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A new real-time RT-qPCR assay for the detection, subtyping and quantification of human respiratory syncytial viruses positive- and negative-sense RNAs

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A B S T R A C T

Human respiratory syncytial virus (RSV) is a major health problem and the main cause of hospitalization due to bronchiolitis. RSV is divided into two antigenic subgroups, RSV-A and -B that co-circulate worldwide. Rapid and sensitive detection is desirable for proper patient handling while assessment of viral load may help to evaluate disease severity and progression. Finally RSV subtyping is needed to determine the prevalence and pathogenicity of each RSV subgroup, as well as their sensitivity to treatment. In this study, we took into account the most recent circulating RSV variants and designed two quantitative TaqMan one-step RT-PCR assays to detect and quantify both RSV subgroups separately. Standard dilutions of transcripts of positive and negative polarities were included in the assay validation to assess potential differences in sensitivity on negative-sense genomes and positive-sense RNAs. In addition, RSV detection in respiratory specimens of different types and sampled in different populations was compared to commercially available RSV diagnostic tools. Altogether, the RSV-A and -B assays revealed sensitive and quantitative over a wide range of viral loads, with a slight improved sensitivity of the RSV-B assay on positive sense transcripts, and allowed accurate RSV subtyping. We thus provide a useful tool for both RSV diagnostics and research.

1. Introduction

Human respiratory syncytial virus (RSV) is an enveloped, negative-strand RNA virus classified in the *Pneumovirus* genus within the *Paramyxoviridae* family. RSV has a single serotype with two antigenic subgroups, RSV-A and RSV-B that co-circulate during the year with an elevated incidence from winter to early spring (Ambrosioni et al., 2013; Haynes et al., 2014; Terletskaia-Ladwig et al., 2005). RSV causes upper respiratory tract infection with frequent involvement of the lower airway and represents the main cause of hospitalization due to bronchiolitis (Hall, 2001; McNamara et al., 2005; Stein et al., 1999). There is currently no vaccine against this virus and ribavirin, the only licensed antiviral, has shown little or no benefit and is not used routinely (Krilov, 2011). Palivizumab, an anti-RSV antibody, has proven to be an effective therapeutic but only in prophylaxis (Tinnion et al., 2014; van Beek et al., 2013; Yi et al., 2014). Rapid RSV detection is thus necessary to apply appropriate infection control measures. Assessment of viral load may further help to evaluate disease severity and progression (Buller, 2013; Chartrand et al., 2015; Vallières and Renaud, 2013). Finally RSV subtyping is needed to assess the prevalence and pathogenicity of each RSV subgroup, as well as their sensitivity to treatments, as these features remain unclear.

Real-time quantitative PCR (RT-qPCR) is the gold standard for both accurate RSV diagnosis and quantification and several assays have been developed during the last decades (Chi et al., 2007; Choudhary et al., 2013; Dewhurst-Maridor et al., 2004; Do et al., 2012; Gueudin et al., 2003; Hu et al., 2003; Kuypers,

Abbreviations: 6-FAM, 6-carboxyfluorescein; BHQ, black hole quencher; CT, threshold cycle; hMPV, human metapneumovirus; hPIV, human parainfluenza virus; L-LOD, lower limit of detection; LNA, locked nucleic acid; MGB-NFQ, minor-groove binder non-fluorescence quencher; QCMD, Quality Control Molecular Diagnostics; RSV, human respiratory syncytial virus; RT-qPCR, real-time quantitative PCR; U- and L- LOQ, upper and lower limit of quantification.

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et al., 2004; Mentel et al., 2003; van Elden et al., 2003), although these have to be adapted constantly to take into account genetic variations among circulating strains. One-step RT-PCR assays are progressively replacing two-step assays for both diagnostics and quantification of RNA viruses due to their rapidity, robustness and reproducibility (Schibler et al., 2012). In these assays, the reverse transcription step uses one of the PCR primers and the reaction efficiency may depend on primer design. Accordingly, assay sensitivity may differ upon amplification of positive or negative strand RNA. This is particularly meaningful and must be taken into account in the design of one-step, RT-qPCR targeting negative-strand RNA viruses. Indeed for these viruses, some specimens (*e.g.* serum or infected cell supernatant) will contain mainly free virions and thus negative-strand genomes, whereas other samples enriched with cells (*e.g.* respiratory specimens or infected cell lysates) will contain both genomic and intracellular RNAs of positive polarity (mRNAs and antigenomes). In the present study, using a large number of sequences and recent circulating clinical strains, we designed and validated two specific and sensitive one-step TaqMan RT-qPCR assays that showed accurate and sensitive for the detection and quantification of RSV-A and -B positive and negative sense RNAs in respiratory specimens.

