

**The Development And Implementation Of A
Molecular Technology Based Service For
The Diagnosis Of Respiratory Viral
Infection In Wales**

*A Submission for the Award of
PhD by Published Work*

to

University of Wales Institute, Cardiff

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ABSTRACT

The manuscripts submitted in this thesis describe the early development and implementation work for a molecular technology based service for the diagnosis of respiratory viral infection in Wales. The service allowed for a greater understanding of the spectrum of disease caused by respiratory viruses as molecular methods were proven rapid and sensitive when compared to traditional laboratory methods. This work transformed the understanding of respiratory virus infection in both vulnerable and healthy patients in the community and hospital setting and eventually informed on the most appropriate way to deliver a 21st century respiratory virus diagnostic service. Implementation of the service has had a direct impact on public health by improving the detection and monitoring of respiratory virus outbreaks and by providing information regarding the circulation of respiratory viruses in the community. The service in Cardiff became part of a network of laboratories that responds to new and emerging respiratory viral infections across the UK and the expertise gained proved instrumental in the Welsh laboratory response to the influenza A (H1N1) 2009 virus pandemic and the issues that arose as a direct consequence including an oseltamivir resistant outbreak. Ongoing developments in the field of molecular diagnostics together with the increasing repertoire of respiratory viruses means that the service is constantly improved to ensure that Wales continues to have a first-class service for the diagnosis of respiratory viral infection.

FOREWORD

Respiratory viruses contribute to significant morbidity and mortality in both healthy and vulnerable individuals. Influenza A virus has been pivotal to respiratory virus research, health planning, and the development of treatment and vaccination. This has been due to its ability to undergo major genetic change leading to the emergence of pandemic strains. Further to this, the zoonotic transmission of influenza A (H5N1) in 1997 and SARS in 2003 focussed global attention upon novel respiratory viral infections and how they were diagnosed and treated. Increasingly it was shown that molecular techniques could provide sensitive and specific detection for respiratory viruses. Importantly, this work led to change in the way all respiratory viruses are diagnosed in the routine clinical diagnostic laboratory.

The body of work presented in this thesis describes the development and implementation process by which the respiratory virus diagnostic service for Wales evolved from one which was delivered using traditional laboratory techniques, to one using entirely molecular techniques.

The European Union Framework 5 Grant

Initial work for the development of the molecular assays was undertaken through a European Union (EU) Framework 5 grant obtained by the Microbiology department of Cardiff University in 2001. The grant facilitated the development of molecular based assays for the diagnosis of respiratory pathogens as causative agents of community acquired pneumonia (CAP). Assays based on real-time reverse transcription polymerase chain reaction (RT-PCR) techniques were developed in Leiden University in the Netherlands, whilst

Cardiff University used the novel Nucleic Acid Sequence Based Amplification (NASBA) technique with technical support from the research and development team at Organon Teknika (now BioMérieux). It became clear as the assays were developed that transfer of the technology to the Molecular Diagnostic Unit within the Wales Specialist Virology Department, Cardiff should be undertaken, so that the assays could form the basis of a routine molecular service for the diagnosis of respiratory viruses.

The Cardiff Molecular Diagnostic Unit

By 1999, a limited number of routine molecular assays were performed by the then Public Health Laboratory Service (PHLS) Microbiology Service in Cardiff, often with direct involvement from Cardiff University. Most were assays developed by commercial companies and available as 'off the shelf' kits, but as these were the early days of molecular diagnostics, they were inherently complex in design and delivery. This problem increased with the introduction of in-house molecular assays where primers and probes for the detection of target pathogens were designed within the laboratory. It was during this time that a central molecular diagnostic unit within the Cardiff PHLS laboratory was envisaged, which would be headed by a clinical scientist with previous experience of molecular assay design, development, and routine diagnostics. Not only would viral targets be brought under the umbrella of the unit but also fungal and bacterial targets, thus making it one of the first routine molecular diagnostic laboratories in the UK staffed from both Scientific and Technical personnel from all microbiology disciplines. As well as transferring the already implemented kit based assays, in-house PCR and NASBA assays to the unit, real-time PCR assays using Lightcycler technology were also introduced. Cardiff became the second laboratory outside the reference laboratory in Manchester to

offer routine molecular diagnosis of meningococcal disease. Later the work towards the development of novel assays for the diagnosis of invasive fungal disease was also brought under the umbrella of the molecular unit, so broadening even further the remit of the unit.

The technology used in molecular diagnostics evolved very quickly during the early part of the new century; this included the expansion of the real-time technologies. It was into this background of rapid change in molecular diagnostics that the respiratory virus assays were developed in Cardiff and introduced into the unit.

The work included in this dissertation comprises the early development work undertaken whilst the candidate was seconded to Cardiff University and demonstrates the process of technology transfer to the Molecular Diagnostic Unit through to service delivery. It also explains how, in light of the first influenza pandemic of the 21st Century, the service has evolved to become an essential component of laboratory service provision.

Acknowledgements

In 1994 I hand-wrote my CV on a piece of paper and sent it to the Microbiology Department at the University Hospital of Wales hoping to get a summer job. I received a reply a week later from Norman Bishop inviting me for an interview with Dr Diana Westmoreland. The career journey that letter has taken me on has been amazing and along the way I met some of the most knowledgeable and inspiring people in the field of virology.

I am very grateful for the advice from Professor Rose Cooper and Dr Lalage Sanders in the construction and content of this thesis and particularly to Rose for starting my interest in microbiology in the first place.

I have acknowledged the work of others throughout the manuscripts submitted as part of this thesis and include all of my co-authors. There are people who should be highlighted as they played an important role in my career progression and consequently in the development of the respiratory virus molecular service.

I was trained to perform the traditional laboratory techniques described in this thesis by Ian Phillips and Brian Mitchell. Norman Bishop provided me with a research project for my BSc degree in respiratory viruses and therefore seeded my interest in this group of viruses. I am particularly grateful to Dr Julie Fox and Dr Sam Hibbitts who allowed me to work with them on the EU Framework 5 project and taught me almost everything they knew about assay development and NASBA. The research and development team in Organon Teknika and BioMérieux supported the transfer of the assays into routine diagnostic service with the help of Pierre van Aarle. In terms of expanding my knowledge beyond the laboratory and into epidemiology I am grateful to Dr Daniel Thomas, Dr Roland Salmon and Dr Simon Cottrell for their support with the community

surveillance of respiratory viruses. Dr Mark Hastings and Dr Eleri Davies invited me to be involved with the outbreak control team during the oseltamivir resistant virus outbreak and I learned a great deal. The support from the laboratory staff during the pandemic is appreciated far more than they realise, Joanne Watkins, Myfanwy Clark, Bree Wilcox, Sara Summerhayes, Michael Perry, Dr Lewis White and the staff who supported from virology and bacteriology are acknowledged for providing a service under very difficult circumstances and for being so patient when I changed the assays halfway through the outbreak.

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My greatest inspiration is my daughter Amy for whom that letter was written so that I could provide for her and then inspire her to achieve her full potential too.

CRITICAL ANALYSIS

The body of work submitted and discussed here demonstrates the positive impact that the development and implementation of a molecular based routine diagnostic service for the detection of respiratory viruses has had on the understanding of respiratory viral disease and patient management in Wales. It was conducted using retrospective studies on well-defined clinical samples, and prospectively with direct impact on patient management.

The content highlights three key points:

1. The complexities of developing novel assays for viral targets that naturally undergo genetic change and for which many types and sub-types exist;
2. The increased detection rate of respiratory viruses using molecular techniques over routine traditional techniques helping to increase our understanding of respiratory viral infection in both healthy (through community surveillance) and vulnerable Welsh patients;
3. How the respiratory virus service has responded to emerging respiratory pathogens, new developments in respiratory virus diagnosis and the rapidly changing field of molecular diagnostics.

1. An Evaluation of the Field of Study

To demonstrate comprehensively the contribution that this research has made to the field of respiratory virus diagnosis and patient management in Wales, it is important to evaluate the status of knowledge about respiratory viruses and to document methods available for respiratory virus diagnosis prior to the

introduction of routine molecular assays. This evaluation will also explain how novel molecular techniques were already beginning to impact on our understanding of respiratory viral infection prior to the development and implementation of the molecular diagnostic service in Wales.

1.1 An overview of Respiratory Viruses

Respiratory viruses are defined as viruses that cause infection that is restricted to the surface of the respiratory tract. Transient but limited viraemia probably occurs. There is limited evidence to suggest that prolonged viraemia may indicate an increase in disease severity, but viral seeding and infection of compartments outside of the respiratory tract is uncommon (Tsuruoka *et al.*, 1997; Xatzipsalti *et al.*, 2005). The clinical syndromes associated with respiratory viral infection ranges greatly from mild upper respiratory tract symptoms (e.g. rhinorrhoea, pharyngitis, or typical common cold symptoms) through to lower respiratory tract infections such as bronchitis and pneumonia. Morbidity and mortality associated with respiratory virus infection is considerable and by the 1990's most of the viruses associated with significant respiratory disease had been identified with most laboratory diagnostic techniques optimised for detecting them routinely (Kilbourne 1992). The respiratory viruses detected using traditional laboratory methods in a routine screen belong to four virus families, the *Orthomyxoviridae* (influenza types A and B), the *Paramyxoviridae* (respiratory syncytial virus (RSV) and the parainfluenza viruses' types 1-3), and the *Adenoviridae* (adenoviruses) and in some laboratories the *Picornaviridae* (rhinoviruses). A complete understanding of the role that each respiratory virus had on the burden of clinical disease was limited in part by the laboratory methods used to isolate and detect them during the acute phase of illness. Rhinoviruses, for example, were primarily associated

with upper respiratory tract infections due to the conditions under which they grew best in cell culture and also because of the infrequency in which they were detected in samples from the lower respiratory tract (Winther *et al.*, 1986). RSV was associated with epidemic disease in the infant with rare reports of severe adult infection (Glezen *et al.*, 1980; Hijazi *et al.*, 1996; Horn *et al.*, 1979). Influenza was associated with a more severe clinical course affecting both healthy as well as high-risk patients (Assaad and Cockburn 1974; Ghendon 1991; McAnerney *et al.*, 1994; Snow, Jr., 1969).

1.1.1 Influenza Viruses

There are three antigenically distinct influenza virus types A, B and C with influenza types A and B considered clinically the most important of the respiratory viruses. The compelling arguments for this have been discussed widely in scientific publications since the first isolation of influenza A virus in the ferret model in 1933 (Easterday 1980; Frank 1982; Knight 1976). Two main factors make influenza viruses unique from the other respiratory viruses. Firstly, they possess a segmented RNA genome, and secondly influenza A has wide host range (Shope 1958; Stockton and Zambon 1999; Stuart-Harris & Schild 1976).

Point mutations accumulate in the viral genome of influenza viruses due to an error prone RNA polymerase based replication cycle. When a number of these changes occur in the genes encoding for the antigenic surface proteins haemagglutinin (HA) and neuraminidase (NA), antigenic drifting is said to have occurred. Prior exposure to closely related influenza viruses to a drifted influenza strain can attenuate any infection so outbreaks that do occur tend to be limited to the very young with no prior exposure, unvaccinated individuals,

and those with some degree of immunodeficiency (Knight 1976; Pereira 1980; Stuart-Harris & Schild 1976; Zambon 1999).

Influenza B viruses exist in two lineages Yamagata and Victoria and are predominantly human pathogens with just one report of influenza B being found in seals (Chakraverty 1971; Kloene *et al.*, 1966; Osterhaus *et al.*, 2000; Schild *et al.*, 1973). The severity of influenza B disease is considered less than that caused by influenza A. However, there is a propensity of published case reports associating influenza B infection with Reye's syndrome (in association with aspirin use) (Corey *et al.*, 1976; Davis *et al.*, 1985; Reynolds *et al.*, 1972), myositis and central nervous system complications (Bayer 1987), as well as severe disease associated with primary infection (Baine *et al.* 1980; Luksza and Jones 1984; Malpas and Stanley 1990; Troendle *et al.* 1992). Therefore, the disease burden associated with influenza B should not and cannot be underestimated.

Influenza A is sub-typed based on the serotype of the HA and NA spike proteins of which 16 HA types and 9 NA types have been identified (Stockton and Zambon 1999; Zambon 1999). The reservoir for most strains of influenza A are aquatic birds who remain asymptomatic but shed high titres of virus in their faeces that can contaminate land with virus that is readily transmitted to poultry. Once infected, most poultry species become symptomatic with the severity of the symptoms correlating to the pathogenicity of the influenza A sub-type with which they are infected. These can be highly pathogenic avian influenza (HPAI) types or low pathogenic avian influenza (LPAI) types (Alexander and Brown 2009; Lupiani and Reddy 2009; Shoham 2006). Strains of influenza A that preferentially infect humans are limited to one of three HA sub-types H1, H3 and H2 viruses. The binding site for these viruses is specific to

human cells in the respiratory tract expressing the sialic acid α 2,6 receptor. Avian strains specifically bind to the sialic acid α 2,3 receptor which is expressed only on human cells deep in the lower respiratory tract and on the epithelial mucosa of the eye making direct transmission to humans difficult but not impossible as seen in recent H5 and H7 outbreaks (Davison *et al.* 2003; Osterhaus 2008).

The segmented nature of the influenza A genome makes it possible for viruses to exchange or re-assort gene segments in a process called antigenic shift. Successful antigenic shift results in a new virus that the host population has no prior immunity towards, resulting outbreaks can occur widely in the population at a global or pandemic level (Pereira 1980). The original hypothesis for the mechanism of antigenic shift put swine in a pivotal position due to the presence of both avian and human influenza A receptors in the swine upper respiratory tract (Dowdle and Hattwick 1977; Easterday 1980). Pandemic strains of influenza A were therefore thought to emerge in areas where humans, pigs, and birds live in close proximity to each other. It is now recognised that human infection with avian strains can occur directly and therefore dual infection with human and avian strains may offer an environment for re-assortment. An alternative hypothesis suggests that evolutionary pressure within the human host could directly select for avian viruses that readily bind to receptors of cells of the upper respiratory tract in humans allowing for onward transmission (Adungo *et al.* 2005; Easterday 1980; Osterhaus 2008). There have been three influenza A pandemics recorded in the 20th Century (H1N1 1918; H2N2 1957; H3N2 1968) and despite concerns about H5N1 virus and its ongoing global circulation, the first pandemic of the 21st Century was a triple reassorted H1N1 virus that emerged in Mexico during 2009. The virus was largely associated with

mild illness in the majority of infected individuals but severe sometimes fatal lower respiratory tract infections in young, previously healthy individuals and pregnant women was a key feature of the pandemic (Adungo *et al.*, 2005; Alexander & Brown 2009; Hamilton 2009).

Prevention of influenza infection is offered through annual vaccination. The seasonal influenza vaccines used in the UK are subunit vaccines containing two currently circulating strains of influenza A H1 and H3 and one of the two lineages of influenza B Victoria or Yamagata (Morgan *et al.*, 2001). The strain of influenza viruses included in the vaccine varies year upon year based on viruses known to be circulating in the Northern and Southern hemisphere. Adjustments are made annually to take into account the genetic drifting the viruses undergo, requiring continuous surveillance of the global circulation of influenza viruses through national community surveillance schemes and laboratory networks (Ellis *et al.*, 1999; Frank 1982; Ghendon 1991; Szecsenyi *et al.* 1995). It is important therefore, that routine laboratory techniques for the detection of influenza are sensitive and specific for all strains of influenza known to infect man.

1.1.2 Respiratory Syncytial Virus

Compared to the sporadic nature of influenza pandemics, RSV is one of the few respiratory viruses with clear annual seasons. It is the most common cause of hospitalisation due to respiratory infection of infants, affecting between 1-2% of the infant population in Wales annually in epidemics that peak between November and March (Brooks *et al.*, 1999; Nicholson 1996; Winter and Inglis 1987). Nosocomial transmission is common during these epidemics causing significant problems on paediatric units, on wards for vulnerable adults and in

nursing homes caring for the elderly (Englund *et al.*, 1991; Falsey and Walsh 2000; Hall *et al.*, 1975; Hall 1981; Hall 2000; Mlinaric-Galinovic *et al.*, 1996) .

There are two sub-types of RSV, A and B, which co-circulate in the population. The severity of disease associated with each sub-type has long been a subject of conjecture with RSV type A more often associated with severe disease than RSV type B (Coggins 1998; Hall *et al.*, 1990; Stockton *et al.*, 1998; Walsh *et al.*, 1997) . However, most routine laboratory methods do not differentiate between the two types.

The role of RSV in adult infection has been significantly under-estimated in the past, particularly in healthy adults. Nevertheless, an increasing number of studies have been published that demonstrate RSV as a cause of viral pneumonia and other severe complications in immunocompetent adults (Connolly, Jr. *et al.*, 1994; Crowcroft *et al.*, 1999; Falsey *et al.*, 1996; Falsey *et al.*, 1999; Mlinaric-Galinovic *et al.*, 1996; Teichtahl *et al.*, 1997). An epidemiological study performed over a 15 year period in the UK published in 1996, whilst acknowledging the considerable burden of influenza during inter-pandemic years, estimated that the annual RSV season contributed to approximately 22000 - 23000 excess deaths annually, which was 60-80% greater than influenza over the entire study period (Nicholson 1996). RSV has been considered an important respiratory virus in terms of disease burden and hospitalisation rates. This fact is reflected in a number of reviews published throughout the 1990s and in the testing repertoire of routine clinical laboratories (Anderson and Jordan 1990; Crowcroft *et al.*, 1999; Englund, Anderson, & Rhame 1991; Falsey 1991; Falsey 1998; Hall *et al.*, 1990; Hall 1999; Long *et al.*, 1997; Mlinaric-Galinovic *et al.*, 1996; Teichtahl *et al.*, 1997; Walsh *et al.*, 1999; Whimbey *et al.*, 1995).

1.1.3 Parainfluenza

Four types of human parainfluenza viruses (hPIV) have been identified belonging to two sub genera of the *Paramyxoviridae* family. Types 1 and 3 belong to the *Respirovirus* genera and types 2 and 4 (4a and 4b) belong to the *Rubulavirus* genera (Henrickson 2003; Vainionpaa and Hyypia 1994). Due to the inherent difficulty in isolating hPIV type 4 virus from clinical samples its role in severe clinical disease has been hard to ascertain (Lindquist *et al.*, 1997; Tyrrell and Bynoe 1969). Most significant disease had been associated with hPIV types 1-3, which is reflected in the tests offered by most routine laboratories (Echevarria *et al.*, 1998). As with other respiratory viruses the greatest disease burden associated with hPIV was shown to be in infants, and it was considered second to RSV as a significant cause of LRTI and hospitalisation in young children (Easton and Eglin 1989; Glezen *et al.*, 1984; Henrickson *et al.*, 1994; Knott *et al.*, 1994; Marx *et al.*, 1997; Reed *et al.*, 1997; Welliver *et al.*, 1986). The role of hPIV in adult respiratory tract infection has been poorly described, although during the 1990s outbreaks of hPIV type 3 were regularly reported amongst patients with haematological malignancies, often associated with long-term viral shedding (Easton & Eglin 1989; Love and Suskind 1961; Marx *et al.* 1999; Whimbey *et al.* 1993). The seasonality of hPIV is less well defined than RSV with summer and autumn epidemics common. There is some evidence of hPIV types 1 and 2 having biennial epidemics with hPIV type 3 epidemics occurring most years (Easton & Eglin 1989; Henrickson, Kuhn, & Savatski 1994; Hope-Simpson 1981; Knott, Long, & Hall 1994; Laurichesse *et al.*, 1999; Marx *et al.*, 1997). As a respiratory virus the impact of disease caused by hPIV beyond the young child was investigated infrequently, as demonstrated by the published literature prior to 1999, therefore there was still much to

determine about the role this virus played on overall morbidity and mortality at the start of the new century.

1.1.4 Adenoviruses

Unlike the other important respiratory viruses, adenoviruses have a DNA based genome. They were first isolated in 1953 from adenoid tissue by Rowe and then a year later they were isolated from troops presenting with a febrile illness (Rowe *et al.*, 1957; van, V *et al.*, 1969). Adenoviruses are ubiquitous in nature with human adenovirus belonging to the *Mastadenovirus* genera; by 2010 there were 53 serotypes described belonging to species A-G (Smith *et al.*, 2010). The diversity of the adenoviruses is therefore comparable to that of the RNA respiratory viruses. Respiratory illness caused by adenoviruses has been predominantly associated with the lower numbered serotypes due the ease to which they were initially isolated from respiratory samples (Falsey 1991; Lehtomaki *et al.*, 1986; Rowe *et al.*, 1957). Severe respiratory illness has been described in infants, young children and the immunocompromised, although notably extensive outbreaks are well documented in military personnel predominantly with adenovirus types 3, 4, 7 and 21 (Hierholzer 1992; Rubin 1993a; van, V *et al.*, 1969). Whilst adenoviruses are unquestionably respiratory pathogens, adenovirus disease can involve other tissue and organ systems with viraemia, asymptomatic and persistent infection commonly reported (Evans 1958; Hierholzer *et al.*, 1975; Hierholzer 1992; Rubin 1993; Sanchez *et al.*, 2001; Smith *et al.*, 2010). Detection of adenovirus in certain sample types therefore, is not always indicative of the cause of the illness being investigated. As a respiratory pathogen however, it remains an important target in a routine respiratory screening panel.

1.1.5 Rhinoviruses

Of the respiratory viruses, the rhinoviruses are probably the most genetically diverse with over 100 prototype strains identified (Savolainen *et al.*, 2002). As the virus associated as being the primary cause of the common cold, it has subsequently been targeted for the development of antiviral therapy to reduce the significant morbidity associated with infection. Often drug trials were part of volunteer challenge studies that were performed throughout the 1970s and into the 1990s (Al-Nakib *et al.*, 1987; Blair *et al.*, 1976; D'Alessio *et al.*, 1976; Dick *et al.*, 1987; Gwaltney, Jr. *et al.*, 1980; Hayden *et al.*, 1992; Henderson *et al.*, 1988; Panusarn 1974; Soto *et al.*, 1973; Stanley *et al.*, 1975; Stone *et al.*, 1992; Winther *et al.*, 1986). These studies increased the overall understanding of the upper respiratory tract illnesses caused by rhinoviruses as well as trialling potential remedies, however as they were performed on healthy individuals the role of rhinoviruses in severe infection was largely undetermined. Data published in the 1990s began to show some causal link between rhinoviruses and lower respiratory tract infection in certain vulnerable groups of individuals, but limitations in routine diagnosis failed to produce concrete data supporting case reports being published (Chidekel *et al.*, 1994; Halperin *et al.*, 1983; Johnston 1995; Las and Swanson 1983; McMillan *et al.*, 1993; Nicholson *et al.*, 1996; Smyth *et al.*, 1995; Teichtahl *et al.*, 1997; Wald *et al.*, 1995). As a result, in the routine clinical laboratory at the start of the new century rhinoviruses were largely associated with mild upper respiratory tract infections and often were not included in front-line screening assays.

1.1.6 Emerging Respiratory Viruses

Perhaps the most significant recent discovery in the field of respiratory viruses was human metapneumovirus (hMPV) in 2001, which coincided with the period

when the research presented in this thesis was being undertaken (van den Hoogen *et al.*, 2001). The role of hMPV as a significant cause of human respiratory illness has been elucidated in numerous global studies (Christensen *et al.*, 2003; Falsey *et al.*, 2003; Kahn 2003; Kahn 2006; van Burik 2006; van den Hoogen *et al.*, 2004; Weinberg *et al.*, 2010). The data published over the last decade has provided strong evidence for the role of hMPV in contributing to significant respiratory disease in all age groups. Whilst this extensive data is compelling, the discovery of hMPV however, made little impact in the way respiratory viral infection was diagnosed routinely. This contrasts greatly when compared to the global effort made to determine the cause of severe acute respiratory syndrome (SARS) (Kuiken *et al.*, 2003; Murphy 2006) the causative agent of which was rapidly elucidated as being a novel zoonotic coronavirus. Thanks to an unprecedented global effort once discovered, SARS was controlled and eradicated from man following its dramatic emergence from Mainland China to Canada via Hong Kong (Lo *et al.*, 2005; Murphy 2006; Osterhaus 2008). Together with the emergence of avian influenza A H5N1 with its pandemic potential and associated severe infection in humans, the development and constant modification of new laboratory methods for the detection of novel respiratory viruses has increased significantly predominantly to the emergence of these two viruses.

The evidence for causal association in significant respiratory disease for hMPV contrasts greatly with other recent human respiratory virus discoveries such as the presumptive respiratory parvovirus, bocavirus and the respiratory polyomaviruses KI and WU for which limited data is available for a strong association with respiratory infection (Manning *et al.* 2006; Norja *et al.*, 2007). Apart from the expanding coronavirus family for which increasing data is

available to demonstrate a contribution to human disease (Kahn 2007) more evidence needs to accumulate to build a case for the routine screening of the new emerging respiratory viruses.

Therefore, it is only influenza, RSV, hPIV, adenovirus, rhinovirus, and hMPV for which consistently good evidence is published showing the significant burden each has towards respiratory viral disease globally. This thesis will demonstrate how the research studies completed in Cardiff have allowed improved diagnosis and understanding of these viruses.

1.2 Traditional Techniques for the Detection of Respiratory Viruses

Prior to the introduction of molecular methods to detect respiratory viruses in clinical samples, the methods utilised by the two laboratories undertaking routine diagnosis of acute respiratory illness caused by respiratory viruses in Wales (Cardiff and Swansea) included traditional cell culture and direct immunofluorescence. The laboratory protocols used were based on methods published largely in the 1950s and 60s which describe the most sensitive and specific methods available for the detection of viruses during acute and post respiratory infection (Oswald *et al.* 1975). Most of the traditional methods described in this evaluation were routinely used in Wales until the late 1990s. This highlights the slow evolution of laboratory diagnostic methods for detecting respiratory viruses prior to the work submitted in this thesis.

1.2.1 Sampling the Respiratory Tract

To increase the likelihood of detecting a respiratory virus during an acute infection a respiratory sample should preferably be collected within 48 hours post symptom onset. Observations reported from both the volunteer infection studies and hospital in-patient studies demonstrated that virus isolation rates

in cell culture increased when respiratory samples were collected during peak virus excretion in the host (Oswald *et al.*, 1975; Sturdy *et al.*, 1969). Sample collection later than 48 hours post symptom onset was shown to reduce the isolation rate significantly in cell culture. This is an important factor for appropriate patient management as patients often present later in the course of infection particularly when presenting with complications (Chidekel *et al.*, 1994; Halperin *et al.*, 1983). This might help explain the poor isolation rate of respiratory viruses observed in some early studies using cell culture when compared to those using molecular techniques (Ireland *et al.*, 1993).

Samples collected from the upper respiratory tract including throat and nose swabs have to be transported to the laboratory in specialised virus transport medium to protect any viable virus present in the sample (Jensen and Johnson 1994). Most respiratory swab samples are routinely collected from adults, and the literature gives plenty of evidence to show that isolation rates in adults is much lower than from samples collected from children (Blanding *et al.*, 1989; Frank 1982; Oswald *et al.*, 1975; Sturdy *et al.*, 1969). The sample of choice in infants, young children, and immunocompromised adults remains the nasopharyngeal aspirate (NPA). Compared to respiratory swabs, viral titres in NPAs particularly from children are generally high leading to a greater virus isolation rate. The added benefit of NPAs is that they contain cellular material that can be used for direct detection techniques, decreasing the time to result from sample collection (Daisy *et al.*, 1979; Heikkinen *et al.*, 2002; Kim *et al.*, 1983).

RSV bronchiolitis, a lower respiratory tract syndrome in infants, is routinely screened for using an NPA sample during the RSV season (Selvarangan *et al.*, 2008). However, complicated respiratory virus infection in children and adults

involving the lower respiratory tract is sometimes confounded by the isolation of viruses from upper respiratory tract samples. It is generally accepted that isolation of a respiratory virus in samples collected from the lower respiratory tract in LRTIs is more strongly associated with a causal relationship (Connolly, Jr. *et al.*, 1994; Legoff *et al.*, 2005; Smith *et al.*, 1993). In children, the lower respiratory tract sample of choice is usually a non-directed broncho-alveolar lavage (NBL) whilst in adults a directed lavage is more commonly collected from areas of consolidation on X-ray. However, until the influenza pandemic in 2009 there was a paucity of studies determining the most appropriate samples to collect for the detection of respiratory viruses during acute infection from either the upper or lower respiratory tract.

1.2.2 Traditional Cell Culture Techniques

The principle of diagnostic cell culture is to expose a permissive cell line to a clinical sample from an acutely infected person with the aim for any virus present to infect the cell. Once viral replication has started *in vitro* the infection then spreads to other cells causing either physical changes (cytopathic effect) that can be observed by the naked eye using microscopy or for antigens present on the surface of the virus to be expressed on the infected cell surface that can be detected using a secondary detection system (Goldstein *et al.*, 1970; Rahman 1973; Walker *et al.*, 1971; Winther *et al.*, 1990). Whilst the principle is simple, the methods involved in producing reliable results using cell culture are complex and there are a number of variables that result in poor isolation rates.

Using influenza as a model to describe some of the difficulties in isolating respiratory viruses using traditional cell culture it becomes easy to demonstrate that whilst isolation rates can be high when the right conditions are met, more often isolation rates are poor to moderate. Important factors that affect virus

isolation rates include the cell line that is inoculated, as well as the type of clinical sample and the timing of the sample collection.

Influenza viruses grow in a limited range of cell lines producing little if any evidence of a cytopathic effect (CPE). The virus life cycle is approximately 48 hours after which time the surface antigens HA and NA are expressed on the surface of the infected cell ready for the progeny virus to bud from the cell surface. In a routine diagnostic laboratory this is exploited to allow for the detection of infected cells by the addition of erythrocytes to the cell sheet which bind to the HA expressed on the infected cell surface. This process called haemadsorption can then be visualised using a light microscope (Baxter *et al.*, 1977; Goldstein *et al.*, 1970; Rahman 1973).

The cell line used routinely for the isolation of influenza viruses in Wales was primary monkey kidney (pMK) cells that were obtained from a specialised cell culture facility. However, it was recognised that the permissiveness of the cell line to influenza was significantly reduced each time the cells were sub-cultured. Once a fresh supply was obtained it was important to ensure that the cells were immediately sub-cultured into the tubes used for laboratory diagnosis (Tobita *et al.*, 1975). This was a skilled job, as failure to ensure that the cells were maintained appropriately influenced the capability of the laboratory to detect influenza. An alternative to primary cells were Madin-Darby Canine Kidney (MDCK) cells that were permissive to influenza especially when inoculated in the presence of trypsin (Tobita *et al.*, 1975). This cell line was never routinely used in Wales for the detection of influenza due to the continued availability of pMK cells. During the 2003 respiratory season in Wales, the routine cells were changed to a continuous human hepatoma cell line (PLC/PRF/5) which could also support the growth of influenza (Bryden 1996). Once inoculated with

clinical material the culture tubes containing the cell sheet and maintenance medium were placed in roller drums and rotated slowly at 37°C using a method described in the 1950s to facilitate viral growth (Rogers and Orr 1970; Yongner *et al.*, 1952) .

A positive haemadsorption result (routinely performed by applying human 'O' type red blood cells to the cell sheet every two or three days) would indicate the presence of HA on the virus infecting the cell sheet. However, a positive haemadsorption result could be indicative of any virus with HA, as a spike protein so as well as influenza A or B, a positive result could have been obtained from cells infected by one of the parainfluenza viruses. Alternatively, if a pMK cell line was being used, a positive haemadsorption result could also indicate a simian paramyxovirus such as SV5 (White 1962). Confirmation of a positive haemadsorption test was required and was routinely performed using viral specific monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) or other fluorescent reagent (Rahman 1973) .

The whole process from inoculation of the cell culture tube until confirmation of a positive haemadsorption result was slow and could take between 4-5 days and up to 14 days for a negative result. Similar isolation rates in traditional cell culture could also be expected for the other respiratory viruses including those with a characteristic CPE such as RSV and adenovirus. To maximise the chances of isolating a virus, several cell lines were inoculated with a single respiratory sample to ensure a broad range of viruses could be isolated in a range of permissive cell lines. Difficult to isolate viruses such as the rhinoviruses were incubated at a lower temperature than the other respiratory viruses in human fibroblast cells (routinely MRC-5 cells). RSV and adenovirus could be grown in the same cell line (routinely HEp-2 cells an epidermoid

carcinoma cell line contaminated with HeLa cells). During the winter season, the workload within the routine virus isolation laboratory would increase significantly and the skill required to identify a positive culture was very high. Therefore, a rapid result for appropriate patient management using traditional cell culture alone was a highly unlikely outcome.

