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A novel multiplex real-time RT-PCR assay with FRET hybridization probes for the detection and quantitation of 13 respiratory viruses

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Abstract

Quantitative multiplex real-time RT-PCR assays utilizing fluorescence resonance energy transfer (FRET) hybridization probes were developed for the detection of 13 respiratory viruses, including well recognized viral causes (respiratory syncytial virus, influenza viruses A and B, parainfluenza viruses types 1, 2, and 3, adenovirus) as well as viruses described recently as causes of acute respiratory tract infections (human coronaviruses NL63, HKU1, 229E, and OC43, human bocavirus, and human metapneumovirus). FRET probes have an improved toleration for single base mismatches than other probe chemistries, reducing the chances of missing highly variable RNA viruses. The assay could detect 2.5–25 DNA/RNA copies/ml (2.5 × 10^1–2.5 × 10^4 copies/ml). Validation on 91 known positive respiratory specimens indicated similar specificity as commercial direct immunofluorescence assays (IFA) or single-round PCRs used in initial identification. Screening of 270 IFA negative respiratory specimens identified new viruses in 40/270 (14.8%) cases and additional 79/270 (29.3%) well recognized viruses missed by routine diagnostic assays including 6.7% co-infections. All viruses could be detected in the clinical screening panel. The assays demonstrate an improved sensitivity and scope of detecting respiratory viruses relative to routine antigen detection assays while the quantitative utility may facilitate investigation of the role of co-infections and viral load in respiratory virus pathogenesis.

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1. Introduction

Acute respiratory tract infection is a leading cause of paediatric morbidity and mortality (Murray et al., 2001). Well recognized viral agents traditionally associated with respiratory tract infections are respiratory syncytial virus (RSV), influenza viruses A and B, parainfluenza viruses (PIV) 1–3, and adenoviruses (Adv). Since 2001, improved virus discovery techniques have seen the identification of several new viruses associated with acute respiratory tract infections including human metapneumovirus (hMPV) (van den Hoogen et al., 2001), human coronaviruses (hCoV) HKU1 (Woo et al., 2005) and NL63 (van der Hoek et al., 2004), and human bocavirus (hBoV) (Allander et al., 2005). At present, the most commonly used technique for the diagnosis of viral respiratory tract infections is cell culture usually in combination with direct immunofluorescence assays (IFA) (Fischbach and Dunning, 2009). Nucleic acid detection methods have recently become more readily available for the diagnosis of virus infections (Watzinger et al., 2006). Comparative studies have shown that for the detection of respiratory viruses real-time RT-PCR is significantly more sensitive than conventional detection methods (Gharabaghi et al., 2008; Kuypers et al., 2006; Smith et al., 2003; van de Pol et al., 2007). The majority of real-time RT-PCR detection assays for respiratory viruses are only qualitative of nature. Even though qualitative real-time PCR in the diagnostic setting has numerous advantages over more traditional detection methods, one of the limitations is that it does not discriminate between current infections or shedding of low concentration of virus from previous infections. The significance of co-infections is also not portrayed. In contrast, quantitative real-time PCR provides qualitative as well as quantitative information. The advantages of quantitative real-time PCR are that it permits the assessment of viral load at a given time point, facilitates monitoring of response to treatment, and offers the possibility to determine the dynamics of virus proliferation (Watzinger et al., 2006). Quantitative tests may aid in the study of virus pathogenesis, development of vaccine and antiviral strategies, and will facilitate investigations of the importance of respiratory viruses when detected by themselves or as co-infections in patients.

Qualitative detection of viral respiratory pathogens by multiplex real-time PCR has been described previously (Brittain-Long et al., 2008; Gunson et al., 2005; Templeton et al., 2004). These studies employ the use of hybridisation probes (Brittain-Long et al., 2008; Gunson et al., 2005) and molecular beacons (Templeton et al., 2004).
as probe chemistry which are highly sensitive to hybridization mismatches. Due to the high mutation frequencies of respiratory viral genomes this may result in false negative results (Gunson et al., 2006). In contrast, fluorescence resonance energy transfer (FRET) hybridization probes can tolerate up to 6 mismatches (Zaayman et al., 2009). In addition, the probes are not hydrolyzed during the amplification reaction, thereby facilitating generation of amplicon-specific melting curves for further discrimination and incorporation of multiple targets per reaction.

