

Development of a highly sensitive semi-quantitative real-time PCR and molecular beacon probe assay for the detection of respiratory syncytial virus

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Abstract

Molecular beacons are a novel class of oligonucleotide probe capable of reporting the accumulation of target amplicon during real-time PCR by the emission of a fluorescent signal. A novel assay for the detection and estimation of respiratory syncytial virus (RSV) nucleic acid in clinical specimens based on real-time PCR utilising such a probe was developed. The probe consisted of two short arm sequences and a central loop sequence complementary to a region of the N gene (the target amplicon). The probe was characterised and a semi-quantitative nested real-time PCR using a LightCycler instrument was optimised. Standard curves were generated using cycle threshold (C_t) values calculated from several assays over a range of logarithmic RSV titres. Linear coefficient correlations were close to one ($r^2 = 0.998$) and the detection limit of the optimised assay was reproducibly shown to be 1×10^{-4} pfu/ml. The intra-assay coefficient of variation (CV) of C_t values of the optimal assay was 0.8% and the CV of quantification data was 6.6%. The interassay CV of C_t values was 2.0% and the quantification CV was 6.7%. The validity of the assay for the detection of RSV in clinical specimens was assessed by analysing ten specimens previously assayed in a different laboratory. Real-time PCR analysis was completely consistent with the results of prior analysis. The rapidity, sensitivity and specificity of the assay should greatly facilitate epidemiological studies, particularly in adults as existing methods perform better on clinical specimens from children.

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1. Introduction

Human respiratory syncytial virus (RSV) is an enveloped, single-stranded, negative-sense RNA *Pneumovirus* that has long been acknowledged as the single most important viral pathogen and the leading cause of severe lower respiratory tract infection in infants and young children throughout the world (Chanock and Parrott, 1965; Selwyn, 1990; Hall, 1997). RSV infection in young children has been extensively studied and was previously regarded as a disease confined to this group. It was rarely considered as an im-

portant pathogen in adults, however, during the last decade RSV has been increasingly recognised as a cause of severe community-acquired lower respiratory tract illness associated with significant morbidity and mortality in certain susceptible adult populations including the institutionalised and community-dwelling elderly, the immunocompromised and those with severe underlying pathology, such as cardiopulmonary disease (Vikerfors et al., 1987; Murry and Dowell, 1997; Falsey and Walsh, 2000). Acute respiratory disease caused by RSV is not, however, restricted to paediatric and specific high-risk adult populations. Acquired immunity to RSV is both partial and transient, and natural re-infection occurs repeatedly throughout life in previously healthy, immunocompetent individuals.

The study of RSV and the diagnosis of acute infection in certain populations has been hindered by the lack of

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availability of rapid and sensitive methods of detection (Falsey and Walsh, 2000). Viral culture and antigen detection are of considerable value in diagnosing primary RSV infection in infants. However, such techniques exhibit greatly reduced sensitivity in adults, even when processing specimens under optimal conditions, due to the transient shedding of significantly lower viral titres in the secretions of the upper respiratory tract of adults, the highly thermolabile nature of RSV and the occurrence of pre-existing nasal antibodies (Falsey and Walsh, 2000; Walsh et al., 2001). Reverse transcription-polymerase chain reaction (RT-PCR) has been described recently for viral RNA detection and the diagnosis of RSV and has been shown to be especially suited to the detection of low viral titres or non-viable viral RNA in respiratory secretions (Walsh et al., 2001). Several studies have found RT-PCR and nested RT-PCR to be more sensitive than culture and antigen detection in infants and adults for the diagnosis of RSV (Van Milaan et al., 1994; Freymuth et al., 1995; Henkel et al., 1997; Gonzalez et al., 2000; Walsh et al., 2001; Falsey et al., 2002).

Real-time PCR couples ultra-rapid thermocycling with fluorescence detection that monitors the accumulation of PCR products in 'real-time' (Wittwer et al., 1997). The process facilitates the accurate quantification of nucleic acids during the exponential phase of amplification, circumventing the difficulties associated with endpoint quantitation at the plateau phase of PCR and eliminating the need for post-amplification analysis by methods such as gel electrophoresis or dot blot analysis (Wittwer et al., 1997).

Molecular beacons are a novel class of single-stranded oligonucleotide probes capable of reporting the presence of specific nucleic acids in homogenous solutions and *in vivo*, and may be used for both endpoint and real-time PCR analysis (Tyagi and Kramer, 1996; Matsuo, 1998; Sokol et al., 1998). These probes possess a stem-and-loop structure; fluorophore and non-fluorescent quencher moieties occupy the 5' and 3' termini of each arm of the stem whilst the central loop portion is a probe sequence that is complementary to a predefined sequence in a target amplicon. In the absence of target nucleic acid, a probe adopts a hairpin structure and the stem maintains the fluorophore and quencher moieties in close proximity to one another, quenching the fluorescence of the fluorophore by fluorescent resonance energy transfer (FRET). On encountering a complementary target molecule, the loop sequence of a probe undergoes complementary binding and forms a probe-target hybrid, forcing the stem to dissociate and inducing the spatial separation of the quencher and fluorophore molecules, and thus leading to the restoration of detectable fluorescence (Tyagi and Kramer, 1996).

Neither the use of molecular beacons in the detection of RSV in respiratory secretions, nor their use in the detection of product formation during real-time PCR, have been reported previously. The following paper describes the development and assessment of such a technique.

2. Methods

2.1. Virus propagation and titration

American type culture collection reference strains RSV A2 (VR-1302), RSV Long (VR-26) and RSV B (VR-1401) viruses were used to optimise reaction conditions. RSV was propagated using standard protocols in HEp-2 cells. The viral titre of multiply-passaged RSV A2-infected cell suspensions was determined using a quantitative shell vial assay, as described previously (Domachowske and Bonville, 1998). Detection of RSV antigens by an indirect immunofluorescence assay was performed and the number of plaque forming units per millilitre (pfu/ml) was determined using fluorescence microscopy.

