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Development of a real-time multiplex RSV detection assay for difficult respiratory samples, using ultrasone waves and MNAzyme technology

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ABSTRACT

Background: Elderly infected with Human Respiratory Syncytial Virus (RSV) often bear low viral loads that stay below the detection limits of commercial assays. A more sensitive detection of RSV infections can improve patient management, guide containment strategies, and possibly prevent morbidity and mortality among populations most severely affected by RSV.
Objective: To test the sensitivity for RSV detection by using an alternative extraction method in combination with a new amplification procedure.
Study design: Nasopharyngeal washes and sputum samples ($n = 78$) form clinical cases, and broncheoalveolar lavages ($n = 27$) from an experimental RSV rat model were obtained. An ultrasone-based RNA extraction method was combined with a multi-component Nucleic Acid enzymes (MNAzyme) amplification procedure for simultaneous detection of RSV-A, RSV-B, and an Internal Extraction control IEC.
Results: Compared to standard real-time PCR technology, this method resulted in an increased detection sensitivity, ranging from 0.9 to 4.93 log (average 2.05 ± 1.01) for RSV-A and 0.76 to 4.28 log (average 1.30 ± 0.92) for RSV-B.
Conclusions: An ultrasone-based extraction method with MNAzyme amplification resulted in improved detection of RSV in different respiratory samples, including sputum. This generic method for nucleic acid extraction should be readily applicable for any other respiratory pathogen.

1. Background

Human Respiratory Syncytial Virus (RSV) infections in children are associated with high viral loads, easily detected in respiratory secretions using commercially available direct antigen assays.[1–3] Adults display much lower viral loads,[4] reducing the sensitivity of these assays. A more sensitive detection of RSV could improve patient management, guide containment strategies, and possibly prevent morbidity and mortality among populations most severely affected by RSV.[5]

Clinical sampling is often done by taking naso(-pharyngeal) swabs or aspirates which involve an inherent dilution due to the use of saline or viral transport medium, which may reduce sensitivity of detection. Sputum samples in that respect are easily obtained and non-diluted, but are difficult to manage for high throughput processing due to the cumbersome manipulations needed prior to downstream processing.[6,7]

For diagnostic assays, nucleic acid detection (real-time PCR) delivers the most precise and sensitive results.[8–11] Although more difficult to optimize,[12] multiplex real-time PCR assays have several advantages over monoplex detection assays. Most important for clinical settings, is the limited amounts of sample required to detect multiple targets. Additionally, internal controls can be analyzed in one run, offering a more controlled assay environment. A novel real-time detection technology, Multi-component Nucleic Acid enzymes (MNAzyme), offers new opportunities for multiplexing since intra-assay competition effects seem to be minimal.[13] An MNAzyme is a DNA molecule with a catalytic restriction activity (DNAzyme). For this application, the MNAzyme has been split in two DNA partzymes (Fig. 1). A partzyme has three distinct regions: a target sensor arm, a catalytic subdomain and a probe reporter arm. In a first step the two partzymes will anneal with the target sequence (amplicon), reconstituting the functional DNAzyme. In a second step the probe will anneal on the reporter arms and

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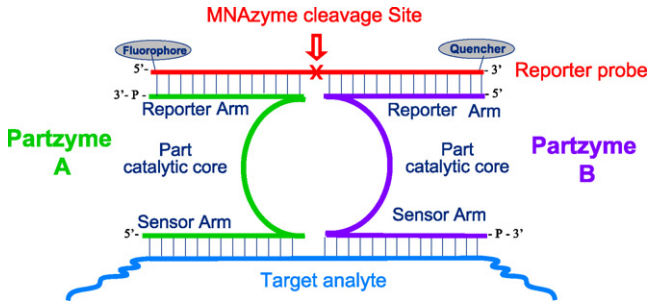

Fig. 1. Schematic representation of an MNAzyme. The MNAzyme consists of two DNA strands, called "Partzymes" bearing three distinct regions: (a) the "sensor arm" targeting one half of the desired DNA motif to be quantified, (b) one half of the "catalytic core" of the DNAzyme and (c) the "reporter arm" targeting one half of the reporter probe. The 3′ end of the Partzyme is phosphorylated to prevent the partzyme from functioning as a primer.

