure.

With the availability of the neuraminidase inhibitors, it has become important for a rapid, specific diagnosis of influenza to be made to ensure appropriate patient management.

Molecular techniques applied to respiratory viral targets have been demonstrated to increase the detection rate compared with traditional laboratory methods such as direct immunofluorescence (IF) and cell culture [Ellis and Zambon, 2002; Harnden et al., 2003]. As influenza has an RNA genome, reverse transcriptase PCR (RT-PCR) has been the technique of choice, usually targeting either the hemagglutinin or neuraminidase gene to enable further virus subtyping. RT-PCR can be multiplexed to detect more than one target in a single reaction. Influenza multiplex assays have been reported that can differentiate influenza A from B and influenza A H3 from H1, reducing both time and overall costs of diagnosis [Stockton et al., 1998; Ellis and Zambon, 2002; Hibbitts and Fox, 2002]. The disadvantage of RT-PCR methods (compared with direct PCR of DNA targets) is that the RT step is often performed separately from the PCR, increasing both assay time and the risk of contamination. This issue has been somewhat resolved with the development of real-time RT-PCR systems; however, in many systems the RT step is again performed independently from PCR [Schweiger et al., 2000; van Elden et al., 2000; Ellis and Zambon, 2002].

Nucleic acid sequence-based amplification (NASBA) is an alternative method to RT-PCR for the detection of RNA targets [Ellis and Zambon, 2002; Hibbitts and Fox, 2002]. Unlike traditional PCR, the amplification step is isothermal and relies on the simultaneous action of three enzymes, avian myeloblastosis virus reverse

transcriptase (AMV-RT), T7 RNA polymerase, and RNaseH. The NASBA amplicon is single-stranded RNA complementary to the original RNA target and is produced via a DNA intermediate. Traditionally, detection of the RNA product is by utilising target specific probes in an amplification end-point electrochemiluminescence (ECL) reaction. A large number of NASBA diagnostic assays have been developed that use ECL detection including those for detecting HIV [Keivits et al., 1991], enteroviruses [Fox et al., 2002], noroviruses [Greene et al., 2003; Moore et al., 2004], and avian strains of influenza [Lau et al., 2004]. Despite being more rapid than traditional RT-PCR, NASBA with ECL detection still requires more hands-on time than real-time PCR techniques [Leone et al., 1998; Ellis and Zambon, 2002; Hibbitts and Fox, 2002].

More recently, real-time NASBA assays have been developed. The principles remain the same as those of standard ECL detection, but the main difference is the introduction of a molecular beacon, a hairpin oligonucleotide probe, labeled with a fluorescent reporter dye and a quencher, into the amplification mix. The reporter dye and quencher are brought into close proximity by a self-complementary stem structure preventing the reporter dye from emitting fluorescence. The target specific portion of the beacon, however, hybridises to complementary sequence in the single-stranded NASBA product as it is amplified, thus opening the hairpin structure, removing the reporter dye away from the quencher allowing it to fluoresce at its characteristic wavelength. By labelling molecular beacons with different dyes, it is possible to detect simultaneously more than one target in any given NASBA reaction. This has been applied successfully in a real-time NASBA assay for the detection of enteroviruses with an internal control and in HIV quantitation using an internal standard [Leone et al., 1998; Deiman et al., 2002; Hibbitts and Fox, 2004].

We describe how a real-time NASBA assay was developed and used to detect influenza A in an outbreak of acute respiratory disease in a nursing home for the elderly and then used extensively during the influenza season of 2003–2004 in Wales to detect the A/Fujian/411/2002 (H3N2) strain of influenza virus.

MATERIALS AND METHODS

Virus Isolates

Reference strains of influenza A were obtained from the American Type Culture Collection (ATCC, University Boulevard, Manassas, VA) and used to develop the NASBA assay. Prototype strains utilized were: A/New Caladonia/20/99 H1N1, A/Moscow/10/99 H3N2, and A/Panama/2007/99 H3N2.

Production of Primary Virus Stocks

Propagation of viruses and confirmation by direct antigen staining was undertaken as described previously [Collins et al., 1996; Shen et al., 1996]. Primary

monkey kidney (pMK) cells (European Collection of Cell Cultures, CAMR, Wiltshire, UK) were used for isolation for of the influenza strains. When a cytopathic effect (CPE) was observed, or after 10 days, the monolayer was scraped and tested for influenza A using an indirect IF assay with specific monoclonal antibodies (Chemicon International, Harrow, UK). Virus titers were determined based on the 50% tissue culture infective dose (TCID₅₀) assay by infecting target cells with serial tenfold dilutions of each virus stock. Infected cells were incubated at 37°C and CPE monitored on a daily basis. The TCID₅₀ was calculated using the method of Reed and Muench [1938].