In this study, the development and application of a novel quantitative real-time RT-PCR assay utilizing FRET probes for the differential diagnosis of well recognized and new respiratory viruses are described. This consisted of four multiplex reactions covering 13 different viruses that may be run individually or concurrently.

2. Materials and methods

2.1. Viruses and clinical specimens

Tissue culture positive controls were available for RSV sub-type A and B, PIV 1–3, influenza A and B, and adenovirus. Clinical specimens that tested positive by PCR for hMPV, hCoV-NL63 and hCoV-OC43 were obtained from Dr. Jan Kimpen, University of Utrecht, The Netherlands, and hBoV from Dr. Heidi Smuts, University of Cape Town, South Africa, while RNA from positive clinical specimens for hCoV-HKU1 and hCoV-229E were obtained from Dr. Astrid Vabret, University Hospital of Caen, France. For the validation of the multiplex assays, 91 respiratory specimens that previously tested positive for each virus were also collected. In addition, respiratory specimens (n = 270) that tested negative for respiratory viruses by direct immunofluorescence with the Light Diagnostics™ Respiratory Panel 1 Viral Screening and Identification Kit for RSV A and B, PIV 1, 2, and 3, Influenza A and B, and Adenovirus (Millipore, USA) or the rapid BD Directigen™ RSV test (BD, Franklin Lakes, NJ, USA) were collected over 1 year by the Department of Medical Virology, University of Pretoria/National Health Laboratory Services Tshwane Academic Division. Specimens included nasopharyngeal aspirates, bronchoalveolar lavage specimens, endotracheal aspirates, and sputum specimens.

2.2. Processing of specimens

Upon receipt phosphate buffered saline (PBS) was added to all nasopharyngeal aspirates to a final volume of 1 ml before vortexing the specimen and clarifying it of cells and mucus at 3000 × g for 5 min. Supernatants were removed and stored at −70 °C for PCR analysis.

2.3. RNA extraction

Nucleic acids from cultured virus controls were extracted from 200 µl cell culture fluid using the High Pure PCR Template Kit (Roche, Mannheim, Germany), according to manufacturer’s instructions. Total nucleic acids from clinical specimens were extracted from 200 µl clarified nasopharyngeal aspirate with the automated MagNA Pure Nucleic Acid Isolation System (Roche, Mannheim, Germany), using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany), according to the manufacturer’s instructions. Negative water controls were included in every run.

2.4. Primer and probe design

For each virus, multiple sequence alignments of all known strains identified by BLAST search analysis were performed in ClustalX (version 1.81) (Thompson et al., 1997), and conserved regions identified for primer and probe design. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) to have melting temperatures of ~60 °C. FRET probes were designed with the support of TIBMOL Biological (Berlin, Germany) and were evaluated using the Light Cycler Probe Design Software 2.0 (version 1.0) on site. The layout of the multiplex assays including primer and probe sequences is indicated in Table 1. Serving as an internal control and reference gene in each multiplex reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was targeted with a separate primer–probe set and individually detected at wavelength of 705 nm.

2.5. Real-time RT-PCR

cDNA was synthesized using Expand Reverse Transcriptase (Roche, Mannheim, Germany), according to the manufacturer’s instructions. Real-time PCR reactions were performed using the Light Cycler FastStart DNA MasterPLUS HybProbe Kit (Roche, Mannheim, Germany) and contained 4 µl of Master Mix, 100 nM of each probe, variable concentrations of primers (indicated in Table 1), and 5 µl of cDNA reaction mixture as template in a final volume of 20 µl. Real-time PCR cycling was performed on a Light Cycler 2.0 (Roche, Mannheim, Germany) as follows: 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 58 °C for 8 s, 54 °C for 8 s, 50 °C for 2 s (ramp rate 5 °C/s), and 72 °C for 11 s, followed by melting curve analysis at 95 °C for 10 s, 60 °C for 30 s and 80 °C for 0 s (ramp rate 0.1 °C/s).

2.6. DNA sequencing

PCR amplicons for DNA sequencing were gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), according to the manufacturer’s instructions. DNA sequencing was performed with specific primers using the ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit (version 3.1) on an ABI PRISM 3130 DNA sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions.