2. Materials and methods

2.1. Primers and probes design

For each antigenic subgroup, genomic sequences of representative RSV strains available in Genbank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) were downloaded and aligned using Geneious Pro software v6.1.6 (Biomatters Ltd.). Primers and probes were designed to match the most recent circulating variants with the help of Primer Express® software (v3.1). Primers and probes sequences are listed in Table 1. Of note, primers and probes numbering is done on negative-sense genomic RNA. The RSV-A probe harbours a locked nucleic acid (LNA) (Vester and Wengel, 2004) while the RSV-B probe contains a minor-groove binder non-fluorescent quencher (MGB-NFQ) (Kutyavin et al., 2000).

2.2. Plasmids and transcripts

After reverse transcription of extracted RNA from RSV-A and -B infected clinical samples with random hexamers and superscript II (Roche #11034731001), the N gene was PCR-amplified using specific primers (RSV-A forward: 5′-TCT TTW GGA TTA AGY TGA TGT TTG-3′; and RSV-B forward: 5′-CTT CTT TGG GRT TGA GTT GAT-3 and common RSV-A/B reverse: 5′-AAA RAT GGC TCT TAG CAA RGT CAA G-3′). PCR products were subcloned in the pCR™2.1-TOPO® vector (TOPO TA Cloning® Invitrogen #K4500-02) according to the manufacturer's instructions. Orientation of the N gene fragment in the plasmid was determined by differential cutting with EcoRV. Plasmids with N in opposite orientations were linearized with BamHI, and transcribed *in vitro* with the MEGAscript T7 Transcription Kit (Ambion #AM1333). Sense and antisense transcripts were then purified with the RNeasy Minikit (Quiagen #74104) and quantified by optical densitometry (Nanodrop®). Serial transcript dilutions (10- and 4-folds) were prepared for each transcript for assay validation and preparation of reference standards.

2.3. Clinical specimens

Nasopharyngeal swabs, aspirations (NPS or NPA) or bronchoalveolar lavages (BAL) were collected from 36 anonymous patients from the University Of Geneva Hospitals (Switzerland). These clinical samples were previously screened positive for RSV using a commercially available kit for multiplex real-time RT-PCR (Fast Track Diagnostics®; # FTD-2), a semi-quantitative method that cannot subtype RSV (Choudhary et al., 2013). Similarly, clinical samples screened positive for human parainfluenza virus (hPIV) or human metapneumovirus (hMPV) were analyzed as specificity controls. Finally, a panel of 8 pre-subtyped RSV infected samples purchased from Quality Control Molecular Diagnostics (QCMD, 2014) were tested to confirm the accuracy of RSV subtyping using the RSVA-N[792-723] and RSVB-N[749-680] assays.

2.4. RNA extraction

Nucleic acids were extracted using the NucliSens easyMAG magnetic bead system (Biomérieux) according to the manufacturer's instructions and stored at −80 °C. For quantitative validation, clinical samples were initially diluted 1:100 and then serially diluted (10-fold completed by 4-fold) in triplicate before the extraction procedure.

2.5. TaqMan RT-qPCR

The assay was performed using the QuantiTect probe RT-qPCR kit (Qiagen #204443) in a StepOne Applied Biosystems thermocycler with the following cycling conditions: 50 °C for 30 min, 95 °C for 15 min, and 45 cycles of 94 °C for 15 s and 60 °C for 1 min. The reaction mixture (25 μl) contains 900 nM of each primer and 200 nM of probe, two units of reverse transcriptase and 5 μl of viral RNA extracts or transcripts. Results were analyzed using the StepOne™ software (Applied Biosystems). Of note the reaction mixture for the commercially available kit of multiplex real-time qPCR (Fast Track Diagnostics®; # FTD-2), contained 10 μl of extracted RNA and 15 μl of master mix, according to the manufacturer's instructions. To establish the standard curve for RNA quantification, four 10-fold serial dilutions of RNA reference standards were included in each batch of test. The threshold cycle (CT) values were used to quantify the RNA copy numbers per reaction using the slope-intercept form. The number of RNA copies per reaction was then corrected for the dilution factor performed between the extraction and the real-time RT-qPCR to obtain a final viral RNA copy number per ml of initial sample.