2.2. RNA extraction, cDNA synthesis and PCR

Viral RNA was extracted from 200 μ l of RSV-infected cell suspensions (infected with RSV A2, RSV Long and RSV B), and from mock-infected cultures, using the RNeasy Mini Kit (QIAGEN) with minor modifications to the manufacturer's protocol. The lysate originating from cell suspensions was homogenised using a QIAshredder column (QIAGEN) in order to reduce viscosity. *In situ* DNase treatment was performed using the RNase-Free DNase Set (QIAGEN) at the end of the extraction procedure. cDNA synthesis was undertaken using Omniscript Reverse Transcriptase (QIAGEN), according to the manufacturer's protocol. Outer and inner primer pairs for PCR amplification of a highly conserved region of the N gene of human RSV A and B isolates were employed (Cane and Pringle, 1991; Singhal et al., 1999), resulting in DNA products of 278 and 126 bp, respectively.

The sequences of the primers were:

Outer primer N1: 5'-GGAACAAGTTGTTGAGGTT-TATGAATATGC-3',

Outer primer N2: 5'-CTTCTGCTGTCAAGTCTAGT-ACACTGTAGT-3',

Inner primer N3: 5'-GTGAAGCAGGATTCTACCAT-3',

Inner primer N4: 5'-CTGTACTCTCCATTATGCC-3'.

The GeneAmp PCR Reagent Kit with AmpliTaq DNA polymerase (Perkin-Elmer) was used for PCR amplification. PCR was performed over 30 amplification cycles using the following parameters: 1 cycle of 95 °C for 2 min; 30 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s; 1 cycle of 72 °C for 10 min.

For conventional nested PCR, 2.5 μ l of first-round PCR product was combined with 47.5 μ l of nested PCR mixture and PCR was performed over 30 amplification cycles using the following parameters: 1 cycle of 95 °C for 2 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; 1 cycle of 72 °C for 10 min.

2.3. Molecular beacon probe design and characterisation

Comparative analysis of the primary nucleotide sequences of the N gene amplicon generated by nested PCR was used for the design of a molecular beacon probe. The sequence of the probe (occupying position 907–932 of the N gene, relative to the translation start site), with fluorophore and quencher moieties, was: Fluorescein-5'-CCAGGGAATG-ATGCTTTTGGGTTGTTCAATATCCCTGG-3'-DABCYL.

The fluorescence emission spectrum of the molecular beacon was measured using a Luminescence Spectrometer LS50B (Perkin-Elmer) at room temperature (25 °C). A fixed excitation wavelength of 491 nm was used and the fluorescence emission was measured at 518 nm. Data were analysed using FL WinLab Software (Perkin-Elmer). All characterisation procedures were performed using PCR buffer conditions, to reflect conditions present during real-time amplification reactions, and in triplicate.

Determination of the signal to background ratio: 100 µl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂) was placed into a 0.1 ml quartz luminescence spectroscopy cell and the fluorescence (F_{buffer}) was measured. Molecular beacon was added to the buffer to a final concentration of 50 nM and the resulting fluorescence (F_{close}) was noted. Subsequently, a 20-fold molar excess of oligonucleotide target was added to the solution and fluorescence (F_{open}) was measured 5 min after the addition of the target. The signal to background ratio was calculated as: $(F_{\text{open}} - F_{\text{buffer}})/(F_{\text{close}} - F_{\text{buffer}})$.

The efficiency of quenching (E_{ff}) of light emitted by fluorescein and absorbed by DABCYL was calculated as: $[E_{\text{ff}} = \{1 - (F_{\text{close}} - F_{\text{buffer}})/(F_{\text{open}} - F_{\text{buffer}})\} \times 100]$.

Assessment of the saturation kinetics of the molecular beacon: oligonucleotide target was added to a buffer solution containing 50 nM molecular beacon at ratios of 1:1, 1:2, 1:4, 1:6, 1:8, 1:10, 1:20 and 1:100 of probe to target. The fluorescence emission of each ratio was measured as previously described. In addition, the fluorescence emission of the molecular beacon was measured over time as follows. F_{buffer} was measured at 60 s intervals over 5 min. After 5 min, 50 nM molecular beacon was added to the solution and F_{close} was measured using the same parameters over 5 min. Finally, a 20-fold molar excess of oligonucleotide target was added to the solution and the F_{open} fluorescence was measured at 60 s intervals over 10 min.

The thermal denaturation profile of the molecular beacon was measured using a LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN, USA). A number of solutions were prepared in LightCycler capillary tubes, each to a final volume of 20 µl. One solution consisted of PCR buffer, another of buffer and molecular beacon at a final concentration of 50 nM, and a third solution contained buffer, 50 nM of molecular beacon and 500 nM of oligonucleotide target. The temperature of each solution was increased from 30 to 95 °C in increments of 5 °C, at a temperature transition rate of 20 °C/s, and incubated at each temperature for

5 s. The fluorescence emission of each solution was determined as a function of temperature by a single fluorescence measurement using detection channel F1 at the end of each incubation. Data were analysed using the LightCycler Software Version 3.3 (Roche Molecular Biochemicals).

Finally, the Zuker DNA folding program was utilised to assess the relationship between the melting temperature of the stem structure and Mg²⁺ concentration. The folding thermodynamics of the molecular beacon at 37 °C, a constant Na⁺ concentration (10 mM) and varying Mg²⁺ concentration (ranging from 0 to 10 mM) were calculated, and the melting temperatures of the stem structure was derived from the free energy of formation of the stem hybrid.