2.7. Quantitation of viral loads

2.7.1. RNA standards for quantitation

Target regions for each respective virus and the internal control (GAPDH) were amplified with the Expand High Fidelity PCR Kit (Roche, Mannheim, Germany) using specific primers. Amplicons were gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and cloned into the pJET1.2/blunt cloning vector (Fermentas, Ontario, Canada) according to the manufacturer’s instructions.

2.7.2. In vitro transcripion

In vitro transcription was performed with the TranscriptAid™ T7 High Yield Transcription Kit (Fermentas, Ontario, Canada), according to the manufacturer’s instructions. Plasmid DNA was removed by addition of 2U of DNase I at the end of the reaction and incubated at 22 °C for 8 h. RNA was purified with the RNeasy Plus Mini Kit (Qiagen, Hamburg, Germany) and the concentration determined with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 nm.

2.8. Analytical sensitivity of multiplex assays

The detection limits of the multiplex assays were determined by testing 10-fold serial dilutions of the quantified RNA transcripts. Detection of each dilution was performed in triplicate. The detection limit was defined as the lowest concentration where all three
Table 1

Design of the multiplex real-time RT-PCR multiplex assays indicating the viruses covered by each of the four multiplex reactions as well as the genes that the primer and probes target and the wavelength where they can be detected.