Primers and Molecular Beacon Design

For design of primers and a molecular beacon specific for influenza A, the nucleoprotein gene sequences of representative strains of the main human and animal types were obtained from GenBank, aligned and conserved regions identified. A BLAST search of the resulting primers and molecular beacon sequences (Table I) revealed the further broad-spectrum nature of the assay (Table II). The stability and predicted structure of the beacon were analyzed by using the European MFOLD server (<http://bibiserv.techfak.uni-bielefeld.de/mfold/>). The 5'-end of the beacon was labeled with fluorescein (FAM) and the 3'-end with the non-fluorescent quencher 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL). Oligonucleotide primers and beacon were synthesized and HPLC purified (Oswel DNA Services, Southampton, UK) before use in RT-PCR or NASBA.

Nucleic Acid Extraction

Extraction of RNA from cultured virus stocks of known titer was carried out according to the method described by Boom et al. [1990] using the NucliSens[®] manual extraction kit according to the manufacturer's instructions (bioMérieux Ltd., Boxtel, Netherlands). One hundred microliters of virus stock was added to 900 µl of lysis buffer in microcentrifuge tubes. To this, 50 µl silica suspension was added, mixed and incubated at room temperature for 10 min, vortexing every 2 min.

The silica was spun down by centrifugation at 10,000g for 1 min and the supernatant discarded. The silica pellets were washed twice with 1 ml guanidinium wash buffer, twice with 70% (v/v) ethanol and once with 1 ml acetone. The silica was then dried and the pellet was resuspended in 50 µl of elution buffer and the nucleic acid eluted by incubating the tubes at 56°C for 10 min. After a final centrifugation at 16,000g, the eluate was transferred to a fresh tube, aliquoted and stored at -80°C.

The clinical samples were extracted using the same technique but utilising the NucliSens[®] automated isolation kit and the NucliSens[®] automated extractor, following manufacturers instructions (bioMérieux, Ltd.) and an input volume of 200 µl into the lysis buffer. The nucleic acid was aliquoted and 5 µl was transferred to a 0.2 ml tube for testing in the influenza A real-time molecular beacon assay. The remaining extract was stored at -80°C following the addition of 1 µl of RNasin (Promega, Southampton, UK).

RT-PCR

The RT reaction was undertaken in a 20 µl reaction volume containing the template extract (5 µl), 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 2 mM DTT (RT buffer, BioGene Ltd., Cambridge, UK), 0.25 mM each deoxynucleoside triphosphate (dNTP) (Amersham-Pharmacia, Little Chalfont, Buckinghamshire, UK), 20 U of RNase inhibitor (Promega, Southampton, UK), 10 U of AMV-RT (BioGene Ltd.), and 1 µM virus specific RT reverse primer (Table I) at 43°C for 1 hr.

The PCR used 5 µl of the synthesized cDNA in a total reaction volume of 50 µl that included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5–3 mM MgCl₂ (optimized for each target), 0.25 mM each dNTP, 2 U of *Taq* DNA polymerase (all from Amersham Biosciences) with 1 µM each virus specific RT-PCR primer (Table I). The PCR protocol involved an initial denaturation step at 94°C for 4 min and then samples were subjected to 30 cycles of amplification, each consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C with a final extension of 72°C for 7 min in an MJ research PCR machine (Genetic Research Instruments, Baintree, Essex, UK). PCR

TABLE I. Sequences of Influenza A Primers, Probe, and Molecular Beacon

Primer/probe ID	Nucleotide no. (influenza A; gene segment 5)	Sequence	Function
Flu A RT	7–26	AGCAGGGTAGATAATCACTC	RT reverse primer
Flu A RT +	7–26	AGCAGGGTAGATAATCACTC	RT-PCR reverse primer
Flu A RT–	100–119	TTGTGCHGCTGTTTGRAATT	RT-PCR forward primer
Flu A T7	7–26	AATTCTAATACGACTCACTATAGG- GAGCAGGGTAGATAATCACTC	NASBA P1 primer T7 RNA polymerase tail
Flu A ECL	100–119	GATGCAAGGTCGCATATGAGA- TYTCRKTGDCATTCTGGCG	NASBA P2 primer ECL detection tail
Flu A MB	55–74	CCAAGCTAAGAYCGTTTGGT- GCCTTGGCTTGG	Influenza A molecular beacon

RT, reverse transcription; PCR, polymerase chain reaction; NASBA, nucleic acid sequence based amplification.