2.4. Molecular beacon semi-quantitative real-time PCR

Detection of an RSV amplicon by a real-time PCR assay employing the molecular beacon was undertaken as follows: 1 µl of primary PCR product derived from RSV A2, RSV Long and RSV B was combined with 19 µl of PCR mix. Probe, at a concentration equal to that of each primer (0.6 µM), was included in the mixture and PCR was performed on a LightCycler over 40 cycles using instrument settings recommended by the manufacturer. PCR product accumulation was monitored during each amplification cycle by a single fluorescence measurement using detection channel F1, made at the end of each annealing step. Data were analysed using LightCycler software. Following amplification, 5 µl of real-time PCR product was removed from the reaction capillaries and analysed by agarose gel electrophoresis to verify product size.

A viral suspension of RSV A2 of known titre was serially diluted 10-fold from 1×10^3 to 1×10^{-5} pfu/ml using viral transport medium (Multi-Microbe Media M4, Micro Test, Lilburn, GA, USA) as diluent. RNA was isolated, cDNA was synthesised, and primary PCR amplifications were performed as described. Either 1 or 5 µl of primary PCR product derived from serially diluted RSV A2, representing 5 and 25% of the final reaction volume (respectively), was combined with either 19 or 15 µl of real-time PCR mixture (respectively) containing the optimised reaction components. Real-time PCR was carried out using the standardised reaction conditions on a LightCycler instrument. Reactions were carried out in triplicate (on separate occasions) and data were analysed using LightCycler software. The lowest viral titre at which RSV was detectable was assigned the detection limit.

Quantification data and cycle threshold (C_t) values, indicative of the quantity of target, were calculated using the second derivative maximum method. This software algorithm identifies the first turning point of the fluorescence curve, corresponding to the first maximum of the second derivative curve (these calculations are automatic and remove subjective operator bias from analysis). These data were used to generate standard reference curves for the

semi-quantitative quantification of RSV titre in clinical specimens by interpolation of sample C_t values.

The variation of both C_t values and quantification data within and between experiments was estimated by calculating the coefficient of variation (CV), the ratio between the standard deviation and the mean of replicate measurements. The significance of the variation was assessed by the F -test.

3. Results

3.1. Molecular beacon probe design

Based upon sequences of the N gene of human RSV generated from a search of GenBank, a generic molecular beacon probe was designed to anneal to a highly conserved region at the 5' end of the sense strand of an amplicon derived from either human RSV A or B. The beacon probe was 38 bp in length and consisted of a central 26 bp loop sequence, complementary to the target amplicon, flanked either side by two complementary 6 bp arm sequences (Fig. 1). The fluorophore, fluorescein, and universal quencher, DABCYL, were coupled to the 5' and 3' ends of the beacon probe, respectively. These two molecules were chosen since DABCYL, the acceptor moiety, possesses an absorption maximum of 491 nm, which overlaps with the emission spectra of excited-state fluorescein (emission maximum 525 nm), the donor. This ensures that fluorescence of the molecular beacon is quenched by FRET when the molecular beacon adopts a closed conformation in the absence of target amplicon. A target loop of 26 bp was designed to tolerate one mismatched base variable nucleotide identified within the amplicon. Sequence complementarity between the beacon probe and inner primers of the nested PCR revealed that no primer–probe interactions existed.

The melting temperature of the probe–target hybrid, without the adjacent arm sequences at the 5' and 3' ends of the

probe, was predicted to be 52.0 °C. This temperature, derived from the percent GC rule, is typically 7–10 °C higher than the PCR annealing temperature (40 °C in this case), allowing the probe to hybridise to target during primer annealing.

The melting temperature of the stem structure was assessed in order to ensure that the probe would remain in a closed conformation in the absence of target. Since the stem structure forms by an intramolecular hybridisation event, the stem melting temperature may not be predicted from the percent GC rule. Thus, the Zuker DNA folding program was used to predict the folding thermodynamics and to assess the free energy of formation of the stem hybrid, from which the melting temperature of the stem structure was derived to be 61.5 °C. In addition, this program was used to predict the closed conformation of the molecular beacon probe in the absence of target, ensuring that a single hairpin structure existed and possessed the correct spatial orientation of the fluorophore and the quencher moieties.

3.2. Molecular beacon probe characterisation

3.2.1. Signal to background ratio and saturation kinetics

Using a fixed excitation wavelength of 491 nm and measuring the fluorescence emission at 518 nm, the signal to background ratio was calculated to be 21.4:1 following hybridisation of a 20-fold molar excess of oligonucleotide target to probe over 5 min at room temperature (Fig. 2). The efficiency of quenching of light emitted from fluorescein by DABCYL under these conditions was 95.3%. Calculations used data derived from experiments performed in triplicate (data not shown). Assessment of the fluorescent emission spectra of the molecular beacon over eight oligonucleotide target concentrations (50 nM to 5 mM) indicated that a 20-fold molar excess of target (1 mM) was sufficient to hybridise to almost all of the probe at a concentration

Fluorescein-5'-CCAGGGAATGATGCTTTTGGGTTGTTCAATATCCCTGG-3'-DABCYL

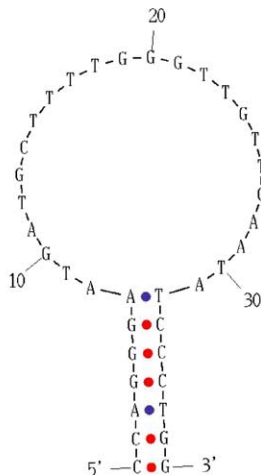


Fig. 1. Molecular beacon probe. Primary nucleotide sequence of the molecular beacon including fluorophore and quencher moieties positioned at the 5' and 3' termini, respectively (underlining indicates complementary stem sequences), and schematic representation of its predicted closed conformation.