will be cut by the DNAzyme, splitting the fluorophore from the quencher. The target sequences are specifically amplified, allowing more MNAzymes to assemble during each round of PCR, generating a real-time signal that can be analyzed in an identical way as for a TaqMan assay. There are several advantages compared to more traditional TaqMan assays. The ability of MNAzymes to discriminate between closely related sequence variants is based on the design of the target-specific sensor arms. With strategic design of the sensor arms, MNAzymes have been shown to discriminate between single base polymorphisms without bias.[13] Additionally, MNAzyme reagents have a higher fidelity compared to TaqMan reagents since a functional MNAzyme requires target-specific binding of two partzymes and two PCR primers (four levels of specificity) while TaqMan assays have only three levels of specificity (two primers and one target-specific probe). The most important contribution of MNAzymes, however, is in their application for multiplex analysis. The catalytic core and reporter arms are generic and have been specifically designed to perform optimally in each other's presence and have no relation to the nature of the target sequence to be quantified.

2. Objective

Here we describe an RSV detection assay suitable for use with sputum samples. This method combines ultrasone waves, RNA extraction and a novel real-time amplification technology, generating a sensitivity that surpasses the currently established real-time RSV detection assays by approximately 2 logs.

3. Study design

3.1. Samples

A total of 78 respiratory samples were collected with informed consent from hospitalized patients with respiratory symptoms (Table 1). A total of 27 BAL samples were collected from cotton rats infected with an RSV-A Long strain. The RSV-A-GFP (rgRSV(224)[14]) and RSV-B-P3-Hep2 (VR1580 ATCC strain grown on Hep2 cells, third passage) strains were used for the development of the External Quantification Controls (EQC).

3.2. RNA extraction methods

3.2.1. Glass beads method

Sputum aliquots were liquefied by vortexing in Viral Transport Medium and glass beads (VWR, Belgium). The liquefied sputum samples were filtered using a 0.45 μm filter (Minisart-plus, Germany). The filtrates were extracted using the QIAamp viral RNA kit, according to the guidelines of the manufacturer (Qiagen, The Netherlands).

Table 1
Sample overview and the corresponding pathogens detected.

Sample type	Datected pathogens[a]						
	RSV	PI[b]1	PI2	PI3	HMPV[c]	NPD[d]	Total
Sputum	7						
BAL[e]						4	
NPS[f]						1	
NPA[g]	28	2	1	3	4	27	
TS[h]			1				
Total	35	2	2	3	4	32	78

[a] Initial clinical screenings were performed by local hospital assays.
[b] Para-influenza.
[c] Human Meta-Pneumo Virus.
[d] No fathogen detected.
[e] Broncheo-Alveolar Lavage.
[f] Naso-Pharyngeal Swab.
[g] Naso-Pharyngeal Aspirate.
[h] Throat Swab.

3.2.2. Ultrasone method

Sputum samples (0.1–1 g) were weighed in a 14 ml polypropylene round-bottom tube (Becton Dickinson, Belgium) and one volume of PBS (Invitrogen, Belgium)/10% DTT (Acros Organics, Belgium) was added with a minimal final volume of 2.2 ml. Samples were immediately transferred to the Adaptive Focused Acoustics (AFA) instrument (Covaris, UK) and treated for 30 s at 1 °C (settings: Duty Cycle 20%; Intensity: 10; Cycles/burst: 100). Subsequently, 4 volumes of NucliSens Extraction Lysis Buffer (BioMérieux, The Netherlands) were added and the whole was incubated for 10 min prior to extraction on the automated EasyMAG extraction platform (BioMérieux). The total minimal volume was 6.2 ml.

A total of 3 ml of the lysed sample volume was transferred to the EasyMAG containers, allowing a repeat extraction if required. A total of 100 μl of extraction control mix (550 μl NucliSens Magnetic Beads (BioMérieux), 110 μl IEC-B RNA (Internal Extraction Control, see below), and 440 μl NucliSens Extraction Buffer 3 (BioMérieux)) was added to each well prior to extraction. Elution of the RNA was done in 110 μl.