Tail sequences for NASBA and stem sequences for the molecular beacon is given in bold italics. Molecular beacon has 5'-FAM and 3'-DABCYL.

TABLE II. Example Influenza A Subtypes With Nucleoprotein Containing the Real-Time NASBA Primer and Molecular Beacon Binding Sites

Strain and subtype	Accession no.
A/Hong Kong/498/97 (H3N2)	AF255749
A/Singapore/1/57 (H2N2)	M63752
A/Hong Kong/427/98 (H1N1)	AF258516
A/Hong Kong/156/97 (H5N1)	AF036359
A/Netherlands/033/03 (H7N7)	AY342426
A/Chicken/Shanghai/F/98 (H9N2)	AY253753
A/Mallard/Astrakhan(Gurjev)/263/82 (H14N5)	M63785
A/Mallard/Alberta/111/99 (H4N6)	AY633127
A/Mallard/Alberta/117/97 (H3N8)	AY633135
A/Mallard/Alberta/203/92 (H6N5)	AY633191
A/GSC Chicken/British Columbia/04 (H7N3)	AY650273
A/Swine/Hong Kong/126/82 (H3N2)	M63771
A/Turkey/Ontario/7732/66 (H5N9)	M63774
A/Duck/Pennsylvania/1/69 (H6N1)	M63775
A/Duck/Beijing/1/78 (H3N6)	M63782

products were analyzed by standard ethidium bromide stained agarose gel electrophoresis (2% w/v).

Cloning and In Vitro Transcription

PCR products obtained from the prototype strains were cloned directly into pCR II-TOPO[®] (Invitrogen, Inchinnan, Renfrewshire) by following the manufacturers' instructions. Plasmids were transformed into high efficiency competent cells (TOP10F⁺; Invitrogen) by chemical transformation. Transformants were 'color' screened on indicator plates (LB plate with 100 mg/ml ampicillin, 80 µg/ml X-gal and 0.5 mM IPTG) and the presence of the expected inserts confirmed by PCR. Plasmids were purified with the SNAP miniprep kit (Invitrogen) and the plasmid copy number estimated by UV spectroscopy at an optical density of 260 nm.

Plasmids (ca. 1–2 µg) containing the inserts were linearized using *EcoRV* and analyzed by agarose gel electrophoresis to ensure complete digestion had occurred. In vitro transcription of each template utilized the SP6 MAXIScript[®] kit (Ambion Europe Ltd., Huntingdon, Cambridgeshire, UK) with 1 µg of DNA in a final reaction volume of 20 µl, according to the manufacturer's instructions. The RNA copy number was estimated by UV spectroscopy at an optical density of 260 nm, using 330 as the estimated molecular weight of each ribonucleotide and Avogadro's number to give the number of copies per milliliter.

Real-Time NASBA Optimisation

NASBA reactions were carried out according to Kievits et al. [1991] with some modifications. The NucliSens[®] Basic Kit (bioMérieux Ltd.) was used according to the manufacturer's instructions. Briefly, to the reagent sphere, 80 µl of diluent, 13.5 µl of molecular grade water, 14–16 µl of stock KCl (final concentration 70–80 mM), 5 µl of each of the required primers (5 µM stock of each), and 2.5 µl of molecular beacon (final concentration 0.05 µM–0.2 µM) were added (Table I). Appropriate

positive and negative controls were included in each assay. Five microliters of extract or synthetic RNA and 10 µl of the master mix was added to 0.2 µl tubes and incubated on the NucliSens[®] EasyQ (bioMérieux Ltd.) incubator for 2 min at 65°C and then 2 min at 41°C. Enzyme was then added to each tube, mixed and briefly centrifuged. Real-time NASBA was undertaken using the NucliSens[®] Easy Q Analyzer (bioMérieux Ltd.) for 90–150 min at 41°C.

The clinical samples were all tested under the optimized reaction conditions and a cut-off value for a positive result was set at 20% above the negative control wild-type signal.

Sensitivity and Specificity of the Real-Time NASBA Assay

The sensitivity of the influenza A NASBA real-time assay was determined using the reference virus strains and in vitro RNA transcripts.

Serial dilutions of the influenza A virus stocks equivalent to final input into the NASBA reaction of 1×10^4 – 1×10^{-2} TCID₅₀ were made in guanidinium lysis buffer before extraction. NASBA was performed on each of these extracts and the cut-off point between a negative and positive real-time fluorescent signal identified (where the signal is repeatedly no longer detected). For the in vitro RNA transcripts, the number of RNA copies was calculated and serial dilutions prepared such that 1×10^4 – 1×10^{-2} RNA copies per reaction were present. These dilutions of RNA were added directly into the NASBA mix.