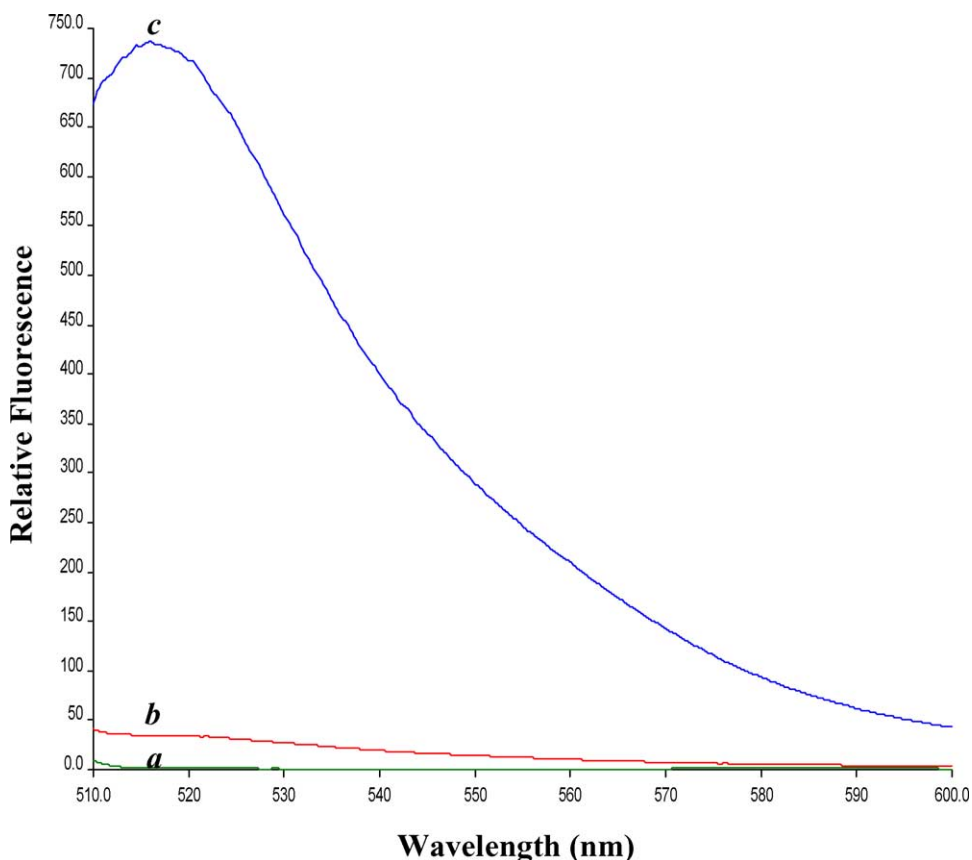


Fig. 2. Representative trace of relative fluorescence intensity vs. emission wavelength of buffer (a), buffer and probe (b), and buffer, probe and a 20-fold molar excess of oligonucleotide target (c). Excitation wavelength = 491 nm; emission wavelength = 518 nm; fluorescence measured over 5 min. Very little background fluorescence is emitted by buffer or probe alone (a and b). Only beacon probe in the presence of oligonucleotide target results in the emission of significant fluorescence (c).

of 50 nM (>99% probe saturation, data not shown). Analysis of emission spectra of the molecular beacon over time demonstrated typical first-order saturation kinetics. Hybridisation periods of 30 s, 1 and 5 min following the addition of a 20-fold molar excess of target to probe resulted in >50, >85 and >99% of maximal fluorescence emission, respectively (data not shown).

3.2.2. Thermal denaturation profile

The probe-target hybrids were found to begin to denature at 55 °C, were 50% denatured at 65 °C and 100% denatured at 90 °C (Fig. 3). The stem of the molecular beacon was denatured in 50% of the probes at 60 °C and in all probes at 80 °C. Between the range of 30–45 °C, the differential between the spontaneous fluorescence of the free molecular beacon (the closed form) and the fluorescence emission of the probe hybridised to target (the open form) is maximal, indicating that the probe is most effective at these temperatures.

3.2.3. Stem structure stability

As determined by the Zuker DNA folding program, an increasing concentration of Mg^{2+} resulted in a more neg-

ative free energy of formation of the stem and a concomitant increase in stem hybrid melting temperature, indicating greater stability of the stem and a theoretical reduction in spontaneous fluorescence of free molecular beacon (data not shown). Both the T_m and ΔG of the stem attained a plateau at 5.0 mM Mg^{2+} , indicating that such a concentration is sufficient to maintain stem stability.

3.3. RSV detection and assay optimisation

The real-time detection of amplicons derived from RSV A2, Long and B reference strains by the molecular beacon during nested PCR was achieved and subsequent analysis of the real-time PCR products by agarose gel electrophoresis established that the amplicons were of the correct size of 126 bp (data not shown). Following the reproducible detection of RSV employing standard reaction parameters, the optimisation of the kinetics of fluorescent signal accumulation was achieved by varying reaction components and conditions. The probe annealing temperature was varied between 35 and 55 °C (5 °C increments) and the final concentrations of molecular beacon and $MgCl_2$ were varied between 0.2 and 1.0 μM , and between 1.0 and 5.0 mM, respectively.