3.3. RNA detection assays

3.3.1. TaqMan design

The primers and probes detecting the fusion (F) gene (RSV-A) and the nucleocapsid (N) gene (RSV-B) are listed in Table 2. Monoplex real-time quantitative RT-PCR reactions (final volume 35 μl) were prepared as follows: 10 μl extracted RNA or standard RNA (see below), 0.360 μl Yeast tRNA (Invitrogen, Merelbeke, Belgium), 150 μl 2× reaction buffer (Euroscript, Liege, Belgium), 0.15 μl Euroscript RT (Euroscript, Liege, Belgium), 0.1 μM probe, 0.9 μM primer each and 1.5 mM $MgCl_2$ and RNase/DNase free water (Invitrogen, Merelbeke, Belgium). Amplification and detection were performed

Table 2
Primer and probe sequences designed for the TaqMan RSV detection assay.

Oligonucleotide	Sequence	Position[a]
RSVA-F-FW656	5′-CTGTGATAGARTTCCAACAAAAGAACA-3′	6315–6341
RSVA-F-RV732	5′-AGTTACACCTGCATTAACACTAAATTCC-3′	6364–6391
RSVA-F-TP684	FAM 5′-CAGACTACTAGAGATTACC-3′ NFQ-MGB	6343–6361
RSVB-N-FW435	5′-GGCTCCAGAATATAGGCATGATTC-3′	1572–1595
RSVB-N-RV508	5′-TGGTTATTACAAGAGCAGCTATACACAGT-3′	1599–1614
RSVB-N-TP460	FAM 5′-TATCATCCCACAGTCTG-3′ NFQ-MGB	1617–1645

[a] Numbering based on AY911262 (RSV-A Long strain) and AY353550 (RSV-B).

Table 3
MNAzyme oligos and probes designed to detect IEC RNA as well as RSV-A and RSV-B RNA in a triplex real-time PCR.

Oligonucleotide	Sequence	Position[a]
5RSVA	5′-GTGATAGAGTTCCAACAAAAGA-3′	6317–6338
3RSVA	5′-AAGTGCTTACAGGTGTAGTTA-3′	6343–6362
RSVAA-P	5′-**CAGACTACTAGAGATTACCA**TACAACGA*GAGGAAACCTT*-3′-P	6363–6382
RSVAB-P	5′-*TGCCCAGGG*AGGCTAGC**GGGAATTTAGTGTTAATGCA**-3′-P	6387–6407
RSV-A probe	FAM 5′-AAGGTTTCCTCguCCCTGGGCA-3′ BHQ1	NAP
5RSVB	5′-GCTCCAGAATATAGGCATGAT-3′	1573–1593
3RSVB	5′-GATCTATCTCCTGCTGCTAAT-3′	1599–1618
RSVBA-P	5′-**AGACTGTGGGATGATAATAC**TACAACGA*GAGGCGTGAT*-3′-P	1619–1638
RSVBB-P	5′-*CTGGGAGGA*AGGCTAGC**TGTGTATAGCTGCACTTGTA**-3′-P	1647–1667
RSV-B probe	JOE 5′-CAGCACAACCguCACCAACCG-3′ BHQ1	NAP
5IECB	5′-CTTGTAATAACCAAAGGGCGA-3′	NAP
3IECB	5′-GGAAACAGCTATGACCATGATT-3′	NAP
IECBA-P	5′-**ACCTGCAGGACTAGTCCCTT**TACAACGA*GGTTGTGCTG*-3′-P	NAP
IECBB-P	5′-*CGGTTGGTG*AGGCTAGC**TAGTGAGGGTTAATTCTGAG**-3′-P	NAP
IEC-B probe	Q6B2 5′-ATCACGCCTCguTCCTCCCAG-3′ BHQ2	NAP

-P: indicates partzyme; underlined: MNAzyme catalytic core; *italics*: probe reporter arm sequences; **bold**: target sensor arm sequence.
[a] Numbering based on AY911262 (RSV-A Long strain) and AY353550 (RSV-B).

in an ABI7900HT (Applied Biosystems, Foster City, CA, USA). All quantifications were performed in duplicate.