2.6. Determination of quantification and detection endpoints

The lower limits of detection (L-LOD) was defined as the lowest dilutions with 100% positive detection rate (N = 9 for both transcripts and clinical specimens). The upper and lower limits of quantification (U- and L-LOQ) were set as, respectively, the lowest and greatest dilutions where the coefficient of variation of CT between the different replicates was less than 2.

3. Results

3.1. Assay design

The RSV-A and -B nucleoprotein (N) gene was selected as target for assay design since it is highly conserved and numerous N sequences are available in GenBank. Six hundred and ten N gene sequences (in negative orientation) representing 480 RSV-A and 130 RSV-B 2014 strains were aligned. Locked nucleic acid bases (LNA) and minor-groove binder non-fluorescent quencher (MGB-NFQ), probes and primers fitting the quality requirement of the Primer Express software and located between nt 792–723 of RSV-A and nt 749–680 of RSV-B N gene were designed. Their sequences are listed in Table 1.

Table 1
Primers and probes sequences. *Sense relative to RSV genome. **Nt positions on the N gene in negative orientation. N: nucleoprotein. LNA: locked nucleic acid. MGB-NFQ: minor-groove binder non-fluorescence quencher. BHQ: black hole quencher. 6-FAM: 6-carboxyfluorescein. {}: LNA modified bases.

		RSVA-N[792-723**] assay	RSVB-N[749-680**] assay
Primers	*Forward	*RSVA_N792-771F***	*RSVB_N749-726F***
		5′-TGCTAAGACYCCCCACCGTAAC-3′	5′-GCA TTC ATA AAC AAT CCT GCA AAG-3′
	*Reverse	*RSVA_N723-746R***	*RSVB_N680-699R***
		5′-GGA TTT TTG CAG GAT TGT TTA TGA-3′	5′-GGC ATT GCA CAA TCA TCC AC-3′
*Probes		*RSVA_N768-753P*** (*LNA*)	*RSVB_N722-702P*** (*MGBNFQ*)
		5′-[Yakima Yellow®]-C{A}C T{T}G C{C}C T{G}{C}W{C}C A-[BHQ-1®]-3′	5′-[6-FAM]-CTT CAA CTC TAC TRC CCC CTC-[MGBNFQ]- 3′

Table 2
Analytical sensitivity of RSVA-N[792-723] and RSVB-N[749-680] assays on transcripts of negative (genomic) or positive (antigenomic) polarity. All mean CT are calculated from the average of CT values of nine replicates at each concentration. Upper (U-) and lower (L-) LOQ (limit of quantification) and L-LOD are expressed in RNA copies per reaction (c/r) or RNA copies/ml (c/ml). UD: undetermined. CV: intra-assay coefficient of variation (%). CV = standard deviation/average CT*100. The dilution factor between 1 ml of RNA transcript and the RNA input per reaction is 200.%pos: Percentage of detected samples. * U-LOQ not reached.