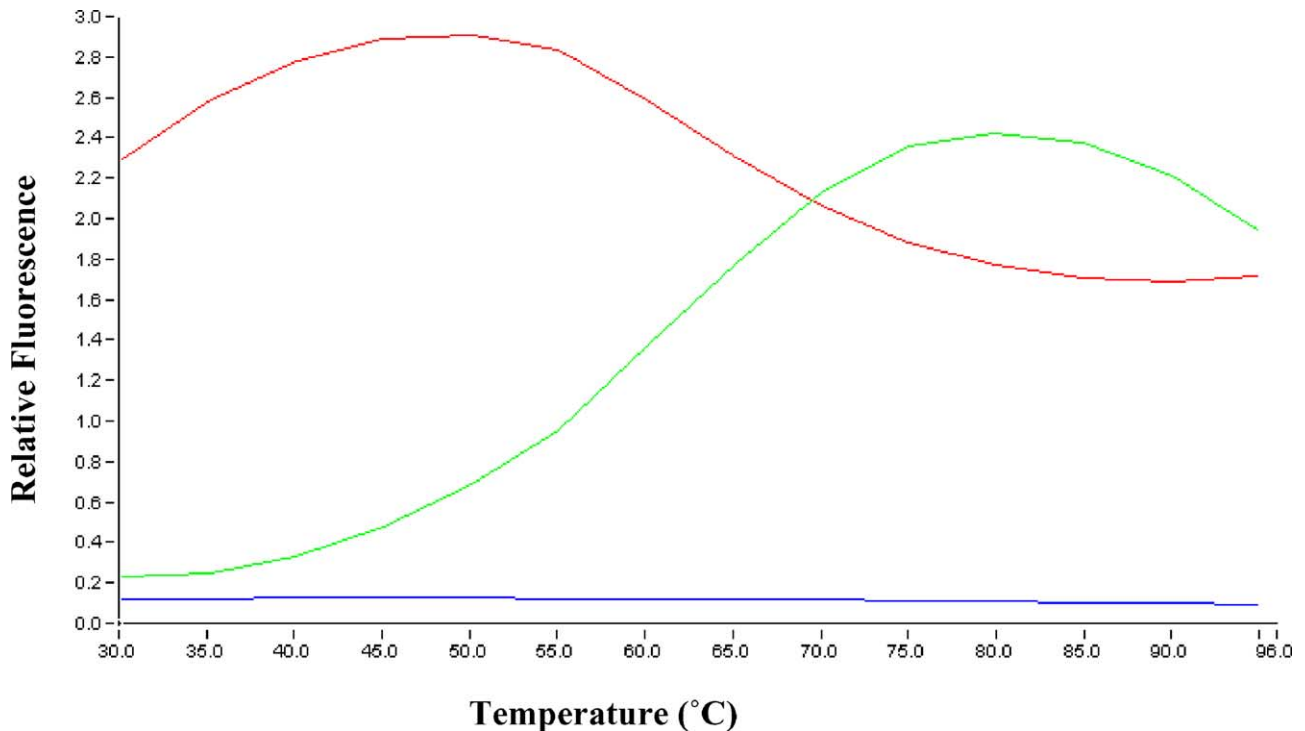


Fig. 3. Thermal denaturation profile of buffer (blue trace), free molecular beacon (green trace), and probe-target hybrid (red trace). Hybrids begin to denature at 55°C, are 50% denatured at 65°C and 100% denatured at 90°C. The stem of the molecular beacon is denatured in 50% of the probes at 60°C and in all probes at 80°C.

It was determined that signal accumulation was approximately proportional to the concentration of molecular beacon over 0–0.6 μM , and reached saturation at 0.8 μM . Due to the negligible increase in fluorescence produced by probe of 0.8–1.0 μM , it was concluded that molecular beacon at a final concentration of 0.6 μM was sufficient to produce satisfactory accumulation of fluorescent signal. A final MgCl_2 concentration of 5.0 mM and an annealing temperature of 40°C were shown to be optimal for signal accumulation, in concordance with values determined previously by theoretical analysis. Thus, optimised reaction mixture consisted of (final concentrations given): 1 \times PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl); dNTP mix (200 μM each); MgCl_2 (5.0 mM); primers N3 and N4 (0.6 μM each); beacon probe (0.6 μM); DNA polymerase (1.0 U); first-round PCR product (5 or 25%); sterile distilled water to a final volume of 20 μl .

3.4. Determination of assay sensitivity and generation of standard curves

The molecular beacon described here corresponded to a highly conserved region of the N gene of human RSV, and was thus capable of detecting all variants of the virus. Following the reproducible detection of amplicons derived from all of the independently prepared RSV reference strains (including two A and one B isolate) using the molecular beacon, a titrated viral stock of RSV A2 was employed for subsequent experiments. First-round PCR products de-

rived from a range of serially diluted RSV A2 of known viral titre (1×10^3 to 1×10^{-5} pfu/ml) were amplified by molecular beacon real-time nested PCR to determine the detection limits of the assay and to establish standard reference curves for the semi-quantitative quantification of RSV RNA titre (the samples would contain both virion RNA and mRNA). A typical amplification plot of relative fluorescence versus cycle number is shown in Fig. 4. PCR amplification plots demonstrated characteristic exponential fluorescence accumulation (reflecting product formation) over time, and a 10-fold increase in viral titre produced a mean increase in C_t value of 3.64 (range = 2.70–4.63). The detection limits of the assay using 1 and 5 μl primary PCR product were consistently shown to be 1×10^{-2} pfu/ml (mean $C_t = 26.83 \pm 0.05$) and 1×10^{-4} pfu/ml (mean $C_t = 32.83 \pm 0.76$), respectively (Table 1).

Standard curves, generated from the mean data of experiments carried out in triplicate on separate occasions, demonstrated a strong linear relationship between C_t values across several logarithmic titres of RSV. Linear coefficient correlations were shown reproducibly to be close to one for both 1 and 5 μl assays, ($r^2 = 0.997$ and 0.998, respectively, Fig. 5). In addition to increasing the sensitivity of the assay, a five-fold increase in the volume of primary PCR product resulted in the earlier detection of nested PCR products. Mean C_t values generated from assays using 5 μl were 2.49 cycle numbers lower (range = 2.17–2.71) than those of 1 μl assays, displacing the standard curve derived from the former to the left.