1.2.3 Direct Immunofluorescence for the Detection of Respiratory Viruses

The application of FITC conjugated viral antibody directly to cells obtained from the respiratory tract was first described in the 1960s. An early study by Sturdy *et al* in 1969 demonstrated the application of the technique to cells obtained from the nasopharyngeal tract for a wide range of respiratory viruses (Sturdy *et al.* 1969). The UK based group went on to describe the methodology in numerous follow up studies highlighting the clinical utility of the method in the routine diagnostic laboratory (Aherne *et al.*, 1970; Downham *et al.*, 1975; McIntosh *et al.*, 1979; Orstavik *et al.*, 1984). Direct immunofluorescence (DIF) soon became the method of choice for the rapid detection of respiratory viruses in infants hospitalised with severe respiratory infection. DIF became more accessible to routine diagnostic laboratories with the commercialisation of immunofluorescence reagents (Blanding *et al.*, 1989; Kim *et al.*, 1983; Landry and Ferguson 2000; Mintz *et al.*, 1979; Pozzetto *et al.*, 1988). As well as DIF being a rapid technique, later studies demonstrated it often remained positive in samples collected later in the course of infection when it was no longer possible to isolate the virus in cell culture. Using DIF a result could still be provided to ensure appropriate patient management (Waner *et al.*, 1990). Today DIF remains a routine technique in many centres, as it is both rapid and sensitive. With the ease in which monoclonal antibodies can be produced, reagents are

widely available for new and emerging respiratory viruses (Landry *et al.*, 2008; Sadeghi *et al.*, 2011).

1.2.4 Enzyme immunoassay and rapid antigen detection methods

Standard enzyme linked immunoassay techniques had been developed for the detection of viral antigen and antibodies to respiratory viruses but they were never widely used in the routine laboratory (Freymuth *et al.*, 1986; van der Logt *et al.*, 1985). Rapid antigen tests developed for the detection of influenza and RSV using a similar principle to pregnancy testing kits were developed in the 1980s but were not widely used in Wales until recently. Primary users remain smaller laboratories and healthcare workers performing tests near to the patient. While the tests are specific and rapid, sensitivity varies greatly and can be affected by user, prevalence of the virus in the population and by variation in the virus antigen being targeted (Casiano-Colon *et al.*, 2003; Cruz *et al.*, 2007; Poehling *et al.*, 2006; Pregliasco *et al.*, 2004; Selvarangan *et al.*, 2008). Despite this, rapid antigen tests remain popular and a number of sites around Wales routinely use them as screening tests during the winter season.

1.2.5 Rapid Detection of Respiratory Viruses using Cell Culture

The next major step in the detection of respiratory viruses was the development of shell vial cultures combined with DIF negating the need to look for a CPE (Bartholoma and Forbes 1989; Engler and Preuss 1997; Johnston and Siegel 1991; Navarro-Mari *et al.*, 1999; Olsen *et al.*, 1993; Schirm *et al.*, 1992). Originally to perform the shell vial technique, cells were seeded and propagated on coverslips or in microtitre plates. The respiratory sample was then inoculated onto the cell layer and after gentle centrifugation, the cells incubated for 24-48 hours. Evidence of viral infection was indicated by cellular fluorescence using

DIF directly onto the cell sheet (Bartholoma & Forbes 1989; Rabalais *et al.*, 1992). The method decreased the time to acquiring a result significantly but failed to become widespread in use because initially the method required seeding of the microtitre plates in-house, which was labour intensive. In addition, single cell lines were often used and so multiple wells for each patient sample were required which increased the complexity and cost of the test when compared to traditional cell culture.

In 2000, a study described the used of two cell lines Mink Lung Cells (Mv1Lu) and A549 (human alveolar adenocarcinoma) cells in a combined shell vial system that increased susceptibility of the cells to all of the major respiratory viruses (Huang and Turchek 2000). This was followed by a commercialised system using the same cell combination (Barenfanger *et al.*, 2001). The next generation of rapid cell culture system was introduced into the routine laboratory with studies demonstrating its improved clinical utility over traditional cell culture (Dunn *et al.*, 2004; Kim *et al.*, 2008). These findings are supported by an unpublished study performed in Cardiff during the winter of 2004-2005; some of the data produced from this study has been presented within the body of work submitted in this thesis.

1.2.6 Early molecular techniques for the detection of respiratory viruses

The original in-house methods utilising the polymerase chain reaction (PCR) for the detection of viral nucleic acids were complicated and performed using a minimum of four separate stages. The steps included a method for purifying the viral nucleic acid, a reverse transcription step to form a complimentary DNA (cDNA) strand for the RNA viruses and the PCR itself that was often repeated as a nest to improve product yield. Finally, a method for the detection of the amplified product most often using gel electrophoresis (but also solid phase

detection techniques) was performed (Ireland *et al.* 1993; Karron *et al.* 1994). As the amplification step was performed using a thermal cycler the methods historically are referred to as 'block-based assays'. The whole process could take over 48 hours and due to the number of manual steps, human error was common and often not discovered until the product detection step was undertaken. The process was heavily controlled to ensure that the amplification step was successful and due to the problem associated with contamination by target, a number of negative template controls were required. The method was therefore sensitive, specific but also labour intensive, and difficult to scale up for high throughput testing.

The purification of viral RNA was itself a difficult process and the yield obtained was often low. In 1990 Boom published a simple method that improved yield of both DNA and RNA significantly and this became the basis for later extraction methods (Boom *et al.*, 1990). The first molecular based assays specifically for the detection of a respiratory virus targeted rhinoviruses (Gama *et al.*, 1988; Ireland *et al.*, 1993). As rhinoviruses were not often considered clinically important, the methods were never widely used in the routine diagnostic laboratory. These early methods did demonstrate the utility of the method and later PCR assays were developed targeting influenza, RSV, adenovirus and parainfluenza often in a multiplex format where multiple targets were amplified in a single PCR.

NASBA was first described in the early 1990s as an alternative method to RT-PCR for the amplification of RNA without the need for a separate reverse transcription step (Compton 1991; Guatelli *et al.* 1990; Kievits *et al.* 1991). The technique itself differs significantly from PCR as the starting template is RNA and the product of amplification is RNA of opposite sense. The method is primer

driven and requires three enzymes (avian myeloblastoma virus (AMV) reverse-transcriptase, RNaseH and T7 polymerase) and a modified primer that as well as being target specific also includes a sequence encoding a T7 RNA polymerase promoter region (Sooknanan *et al.*, 1994). Like PCR, amplification of target is performed in a cyclical process but under isothermal conditions. Detection of the amplified RNA product was either by solid phase capture or by bead based capture with detection of the product by electrochemiluminescence (ECL) (Compton 1991; Malek *et al.*, 1994; Romano *et al.*, 1996).

Simplification of the PCR process was made by the introduction of real-time techniques and systems that performed the amplification process with simultaneous detection of the amplified product by the incorporation of a dye labelled target-specific probe or a simple DNA intercalating dye such as SYBR green into the reaction tube (Aldea *et al.*, 2002). The method became widely used from the late 1990s in routine laboratories especially for the detection of DNA viruses (Kessler *et al.* 2000). At the same time, methods describing the detection of respiratory viruses using real-time PCR were being published, often involving a separate reverse transcription step with only the cDNA being amplified in real-time (Echevarria *et al.*, 1998; Stockton *et al.*, 1998; van Elden *et al.*, 2001; van Elden *et al.*, 2003).

Real-time NASBA utilising molecular beacon probes as a detection system was first described in 1998 (Leone *et al.*, 1998) and it was this novel technique that became the basis for the development of the respiratory virus assays and later the molecular diagnostic service in Wales. However, the further development of reagents to allow the development of rapid and sensitive single-tube, real-time RT-PCR assays saw the service for the detection of respiratory viruses in Wales

evolve to encompass these advancements in molecular techniques during the influenza A (H1N1) 2009 pandemic.

2. Aims and Objectives

2.1 Aims

The research presented here describes the stepwise development and introduction of molecular assays into a routine diagnostic service, with the aim to improve the diagnosis of respiratory viruses in community and hospitalised patients across Wales. As the work was performed over an 8-year time period several further aims were identified.

1. To determine those individuals at greatest risk from complications of respiratory viral infection
2. To determine whether a routine service for the detection of respiratory viruses using molecular techniques could be targeted taking into account the seasonality of respiratory viruses and the patient group being screened
3. To provide a diagnostic service that could rapidly respond to outbreaks or epidemics of specific respiratory viruses
4. To develop significant expertise in the field of respiratory viral diagnosis using the latest molecular techniques to be able to respond to emerging threats

2.2 Objectives

These aims were achieved by the following objectives;

1. The demonstration of the clinical utility of molecular methods to detect genetically diverse RNA viruses to enhance patient management (Manuscripts 1,2,3,4,5,6)

2. By describing the methods used to develop a molecular assay for the detection of influenza A and its implementation into routine service with emphasis on the emergence of a drifted strain of influenza A H3N2 virus (Manuscript 2)
3. By describing methods used to take an assay for the detection of RSV developed in-house to commercial production and by demonstrating the marked increase in the detection rate of RSV in a range of clinical samples when compared to traditional laboratory techniques to improve patient management in both children and adults (Manuscripts 3, 4)
4. By describing a novel respiratory sampling technique to improve the detection rate of respiratory viruses in adults and in community surveillance samples (Manuscript 5)
5. By describing the development of a molecular test and service for the detection of influenza A H5N1 in human infection (Manuscript 6)
6. By demonstrating how the molecular respiratory virus service in Wales contributes to knowledge in terms of circulating viruses detected in the community surveillance scheme during the influenza A (H1N1) 2009 pandemic (Manuscript 7)
7. By demonstrating the clinical utility of the service when responding to an outbreak of oseltamivir resistant influenza A (H1N1) 2009 virus highlighting how the service continues to develop in light of recent improvements in molecular techniques. This includes the use of both traditional Sanger based genome sequencing and rapid pyrosequencing to prove epidemiological links during the outbreak investigation. (Manuscript 8)

3. The Manuscripts submitted for PhD by Published Works

3.1 Manuscript 1. Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses



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Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in faecal material

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Abstract

A recently described nucleic acid sequence based amplification (NASBA) assay for the detection of genogroup I (GI) and genogroup II (GII) norovirus RNA in faecal samples was evaluated against a reverse transcription polymerase chain reaction (RT-PCR). Both assays were used to screen a panel of 38 faecal samples known to contain 17 different norovirus strains and 131 clinical samples collected from 60 gastroenteritis outbreaks of unknown aetiology.

The NASBA assay detected 13 out of the 17 strains of norovirus in the characterised panel, failing to detect a single GII strain and three GI strains. There was 90% agreement between the two assays used to detect norovirus in clinical samples from outbreaks. NASBA detected norovirus RNA in all 64 samples positive by RT-PCR and also detected norovirus RNA in additional 13 samples that were negative by RT-PCR. The sensitivity and specificity of NASBA was 100% and 80%, respectively, compared to RT-PCR results.

The norovirus NASBA assay was shown to be highly sensitive and specific, and its ease of use and rapid turnaround time makes it a favourable alternative to RT-PCR for the investigation of norovirus outbreaks.

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1. Introduction

Noroviruses (previously called Norwalk-like viruses, NLVs) are an important cause of viral gastroenteritis: with symptoms of acute diarrhoea and vomiting lasting for 48 h in otherwise healthy individuals (Caul, 1996). Virus transmission can be via the faecal/oral route, through contaminated food and water (Cowden, 2002; Green et al., 2001) or within aerosols produced during vomiting (Chadwick and McCann, 1994). Due to their highly infectious nature and rapid secondary spread, large outbreaks have been described in closed community settings such as hospitals and care homes (Caul, 1996; Cunney et al., 2000). Norovirus outbreaks present a major challenge in infection control and require aggressive intervention measures. These include the exclusion of infected workers and disinfection of contam-

inated surfaces with chlorine-based products. Outbreaks occur predominately in the winter months, although an increase in confirmed outbreaks across the UK, during the summer months of 2002 (PHLS data) was reported. The cost to the healthcare sector due to norovirus outbreaks, which lead to ward closures, reduced theatre goers and staff presence due to sickness absence is immense. The lack of long term immunity and frequent occurrence of large outbreaks, makes the management of viral gastroenteritis, one of the major infection prevention and control challenges currently faced by the National Health Service. The impact of norovirus outbreaks in commercial and community care settings is also both expensive and disruptive. Although generally mild, in the elderly and vulnerable, norovirus infection can cause significant morbidity and occasionally mortality.

Noroviruses are genetically diverse and divided into genogroups based on sequence comparison of the RNA polymerase and capsid region of the genome. Genogroups I and II are associated with human infection. Within the two genogroups, different genotypes or strains exist and any

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given number of these may co-circulate in the population at any time (Richards et al., 2003).

Historically, the electron microscope (EM) was the only method available for the laboratory diagnosis of noroviruses in faecal samples (Caul, 1996). Despite being labour intensive and relatively insensitive (relying on the visualisation of viral particles), EM is still widely used in many diagnostic microbiology departments.

A commercial norovirus ELISA (Dakocytomation, UK) has recently been launched which allows laboratories to screen large numbers of faecal samples for noroviruses with relative ease. Compared to reverse transcription polymerase chain reaction (RT-PCR), in a recent evaluation by Richards et al. (2003), ELISA gave a sensitivity of 69.2% if six or more samples were tested, but only 52.2% if only two samples from an outbreak were available. Because of the relatively low sensitivity observed with the ELISA, the authors recommended that all negative samples should be examined by RT-PCR, to ensure that no norovirus outbreak remains undetected.

Molecular based assays have made a dramatic impact on the diagnosis of viral infections, in particular, for those such as noroviruses, which had only insensitive methods available for routine diagnosis (Atmar and Estes, 2001). In recent years, a number of broadly reactive norovirus RT-PCR assays have been developed, which amplify and detect a wide variety of circulating norovirus genotypes from faecal material (Green et al., 1995a, 1995b; Jiang et al., 1995; Vinje and Koopmans, 1996; Vinje et al., 2003). However, although highly sensitive and specific for noroviruses, it is labour intensive, making it largely impractical outside a laboratory setting.

The nucleic acid sequence based amplification (NASBA) method has been successfully applied to a wide range of RNA targets, including the RNA genomes of viruses such as HIV (Niesters, 2001), enteroviruses (Fox et al., 2002) and more recently, noroviruses in a GI specific assay (Greene et al., 2003) and a broadly reactive assay (Greene et al., 2002). The principles of NASBA have been extensively described elsewhere (Deiman et al., 2002; Fox et al., 2002). The NASBA assay reagents are supplied in an easy to use basic kit format containing reagents and enzymes (bioMérieux, UK) and the assay can be made target specific by the addition of appropriate primers and capture probes and offers a much simpler molecular format for norovirus detection during outbreaks than RT-PCR.

The norovirus RT-PCR assay was introduced as a routine assay in the Welsh molecular diagnostics unit in January 2002 and immediately led to a dramatic increase in the proportion of outbreaks successfully confirmed as caused by a norovirus (Welsh 2002, Personal Communication). This proved to be a tremendous increase in workload for the unit and this problem led to the assessment of NASBA as an alternative molecular method, that would not only equal the sensitivity of the RT-PCR and also importantly, decrease the time spent for performing the assay.

In this study, the recently described broadly reactive NASBA assay for the detection of noroviruses (Greene et al., 2002) was evaluated against RT-PCR in terms of overall sensitivity, specificity and ease of use in a routine diagnostic laboratory.

2. Materials and methods

2.1. Characterised norovirus panel

The Enteric Virus Unit (EVU) at Central Public Health Laboratory (CPHL), Colindale, London, provided a panel of 29 faecal samples containing 15 known norovirus strains and a sapovirus strain. Another nine samples containing two further norovirus strains were obtained from samples stored in Cardiff (Fig. 1 and Table 2).

2.2. Clinical samples from outbreaks of viral gastroenteritis

One hundred and thirty-one faecal samples from 60 outbreaks referred to the Wales Specialist Virology Centre, Cardiff, between September and November 2002 were selected for the study. Thirteen outbreaks had one sample tested by both RT-PCR and NASBA, 25 outbreaks had two samples tested by both methods, 20 outbreaks had three samples tested by both methods and 2 outbreaks had four samples tested by both methods. Of the 13 outbreaks in which one sample was tested by both methods, at least one more sample from the outbreak had also been tested by RT-PCR. Any sample giving discordant results in the evaluation were re-extracted and repeated by both amplification methods to confirm the findings.

2.3. Sample extraction

Faecal samples were extracted using the NucliSens[®] Automated Isolation Kit (bioMérieux, UK), based on the silica slurry method of Boom et al. (1990). Briefly, 100 µl of the supernatant from a 10% faecal suspension in phosphate buffered saline (PBS) was thoroughly mixed with 0.9 ml of lysis buffer containing guanidine thiocyanate. Fifty microlitres of silica was added to each sample and the slurry transferred to a cartridge on the NucliSens[®] automated extractor, which completed the extraction process. For each sample, the RNA extracted was eluted into a volume of 50 µl.

One microlitre of RNAsin (40 000 U/ml; Promega, UK) was added to each extract, 40 µl of which was used in the RT-PCR assay and 5 µl was used in the NASBA assay.

2.4. RT-PCR

The RT-PCR was performed following the method and cycling parameters of Richards et al. (2003), utilising the

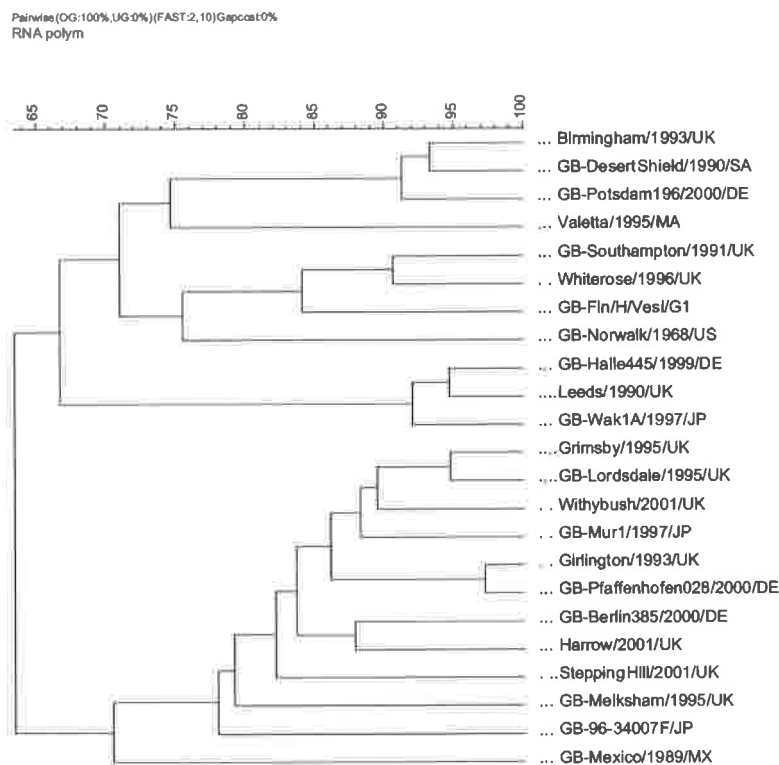


Fig. 1. Generation of contiguous sequences and pairwise alignments of the 76 bp inter-primer region (Ni/E3) and 109 bp inter-primer region (SG1/D1) of the NV ORF sequences were performed using Genebuilder and Clustal in Bionumerics 2.5 (Applied Maths, Belgium). GenBank strains are 96-34007F/JP (AB044358), Wak1A/1997/JP (AB010278), Berlin385/2000/DE (AF312524), Mur1/1997/JP (AB019269), Pfaffenhofen028/2000/DE (AF312519), Halle445/1999/DE (AF312513), Potsdam196/2000/DE (AF312724), Southampton/1991/UK (L07418), other genbank strains are reference strains Mexico/1989/MX (U22498), Lordsdale/1995/UK (X86557), Norwalk/1968/US (M87601) and Desert Shield/1990/SA (U04469). Norovirus strains from the Enteric Virus Unit include, Girlington/1993/UK, Grimsby/1995/UK, Birmingham/1993/UK, Valetta/1995/MA, Whiterose/1996/UK. (Vinje et al., 2000). Other strains are from unpublished data (Gallimore et al., Personal Communication) and include, Harrow/2001/UK, Stepping Hill/2001/UK and Withybush/2001/UK.

previously described broadly reactive primers Ni/E3 and SG1/D1 targeting the RNA-dependant RNA polymerase gene (Green et al., 1995a, 1995b).

The PCR product was detected by gel electrophoresis for 2 h on a 4% gel (Nusieve 3:1, Flowgen, UK) and post-staining with SYBR gold (Molecular Probes, UK). The size of the amplicon was compared to a DNA 25 bp molecular size ladder (Invitrogen, UK), the expected size for the Ni/E3 amplicon and SG1/D1 amplicon were 113 and 150 bp, respectively.

For genotyping purposes, Ni/E3 preferentially amplifies GII viruses and SG1/D1 amplifies GI viruses and some GII viruses and since, cross-reaction may occur, selected amplicons were sent to the EVU for DNA sequencing to confirm genogroup.

2.5. NASBA

Amplification and detection was performed using the NucliSens® basic kit amplification reagents (bioMérieux, UK) following manufacturers instructions and the NLV NASBA conditions described by Greene et al. (2002). Briefly, two lyophilised spheres of reagents, nucleotides and MgCl₂ and enzymes (AMV-RT, RNaseH, and T7 RNA polymerase) were reconstituted in the diluent provided, as first and second steps, respectively. Thirty microlitres of KCl at a concentration of 80 mM and 10 µl of primer mix containing final concentrations of 5 µM BRNVP1.1, 2.5 µM of BRNVP2.1 and BRNVP2.2 (Oswel, UK; Table 1) were added to the reagent mix. Ten microlitres of this mix was added to 5 µl of RNA extract and incubated at 65 °C for

Table 1
Primer and probe sequences used in the NASBA assay (Greene et al., 2002)

Oligonucleotide	Sequence	Primer/reference	Application
5'-attctaatacgcactactataggagaaggatc	TCATCATCACCATA ^a	BRNVP1.1	Primer E3 (Green et al., 1995a, 1995b)
5'-gatgcaaggtcgcataatgag	GAATTCATCGCCCACTGGCT ^a	BRNVP2.1	Primer Ni (Green et al., 1995a, 1995b)
5'-gatgcaaggtcgcataatgag	ATACCACTATGATGCAGATTA ^a	BRPNV2.2	Primer JV12 (Vinje and Koopmans, 1996)
5'-BIOTIN-ACAGGCCTATCACCCGAT		NVG1.1	Ando et al. (1995)
5'-BIOTIN-ACTGGCTTATCACCTGATG		NVG1.2	Ando et al. (1995)
5'-BIOTIN-TATCACCTGATGTTATACAATCC		NVG1.3	Gray et al. (1997)
5'-BIOTIN-GTCCCCTGACATCATACAGGCT		NVG2.1	Gray et al. (1997)
5'-BIOTIN-ACAGGACTAGGCCCGACAT		NVG2.2	Ando et al. (1995)
5'-BIOTIN-TCAGGTCTCTCACAGAT		NVG2.4	Ando et al. (1995)

^a Lowercase letters show T7 promoter sequence and the ruthenium-labelled oligonucleotide detection sequence.

5 min in a dry heating block, followed by a further incubation for 5 min at 41 °C. After this step, 5 µl of the reconstituted enzyme sphere was added to each reaction tube and incubated for further 5 min at 41 °C, before being transferred to a circulating waterbath maintained at 41 °C for 2 h.

Following amplification, GI and GII norovirus detection was performed independently utilising two multiplex detection mixes containing 10 µl of electrochemiluminescence (ECL) generic probe (supplied) and each capture probe bead mix (either GI or GII; Table 1) at a final concentration of 4.5 µM per sample. The NASBA amplification product was diluted at a ratio 1:5 (5 µl of product and 20 µl of diluent (supplied)) and 5 µl was added to 20 µl of each detection mix, this was then incubated for further 30 min at 41 °C with shaking every 10 min to allow hybridisation of the amplicon to the capture probe.

Three hundred microlitres of assay buffer (bioMérieux, UK) was added to each reaction tube and the level of fluorescence signal was read on the ECL reader. An instrument reference solution (RS) provided with the basic kit detection reagents was included on every NASBA assay run following manufacturers instructions, and the cut-off value for the assay was defined as 0.1 times the instrument RS ECL reading.

2.6. Statistical analysis

Statistical analysis was performed using the software package GraphPad InStat Version 3.05 for Windows. The results of the RT-PCR were used as the standard to calculate sensitivity, specificity and positive and negative predictive values. Fishers exact test was used to compare the two assays.

Table 2
Norovirus subtypes supplied in a faecal panel from the EVU

Hu/NLV strain	Genbank /reference	Cluster (Ando et al., 2000)	RT-PCR result (primer reactivity)	NASBA result	GI signal (log ₁₀)	GII signal (log ₁₀)	Genogroup (by NASBA)
96-34007F/JP	AB044358	GII.5?	Detected (Ni/E3)	Detected	1 (0)	739994 (5.6)	GII
Girlington/1993/UK	Vinje et al. (2000)	GII.1	Detected (both)	Detected	39341 (4.6)	10000001 (7.0)	GII
Harrow/2001/UK	Gallimore et al. (unpublished)	GII.1# or GII.3#	Detected (Ni/E3)	Detected	3078 (3.5)	541055 (5.7)	GII
Wak1a/1997/JP	AB010278	GII.7?	Detected (Ni/E3)	Detected	1 (0)	3129 (3.5)	GII
Berlin385/2000/DE	AF312524	GII.2?	Detected (Ni/E3)	Detected	699 (2.8)	1721862 (6.2)	GII
Mur1/1997/JP	AB019269	NK	Detected (both)	Detected	6668 (3.8)	18485 (4.3)	GII
Pfaffenhofen028/2000/DE	AF312519	GII.1	Detected (both)	Detected	2967 (3.5)	355362 (5.6)	GII
Stepping Hill/2001/UK	Gallimore et al. (unpublished)	GII.11	Detected (both)	Detected	24279 (4.4)	209430 (5.3)	GII
Halle445/1999/DE	AF312513	GII.7	Detected (SG1/D1)	Detected	1 (0)	43069 (4.6)	GII
Grimsby/1995/UK	Vinje et al. (2000)	GII.4	Detected (both)	Detected	59742 (4.2)	1050914 (6.0)	GII
Potsdam196/2000/DE	AF312724	GI.3?	Detected (SG1/D1)	Detected	1872 (3.3)	27 (1.4)	GI
Birmingham/1993/UK	Vinje et al. (2000)	GI.3	Detected (SG1/D1)	Detected	3752973 (6.6)	1 (0)	GI
Southampton/1991/UK	L07418	GI.2	Detected (SG1/D1)	Detected	248987 (5.4)	73 (1.9)	GI
Withybus/2001/UK	Gallimore et al. (unpublished)	GII.4	Detected (low positive) (Ni/E3)	Not detected	1 (0)	14 (1.1)	-
Fin/H/Vesi/G1	AJ243787	GI.2?	Detected (SG1/D1)	Not detected	37 (1.6)	1 (0)	-
Valetta/1995/MA	Vinje et al. (2000)	GI.4	Detected (SG1/D1)	Not detected	1 (0)	1 (0)	-
Whiterose/1996/UK	Vinje et al. (2000)	GI.2	Detected (SG1/D1)	Not detected	1 (0)	27 (1.4)	-

Showing RT-PCR, NASBA result, signal and genogroup interpretation for each strain. ? : not confirmed; NK: not known; #: recombinant NV.

3. Results

3.1. Results from the characterised norovirus panel

The results obtained by NASBA and RT-PCR are shown in Table 2. Each virus had been characterised by DNA sequencing and assigned to either GI or GII by the EVU.

There was a degree of cross-reactivity observed between the RT-PCR primer sets and the NASBA detection mix. In particular, many positive GII strains also gave a weaker GI band and a lower signal in the NASBA GI detection mix. Such cross-reactivity was not observed with the GI strains in either the RT-PCR or the NASBA. GI virus strains generally gave a much lower signal overall by NASBA.

The GII Halle/445/1999/UK strain of norovirus is closely related to the Leeds/1990/UK (Vinje et al., 2000) strain of norovirus and gave a band in the SG1/D1 reaction of the RT-PCR, however, it only gave a positive signal in the GII detection mix of the NASBA.

Overall, the NASBA assay detected 10 out of 11 GII strains, but only 3 out of 6 GI strains were included in the faecal panel. The NASBA assay did not detect the sapovirus strain SV/Lyon/1998/Fr (Genbank AJ251991).

3.2. Results from faecal samples submitted from gastroenteritis outbreaks

Of the 131 clinical samples, norovirus RNA was detected in 64 samples by both RT-PCR and NASBA and all of them were GII viruses. Another 54 samples were found negative for norovirus RNA by both methods. Thirteen samples from 12 outbreaks gave discordant results: all were found negative by RT-PCR, but positive by NASBA giving an overall agreement of 90%. Compared to RT-PCR, the sensitivity and specificity of NASBA was found to be 100% and 80%, respectively ($P \geq 0.0001$).

Of the 13 discordant samples (Table 3), eight came from outbreaks in which at least one sample had been found posi-

tive for noroviruses by RT-PCR. The remaining five samples found positive by NASBA came from outbreaks in which no other sample was found positive by either RT-PCR or NASBA. One of these samples gave high ECL signals suggesting true positive results and the remaining samples gave a very low GI signal that was just above the assay cut-off value.

4. Discussion

RT-PCR has been shown in recent evaluations to be of much greater sensitivity in diagnosing norovirus infections, compared with the traditional diagnostic methods of EM and ELISA. However, the validated method is based on block based PCR followed by gel detection and as a result, is time consuming and labour intensive. These disadvantages are offset, by the need only to investigate a few samples from each outbreak and to establish the cause of infection, made possible by the increased sensitivity of this method, compared with ELISA and EM. Ideally, a routine diagnostic method should combine high sensitivity and specificity with ease of use in the diagnostic laboratory. RT-PCR does not fit ideally into the routine diagnostic setting, particularly if the detection system is based on gel detection.

Other RT-PCR detection systems have been described including southern blotting (Vinje and Koopmans, 1996) microplate hybridisation of the PCR product (Maunula et al., 1999) and more recently, real-time RT-PCR (Kageyama et al., 2003). Either of these methods may offer a less labour intensive and more specific detection method than traditional gel detection.

In most RT-PCR methods however, performing the reverse transcription step separately from the PCR step leads to increase in the overall time of performing the assay and may also increase the chances of cross-contamination. Conversely, the NucliSens[®] NASBA is a sensitive single tube isothermic amplification assay, that is largely kit based

Table 3
Discordant sample results

Sample	Outbreak	NASBA GI signal (log ₁₀)	NASBA GII signal (log ₁₀)	Genogroup	Number of samples from same outbreak (RT-PCR positive)
N5128	1	73539 (4.9)	1510131 (6.2)	GII	3
N3847	2	8860 (3.9)	1936591 (6.3)	GII	2
N3967	3	51919 (4.7)	2323339 (6.4)	GII	2
N4073	4	1012 (3.0)	2341331 (6.4)	GII	2
N4872	5	1 (0)	9220 (3.9)	GII	2
N4922	6	65 (1.8)	39845 (4.6)	GII	2
N4868	7	1 (0)	780 (2.9)	GII	1
N4870	7	1 (0)	97349 (5.0)	GII	1
N3844	8	2992 (3.5)	1791093 (5.3)	GII	0
N3948	9	8686 (3.9)	10000001 (7.0)	GII	0
N4871	10	1 (0)	71615 (4.9)	GII	0
N5121	11	11552 (4.1)	1271216 (6.1)	GII	0
N4615	12	182 (2.3)	3 (0.5)	?GI	0

All samples found RT-PCR negative.

(requiring only specific primer and probe design), which makes it both user friendly and less time consuming than RT-PCR. Result interpretation is also much simpler, as it is based on a cut-off signal rather than visualising a PCR product on a gel.

Early evaluations of the NASBA assay performed in the USA demonstrated it to be broadly reactive against a wide range of norovirus strains (Greene et al., 2002). This evaluation however, is the first to use largely European norovirus strains in clinical samples.