| Multiplex Target | Primer | Sequence (5'-3') [concentration in nM] | Gene | Authors (Ref.) |
|------------------|--------|----------------------------------------|------|----------------|
| 1 | Respiratory syncytial virus | Sense primer | ATG GCT CTT AGC AAA GTC AA [800 nM] | Nucleoprotein | This study |
| | Antisense primer 1 | TTT TCG ACA TCA TTA TAA GTA G [400 nM] | | Gunson et al. (2005) |
| | Donor probe | AAT GTA ACA TGT TCA ACC AAC AAA TAC AC-FL | | This study |
| | Acceptor probe 1 | LCRed610-TGC TAA CCA GCA TAC GAG ATG ATA TGG PH | | This study |
| | Donor probe | LCRed610-TGC TAA CCA GCA TAC GAG ATG ATA TGG PH | | This study |
| | Acceptor probe 2 | LCRed610-TGC TAA CCA GCA TAC GAG ATG ATA TGG PH | | This study |
| Human metapneumovirus | Sense primer | CAA TGT TTT ACT ACC CAA ATG A [300 nM] | Fusion protein | This study |
| | Antisense primer | ATA GGG TGT CTT GCT TGC C [300 nM] | | This study |
| | Donor probe | TGT TGC ATT CTT TGG AYT CTT CAG CAA C-FL | | This study |
| | Acceptor probe | LCRed640-4TIG ATG CGT TGC CTT CGG GTG CA-CA | | This study |
| Human bocavirus | Sense primer | GGG TGT GTT AAT CAT TGT AAC [400 nM] | NS1 protein | This study |
| | Antisense primer | CAC TGT TCT GCT TGA CG [400 nM] | | This study |
| | Donor probe | CTT TGC AGG TCT CAC CAA ATC C-FL | | This study |
| | Acceptor probe | LCRed670-TGC ATT AAG CAC TCC TCC CAC CAA C-FL | | This study |
| 2 | Parainfluenza virus type 1 | Sense primer | TAT CAA TGG GTG ATG CAA TAT AT [300 nM] | Hemagglutinin | This study |
| | Antisense primer | TCC TGT TCT TGA TGT C [300 nM] | | Neuraminidase |
| | Donor probe | TAA CCT ATG TGT AAA ACC Tgc CCT ATA TAC AC-FL | | This study |
| | Acceptor probe | LCRed610-CAC TAC TGT CTA GAG GTA TTA AGT TGG ATG GTA PH | | This study |
| Parainfluenza virus type 2 | Sense primer | CCA TTT ACC TAA GTG ATG AA [300 nM] | Hemagglutinin | This study |
| | Antisense primer | ATG AGA CCA CCA TAT ACA AGA [300 nM] | | Neuraminidase |
| | Donor probe | CAT CTA GCT GAA ATG AGA CTT CAC TCT TAC TAT TFL | | This study |
| | Acceptor probe | LCRed610-TGA CCC TAC T TD TAT TGA AAG AGT CAT ATC TCT TCC PH | | This study |
| Parainfluenza virus type 3 | Sense primer | TGT ATT TAT CAA AGG GAC CAC [300 nM] | Nucleoprotein | This study |
| | Antisense primer | TCC AGA TAT GAT CGT TGA TTA GCC ACC AG-FL | | This study |
| | Donor probe | CAT ACA TGG TGA GTT CCT CCA ACC AG-FL | | This study |
| | Acceptor probe | LCRed640-CAA CTA TCG TCC CAT AGT GAG YTA TGG PH | | This study |
| Human coronavirus NL63 | Sense primer | ATA AAC TGG GTA ATG TAG CAC AA [400 nM] | ORF1a | This study |
| | Antisense primer | TCT AAT GTT ATA CTT AAA ACT ACG GTC G [400 nM] | | This study |
| | Donor probe | CTA TTA TAG ATG ATA TTA TAG GAG AGT TGG TTA CC-FL | | This study |
| | Acceptor probe | LCRed670-AGG TCT CTA AAT CAG TGA CAG TG TTA TTT PH | | This study |
| 3 | Influenza A | Sense primer | GAG CTA TCC TGT CAC CTC TGA C [1000 nM] | N51 protein | WHO recommended |
| | Antisense primer | AGG GCA TTY TGG ACA AAK GCT CTA [1000 nM] | | WHO recommended |
| | Donor probe | GGG ATT TTR GGR TTT TTT TAC TAC ACC ACC-FL | | This study |
| | Acceptor probe | GCRed640-CAA CTA CTC TGG CTA GGA CTA CAG C-CH | | This study |
| Influenza B | Sense primer | AGA GTT GGA CTT GAY CC [300 nM] | Nucleoprotein | Smith et al. (2003) |
| | Antisense primer | CAT AGG CAG CTT TCT CTA [300 nM] | | Smith et al. (2003) |
| | Donor probe | GGA AGC ACA MTC CCC AGA AGA TCA GGT FL | | Smith et al. (2003) |
| | Acceptor probe | LCRed640-AAC TGC ACC AGC CCG GG-FL | | Smith et al. (2003) |
| Adenovirus | Sense primer | GCC CCA GTG GKC TTA CAT GCA CAT C [300 nM] | Hexon protein | Heim et al. (2003) |
| | Antisense primer | GCC AGG GTG GGT TTT CTA AAT TT [300 nM] | | Heim et al. (2003) |
| | Donor probe 1 | GGA AGC ACA MTC CCC AGA AGA TCA GGT PH | | This study |
| | Acceptor probe 1 | LCRed670-TGC TAC TGA CCA GCA TCG TCT CC-PH | | This study |
| | Donor probe 2 | AAC TGC ACC AGC CCG GG-FL | | This study |
| | Acceptor probe 2 | LCRed670-CGC TAC TCT GCG GTC TGC TCC PH | | This study |
| 4 | Human coronavirus OC43 | Sense primer | CAT GCA ATG GCC GTA ATA TGA T [400 nM] | ORF1b | This study |
| | Antisense primer | TGT AAA GGC ACT TAT ATT AGC A [400 nM] | | This study |
| | Donor probe | CAA AGG TTT AAT CAA CCT TAC TAC TCT T-FL | | This study |
| | Acceptor probe | LCRed610-CAC ATG TGA TTA GGA GCT TGC TTA GAG-PH | | This study |
| Human coronavirus HKU1 | Sense primer | TTT GTG TTT ACA TTA TGC TTA [500 nM] | ORF1b | This study |
| | Antisense primer | TGT CGT ATA CTG AAA ACG ATG TAC [500 nM] | | This study |
| | Donor probe | CTT GTA GCA CCA GCA TGC CAT AAC ATC TAC T-FL | | This study |
| | Acceptor probe | LCRed640-CGC CAC ACA TAA TAC TAT CA-PH | | This study |
| Human coronavirus 229E | Sense primer | CTC AAT CTC GGA ATC CCT C [300 nM] | | Nucleoprotein |
| | Antisense primer | CTC TGA GAA CGA CGA ATG CT [300 nM] | | This study |
| | Donor probe | GGG TAC TCT TCA GCC TGC TGA TTA TAC FL | | This study |
| | Acceptor probe | LCRed670-TGC TCC TTT TCA AAT TCT CGC PH | | This study |