The specificity of each assay was evaluated using a cross-reactivity panel of other respiratory viruses including parainfluenza (PIV) types 1–4, respiratory syncytial virus (RSV) and influenza B positive material.

Patients and Samples

The assay evaluation was undertaken in a routine diagnostic laboratory following standard operation procedures. Two groups of patient samples were included in the assay evaluation. Samples from both groups were referred to the laboratory for respiratory virus investigations from both hospitals and primary care centers in the community from across Wales.

Patient Group 1

Sixteen patients out of a total of 58 patients living in a nursing home for the elderly developed an acute febrile illness between the March 13 and April 1, 2003. Of these, six patients died. Nose and throat swabs collected into virus transport medium (VTM) composed of Hanks BSS × 1 (BioWhittaker[™], Wokingham, Berkshire, UK) 7.5% bovine albumen (Sigma-Aldrich, Poole, Dorset, UK) penicillin (Britannia Pharmaceuticals, Redhill, Surrey, UK) and streptomycin (Sigma) together with acute serum samples were obtained from 11 patients for virological investigations. Two weeks later, a convalescent serum sample was obtained from seven of the patients.

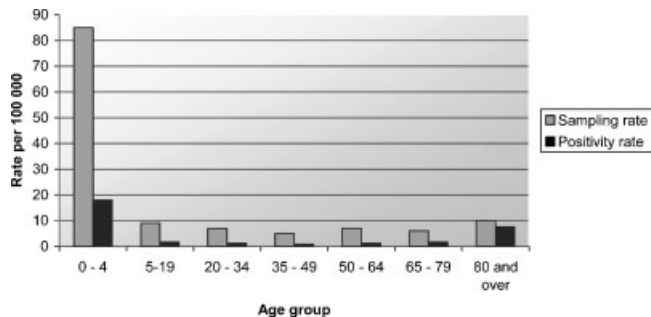


Fig. 1. Samples taken by age group (patient group 2). The sampling rate is calculated based on the total population in Wales of each age group.

Patient Group 2

Three hundred seventy-eight clinical samples were collected from 337 patients from across Wales during October 2003–February 2004. Of these, 249 were hospital in-patients, 76 were patients in the community and 12 were patients who had died. All had a history of an acute febrile illness, a respiratory tract infection or viral pneumonia. The sampling rate in each age group is shown in Figure 1.

Of the samples received, 159 (42%) were respiratory swabs, most were collected and put into VTM prior to being sent to the laboratory, although three dry swabs were also received for testing. A further 174 (46%) samples were nasopharyngeal aspirates, 24 (6.3%) were broncho-alveolar lavages, 6 (1.6%) were induced sputum samples, 1 (0.3%) was a CSF and 14 (3.7%) were post mortem tissue samples or swabs.

Sample Processing

Samples received in VTM were vortexed on arrival in the laboratory and treated with antimicrobials prior to inoculation in cell culture. An aliquot of the medium was sent for real-time NASBA testing.

The nasopharyngeal aspirates, broncho-alveolar lavages and sputum were washed in phosphate buffered saline (PBS) to remove excess mucus and centrifuged for 5 min at 1,000g to pellet any cells. The washing and centrifugation procedure was repeated three times, before the cells were finally re-suspended in 100 µl of PBS. Small aliquots of the cell suspension were spotted onto a slide, air-dried and fixed in acetone ready for direct IF testing. A further aliquot of the cell suspension was put into virus transport media for cell culture and NASBA testing.

The post mortem tissue samples were dispersed into cell culture media, and frozen overnight at -20°C , prior to being centrifuged. The supernatant was used for inoculation into cell culture and for real-time NASBA testing. Twelve of the post mortem samples received were swabs taken from the lung, these were treated in the same way as respiratory swabs in VTM.

Direct IF

Samples received for direct IF were tested initially using the Simulfluor[®] respiratory IF screen (Light Diagnostics[™], Chemicon Europe Ltd., Chandlers Ford, Hampshire, UK) following manufacturers' instructions. Using this assay, RSV could be differentiated rapidly from adenovirus, influenza A, influenza B, PIV1, PIV2 and PIV3 by the use of monoclonal antibodies coupled to different fluorescent dyes.

Samples positive by IF but negative for RSV were tested further using individual monoclonal reagents (Imagen[™], Dakocytomation Ltd., UK) directed against adenovirus, influenza A, influenza B, PIV1, PIV2, and PIV3.

Cell Culture and Serology

All of the samples received for virus isolation were inoculated into pMK, MRC5, and HEp-2 cell lines. Soon after the start of the evaluation, pMK cells were substituted by PLC cells (human liver hepatoma cells, Alexander cell line [kindly provided by HPA Cambridge laboratory]) in an attempt to improve the influenza isolation rate.