RNA concentration		RSVA-N[792-723] assay								RSVB-N[749-680] assay							
		Genomic (sense)				antigenomic (anti-sense)				genomic (sense)				antigenomic (anti-sense)			
C/r	C/ml	Mean CT	SD	CV (%)	%pos	Mean CT	SD	CV(%)	%pos	Mean CT	SD	CV(%)	%pos	Mean CT	SD	CV (%)	%pos
2.50E + 10	5.00E + 12	4.18	0.08	1.98	100%	6.03	0.11	1.77	100%	8.24	1.03	12.47	100%	6.41	0.24	3.67	100%
2.50E + 9	5.00E + 11	7.13	0.05	0.71	100%	9.07	0.09	0.97	100%	11.60	0.31	2.68	100%	9.00	0.37	4.13	100%
2.50E + 8	5.00E + 10	10.94	0.11	1.01	100%	12.91	0.07	0.52	100%	14.83	0.20	1.35	100%	12.21	0.27	2.25	100%
2.50E + 7	5.00E + 09	14.40	0.13	0.93	100%	16.37	0.06	0.38	100%	18.17	0.16	0.86	100%	15.62	0.12	0.78	100%
2.50E + 6	5.00E + 08	17.82	0.12	0.66	100%	19.87	0.11	0.55	100%	21.60	0.20	0.92	100%	19.59	0.13	0.66	100%
2.50E + 5	5.00E + 07	21.26	0.37	1.75	100%	23.51	0.09	0.40	100%	24.58	0.42	1.72	100%	23.01	0.16	0.71	100%
2.50E + 4	5.00E + 06	24.79	0.40	1.60	100%	27.02	0.07	0.27	100%	28.53	0.14	0.50	100%	26.55	0.15	0.58	100%
2.50E + 3	5.00E + 05	27.84	0.12	0.44	100%	30.57	0.10	0.32	100%	31.96	0.20	0.63	100%	28.67	0.26	0.89	100%
625	1.25E + 05	29.77	0.10	0.34	100%	32.67	0.12	0.36	100%	33.63	0.31	0.91	100%	28.96	0.29	1.02	100%
156.25	3.13E + 04	31.79	0.13	0.42	100%	35.02	0.40	1.14	100%	35.18	0.28	0.80	100%	30.94	0.13	0.41	100%
39.06	7.81E + 03	33.87	0.31	0.91	100%	37.47	0.99	2.63	100%	38.06	1.24	3.27	78%	32.95	0.23	0.69	100%
9.77	1.95E + 03	36.16	0.69	1.90	100%	39.27	0.18	0.45	100%	38.79	0.03	0.08	23%	34.76	0.39	1.13	100%
2.44	4.88E + 02	37.89	0.78	2.06	89%	40.02	0.62	1.55	33%	38.85	0.06	0.16	23%	37.58	0.78	2.07	89%
0.61	1.22E + 02	39.43	1.47	3.72	56%	40.71			33%	UD			0%	39.17	1.20	3.06	67%
LOD	L-LOD	9.77 c/r (1.95E + 03 c/ml)				9.77 c/r (1.95E + 03 c/ml)				156.25 c/r (3.13E + 04c/ml)				9.77 c/r (1.95E + 03 c/ml)			
LOQ	L-LOQ	9.77 c/r (1.95E + 03 c/ml)				156.25 c/r (3.13E + 04 c/ml)				156.25 c/r (3.13E + 04c/ml)				9.77 c/r (1.95E + 03 c/ml)			
	U-LOQ	>2.50E + 10 c/r (5.00E + 12 c/ml)*				>2.50E + 10 c/r (5E + 12 c/ml)*				2.50E + 8 c/r (5.00E + 10 c/ml)				2.50E + 7 c/r (5.00E + 09 c/ml)			

3.2. Analytical sensitivity and linear range of the RSVA-N[792-723] and RSVB-N[749-680] assays on positive and negative sense transcripts and on clinical specimens

The analytical sensitivity and the linear range of the RSVA-N[792-723] and RSVB-N[749-680] assays was first tested on dilution series of RSV-A and RSV-B transcripts of positive and negative senses. Nine replicates were analyzed for each dilution by Real time RT-qPCR (Table 2). The RSVA-N[792-723] assay showed detection sensitivity from 9.77 copies per reaction (c/r) (equivalent to 1.95E + 03 c/ml) up to 2.5E + 10 c/r (5E + 12 c/ml) on genomic transcripts (negative polarity). The LOD on transcripts of positive polarity was similar but the L-LOQ was 16 fold higher (156.25c/r or 3.13E + 04 c/ml). In contrast, RSVB-N[749-680] assay showed higher detection and quantification sensitivity on antigenomic RSV-B transcripts since the L-LOD and L-LOQ were 16 fold higher in the genomic RSV-B transcripts compared to antigenomic trancripts (156.25 c/r or 3.13E + 04 c/ml for sense transcripts versus 9.77 c/r for 1.95E + 03 c/ml for antisense transcripts). The U-LOQ was ten-fold higher in the genomic transcripts (2.50E + 8 c/r or 5.00E + 10 c/ml versus 2.50E + 7 c/r or 5.00E + 09 c/ml in RSV-B). The coefficient of correlation (R^2) ranged between 0.993 and 0.999, the PCR efficiency (E) between 90.92% and 93.92%, and the slope between −3.56 and −3.47 in these first validation experiments. To establish a standard curve for RSV RNA quantification in clinical specimens, four 10-fold serial transcript dilutions were selected as reference standards.