The NASBA assay detected 10 out of the 11 GII strains in the characterised faecal panel, but detected only 3 out of 6 GI strains. One of the missed GI strains had caused a cluster of outbreaks in Wales during the summer of 2002. Overall, the NASBA assay performed well on the clinical samples examined, detecting norovirus RNA in all of those which were found to be positive by RT-PCR. All noroviruses detected in the clinical samples were GII virus strains, this finding is consistent with the majority of norovirus outbreaks across the UK, that are also found to be caused by GII viruses. Furthermore, the NASBA assay was able to detect norovirus RNA in six additional samples that were RT-PCR negative and from outbreaks, where two or three other samples were shown to contain norovirus RNA by RT-PCR. The assay also detected norovirus RNA in two samples from an outbreak, where only one sample had previously been found positive. Most outbreak criteria state that at least two samples must be positive before an outbreak and can be confirmed as having norovirus as the causative agent. Thus in this case, the results obtained help confirm an otherwise ambiguous result and suggest that the NASBA may have an increased sensitivity compared to the RT-PCR used.

Five further samples had detectable norovirus RNA by NASBA, despite having a negative RT-PCR result. In these cases, no other sample from the same outbreak was shown to be positive for norovirus RNA and as NASBA products do not lend themselves well to post amplification analysis, it makes it difficult to confirm noroviruses as a cause, despite clinical information suggesting viral gastroenteritis.

The expense and time involved in sample testing all patients involved in an outbreak of viral gastroenteritis are overwhelming. Often numerous people become unwell in a very short time period and establishing the cause of the outbreak is imperative, particularly in a semi-closed or closed community, to enable appropriate outbreak management. By using a sensitive molecular assay, it is no longer necessary to examine samples from all affected patients for norovirus. By examining a small number of carefully collected acute stool samples, the cause of the outbreak can be determined cost effectively.

This approach, in particular, has improved the surveillance of outbreaks in the community, where it is often difficult for environmental health departments to collect many good quality samples. It also means that poor quality samples from an outbreak can be stored without testing in favour of good quality samples, saving both time and resources.

The results of this study, show that, for GII norovirus strains, replacing RT-PCR with NASBA, provides the laboratory with an assay that has equal or even greater sensitivity and is much easier to integrate into a routine service.

A problem highlighted by this study is that the NASBA assay performed less well in detecting GI norovirus strains. Three out of six strains supplied in the characterised panel were not detected and one strain that was known to have caused several outbreaks in Wales as mentioned previously, was only detected by RT-PCR. The lower ECL signals observed in the NASBA assay could be due to a low titre of virus in the samples tested, although in most cases, there was a strong RT-PCR product and sequence analysis did highlight primer and probe mismatches in some of the GI virus strains. Attempts were made to increase the sensitivity of the NASBA assay to GI viruses by introducing a fourth NASBA primer based on the RT-PCR primer SG1: this has showed some improvement, but needs to be investigated further before a modified NASBA assay can be introduced.

An interesting observation during the study was that the degree to which strains of GII norovirus cross-reacted in the GI assay not only varied between outbreaks, but also, the strain of virus from a particular outbreak appeared to have a stable cross-reactivity seen in each sample from that outbreak. This phenomenon was reproducible, not only when the extract was re-amplified, but also when the sample was re-extracted. This suggests that the ECL signal may correlate with the sequence variation of the virus at the probe-binding site and may help indicate whether more than one strain of norovirus is involved in the larger, protracted outbreaks. To investigate this, further sequence analysis needs to be performed on viruses from different outbreaks that show different cross-reactivity patterns.

A point for further consideration is that, as the study demonstrates RT-PCR can detect norovirus strains, while the NASBA assay is unable to, it may also be possible that the NASBA assay may detect norovirus strains that are not detected by RT-PCR, due to the different primer combinations used. This may explain why the RT-PCR failed to detect some of the samples from outbreaks, where only a single sample was found positive by NASBA. Confirming this point may be difficult and certainly requires further investigation, but does highlight the difficulty in developing any molecular based assay, that will detect every circulating strain of norovirus in the population.

5. Conclusion

This evaluation has demonstrated the norovirus NASBA assay to be highly sensitive and specific, when compared to RT-PCR, and has shown that it can detect a wide variety of norovirus strains. Its ease of use and rapid turnaround time when compared to RT-PCR makes it a favourable alternative within a busy routine molecular diagnostics unit for the

investigation of norovirus outbreaks. However, lack of sensitivity in detecting GI viruses remains a problem.

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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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KEY WORDS: influenza A; NASBA; real-time NASBA; molecular beacon; rapid diagnosis

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 (TamifluTM), 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 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- GCCTTGCTTGG	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 (TOP10[®]; 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.

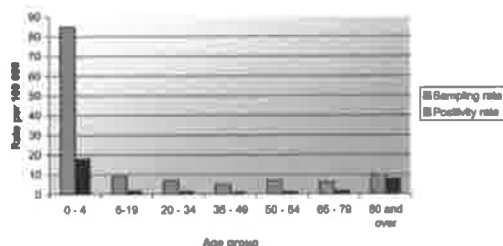


Fig. 1. Samples taken by age group (patient group 2). The sampling rate is calculated based on the total population in Wales of each age group.

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

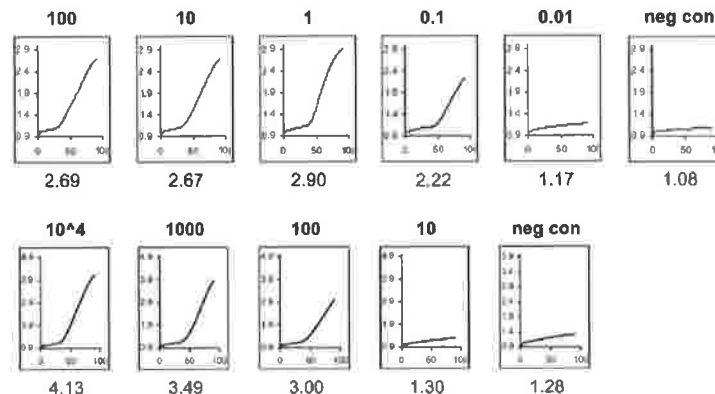


Fig. 2. Influenza A real-time NASBA results. Influenza A virus (H1N1) titer. Influenza A virus (H1N1) synthetic RNA (RNA copy number). The x-axis indicates the time in minutes and the y-axis represents the raw fluorescence signal. The virus input or RNA copy number input into the reaction is listed above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

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

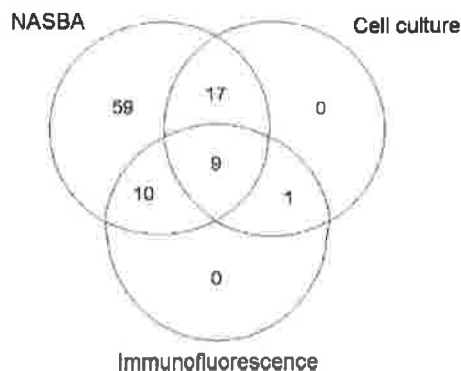


Fig. 3. Positive results by assay (patient group 2).

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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Rapid and highly sensitive qualitative real-time assay for detection of respiratory syncytial virus A and B using NASBA and molecular beacon technology

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Abstract

The performance of a sensitive and specific qualitative respiratory syncytial virus (RSV) assay based on NASBA technology and real-time molecular beacon detection is presented. Very low detection limits for both RSV A and RSV B were determined: 95% detection hit-rate of 95 and 47 copies/input in isolation for RSV A and RSV B, respectively. RSV was detected in a wide variety of clinical samples including respiratory swabs, nasopharyngeal aspirates (NPA), bronchoalveolar lavages (BAL), endotracheal secretions, and sputum samples. In total 779 clinical samples were tested and a valid result was obtained for 765 (RSV NASBA assay), 765 (cell culture), and 529 (rapid direct immunofluorescence testing (IF)) samples. Of these samples, 229 (RSV NASBA assay), 61 (cell culture), and 122 (IF) samples were positive for RSV. In addition, 106 samples were reported as RSV negative using the NOW[®] RSV assay (Binax). Subsequent testing using the RSV NASBA assay demonstrated that 32 (30%) of these samples were RSV positive. The RSV NASBA assay includes a homologous internal control, which offers a high degree of standardization and quality control. When the RSV NASBA assay was performed on the NucliSens EasyQ platform (bioMérieux), test results of 48 sample extracts were obtained in less than 2 h.

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1. Introduction

Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract infections in infants, vulnerable older children, and adults with underlying conditions. It is associated with annual epidemics in the winter months in temperate climates leading to high rates of morbidity and hospitalization in these patient groups. Fast and accurate diagnosis is crucial for appropriate patient management and infection control measures to prevent nosocomial transmission. Antigen detection assays, such as direct immunofluorescence assays (DFA), enzyme immunoassays (EIA), and more recently immunochromatography point of care tests (POCTS) on nasopharyngeal aspirates (NPA) have become common rapid methods for RSV detection particularly in samples from infants (Lipson, 2002;

Ohm-Smith et al., 2004; Aldous et al., 2004). With these assays, results are obtained within an hour of sample receipt and the associated costs are moderate (Barenfanger et al., 2000; Lipson, 2002). However, these assays often have limitations in sensitivity and specificity as compared to molecular tests (Abels et al., 2001; Liolios et al., 2001; Falsey et al., 2002; Boivin et al., 2004; Kuroiwa et al., 2004), particularly on samples of poor quality or those from adult patients, and DFA, the rapid method of choice in many laboratories, requires good technical skills, high quality reagents, and equipment for optimal sensitivity. Because of the higher costs and longer turnaround time of the molecular tests, as compared to the antigen-detection tests, some laboratories use antigen detection to screen incoming clinical samples and only perform a more sensitive molecular test on the negative samples (Gruteke et al., 2004). Some laboratories use an 'in-house' developed assay (Abels et al., 2001; Falsey et al., 2002; Whiley et al., 2002; Hu et al., 2003; Boivin et al., 2004; Dewhurst-Maridor et al., 2004; Templeton et al., 2004). However, many laboratories do not have the appropriate facilities to

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develop their own molecular test for RSV or prefer to work with a quality controlled standardized molecular test, emphasizing that there is a need for commercial molecular RSV assays.

This paper describes the performance of a qualitative real-time assay for detection of RSV A and B using NASBA (for review see Deiman et al., 2002) and real-time molecular beacon detection technology. The RSV assay includes a homologous internal control (IC) to control both the isolation and amplification step. The assay is highly sensitive and specific, and delivers a same-day result, which leads directly to rapid appropriate patient management and the implementation of infection control measures to prevent further transmission events.

2. Materials and methods

2.1. Alignments

Alignments were made, using DNAMAN software Version 3.2 (Lynnon BioSoft), on sequences taken from EMBL/GenBank database. The accession numbers that were used for the selection of the primers and molecular beacon are as follows: RSV A: AF512538, AY198177, Z26524, L25351, U39661, U31562, U50362, M22643, X02221, AY198176, AY198175, AY114151, AY114150, AY114149, U39662, U63644, AF035006, U31561, U31560, U31559, U31558, D00151, M74568, M11486, AF067125 and for RSV B: NC_001781, AF013254, AF013255, and D00334.

2.2. Primer and molecular beacon design

Primers and molecular beacons were designed in a conserved part of the F-gene region of RSV A and RSV B. One set of primers was used to amplify both RSV A and RSV B and the IC. One generic molecular beacon (MB) probe was designed to detect both RSV A and RSV B. An additional beacon, RSV MB IC was designed for detection of the IC. The generic RSV beacon was labeled with FAM and the IC beacon was labeled with ROX at the 5' ends and Dabsyl was used as the quencher at the 3' ends. The molecular beacons were designed making use of the DNA folding program Mfold to predict the structures of the resulting molecules (<http://www.bioinfo.rpi.edu/applications/mfold>). The purity of the primers and beacons was determined to be at least 90% by capillary gel electrophoresis. For theoretical determination of specificity, primers and beacons were aligned in NCBI BLAST (<http://www.ncbi.nlm.nih.gov/blast/>).

2.3. Wild type and internal control RNA

RSV A wild type (WT) and RSV B wild type plasmids were constructed for the *in vitro* production of the corresponding wild type transcripts. Supernatant of tissue culture material of RSV A was obtained from the Wales specialist virology center of the University Hospital of Wales (Cardiff, United Kingdom) and of RSV B (no. V3976) was obtained from the Centre d'Immunologie Pierre-Fabre (Saint-Julien en Genevois, France). An RNA fragment of 249 bp of the F-gene was multiplied by RT-PCR and cloned in-between the *EcoRI* and *Csp45I*

sites of a pGEM-3X-based vector. Sequencing of the plasmid showed that the sequence of the RSV A fragment corresponded to EMBL accession number Hru39662, position 6640–6889, and the RSV B fragment corresponded to EMBL accession number D00334, position 1014–1271.

The RSV A wild type plasmid was used for the construction of the IC plasmid from which the IC RNA could be transcribed. The IC plasmid is identical to the WT plasmid except for a fragment of 20 bp including the molecular beacon-binding site for the generic RSV detection which was replaced by a random sequence, not related to RSV, of 20 bp including the IC molecular beacon-binding site, using fusion PCR. The correct sequence was confirmed by sequencing (Baseclear).

The WT and IC plasmids were digested with Bam HI prior to run-off T7 transcription (Ribomax™ large scale RNA production system T7, Promega). The transcripts were purified (RNeasy mini kit, Qiagen), and the concentration was determined by OD (A_{260}) measurement. The length of the RNA (1178 nt for both the WT and IC) was checked by gel electrophoresis and bioanalyzer (Agilent Technologies). All transcripts were stored at -70°C .

2.4. Isolation

Nucleic acid was isolated from clinical samples or transcript samples using the NucliSens miniMAG (bioMérieux), in combination with the NucliSens magnetic extraction reagents (bioMérieux) and NucliSens Lysis buffer (bioMérieux) according to the manufacturers instructions. For transcripts in water, 1 ml defibrinated human plasma (Seracon I, Serologicals corporation) was added to mimic sample background. Fifty to two hundred microlitres of sample, was added to 2 ml of Lysis buffer. The mixture was incubated at RT for 10 min, and 3000 cp IC was added. To this, 50 μl of magnetic silica was added and the mixture incubated for another 10 min at RT. The silica was then washed several times and the nucleic acid eluted in 25 μl elution buffer following incubation for 5 min at 60°C . The extracts were used directly in amplification or stored at -80°C .

2.5. Amplification, detection, and data analysis

A primer mixture was prepared using the reagents from the NucliSens EasyQ basic kit Version 2 (bioMérieux) and the RSV primers and probes. The WT and IC targets are amplified with the same RSV related primer pair. Amplification reactions were performed at 100 mM KCl. The final concentration in the reaction mixture of each primer was 0.2 μM , and the final concentrations of the FAM and ROX molecular beacons were at 0.01 μM and 0.1 μM , respectively. For each amplification reaction, 5 μl of extract was added directly to the primer mixture. Removal of secondary RNA structure and primer hybridization was performed by a two-step incubation of 2 min at 65°C and 2 min at 41°C . To each reaction, 5 μl of enzyme mixture was added, amplification and simultaneous detection was then performed using the NucliSens EasyQ analyzer (bioMérieux) at 41°C for 90 min. The fluorescence signal was measured with an interval time of 30 s for each independent reaction at two wavelengths using

the accompanying NucliSens EasyQ Director software (Version 2.0). For data analysis the QL1 calculation engine was used and the following parameters were defined: 'target detection threshold': 1.1, 'IC lambda threshold': 3.0, 'Growth threshold': 50, 'Maximum time to primer depletion': 60.

2.6. Assay sensitivity

For determination of assay sensitivity, a dilution series of RSV A and RSV B transcript of 125, 100, 75, 50, 35, 25, and 12.5 copies/input in isolation (independent dilutions), were tested. The highest input concentrations (125 and 100 copies/input) were replicated 12 times. The other inputs were tested in 24 replicates. To each dilution of WT RNA, 3000 cp IC RNA, 2 ml Lysis buffer and 1 ml defibrinated human plasma were added. To control for contamination and inhibition from extraction through to amplification and detection, each isolation run ($n = 24$) included two no template controls (NT) (=no WT, no IC input) and three no sample controls (NS) (=no WT, normal IC input) in 2 ml Lysis buffer and 1 ml defibrinated human plasma. In all amplification runs extra NT (amplification NT*) were tested. For these NT* 5 μ l NASBA water were used instead of eluted clinical or control material.

2.7. Sample type comparison study

Twenty-four RSV negative nasopharyngeal swab samples obtained from the National flu center (Geneva) were pooled together. From this 200 μ l aliquots were spiked with 45 copies of RSV A transcript, and tested in 20 replicates using the RSV NASBA assay, from this the 80% hit-rate as determined by probit analysis.

In addition, six RSV negative bronchoalveolar lavages (BAL) samples were obtained from the *Centre national de référence des légionelles (hôpital Edouard-Herriot, Lyon, France)* and 200 μ l aliquots of these were spiked with 3000 copies IC and tested for RSV.

To determine the assay sensitivity in BAL samples, 11 RSV negative BAL samples obtained from the *Centre national de référence des légionelles (hôpital Edouard-Herriot, Lyon, France)* were pooled. From this pool, 100 μ l was treated with 10 units DNase I (Promega, Southampton, UK) in DNase I buffer (provided with the enzyme) for 1 h at 37 °C or with 20 units of DNase I in DNase buffer for 30 min at 37 °C to degrade cellular DNA before isolation. The BAL samples were then spiked with 45 copies (80% hit-rate) of RSV A transcript and tested in 20-fold. For comparison, 1 ml defibrinated human plasma samples were spiked with the same input and tested in 20 replicates. To all samples IC (3000 copies) and 2 ml Lysis buffer was added before isolation. Three types of NT have been included: 2 ml Lysis buffer with 1 ml defibrinated human plasma, 200 μ l of RSV negative nasopharyngeal swab pool, or 100 μ l DNase-treated BAL.

2.8. Cross-reactivity

Assay specificity was tested using the following targets: parainfluenza virus (PIV 1: V4036: from ATCC ref

VR-907 strain sendai/cantell, PIV 2: V4035: from ATCC ref VR-92 strain greer, PIV 3: V4029: strain unknown), human metapneumovirus (hMPV A1: NL/1/00, hMPV A2: NL/17/00, hMPV B1: NL/1/99, hMPV B2: NL/1/94) (acquired from Vironovative (Rotterdam, the Netherlands)), Measles (V4158 from ATCC ref VR-24 strain Edmonster 97-08). In addition a 'respiratory viruses' panel (diagnostic hybrids, Athens, OH, USA) was tested including: RSV (RSV long, RSV9320, and RSV/B/wash/18537/62), influenza virus (Influenza A: A/WS/33, A/Mal/302/54, A/Victoria/3/75 and A/PortChalmers/1/73, Influenza B: B/Taiwan/2/62, B/GL/1734/54), PIV (PIV 1: C-35, PIV 2: Greer, PIV 3: C234), Adenovirus (Type 1: Adenoid 71, Type 5: Adenoid 75). For each sample, 200 μ l was used for the extraction.

2.9. Clinical samples

Seventeen respiratory clinical samples (nasopharyngeal swabs) were obtained from the Rockefeller virology laboratory (Lyon, France). These samples were found to be RSV positive, and some were further characterized as RSV A or RSV B. The samples were received frozen and were stored at -70 °C until testing. For RNA extraction, 200 μ l of each sample was added to 2 ml of Lysis buffer. Before extraction, 3000 cp IC RNA was added to the sample once added to the Lysis buffer.

In addition, 116 respiratory swabs, 586 nasopharyngeal aspirates, 12 endo-tracheal secretions, 59 bronchoalveolar lavages, and six sputum samples, collected during the winter 2004–2005 season, were tested in the Wales specialist virology center (UHW, Cardiff, United Kingdom). Most samples were received in virus transport medium comprising of Hanks BBS \times 1 (BioWhittaker™, Worthingham, Berkshire, USA), 7.5% bovine albumen (Sigma-Aldridge, Poole, Dorset, Surrey, UK), penicillin (Britannia pharmaceuticals, Redhill, Surrey, UK) and streptomycin (Sigma-Aldridge). All samples were tested as soon as they arrived in the laboratory and were not stored for any length of time. The samples were incubated with 20 units of DNase I (Promega, Southampton, UK) at 37 °C for 30 min in DNase buffer (provided with the enzyme) prior to isolation. Some of the samples were initially screened in external laboratories using the NOW® RSV Assay (Binax). Samples from which cells could be obtained were screened with IFA using Simulfluor® immunofluorescence screening assay (Light diagnostics, Chemicon Europe Ltd, Hampshire, UK). In addition, most samples were tested by cell culture using primary monkey kidney cells, human hepatoma cells (PLC), MRC-5 cells or Hep-2 cells (for details see Materials and methods of the retrospective study, Moore et al., 2006).

3. Results

3.1. Analytical sensitivity of the real-time RSV NASBA assay

Based on the alignment of sequences of RSV A and RSV B, a real-time RSV NASBA assay was designed on the F-gene of

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the viral genome. The assay includes an IC to control the entire process from nucleic acid isolation through to amplification and detection. To determine assay sensitivity a dilution series of *in vitro* transcribed WT RNA of RSV A and RSV B of concentrations of 12.5, 25, 35, 50, 75, 100, and 125 copies/input in isolation were tested. To mimic sample type background, commercially available defibrinated human plasma was added to the transcript dilution series. To each dilution 3000 copies/input in isolation of IC RNA was added. By probit analysis, a 50% hit-rate of 21 copies/input in isolation and a 95% detection hit-rate of 95 copies/input in isolation for RSV A (Fig. 1A), and a 50% hit-rate of 15 copies/input in isolation and 95% detection hit-rate of 47 copies/input in isolation for RSV B (Fig. 1B) was determined. By testing a dilution series of *in vitro* transcribed WT RNA of RSV A and RSV B directly in amplification, it was shown that this corresponds to a 95% hit-rate of approximately 10 and 5 copies/amplification reaction of RSV A and RSV B, respectively. This Factor 10 is explainable as, when using the NucliSens miniMAG for isolation, only 1/5th of the extract is used in amplification, and some of the nucleic acid can be lost in extraction (van Deursen et al., 2004).

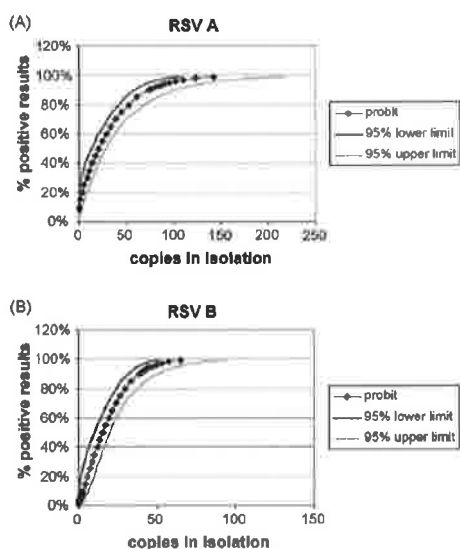


Fig. 1. An assay sensitivity for RSV. (A) *In vitro* transcribed WT RNA of RSV A of input concentrations of 12.5, 25, 35, 50, 75, 100, and 125 copies/input in isolation was tested. The highest inputs (125 and 100 copies/input in isolation) were tested in 12-fold. The other inputs were tested in 24-fold. The percentage of positive results per input is presented. The probit and the 95% lower limit and 95% upper limit are presented. Fig. 1. (B) Assay sensitivity for RSV B. *In vitro* transcribed WT RNA of RSV B of input concentrations of 12.5, 25, 35, 50, 75, 100, and 125 copies/input in isolation was tested. The highest inputs (125 and 100 copies/input in isolation) were tested in 12-fold. The other inputs were tested in 24-fold. The percentage of positive results per input is presented. The probit and the 95% lower limit and 95% upper limit are presented.

Table 1
Sample type comparison

Matrix	Hit-rate (% positive reactions) ^a
Defibrinated human plasma/nasopharyngeal swab	
Defibrinated human plasma I	72
Nasopharyngeal swab	70
Defibrinated human plasma/BAL	
Defibrinated human plasma I	70
BAL	90

^a Input used is 45 copies/input in isolation of RSV A, which is the 80% hit-rate in a defibrinated human plasma background as determined by probit analysis.

3.2. Sample type comparison study

To determine assay sensitivity, defibrinated human plasma was added to the transcript dilution series to mimic sample type background. To investigate assay sensitivity in RSV samples types, nasopharyngeal swab was compared to defibrinated human plasma. A pool of RSV negative nasopharyngeal swabs samples was used. To both matrices 45 copies/input in isolation of RSV A transcript, that is, 80% hit-rate in defibrinated human plasma as determined by probit analysis, was added. This input was tested in 20-fold. Results show that the number of positive reactions in nasopharyngeal swab background is 70%, which is comparable to that in defibrinated human plasma background, determined to be 72% in this experiment (Table 1). This indicates that assay sensitivity in nasopharyngeal swab samples is comparable to that in defibrinated human plasma background.

In addition, assay sensitivity was determined in BAL samples. Initially, six RSV negative BAL samples were spiked with 3000 copies/input in isolation of RSV IC. However, in only two out of six sample, IC was detectable (result not shown). BAL samples may contain a large amount of cellular DNA that could inhibit NASBA. Therefore, a pool of BAL samples was treated with DNase I. To investigate assay sensitivity, this pool was compared to defibrinated human plasma. To both matrices 45 copies/input in isolation of RSV A transcript (80% hit-rate), was added and tested in 20-fold. Results showed that the percentage of positive reactions in BAL samples treated with DNase I is 90%, which was not significantly different from that in defibrinated human plasma background, determined to be 70% in this experiment (Table 1).

Additional experiments showed that the DNase I sample pretreatment also improved RSV detection in nasopharyngeal aspirate samples and sputum samples containing high levels of mucus.

3.3. Assay specificity

To investigate assay specificity of the RSV NASBA assay, pathogens isolated from the same sample types were tested. No cross-reactivity could be observed for PIV1-3, Influenza A and B, measles, Adenovirus Types 1 and 5, hMPV A1, A2, B1, and B2, indicating that the assay was specific for RSV.

Table 2
Clinical samples

Sample type	Number	Number of RSV positives by method			
		Culture ^a	Binax NOW	IFA	NASBA
Respiratory swabs	116	2	Not tested	Not tested	12
NPA	586	59	0	109 ^b	208
Endo-tracheal secretions	12	0	Not tested	1	1
BAL-directed	32	0	Not tested	2	4
BAL-non directed	27	0	Not tested	4	4
Sputum	6	0	Not tested	0	0
Samples tested	779	765 ^c	106 ^d	529 ^e	765
Total positive		61	0	122	229
Percentage positives		8	0	23.1	29.9

^a All samples positive by culture were also positive in the RSV NASBA assay.

^b One sample was tested negative for RSV by the RSV NASBA assay. This sample was tested three times by IFA and only once the result was positive for RSV.

^c 29.5% of these samples were tested RSV positive by the RSV NASBA assay.

^d 30.2% of these samples were tested RSV positive by the RSV NASBA assay.

^e 32.7% of these samples were tested RSV positive by the RSV NASBA assay.

3.4. Evaluation of clinical samples

Initially seventeen clinical nasopharyngeal swab samples, indicated to be positive for RSV of unknown concentration, were tested. As shown in Fig. 2, RSV (RSV A, RSV B or nontyped RSV) was detectable in all samples.

In addition, clinical respiratory swabs, nasopharyngeal aspirates, endo-tracheal secretions, bronchoalveolar lavages and sputum samples were tested as part of a prospective study at the Wales specialist virology center (UHW, Cardiff, United Kingdom). In this study traditional laboratory techniques were directly compared with the RSV NASBA assay. The samples were obtained from various external laboratories referring samples to Cardiff for testing. Some samples were initially tested by the NOW[®] RSV assay (Binax, Inc., Portland, Maine) and all samples from which cells could be obtained such as nasopharyngeal aspirates and bronchoalveolar lavages were screened by the simufluor immunofluorescence-screening assay (Light diagnostics, Chemicon Europe Ltd, Eastleigh, UK). In addition

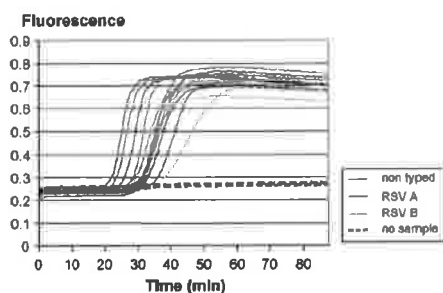


Fig. 2. Clinical samples. Seventeen nasopharyngeal swab samples indicated to be positive for RSV (A, B or non-typed) were tested. The fluorescence curves of real-time NASBA are presented.

most samples were tested in cell culture. As BAL and sputum samples were included, all samples were pretreated with DNase I before isolation. The number of RSV positive samples, as determined by the different methods, is presented by sample type (Table 2). A total of 779 samples were tested during this study, of which 106 were initially screened with the NOW[®] RSV assay (Binax), and 529 were screened with immunofluorescence (IF). Of these, cell culture results were available for 765 samples. All samples were tested with the RSV NASBA assay. A result was obtained for 765 samples giving an inhibitory rate of 1.8%. From a group of 106 NPA samples initially tested to be negative for RSV with NOW[®] RSV Assay (Binax), RSV was detectable with the RSV NASBA assay in 32 samples. Two of these samples were also found positive by cell culture. Samples initially determined to be positive for RSV by IF, were also positive for RSV using the RSV NASBA assay, except for one sample. However, this sample was tested three times by DFA and was only found RSV positive once. The greatest discrepancy of results came from NPAs tested by DFA, in total 354 were found to be negative by DFA in the initial screen, however on repeat testing using the RSV NASBA assay 62 (17%) were found to be RSV positive. In addition, 30 BAL samples that were initially reported as being negative for RSV by DFA and cell culture, an RSV positive result was obtained for another two samples using the RSV NASBA assay. Only 61 out of 765 samples were found RSV positive using cell culture whereas 229 out of 765 samples were found RSV positive using the RSV NASBA assay.

3.5. Clinical significance of clinical samples found to be RSV positive by NASBA alone

Of the clinical samples tested by all methods, 102 were found to be RSV positive by NASBA but not by any other method. In most cases a second sample from the patient was tested to confirm the initial positive result. These samples came from

89 patients. Nine of these patients had repeat samples tested during the study that were only RSV positive by NASBA. All nine patients presented with haematological malignancies and respiratory tract infections. In all cases RSV was the most significant pathogen repeatedly detected. A further 13 patients had a previous sample tested positive for RSV by both NASBA and traditional assays. The detection of RSV by NASBA alone in this group of patients probably represents the end of the infection and a low viral load detected only by molecular method. Conversely eight patients had repeat samples taken following their first test positive which were subsequently positive by other methods as well as NASBA. Together this data suggests that NASBA is effective in detecting early and late infection when compared to the less sensitive traditional methods. Again these 21 patients had underlying haematological malignancies highlighting the importance of RSV infection in this patient group. The remaining 59 patients had a single test RSV positive. By looking at the clinical picture of the patients and the results of other tests it is possible to determine the clinical significance of the RSV result. As described, RSV has its greatest burden in infants below the age of one year. Of the 59 patients, 49 were in this age group, all were hospitalized and of these, the primary clinical diagnosis in 36 was bronchiolitis. As all of the cases fell within the RSV season of 2004–2005 and without the detection of any other pathogen, it would be difficult to rule out RSV as the causative pathogen. The remaining infants presented with a range of respiratory symptoms including wheeze, shortness of breath cough, coryzal symptoms, and poor feeding. One infant was preterm and two required mechanical ventilation. Again as all presented within the defined RSV season and with the absence of an alternative diagnosis, the RSV result was considered clinically significant in all cases. The remaining 10 patients were older children and adults presenting with a range of respiratory symptoms, apart from one patient, all had underlying chronic conditions or trauma. Four were children below the age of 16 with haematological malignancy, one child presented with a confirmed parainfluenza Type 3 dual infection who required ITU treatment. The remaining six patients included one community patient presenting with influenza like illness and five hospitalized patients. Two patients had chronic obstructive pulmonary disease, one patient subsequently died. The remaining patients were haematology patients with respiratory tract infections. In all cases the RSV result was considered significant and the patients were managed accordingly.