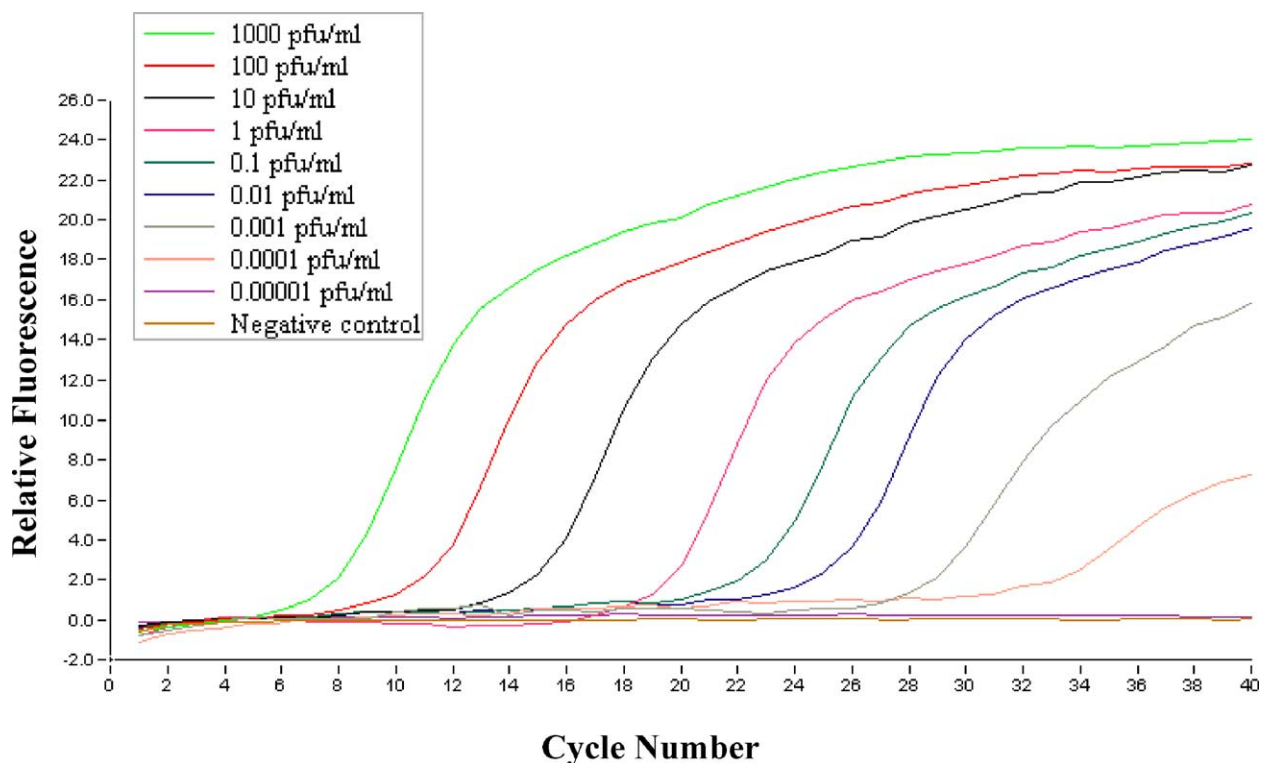


Fig. 4. Typical amplification plot of molecular beacon real-time nested PCR of the N gene of RSV A2. Reactions were performed over 40 cycles using 5 μ l primary PCR product over 9 log viral titres (1×10^3 to 1×10^{-5} pfu/ml).

The limit of detection of molecular beacon real-time PCR for RSV-derived amplicons was compared with the sensitivity of qualitative nested PCR. 2.5 μ l or 12.5 μ l of first-round PCR product derived from each of the standard RSV titres was combined with 47.5 μ l or 37.5 μ l of conventional PCR mixture, respectively (representing 5 and 25% of the final reaction volume, respectively) and nested PCR and product analysis subsequently performed. The detection limits of qualitative nested PCR for amplicons derived from the 5 and 25% of primary PCR product assays were 1×10^{-1} and 1×10^{-2} pfu/ml, respectively, 10- to 100-fold less sensitive than the corresponding real-time assays (data not shown).

Thus, the assays employing 5 μ l of primary PCR product in molecular beacon real-time nested PCR were more sensitive and resulted in both speedier and better defined

amplicon detection than 1 μ l assays and conventional PCR.

3.5. Accuracy, repeatability and reproducibility

The correlation between actual viral RNA (which would include both genomic and mRNA) and RSV titre calculated from cycle threshold values demonstrated a high degree of predictive accuracy in both 1 and 5 μ l assays. The accuracy error (the difference between mean experimental and actual viral RNA load, reported as a function of the latter) was calculated for each titre (data not shown). The pattern of accuracy error over the range of RSV titres was similar in both assays. The mean accuracy error for the 1 μ l assay was 2% (range = -25 to 17%) whilst for the 5 μ l assay

Table 1

Mean cycle threshold data (C_t) and semi-quantitative quantification of viral load (pfu/ml) of 1 and 5 μ l primary PCR product assays over 6 and 8 RSV titres (respectively)

RSV titre (pfu/ml)	1 μ l Assay		5 μ l Assay	
	Mean C_t (S.D.)	Mean pfu/ml (S.D.)	Mean C_t (S.D.)	Mean pfu/ml (S.D.)
1×10^3	9.20 (0.14)	9.9×10^2 (10.5)	6.60 (0.28)	9.4×10^2 (32.4)
1×10^2	12.38 (0.20)	1.2×10^2 (6.8)	9.86 (0.25)	1.2×10^2 (0.5)
1×10^1	16.29 (0.21)	1.4×10^1 (0.6)	13.59 (0.30)	1.1×10^1 (1.0)
1.0	20.33 (0.20)	0.8 (4.3×10^{-2})	17.94 (0.25)	1.0 (0.1)
1×10^{-1}	23.75 (0.20)	9.9×10^{-2} (1.5×10^{-2})	21.58 (0.25)	1.3×10^{-1} (2×10^{-2})
1×10^{-2}	26.83 (0.05)	1.2×10^{-2} (1.1×10^{-3})	24.28 (0.36)	1.3×10^{-2} (1.5×10^{-4})
1×10^{-3}			28.20 (0.11)	1.5×10^{-3} (9.8×10^{-5})
1×10^{-4}			32.83 (0.76)	1.6×10^{-4} (5.6×10^{-6})