a The donor fluorophore, fluorescein (FL).
b The acceptor fluorophore. The number following LCRed is the wavelength at which fluorescence is emitted.
c Phosphate (PH) on 3'-end to prevent extension of probe by DNA polymerase.
d These primers were substituted with the WHO primers that detect all influenza A strains, including novel influenza A H1N1 and corresponding probes designed. Testing on seasonal H3N2, H1N1 and novel H1N1 confirmed the specificity of the new probes.
replicates could be detected. For DNA viruses dilution series of plasmid DNA was used.

2.9. Statistical analyses

The confidence level of data analysed was determined with the Student’s t-test with two-tailed distribution.

3. Results

3.1. Multiplex development

Optimization of the multiplex assays was done on culture controls or on specimens that previously tested positive by PCR in independent laboratories. The newly designed real-time RT-PCR assays were initially optimized as monospecific assays. To assess the specificity of the primers and probes, the assays were tested for cross-reactivity among the 13 viruses using control specimens with viral loads of approximately 1 × 10^3–2.5 × 10^4 copies/ml. In all cases each virus was only detected by its specific primer and probe set, no cross-reaction was observed. The monospecific assays were subsequently combined to form four multiplex assays comprising of the following viruses: Multiplex 1—RSV A and B, hMPV, bHBoV; Multiplex 2—parainfluenza virus (PIV) 1, 2, and 3, hCoV-NL63; Multiplex 3—influenza A and B, adenovirus; Multiplex 4—hCoV-OC43, hCoV-HKU1, and hCoV-229E. Viruses were combined into the various multiplex assays according to shared similarities in clinical manifestations and susceptible age groups. The sensitivity of the assays was determined in triplicate through the amplification of serially diluted in vitro transcribed RNA from cloned controls. Detection limits for RNA viruses ranged from 2.5 to 25 in vitro transcribed RNA copies/μl (corresponds to 2.5 × 10^2–2.5 × 10^4 copies/ml of specimen) and 25 plasmid DNA copies/μl for DNA viruses (corresponds to 2.5 × 10^4 copies/ml of specimen) (Table 2). Lower concentrations were detected, however, at these concentrations detection of all replicates could not be achieved and was therefore not considered true detection limits. To assess the inter-run precision of the respective assays, extraction and real-time RT-PCR detection were performed on triplicate aliquots from viral suspensions. Three independent real-time RT-PCR runs performed showed a coefficient of variation of between 0.03% and 3.05% (Table 2), indicating high reproducibility of the real-time RT-PCR assays. All four multiplexes were optimized to be performed under the same cycling conditions for simultaneous use. In the event that a 60–nt conserved region could not be identified, a technique described by Pont-Kingdon and Lyon (2005) was used to overcome this. This technique was applied to probes used for the detection of RSV A and B, and PIV-2.

To determine if amplification competition may lead to interference during simultaneous quantitation of more than one virus in a single reaction, quantified controls were added together at varying concentrations and quantified concurrently. The results indicated that simultaneous quantitation of two viruses with varying concentrations >2.5 × 10^4 copies/ml (i.e. detected before amplification cycle 28), showed a coefficient of variation of <3.5%, indicating that the multiplex assays can accurately quantify two viruses in a single reaction. However, when a second virus is detected after amplification cycle 28 (i.e. virus concentration <2.5 × 10^4 copies/ml) or a third virus was present, accurate quantitation of viral load could not be achieved. In these events the viruses will have to be quantified separately to ensure accurate quantitation of viral load. However, for diagnostic purposes simultaneous qualitative detection of more than one virus can be achieved to a detection limit of approximately 2.5 × 10^4 copies/ml.