The inoculated cells were incubated at 37°C on a rolling drum for 14 days and inspected on alternate days for evidence of a CPE. The pMK/PLC cells were treated further with human 'O' type red blood cells and checked for evidence of hemadsorption that might indicate the growth of influenza or parainfluenza (PIV), confirmation of a positive CPE, or hemadsorption was by direct IF.

The acute and convalescent serum samples received from patient group 1 and any from patient group 2 were screened for respiratory viruses, *Mycoplasma pneumoniae* and *Chlamydia spp.* by complement fixation test (CFT).

Influenza A Sub-Typing

Samples positive for influenza A by any method were sent for further typing studies at the Enteric, Respiratory and Neurological Virus Laboratory (ERNVL), Health Protection Agency (HPA), Colindale, London. Influenza A sub-typing was performed by either the hemagglutination inhibition assay or by direct sequencing of the influenza hemagglutinin and neuraminidase genes.

Statistics

The Kappa statistic comparing the three assays for agreement was calculated using the online Javastat package [www.statistics.com/content/javastat.html, accessed March 15, 2004] and the software package GraphPad InStat[®] version 3.05 for windows (Graphpad software, San Diego, CA) was used to determine sensitivity, specificity, and positive and negative predictive values. Cell culture and direct IF were each used as gold standards to compare the NASBA results against.

RESULTS

Optimization of the Real-Time NASBA Assay

Final reaction conditions for the real-time NASBA assay were determined as 70 mM KCl and 0.1 μ M molecular beacon with an amplification time of 90 min. These conditions gave consistently robust kinetic curves and good raw fluorescent signals for positive samples using the least amount of KCl and molecular beacon. All of the clinical samples in the assay evaluation studies were tested using these conditions.

Sensitivity and Specificity of the Real-Time NASBA Assay

The sensitivity of the influenza A real-time assay was found to be within the range of 0.1–0.01 TCID₅₀ virus input and 10–100 copies of synthetic RNA, example results are given in Figure 2.

No signal above background was detected in this assay with the samples from the cross-reactivity panel (data not shown). This primer set and molecular beacon led to specific detection of influenza A. Overall, the average raw fluorescence signal (the level at which the sigmoid curve generated by the software reaches a plateau) for positive clinical samples was 3.5 and the average baseline signal (together with the absence of a sigmoid curve) for negative samples was 1.1 allowing for clear discrimination between a positive and negative result in the assay. On the basis of this, the cut-off threshold for a positive result was defined as 20% above the negative control wild-type signal; this threshold value was on average around 1.3.

ASSAY EVALUATION

Results From Patient Group 1

Following 14 days incubation, no viruses were isolated from the inoculated cell lines. However, the real-time

influenza A assay was positive for 7/11 (64%) samples tested, indicating that influenza A was the cause of the outbreak. The results of the real-time NASBA assays were confirmed by serology. Six of seven patients from whom acute and convalescent sera were obtained had evidence of a greater than fourfold increase in antibody titer and all were shown to have influenza A by real-time NASBA. The seventh patient followed serologically was negative for influenza A by NASBA and had no evidence of an increase in antibody titer (Table III).

One sample from the outbreak was sent for typing by RT-PCR and sequencing. The virus causing the outbreak was shown to be an A/Panama/2007/99-like H3N2 virus.

Results of Patient Group 2

In total, 367 (97%) samples were tested by both cell culture and real-time NASBA; of these samples, 158 (43%) also had an additional direct IF test. The remaining 11 (3%) samples were tested only by real-time NASBA.

Two hundred seventy-six samples (73%) were negative for influenza A by all of the methods used, and viruses other than Influenza A were isolated from a further 6 (2%). Overall, 96 (25%) samples from 87 patients were positive for influenza A by one or more methods. One sample shown to be positive by both immunofluorescence and cell culture was repeatedly negative for influenza A by real-time NASBA; in addition, influenza A was not detected in the sub-typing assays performed on this sample in the reference laboratory.