The analytical sensitivity and linear range of the two assays were then tested on clinical specimens. Nasopharyngeal swabs of patients infected with RSV-A or-B were serially diluted in triplicate and tested with the two assays. The L-LOQ and L-LOD were 43.7 c/r (1.09E + 03 c/ml) and 19.4 c/r (4.85 E + 02 c/ml) for RSVA-N[792-723], and 408 (1.02 +04 c/ml) and 30.7 (7.68 E + 02 c/ml) c/r for RSVB-N[749-680] (Table 3). In these experiments, the coefficient of correlation (R^2) ranged between 0.997 and 1, the PCR efficiency (E) between 87.3% and 99.16%, and the slope between −3.66 and −3.34 (data not shown).

3.3. Assay validation on clinical specimens and external QCMD quality controls

Eight NPA, 26 NPS and 2 BAL specimens collected in 2014 and screened positive for RSV by the Fast Track Diagnostics multiplex PCR kit in the Geneva University Hospitals were tested with the RSVA-N[792-723] and RSVB-N[749-680] assays (Table 4). Despite a 2-fold lower RNA input in the reaction and a potential alteration of RNA quality due to extra freeze-thaw cycle(s), the in-house assays showed similar detection sensitivity compared to the commercial assay. Non-specific cross-detection of RSV-A positive samples with the RSVB-N[749-680] assay occurred for 5 samples with high RSV-A RNA loads (CT values between 17.9 and 24.4). This non-specific cross-detection (dCT ≥ 16.6 between the two assays) does not prevent accurate RSV subtyping. The subgroup-specificity of the 2 assays was further validated on QCMD samples. All tested samples were accurately detected as RSV-A or-B except for 2 samples that were below the L-LOD in both commercial and in-house assays. Of note, these two samples were also infrequently detected in other laboratories (Table 5).

Finally the specificity of our assays for pneumoviruses was confirmed by non detection of human metapneumovirus (hMPV) and human parainfluenza virus 1 (hPIV-1) −infected samples (Table 4).

Table 3
Analytical sensitivity of RSVA-N[792-723] and RSVB-N[749-680] assays on RSV-A and-B infected clinical specimens. All values are calculated from CT values of three extraction replicates and three PCR replicates at each concentration. For RNA quantification, four 10-fold serial dilutions of RNA reference standards were included in each run. L-LOD and L-LOQ are expressed as RNA copies/reaction. CV: intra-assay coefficient of variation of CT (expressed in%). CV = standard deviation/average CT*100. The dilution factor between 1 ml of clinical specimen and the RNA input per reaction is 25.%pos: Percentage of detected samples.

Clinical sample dilution factor	hRSVA-N[792-723] assay						hRSVB-N[749-680] assay					
	Mean Ct	% pos	SD	CV (%)	RNA copies/ reaction	RNA copies/ml	Mean Ct Value	% pos	SD	CV (%)	RNA copies/ reaction	RNA copies/ml
100	22.8	100	0.2	0.9	6.70E + 04	1.68E + 06	25.6	100	0.1	0.5	1.39E + 06	3.48E + 07
1000	26.4	100	0.2	0.8	6.96E + 03	1.74E + 05	29.0	100	0.1	0.5	1.37E + 05	3.43E + 06
10000	30.2	100	0.2	0.9	8.43E + 02	2.11E + 04	33.0	100	0.3	0.7	4.08E + 02	1.02E + 04
40000	32.3	100	0.2	0.7	2.04E + 02	5.10E + 03	34.8	100	0.9	2.5	1.42E + 02	3.55E + 03
160000	34.7	100	0.2	0.6	43.7	1.09E + 03	37.1	100	1.2	3.2	30.7	7.68E + 02
640000	36.1	100	0.8	2.2	19.4	4.85E + 02	38.3	44			10.9	2.73E + 02
2560000	39.0	44	1.3		3.4	84.50	38.4	11			9.8	2.46E + 02
L-LOQ					43.70	1.09E + 03	L-LOQ				4.08E + 02	1.02E + 04
L-LOD					19.40	4.85E + 02	L-LOD				30.7	7.68E + 02