4. Discussion

Routine screening for RSV by molecular methods is becoming increasingly popular due to the low sensitivity and specificity of traditional assays, particularly in samples taken from adult patients, where the viral load was shown to be reduced when compared to infant samples. This paper describes the performance of a highly sensitive and specific RSV A + B real-time NASBA assay utilizing molecular beacon technology. By testing dilution series of transcripts of both RSV A and RSV B it is demonstrated that assay sensitivity for RSV A and RSV B transcripts is comparable, a 95% detection hit-rate of 95

and 47 copies per input in isolation for RSV A and RSV B, respectively. This assay sensitivity was confirmed in two RSV sample types: nasopharyngeal swab samples and BAL samples.

For most BAL material sample pretreatment with DNase I was required to remove extraneous DNA that inhibited the NASBA reaction. DNase I pretreatment also improved the detection of RSV in some nasopharyngeal aspirate and sputum samples.

The assay was shown to be highly specific for RSV, when tested against a panel of common respiratory pathogens including; PIV1-3, Influenza A and B, measles, Adenovirus Types 1 and 5, hMPV A1, A2, B1, and B2. No cross-reactivity was observed with the RSV assay. This was in agreement with results published previously (Moore et al., 2006) showing that a number of clinical sample tested negative with the RSV NASBA assay were tested positive for Influenza A, parainfluenza Type 1, parainfluenza Type 3, adenovirus, human metapneumovirus or herpes simplex Type 1.

RSV could be detected in a wide range of clinical sample types including nasopharyngeal swabs, and as part of a prospective study, respiratory swabs, NPAs, BALs, endotracheal secretions, and sputum samples. Of the 779 samples tested in the prospective study, a valid result was obtained with the RSV NASBA assay for 765 samples, giving an overall inhibition rate of 1.8%. Of these 765 samples, a positive result was obtained for 229 samples (29.9%) by the RSV NASBA assay. In comparison, the RSV isolation rate was only 8% in cell culture (61/765). Direct immunofluorescence on appropriate samples gave a detection rate of 23.1% (122/529) although some samples gave insufficient cells for an immunofluorescence test to be performed. The detection rate in these samples was increased using the NASBA assay giving an overall detection rate of 32.7% compared to 23.1% when using DFA alone. POCT are becoming routine in many centers. Of the 106 samples that were tested by the NOW[®] RSV assay (Binax) for which a negative result was obtained, 32 tested positive by the RSV NASBA assay (30.2%), and RSV was isolated in cell culture from two of these samples.

In total, 102 samples were tested RSV positive by the RSV NASBA alone. For most of these samples a second patient sample was tested to confirm the initial result. For all of these samples the validity of this positive result was supported by the information of the condition of the patient and the fact that samples were taken during the RSV season period and no other pathogen was detectable. In addition, some of these samples are part of a sample collection obtained from one and the same patient used for repeat testing during infection of which the previous samples or the subsequent samples were tested RSV positive also by the traditional methods, proving that the patient was indeed infected with RSV. In addition, the results of a retrospective study using the same RSV NASBA (Moore et al., 2006) showed that the presence of RSV RNA in samples tested positive by the RSV NASBA assay but negative by the traditional methods, could be confirmed by RT-PCR. However, as for all test systems, it should be mentioned that there is always a potential for any of these results to be false positives by NASBA.

In conclusion, the data presented in this paper demonstrated the RSV NASBA assay to be highly sensitive compared with cell culture, DFA, and the NOW[®] RSV assay (Binax). In addition, most respiratory sample types could be processed, using a combination of the NucliSens magnetic extraction reagents (bioMérieux) and the NucliSens EasyQ miniMAG (bioMérieux). It is recommended that a DNase I sample pretreatment step is used for samples containing high levels of mucus such as BAL, NPAs, and sputum samples.

The results of a retrospective study that was recently published (Moore et al., 2006), using the RSV A + B NASBA assay also demonstrated that this assay is indeed highly specific for RSV and highly sensitive compared to traditional assays. This retrospective study was a pilot study to show the performance of the RSV A + B NASBA assay on a large amount of clinical samples that had been frozen and tested some months after collection. The prospective study as presented in this paper shows how well the assay works in practice in a routine laboratory on freshly received samples. Together, it could be concluded that assay sensitivity was not affected by sample storage.

The RSV NASBA assay includes a homologous IC, which offers a high degree of standardization and quality control. In addition, using the NucliSens EasyQ Director software used for data analysis requires the specification of various assay specific software parameters what leads to a high degree of validity control and prevention of false negative results.

Using the RSV NASBA assay on the NucliSens EasyQ system, test results from 48 sample extracts were obtained in less than 2 h. Using the NucliSens EasyQ miniMAG as front end, 48 samples were processed and extracted within 2 hours and test results from 48 samples were obtained within 4 h. In case a DNase I pretreatment needs to be performed, the total sample to result time is approximately 4 h and 30 min. This rapid turnaround time and the ease of use of the assay, makes it an attractive alternative for routine RSV testing and a valuable addition to traditional testing.

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Except for USA, the RSV NASBA assay is worldwide commercially available as NucliSens EasyQ RSV A + B assay (bioMérieux). For USA, the NucliSens EasyQ RSV A + B ASR (bioMérieux) is commercially available.

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ARTICLE

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Enhanced clinical utility of the NucliSens EasyQ RSV A+B Assay for rapid detection of respiratory syncytial virus in clinical samples

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Abstract The aim of the present study was to compare traditional methods for the detection of respiratory syncytial virus with a newly developed commercial assay based on real-time nucleic acid sequence based amplification. Respiratory syncytial virus is a major cause of severe respiratory infection in infants and in certain groups of older children and adults. Treatment options are limited, but a rapid diagnosis improves patient management and infection control. The rapid diagnosis of respiratory syncytial virus currently relies on antigen detection assays. These tests are limited to use in certain good-quality types of samples, which are rarely obtained from adult patients. Molecular-based assays for the detection of respiratory syncytial virus are shown to be highly sensitive, specific, and more rapid than cell culture techniques. This retrospective study compared traditional laboratory techniques for the detection of respiratory syncytial virus in 508 respiratory samples collected during the winter months of 2003–2004 against the recently developed, commercially available NucliSens EasyQ Respiratory Syncytial Virus A+B assay (bioMérieux, Marcy l'Etoile, France), which is based on real-time nucleic acid sequence based amplification using molecular beacons and an internal control. Using traditional techniques, the prevalence of respiratory syncytial virus in the samples tested was found to be 21%. Using the real-time nucleic acid sequence-based amplification assay, an additional 41 samples from patients with a clinically diagnosed respiratory illness were found to be positive for respiratory syncytial virus. The NucliSens EasyQ assay was shown to be sensitive and specific for the detection of respiratory syncytial virus A+B in different

types of respiratory samples. Moreover, the time required to complete the assay was <4 h, so results could be obtained on the same day as sample receipt in the laboratory.

Introduction

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection in infancy and early childhood and is acknowledged as being an important cause of morbidity in certain adult populations [1, 2]. It is the single most common cause of bronchiolitis in infants, with around 2% of those infected requiring hospitalisation in developed countries annually [1, 3]. In adult populations, it is estimated that up to 15% of pneumonia hospitalisations during the winter months are caused by RSV infection [2].

RSV epidemics are highly predictable annual occurrences. In temperate climates, the "RSV season" generally peaks during November to March, with RSV rarely isolated during the summer. In tropical or subtropical regions, an increased incidence of RSV occurs during rainy seasons [4]. Two subtypes of RSV exist, A and B, which co-circulate within the population. Epidemiological studies suggest that RSV type A may be associated with a more severe infection than RSV type B, although this has not been proven conclusively [1, 5, 6]. No vaccine is currently available, and treatment options for RSV are limited. A rapid diagnosis of RSV, however, allows for appropriate patient management and infection control measures [7].

RSV is a labile virus, which has implications for the laboratory diagnosis of infection, particularly if the method of choice is cell culture [8]. The cell line used in most routine diagnostic laboratories for isolation of RSV is HEP-2, with viral growth being identified by the characteristic syncytia (giant cell) formation from which the virus derives its name. Whilst RSV can be isolated from most respiratory samples, more rapid results can be achieved by using an RSV antigen detection assay such as direct immunofluorescence of washed epithelial cells obtained from clinical samples such as nasopharyngeal aspirates and bronchoal-

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veolar lavage fluids. These samples must be obtained invasively, and good-quality aspirates are rarely achieved from adult patients, particularly from those who are immunocompromised [8]. There is also evidence that the amount of virus shed during an RSV infection in an adult is less than that shed during acute infections in young children [1, 8–10]. This means that both direct immunofluorescence and cell culture have reduced sensitivity in adult populations [2, 10]. Recent developments include rapid antigen detection kits for RSV that can be used at patient point-of-care as well as in the routine diagnostic laboratory. The drawback for many of these, however, is their relative insensitivity when compared to methods such as direct immunofluorescence [11].

Molecular techniques for the detection of RSV have been developed but have yet to find wide appeal in the routine diagnostic setting. This is because screening assays such as direct immunofluorescence offer rapid results for a fraction of the cost [12]. Molecular assays based on reverse-transcription polymerase chain reaction (RT-PCR), have been demonstrated to have an increased sensitivity and specificity over traditional laboratory methods for the diagnosis of RSV on most types of samples, including respiratory swabs [10–13]. Furthermore, the development of real-time RT-PCR assays has reduced the time from sample receipt to obtaining a result when compared to traditional endpoint detection RT-PCR assays [14–17].

It is difficult to obtain good-quality nucleic acid from respiratory samples because PCR-inhibiting substances are often extracted along with the nucleic acid. To this end, it is important that some form of internal control is used to ensure the absence of any inhibiting substance from the reaction and to avoid false-negative results that might result from human error during the extraction procedure [18]. Few of the molecular assays described that have been developed "in-house" and that specifically target respiratory viruses incorporate an internal control for the assay.

Nucleic acid sequence-based amplification (NASBA) technology with endpoint detection has been well described elsewhere [19, 20]. Real-time NASBA is a recent development, and already a number of assays have been described and utilised for a range of viral targets. Such assays include the commercially available NucliSens HIV- and enterovirus-specific assays [21] as well as assays developed "in house" for the detection of respiratory viruses such as parainfluenza types 1, 2, 3, and 4 [22, 23] and influenza A [24]. Real-time NASBA differs from traditional endpoint detection by incorporating target-specific molecular beacon probes into the reaction mix, thereby allowing simultaneous amplification and detection of the target to occur in the same way as for real-time PCR techniques [21].

The aim of this study was to compare the performance of a recently developed NucliSens EasyQ RSV A+B real-time NASBA assay (bioMérieux, Marcy l'Etoile, France) against traditional laboratory techniques for the detection of RSV in a variety of clinical specimens. In addition, the ease of use and clinical utility of the assay within a routine diagnostic laboratory was also determined.

Materials and methods

Clinical material

The study was performed retrospectively on a total of 508 respiratory samples of different types collected from October 2003 to March 2004. Most samples were received in virus transport medium comprising Hanks balanced salt solution $\times 1$ (BioWhittaker, Wokingham, Berkshire, UK), 7.5% bovine albumen (Sigma-Aldridge, Poole, Dorset, Surrey, UK), penicillin (Britannia Pharmaceuticals, Redhill, Surrey, UK), and streptomycin (Sigma-Aldridge); both antibiotics were used at a final concentration of 100 U/ml. Samples not received in transport medium and those in other types of transport medium (commercially available and otherwise) were also included to ensure a broad range of sample types that are received routinely in the laboratory (Table 1). Respiratory swabs received dry into the laboratory were broken directly into a 0.9 ml NucliSens lysis buffer tube (bioMérieux, Boxtel, The Netherlands), vortexed, and left at room temperature for 10 min. The lysis buffer was then processed as for virus transport medium.

Following routine testing at the time of receipt, separate aliquots of the samples together with the remaining sample in the original transportation vial were frozen and stored at -80°C . Every effort was made to ensure that each sample had minimal freeze/thaw cycles to ensure the integrity of both virus and nucleic acid.

Table 1 Clinical samples tested for RSV. Showing the total number of each sample type tested and the number of each sample type found to contain RSV by any of the methods used in the study and the number of each sample type found positive by real-time nucleic acid sequence-based amplification (NASBA) alone

Type of specimen	Number of samples tested (percentage of total)	Number positive (%)	Number positive by NASBA only
Upper respiratory tract samples			
Nasopharyngeal aspirates	324 (64%)	135 (92%)	35
Endotracheal secretions	9 (2%)	3 (2%)	0
Throat swabs	85 (17%)	2 (1%)	2
Nasal swabs	23 (5%)	3 (2%)	3
Combined nose and throat swabs	9 (2%)	0	0
Lower respiratory tract samples			
Sputum	10 (2%)	1 (0.5%)	1
Bronchoalveolar lavage fluids	38 (7%)	4 (2.5%)	0
Post-mortem samples			

Traditional laboratory assays

The majority of samples included in the study had been tested by more than one method on initial receipt in the laboratory; these results were used for comparison with the results of the real-time RSV NASBA.

Where possible, samples were screened by direct immunofluorescence using the Simulfluor immunofluorescence screening assay (Light Diagnostics, Chemicon Europe, Eastleigh, UK). If the sample was positive for a virus other than RSV, identification of the specific virus was performed using individual monoclonal immunofluorescence reagents (Imagen; Dakocytomation, Glostrup, Denmark). Some external laboratories that referred samples for testing also screened similar samples using the Binax NOW RSV Assay (Binax, Scarborough, ME, USA). In total, 379 of 508 (75%) of the samples were tested by either method.

Most of the samples, 492 of 508 (97%), were also inoculated into cell culture, namely, primary monkey kidney cells or PLC (human hepatoma) cells, MRC-5 cells, and HEp-2 cells. The cell culture tubes were incubated on a rolling drum at 37°C and observed every 2-3 days for signs of cytopathic effect (CPE). In addition, haemadsorption was performed using human "O" type erythrocytes to indicate growth of influenza or parainfluenza. Immunofluorescence was used to confirm a CPE.

Extraction of nucleic acid

Two hundred microlitres of sample was added to 2 ml of NucliSens Lysis Buffer (bioMérieux) for extraction. The NucliSens EasyQ RSV A+B assay incorporates an RSV-specific internal homologous control RNA, which is added to the sample in lysis buffer prior to extraction and is optimised to monitor the extraction, amplification, and detection procedure at the individual sample level.

Following addition of the internal control, nucleic acid was extracted using the NucliSens miniMAG extraction system and NucliSens Magnetic Extraction Reagents (bioMérieux) following the manufacturer's instructions. The method is based on the Boom silica slurry technique [25] utilising magnetic silica. Rather than using centrifugation, magnets were applied to pull the silica out of the buffer solutions, which could then be easily removed without disrupting the silica.

Three wash buffers containing decreasing amounts of salt solution were used during the extraction procedure. Total removal of wash buffer 1, which contains the highest salt concentration, was paramount to ensure the assay worked effectively. To facilitate this, the first wash with buffer 2 was performed in such a way to ensure any residual droplets of wash buffer 1 were put into solution for easy removal. In addition, careful manipulation of the silica pellet by gentle pipetting with wash buffer 2 helped reduce silica aggregation and improved the overall washing procedure. Nucleic acid elution was performed by the addition of 25 µl of elution buffer followed by agitated

incubation at 60°C for 5 min. The tubes were transferred to a magnetic rack and the eluate removed to a fresh 1.5-ml tube ready for testing.

Amplification and detection of nucleic acid sequences

The NucliSens EasyQ RSV A+B assay was provided as a 48-sample test kit containing the internal control and specific primer and a molecular beacon mix targeting the fusion protein of RSV [26]. This was then used in conjunction with the generic 48-reaction NucliSens EasyQ Basic Kit Amplification Reagents (containing lyophilised spheres of enzymes and reagent, diluent for both, KCl, and molecular-grade NASBA water).

The RSV primer-binding sites also flank a portion of the internal control sequence, so only a single primer set was needed for the amplification of both targets. Two specific molecular beacons labelled with the fluorescent dyes FAM (wild-type RSV) and ROX (internal control) were used to differentiate between the two amplification products. Real-time amplification and detection was performed using the NucliSens EasyQ Analyser and NucliSens EasyQ Director software. The lower detection limit of the assay was determined as being 22 input copies of wild-type RSV RNA during the assay development [26].

The assay was performed following the manufacturer's instructions. Briefly, 5 µl of eluate was transferred to a 0.2-ml reaction tube, to which 10 µl of mastermix was added. This was then incubated for 2 min at 60°C followed by 2 min at 41°C. The enzyme mix was prepared 20-30 min earlier to allow full reconstitution from the lyophilised bead. Five microlitres of enzymes was then pipetted into the appropriate number of 0.2-ml reaction tube lids, and these were then used to cap the reaction tubes. The reaction tubes were pulse centrifuged, flicked to mix the enzymes with the reaction mix, and recentrifuged. The reaction tubes were then transferred to the NucliSens EasyQ Analyser and the amplification run started.

Analysis of results

The NucliSens EasyQ Director software allowed for continuous monitoring of each individual amplification reaction by continuously plotting an amplification curve based on the fluorescent signal emitted by either the internal control or the wild type molecular beacon during target amplification.

Once completed, the results of each RSV run were analysed and validated by the software. Valid results were obtained when there was a wild-type RSV signal or internal control signal above a defined threshold present at the end of the assay. Due to the competitive nature of the amplification reaction, a very high wild-type RSV signal often occurred in the absence of any internal control signal; these results were still valid. Invalid results occurred when there was a poor internal control signal or when no internal control signal occurred in the absence of a wild-type signal.

Invalid and discordant results

All samples found to give invalid results were re-extracted and tested again. Those samples that gave discordant results in the real-time NASBA assay (negative for RSV when one or more other tests were positive) were always retested from the original sample vial to ensure that there

wasn't an error during the original sample aliquoting and storage.

A molecular test for RSV was not established at the time of the evaluation in the laboratory. A previously published nested RT-PCR assay [27] was introduced to confirm the results of 49 of the samples. The size of the second round product obtained allowed for subtyping of the RSV present

Table 2 Clinical details of patients positive for RSV by real-time nucleic acid sequence-based amplification (NASBA) alone. All patients were admitted to hospital unless otherwise stated

Patient number	Age	Sex	RSV type	Clinical details
1 ^a	5 months	F	B	Bronchiolitis
2	37 years	M	A	URTI, pyrexia, AML, chemotherapy
3 ^a	9 months	M	A	Pyrexia, influenza-like illness
4 ^a	4 months	F	NT	Wheeze
5	6 years	M	A	Low-grade fever, URTI, HIV, community infection
6	1 year	M	A	Pneumonia, ICU, ventilated
7 ^b	67 years	M	NT	Pyrexia, URTI, AML
8	11 months	M	A	Pneumonia, ICU, ventilated
9 ^a	2 months	F	NT	Bronchiolitis
10 ^a	2 weeks	F	NT	Bronchiolitis
11 ^a	2 months	F	NT	Bronchiolitis
12 ^a	2 months	F	A	Bronchiolitis
13 ^a	4 months	M	NT	Bronchiolitis
14 ^a	3 months	M	B	Bronchiolitis
15 ^a	3 months	F	A	Wheeze, creps, increased O ₂ requirement
16 ^a	1 year	M	A	Bronchiolitis, increased O ₂ requirement, sister RSV positive
17 ^a	11 months	M	A	Bronchiolitis
18 ^a	1 month	M	A	Bronchiolitis
19	4 months	F	NT	Chronic lung disease, ICU, ventilated
20	1 year	M	NT	Pneumonia, ICU, ventilated
21	1 year	M	NT	Bronchiolitis, premature at birth
22	11 years	F	A	Congenital heart defect, ICU, ventilated
23	61 years	F	B	Influenza-like illness, community-acquired infection
24	3 months	F	B	Cough, HIB also isolated
25	39 years	M	A	Pyrexia
26 ^a	1 year	M	A	Bronchiolitis
27 ^a	4 months	M	A	Bronchiolitis
28 ^a	7 months	M	NT	Bronchiolitis, respiratory distress
29 ^a	6 months	F	A	Bronchiolitis
30	3 months	M	B	Severe cough, <i>Bordetella pertussis</i> also isolated
31	70 years	M	B	Community-acquired pneumonia, ICU, ventilated
32 ^a	3 months	M	NT	Bronchiolitis
33	7 months	F	NT	Pyrexia, congenital heart defect
34	6 months	F	NT	Bronchiolitis, congenital heart defect
35	51 years	F	NT	Respiratory failure, COPD, ICU, ventilated
36	3 years	F	NT	URTI, pyrexia, ALL, chemotherapy
37 ^a	1 year	M	NT	Bronchiolitis
38 ^a	9 months	F	A	Bronchiolitis
39 ^a	6 months	M	A	Bronchiolitis
40 ^a	2 months	F	A	Bronchiolitis
41 ^a	4 months	F	NT	Respiratory distress

URTI upper respiratory tract infection, AML acute myelogenous leukemia, ICU intensive care unit, NT not typed, COPD chronic obstructive pulmonary disease, ALL acute lymphocytic leukemia

^aNo underlying condition, made full recovery

^bSeparate nasopharyngeal aspirate from patient; RSV positive by direct immunofluorescence

in the sample to validate the assay's ability to detect both RSV subtypes.

Samples found repeatedly inhibitory by NASBA were investigated further using a pretreatment step to remove extraneous DNA that might be inhibiting the NASBA reaction. Briefly, for each 100 μ l of sample tested, 20 μ l of DNase I (1 U/ μ l) (Promega, Southampton, UK) was added, together with 24 μ l of DNase I buffer (provided with the enzyme). The mixture was then incubated at 37°C for 30 min in a water bath prior to the addition to lysis buffer. From then, the same extraction and amplification protocol was followed as previously described.

Statistical analysis

Sensitivity and specificity were calculated using Fisher's exact test and by comparing the results of real-time NASBA with those of the rapid RSV screening and cell culture tests. From these results, the positive and negative predictive values of the real-time NASBA assay were calculated. In addition to Fisher's exact test, both Kappa and Newcombe statistics [28], which compare different assays directly without a "gold standard," were also applied to allow for the expected increase in sensitivity seen with most molecular assays when compared to traditional techniques, which can lead to poor scores for specificity and positive predictive value. The Fisher's exact and Kappa statistical analysis was performed using GraphPad Instat and Prism 4 (GraphPad Software, San Diego, California, USA).

This study complies fully with the current laws of the UK.

Results

A total of 508 samples were tested by real-time NASBA in the study. Over half of the samples 280 of 508 (55%) were collected from infants. The remaining samples were collected from older children and adults. Most samples (95%) were collected from hospital inpatients, whilst the

remaining samples were obtained from outpatient clinics or from the community. In all cases, the patients presented with a respiratory illness of varying severity.

Traditional laboratory assays

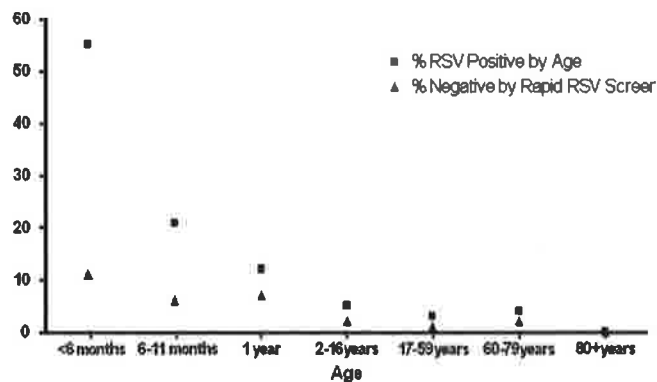
In total, 339 of 508 (67%) samples were initially screened for RSV by immunofluorescence. Of those, 14 (4%) were reported as having insufficient cells for interpretation when processed and so were included with the samples tested by cell culture alone. Of the remaining samples, 101 of 325 (31%) were RSV positive. In addition, the laboratory received 45 (9%) samples that had already been screened by the Binax NOW assay at the referring laboratory, one of which was already screen positive for RSV. Four hundred ninety-two (97%) of the samples were inoculated into cell culture, and of these, RSV was isolated from 62 (13%).

Of the 106 of 508 (21%) samples positive in the traditional laboratory assays, 58 (55%) were positive for RSV by both the rapid antigen screening assay and cell culture. Of the remaining 48 RSV positive samples, 44 of 106 (42%) were detected by direct immunofluorescence alone, while 4 of 106 (3%) were RSV positive by cell culture alone. This initial data demonstrated that immunofluorescence was nearly twice as good as cell culture in detecting RSV in clinical samples ($p < 0.0001$). In addition, RSV was not detected in any of the respiratory swabs received in the laboratory using cell culture. Other viruses were found in 21 samples, including influenza type A, parainfluenza type 1, parainfluenza type 3, adenovirus, human metapneumovirus, and herpes simplex virus type 1.

NucliSens easyQ RSV A+B assay

Using the real-time NASBA assay, RSV was detected in a further 41 samples (Table 2), which resulted in an increase in the positivity rate of 38% when compared to traditional assays alone. This included the detection of RSV in 35 of 324 additional nasopharyngeal aspirate samples tested by direct immunofluorescence and culture (an increase in the

Fig. 1 Data on RSV-positive patients, shown grouped according to age. "Percent RSV positive by age" refers to the percentage of samples received in each age group and found to be RSV positive by any method. "Percent negative by rapid RSV screen" refers to those samples negative by rapid assay (immunofluorescence or Binax Now assay) but positive by real-time NASBA and cell culture or RT-PCR. These samples are shown to illustrate that in some age groups up to 10% of RSV-positive samples can be missed if detection is reliant on a rapid RSV screen alone



positivity rate of 35% in this sample type alone) and in 5 of 119 (4%) respiratory swabs (two of which were dry when received into the laboratory). The NASBA assay also detected RSV in 6 of 44 (14%) samples found to be negative by the Binax NOW RSV assay. RSV was detected in all age ranges from which samples were received, except in the age group >80 years (Fig. 1).

Statistical analysis

The real-time NASBA assay detected RSV in 131 of 325 (40%) samples tested by immunofluorescence, which includes the 35 additional nasopharyngeal aspirate samples in which RSV was detected. Compared to immunofluorescence, therefore, the real-time NASBA assay had a sensitivity and specificity of 99% (95%CI 94–99) and 87% (95% CI 81–90), respectively, with a positive predictive value of 77% and a negative predictive value of 99% ($p < 0.0001$). The Kappa statistic was calculated at 0.79, with a 95%CI of 0.7–0.9 indicating good agreement between the two methods. Using Newcombe statistics, the real-time NASBA assay was shown to be 9% more sensitive than direct immunofluorescence alone, with a 95%CI of 5–12%.

Of the 492 samples tested by cell culture, the real-time NASBA assay detected RSV in an additional 84 samples above the 62 detected by cell culture alone. Because cell culture proved relatively insensitive for RSV isolation, the sensitivity of the real-time NASBA assay was calculated at 98% (95%CI 91–99). The specificity was shown to be only 77% (95%CI 76–84), with a positive predictive value of 42% (95%CI 33–51) and a negative predictive value of 99% (95%CI 98–99) ($p < 0.0001$). The Kappa statistic for cell culture versus NASBA was calculated at 0.50, with a 95%CI of 0.41–0.50 indicating moderate agreement. Using Newcombe statistics, the increased sensitivity of real-time NASBA over cell culture was calculated at 17%, with a 95%CI of 11–22%.

Only 7 of 45 samples tested by both the Binax NOW RSV assay and the real-time NASBA were positive for RSV, which made meaningful statistical analysis difficult. Using Binax as the "gold standard," the sensitivity of the real-time NASBA was calculated as 100% (95%CI 2–100), the specificity as 86% (95%CI 72–94), the positive predictive value as 14% (95%CI 3–57), and the negative predictive value as 100% (95%CI 90–100). As expected, these findings were found not to be statistically significant ($p = 0.155$). This was reflected in the Kappa statistic, which was 0.22 with a 95%CI of 0.01–0.2, indicating poor agreement between the two assays. The Newcombe statistic showed an increase in sensitivity of the real-time NASBA assay of 13%, with a 95%CI of 2–26%.

Discordant and inhibitory results

Two samples gave negative results by real-time NASBA that were discordant with the results of traditional methods.

One sample was found to be RSV positive by cell culture alone. This sample was re-extracted and tested by real-time NASBA and was repeatedly negative. In an attempt to resolve the results, the eluate was amplified using the RT-PCR assay, which also failed to detect RSV. The second sample was positive by immunofluorescence alone. Again, despite re-extraction and testing both from the aliquot stored at -80°C and the original transportation vial, RSV was not detected by the real-time NASBA assay.

Thirteen samples gave inhibitory results by the real-time NASBA assay that could not be resolved by re-extraction. Of these, the most problematic sample type was sputum, with 4 of 10 (40%) received being inhibitory. Respiratory swabs and nasopharyngeal samples received in skimmed-milk-based transport medium also proved problematic and remained inhibitory even after extra wash steps. However, following the pretreatment step using DNase I, the inhibition effect was removed in all cases, and all samples were found to be negative for RSV.

Confirmation and subtyping by reverse transcriptase-polymerase chain reaction

Forty-nine eluates were amplified using RT-PCR to confirm the real-time NASBA results, including 44 positive samples and five negative samples. Of the 44 positive samples, 24 were positive by real-time NASBA alone. All of the NASBA results were confirmed. The RT-PCR detected 31 RSV type A viruses and 13 RSV type B viruses. The sample RSV positive by cell culture could not be confirmed by either real-time NASBA or RT-PCR.

Discussion

Real-time molecular assays based on RT-PCR for the detection of RSV have been widely described in the literature, but this is the first clinical evaluation of a commercially available real-time assay based on NASBA technology with molecular beacon detection. The modular approach to sample extraction, amplification, and detection afforded by the NucliSens miniMAG together with the NucliSens EasyQ Analyser and software was well suited to a busy routine diagnostic laboratory. Compared to other molecular techniques, the methodology was simple to learn and easy to perform. The rapid turnaround time enabled 24 samples to be received in the laboratory, processed, and amplified and the results reported within 4 h.

The NucliSens RSV A+B assay was highly sensitive and specific when compared to direct immunofluorescence and cell culture techniques routinely used in the laboratory. The increase in sensitivity demonstrated by the real-time NASBA assay over both of these methods was significant and similar to that seen in a recent evaluation of another real-time RSV assay [14].

The incorporation of a specifically designed internal control that is added prior to sample extraction ensured greater confidence in the negative results obtained.

Consequently, the use of the internal control served to highlight specific sample types in which inhibition rates were higher than expected. These included samples that contained high levels of mucus such as bronchoalveolar lavages and sputum. This precipitated a pretreatment step using DNase I, which was shown to significantly reduce the inhibition rates seen in these particular samples.

Few studies describing real-time molecular assays for RSV detection include the range of different respiratory sample types from adults and children, as described in this study. Two recently described real-time multiplex RSV and influenza assays [14, 16] were evaluated on nasopharyngeal aspirates from young children. In another real-time RT-PCR assay [17], the results were compared only with cell culture, which has a lower sensitivity than direct immunofluorescence for the detection of RSV.

Overall, direct immunofluorescence for the detection of RSV in nasopharyngeal aspirates from young infants compares favourably against real-time molecular methods [14]. However, there is some merit in testing immunofluorescence-negative samples by a molecular assay for RSV, particularly if the child has an underlying condition or requires intensive care. This should also apply to samples that test negative by a rapid RSV antigen immunoassay.

Of the 41 samples found RSV negative by traditional assays but positive by real-time NASBA in this study (Table 2), 32 (76%) came from infants. Of these, 10 of 32 (32%) were obtained from infants that had either an underlying medical condition or required intensive care treatment and mechanical ventilation for pneumonia. The remaining 22 (68%) infants had uncomplicated clinical bronchiolitis and required a short hospital stay. The remaining 9 of 41 (21%) came from older children and adults. All of the patients presented with a respiratory illness (in 2 cases, later samples were found to be RSV positive by a traditional assay). In total, 8 of 9 (89%) patients had an underlying condition or had community-acquired pneumonia and required intensive care and ventilation. One sample came from a patient in the community who presented with an influenza-like illness.

A major advantage of the real-time NASBA assay over traditional techniques is that it can provide a rapid result using a respiratory swab. During this study, all of the respiratory swabs found positive by real-time NASBA for RSV were taken from individuals who were immunocompromised with upper respiratory tract infections. These included three adult haematology patients who had undergone recent bone marrow transplants and were all on a ward at the same time. This highlights the importance of sensitive molecular assays for the detection of respiratory viruses in this vulnerable group of people as well as the advantage offered by the real-time NASBA assay to provide more effective testing for RSV in adults in general.