3.2. Evaluation of multiplex assays

3.2.1. Clinical evaluation

The multiplex assays were validated on a total of 91 clinical specimens that previously tested positive for the presence of respiratory viruses by IFA, PCR or semi-nested PCR. With the exception of bHBoV, that was only detected in 5/9 specimens, viruses were detected in all the known positive specimens evaluated (Table 2). bHBoV was previously identified in the positive control specimens by semi-nested PCR. In 4/9 specimens bHBoV could not be detected by a single-round PCR. The detection limit of bocavirus by the real-time PCR is 25 copies/μl (2.5 × 10^4 copies/ml of specimen), suggesting high sensitivity although this is not as sensitive as the conventional semi-nested PCR by which it was initially detected (Smuts and Hardie, 2006). Application of the quantitative feature of this assay was demonstrated on RSV positive specimens from 32 children with mild disease (infants who visited outpatients but did not require hospitalization) and 26 children with severe disease (patients admitted to the intensive care unit) (Table 3). An association between higher viral loads and severe disease is

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**Table 2**

| Virus and method of control detection | Number of known positive clinical samples | Samples detected with multiplex assays | Detection limit | Inter-run coefficient of variation (%) |
|--------------------------------------|------------------------------------------|----------------------------------------|----------------|----------------------------------------|
| Direct immunofluorescence assay (IFA) |                                          |                                        |                |                                        |
| RSV                                  | 30                                       | 30                                     | 2.5 RNA copies/μl | 0.42                                    |
| PIV 1                                | 8                                        | 8                                      | 2.5 RNA copies/μl | 0.31                                    |
| PIV 2                                | 1                                        | 1                                      | 2.5 RNA copies/μl | 1.46                                    |
| PIV 3                                | 8                                        | 8                                      | 25 RNA copies/μl  | 0.91                                    |
| Influenza A                          | 8                                        | 8                                      | 25 RNA copies/μl  | 0.29                                    |
| Influenza B                          | 4                                        | 4                                      | 25 RNA copies/μl  | 0.44                                    |
| Adv                                   | 5                                        | 5                                      | 25 DNA copies/μl  | 0.38                                    |
| PCR                                  |                                          |                                        |                |                                        |
| hMPV                                 | 7                                        | 7                                      | 2.5 RNA copies/μl | 3.05                                    |
| hCoV-NL63                            | 5                                        | 5                                      | 2.5 RNA copies/μl | 0.21                                    |
| hCoV-HKU1                            | 1                                        | 1                                      | 2.5 RNA copies/μl | 0.31                                    |
| hCoV-OC43                            | 4                                        | 4                                      | 2.5 RNA copies/μl | 2.93                                    |
| hCoV-229E                            | 1                                        | 1                                      | 2.5 RNA copies/μl | Not done b                             |
| Nested PCR                           | hBoV                                     | 9                                      | 25 DNA copies/μl  | 1.14                                    |

*a* Only one clinical specimen was available for validation of this virus. However, in addition, three PIV 2 positive specimens that previously tested negative by routine immunofluorescence were detected in the negative specimen screen.

*b* Due to limited sample volume the inter-assay coefficient of variation for hCoV-229E could not be determined.

*c* In 4 specimens, bHBoV could only be detected by semi-nested PCR.
Combination of respiratory virus co-infections as identified and quantified by multiplex real-time RT-PCR.

Table 4b
Additional respiratory viruses detected in 270 respiratory specimens that tested negative by routine diagnostic assays.

| Virus                  | Number of detections | Number of co-infections (%) |
|------------------------|----------------------|-----------------------------|
| **Viruses routinely screened for by IFA panel** |
| RSV                    | 35                   | 6 (17.1%)                   |
| PIV 1                  | 2                    | 1 (50%)                     |
| PIV 2                  | 3                    | 1 (33%)                     |
| PIV 3                  | 14                   | 2 (14.3%)                   |
| Influenza A            | 4                    | 2 (50%)                     |
| Influenza B            | 3                    | 0                           |
| Adv                    | 18                   | 7 (38.9%)                   |
| Total                  | 79                   | 29.3%                       |
| **Viruses not in routine diagnostic assays** |
| hMPV                   | 14                   | 4 (28.6%)                   |
| hBoV                   | 15                   | 8 (53.3%)                   |
| hCoV-NL63              | 5                    | 4 (80%)                     |
| hCoV-HKU1              | 0                    | 0                           |
| hCoV-229E              | 0                    | 0                           |
| hCoV-OC43              | 6                    | 5 (83.3%)                   |
| Total                  | 40                   | 14.8%                       |