3.3.2. MNAzyme design

The principle of the MNAzyme technology is presented in Fig. 1. In total three MNAzyme complexes were designed to detect the IEC-B, the RSV-A and RSV-B RNA in a triplex real-time PCR setting (Table 3).

The triplex MNAzyme mix (final volume 20 μl) contained "Immobuffer + Mg" (1×, Bioline, USA), $MgCl_2$ (6.5 mM), dNTPs (200 μM-each, Bioline, USA), probes (0.2 μM-each), partzymes (0.2 μM-each), forward primers (0.04 μM-each), reverse primers (0.2 μM-each), Rox (1×, Invitrogen, Belgium), Immolase (1 U, Bioline, USA), Protector RNase Inhibitor (10 U, Roche, Belgium), MMLV(-H) (40 U, Promega, The Netherlands) and water. A total of 10 μl RNA eluate or RNA standard (see below) was added to this mix. All quantifications were performed in duplicate. Amplification and detection was performed on a LightCycler® (Roche, Belgium) under the following conditions: Reverse Transcription (RT) at 50 °C for 30 min, ending with 95 °C for 7 min. RT was followed by two cycling stages: first 10 cycles of 95 °C for 15 s and 65 °C for 30 s, lowering the temperature by 1 °C per cycle, then 50 cycles at 95 °C for 15 s and 50 °C for 1 min.

3.4. RNA controls

3.4.1. Construction

pIEC-B is a TOPO-TA (Invitrogen, Belgium) based plasmid harboring a 74 bp artificial sequence: 5′-GGCTCCAGAATATAGGCA-TGATTCTCTACCGTACTCTAGCCTAATA *CTGTGTATAGCAGCACTTGTA-ATAACCA*-3′. The flanking sequences of this insert are identical to the RSV-B amplification primer sequences (*italics*) (Table 2). pIEC-B was used for *in vitro* RNA transcription.

pEQC (External Quantification Control): RNA was extracted from 1000 μl RSV-A-GFP and RSV-B-P3-Hep2 virus stocks using the EasyMAG (BioMérieux) and eluted in 55 μl. A partial RSV-A F-gene (nt position 5631–6615 of AY911262) and RSV-B N-gene fragment (nt 908–2286 of AY353550) was amplified in an RT-hemi-nested PCR. The obtained amplicons were cloned using the TOPO-TA kit (Invitrogen). For each construct, one clone – pEQC-A and pEQC-B – was used for *in vitro* RNA transcription.

3.4.2. RSV standard evaluations

The RNA transcripts of pIEC-B were processed in duplicate with the TaqMan assay using probe FAM-5′-TACCGTACTCTAGCCTA-3′-NFQ-MGB. On a total of 870 measurements, the average IEC-B Ct value was 28.09 ± 0.36 (Batch 1). Other batches of IEC-B were produced with average Ct values of 26.0 ± 0.71 (Batch 2) and 22.85 ± 0.53 (Batch 3). IEC RNA standard was added (100 μl of extraction control mix, see above) to 3 ml of ultrasone-treated samples, just prior to the extraction on the EasyMAG. To correct for loss of RNA during the extraction process, the following formula was used: $Ct_{Sample} - (\text{IEC-B } Ct_{Sample} - \text{IEC-B } Ct_{Batch\,Value})$. All Ct values reported here have been corrected by means of their IEC-B Ct_{Sample} value.

Several RNA standard dilution series (EQC-A and EQC-B) were taken from −80 °C storage and tested by different operators on different days to check the reproducibility and the dynamic range. For each dilution of the standard series, two aliquots of 10 μl were submitted to the real-time assay in duplicate for RSV-A (EQC-A) or

Table 4
Comparison of two extraction methods (glass beads/Qiagen vs. Ultrasone/EasyMAG) on sputum samples. The quantity of viral RNA is derived from TaqMan q-RT-PCR.