The role of RSV in community infections is not entirely clear, although interest in RSV as a cause of community-acquired infection is increasing. Enhanced community surveillance of RSV is now a realistic proposition with the advent of molecular assays such as the real-time NASBA assay, which can rapidly detect RSV from swabs received

in virus transport medium and, as demonstrated by this study, from swabs received dry in the laboratory.

During the whole study, there were two discordant samples. These were found to be RSV positive by either cell culture or direct immunofluorescence but gave repeated negative results in the real-time NASBA assay. It is possible that in both cases the amount of RSV RNA present in the sample was below the detectable threshold of the RSV real-time NASBA assay, but it also cannot be ruled out that the original result was incorrect, as neither could be confirmed.

Taking the confirmatory RT-PCR results, the clinical information of the patients found RSV positive by real-time NASBA alone, the high number of negative samples, and those samples in which other viruses were detected into account, the real-time NASBA assay was shown in this study to be highly specific for RSV, with no known false-positive results obtained.

Because the real-time NASBA assay could not type the RSV detected, typing was performed by RT-PCR. Of the 31 patients from whom RSV type A viruses were detected, 8 (26%) developed pneumonia that required intensive care therapy. Of the 13 patients with positive samples typed as RSV type B, 3 (23%) also developed pneumonia and required ventilation in an intensive care unit. These results indicate that RSV type B can give rise to severe infection in the same way as RSV type A. This suggests that the development of assays that detect RSV type A alone cannot be justified [15]. Typing RSV has little clinical significance in terms of patient management, and the increased cost associated with typing is not justified in a routine clinical diagnostic context. RSV typing assays do, however, have some utility as epidemiological tools.

Multiplex assays are popular, and several are described for the detection of respiratory viruses. These assays are favourable in terms of overall cost and time management, and so the further development of multiplex real-time NASBA assays would be welcome. It is vital, however, that overall assay sensitivity is not compromised due to the complexity and difficulties associated with fully optimising multiplex assays.

This study has demonstrated that the NucliSens EasyQ RSV A+B assay has significantly improved sensitivity and specificity when compared to cell culture and rapid RSV antigen detection assays on all types of respiratory samples tested in this study, including those from adult patients. Using the real-time NASBA assay, RSV was detected in samples reported as RSV negative by other traditional assays. In some of these cases, RSV was shown to be the most significant pathogen isolated in patients presenting with severe respiratory illness. This demonstrates the enhanced clinical utility of this assay over traditional laboratory techniques. Overall, the ease of use of the assay and the rapid turnaround time mean that the NucliSens EasyQ RSV A+B Assay would make a valuable addition to the molecular repertoire of any routine laboratory wishing to introduce molecular assays for the detection of respiratory viruses.

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3.5 Manuscript 5. Dry Cotton or flocked swabs as a simple collection technique for the molecular detection of respiratory viruses.

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Dry cotton or flocked respiratory swabs as a simple collection technique for the molecular detection of respiratory viruses using real-time NASBA

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ABSTRACT

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This paper describes the molecular detection of influenza A, influenza B, respiratory syncytial virus and human metapneumovirus using real-time nucleic acid sequence based amplification (NASBA) from respiratory samples collected on simple dry cotton swabs, non-invasively and in the absence of transport medium. Viral RNA was detectable on dry cotton and flocked swabs for at least 2 weeks at room temperature and was readily extracted using magnetic silica extraction methods. Dry cotton respiratory swabs were matched with traditionally collected respiratory samples from the same patient, and results of traditional laboratory techniques and real-time NASBA were compared for all four viral targets. The results not only showed a significant increase in the detection rate of the viral targets over traditional laboratory methods of 46%, but also that dry swabs did not compromise their recovery. Over two subsequent winter seasons, 736 dry cotton respiratory swabs were collected from symptomatic patients and tested using real-time NASBA giving an overall detection rate for these respiratory virus targets of 38%. The simplicity of the method together with the increased detection rate observed in the study proves that transporting a dry respiratory swab to the laboratory for respiratory virus diagnosis using molecular methods is a suitable and robust alternative to traditional sample types.

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1. Introduction

Respiratory viruses contribute to significant morbidity and mortality in healthy and vulnerable individuals. The introduction of improved antiviral treatments for respiratory viral infection in recent years has meant that rapid diagnosis of respiratory viral infection is vital to ensure patients are treated and managed appropriately (Englund et al., 1996; Boivin et al., 2004; Templeton et al., 2004; Moore et al., 2004; Deiman et al., 2007).

Achieving a rapid result that is both sensitive and specific is challenging. The timing of sample collection in relation to the onset of symptoms together with the quality of the sample is crucial. For example, although direct immunofluorescence allows results to be available within 1 h of sample receipt, compared to collecting a respiratory swab, more invasive sampling is required to collect a nasopharyngeal aspirate or broncho-alveolar lavage. Also, these samples in adult patients can give poor results due to reduced viral shedding and reduced amounts of cellular material when compared to infants (Hall et al., 1976; Englund et al., 1996; Falsey, 2007). More rapid point of care testing for certain targets like influenza and

RSV have their place, but these tests are generally less sensitive than traditional laboratory tests and so it is important that negative results from these assays are later confirmed by a more sensitive test (Moore et al., 2006; Dwyer and Sintchenko, 2007).

For the laboratory diagnosis of respiratory viral infection, cell culture has been historically the gold standard. But for successful isolation it is important that the clinical specimen is collected from the patient close to initial symptom onset and be transported under appropriate conditions to the laboratory. For swabs taken from the respiratory tract, this requires the use of virus transport medium. Using traditional cell culture techniques, a positive result may take several days to become positive depending on the quality of the sample and the viral load, but a negative result may not be available for up to 2 weeks. However, recent advances in cell culture techniques, in particular the commercial shell vial techniques, such as ready-cells (R mix) have reduced the time to result to just 48 h (LaSala et al., 2007).

Molecular tests for respiratory virus detection are being used increasingly in routine diagnostic laboratories (Hibbitts and Fox, 2002). Numerous studies have shown that molecular techniques based on conventional PCR or nucleic acid sequence based amplification (NASBA) vastly improves the detection rate for respiratory viruses over traditional laboratory techniques. Using real-time detection of the amplified product, same day results are now a

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reality (Englund et al., 1996; Boivin et al., 2004; Templeton et al., 2004; Moore et al., 2004, 2006; Deiman et al., 2007). However, there has been very little work reported on evaluating the most appropriate way of collecting and transporting respiratory samples to the laboratory for molecular testing. The evaluation of new molecular assays is often based on samples received for virus isolation allowing for testing by more than one method and thus a direct comparison of results. By using this approach routinely it means that the sample received might be contaminated with the target virus if processed in a room where cell culture is performed. In an ideal situation, a dedicated respiratory sample for molecular testing would be collected non-invasively, the transportation method would be simple and inexpensive; with viral nucleic acid stability guaranteed allowing for accurate detection of the target virus for several days. Dedicated samples for molecular testing would reduce the risk of contamination in laboratories also performing traditional methods for virus detection and the ease of sample collection would facilitate clinical teams in confirming a diagnosis of respiratory infection. Removing the need for virus transport medium would reduce its associated cost, storage requirements and risk of leakage during transportation.

Other simple collection and transportation methods have been described. One of the most effective methods is a dried blood spot collected onto filter paper (Karapanagiotidis et al., 2005). This method was used in the late 1990s for HIV-1 RNA detection (Cassol et al., 1997; Mwaba et al., 2003; Alvarez-Munoz et al., 2005; Li et al., 2005) genotyping (Plantier et al., 2005) and measles surveillance schemes in Africa (El Mubarak et al., 2004). These showed that the integrity of RNA can be maintained for long periods and at varying temperatures (Abe and Konomi, 1998). A more complex, but commonly used approach is to use FTA[®] filter paper, which is impregnated with lyophilised chemicals which lyses both viruses and bacteria rendering them non infectious (Moscoso et al., 2005). Filter paper strips have also been used to collect faecal material which is then fixed prior to sending for molecular testing and sequencing studies (Vilcek et al., 2001; Woollants et al., 2004). Recently, a method has been described for collecting respiratory samples for surveillance using swabs fixed in ethanol (Krafft et al., 2005). Similarly, in an outbreak of parainfluenza 3 on a haematology ward at the University Hospital of Wales where nasal samples were collected directly into guanidine thiocyanate lysis buffer for transportation and nucleic acid extraction (Hibbitts et al., 2003).

In the winter season of 2003–2004, a real-time nucleic acid sequence based amplification assay for the detection of influenza A was developed. During the clinical evaluation studies a small number of dry respiratory swabs were received for testing transported in the outer sterile protective cover alone. Rather than discard the swabs as unsuitable for testing, they were broken into lysis buffer on receipt in the laboratory, extracted and tested for influenza as for other samples. In more than one instance influenza A was detected (Moore et al., 2004).

This paper includes three constituent studies that together demonstrate the validity of dry swabs for the detection of respiratory viruses. The first describes a simple RNA stability study comparing the widely available cotton tipped wooden swab and the recently introduced flocked swab designed to increase the surface area of the swab allowing for improved sample collection and yield. The second describes a pilot study comparing dry cotton tipped respiratory swabs with matching nasopharyngeal aspirates or respiratory swabs transported in viral transport medium collected at the same time from the same patient using both real-time NASBA and traditional laboratory techniques and the final part demonstrates how dry cotton respiratory swabs can be used as a simple effective collection technique for routine molecular testing.

2. Methods

2.1. Study to determine viral stability

To demonstrate viral stability, dry sterile cotton tipped, wooden swabs and flocked swabs (Bibby Sterilin, Copan, Italy) were compared using serially diluted viral isolate. RSV was selected for this work as it was considered to be most environmentally labile virus being targeted in the study. RSV positive virus culture supernatant was obtained from the Welsh Specialist Virology Centre and its TCID₅₀ was calculated to be 1×10^5 using the method of Reed and Meunch (1938). The virus culture fluid was diluted to 10^{-2} into sterile phosphate buffered saline (PBS). To a series of matching cotton and flocked swabs marked day 0 to day 15, 50 μ l of the diluted virus culture fluid was added to the tip of the swab and the swab re-sheathed and allowed to dry at room temperature. After 1 h the swabs marked day 0 were broken into a 0.9 ml Lysis buffer tube (bioMérieux, Marcy l'Étoile, France), vortexed and left to stand for 10 min before freezing at -80°C . After 24 h at room temperature, day 1 swabs were processed in the same way as for day 0. This was repeated on a daily basis until the swabs inoculated on day 15 had been processed. All of the swabs were then defrosted and RNA was extracted from 200 μ l of the Lysis buffer as described below for molecular testing by real-time NASBA.

2.2. Comparison study of dry respiratory swabs and matching clinical respiratory specimens

Dry cotton tipped wooden ended swabs were utilised as these were used routinely by all clinicians and available widely on all clinical ward areas. The swab was collected from either the nasal passage or the throat and returned to its original holder for transportation back to the laboratory. Samples were collected from children and adult oncology and haematology patients presenting with respiratory illness and from patients in community outbreaks where respiratory virus infections were suspected.

From one cohort of patients, clinicians were asked to collect two respiratory swabs from either the throat or nasal passage, one was put in virus transport medium for virus isolation in cell culture and molecular testing by real-time NASBA (Moore et al., 2004) and the second sent dry for real-time NASBA only.

In a second patient cohort, nasopharyngeal aspirates were taken following the usual local protocols for rapid virus detection in acutely unwell patients using direct immunofluorescence followed by cell culture. These samples were also tested and by real-time NASBA. In addition a corresponding dry respiratory swab for real-time NASBA testing only was also collected from each patient.

2.3. Dry respiratory swab collection for routine NASBA testing study

Dry cotton respiratory swabs received in the laboratory up to 5 days after collection from the throat or nasopharyngeal passage were accepted for testing by real-time NASBA only. These samples were obtained from a wide range of patient groups including adults and children both hospitalised, attending out-patient clinics and from the community.

2.4. Direct immunofluorescence and cell culture

The routine laboratory methods used for direct immunofluorescence and cell culture have been described previously (Moore et al., 2004, 2006). Briefly, all nasopharyngeal aspirates received were tested by both direct immunofluorescence and then inoculated into cell culture (PLC, MRC5 and Hep-2 cells), whilst all throat

swabs received in virus transport medium were inoculated directly into cell culture. A proportion of the samples were also inoculated into R Mix cell culture (Diagnostics Hybrids inc, Athens, OH) as part of a parallel evaluation following manufacturer's instructions.

2.5. Real-time NASBA assays

During the 2004–2005 (October–April) season, all samples were tested for four respiratory viruses (influenza A, influenza B, RSV and hMPV) by real-time NASBA; this included the nasopharyngeal aspirate and respiratory swabs received in virus transport medium. This season included all of the samples collected for the pilot study. The following winter season (2005–2006) only dry respiratory swabs were collected and tested for RSV, influenza A and influenza B only.

2.5.1. Influenza A and influenza B molecular testing

Both influenza A and B real-time NASBAs were developed in-house and evaluated against a panel of positive material including the 2005 QCMD respiratory panel (Templeton et al., 2006). The Influenza A assay has been described previously (Moore et al., 2004). The influenza B assay was developed to target the polymerase PA gene (segment 3). The influenza B primer sequences used were P1; 5' AAT-TCT-AAT-ACG-ACT-CAC-TAT-AGG-GAG-AAG-GCT-ATT-CAA-CAT-CTG-CGT-CCA-TC 3' (including the T7 promoter sequence) and P2; 5' ATY-ACT-TCA-TAY-TGT-GGT-CTC-A 3' together with a specific molecular beacon 5' FAM-CCA-TGC-CCC-TTG-TCC-TTC-TAA-TGC-TGT-ATA-GGC-ATG-G-DABCYL 3'. During the development of the assay the sensitivity and specificity of the assay on RNA transcripts and virus stock dilutions were shown to be comparable to that of the influenza A assay.

2.5.2. RSV and human metapneumovirus (hMPV) molecular testing

The commercial NucliSens RSV A+B assay and hMPV assay (bioMérieux, Marcy l'Étoile, France) was used throughout the season. The principle behind these assays has been described previously (Moore et al., 2006; Manji et al., 2006; Deiman et al., 2007)

2.6. Sample processing for molecular testing

To reduce the effect of inhibitory substances the nasopharyngeal aspirates and samples received in transport medium were either diluted 1/10 in sterile phosphate buffered saline or if repeatedly inhibitory were further treated with DNase I as previously described (Moore et al., 2006).

On receipt in the laboratory the dry cotton swab was broken into a 0.9 ml tube of NucliSens Lysis Buffer. The vial was vortexed and left to stand for 10 min at room temperature. A 200 µl aliquot of Lysis buffer containing either the pre-treated sample or swab was then transferred to a 2 ml NucliSens Lysis buffer tube for extraction. The remaining Lysis buffer was then stored frozen at –80 °C.

2.7. Nucleic acid extraction

For each extraction a positive control consisting of a mixture of all four respiratory targets, influenza A (H1N1 and H3N2), influenza B (unknown strain type), RSV and hMPV was used. The influenza A and B and RSV isolates were obtained from The Welsh Specialist Virology Centre. The hMPV control was obtained from The bioMérieux R&D laboratory (Grenoble, France). A negative control was also included in each extraction run.

All samples and controls during the 2004–2005 season were processed as follows; the internal control for the RSV and hMPV was reconstituted by combining the lyophilised spheres for both assays into one tube and adding 220 µl of elution buffer. The vial

was vortexed until the buffer was clear and the spheres were fully dissolved. To each 2 ml lysis buffer tube containing sample, 20 µl of the internal control mix was added and the tube vortexed. The lysis buffer was extracted using the NucliSens miniMAG magnetic silica extraction method following manufacturer's instructions (Moore et al., 2006).

Samples received during the 2005–2006 season were processed in the same way but with addition of just the RSV internal control, which was reconstituted in 550 µl of wash buffer 3 followed by 550 µl of magnetic silica. The nucleic acid extraction was performed on the fully automated bioMérieux NucliSens easyMAG system following the manufacturer's protocol.

The nucleic acid was eluted from the magnetic silica from either extraction method into 25 µl of elution buffer; this was either amplified immediately or stored at –80 °C.

2.8. Amplification and detection

For each target, a 5 µl aliquot of nucleic acid eluate was used for each reaction. The bioMérieux NucliSens basic kit version 2 reagents along with the NucliSens EasyQ system were used to perform the amplification and detection of the real-time NASBA assays. The manufacturer's instructions were followed to perform the RSV and hMPV assays (Moore et al., 2006). For the influenza assays optimised conditions were followed as determined during assay development and evaluation (Moore et al., 2004).

For more detailed analysis, the RNA stability assay was performed using the EasyQ analyzer 1.2 software. Unlike other real-time PCR platforms, real-time NASBA reports as fluorescence signal over time, taking 120 reads over 90 min. The threshold level set for the RSV assay wild type signal is 1.2, by using the analyzer software it is possible to determine the time at which the signal reaches this threshold and thus becomes positive. The time to positive for both dry cotton and flocked swabs were analysed for each day and plotted graphically to determine whether there was a difference with storage time.

2.9. Statistical analysis

The javastat online statistics package (<http://statpages.org/ctab2x2.html>) was used to determine the kappa statistic for agreement of methodologies, sensitivity, specificity, positive predictive value and negative predictive values. The unpaired difference between the methods used was calculated using Newcombe statistics (Newcombe, 1998).

3. Results

3.1. Virus stability results

RSV RNA could successfully be extracted and detected from all of the swabs. The amount of RNA detected on the cotton tipped swabs decreased towards day 15 with a difference in 'time to positive' at day 15 of 8 min in favour of the flocked swab. The consistency in the time to positive results was significantly improved using the flocked swab, which varied very little over the 15 days resulting in a more linear graph over time when compared to the cotton swab (Fig. 1).

3.2. Dry respiratory swabs and matching clinical respiratory specimen study

In total 164 dry respiratory swab results were compared to the results of respiratory swabs received in virus transport medium (56) and nasopharyngeal aspirates (108). Of these, 37 swabs (66%)

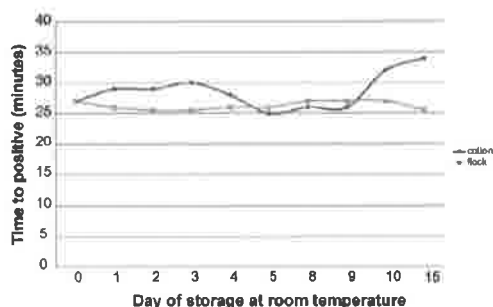


Fig. 1. Graph comparing the time taken (time to positive) in minutes to obtain a FAM threshold of 1.2 for wild type RSV eluted from dry cotton tipped swabs and dry flocked swabs inoculated with equal amounts of RSV control virus stored at room temperature for up to 15 days. This shows the high variability seen with the cotton tipped swab when compared to the flocked swab.

were received from patients involved in respiratory illness outbreaks.

The results of the comparison work has been summarised in Fig. 2 showing the increased detection rate of the four respiratory viruses using dry cotton swabs and real-time NASBA over the traditional laboratory techniques.

When compared to swabs received in virus transport media for cell culture, the dry cotton swabs yielded 34 positive results using real-time NASBA giving an overall positivity rate of 60% (38% influenza A, 20% influenza B and 2% RSV) compared to just 5% if traditional cell culture was used. The isolation rate increased to 23% if R mix was used which detected more influenza A than traditional cell culture.

Compared to direct immunofluorescence and cell culture on nasopharyngeal aspirates, the dry cotton swabs yielded 25 positive results using real-time NASBA giving an overall detection rate of 24% (11% influenza A, 11% RSV and 2% dual influenza A and RSV) compared 5% for both IF and cell culture. The detection rate again increased to 12% if R mix was used in conjunction with immunofluorescence.

Aliquots of all influenza positive samples were sent to the Influenza Reference Laboratory based at the Centre for Infections, London for further strain analysis. The results obtained confirmed the real-time NASBA results obtained. Further to this, real-time NASBA assays were also later performed on the swabs in virus transport media tested and on the nasopharyngeal aspirates, the results

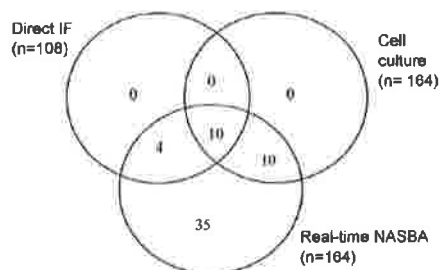


Fig. 2. Results of the direct comparison study between NASBA on dry cotton swabs and traditional laboratory techniques showing the increased detection of four respiratory viruses when using real-time NASBA.

of which showed 100% correlation with the dried swab result, indicating that the virus was present in both samples.

One of the viruses isolated in cell culture was parainfluenza 3 and was removed from the statistical analysis as this virus was not included in the real-time NASBA virus panel.

3.2.1. Statistical analysis

For the comparison between dry cotton swabs and swabs received in virus transport medium, using traditional cell culture as the gold standard, dry respiratory swabs in combination with real-time NASBA gave a sensitivity of 100% (95% CI 35–100) and a specificity of 41% (95% CI 38–41). This translated to a positive predictive value of 59% (95% CI 21–59) and a negative predictive value of 100% (95% CI 94–100). This indicates that real-time NASBA detected all the relevant viruses isolated in cell culture, but that the additional samples giving positive results are considered false positive by comparison with the gold standard. By applying the kappa statistic and removing the need for a gold standard, a better reflection of agreement can be established. The kappa statistic was shown to be 0.047 (95% CI 0.031–0.047) showing a poor agreement between the two testing strategies. Using the Newcombe statistic it is possible to show how one method compares directly with another on the same cohort of samples. Against traditional cell culture; real-time NASBA using dried swabs showed an increased detection rate of 55% (95% CI 39–68) but compared to R mix this was reduced to 36% (95% CI 17–51).

For the comparison between dry cotton swabs and nasopharyngeal aspirates, using the traditional assays in combination as the gold standard, dry respiratory swabs with real-time NASBA detection gave a sensitivity of 100% (95% CI 52–100) and a specificity of 80% (95% CI 74–80). This translated to a positive predictive value of 23% (95% CI 7–23) and a negative predictive value of 100% (95% CI 97–100). The kappa statistic was 0.28 (95% CI 0.060–0.19) showing fair to poor agreement between the two methodologies. Using the Newcombe statistic on immunofluorescence and traditional culture there was a detection rate improvement of 18% (95% CI 8–27) when real-time NASBA is used on dry swabs. However, if R mix was used in combination with IF this changed to 10% (95% CI 1–20).

3.3. Routine testing of dry cotton respiratory swabs

In total, 344 swabs were collected from the period of the 1st October 2004 to the 1st April 2005 and tested for the four viral targets over this season. Positive results from one or more of the four targets were obtained from 127/344 (37%) samples. During the period from 1st October 2005 to 1st April 2006, 392 dry respiratory swabs were taken for routine testing for three respiratory targets. Of these, 150/392 (39%) samples were found to be positive for one or more of the respiratory targets. Fig. 3 summarises the viruses detected by season, during 2004–2005, hMPV accounted for 2% of all positive results. Dual infections with influenza A and RSV (5%) were detected where the RSV and influenza A seasons crossed in each season.

In patients with underlying malignancies, RSV was the most common virus detected across both seasons. In haematology patients RSV was detected in 18% and 20% of samples collected each year. Clinical symptoms ranged from mild upper respiratory tract infections to severe lower respiratory tract infections often associated with prolonged shedding of virus. Repeat testing of patients demonstrated that prolonged shedding of virus was more commonly associated with RSV than with any of the other viral targets in the study. One patient who had prolonged shedding was a 5-year-old female who had a previous RSV positive result by direct immunofluorescence before molecular screening was performed. Subsequent sampling of this patient showed prolonged shedding

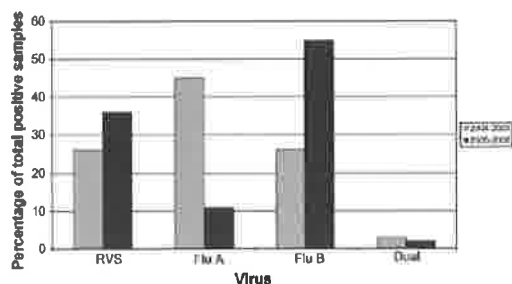


Fig. 3. The prevalence of three respiratory viruses tested for routinely by real-time NASBA over two seasons in a total of 736 dry cotton respiratory swabs. The overall detection rate was 37% for these targets. This demonstrates the utility of the dry swab for respiratory virus molecular detection in routine practice.

of RSV over a period of 3 months, which was further confirmed by virus isolation and direct immunofluorescence in matching samples.

Another 15-year-old child initially presented with a cough and coryzal signs following an allograft for anaplastic large cell non Hodgkin's lymphoma. Her first screen was positive for RSV and she was started on ribavirin therapy. However, her illness progressed to pneumonia despite treatment and four follow up dry cotton respiratory swabs were also shown to be RSV positive. She continued to excrete RSV for a month following treatment onset before finally clearing the virus. Throughout this time, she remained symptomatic.

Using dry cotton swabs and real-time NASBA the cause of 11 respiratory virus outbreaks was identified. Influenza A was the causative agent in 6/11 (75%) of the outbreaks as defined by two or more samples from the outbreak being found positive. Influenza B was found to be the causative agent in 4/11 (25%) of the outbreaks, including 2 outbreaks in schools. Additionally, RSV was found to be the causative agent of an outbreak in a neonatal unit and also responsible for a protracted outbreak in a haematology ward.

Over the two seasons, nine samples were received from patients who had died following a history of respiratory illness. The only virus detected in these patients was hMPV that was detected in the lung swab of an infant who died of suspected sudden infant death syndrome following a short illness.

3.4. Inhibition rates

An important consideration when processing respiratory samples for molecular testing is inhibitory substances that might be present in the samples. This is a significant problem for samples such as nasopharyngeal aspirates, sputum and broncho-alveolar lavages, which in this study and in previous studies have needed to be pre-treated prior to extraction to reduce inhibition rates (Moore et al., 2006). The work performed using dry respiratory swabs has shown that inhibition rates are negligible (<0.5%) and are rarely encountered using this method.

4. Discussion

As more routine laboratories introduce molecular testing for the detection of respiratory viruses the most appropriate way of collecting respiratory samples to ensure a rapid and sensitive delivery of results also needs to be assessed.

The stability study in this paper demonstrates that nucleic acid from RSV is stable over a long period of time on a dry swab and that

the nucleic acid is readily released once the swab is vortexed into the guanidinium based lysis buffer. This means that dry swabs can be sent directly to the laboratory without need of any form of transport medium, or fixative reagent to ensure nucleic acid integrity. These findings are supported by the increased detection rate seen using dry cotton swabs compared to traditional respiratory samples demonstrated in the comparison study.

Viral RNA was extracted successfully from conventional cotton tipped or flocked swab types even after storage at room temperature for 15 days. The results of the flocked swab showed that this type of swab not only kept the RNA stable for prolonged periods of time, but that the amount of nucleic acid released from the swab was highly consistent as demonstrated in the time to positive curve. Whilst cotton tipped swabs can still be recommended on the basis of the study and routine diagnostic work results, the use of flocked swabs in combination with a molecular test such as real-time NASBA will improve the detection rate of respiratory viruses further. This is particularly true if samples are to be transported over a distance to the laboratory or if there was a delay in sample processing.

Over two winter seasons, 736 dry respiratory swabs were collected and tested for up to four respiratory targets. Of these, one or more viral targets were detected in 277 samples (38%). The majority of samples received were from older children and adult patients with an underlying condition in whom rapid respiratory virus detection has in the past been complicated by the difficulties in obtaining good quality clinical samples. Rapid molecular testing has allowed for improved understanding and management of respiratory viral infections in different patient groups. RSV in particular, was shown to be a significant cause of respiratory symptoms and was associated with prolonged shedding of virus in immunocompromised children and adults. By using a non-invasive method of sample collection that is acceptable to both patients and staff, samples can quickly and easily be collected and sent directly to the laboratory for testing. The laboratory can also request repeat samples to be taken from patients for same day testing to monitor the course of infection or to confirm the presence of a virus, particularly in vulnerable patients who may require strict infection control measures and appropriate antiviral treatment.

By further expanding the molecular panel of respiratory viruses it is has been shown that the detection rate of respiratory viruses increases further to 60% as retrospective work performed on stored dry respiratory swabs in lysis buffer (unpublished in-house data) has shown that enteroviruses, rhinoviruses, parainfluenza types 1–4 and adenovirus can also be detected from the swabs. This retrospective work also demonstrated that once frozen, the nucleic acid in lysis buffer remains stable for many months.

Although real-time NASBA was the method of choice for detecting the respiratory virus targets, the extracted nucleic acid could be amplified using traditional PCR and reverse transcription-PCR methods. This makes the collection method appropriate for most molecular technologies, although in combination with real-time NASBA a result can be obtained within 4 h. This is particularly useful for same day testing when investigating the cause of severe acute infections and outbreaks.

The disadvantage of this method is the lack of virus for culture, particularly for influenza. It is possible to type a virus using gene sequencing but the role of genetic drift in immune escape can only be determined using neutralisation techniques, which require the live virus. It is also impossible to ascertain whether the virus being detected by a molecular method is still infectious or is non-viable virus at the end of an infection. Symptomatic patients often have respiratory swabs taken that give no virus growth in cell culture or by direct immunofluorescence, but are repeatedly positive by molecular testing using the dry respiratory swab collection method.

All positive results should therefore be taken in context with clinical features.

The application of this simple collection method using dry respiratory swabs has consistently been shown to be sensitive and specific for the detection of a range of respiratory viruses by molecular methodology. It is now used routinely across Wales for the community surveillance of influenza, respiratory virus outbreak investigation, screening of high-risk patients presenting with respiratory illness and also for monitoring the course of the infection. The expansion of the range of viruses being targeted by molecular technology in combination with this method of sample collection will further enhance clinical management of respiratory infections in Wales.

5. Conclusion

Collecting a nasal or throat swab and transporting it dry to the laboratory as a dedicated molecular specimen is a simple and robust method for detecting a wide range of respiratory viral targets.

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3.6 Manuscript 6. Development and validation of a commercial real-time NASBA assay for the rapid confirmation of influenza A H5N1 in clinical samples

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Short communication

Development and validation of a commercial real-time NASBA assay for the rapid confirmation of influenza A H5N1 virus in clinical samples

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ABSTRACT

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A real-time NASBA assay for the specific confirmation of influenza A H5N1 infection was developed and evaluated using proficiency panels distributed to the UK influenza network of laboratories and clinical samples received through the Chinese National Influenza Centre in Beijing. The aim of the proficiency panels was to determine the sensitivity and specificity of the assay on a range of influenza virus types and subtypes including different clades of influenza A H5 viruses. The assay was then evaluated using 19 clinical samples obtained from seven confirmed human cases of influenza A H5N1 infection in China. The assay was shown to have a level of sensitivity of 0.01 TCID₅₀ and 10 copies/μl using RNA transcripts of the A/VietNam/1194/2004 H5N1 virus. During the evaluation the assay successfully detected H5N1 viruses known to infect humans from clades 1, 2.1, 2.2 and 2.3 as well as low pathogenic H5N3 avian influenza viruses. The clinical utility of the real-time NASBA assay was proven on a range of clinical samples from patients with confirmed H5N1 infection collected during 2005 and 2006. The real-time NASBA assay was demonstrated to be sensitive and rapid allowing for same day confirmation of a H5N1 infection direct from clinical samples.

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Since the emergence of pandemic influenza A (H1N1) 2009 virus there has been a reduction in the media coverage of human infections with highly pathogenic avian influenza A (H5N1) viruses. It is well recognised that H5N1 remains a potential pandemic virus through adaptation via genetic drift to infect humans preferentially or via re-assortment with a human influenza A virus if a dual infection occurs (WHO writing committee, 2008). H5N1 continues to circulate and outbreaks have occurred in birds throughout Asia and into Europe and Africa with the viral H5 haemagglutinin evolving into phylogenetically distinct clades and subclades (Webster and Govorkova, 2006). Reports to WHO show that human cases continue to occur and although the vast majority are associated with contact with infected birds there are a few reports of limited human to human transmission (Wang et al., 2008). Human H5N1 infection often leads to a severe systemic infection which is associated with a high mortality rate. During 2009 into early 2010 there were 84 human cases of H5N1 virus reported in Egypt, Indonesia, Vietnam, and China. The overall mortality

rate was 46%, which remains greater than any pandemic human influenza A (WHO website accessed March 2010). For this reason, rapid diagnosis and global surveillance of H5N1 influenza A is still as important today as when the number of reported cases peaked at 115 with an associated mortality of 69% in 2006 (WHO writing committee, 2008; WHO website accessed March, 2010).