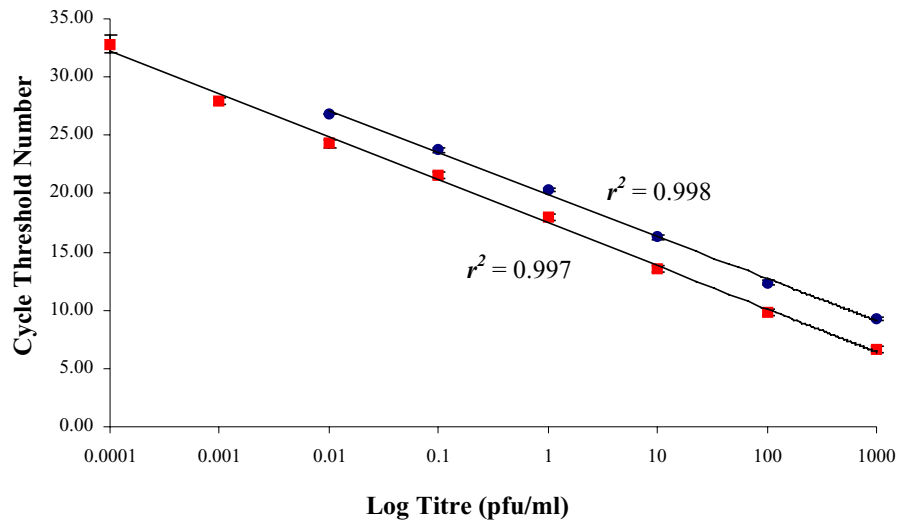


Fig. 5. Standard curves for the semi-quantitative quantification of RSV viral load using 1 μ l (●) and 5 μ l (■) primary PCR product. Plots are cycle threshold number (C_t) vs. log viral titre (pfu/ml) generated from mean data of experiments performed in triplicate on separate occasions.

it was 25% (range = -6 to 56%). There was no significant difference in error of accuracy over the range of viral titres between the assays ($P = 0.39$ by covariance analysis). The variation of C_t values and quantification data, both within and between experiments, was assessed in order to determine the repeatability and reproducibility of the real-time PCR assay. Intra-assay and interassay variation was determined by performing real-time PCR in triplicate on a single occasion and under identical conditions on three or more different occasions, respectively. The mean intra-assay coefficients of variation (CVs), indicating variability within a single experiment, were highly comparable for both 1 and 5 μ l primary amplicon reactions. The CVs of C_t values for each of these assays were 0.6% (range = 0.2–1.0%) and 0.8% (range = 0.2–3.0%), respectively, whilst mean CVs of quantification data were 7.0% (range = 2.8–14.1%) and 6.6% (range = 2.0–17.6%) for 1 and 5 μ l assays, respectively. No significant difference between the CVs of either cycle threshold ($F = 1.3$ and $P = 0.35$) or quantification values ($F = 1.1$ and $P = 0.44$) between 1 and 5 μ l assays within a single experiment was noted.

The mean interassay CVs (indicating the variability between several experiments) of C_t values of 1 and 5 μ l primary PCR product assays were 1.1% (range = 0.2–1.6%) and 2.0% (range = 1.1–4.2%), respectively, whilst mean quantification CVs were 7.4% (range = 1.1–15.5%) and 6.7% (range = 0.4–16.1%) for 1 and 5 μ l assays, respectively. There was no significant difference between the CVs of cycle threshold ($F = 1.8$ and $P = 0.23$) or quantification values ($F = 1.1$ and $P = 0.44$) between 1 and 5 μ l assays performed on different occasions.

3.6. Application to clinical specimens

Verification of the validity of molecular beacon semi-quantitative real-time PCR for the detection of RSV in

clinical specimens was performed by analysing specimens assayed previously in a different laboratory (generously donated by Dr. D. Erdman of the Centers for Disease Control and Prevention, Atlanta, GA, USA). Ten clinical specimens originating from infants presenting with symptoms of acute respiratory illness during the previous RSV season were tested using the assay and the results of prior analysis remained undisclosed until analysis with molecular beacon real-time PCR was complete. The specimens consisted of RNA isolated from 200 μ l of nasopharyngeal swab material using the Nuclisens kit (Organon Teknica, Fresnel, France). cDNA was synthesized and primary PCR amplifications were performed as described earlier. 5 μ l of primary PCR product derived from each sample was combined with 15 μ l of real-time PCR mixture containing optimised reaction components and real-time PCR was performed in triplicate (on different occasions) using a LightCycler instrument. Data were analysed using LightCycler software and C_t values were calculated automatically using the second derivative maximum method. Semi-quantitative estimation of RSV RNA levels in positive samples was calculated using the 5 μ l standard reference curves generated previously and interpolation of specimen C_t values. Real-time PCR analysis determined that eight of the ten samples were positive for RSV, whilst the final two samples were negative for the virus, consistent with the results of prior analysis. Mean C_t values ranged from 14.38 to 31.04 and the mean viral RNA load of positive samples was 0.91 pfu/ml (range = 2.0×10^{-4} to 7.0 pfu/ml). A correlation between early C_t values and samples containing a high viral load was noted.