Sample	Glass beads/Qiagen (A)		Ultrasone/EasyMAG (B)		ΔCt (A − B)		Log increase sensitivity
	Ct RSV-A	Ct RSV-B	Ct RSV-A	Ct RSV-B	RSV-A	RSV-B	
1	19.8	ND	16.45	ND	3.35	–	1.0
2	22.6	ND	12.5	ND	10.1	–	3.1
3	ND	27.66	ND	16.55	–	11.11	3.4
4	27.55	ND	20.1	ND	7.45	–	2.3
5	ND	31.69	ND	18.57	–	13.12	4.0
6	ND	20.8	ND	14.95	–	5.85	1.8
7	31.16	ND[a]	26.75	33.35	4.41	6.65	1.3 (A)/2.0(B)

ND, not detected.
[a] Theoretical detection limit of 40 was taken.

Table 5
Comparison of real-time detection methods for RSV-A (TaqMan vs. MNAzymes) following ultrasone/EasyMAG extraction of rat BAL samples.

	TaqMan Ct[a]	MNAzyme Ct[a]	ACt (Taq-MNA)
BALF 1	27.79	23.36	4.43
BALF 2	25.66	22.07	3.60
BALF 3	26.52	21.87	4.65
BALF 4	24.89	22.38	2.52
BALF 5	24.96	20.69	4.27
BALF 6	28.24	24.57	3.68
BALF 7	26.24	21.77	4.47
BALF 8	25.21	20.52	4.69
BALF 9	25.73	22.40	3.33
BALF 10	24.37	20.96	3.41
BALF 11	27.04	23.99	3.05
BALF 12	24.06	20.48	3.57
BALF 13	25.69	22.61	3.08
BALF 14	23.47	19.99	3.49
BALF 15	24.51	21.14	3.37
BALF 16	22.76	20.21	2.55
BALF 17	25.35	22.28	3.07
BALF 18	23.18	19.04	4.14
BALF 19	22.27	19.78	2.49
BALF 20	21.74	18.58	3.16
BALF 21	23.30	19.40	3.90
BALF 22	21.31	18.09	3.22
BALF 23	21.12	18.40	2.72
BALF 24	21.76	18.02	3.74
BALF 25	22.13	18.55	3.58
BALF 26	20.38	16.90	3.48
BALF 27	21.49	17.57	3.92
Mean difference			3.54
Standard deviation			0.63

[a] All Ct values have been corrected for loss of RNA (IEC-B Ct = 26.0 ± 0.71).

RSV-B (EQC-B). The average RSV-A slopes were 3.43 ± 0.05 (TaqMan) and 3.23 ± 0.05 (MNAzymes) with an average dynamic range of 7.2 ± 0.15 log and 6.98 ± 0.09 log, respectively. The average RSV-B slopes were 3.30 ± 0.05 (TaqMan) and 3.41 ± 0.02 (MNAzymes) with an average dynamic range of 6.9 ± 0.25 log and 6.9 ± 0.17 log, respectively.

The standard RNA dilution series were processed on each real-time PCR plate, with the same reaction mix used to process the samples, in order to determine the dynamic range and the performance of the real-time PCR reagents during each run.

3.5. N-gene sequencing

RSV RNA derived from clinical respiratory samples using the Ultrasone/EasyMAG method was submitted to a 1-step RT-PCR (Superscript III, Invitrogen) using 10 μl RNA and 0.2 μM primer each (N-gen-FW: 5′-GAAATGAAATTCGAAGTATTAACAT-3′ and N-gen-RV: 5′-TGAGGGTGTTTTTCAAACACTTCATA-3′). Amplicons were TOPO-TA cloned (Invitrogen) and sequenced using the same primers. In this way the N-gene region was covered where the MNAzymes and the TaqMan reagents for the detection of RSV-B annealed.

4. Results

4.1. Effect of ultrasone treatment on real-time RNA detection

One aliquot of RSV-A-GFP in PBS was subjected to 60 min of ultrasone treatment at 1 °C while a second aliquot was left untreated. Both aliquots were then extracted and tested by the RSV-A TaqMan assay. An average Ct of 21.28 ± 0.08 was observed illustrating that ultrasone treatment under these conditions had no negative effect on the detection sensitivity.