H5N1 infection in returning travellers from an endemic region, or infection in poultry with onward transmission to poultry workers, are considered to be the major routes of introduction of H5N1 to non-endemic regions such as Western Europe and the Americas. Once this occurs; a rapid confirmation of infection is required to ensure appropriate management of the patient and potential contacts (Curran et al., 2007; Ellis et al., 2007; HPA guidelines accessed March, 2010). Assays designed to detect and confirm H5N1 virus must be rapid, sensitive and specific for all known circulating clades. Molecular techniques are preferable to traditional laboratory techniques such as culture for the detection of H5N1 in clinical samples, with most current molecular assays developed using real-time RT-PCR methods (Ellis et al., 2007). An alternative molecular method for the amplification and detection of respiratory viruses is real-time nucleic acid sequence based amplification (NASBA) the

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detailed method of which has been described previously (Leone et al., 1998; Moore et al., 2004; Fox et al., 2009).

The NucliSens EasyQ Influenza A H5 and N1 NASBA assay (bioMérieux, Grenoble, France) was developed as a rapid and specific sub-typing assay for the detection of H5N1 virus in patients positive for influenza A who have travelled to or live in an H5N1 endemic regions and have been in close contact with sick-poultry or confirmed human H5N1 cases.

The assay provides primer and probes to detect H5 and N1 in separate reactions in combination with the NucliSens EasyQ basic kit version 2 on the EasyQ analyser. It was developed using sequence alignment of the haemagglutinin and neuraminidase genes of all known H5 viruses circulating in 2004. Sensitivity of the assay was determined using *in vitro* RNA transcripts of the reference strain A/Vietnam/1194/2004 and serial dilutions of cultured H5N1 virus with known TCID₅₀.

Specificity of the assay was determined by performing the assay on non H5 strains of influenza A, influenza B, RSV, parainfluenza and adenovirus (Telles et al., 2007).

Further assay validation and specificity testing was performed by the Public Health Wales Cardiff Laboratory as part of the UK Influenza Laboratory Network (Curran et al., 2007). Validation of the NucliSens H5N1 NASBA assay was performed using influenza proficiency panels containing inactivated strains of influenza A and B from a range of hosts (human, avian, equine and swine) provided by the Health Protection Agency (HPA), Centre for Infections (CFI), London. The panels were sent to each laboratory within the network simultaneously and preliminary results were expected to be returned within 6 h of panel receipt. An aliquot of inactivated A/Duck/Malaysia/F119/3/97 (H5N3) culture fluid was provided by CFI to all laboratories in the UK network performing H5N1 testing for use as a H5 positive run control.

In Wales; four samples from suspected human cases of H5N1 infection were also tested in addition to the proficiency panels. Each suspected case fulfilled the UK Health Protection Agency (HPA) case definition for a suspected H5N1 infection (HPA guidelines accessed March, 2010). Each patient was sampled using dry respiratory swabs, as previously described to reduce the risk of infection during transportation to the laboratory and during sample processing (Moore et al., 2008).

All samples, proficiency panels and controls were processed in a category III facility. Guanidinium thiocyanate based lysis buffer (bioMérieux, Grenoble, France) was used to inactivate any virus present in 200 µl of sample. Nucleic acid extraction was performed on the NucliSens automated EasyMag system and eluted into 60 µl following manufacturers instructions. The entire extraction procedure for up to 24 samples following sample processing was completed in 40 min. All samples were tested by a generic influenza A assay, an influenza B assay and a full respiratory screen using real-time NASBA to rule out other causes as described previously (Moore et al., 2008). All Influenza A positive samples were sub-typed to seasonal H1, H3, pandemic H1 and H5 using real-time molecular assays developed by CFI (Ellis et al., 2007; Moore et al., 2008). The NucliSens H5N1 real-time NASBA assay was used as the second line test to confirm an H5 positive sample as it allowed discrimination between H5N1 and any other neuraminidase sub-type, e.g. N3.

Due to the small number of human H5N1 cases each year, validation of any H5N1 assay using clinical samples is challenging, particularly in nonendemic regions. A clinical validation of the real-time NASBA assay was performed in China on 19 respiratory samples collected from seven confirmed cases of H5N1 infection between 2005 and 2006. The clinical validation included five sequential samples collected from a single patient. The samples tested included throat swabs, tracheal secretions, bronchoalveolar lavages and lung tissue. All samples were cultured for H5N1 virus by isolation in specific pathogen free (SPF) class eggs and confirmed by

in-house PCR targeting the haemagglutinin of H5 viruses. Cases 1–6 were tested by a traditional block-based RT-PCR with end-point gel detection and case 7 was tested by real-time RT-PCR as described previously (Yu et al., 2008; Zou et al., 2007). Nucleic acid extraction was performed on 200 µl of clinical sample using the RNeasy Mini Kit (Qiagen, Germany), with elution into a 50 µl volume of nuclease free water following the manufacturers instructions. All sample extracts were stored at –20 °C and tested retrospectively by real-time NASBA following 1 year of storage.

The NASBA reaction was performed in both centres on 5 µl of extracted nucleic acid as described previously using the basic kit version 2 with a KCl concentration of 70 mM (Moore et al., 2004). Amplification and simultaneous detection was performed using the NucliSens EasyQ analyser with a wild-type (FAM) threshold of 1.2 for each of the targets (Telles et al., 2007).

During development, the lower level of sensitivity for the assay was shown to be 10 copies/µl of A/Vietnam/1194/2004 RNA transcript. For manufacturing and stability purposes each batch of primers and probe mix was tested at 0, 6, 12 and 18 month intervals on 42 replicate reactions of the RNA transcript at 20 copies/µl for both H5 and N1. As each primer and probe batch is only released if the reactions are positive in all of the replicates, the assay has consistently been shown to be 100% sensitive at 20 copies/µl and is stable for 18 months.

Serial dilutions of cultured A/Turkey/582/06 (H5N1) at stock concentration of 10^{7.63} TCID₅₀, A/Turkey/073/06 (H5N1) at a stock concentration of 10^{7.8} TCID₅₀ and A/VietNam/1194/04 (H5N1) (no data on TCID₅₀) were also tested in triplicate. The consistent level of sensitivity was at a dilution of 10⁻⁷ in both the H5 and N1 assays for the Turkey strains and 10⁻⁷ in the H5 assay and 10⁻⁸ in the N1 assay for the Viet Nam strain.

The results of the proficiency panels are summarised in Table 1, the H5 assay was shown to be sensitive and specific for a range of H5 viruses from clades 1, 2.1, 2.2 and 2.3 as well as low pathogenic avian strains of H5N3. The N1 assay was relatively specific although some cross reactivity was noted in samples containing high titre pandemic (H1N1) 2009 viruses, an avian H6N1 virus and a swine lineage H1N1 virus.

Of the four clinical samples received for H5N1 testing in the Cardiff laboratory all were negative for influenza A. The ease in assay set-up meant that the influenza A screening tests, H5 specific RT-PCR and the H5N1 real-time NASBA assays could be run simultaneously reducing the time to confirmed result significantly. The average turnaround time from sample receipt to result reporting was 4 h.

The clinical validation performed in China demonstrated that samples positive by egg culture and/or H5 RT-PCR were also positive by both the H5 and N1 NASBA reagents despite long term storage of the RNA extracts (Table 2).

Compared to the RT-PCR H5 specific assays used for screening in both the UK and China the real-time NASBA H5 assay was demonstrated to have equal sensitivity during the validation. The N1 assay was shown to be marginally more sensitive than the H5 assay during development and during the clinical validation.

The development and validation data from both the UK and China thus demonstrates that the NucliSens H5N1 real-time NASBA assay to be rapid, sensitive and specific for confirming the presence of H5N1 virus in clinical samples. As the assay is available for research use only its use is limited to being a second line confirmatory test despite having comparable sensitivity to the front-line H5 assay being used routinely by the two evaluation centres. The added benefit of the H5N1 reagents is the ability to rapidly sub-type to the N1, thus confirming a H5N1 infection. The limited cross reaction seen with the N1 assay is not surprising due to considerable sequence identity between N1 viruses of avian lineage which includes the N1 of the pandemic influenza (H1N1) 2009 virus.

Table 1

The results of the real-time NASBA H5N1 assay on a range of influenza A strains received through the Chinese National Influenza Centre* and through the UK National Influenza Laboratory Network**.

Influenza A strain (H5N1 Clade)	H5 RT-PCR result	H5 NASBA result	N1 NASBA result
A/Guangdong/2/1991 (H3N2)	–	–	–
A/Anhui/318/2003 (H3N2)	–	–	–
A/Wisconsin/67/05 (H3N2)	–	–	–
A/Brisbane/10/2007 (H3N2)	–	–	–
A/Perth/16/2009 (H3N2)	–	–	–
A/Swine/Italy/1477/96 (H3N2)	–	–	–
A/Equine/Miami/63 (H3N8)	–	–	–
A/Jingshen/1/1956 (H1N1)	–	–	–
A/Gui/387/2002 (H1N1)	–	–	–
A/New Caledonia/20/99 (H1N1)	–	–	–
A/Solomon Islands/3/2006 (H1N1)	–	–	–
A/Brisbane/59/2007 (H1N1)	–	–	–
A/England/195/2009 (H1N1)	–	–	–
A/Aragon/3218/2008 (H1N1)	–	–	–
A/England/935240/2009 (H1N1)	–	–	–
A/Scotland/8/2009 (H1N1)	–	–	–
A/Swine/IV/1455/99 (H1N1)	–	–	–
A/Turkey/England/198/2009 (H6N1)	–	–	–
A/Eng/481/06 (H7N3)	–	–	–
A/Av/1306/2007 (H7N2)	–	–	–
A/Chicken/HK/G9/97 (H9N2)	–	–	–
A/HK/1073/99 (H9N2)	–	–	–
A/Duck/Malaysia/F119/3/97 (H5N3)	+	+	+
A/Teal/Eng/06 (H5N3)	+	+	+
A/Vietnam/1194/2004 (H5N1) (Clade 1)	+	+	+
A/Indonesia/5/05 (H5N1) (Clade 2.1)	+	+	+
A/Turkey/Turkey/05 (H5N1) (Clade 2.2)	+	+	+
A/Anhui/2005 (H5N1) (Clade 2.3)	+	+	+
A/Guangdong/1/2006 (H5N1) (Clade 2.3)	+	+	+
A/Heishan/1/2006 (H5N1) (Clade 2.3)	+	+	+

Conversely, it has been shown that assays designed to detect the pandemic H1N1 2009 neuraminidase gene will often cross react into the avian influenza A N1 viruses (Ellis et al., 2009). However, there is no evidence of the assay detecting seasonal human H1N1 influenza viruses and as the N1 assay would never be used in isolation to confirm the sub-type of an influenza A positive sample the limited cross reactivity observed should not be problematic.

With such a limited number of reported human infections with H5N1 virus globally, there is increased need to ensure that appropriate testing strategies are in place that will rapidly identify and

confirm an infection. Recent data from China has shown that viruses from the Asian H5N1 2.3 clade have a predilection for infecting the lower respiratory tract with severe illness and prolonged viral shedding in survivors (Yu et al., 2008). This data is supported by this clinical validation with respect to cases 2, 5 and 7. Case 2 had H5N1 virus detected only in the lower respiratory tract and prolonged viral shedding was demonstrated with the detection of H5N1 virus in samples collected 12 and 14 days post symptom onset in cases 5 and 7 respectively. Whether it is adequate to collect swabs from the upper respiratory tract only from cases that may

Table 2

Results of the real-time NASBA H5N1 assay on clinical samples received from confirmed H5N1 infected Chinese patients 2005–2006.

Case	Sample type	Time since onset (days)	H5 NASBA result	N1 NASBA result	H5 RT-PCR (c _t value if available)	Virus isolation
1	Throat swab	7	+	+	+	+
	Throat Swab	8	+	+	+	+
2	Throat Swab	10	–	–	–	–
	Throat Aspirate	10	+	+	+	+
	Tracheal Aspirate	10	+	+	+	+
	Lung tissue	10	+	+	+	+
3	Throat swab	6	+	+	+	+
	Mouth wash	6	–	–	–	–
4	Tracheal aspirate	9	+	+	+	+
	Tracheal aspirate	9	+	+	+	+
5	BAL	12	+	+	+	+
6	Throat swab	9	+	n/t	+	+
	Throat swab	9	+	+	–	–
	Tracheal aspirate	9	+	n/t	+	+
7	Tracheal aspirate	8	+	n/t	+(29.8)	+
	Tracheal aspirate	9	+	n/t	+(29.8)	+
	Tracheal aspirate	14	+	n/t	+(28.3)	+
	Tracheal aspirate	20	–	n/t	–	–
	Tracheal aspirate	27	–	n/t	±(39) ^a	–

BAL: Bronchoalveolar lavages. n/t: Insufficient RNA extract to test.

^a Weakly reactive with late c_t value not confirmed by other assays.

have acquired infection from areas where clade 2.3 viruses circulate is questionable especially as they often present greater than 7 days post symptom onset.

Overall, the NucliSens H5N1 real-time NASBA assay has been proven to be sensitive in detecting all circulating clades of H5 virus known to infect humans from both cultured virus and directly from clinical material, including difficult to process lower respiratory tract samples and lung tissue. When used as a second line test in combination with a sensitive generic influenza A assay (with an internal control such as RNaseP) and an alternative H5 specific assay; H5N1 infection can be detected rapidly and confirmed within 4 h of sample receipt in the laboratory.

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3.7 Manuscript 7. Two waves of pandemic influenza A (H1N1) 2009 in Wales - The possible impact of media coverage on consultation rates.

SURVEILLANCE AND OUTBREAK REPORTS

Two waves of pandemic influenza A(H1N1)2009 in Wales – the possible impact of media coverage on consultation rates, April – December 2009

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In the United Kingdom, the influenza A(H1N1)2009 pandemic had a distinct two-wave pattern of general practice consultations for influenza-like illness (ILI). We describe the epidemiology of the influenza pandemic in Wales between April and December 2009 using integrated data from a number of independent sources: GP surveillance, community virology surveillance, hospital admissions and deaths, and media enquiries monitoring. The first wave peaked in late July at 100 consultations per 100,000 general practice population and attracted intensive media coverage. The positivity rate for the A(H1N1)2009 influenza did not exceed 25% and only 44 hospitalisations and one death were recorded. By contrast, the second wave peaked in late October and although characterised by lower ILI consultation rates (65 consultations per 100,000 general practice population) and low profile media activity, was associated with much higher positivity rates for pandemic influenza A(H1N1)2009 (60%) and substantially more hospital admissions (n=379) and deaths (n=26). The large number of ILI-related consultations during the first wave in Wales probably reflected the intensive media activity rather than influenza virus circulating in the community. Data from community surveillance schemes may therefore have considerably overestimated the true incidence of influenza. This has implications for the future interpretation of ILI surveillance data and their use in policy making, and underlines the importance of using integrated epidemiological, virological and hospital surveillance data to monitor influenza activity.

Introduction

The media are major sources of health information. They can generate awareness of health issues and play key roles in health behaviour change [1]. Studies suggest that media reports are the main source of most parents' information about health problems [2]. The media can also influence the behaviour of healthcare

professionals, for example by increasing awareness and reporting of communicable diseases especially during outbreaks [3,4].

In mid-April 2009, a new strain of influenza A(H1N1) was identified in the United States (US). The same strain was identified in Mexico and Canada and later elsewhere. By late April the virus, then named novel influenza A/H1N1, had spread worldwide [5]. Within Europe, the United Kingdom (UK) and Spain were the countries initially most affected [6]. On 11 June 2009, after confirming community transmission of influenza A(H1N1)2009 virus in two of its regions, the World Health Organization (WHO) declared an influenza pandemic [7].

On 29 May 2009, the first confirmed case of influenza A(H1N1)2009 was diagnosed in Wales (a man returning from the US with a respiratory illness). In response, measures were taken in Wales to strengthen case finding and reporting of influenza-like illness (ILI) among travellers returning from affected areas [8]. All suspected cases were tested for the virus by specific real-time reverse transcription – polymerase chain reaction (RT-PCR) and confirmed by sequence analysis. All household contacts were given antiviral prophylaxis, oseltamivir, as part of an initial containment strategy.

On 6 July 2009, the Welsh Assembly Government announced a move from containment to mitigation after community transmission of influenza A(H1N1)2009 had been confirmed in several parts of Wales [9]. Active case finding and routine diagnostic testing for influenza were discontinued and tracing and prophylaxis of contacts ceased. All patients who were diagnosed clinically with influenza A(H1N1)2009 by a GP were given antiviral treatment and diagnostic laboratory testing was confined to suspected influenza cases admitted to hospital or presenting to a network of sentinel general

practices. Thereafter, influenza activity in the general population was monitored using a variety of community surveillance systems.

In England, the National Pandemic Flu Service (NPFS) was introduced in mid-July 2009 in order to relieve pressure on primary care services [10]. Patients with influenza symptoms were advised not to consult their general practitioner (GP), but to contact the NPFS either online or by telephone in order to obtain antiviral drugs. This meant that GP surveillance data no longer provided a reliable indicator of influenza activity in England. However, in Wales, no change was made to usual arrangements for clinical influenza diagnosis and antiviral prescribing by GPs.

We investigated the impact of media coverage of the influenza pandemic in Wales between April and December 2009 on surveillance systems using integrated data from a number of independent sources.

Methods

We examined data on ILI consultation rates generated by NHS Direct Wales, two independent GP surveillance systems (GP sentinel surveillance of infection and rapid automated GP surveillance) in conjunction with laboratory data (community virology surveillance), hospital admissions and deaths in order to define the epidemic period of influenza and the distribution of other circulating viruses. We also analysed media interest in influenza A(H1N1)2009 over the same time period. The data sources used are detailed below.

NHS Direct Wales

This is a nurse-led telephone helpline that provides health information and advice to callers. Anyone may call the helpline at any time and symptoms are classified based on a series of clinical algorithms. Call data can be used for syndromic surveillance and symptoms that correspond to the influenza/colds algorithm provide the basis for real-time, daily monitoring of ILI in the community [11].

GP sentinel surveillance of infection

Influenza activity is reported to Public Health Wales according to the GPs' clinical diagnosis of the patients' ILI symptoms (upper respiratory tract symptoms, fever, chills, myalgia and cough). The resulting data is reported on a weekly basis by 44 volunteer, sentinel general practices, approximately 9% of practices in Wales, covering some 356,000 people. Weekly clinical consultation rates are calculated per 100,000 general practice population by age group. The scheme has operated since 1985 with no change in case definition or reporting procedure, thus allowing historical comparisons to be made.

Laboratory-based surveillance

Virological surveillance was carried out to monitor the circulation of seasonal respiratory viruses. A volunteer subset of sentinel practices collected dry nasal/ throat

swab samples from the first patients presenting with ILI symptoms each week (maximum five samples per week). These specimens were sent to the regional virus laboratory and tested for influenza A, influenza B, respiratory syncytial virus (RSV) and rhinovirus using real-time molecular techniques. All influenza A positive samples were subtyped as A(H1N1)2009 or seasonal H1 or H3 viruses using real-time RT-PCR.

Rapid automated GP surveillance

Around 400 general practices across Wales (approximately 80% of practices in Wales) report clinical diagnoses of ILI, classified according to Read codes [12], on a daily basis using an automated computer system called Audit+ (Informatica Systems Ltd [13]). We used these data to calculate ILI consultation rates per 100,000 general practice population. Rates were calculated as rolling weekly rates based on the seven day period leading up to and including the report submission date. This scheme started in late April 2009 specifically to monitor the influenza pandemic in Wales.

Hospital admissions and deaths

All acute hospitals were asked to report admissions and deaths in hospital of people with laboratory-confirmed influenza A(H1N1)2009. GPs were asked to report any deaths from suspected influenza occurring outside hospital and post-mortem testing was carried out to confirm the diagnosis.

Media coverage of pandemic influenza

Google News captures articles from printed press, television, radio and internet sources. The keyword 'swine flu' was used to search Google News for media references between 1 January and 30 December 2009. Searches were conducted on a worldwide, UK, and Wales basis. A record of influenza-related media enquiries received by Public Health Wales was also maintained throughout the pandemic. These include only a fraction of media coverage of the influenza A(H1N1)2009 pandemic in Wales, but they tend to reflect levels of media coverage nationally.

Results

Surveillance of ILI-related calls to NHS Direct Wales

NHS Direct in Wales recorded a small peak in the percentage of calls related to influenza in early May 2009 (about 25% of total calls), followed by a rapid rise to a peak of more than 50% of calls by mid-July. A second peak occurred in mid-October 2009 (30% of calls). This level of influenza calls to NHS Direct Wales was higher than at any time during the previous four years (January 2006-December 2009), superseding the peak in December 2008 (28% of calls).

Surveillance of ILI consultations by the GP schemes

The GP sentinel surveillance scheme detected an increase in ILI consultations that exceeded the threshold for normal seasonal activity by mid-July 2009

(week 29) (Figure 1). The first wave of ILI lasted from weeks 27 to 34 and reached a peak of nearly 100 consultations per 100,000 general practice population at the end of July (weeks 30–31). This was followed by a period of quiescence during August before the development of a second wave of ILI in the autumn, which started in early September (week 38), peaked in late October (week 42) and receded at the end of December (week 52). The second wave was more prolonged than the first, with a lower peak in consultation rate of 65 consultations per 100,000 general practice population. Neither of the waves exceeded an ILI rate of 100 consultations per 100,000 general practice population, the threshold used by the scheme for higher than average seasonal activity. During both waves, rates were recorded well below those in winter 1999/2000, the last winter season when substantial influenza activity occurred in Wales.

ILI consultation rates by sex were similar for both waves with females accounting for 58% of consultations in the first wave and 56% in the second. The mean age for ILI consultations was 32.1 years (standard deviation 19.9 years) and 75% of consultations were in people under 45 years of age. There was a difference in the age distribution of patients consulting with ILI during the two waves (Figure 2). In the first wave, consultation rates were highest in children aged 0–4 years and lowest in the 5–19 age group, while in the second wave rates were highest in the 10–14 age group.

Virological surveillance of GP sentinel samples

The two waves of ILI activity also differed with respect to a number of other epidemiological characteristics. Both the number of people being tested and the proportion testing positive for influenza A(H1N1)2009 were much higher during the second wave than the first (Figure 3). The proportion testing positive remained below 25% during the first wave, but reached almost 60% at the peak of the second wave (week 43). Neither of the two waves was associated with substantial numbers of positive tests for other respiratory viruses, and the influenza A(H1N1)2009 virus was the only influenza strain identified. During the first wave, samples were as likely to test positive for rhinovirus as influenza A(H1N1)2009. However, from early October (week 40) the majority of positive tests were for influenza A(H1N1)2009, until late November (week 48) when RSV became the dominant virus identified (Figure 3).

Surveillance of hospitalisations and deaths

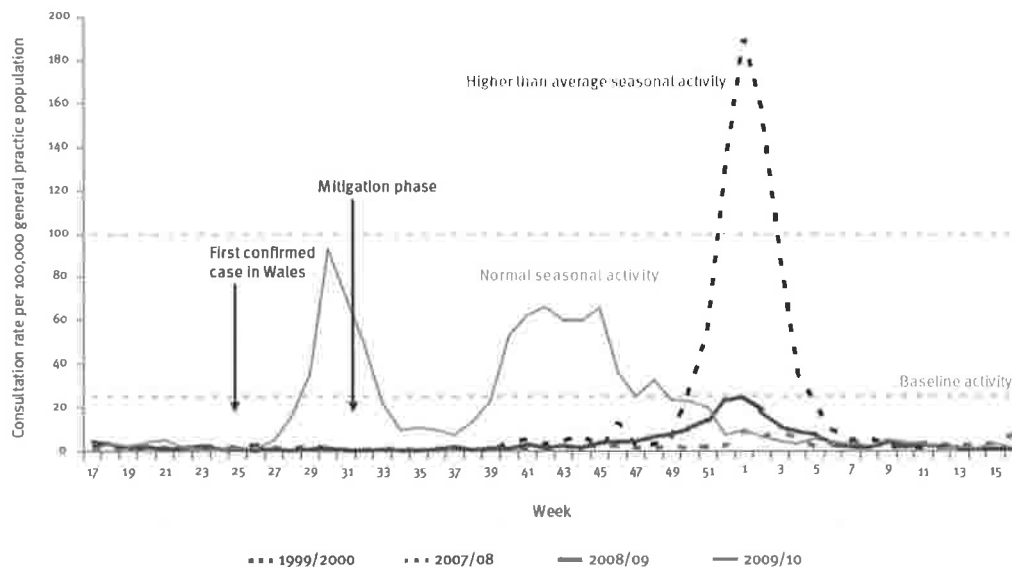
During the first wave, there were 44 hospital admissions and one patient died from confirmed influenza A(H1N1)2009. By contrast, the second wave resulted in substantially more hospital admissions (n=379), despite lower ILI consultation rates in GP, including over 60 admissions to intensive care units and 26 deaths (Figure 4).

Surveillance of media reports and enquires

The Google News search for news articles showed that the highest concentration of media reports on

FIGURE 1

Weekly consultation rates for influenza-like illness per 100,000 general practice population in Wales, United Kingdom, 1999/2000 and 2007/08–2009/10*



* Key events in 2009/10 are shown on the graph.

Source: Public Health Wales (general practitioner sentinel surveillance scheme).

pandemic influenza occurred during May 2009 with 34,300 reports internationally and 2,560 in the UK. The second highest month for articles in the UK was July 2009 with 2,330 reports.

Public Health Wales received 344 influenza-related media enquiries between April and December 2009. Of these, 172 came from print media, 92 from radio, 76 from television, and four from other sources. The highest peak in media coverage was recorded in week

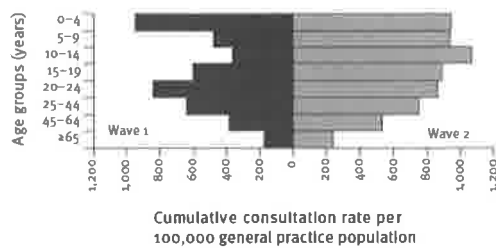
18 when WHO raised the level of influenza pandemic alert to phase 4 and later to phase 5 (Figure 5). Media interest dropped considerably after this week. Another wave of media interest began in week 26, preceding the first wave. A third period of media activity occurred at the end of October and beginning of November, coinciding with the launch of influenza A(H1N1)2009 vaccine in the UK.

Discussion

The influenza A(H1N1)2009 pandemic in Wales was characterised by two waves in ILI consultation rates that peaked in late July and late October 2009 respectively. However, the two waves were strikingly different in their epidemiological features. During the first wave, the highest ILI rates were in preschool children and the lowest rates in school children. During the second wave, the highest ILI rates were in school children. The first wave was also characterised by a much lower proportion of confirmed infections, and far fewer hospital admissions and deaths. These findings led us to question whether the first wave of ILI consultations in Wales was a genuine reflection of large numbers of infected people or mainly a consequence of extensive media coverage. A number of possible explanations for the differences observed between the two waves are considered below.

FIGURE 2

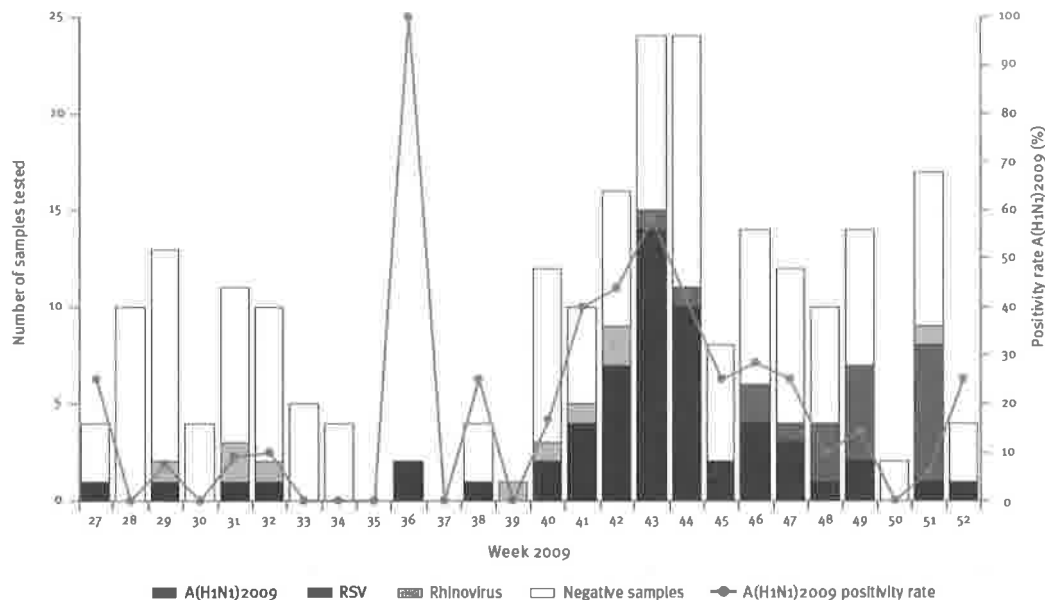
Consultation rates by age group during the first and the second pandemic influenza A(H1N1)2009 wave, Wales, United Kingdom, weeks 27–52, 2009



Source: Public Health Wales (Rapid general practitioner surveillance of influenza using Audit+).

FIGURE 3

Community virological surveillance showing tests for respiratory viruses and proportion positive for influenza A(H1N1)2009, Wales, United Kingdom, weeks 27–52^a, 2009



RSV: respiratory syncytial virus.

^a In week 36 only two samples were tested, both were positive.

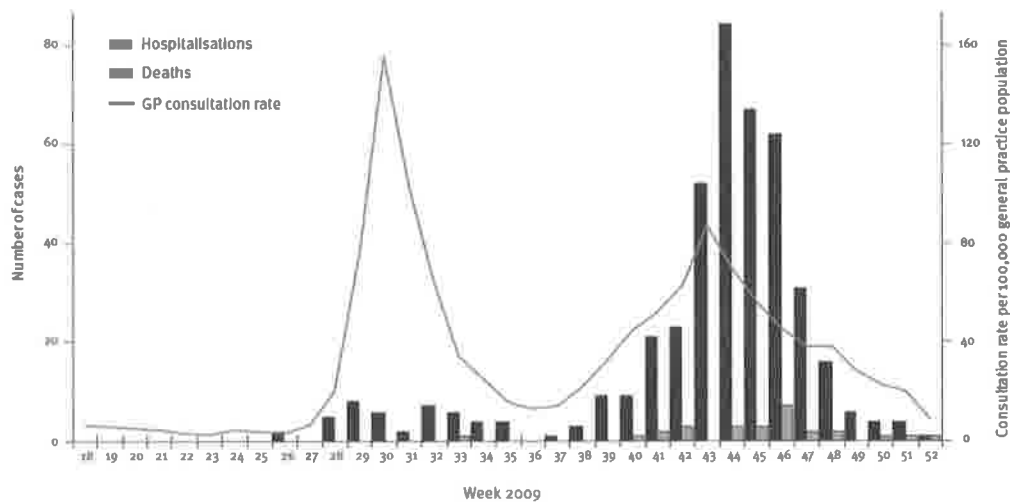
Source: Public Health Wales (regional virus laboratory).