Table 4
Detection and quantification of RSV-A and RSV-B in clinical specimens. CTs obtained with RSVA-N[792-723] and RSVB-N[749-680] assays and commercially available Fast Track Diagnostics multiplex PCR (Choudhary et al., 2013) are indicated as well as viral loads determined with the in-house assays. Viral loads above or below the validated LOQ (from Table 2) are shown in parenthesis. Human parainfluenza virus-1 (hPIV-1) and human metapneumovirus (hMPV) positive samples were also tested as specificity controls. Of note, the RNA input is twice higher in the commercial kit (10ul of extracted RNA versus 4ul in our assay), which theoretically corresponds to 1CT value. NPS: nasopharyngeal swab; NPA: nasopharyngeal aspiration; BAL: bronchoalveolar lavage. UD: undetermined. dCT: CT value obtained with the commercial multiplex PCR minus the CT value obtained with the in-house RSV-A or -B assays. * genogroup confirmed by sequencing.

Sample type	Sample N°	Commercial kit (CT value)	RSVA-N[792-723] assay(CT value)	RSVB-N[749-680] assay(CT value)	dCT	Subgroup	Viral load (RNA c/ml)
NPA	1	RSV:25.63	24.44	43.04	1.19	*A	3.44E + 07
	2	RSV:21.41	UD	21.81	−0.4	B	3.38E + 08
	3	RSV:18.11	UD	18.33	−0.22	B	3.59E + 09
	4	RSV:22.97	UD	21.80	1.17	B	5.34E + 08
	5	RSV:17.95	UD	16.90	1.05	B	1.04E + 10
	6	RSV:38.35	38.28	UD	0.07	A	(7.84E + 02)
	7	RSV:22.48	20.92	43.34	1.56	A	2.76E + 08
	8	RSV:21.41	UD	21.09	0.32	B	3.04E + 07
NPS	9	RSV:20.74	UD	19.89	0.85	B	6.86E + 07
	10	RSV:26.6	UD	30.84	−4.24	B	1.14E + 06
	11	RSV:27.7	UD	28.94	−1.24	B	4.07E + 06
	12	RSV:27.9	UD	28.07	−0.17	B	7.24E + 06
	13	RSV:31	UD	35.39	−4.39	B	5.45E + 04
	14	RSV:27.9	29.41	UD	−1.51	A	1.05E + 06
	15	RSV:18.1	UD	21.75	−3.65	B	4.95E + 08
	16	RSV:26	UD	27.07	−1.07	B	1.42E + 07
	17	RSV:18.8	18.80	39.40	0	*A	9.45E + 08
	18	RSV:22.26	UD	21.52	0.74	B	4.12E + 08
	19	RSV:24.02	21.82	38.45	2.2	*A	1.72E + 08
	20	RSV:20.74	UD	22.92	−2.18	B	1.59E + 08
	21	RSV:23.72	UD	22.93	0.79	B	1.58E + 08
	22	RSV:23.63	UD	24.84	−1.21	B	4.30E + 07
	23	RSV:19.80	UD	19.40	0.4	B	2.28E + 09
	24	RSV:25.54	UD	22.72	2.82	B	3.06E + 08
	25	RSV:31.41	29.78	UD	1.63	A	4.08E + 05
	26	RSV:26.27	UD	25.09	1.18	B	7.24E + 07
	27	RSV:16.09	UD	19.80	−3.71	B	7.30E + 07
	28	RSV:22.49	UD	17.93	4.56	B	2.59E + 08
	29	RSV:22.26	UD	23.01	−0.75	B	8.28E + 06
	30	RSV:23.49	25.89	UD	−2.4	A	5.22E + 06
	31	RSV:29.70	UD	29.72	−0.02	B	8.86E + 04
	32	RSV:23.63	UD	25.92	−2.29	B	1.16E + 06
	33	RSV:37.28	UD	38.80	−1.52	B	1.79E + 04
BAL	34	RSV:18.25	17.90	35.49	0.35	*A	1.93E + 09
	35	RSV:28.00	UD	27.62	0.38	B	6.53E + 06
NPA	36	hHPIV:25.47	UD	UD		–	–
NPS	37	hMPV:27.55	UD	UD		–	–
	38	hMPV:33.17	UD	UD		–	–