Demonstrated as the RSV viral load in patients with severe disease was significantly higher ($p = 0.014$) (mean: $3.35 \times 10^{10}$ copies/ml, median $6.04 \times 10^{9}$ copies/ml) compared to patients with mild disease (mean: $3.44 \times 10^{6}$ copies/ml). The internal control (GAPDH) was continuously detected in all supernatant fractions ranging from $1.59 \times 10^{5}$ and $1.41 \times 10^{9}$ copies/ml of processed specimens (mean: $2.69 \times 10^{6}$ copies/ml, median: $1.03 \times 10^{6}$ copies/ml), confirming its value as a nucleic acid extraction control.

3.3. Identification of nucleotide base changes

Melting curve analysis of RSV positive clinical specimens revealed four distinct melting curves with melting temperatures ($T_m$) of 59 °C, 54 °C, 51 °C, and 47 °C (Fig. 1) identified in 100/131 (76.3%), 20/131 (15.3%), 4/131 (3.1%), and 7/131 (5.3%) of RSV infected patients, respectively. Sequencing of the RSV target regions of positive specimens, hCoV-HKU1 as a co-infection with PIV 3 and hCoV-229E occurred as a co-infection with RSV. In addition, 79 (29.3%) of the IFA negative specimens tested positive for viruses already included in the IFA panel, suggesting a marked improved sensitivity over the IFA. To rule out the possibility of false positive results, one-third of all amplicons were randomly selected and sequenced to confirm the specificity of the assay. All sequencing reactions were successful. Non-specific detection was not observed. Co-infections were detected in 6.7% of specimens (Tables 4a and 4b). Viral loads were quantified where co-infections occurred with hMPV, hBoV and coronaviruses (Table 4b), though the clinical relevance of these findings will be described elsewhere. Viruses could be detected in all the different types of respiratory specimens (nasopharyngeal aspirate specimens, bronchoalveolar lavage specimens, endotracheal aspirate specimens, and sputum specimens).

Table 4a
Combination of respiratory virus co-infections as identified and quantified by multiplex real-time RT-PCR.

| Co-infecting viruses | Number of detections | Quantification of co-infections (copies/ml) |
|----------------------|----------------------|---------------------------------------------|
| **Double infections** |
| Adv + hCoV-OC43      | 2                    | Adv: $2.78 \times 10^{4}$ and $3.35 \times 10^{10}$; hCoV-OC43: $2.80 \times 10^{4}$ and $5.48 \times 10^{8}$ |
| Adv + hBoV           | 2                    | Adv: $3.53 \times 10^{10}$ and $7.40 \times 10^{9}$; hBoV: $3.58 \times 10^{6}$ and $6.10 \times 10^{9}$ |
| hBoV + hMPV          | 2                    | hBoV: $5.98 \times 10^{6}$ and $3.35 \times 10^{10}$; hMPV: $1.45 \times 10^{5}$ and $2.33 \times 10^{8}$ |
| Adv + RSV            | 1                    | Not determined                             |
| hBoV + hCoV-NL63     | 1                    | hBoV: $5.28 \times 10^{9}$; hCoV-NL63: $1.21 \times 10^{6}$ |
| hBoV + hCoV-OC43     | 1                    | hBoV: $4.30 \times 10^{6}$; hCoV-OC43: $1.58 \times 10^{6}$ |
| hBoV + PIV3          | 1                    | hBoV: $2.30 \times 10^{6}$; PIV3: $8.38 \times 10^{5}$ |
| hCoV-OC43 + PIV1     | 1                    | hCoV-OC43: $3.43 \times 10^{5}$; PIV1: $2.58 \times 10^{4}$ |
| hCoV-OC43 + PIV2     | 1                    | hCoV-OC43: $4.35 \times 10^{5}$; PIV2: $4.20 \times 10^{4}$ |
| hCoV-NL63 + RSV      | 1                    | hCoV-NL63: $2.50 \times 10^{3}$; RSV: $4.15 \times 10^{5}$ |
| hMPV + RSV           | 1                    | hMPV: $1.03 \times 10^{4}$; RSV: $1.30 \times 10^{11}$ |
| Influenza A + PIV3   | 1                    | Not determined                             |
| Influenza A + RSV    | 1                    | Not determined                             |
| **Triple infections** |
| hBoV + hMPV + hCoV-NL63 | 1 | hBoV: $3.23 \times 10^{3}$; hMPV: $1.36 \times 10^{5}$; hCoV-NL63: $8.15 \times 10^{6}$ |
| Adv + RSV + hCoV-NL63 | 2 | Adv: $1.34 \times 10^{1}$; RSV: $9.53 \times 10^{6}$; hCoV-NL63: $8.09 \times 10^{7}$ |
|                      |                      | Adv: $3.75 \times 10^{5}$; RSV: $9.83 \times 10^{7}$; hCoV-NL63: $3.75 \times 10^{9}$ |
Fig. 1. Melting curve analysis illustrating the detection of four different RSV A strains identified with the RSV specific fluorescence resonance energy transfer (FRET) hybridization probes. Comparison of the nucleotide sequences of the RSV A strains relative to a reference strain (RSV A\textsuperscript{\textregistered}, GenBank accession number X00001) indicating the single nucleotide mismatches corresponding to the four different melting curve profiles. Dots indicate conserved bases, whereas sequence variations are indicated by the nucleotide base.