Firstly, there may have been a lower threshold for contacting NHS Direct or consulting a GP during the first wave. This may have been influenced by extensive media coverage early in the pandemic, also observed

in other countries [14,15], and perhaps by general public anxiety and fear of the unknown. Additionally, the public health message delivered by the public health authorities to consult promptly in order to obtain

FIGURE 4

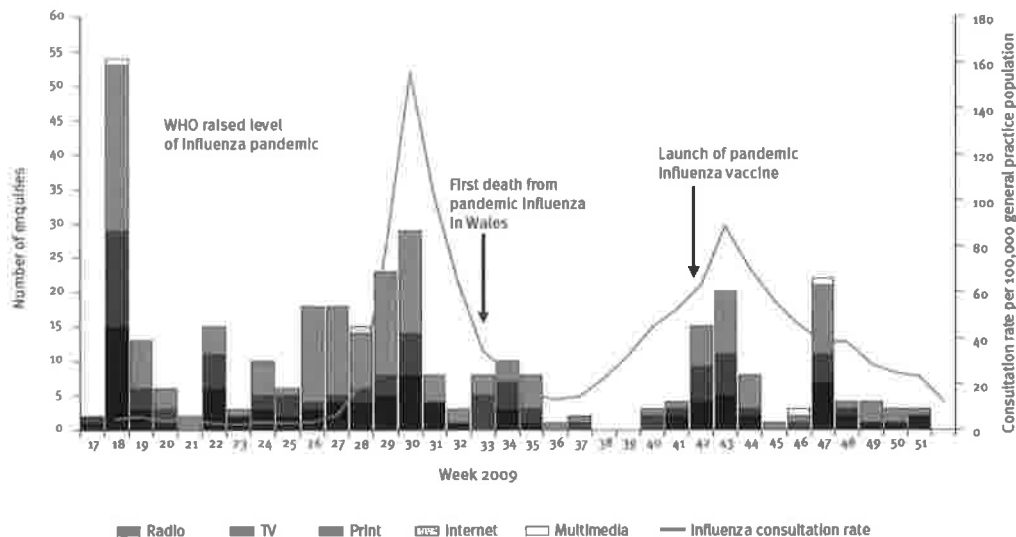
Consultation rates for influenza-like illness, and admissions to hospital and deaths from Influenza A(H1N1)2009, Wales, United Kingdom, weeks 18–52, 2009



Source: Public Health Wales (Health Protection Services and Audit+).

FIGURE 5

Media enquiries about influenza A(H1N1)2009 received by Public Health Wales, April - December 2009



TV: television; WHO: World Health Organization.
Source: Public Health Wales (Communications team and Audit+).

medical advice and treatment with antiviral medication may have led patients with minor upper respiratory infections, who would not normally consult, to seek medical care [16]. This would account for the low positivity rate for influenza A(H1N1)2009 in community samples in the first wave.

Secondly, GPs may have had been more likely than usual to suspect influenza in patients presenting with non-specific respiratory symptoms, particularly since public health authorities encouraged a low diagnostic threshold as part of the case-finding approach used during the initial stages of the pandemic. Moreover, GPs may have also been influenced by the extensive media coverage. As a result they may have obtained samples from patients with mild respiratory symptoms, accounting for the low proportion of positive tests.

Thirdly, the difference between the two waves may be an artefact of surveillance. However, unlike in England where the introduction of the NPFS substantially altered the pattern of GP consultation (and hence make it difficult to interpret GP sentinel surveillance data), no such changes were made in Wales. New diagnostic codes were introduced for influenza A(H1N1)2009 by some GP software providers but similar patterns in ILI rates were recorded by both GP surveillance systems in Wales even though they operate independently and used different methods: one based on a weekly return of cases meeting a clinical case definition and the other based on automated extraction of coded diagnoses from general practice computers. Triangulation of data from both GP surveillance schemes and from NHS Direct Wales shows synchronous timing in the peaks, indicating that the three data sources were recognising the same phenomenon.

Fourthly, there may have been other respiratory viruses giving rise to ILI symptoms circulating at the time of the first wave. Some virological specimens were positive for other viruses, particularly rhinovirus which accounted for half of the samples testing positive during the first wave. It is possible that viral interference could have affected the spread of influenza A(H1N1)2009 virus during the first wave in Wales, as occurred elsewhere in the autumn [17,18]. However, this rhinovirus activity is more likely to represent background levels rather than a coincident epidemic, though there are no historical Welsh data from the summer months available for comparison as community samples are normally only tested during the influenza season. During the second wave, influenza A(H1N1)2009 was the predominant virus identified until the onset of the RSV season in late November.

Fifthly, influenza A(H1N1)2009 may have been underestimated during the first wave because of false negative laboratory tests. The reliability of virological testing depends on the timing of the sample (negative tests are more likely five or more days after symptom onset), the quality of the sample, and the sensitivity

and specificity of the test [19]. Sample quality might be affected if primary care staff improved their sampling technique as the pandemic progressed. However, sample quality is routinely checked by the laboratory using a housekeeping gene probe to confirm the presence of human RNA and there was no change in the proportion of samples with inadequate cells. This explanation is therefore unlikely.

Finally, the much higher number of hospital admissions and deaths of people with confirmed influenza during the second wave might be due to a change in the virulence of the virus or to a change in hospital testing policy. There is no evidence for increased virulence of the influenza A(H1N1)2009 virus during the second wave and hospital testing policy remained consistent throughout the pandemic. The simplest explanation is that there were higher levels of influenza A(H1N1)2009 circulating in the community during the second wave in Wales, as demonstrated by the much higher influenza positivity rate in community samples.

There are several strengths as well as limitations to our study. We used a number of independent data sources to analyse the two waves of influenza A(H1N1)2009 in Wales, and all reflect the same phenomenon. Health service arrangements for clinical diagnosis and treatment of influenza remained consistent in contrast to England where the NPFS was introduced partway through the pandemic. Virological surveillance was also carried out consistently throughout the pandemic with participating practices instructed to send a maximum of five specimens per week from patients meeting the ILI case definition.

The main limitation of the study is the absence of detailed information on the symptoms of the patients consulting with ILI. The GP surveillance schemes rely either on an imprecise clinical case definition of ILI or automated extraction of relevant Read codes, neither of which capture subtle changes in presenting symptoms. Virological surveillance was restricted to five viruses, (influenza A, influenza B, influenza A(H1N1)2009, RSV and rhinovirus), so we cannot tell if some ILI consultations were due to other respiratory viruses, such as parainfluenza virus or adenovirus.

In conclusion, Wales experienced two waves of pandemic influenza during mid-summer and mid-autumn 2009 respectively. Each wave presented a different epidemiological profile. The first wave had a lower proportion of ILI cases confirmed as influenza and fewer hospital admissions and deaths compared with the second. These differences are most likely to be due to the different thresholds for contacting a GP that existed during the period of the pandemic and the different risk perceptions of the population over time. This was probably triggered by changes in media coverage throughout the pandemic and especially the high media profile during the initial stages of the pandemic, causing public anxiety. What is clear is

that most patients presenting with ILI during the first wave in Wales do not appear to have had influenza and therefore did not require antiviral treatment. This has implications for the interpretation of surveillance data on ILI and on its use in policymaking. Above all, our study underlines the importance of using integrated epidemiological, virological and hospital surveillance data to routinely monitor influenza activity.

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3.8 Manuscript 8. Evidence of person to person transmission of oseltamivir resistant pandemic influenza A (H1N1) 2009 virus in a hematology unit.

BRIEF REPORT

Evidence of Person-to-Person Transmission of Oseltamivir-Resistant Pandemic Influenza A(H1N1) 2009 Virus in a Hematology Unit

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We describe the first confirmed person-to-person transmission of oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus that occurred in a hematology unit in the United Kingdom. Eleven cases of (H1N1) 2009 virus infection were identified, of which, ten were related as shown by sequence analysis of the hemagglutinin and neuraminidase genes. H275Y analysis demonstrated that 8 of 10 case patients had oseltamivir-resistant virus, with 4 of 8 case patients infected by direct transmission of resistant virus. Zanamivir should be considered as first-line therapy for influenza in patients with lymphopenic hematological conditions and uptake of influenza vaccination encouraged to further reduce the number of susceptible individuals.

Pandemic influenza A(H1N1) 2009 virus emerged in Mexico during April 2009 and has caused 2 successive pandemic waves [1]. Analysis of the (H1N1) 2009 virus showed that it was

inherently resistant to the adamantane group of antivirals due to a serine to asparagine mutation at amino acid 31 (S31N) in the M2 ion channel [2]. Unlike the previously circulating seasonal influenza A(H1N1) virus, which is fully resistant to oseltamivir due to the presence of the H275Y mutation in the neuraminidase (NA) gene [3], the (H1N1) 2009 virus was shown to be susceptible to both oseltamivir and zanamivir [2].

However, by October 2009, the sporadic emergence of oseltamivir-resistant (OR) (H1N1) 2009 virus was reported in immunocompromised patients who were receiving oseltamivir therapy and in some individuals who had received oseltamivir prophylaxis [4]. Fewer than 60 isolates of OR-(H1N1) 2009 had been reported to the World Health Organization by the end of October 2009, and, of these, only 3 isolates had been identified in the United Kingdom. There were no confirmed reports of person-to-person transmission of OR-(H1N1) 2009 virus except for 1 incident involving 2 cases of resistant virus in adolescents at a summer camp in the United States, where evidence of spread was inconclusive [5]. We describe the emergence of OR-(H1N1) 2009 virus during a nosocomial outbreak on a hematology unit between October and November 2009 with epidemiological and molecular evidence of person-to-person transmission.

METHODS

Setting and patients. During October 2009, an outbreak of OR-(H1N1) 2009 virus began in an adult hematology unit at the University Hospital of Wales (UHW), Cardiff, United Kingdom. The hematology unit provides hematology services to a local population of 770, 000 and hematopoietic stem cell transplant (HSCT) service for Wales (population 3 million). The unit has a general hematology section for nontransplant recipients (17 beds) and a dedicated HSCT section for patients immediately after transplantation (10 beds). The HSCT section has its own positive pressure, high efficiency particulate (HEPA) filtered air supply. Elective admissions are for planned chemotherapy, transplantation, or central line insertion, whereas emergency admissions are mostly associated with neutropenic sepsis or chemotherapy complications.

Upper respiratory tract samples were collected from inpatients presenting with an influenza-like illness (ILI), defined by the Health Protection Agency (HPA) as a fever >38°C with 2 or more of the following: cough, coryza, sore throat, headache, myalgia, diarrhea, and from patients with an unresolving pyrexia. Patients who received mechanical ventilation had

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diagnostic bronchoalveolar lavages (BALs) performed. Samples were obtained from case patients every 3–5 days after treatment until 2 sequential test results were influenza A negative. Patients who continued to have symptoms or who became symptomatic were retested regardless of previous polymerase chain reaction (PCR) results. All patients in contact with confirmed case patients were monitored for symptoms with a low threshold of suspicion to ensure early sampling.

Retrospective review of admission records was undertaken by the attending physician for each of the case patients to determine clinical characteristics, date of admission, and length of time on the unit (Table 1). Lymphocyte counts were recorded (reference range, $1.0\text{--}4.0 \times 10^9$ cells/L) and compared for each of the case patients during viral excretion and post viral clearance. Statistical analysis was undertaken using the Mann-Whitney 2-tailed nonparametric test.

Community surveillance and controls. Positive (H1N1) 2009 virus isolates from across Wales were tested for the H275Y mutation by pyrosequencing to determine whether OR-(H1N1) 2009 virus was circulating in the wider community [6]. These data were used to determine whether the emergence and transmission of OR-(H1N1) 2009 virus in the UHW was an isolated event in Wales.

Outbreak management. Early cases in the outbreak were managed following standard outbreak interventions, including isolating and cohorting of case patients and the reinforcement of the use of personal protective equipment by health care workers (HCWs) when working with symptomatic patients. HCWs on the unit were offered both seasonal and pandemic influenza vaccination.

Additional control measures were initiated when later cases of OR-(H1N1) 2009 virus were identified. Elective admissions were delayed, and the HSCT unit was used as an isolation facility. Prophylaxis was stopped, and zanamivir was used as dual therapy with oseltamivir. Patients who could not tolerate inhaled zanamivir or who were receiving mechanical ventilation were prescribed intravenous zanamivir on a named-patient basis. Seasonal and pandemic vaccination was extended to include all patients and close contacts. Finally, screening of all inpatients on the unit was undertaken to locate additional cases; this was repeated 72 h later to show no further transmission events had occurred.

Molecular diagnostics. Generic influenza A testing was performed by real-time reverse-transcription PCR (RT-PCR) targeting the matrix gene. To ensure sample quality and lack of inhibition, an assay targeting human RNaseP was used; a sample with a crossing threshold (ct) value of <40 in the influenza A assay as described in the Centers for Disease Control and Prevention (CDC) protocol indicated a positive test result for influenza A [7]. Influenza A-positive samples were subtyped using a specific (H1N1) 2009 duplex real-time RT-PCR assay designed by the Health Protection Agency

(HPA), Centre for Infections (CfI) (Colindale, London, United Kingdom) [8].

Antiviral susceptibility testing. Positive samples from case patients who did not clear virus despite treatment and from cases whose first diagnosis occurred after evidence of viral resistance was found on the unit were forwarded to the respiratory virus unit at CfI for pyrosequencing analysis for the H275Y mutation using (H1N1) 2009 specific primers. When virus could be isolated in cell culture, phenotypic testing for oseltamivir and zanamivir was undertaken [6].

Sequencing and phylogenetic analysis. Amplification and sequencing of the complete coding regions of the haemagglutinin (HA) and NA genes was performed directly from original respiratory material with a 2-step RT-PCR using Superscript III RT reverse transcriptase and Platinum Pfx polymerase (Invitrogen Ltd), following manufacturer's instructions. The HA gene was amplified in 2 overlapping fragments (1.2–1.0 kb), whereas NA was amplified in a single fragment (1.4 kb). Primers are available upon request. Sequencing was performed on a 48-capillary ABI 3730 Genetic Analyser (Applied Biosystems). Raw sequencing data were edited and assembled using Sequencher software (version 4.9).

Nucleotide sequences were aligned, trimmed to include coding regions, and concatenated in the order HA-NA. Before phylogenetic analysis, the triplet coding for position 275 associated with drug resistance was deleted from all NA sequences. A maximum likelihood (ML) phylogenetic tree of the concatenated HA-NA segments was inferred using PAUP software package, version 4.0 (Swofford DL). The best-fit model of nucleotide substitution was identified using Modeltest [9] as the HKY85+ Γ model, with parameters estimated from the empirical data. ML trees were determined through an heuristic search. Bootstrap analysis was performed through neighbor joining algorithm with 1000 replicates, incorporating the ML substitution model previously determined. Accession numbers and standard strain names are provided in Suppl Table 1.

RESULTS

Case identification. From 29 October through 25 November 2009, 11 cases of (H1N1) 2009 virus were found in the hematology unit (defined as an in-patient in the hematology unit with a virological diagnosis of [H1N1] 2009). Epidemiological analysis suggested an outbreak of (H1N1) 2009 virus had occurred because the patients involved could be linked in time and place. The outbreak affected male patients with underlying hematological malignancy, and sequence analysis of the isolates showed that 10 cases were virologically linked (Table 1 and Figure 1). The probable index case of the outbreak (case 1) was identified retrospectively on the basis of the HA and NA sequencing of this virus.

Table 1. Clinical and Virological Background of the 16 Cases Involved in the Oseltamivir-Resistant (H1N1) 2009 Virus Outbreak, University Hospital of Wales, Cardiff, United Kingdom

Case	Age	Underlying Condition	Admission	Reason for admission	Length of admission	Date of first Influenza PCR Positive	Date of sample positive with H275Y mutation	Minimum time of viral shedding	Treatment	Other information
1	19	Acute Lymphoblastic leukaemia	10/18/09	Air	<1 day	10/19 (Oseltamivir sensitive)	N/A	N/A	5 days oseltamivir 75 mg/BID	Admitted for sampling Recovered
2	20	Acute myeloid leukaemia	10/11/09	Fever	>40 days (transferred to ICU 11/05)	10/20 (Oseltamivir sensitive)	11/04 (70% H275Y) (result available for clinician 11/12)	30 days	5 days oseltamivir 75 mg/BID 4 days zanamivir 150 mg/BID 10 days intravenous zanamivir	Recovered
3	32	Merckle cell lymphoma	10/05/09	Induction chemotherapy	7 days (readmitted 11/09 for 8 days)	10/20 (Oseltamivir sensitive)	11/06 (80% H275Y) (result available for clinician 11/12)	12 days	5 days oseltamivir 75 mg/BID 10-day treatment 1 day 10 days oseltamivir 150 mg/BID + intranasal zanamivir	Recovered
4	56	Multiple myeloma	10/28/09	Consolidation chemotherapy	8 days	11/02 (Oseltamivir sensitive)	No evidence of H275Y mutation in repeat samples	<8 days	5 days oseltamivir 75 mg/BID	Earlier sample 10/30 negative Recovered
5	65	Multiple myeloma	10/28/09	Fever	25 days	11/07 (Oseltamivir resistant)	11/07 (100% H275Y) (result available for clinician 11/19)	23 days	3 days Oseltamivir 75 mg/BID 9 days zanamivir (150 mg/BID) 1 day oseltamivir 150 mg/BID + intranasal zanamivir 4 days intranasal zanamivir	Recovered
6	27	Non-Hodgkin lymphoma	10/25/09	Induction chemotherapy	24 days	11/09 (Oseltamivir sensitive) Negative PCR 11/17	Viral rebound 11/10 (100% H275Y) (result available for clinician 11/06)	10 days	10 days Oseltamivir 150 mg/BID	Recovered
7	48	Burkitt's lymphoma	11/02/09	Consolidation chemotherapy	9 days (readmitted 11/12 for >14 days)	11/09 (Oseltamivir resistant)	11/09 (100% H275Y) (result available for clinician 11/19)	20 days	9 days Oseltamivir 150 mg/BID 5 days oseltamivir 150 mg/BID + intranasal zanamivir 10 days intravenous zanamivir	Recovered

Case No.	Diagnosis	Onset Date	Induction/Therapy	Time to Illness	Genetic Findings	Antiviral Treatment	Outcome
8	Acute myeloid leukemia	10/24/09	Induction chemotherapy	25 days	11/09 (Osetamivir resistant)	11/09 (100% H275Y) result available for clinician 11/19	10 days oseltamivir (150 mg/BD) Earlier sample 11/02 negative Recovered
9	Acute myeloid leukemia	10/26/09	Perianal abscess	11 days readmitted 11/16	11/17 (Osetamivir resistant)	11/17 (100% H275Y) result available for clinician 11/19	2 days oseltamivir (150 mg/BD), 8 day oseltamivir (150 mg/BD) + inhaled zanamivir 2 days intravenous zanamivir Readmitted with ILI Recovered
10	Chronic lymphocytic leukemia	11/05/09	Expressive dysphasia, poor coordination	>25 days	11/20 (Osetamivir resistant)	11/20 (100% H275Y) result available 11/26	Osetamivir prophylaxis from 11/10, 10 days Osetamivir (150 mg/BD) + inhaled zanamivir Earlier sample 11/07 negative Recovered

NOTE. ILI, influenza-like illness.

The 11th case patient was admitted in late October 2009 but was ruled out of the outbreak by sequence analysis. He was symptomatic and had been admitted directly into isolation where he was managed for his entire admission. No evidence of onward transmission of his virus was found, suggesting appropriate infection control measures were being undertaken. Overall, outbreak management was challenging because of the difficulty in clinically diagnosing influenza in this patient cohort using standard definitions of ILI. Mild symptoms and asymptomatic illness were main features of this outbreak.

During the first 2 weeks of the outbreak, 6 HCWs on the unit were absent from work with a multitude of different symptoms, including lethargy, diarrhea, and coryza. No respiratory sampling of HCWs had been undertaken because symptoms were generally mild and nonspecific.

Antiviral susceptibility findings. Eight of the 10 outbreak case patients had (H1N1) 2009 virus expressing the H275Y mutation (Table 1). Where phenotypic testing was performed, the presence of this mutation correlated with high-level oseltamivir resistance (200–600-fold increase in oseltamivir IC50). Three of 8 case patients developed resistant virus while receiving treatment. Pretreatment samples from these patients showed sensitive virus by pyrosequencing and phenotypic assays analysis. Cases 2 and 3 developed a mixed majority populations of H275Y (Table 1), which did not alter on repeat testing. No other case patients in the unit with OR-(H1N1) 2009 were shown to have a similar mixed population. Case 6 had viral rebound with OR-(H1N1) 2009 that was fully resistant to oseltamivir. Case 10 was on oseltamivir prophylaxis due to being in contact with 2 cases, and this possibly drove the emergence of OR-(H1N1) 2009 virus. No other patients had prior exposure to oseltamivir, suggesting that person-to-person transmission of oseltamivir-resistant virus had occurred.

Prophylaxis and treatment. Treatment and prophylaxis used in the outbreak is summarized in Table 1. At the start of the outbreak, all case patients were treated with 5 days of standard dose oseltamivir (75 mg/BD). Subsequently, all case patients were treated with 10 days double-dose oseltamivir (150 mg/BD) and inhaled or intravenous zanamivir added if resistant virus was suspected. Oseltamivir prophylaxis was used sparingly and was stopped when it became apparent that the outbreak involved an OR-(H1N1) 2009 virus.

Viral excretion and immune status. OR-(H1N1) 2009 virus was excreted by case patients for a mean of 17 days (range, 6–30 days). Prolonged positivity was seen in patients in whom lymphocyte counts were $<0.5 \times 10^9$ cells/L, with a median of 0.27×10^9 cells/L compared with 0.65×10^9 cells/L when virus was not detected ($P < 0.001$).

Sequence analysis results. Phylogenetic analysis of the concatenated HA and NA genes from the outbreak influenza viruses showed that they clustered together in a separate branch from other (H1N1) 2009 viruses isolated in Wales and the

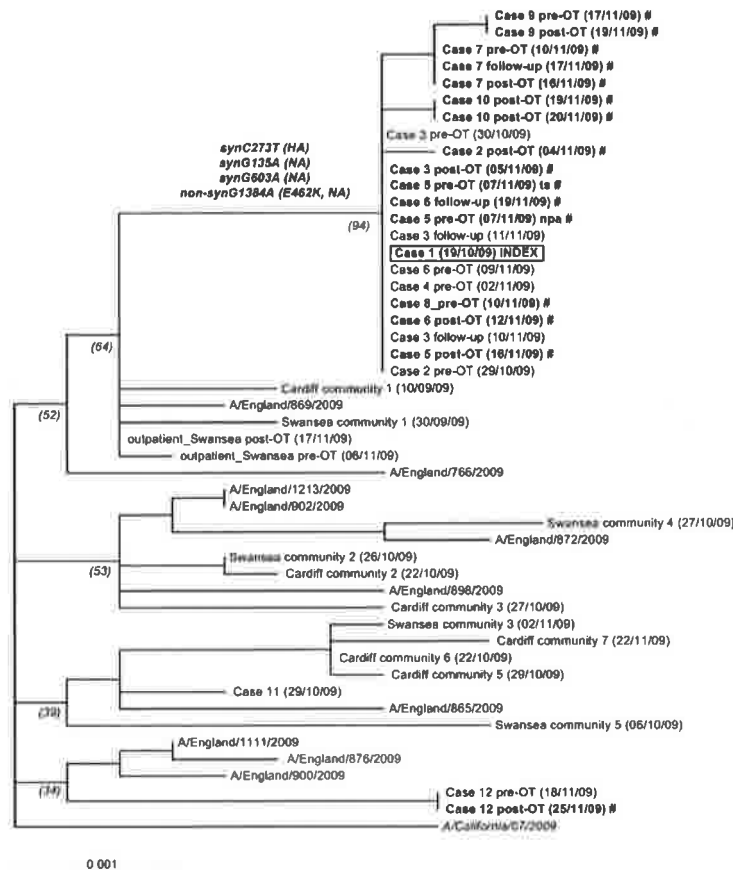


Figure 1. Maximum-likelihood tree of the nucleotide coding region of the concatenated HA and NA genes of pandemic H1N1 2009 influenza viruses from the outbreak and the community in Wales and the United Kingdom. Tree was rooted using A/California/07/2009 as out-group. Branch lengths are drawn to scale. Oseltamivir-resistant viruses are in bold marked with #. Bootstrap values are displayed in brackets below the nodes. Signature mutations are annotated in bold italics and refer to changes seen in the nucleotide sequence of the HA and NA gene using as reference the sequence of A/California/07/2009. OT = oseltamivir treatment.

United Kingdom during the same period (October – November 2009) (Figure 1). This cluster was characterized by a bootstrap value of 94% and the presence of 4 nucleotide substitutions: 1 synonymous change in the HA gene (C273T), 2 synonymous changes in the NA gene (G603A, T645C), and a nonsynonymous mutation also in NA (G1384A) leading to an amino acid replacement (E462K). After deletion of codon position 275, all the NA sequences from this cluster were shown to be identical. Given the higher variable nature of the HA gene, a few additional single mutations had accumulated in all the viruses sampled from case patients 9 (G751A), 7 (A330G), and 10

(G1035A). Also, posttreatment sample from case patient 2, taken 5 days after the pretreatment sample, had accumulated single substitution A716G. To further confirm the distinctive feature of the 4 mutations characterizing the outbreak cluster, a thorough search through all the sequences deposited in public influenza sequencing databases (NCBI, GISAID) was carried out, revealing that these mutations were present in <1% of global sequences, none of them from the United Kingdom, and not concomitantly seen in any NA global sequence deposited to date. This striking finding strongly supports our hypothesis of transmission of OR viruses among the outbreak patients.

No evidence of the H275Y mutation was found in any other (H1N1) 2009 virus isolate collected over the same period as the outbreak from Wales. One further hematology patient (case 12, Figure 1) later developed a minority mixed population of OR-(H1N1) 2009 virus on treatment, but this virus was shown to be genetically distinct from the outbreak cluster.

DISCUSSION

This is the first confirmed outbreak to our knowledge of OR-(H1N1) 2009 virus with person-to-person transmission, as demonstrated by the presence of OR virus in pretreatment and posttreatment samples of 2 patients. Subsequent genetic analysis of the HA and NA genes proved that the same virus was involved in the outbreak.

It occurred during the peak of the second wave of the (H1N1) 2009 pandemic in Wales, when 60% of community surveillance samples tested positive for (H1N1) 2009 virus [10]. (H1N1) 2009 virus was introduced to the hematology unit from the community with oseltamivir resistance being driven by treatment in 2 lymphopenic patients with onward transmission of OR-(H1N1) 2009 virus to other patients on the unit. Case patients were all men, and their admissions on the ward overlapped.

Treatment guidelines for hematology patients with (H1N1) 2009 virus infections were revised in light of this outbreak. Dual treatment with oseltamivir and zanamivir was used when resistant virus was suspected or confirmed. Although all of the cases recovered from influenza using this approach, recent data suggest there is possibly no synergy to be gained from this approach [11]. Zanamivir alone therefore would be preferable as a frontline treatment in particularly high-risk groups. Prophylaxis should be used with caution in patients with lymphopenia, because they may have asymptomatic infection with prolonged viral excretion; both of which are significant factors in the emergence of resistance [12]. Viral clearance in this outbreak was associated with immune reconstitution, supporting previous findings [12, 13]. Case 6 transiently had a lymphocyte count above 0.5×10^9 cells/L and became PCR negative, suggesting that a combination of antivirals and a degree of immune reconstitution temporarily suppressed viral replication. Viral rebound may have occurred because of the presence of a minority population of OR-(H1N1) 2009 virus that replicated despite the presence of oseltamivir [6]. Drug resistance should therefore always be considered in any immunosuppressed patient who does not clear virus.

Transmission of infection on hematology units might be reduced by protective isolation of neutropenic patients using single cubicles, but this approach is controversial. Although some studies show reduced infection rates, none have been able to demonstrate reduced mortality [14]. Patients treated in isolation were shown to have a significant psychological burden,

including increased levels of insomnia and depression [15]. An alternative to isolation is increasing vigilance for the introduction of respiratory infections into areas where immunocompromised patients are cared for, together with good infection control procedures to prevent transmission events.

There were no deaths and only limited morbidity associated with (H1N1) 2009 infection regardless of oseltamivir susceptibility in this outbreak. Despite the ease of transmission of the OR-(H1N1) 2009 virus on the unit, the mild symptoms associated with this outbreak led to difficulties in determining when a case patient acquired their infection, making the production of a meaningful outbreak curve impossible. Because screening of the unit did not occur until late into the outbreak, it is possible that an individual with mild symptoms or asymptomatic shedding played a role in the continued transmission of the OR-(H1N1) 2009 virus.

Pandemic influenza vaccine was available in the United Kingdom in late October 2009; when the outbreak was identified, vaccination of hematology patients, their families, and hospital staff was undertaken. Vaccination against influenza remains the best way of preventing infection in these patients, and therefore, vaccine uptake must be strongly encouraged to reduce the pool of susceptible individuals prior to the start of each influenza season.

Supplementary Data

Supplementary data are available at http://www.oxfordjournals.org/our_journals/jid/ online. Supplementary Table 1 contains GenBank accession numbers of nucleotide sequences of pandemic influenza (H1N1) 2009 used for phylogenetic analysis.

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4. Research Overview

4.1 The Methods Used.

4.1.1 NASBA as an Amplification Method for the Detection of RNA Viruses

NASBA at the inception of the Framework 5work was a novel technique with relatively few studies published showing its clinical utility beyond HIV viral load monitoring and mRNA detection (Darke *et al.*, 1998; Heim *et al.*, 1998; Romano *et al.*, 1996). In-house NASBA assays were infrequently described, and direct comparison of the method against in-house RT-PCR was even more infrequent (Malek *et al.*, 1994). The Cardiff virology laboratory was already routinely using a commercial NASBA for HIV viral load monitoring and due to links between Cardiff University and the research and development team in Organon Teknika, further in-house NASBA assays were being developed. Therefore, a research and routine diagnostic background was already in place, with an expanding knowledge base of the technique. Hence, it seemed logical that NASBA could offer an alternative molecular diagnostic method to RT-PCR for viruses with RNA based genomes.

During 2001, the molecular service for the detection of noroviruses in faecal samples from outbreaks across Wales was introduced in Cardiff. Noroviruses represent one of the most genetically diverse groups of viruses that cause diarrhoea and vomiting outbreaks that impact significantly upon health services due the resulting ward closures. The detection of these viruses prior to the advent of molecular diagnostics was difficult as they do not replicate in routine diagnostic cell lines and the assays developed for the detection of viral antigens lacked sensitivity. Electron microscopy was therefore the 'gold standard' for

their detection but the method required a high level of skill together with the requirement of a high viral titre in the clinical sample to allow visualisation of the virus (Ando *et al.*, 2000; Caul and Appleton 1982; Green *et al.*, 1995; Herrmann *et al.*, 1985; Jiang *et al.*, 1995).

The assay initially introduced was a block based PCR following a randomly primed reverse transcription step with gel detection of amplified product. The use of NASBA for the detection of noroviruses until this point had been described in very limited studies from the USA and the method had not yet been fully validated on clinical samples. The Cardiff study (manuscript 1) comparing the two methods on clinical samples and prototype norovirus strain demonstrated not only the improved sensitivity and specificity of NASBA over traditional block based PCR but also the decreased time to result. The reduced number of manual steps required to perform the NASBA assay gave it enhanced utility in the routine diagnostic laboratory and the method quickly replaced the block-based PCR assay in routine use.

Publication of this validation supported an earlier published study describing the development of an enterovirus NASBA assay from the Cardiff University group in collaboration with the diagnostic laboratory (Fox *et al.*, 2002). Both studies together demonstrated a sound basis for the further development of assays for the detection of genetically diverse RNA viruses using NASBA technology. The respiratory viruses were prime candidates and it was by obtaining the EU Framework 5 grant that this work could be undertaken.

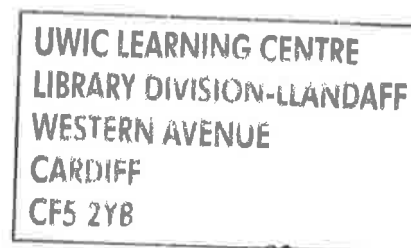
The process of developing and validating an in-house NASBA assay was described in particular detail in manuscript 2. This format was followed for each target virus throughout the study with three assays being developed per target including end-point detection NASBA and a block based PCR assay. The block-

based PCR assay was used as the foundation for the production of transcript RNA for sensitivity testing as part of the development of the real-time NASBA assays, which utilised molecular beacons as the target specific detection probe.

The first research study published from the Framework 5 grant described the development of parainfluenza 1-4 assays (Hibbitts *et al.*, 2003). Real-time NASBA was shown to have comparable sensitivity and specificity to end-point detection NASBA. Furthermore, the study also demonstrated the potential of duplexing NASBA assays by using different reporter dyes for the detection of parainfluenza types 1 and 3. At that time parainfluenza, as a target for routine diagnostics was not yet considered important enough for the assay to be immediately validated for transfer to the molecular diagnostics unit.