4. Discussion

Real-time PCR is the most widely used method for RSV diagnostic, with proven sensitivity and specificity (Chi et al., 2007; Choudhary et al., 2013). Different RSV genes have been targeted in these assays, such as the matrix, the polymerase (Kuypers et al., 2004), or the fusion protein genes (Mentel et al., 2003; van Elden et al., 2003), but the N gene is the most widely used due to its high conservation and the number of N sequences available in Genbank. Several assays were designed to subtype RSV, but most were validated more than 10 years ago (Dewhurst-Maridor et al., 2004; Do et al., 2012; Gueudin et al., 2003; Hu et al., 2003). In order to include

Table 5
Validation of subtyping efficiency. Quality control samples from QCMD (Quality Control Molecular Diagnostics) with defined RSV subgroups were analyzed *According to QCMD. **CT obtained at the laboratory of diagnostic at the University Of Geneva Hospitals using Fast Track Diagnostics multiplex PCR (Choudhary et al., 2013). UD: undetermined. In our assays, two-fold less extraction (1CT) material was used for Taqman detection compared to the multiplex PCR. # infrequently detected according to QCMD data sheets.

	QCMD results		Commercial kit	RSVA-N[792-723] assay		RSVB-N[749-680] assay	
	Subtype*	CT*	CT**	CT	RNA copies/reaction	CT	RNA copies/reaction
QCMD-01	Negative control		UD	UD	–	UD	–
QCMD-02	A	32.1	36.85	37.33	20.5	UD	–
QCMD-03	B	26.5	31.84	UD	–	32.61	367
QCMD-04	A	34.0#	UD	UD	–		
QCMD-05	B	32.9#	UD	UD	–	UD	
QCMD-06	B	29.8	36.11	UD	–	36.73	22.5
QCMD-07	B	29.9	35.15	UD	–	35.20	63.6
QCMD-08	A	28.8	32.36	34.07	153	UD	–
QCMD-01	Negative control		UD	UD	–	UD	–

recent RSV strains, an update of the viral subtypes is necessary. A one-step multiplex RSV-A and -B assay was validated recently, but the low annealing temperature of 45 °C does not fit with standards used in diagnostic laboratories (Do et al., 2012).

In this study, we designed and validated two one-step TaqMan RT-qPCR assays to efficiently detect, quantify and subtype RSV. These assays target the RSV-A and -B N gene and were designed to take into account a large number of sequences, including recent circulating strains. Our validation experiments proved the robustness of the assays both with *in vitro* transcripts and clinical specimens of different types and with a wide range of viral loads. We showed that our assays were as sensitive as the commonly used commercial Fast Track diagnostic kit. Importantly, we demonstrated that both the RSV-A and -B assays efficiently detected negative- and positive-sense RNA and thus RSV genomes, antigenomes and transcripts. The sensitivity of the RSV-A assay was similar, independent of transcript polarity while the L-LOD of the RSV-B assay was 16-fold lower on positive sense RNA. Though the difference is low, other assays may present higher discrepancy. Strand polarity should thus be taken into account upon design and validation of one-step RT-qPCR assays targeting single strand RNA viruses and particularly negative strand viruses. In addition, RSV quantification results should be expressed as RNA copies/ml rather than genome equivalent when obtained with assays designed in the coding sequence.

We proved the specificity of our assays for RSV but observed cross detection of RSV-A infected samples with the RSV-B assay. However, this cross-detection did not prevent accurate subtyping since the sensitivity of the RSV-B assay was 5 logs lower than RSV-A assay on RSV-A infected samples and occurred only with RSV-A RNA loads greater than 10^7 c/ml. In addition, accurate subtyping was confirmed on QCMD quality controls.

In conclusion, we propose two RSVA-N[792-723] and RSVB-N[749-680] assays as reliable tools to detect RSV, and also to quantify and subtype the virus. We hope that these tools will contribute to more accurate diagnostics and development of effective anti-RSV therapies.

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