4. Discussion

The early diagnosis of acute RSV infection has been hindered by the lack of rapid and highly sensitive diagnostic

methods, hampering early definitive therapy and the study of RSV infection, particularly in adults (Falsey and Walsh, 2000). Viral culture and antigen detection demonstrate reduced sensitivity for diagnosing RSV infection in adults, due primarily to the low viral titres shed in the respiratory secretions of adults over short durations and compounded by the extreme lability of the virus. While serology demonstrates greater sensitivity, this method is limited to the retrospective diagnosis of RSV infection and use in epidemiological studies.

The detection of viral RNA by RT-PCR offers several advantages over other diagnostic tools. The exquisite sensitivity of RT-PCR is especially suited to the detection of low viral titres or non-viable viral RNA in respiratory secretions (Walsh et al., 2001). In addition, RT-PCR may be completed within 1–2 days, providing a diagnosis more rapidly than conventional methods.

However, the detection limits of RT-PCR procedures described currently could be improved further, affording the possibility of identifying RSV infection in patient groups in which low viral titres are found and in environments in which optimal conditions of sample collection, storage and transportation are not available. In addition, even faster results should be achievable using real-time PCR, making same-day diagnosis possible and facilitating the rapid screening of large numbers of clinical specimens in epidemiological studies. Real-time PCR would also allow the quantification of viral RNA load, with potential applications in the assessment of disease severity, the study of the immunopathology of RSV infection and prognostic indicators of different treatment regimens, especially relevant to paediatric and immunocompromised patients.

Nested PCR is up to 100-fold more sensitive than standard single primer pair PCR and therefore an assay employing outer and inner primers targeted against a region of the highly conserved N gene of human RSV was used. The mRNA for the N gene is highly abundant in infected cells. An optimised method for viral RNA extraction was devised and optimal reaction components and conditions for the PCR were determined. Assays employing 25% primary PCR product were found to be up to 100-fold more sensitive than those using 5% primary product. In addition, no significant differences in the accuracy, repeatability or reproducibility between the assays were noted and therefore the former was used in the analysis of clinical specimens. Under optimal conditions, the detection limit of standard qualitative nested PCR using 25% primary PCR product followed by amplification analysis by agarose gel electrophoresis was found to be equivalent to 0.01 pfu/ml of tissue culture-passaged virus.

The recent advent of real-time quantitative PCR has proven useful in various applications, including pathogen detection, gene expression and regulation, and allelic discrimination (Takeuchi et al., 1999; Das et al., 2000; Fujii et al., 2000). Fluorescence detection monitors the accumulation of PCR products in 'real-time' during the exponential phase of amplification, allowing the rapid detection and

quantitative assessment of specific targets using external reference curves. High degrees of reproducibility and accuracy with extreme sensitivity over extended detection ranges have been demonstrated, and the requirement for post-amplification analysis is eliminated, thus avoiding potential cross-contamination, reducing the labour-intensive nature of the procedure and increasing the speed of the assay.

Several research groups have described recently real-time PCR techniques using hybridisation probes for the detection of RSV in clinical samples (Whiley et al., 2002; Borg et al., 2003; Gueudin et al., 2003; Van Elden et al., 2003). Molecular beacons afford several advantages over other methods of amplicon detection using fluorescence during real-time PCR. The presence of the loop sequence conveys an additional level of specificity not available to intercalating dyes such as SYBR Green I and ethidium bromide which bind non-specifically to dsDNA (Tyagi and Kramer, 1996; Cayouette et al., 2000). The hairpin structure formed by molecular beacons also provides advantages over linear hybridisation probes, such as FRET probes and those employed in the 5'-nuclease TaqMan assay (Tyagi and Kramer, 1996; Nazarenko et al., 1997). The fluorescence of non-hybridised molecular beacons is quenched with greater efficiency than the fluorescence of uncleaved non-hybridised linear probes, thus the background emission of molecular beacons is significantly less than that of such linear probes. Also, molecular beacons allow more versatile thermal cycling parameters than are available to TaqMan and FRET probes (Cayouette et al., 2000).

The assay described above was considered semi-quantitative as it was a nested PCR in which final amplicons, and thus quantification data, were derived from products synthesised during a preceding PCR. Another issue pertaining to the validity of the quantitation of RSV RNA relates to the methods of preparation of nucleic acid and the acquisition of clinical material. The method of nucleic acid extraction described in this study resulted in the isolation of total viral RNA (both virion RNA and mRNA). The strategy of detecting both cell-associated and virion nucleic acid was employed in an attempt to increase the limit of sensitivity of the assay. In addition, the use of nasopharyngeal swabs for the isolation of virus would introduce an element of variation between specimens in terms of the quantity of viral nucleic acid obtained. Thus, this method may be considered as a semi-quantitative estimation of RSV viral nucleic acid in clinical specimens that can provide some comparison of viral loads between samples.

A very strong inverse association between cycle threshold number and viral load was noted and standard reference curves demonstrated linear correlation coefficients of ~ 1.00 over several logarithmic titres of RSV. The detection limit of the assay was up to the equivalent of 1×10^{-4} pfu/ml of tissue culture-passaged virus, significantly more sensitive than standard qualitative nested PCR and other currently described methods. Furthermore, the assay demonstrated high

degrees of accuracy, repeatability and reproducibility. Mean intra- and interassay coefficients of variation of quantification data were 6.6 and 6.7%, respectively, indicating the minimum error associated with the assay. Finally, the assay developed was rapid, with the analysis of 30 specimens, from RNA extraction to the completion of real-time nested PCR, being completed within 6 h. The specificity and extraordinary sensitivity of the assay over conventional qualitative PCR is directly attributable to the use of a molecular beacon probe and its robustness was further illustrated when applied to the analysis of clinical samples previously tested for the presence of RSV in a different laboratory, producing concordant results.

The development and assessment of a highly sensitive molecular beacon real-time semi-quantitative nested PCR assay for the detection of RSV viral RNA described in this paper will have applications in the detection of RSV RNA particularly in samples where the amounts of virus are low, and in the estimation of viral load in clinical samples.

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