The highest influenza detection rate of 38% (9/24) was in broncho-alveolar lavage samples, although the less invasive respiratory swab samples had a comparable detection rate of 33% (52/159). Perhaps, surprising was the relatively low detection rate of 19% (34/174) seen in

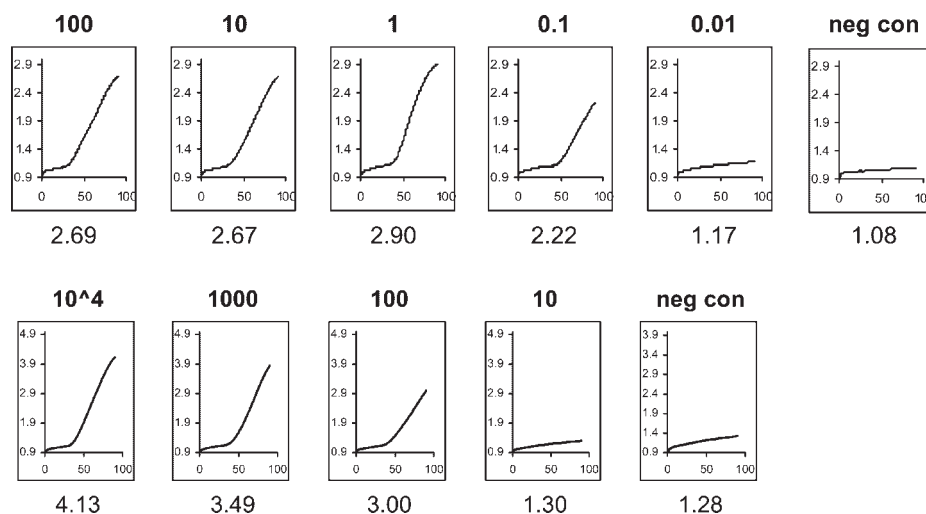


Fig. 2. Influenza A real-time NASBA results. Influenza A virus (H1N1) titer. Influenza A virus (H1N1) synthetic RNA (RNA copy number). The x-axis indicates the time in minutes and the y-axis represents the raw fluorescence signal. The virus input or RNA copy number input into the reaction is listed above each graph. The wild-type fluorescence signal reached is indicated beneath each figure.

TABLE III. Results of Patient Group 1

Patient	Age	Influenza A real-time NASBA result	Serology titer (acute-convalescent)
1	81	Detected	<8-128
2	80	Detected	<8-64
3	89	Not detected	<8-<8
4	84	Detected	<8-128
5	91	Not detected	No convalescent sample
6	89	Detected	<8-512
7 ^a	76	Detected	No convalescent sample
8	79	Not detected	No convalescent sample
9	82	Detected	<8-512
10	86	Detected	<8-128
11	N/K	Not detected	No convalescent sample

All samples taken were nose and throat swabs. No viruses were isolated from any of the samples in cell culture. All serology samples demonstrated a fourfold increase in titer.

^aPatient died.

nasopharyngeal aspirate samples particularly as 77% (134/174) were tested by all three methods. One post mortem lung swab had detectable influenza A RNA by NASBA.

Of the 96 samples positive for influenza A, 27 were isolated in cell culture, 20 were positive by direct IF, and 95 had influenza A RNA detected by real-time NASBA. Figure 3 summarises how results for the different assays correlate.

The Kappa statistic for NASBA versus culture was 0.35 (0.27-0.37; 95% CI) indicating that there was poor agreement between the two tests. The sensitivity and specificity of the real-time NASBA versus cell culture were 96% (79.9-99.8; 95% CI) and 80% (78.7-80.3; 95% CI), respectively. The negative predictive value of the NASBA assay was good at 0.99 (0.98-0.1; 95% CI). However, the positive predictive value of 0.27 (0.22-0.28; 95% CI) was reduced by the low number of infections detected by cell culture. Of the 367 samples

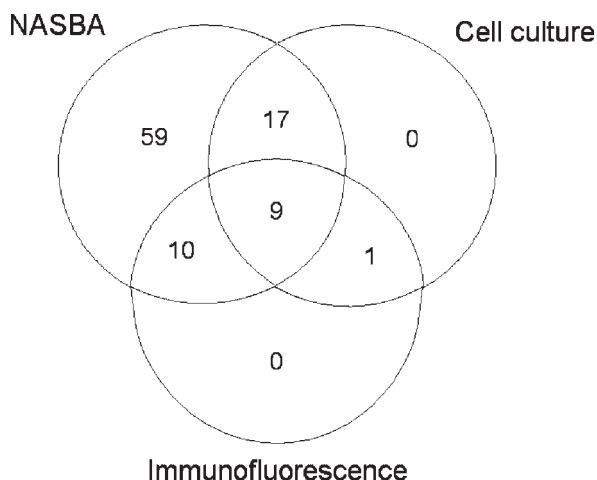


Fig. 3. Positive results by assay (patient group 2).

tested by culture and NASBA, only 7% were positive by both methods with an additional 19% being detected by NASBA alone.