The Framework 5 grant facilitated the development of assays for the detection of influenza A, influenza B, RSV, parainfluenza viruses, and rhinoviruses. The assays underwent further optimisation as the enzyme and buffers used to develop the assays were modified and improved by the manufacturer. The opportunity was taken at this time to duplex the assays where possible and to standardise the methods allowing the assays to be run concurrently using the same amplification conditions. The sensitivity and specificity of the fully optimised NASBA assays was demonstrated when a pilot external quality panel for the detection of respiratory viruses using molecular techniques was distributed in 2005 to 17 laboratories across Europe who had developed in-house respiratory viral molecular assays. The results of the panel were finally published in 2006 and demonstrated that the NASBA assays performed well in comparison to other centres (Templeton *et al.*, 2006).

4.1.2 Proving Clinical Utility in the Routine Setting



A newly developed assay, regardless of the method utilised, will always have limited validity as a diagnostic test unless it can be proven both sensitive and specific in a clinical background. As well as being involved in the development of the assays, it was the primary role of the candidate to prove clinical utility of each test and to show an improvement over any current assays. This was not difficult to achieve in terms of the norovirus assay as not only was it possible to show an improvement in sensitivity over the block-based assay in use, but by introducing a strict algorithm and limiting the number of samples tested, the burden of testing large numbers of samples from outbreaks for the laboratory was significantly reduced.

For respiratory viruses, the task of introducing a molecular based service was challenging due to the ambivalent clinical importance afforded to respiratory viral infection together with the limited treatment options. Traditional laboratory techniques (in particular DIF) were therefore considered adequate for routine diagnostics. For influenza however, the development and introduction of the neuraminidase inhibitors (NAI) zanamavir and oseltamivir meant that rapid and sensitive assays were required as although both drugs had been given NICE approval, they could only be prescribed within 48 hours of symptom onset and when influenza was known to be circulating in the community <http://www.nice.org.uk/nicemedia/live/11774/43268/43268.pdf>. Whilst DIF was an option for rapid diagnosis in hospitalised patients, the diagnosis of acute influenza infection in the community was reliant on cell culture or clinical judgement.

During early 2003, an outbreak of influenza like illness was reported from a home for the elderly in West Wales. The outbreak had resulted in significant illness in the residents and several had subsequently died. This outbreak offered

the first opportunity to use the newly developed influenza A NASBA assay in a clinical setting. Respiratory samples collected from residents were inoculated into cell culture and were tested by both end-point and real-time NASBA assays. Despite the cell culture being negative for respiratory viruses, the results of the NASBA assays demonstrated that influenza A was indeed the cause of the outbreak; this was subsequently confirmed using serology on acute and convalescent serum. The results of this investigation provided the basis for a business case that constituted a full validation of the assay on clinical samples during the 2003-2004 Winter respiratory season. This was fortuitous due to the emergence of a drifted strain of influenza A (H3N2) virus that grew less well in routine cell culture. This work provided the clinical validation required to introduce the test into routine use for the next respiratory season (manuscript 2).

During 2004, there was a significant publication by Templeton and colleagues from Leiden University who were developing the real-time PCR based assays as part of the Framework 5 grant (Templeton *et al.*, 2004). The group described the first multiplex real-time RT-PCR assay for the detection of seven major respiratory viruses and like the work in Cardiff demonstrated the improved sensitivity of the assays over cell culture and direct immunofluorescence. However, unlike the Cardiff study that was largely undertaken prospectively and directly influenced patient management, the study in Leiden was retrospective and so it was difficult to fully ascertain clinical utility in a routine diagnostic setting.

It was soon found that one of the major difficulties with the introduction of molecular techniques for respiratory viruses was that the gold standards being used for the comparison work were less sensitive traditional techniques. The

assumption therefore had to be made that every extra positive result by the molecular method was a 'false positive' that required further evidence to prove the result was true. A retrospective study performed in Cardiff using a commercialised version of the RSV assay developed during the Framework 5 grant highlighted this issue (manuscript 4). During this study, 41 samples that gave a positive result by NASBA were negative by cell culture and/or DIF. These 41 samples were subsequently re-extracted and retested to prove the NASBA result was reproducible. Following this, the extract was then tested by a previously published block-based PCR. Further to this, the clinical details were assessed to determine whether the cases matched a diagnosis of RSV. Only if this criterion were met would a sample be considered a true positive. An extra layer of complexity was added if a traditional method was positive but the molecular techniques gave a negative result. In this study, two samples were discordant, one was cell culture positive and one was DIF positive. Results such as this meant that the sensitivity and specificity of molecular techniques were rarely 100%. This made validation of molecular techniques difficult and protracted as well as expensive. This problem was a feature of many early studies comparing molecular techniques to traditional laboratory methods and this together with funding issues contributed to the limited development of molecular diagnostics for respiratory viruses in Europe. By 2005, few laboratories in Europe had published data showing the clinical utility of a respiratory virus screen in routine diagnostics but those that had were beginning to add to the depth of knowledge about the burden of acute respiratory infection caused by respiratory viruses in the community and hospitalised patient (Coyle *et al.*, 2004; Gunson *et al.*, 2005; Templeton *et al.*, 2004; Templeton *et al.*, 2006).

Proving clinical utility of the assay developed for the detection of influenza A H5N1 proved a significant problem. Cardiff had and continues to play a role within the UK network of laboratories for the early detection of emerging influenza viruses and in particular for H5N1 (Curran *et al.*, 2007). Prototype strains of influenza were regularly provided as part of an external diagnostic panel, however due to the lack of imported cases into the UK, the assay was never validated on clinical material. This meant that a collaborative study was required at a site external to the UK where H5N1 was endemic and frequently infected humans. This collaboration was finally set up with a reference laboratory in China and the utility of the H5N1 NASBA was proven on clinical material (manuscript 5).

Whilst the 'extra' positive results obtained using molecular techniques over traditional methods were logistically problematic to confirm in the laboratory setting, at least a scientific understanding of why discordant results might occur when using highly sensitive methods was likely. Therefore, perhaps the greatest dilemma originally facing the service was not in showing that the assays worked in a clinical setting, but in the clinical interpretation of the results and applying it to patient management. Suddenly frontline clinicians were faced with patients with clinical influenza but who were found to have an infection with rhinovirus instead. With patients who still excreted RSV despite being symptom free for weeks and with patients who despite two full courses of oseltamivir failed to clear an influenza infection.

The body of work submitted contributed not only to new knowledge in the field of respiratory virus diagnostics in Wales but also towards the clinical application of increasingly sensitive and rapid testing, the questions that arose as a result, and in the ongoing attempts to provide clarity through clinical

evidence gathering to overall improve the management of respiratory viral infection.

4.2 Contribution to New Knowledge in the Field of Respiratory Virus Diagnosis

4.2.1 Evidence for the Clinical Utility of Molecular Testing for Respiratory Viruses

The research submitted in this thesis represents a small part of the work that contributed to the introduction of the molecular diagnostics service in Wales. The winter of 2004-2005 saw a large study undertaken in Wales where all samples received in the laboratory in Cardiff for respiratory virus diagnosis were tested by traditional and molecular techniques. The study was supported by the Welsh Assembly Government and BioMérieux as Cardiff was undertaking the validation of the commercial RSV assay (manuscript 3) and the virological surveillance of influenza in Wales was being transferred to an all molecular service (albeit just for the detection of influenza A and B). In total, 1058 samples were tested in the study; the results were submitted as a business case to the Welsh Assembly Government for the implementation of a respiratory virus service. Data from this study was also used in publications submitted as part of this thesis (manuscripts 3 and 5).

The clinical validation work whilst demonstrating the improved detection rate of respiratory viruses over traditional techniques also provided valuable information regarding the spectrum of respiratory viral disease. The role of RSV as a significant illness in adults was an unexpected finding but supported earlier studies (Crowcroft *et al.*, 1999; Ellis *et al.*, 1997; Nicholson 1996). This meant that a shift in the understanding of the clinical impact that RSV had on

the Welsh population as a whole needed to occur since both the retrospective and prospective studies in Wales had demonstrated a significant minority of severe cases in adults and children outside the high-risk age groups. The data also supported the role of RSV as a community acquired illness presenting with clinical features of influenza, this prompted the inclusion of RSV as a target in the community surveillance of respiratory viral infection. This would allow RSV to be factored into the consultation rates during the winter seasons with low influenza circulation, so that answers at least could be given in response to questions regarding increased consultations to primary care due to respiratory illness when there was little virologically confirmed influenza in the community. All of the research performed in Cardiff on respiratory viruses, highlighted the significant impact that respiratory viruses had in particularly on patients with a haematological malignancy over all other patient groups. The problem however, with the diagnosis of respiratory viruses in this group of individuals was the quality of the samples obtained, particularly whilst they were significantly immunocompromised.

4.2.2 Respiratory sampling and transportation using dry swabs improves access to the molecular diagnostic service

For rapid diagnosis, NPAs were routinely collected from symptomatic haematology patients however, these often contained few cells making diagnosis by DIF impossible. During the 2003-2004 season a GP in the community had collected a dry respiratory swab from an elderly gentleman in the community which had been tested by NASBA giving a positive result. This result provided an unexpected but interesting research question about whether dry respiratory swabs could be used as an alternative collection method to those collected into virus transport medium (VTM). As Cardiff also provided the molecular

diagnostic service for North Wales, including the service to respond to emerging infections, the logistics for providing VTM in an emergency was often inadequate and transport of liquid VTM was considered high risk for viruses such as H5N1 that remained viable. Collecting and transporting dry respiratory swabs could prove to be a good alternative. The study described in manuscript 5, not only showed the excellent recovery of viruses from a dry swab, but also demonstrated the stability of RNA on a dry matrix. Until this point, RNA was considered highly labile in the environment due to the ubiquitous nature of RNase enzymes. As part of the study, RSV virus positive culture medium was applied to dry swabs and left at room temperature for 15 days without loss of RNA. This data was compelling and led to a radical move in Wales, to use dry respiratory swabs as the sample of choice in all adults, community surveillance and in outbreak investigations. The data from the dry swab work showed that RSV was the second most frequent virus detected in adult haematology patients, that asymptomatic infection was common and that long term shedding of respiratory viruses was frequent. This in turn led to problems in terms of infection control and patient management, an issue that is still a matter of debate particularly when DIF or cell culture is negative.

4.2.3 The Introduction of the Routine Molecular Diagnosis of Respiratory

Viruses in Wales

By 2007, all of the respiratory in-house NASBA assays were in routine use in the Cardiff laboratory. The time to a result was 3 hours from sample arrival in the laboratory to the final result post amplification, approximately half the time of many of the routine in-house real-time RT-PCR assays being performed elsewhere (Gunson *et al.* 2005; Templeton *et al.* 2004; Templeton *et al.* 2006). The assays produced data that impacted not only on patient management in

Wales but also allowed the rapid detection of influenza as a cause of outbreaks (Carnicer-Pont *et al.* 2005). They informed on emerging infections such as H7N2 transmission from poultry to humans in North Wales http://www.who.int/csr/don/2007_05_29/en/index.html and facilitated the switching on of oseltamivir prescribing in Wales outside the normal respiratory season in response to an increased circulation of influenza B (Mook *et al.*, 2008)

4.2.4 Pandemic Influenza A (H1N1) 2009 and the Subsequent Emergence of Oseltamivir Resistance in Wales

The full impact of the respiratory virus service was not realised until the emergence of influenza A (H1N1) 2009 virus (Hamilton 2009). During the first wave of the pandemic, the data of previous seasons that had suggested that the circulation of other respiratory viruses influenced influenza consultation rates was realised with the data showing that patients were presenting to GPs with suspected pandemic influenza but were actually presenting with other viruses (manuscript 7). Rhinoviruses in particular were found frequently not only in community samples but also in cases that required hospitalisation. This phenomenon was confirmed in data collected from other sites in Europe (Anestad and Nordbo 2009; Casalegno *et al.*, 2009; Linde *et al.*, 2009). The molecular respiratory virus service for the surveillance of respiratory viruses in the community using dry respiratory swabs allowed for the close monitoring of the pandemic virus across Wales, informing the Minister for Health, the Welsh Assembly Government and ultimately the UK government of the impact of the virus in the community in Wales.

The emergence of the new influenza virus however saw the introduction of a new assay based on real-time PCR for the detection of influenza A. The virus was proven genetically different to seasonal influenza even in the usually

conserved genes; matrix and nucleoprotein used as targets in the UK network and NASBA assays (Klungthong *et al.*, 2010; Pabbaraju *et al.*, 2009). Although the virus could be detected using both assays the concern was that sensitivity would be compromised due to point mutations in the region coding for the molecular beacon. The decision was made to introduce influenza A assays developed by the Centres for Disease Control (CDC) in the US where the H1N1 (2009) virus was first detected outside Mexico. Wales introduced the assay in early May 2009 following a rapid validation of the test. Despite both the in-house NASBA assay and CDC assay detecting the first case in Wales on the 28th May 2009, the CDC assay was introduced into routine use. The assay was in use when global attention was turned to Cardiff after the detection of an oseltamivir resistant H1N1 (2009) virus outbreak on the haematology unit (manuscript 8). The data observed in the early manuscripts submitted in this thesis was confirmed during the outbreak, immunocompromised haematology patients rarely presented with typical influenza symptoms, long term shedding of virus was frequent, and transmission of virus was insidious and continued despite enhanced infection control procedures being introduced. The outbreak was first suspected after two patients on the unit failed to clear the virus despite a full course of oseltamivir prompting a full screening of the unit. Importantly, by using a rapid molecular technique, the outbreak could be confirmed and reported in real-time to the WHO resulting in the use of oseltamivir being questioned in this group of patients. Global recommendations for the treatment and prophylaxis of highly immunocompromised patients were revised to make zanamavir the first line drug of choice http://www.who.int/csr/disease/swineflu/notes/briefing_20091202/en/index.html.

4.2.5 Impact of the Respiratory Virus Molecular Service to Patient

Management and Community Surveillance

The changes to respiratory virus diagnosis and understanding of respiratory viral disease in Wales due to the introduction of the molecular diagnostic service have been far reaching. There is now a general expectation that a patient who presents acutely with a viral respiratory illness will have a respiratory virus diagnosed. Community surveillance has confirmed that during the winter influenza and RSV are the predominant viruses circulating and are most likely to be the cause of respiratory infections regardless of age and in the autumn, spring and summer parainfluenza or rhinovirus circulate. Long-term detection of respiratory viruses with or without symptoms is now accepted as being a common occurrence. Patient management of these cases is usually based entirely on the vulnerability of the patient together with the risk of transmission of infection to others, for example a relatively well or asymptomatic person is more often sent home rather than being kept in hospital with viral monitoring being performed through an out-patient clinic. Dual and even triple infections are frequently found in both severely ill and community surveillance samples. Rhinoviruses have caused outbreaks and severe complications and influenza infections have been incredibly mild. The point being, that molecular testing has blurred the edges of the spectrum of disease associated with respiratory viral infection and each virus can no longer be associated with specific illnesses as each has been shown to cause significant disease regardless of whether the host is otherwise healthy or vulnerable. The greatest contribution to new knowledge afforded by this work is to show that each respiratory virus can impact significantly on patient morbidity and mortality in Welsh patients.

4.2.6 Limitations of the Submitted Work

The limitation of the work, proved to be NASBA itself and in particular, the system used to detect the product. Whilst RT-PCR had continuously moved forward with the introduction of improved, more stable reverse transcription enzymes that allowed single tube RT-PCR, the progress with NASBA stalled. Whilst the technique will always remain extremely sensitive for the detection of RNA, the system itself has not progressed beyond that used for the development of the in-house assays a decade ago. Compared to developing assays for the newer real-time systems such as the ABI 7500 FAST (now routinely used in Cardiff), developing NASBA assays are complicated and the cost of the reagents required to perform NASBA has increased over time. Commercial NASBA assays are relatively easy to set-up and interpret, which is a stark contrast to an in-house NASBA assay that needs local expertise to troubleshoot initial problems that might arise with optimisation and interpretation. The expertise associated with developing in-house NASBA assays has decreased over the decade since the start of the Framework V work, meaning that the candidate remains one of the only scientists in the UK that is able to validate and troubleshoot in-house NASBA assays. Unless there is significant investment in the systems and software required for developing and validating in-house NASBA assays, regrettably the future of NASBA can only be in commercially developed assays in the routine diagnostic field.

In response to this problem, the candidate developed and introduced real-time RT-PCR respiratory virus assays for each target and transferred the service over to the ABI system in time for the third wave of H1N1 (2009) virus in 2011. This work further reduced the time to result to 1.5 hours and allowed for the transfer of the assays to the Virology laboratory in Swansea where the respiratory

samples from across West Wales are now tested. This alleviating another limitation that arose from the work in that by developing a successful service the significant increase in service demands could not be managed by a single laboratory.

An important limitation of this work is the gradual erosion of the cell culture facility in Wales. The molecular detection of respiratory viruses can only be accomplished if the genome of the circulating respiratory viruses is known and that the viruses can be shared. The global surveillance network ensures that influenza viruses are monitored for the major changes that might affect routine molecular tests; however, the same cannot be said for the other respiratory viruses. Cell culture provides a means to ensure that current circulating respiratory viruses can be stored at a high titre so that as well as providing material for sequencing and control material for molecular diagnostics a limited cell culture bank could also potentially provide viruses for future vaccine development work.

4.2.7 Future Work

The respiratory virus mentioned the least in the critical analysis has been hMPV. Early work with hMPV was promising but failed to demonstrate a real clinical need for the assay during 2004-2005. During 2011, the addition of an hMPV assay to the respiratory virus panel will complete the full screen for respiratory viruses in Wales.

Respiratory virus detection in post pandemic Wales remains as important as the years leading up to the pandemic and it is now accepted in the UK that respiratory viral infection should be diagnosed using molecular techniques. The challenges now facing virologists interested in respiratory viruses involves

elucidating further the clinical spectrum of respiratory viruses, treatment options, and whether an infection should be monitored using a quantified molecular technique and if so what would be the most appropriate sample type.

In terms of influenza, apart from preparing for the next pandemic, the immediate challenge is to determine the extent in which oseltamivir resistance will impact on treatment, an assay to detect the H275Y mutation has already been introduced into Cardiff using pyrosequencing as described in manuscript 8 (Deyde *et al.* 2010), the data from the first years work has already contributed to a UK wide publication looking at the increasing incidence of oseltamivir resistance (Lackenby *et al.* 2011). The next step would be to develop an assay for the routine monitoring of zanamavir resistance.

Pyrosequencing potentially offers an alternative approach for the monitoring of point mutations in target genes of respiratory viruses by rapidly sequencing short fragments encompassing the region targeted by primers and importantly the probe. This work would help ensure that even small changes that might affect the sensitivity of the assay would be detected allowing for assay modification.

As data accumulates regarding the significance of the newer respiratory viruses, the service will be expected to continue to expand and adapt to offer the people of Wales an excellent diagnostic service for respiratory viral infection, building on the expertise and service afforded in the early stages of the development and implementation of the service.

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Appendix 1. Contribution of the Candidate towards the Submitted Manuscripts

Manuscript Number	Conception and Design of the Investigation (%)	Conduct of the Research (%)	Analysis of the data (%)	Preparation for publication (%)
1	80	100	100	90
2	40	50	90	70
3	40	40	50	50
4	50	50	60	90
5	90	80	90	100
6	60	60	60	100
7	10	10	20	15
8	50	50	40	70

May 1st 2011

I hereby confirm that Catherine Moore contributed to the following publications of which I am a co-author with the indicated percentages.

1. Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in faecal material. *J Clin Virol.* 2004 Apr;29(4):290-6. Moore C, Clark EM, Gallimore CI, Corden SA, Gray JJ, Westmoreland D.

Concept and design of the Investigation	80%
Conduct of research	100%
Analysis of data	100%
Preparation for publication	90%

2. Development and evaluation of a real-time nucleic acid sequence based amplification assay for rapid detection of influenza A. *J Med Virol.* 2004 Dec;74(4):619-28. Moore C, Hibbitts S, Owen N, Corden SA, Harrison G, Fox J, Gelder C, Westmoreland D.

Concept and design of the Investigation	40%
Conduct of research	50%
Analysis of data	90%
Preparation for publication	70%

3. Rapid and highly sensitive qualitative real-time assay for detection of respiratory syncytial virus A and B using NASBA and molecular beacon technology. *J Virol Methods.* 2007 Dec;146(1-2):29-35. Deiman B, Schrover C, Moore C, Westmoreland D, van de Wiel P

Concept and design of the Investigation	40%
Conduct of research	40%
Analysis of data	50%
Preparation for publication	50%

4. Enhanced clinical utility of the NucliSens EasyQ RSV A+B Assay for rapid detection of respiratory syncytial virus in clinical samples. Eur J Clin Microbiol Infect Dis. 2006 Mar;25(3):167-74. Moore C, Valappil M, Corden S, Westmoreland D.

Concept and design of the Investigation	50%
Conduct of research	50%
Analysis of data	60%
Preparation for publication	90%

Yours sincerely

Diana Westmoreland.

Dr Diana Westmoreland
 Consultant Clinical Virologist and Head of Molecular Diagnostics (retired)
 Department of Virology
 National Public Health Service, Wales
 University Hospital of Wales
 Heath Park
 Cardiff CF4 4XW

Catherine Moore has been part of The Molecular Diagnostic Unit in Cardiff since its manifestation as a Unit in 2000. During her time within the Unit Catherine has been instrumental in the development and introduction of numerous routine viral diagnostic assays. Catherine has developed a particular interest in Respiratory Virus Diagnostics and is continually monitoring and improving routine respiratory virus diagnostics. Catherine is actively looking to expand the current service to encompass influenza antiviral susceptibility testing.. The publications reflect Catherine's ongoing interest within a challenging field.

I hereby confirm that Catherine Moore contributed to the following publications of which I am a co-author with the indicated percentages.

1. Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in faecal material. *J Clin Virol.* 2004 Apr;29(4):290-6. Moore C, Clark EM, Gallimore CI, Corden SA, Gray JJ, Westmoreland D.

Concept and design of the Investigation	80%
Conduct of research	100%
Analysis of data	100%
Preparation for publication	90%

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Concept and design of the Investigation	40%
Conduct of research	40%
Analysis of data	50%
Preparation for publication	50%

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Concept and design of the Investigation	50%
Conduct of research	50%
Analysis of data	60%

Preparation for publication 90%

5. Dry cotton or flocked respiratory swabs as a simple collection technique for the molecular detection of respiratory viruses using real-time NASBA. J Virol Methods. 2008 Nov;153(2):84-9 Moore C, Corden S, Sinha J, Jones R.

Concept and design of the Investigation 90%
Conduct of research 80%
Analysis of data 90%
Preparation for publication 100%

6. Development and validation of a commercial real-time NASBA assay for the rapid confirmation of influenza A H5N1 virus in clinical samples. J Virol Methods 2010 Dec;170(1-2):173-6. Moore C, Telles JN, Corden S, Gao RB, Vernet G, Van Aarle P, Shu YL.

Concept and design of the Investigation 60%
Conduct of research 60%
Analysis of data 60%
Preparation for publication 100%

Yours sincerely

Sally Corden

Head of Molecular Diagnostics Unit
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To whom it may concern:

I hereby confirm that Catherine Moore contributed to the following publications of which I am a co-author with the indicated percentages.

1. Dry cotton or flocked respiratory swabs as a simple collection technique for the molecular detection of respiratory viruses using real-time NASBA. J Virol Methods. 2008 Nov; 153(2):84-9
Moore C, Corden S, Sinha J, Jones R.

Concept and design of the Investigation	90%
Conduct of research	80%
Analysis of data	90%
Preparation for publication	100%

Catherine has contributed to the development of a number of assays that have greatly facilitated the improvements in the provision of diagnostic service for the population in Wales. Areas in particular that have added significantly to the development of molecular diagnostic services are the evaluation of dry swabs for the clinical diagnosis of infection. Given the geographical nature of Wales and the recent devolution of the Principality this initiative alone allowed the development of a national diagnostic service that ensured the integrity of samples across Wales in a cost effective manner, with reduced concerns on health and safety, due to the absence of lysis buffer. This also greatly facilitated the delivery of the respiratory service during the recent pandemic. In addition Catherine has continued to develop this area with the introduction of flocked swabs.

Catherine is also active in monitoring the progression and changes seen with influenza viruses, in particular relating the changes in sensitivity of the viruses to anti-viral agents. She has published in this area and her contributions at national level in the management of resistant H1N1 (2009) virus in an immunocompromised population within Wales was both balanced and insightful, facilitating sensible containment and management of a politically sensitive issue.

Yours sincerely

Consultant Virologist
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16 May 2011

To whom it may concern

Re: Catherine Moore

I hereby confirm that Catherine Moore contributed to the following publications of which I am a co-author with the indicated percentages.

7. Two waves of pandemic influenza A(H1N1)2009 in Wales. The possible impact of media coverage on consultation rates, April-December 2009. *Euro Surveill* 2011;**16(3)**: pii=19772.
Keramarou M, Cottrell S, Evans MRh, Moore C, Stiff RhE, Elliott C, Thomas DRh, Lyons M, Salmon RL

Concept and design of the Investigation	10%
Conduct of research	10%
Analysis of data	20%
Preparation for publication	15%

8. Evidence of person-to-person transmission of oseltamivir resistant pandemic influenza A (H1N1) 2009 virus in a hematology unit. *J Infect Dis* 2011;**203**:18-24.

Catherine Moore, Monica Galiano , Angie Lackenby, Tamer Abdelrahman, Rosemary Barnes, Meirion R Evans, Christopher Fegan, Susannah Froude, Mark Hastings, Steven Knapper , Emma Litt , Nicola Price, Roland Salmon, Mark Temple and Eleri Davies

Concept and design of the Investigation	50%
Conduct of research	50%
Analysis of data	40%
Preparation for publication	70%

Yours faithfully

Dr Meirion Evans
Senior Lecturer in Epidemiology and Public Health
Department of Primary Care and Public Health
Cardiff University

Regional Epidemiologist
Communicable Disease Surveillance Centre
Public Health Wales

Public Health Wales Microbiology Cardiff
University Hospital of Wales
Cardiff

Email: Eleri.Davies4@wales.nhs.uk
May 10th 2011

I hereby confirm that Catherine Moore contributed to the following publications of which I am a co-author with the indicated percentages.

7. Evidence of Person-to-Person Transmission of Oseltamivir Resistant Pandemic Influenza A (H1N1) 2009 Virus in a Hematology Unit. *J Infect Dis* 2011;**203**:18-24. Catherine Moore, Monica Galiano , Angie Lackenby, Tamer Abdelrahman, Rosemary Barnes, Meirion R Evans, Christopher Fegan, Susannah Froude, Mark Hastings, Steven Knapper , Emma Litt , Nicola Price, Roland Salmon, Mark Temple and Eleri Davies

Concept and design of the Investigation	50%
Conduct of research	50%
Analysis of data	40%
Preparation for publication	70%

I can also confirm that during the course of the investigation of the outbreak of Oseltamivir Resistant Pandemic Influenza A (H1N1)2009 virus, Catherine Moore was an essential part of the outbreak team. Her expertise in diagnosis and resistance detection allowed the outbreak control team to systematically work through the incidents of infection that we had, taking appropriate control measures and evaluating the epidemiology.

Catherine Moore contributes high level scientific knowledge in Virology, coupled with hands on experience of outbreak management and is an asset to outbreak control teams at a local level in Cardiff and Vale University Health Board, but also at a National level within Wales.

Yours sincerely,



Dr. Eleri Davies
Consultant Microbiologist and Director Infection Prevention and Control,
Cardiff and Vale University Health Board.
Director of the Healthcare Associated Infection Programme, Public
Health Wales NHS Trust.



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Catherine Moore

PhD by Published Work Statement

Catherine works as a Clinical Scientist in the Molecular Diagnostics/ Specialist Virology Service in Cardiff Microbiology, Public Health Wales. The service provides specialist molecular diagnostics for all of Wales. Catherine heads up the respiratory virus section of the service and is directly responsible for the development of molecular diagnostics for respiratory viruses. This includes horizon scanning, assay development, scientific evaluation, specialist input into the Public Health Wales Respiratory Programme (epidemiology, diagnostics, management, control), and collaboration with other specialist centres in the UK.

During the recent H1N1 (2009) pandemic Catherine was a key member of the Public Health Wales Senior Response Team and played an important role in managing the outbreak of oseltamivir-resistant H1N1 (2009) on the Haematology Unit at the University Hospital of Wales. Catherine was the principle author of the published account of this outbreak.

(Evidence of Person-to-Person Transmission of Oseltamivir Resistant Pandemic Influenza A (H1N1) 2009 Virus in a Hematology Unit (JID in Press) Catherine Moore, Monica Galiano, Angie Lackenby, Tamer Abdelrahman, Rosemary Barnes, Meirion R Evans, Christopher Fegan, Susannah Froude, Mark Hastings, Steven Knapper, Emma Litt, Nicola Price, Roland Salmon, Mark Temple and Elen Davies)

Signed

Dr J G M Hastings MB BS MD, FRCPath

Microbiology Services Lead for Public Health Wales NHS Trust

Laboratory Director and Consultant Microbiologist Public Health Wales Microbiology Cardiff



Appendix 2. Statement Describing the Contribution of the Candidate Towards the Work of the Molecular Diagnostics Unit

May 12th 2011

Catherine Moore's contribution to the Molecular Diagnostics Unit, Cardiff

The establishment of the Molecular Diagnostics Unit in Cardiff depended upon the vision and hard work of a handful of people. Catherine Moore was (and is) one of those who were pivotal in the establishment of a premier molecular diagnostic service for infectious diseases in Wales.

Molecular diagnostics is an area of rapid expansion with a fiercely competitive commercial sector; consequently, a commitment to research and development is crucial to the success of the unit. At its inception in 1999 the Molecular Unit worked very closely with staff of Cardiff University and the College of Medicine as knowledge and skills necessary in the academic research laboratory were also crucial to the diagnostic molecular laboratory. However, the main drive of diagnostics is the reliable and timely delivery of accurate diagnoses and the relentless demands of service provision demand a rather different skill mix from laboratory staff. A challenge faced by every research based diagnostic service is to blend high quality science and innovation with routine service delivery.

It was the great good fortune of the molecular unit in Cardiff that Catherine was already employed as a young biomedical scientist in virology. As a biomedical scientist she had already established a reputation for intelligence, technical skill, reliability and hard work. She was ambitious, interested in taking on the challenge of molecular diagnostics, and showed an aptitude for assay development and scientific experiment which the unit was happy to exploit.

Quite quickly Catherine carved for herself a unique role within the unit as the person with particular responsibility for the development and modification of new assays to fit them for use in the routine laboratory. Perhaps unsurprisingly, because of her background and training, Catherine well understands the requirements of assays fit for use in a diagnostic service and this understanding informed her development work.

More importantly however was her real talent as an experimental scientist. She understands the science behind molecular technology and brought the same qualities of technical skill, reliability and hard work into her new role as research innovator for the molecular diagnostic service.

This was (and undoubtedly still is) a huge benefit to the unit, and was recognized by Catherine's appointment to a Clinical Scientist post dedicated to the development and introduction of new molecular assays.

Catherine started to build a significant scientific portfolio. From the start she was unafraid of difficult projects; her MSc project on drug resistant HIV genotypes in Wales was a major achievement attained with very modest local support. Indeed this project demonstrated another of Catherine's qualities, the ability to work with

and learn from colleagues and fellow scientists in academia, service work and industry.

She started to attend scientific meetings and congresses, no longer just in the audience but as an active participant, presenting and defending her work to a national and international audience.

She became an expert on the use of various molecular formats used in diagnosis and disease monitoring. She understood how far a new method could be developed pre-launch and how to prepare new techniques for routine roll out. She has trained many colleagues both locally and elsewhere in Wales and has been instrumental in ensuring robust assay quality carefully and reliably delivered.

As a named collaborator in several grant-aided research projects she had to design and carry out work of uncertain outcome and to interpret and explain her data to colleagues, she had to train junior scientists and be responsible in part for their work as well as her own. Without Catherine's research input the molecular unit runs the risk of losing its scientific edge and ability to embrace new technologies as they emerge.

Her submitted papers show just a small part of the scientific work she has undertaken during the last decade but they do demonstrate her originality, her technical soundness, her ability to perceive clinical context and to work with colleagues from other fields in demonstrating the "big picture", and of course, her capacity for sheer hard work.

She is a quite exceptional scientist; her participation in the Molecular Diagnostics Unit is one of its major strengths.

A handwritten signature in black ink that reads "Diana Westmoreland." The signature is written in a cursive, flowing style.

Diana Westmoreland MA, MSc, BM, BS, DPhil, FRCPath
Consultant Clinical Virologist
Head, Molecular Diagnostics Unit (1999-2006)

National Public Health Service Wales
Cardiff