4. Discussion

In this study we described the development of four quantitative multiplex real-time RT-PCR assays that can be run concurrently for the detection of 13 respiratory viruses. For the detection of respiratory viruses targeted by routine IFA, the multiplex assays were shown to be as specific as the routinely used commercial IFA but also to be significantly more sensitive, increasing the detection of these viruses by 29.3%. Inclusion of the additional respiratory viruses in the detection assay increased the overall detection frequency by a further 9.7%. Discrepant results in the study may be attributable to the increased sensitivity of real-time PCR. For the detection of RSV, a previous study reported 50–90% of specimens positive by PCR but negative by IFA had viral loads less than $1 \times 10^6$ copies/ml, suggesting a lower limit of sensitivity for IFA in this range (Kuypers et al., 2004). In this study, the assay demonstrated a detection limit ranging between $2.5 \times 10^3$ and $2.5 \times 10^4$ copies/ml, which is $>2$ logs lower than the approximate IFA detection limit. These detection limits should be fitting for the detection of respiratory viruses, as previously reported viral loads in respiratory specimens ranged between $1 \times 10^3$ and $1 \times 10^{11}$ copies/ml (Kuypers et al., 2006). Viral loads less than $1 \times 10^3$ copies/ml have been described, though the clinical relevance at such low levels has been questioned (Allander et al., 2007; van der Hoek et al., 2005).

Respiratory specimens are non-homogenous and may vary in consistency (degree of viscosity due to the presence of mucous), volume, and manner of collection, thus making quantitative analysis of respiratory specimens methodically difficult. Since respiratory specimens are difficult to standardise, quantitation of respiratory viruses by PCR is better described as semi-quantitative (Schildgen et al., 2008). In this study, semi-quantitative results of RSV positive patients with mild and severe disease demonstrated a significantly higher RSV viral load in patients with severe disease confirming the relevance of this feature and confirms previous findings for RSV (DeVincenzo et al., 2005; Fodha et al., 2007; Martin et al., 2008). Continuous detection of the internal control (GAPDH) indicated that quantitation was not influenced by poor RNA extraction or PCR inhibitors.

In comparison to conventional detection methods, real-time probe based PCR assays have been shown to detect respiratory viruses with increased sensitivity and specificity (Gharabaghi et al., 2008; Kuypers et al., 2006; Smith et al., 2003; van de Pol et al., 2007). A pitfall of real-time PCR is that the more commonly used probe chemistries (hydrolysis probes and molecular beacons) are highly sensitive to mismatches, which may lead to false negative results if point mutations occur (Gunson et al., 2006). In contrast, the assays described in this study utilize two FRET hybridization probes which have the advantage of being able to tolerate a greater number of mismatches than hydrolysis probes or molecular beacons, decreasing the occurrence of false negative results substantially. The benefit of FRET probes was illustrated here; where point mutations were detected in the sites where the probe hybridized to the target template in several clinical specimens despite complete conservation of this area on GenBank. An obstacle when designing FRET hybridization probes is that a conserved area of approximately 60 nt is needed for the two probes to bind. In highly variable virus
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