The correlation between NASBA and IF was much better, with a Kappa statistic of 0.64 (0.47-0.68; 95% CI) indicating fair to good agreement between the two tests. The sensitivity and specificity of the NASBA assay compared to IF was 95% (75.5-99.7; 95% CI) and 89% (86.3-89.8; 95% CI), respectively. The negative predictive value was 0.99 (0.96-0.1; 95% CI) and the positive predictive value was 0.56 (0.44-0.59; 95% CI) reflecting the additional 9% of samples detected by NASBA alone over the 12% detected by both NASBA and IF.

The hemagglutinin and neuraminidase genes from the influenza virus detected in seven samples were sequenced and sub-typed from different demographic regions across Wales. All were shown to be A/Fujian/411/2002-like H3N2 viruses. Three of these samples were positive by the real-time NASBA assay alone. Unlike group 1 patients, follow-up serology was rarely performed for patients in group 2. Therefore, serology results were only available for three positive samples and for none of the negative samples. Of these, all three showed a single high influenza A titer, although two had only been positive by real-time NASBA previously. These results suggest that compared to the other traditional techniques, real-time NASBA was more sensitive for detection of influenza A.

Overall, 25% of all samples submitted for influenza testing were positive by one or more methods. The rate of influenza infection overall in each age group is summarized in Figure 1. The highest influenza rates seen in this study population were at either ends of the age spectrum mirroring the high number of positive samples received from hospital in-patients (71% [60/84]). It was in the extremes of age that hospitalization due to an influenza-like illness (ILI) was more commonly seen. In particular, a high proportion of children of less than 1 year of age were admitted to hospital during the study period and it was in the 0-4 age group that the highest rate of influenza A occurred with 18 cases per 100,000 of the total population.

Of the hospitalized patients, 13% (11/58) were diagnosed with influenza A whilst on an intensive care unit (ICU). Again, the majority of the patients admitted to the ICU were from the 0-4 year age group (45% [26/58]). Of these, 11.5% (3/26) were found to have influenza A. This, however, was much lower than the incidence seen in the 50-79 age group where 40% (8/20) of those admitted to ICU were shown to have influenza A.

Most of the samples received from the 5-64 age groups came from primary care centers and general practitioner (GP) influenza spotter practices in the community; in this cohort, the incidence rate was 1.66 cases per 100,000 of the population.

In patient group 2, oseltamivir was used successfully on a number of patients including one patient who had an underlying hematological malignancy. During her oseltamivir treatment, serial nose and throat swabs

TABLE IV. Example Results of Patient Sampled During Oseltamivir Treatment

VH, 42 years old, myelodysplastic syndrome				
Date	Sample	Flu A result IF	Flu A result NASBA	Oseltamivir
October 19, 2003	NPA	Positive	Positive	No
October 27, 2003	NPA	Negative	Positive	No
October 29, 2003	NPA	Positive	Positive	No
November 3, 2003	Nose/throat swab	N/A	Negative	Yes
November 5, 2003	Throat swab	N/A	Negative	Yes
November 10, 2003	Throat swab	N/A	Positive	No

IF, immunofluorescence; NPA, nasopharyngeal aspirate; N/A, not applicable.

A third throat swab was taken between November 5, 2003 and November 10, 2003 during treatment that was also negative for influenza A. It was following this result that treatment was stopped. Treatment was restarted following the swab taken on November 11, 2003.

were taken to monitor viral shedding (Table IV). Whilst on treatment, she stopped shedding virus and following three negative swabs, her treatment was stopped. A subsequent swab taken 2 days following treatment cessation showed that she was once again shedding virus and her treatment was restarted. Unfortunately, further swabs were not taken, following the re-initiation of her treatment but she eventually made a full recovery.

DISCUSSION

Real-time NASBA was shown to be a rapid, easy to use kit-based molecular method, and a good alternative to RT-PCR techniques. The evaluation demonstrated it to be sensitive and specific for the detection of influenza A in a wide range of respiratory samples. Compared to traditional methods, the NASBA assay detected higher numbers of influenza A cases overall.

The outbreak of influenza A described in patient group 1 occurred in an elderly population towards the end of a season with little documented influenza. During the outbreak investigation, it was noted that 79% (46/58) of the residents had been immunized against influenza during the previous autumn. There was some delay in sampling the patients during the outbreak, as the first cases were not reported immediately to the local consultant in communicable disease control (CCDC) and due to the rural location where the outbreak occurred, there was also some delay in receiving the samples in the laboratory. Consequently, none of the nose and throat swabs taken yielded influenza A in cell culture, probably due to a loss in viral infectivity, as the influenza A strain identified as causing the outbreak readily grows in routine cell lines. It is likely that if traditional laboratory techniques were used alone, the cause of the outbreak would not have been known until the results of the serology became available. By utilizing the real-time NASBA assay, viral RNA could still be detected in the samples and the results were available within 24 hr of sample receipt in the laboratory providing the opportunity for some prophylactic intervention.

