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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  $-80^{\circ}\text{C}$ . 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 <sup>a</sup>	5 months	F	B	Bronchiolitis
2	37 years	M	A	URTI, pyrexia, AML, chemotherapy
3 <sup>a</sup>	9 months	M	A	Pyrexia, influenza-like illness
4 <sup>a</sup>	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 <sup>b</sup>	67 years	M	NT	Pyrexia, URTI, AML
8	11 months	M	A	Pneumonia, ICU, ventilated
9 <sup>a</sup>	2 months	F	NT	Bronchiolitis
10 <sup>a</sup>	2 weeks	F	NT	Bronchiolitis
11 <sup>a</sup>	2 months	F	NT	Bronchiolitis
12 <sup>a</sup>	2 months	F	A	Bronchiolitis
13 <sup>a</sup>	4 months	M	NT	Bronchiolitis
14 <sup>a</sup>	3 months	M	B	Bronchiolitis
15 <sup>a</sup>	3 months	F	A	Wheeze, creps, increased O <sub>2</sub> requirement
16 <sup>a</sup>	1 year	M	A	Bronchiolitis, increased O <sub>2</sub> requirement, sister RSV positive
17 <sup>a</sup>	11 months	M	A	Bronchiolitis
18 <sup>a</sup>	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 <sup>a</sup>	1 year	M	A	Bronchiolitis
27 <sup>a</sup>	4 months	M	A	Bronchiolitis
28 <sup>a</sup>	7 months	M	NT	Bronchiolitis, respiratory distress
29 <sup>a</sup>	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 <sup>a</sup>	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 <sup>a</sup>	1 year	M	NT	Bronchiolitis
38 <sup>a</sup>	9 months	F	A	Bronchiolitis
39 <sup>a</sup>	6 months	M	A	Bronchiolitis
40 <sup>a</sup>	2 months	F	A	Bronchiolitis
41 <sup>a</sup>	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

<sup>a</sup>No underlying condition, made full recovery

<sup>b</sup>Separate 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 µl of sample tested, 20 µl of DNase I (1 U/µl) (Promega, Southampton, UK) was added, together with 24 µl 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

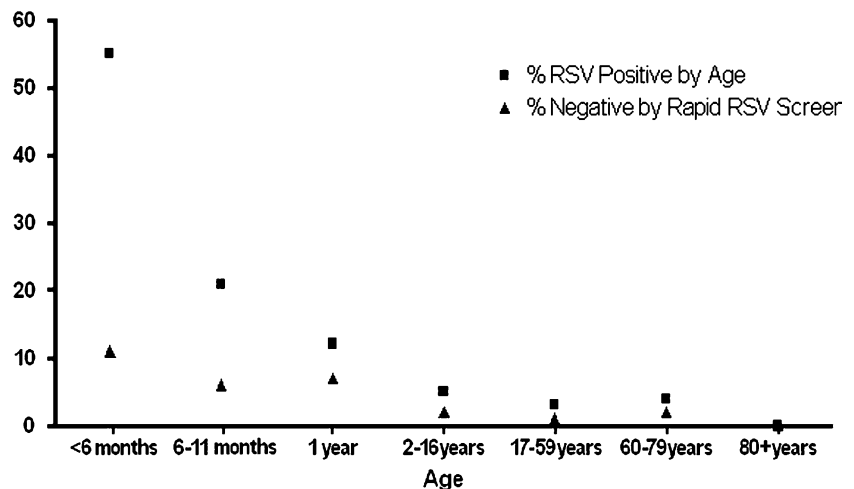
Sensitivity and specificity were calculated using Fisher's exact test and by comparing the results of real-time NASBA with those of the rapid RSV screening and cell culture tests. From these results, the positive and negative predictive values of the real-time NASBA assay were calculated. In addition 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

**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



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

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  $-80^{\circ}\text{C}$  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.

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## 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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