The need for molecular techniques for the diagnosis of influenza virus was highlighted during the 2003/2004

influenza season. From sequencing studies, the strain of virus causing most infections in the UK was the drifted Fujian strain of H3N2 influenza A. The pattern of disease was typical of that observed for other influenza epidemics [Treanor, 2004]. This included an early start to the season and an increased number of very young children being severely infected due to a lack of prior exposure to other H3N2 viruses. There were significant difficulties in diagnosing the virus effectively using cell culture techniques, as the virus did not grow well, this meant that early infections during the season were probably missed. This feature of the virus is reflected in the data presented for patient group 2 with the low number of influenza isolations seen overall compared to the number of samples found positive by real-time NASBA. As a result a better correlation between cell culture and the real-time NASBA assay may be achieved during a year when a different influenza A virus circulates.

Most of the discrepant results seen in the evaluation were due to samples shown to be positive for influenza A by real-time NASBA but negative by the traditional laboratory techniques. One sample was, however, found to be negative by NASBA on repeat extraction and testing despite having positive IF and cell culture results. This discrepancy may be due to the presence of an inhibitor in the sample affecting the molecular assays used. Overall, the inhibition rates are not known for each sample type included in the evaluation and the only way to determine this and to ensure that the assay is performing optimally is to develop and include an internal control with each sample extraction and amplification.

Confirmation of clinical samples found to be positive by NASBA but negative by other methods was only carried out for 17% (12/67) of the samples overall and the methods included RT-PCR and sequencing (four samples) and serology (eight samples). As all were confirmed, a high level of confidence was given to a NASBA positive result. With the absence of an internal control, however, such confidence cannot be afforded to a negative result, although as demonstrated only one positive sample seemed to be missed by the real-time

NASBA assay and in patient group 1, a negative result was confirmed by serology giving some degree of confidence.

An observation made during the evaluation was how the introduction of sensitive molecular techniques can impact on epidemiological surveillance. For example, during the 2003/2004 season, data from primary care centres in Wales remained well below normal activity for influenza consultations [www.cdsc.wales.nhs.uk, accessed March 17, 2004]. However, during the same period, the laboratory data produced a different picture with more influenza being detected than in the three previous seasons due to the introduction of the NASBA assay. This was particularly important in terms of the National Institute of Clinical Excellence (NICE) treatment guidelines that state that oseltamivir should only be prescribed if there is evidence of influenza circulating in the community.

As a result of the NASBA assay, many patients were prescribed oseltamivir during the evaluation, including the patient described previously. With this patient in particular, the pattern of results observed demonstrates the mode of action of drugs like oseltamivir in suppressing viral spread to allow the immune system to recognise and 'mop up' infected cells and also suggests that immunocompromised patients should be given longer treatment regimens of antivirals to ensure that all virus is cleared before the treatment is stopped.

Traditional techniques, such as cell culture and IF, still play an important role in the diagnosis of respiratory infection particularly in terms of the much lower cost involved in providing these services. However, with the emergence of SARS and avian influenza in the Far East, it has become imperative that rapid, sensitive, and specific techniques are developed to provide a differential diagnosis for common respiratory viruses in the community. For example, the influenza outbreak investigated (patient group 1) occurred during a time when Britain was on high alert for the importation of the SARS virus. By providing a rapid diagnosis using molecular assays such as the real-time NASBA, outbreaks such as this can be investigated quickly helping in part to allay any fears regarding more sinister causative agents. In addition, there is the potential of taking a nose or throat swab and putting it directly in lysis buffer. In this way, the sample can be more safely transported to the laboratory for testing, as any virus present will be rendered non-infectious.

A molecular technique that targets a conserved gene, such as the influenza nucleoprotein gene, will perform well despite antigenic changes in variable genes and many different strains including avian influenza should be detected easily. The disadvantage of this approach is that sub-typing the detected virus is impossible and further techniques, such as sequencing of the HA and NA genes, are needed to confirm the strain. NASBA also has its limitations in that the products of amplification cannot be sequenced further to detect mutations that might give rise to antiviral resistance. However, by being able to detect such a broad range of influenza A

strains in a single assay is extremely useful in screening large numbers of clinical samples in a routine diagnostic setting.

In conclusion, this study has demonstrated the clinical use of the real-time NASBA to enhance both the detection and surveillance of influenza A in both hospital and community populations.

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