4.2. Comparison of glass beads/Qiagen and ultrasone/EasyMAG processing

Seven sputum samples were processed in parallel, using either the glass beads/Qiagen or the ultrasone/EasyMAG extraction method. An increase in detection sensitivity was observed ranging from 1.0 to 4.0 log/input RNA quantity for the ultrasone/EasyMAG-treated aliquots (Table 4). Sample 7 revealed an additional RSV-B infection using the ultrasone/EasyMAG extraction method that was not detected using the glass beads/Qiagen method (Table 4).

4.3. Comparison of TaqMan and MNAzymes on rat BAL samples

A total of 27 rat BAL samples were processed by the ultrasone/EasyMAG extraction method. The RNA was tested in parallel by the monoplex TaqMan assays, and the triplex MNAzyme assay. The average difference in IEC-B Ct values between the TaqMan and the MNAzyme assay was 0.03 Ct ± 0.41. The average difference between the TaqMan and the MNAzyme assay was 3.54 ± 0.63 Ct or approximately 1 log (Table 5).

4.4. Comparison of TaqMan and MNAzymes on clinical samples

To extend the previous observations, 71 clinical samples were processed via ultrasone/EasyMAG, followed by parallel processing with the monoplex TaqMan and the triplex MNAzyme assay. The average difference in IEC-B Ct values between the TaqMan and the MNAzyme assay was 0.21 Ct ± 0.94. The TaqMan assays detected 17 RSV-A infections and 18 RSV-B infections (Table 6) in agreement with previous clinical evaluations (Table 1), except for Sample 1 which was previously not diagnosed as RSV positive. There was no cross reactivity of the RSV reagents with other pathogens present in these clinical samples. The MNAzymes detected RSV-A with a higher sensitivity (Ct range 2.97–16.26, Table 6) corresponding to an increase in sensitivity of 0.90–4.93 logs (average 2.05 logs ± 1.01). The MNAzyme RSV-B assay detected 13/19 infections (one of which was not detected in the TaqMan assay and six samples detected in the TaqMan assay which were not detected by MNAzymes, Table 6), all with a higher sensitivity ranging from 2.50 to 14.13 Ct, corresponding to a sensitivity increase of 0.76–4.28 logs (average 1.30 logs ± 0.92, Table 6).

4.5. Analysis of TaqMan/MNAzyme RSV-B discordant samples

To further investigate the difference in RSV-B detection between the TaqMan assay and the MNAzyme assay (samples 12–17, Table 6), the MNAzyme assay was performed as a monoplex RSV-B real-time PCR confirming the initial results (Table 6) and ruling out any intra-assay competition effects. Subsequently, partial RSV-N-gene (332 bp) fragments covering the region targeted by the MNAzymes and the TaqMan assay were TOPO-TA cloned and sequenced (Fig. 2). Sample 11 revealed a clear RSV-B N-gene consensus sequence (AY353550) without mismatches for RSV-B primer and probe sequences. The other 6 samples (samples 12–17, Fig. 2) showed clear resemblance with RSV-A rather than RSV-B (consensus Y911262, Long strain), also outside the target region of the real-time reagents shown in Fig. 2 (data not shown) and were most homologous with genotype GA2. Relative to the RSV-B primer and probe sequences designed for the TaqMan, samples 14–17 showed a difference in 8 nucleotides and samples 12 and 13 in 9 nucleotides (Fig. 2).

5. Discussion

An ultrasone-based extraction with MNAzyme amplification resulted in an improved detection of RSV (2 logs on average) in

Table 6
Validation of the ultrasone/EasyMAG RNA extraction in combination with TaqMan and MNAzymes on 71 clinical samples.

	RSV-A Ct[a]			RSV-B Ct[a]			ΔCt (Taq-MNA)		log increase sensitivity	
	Viscous[b]	TaqMan	MNAzymes	TaqMan	MNAzymes	MNAzyme Monoplex RSV-B	RSV-A	RSV-B	RSV-A	RSV-B
1[c]	Yes	31.57	26.28				5.29		1.60	
2	No	27.55	20.11				7.44		2.25	
3	No	22.64	19.16				3.48		1.05	
4	No	23.11	18.23				4.88		1.48	
5	No	23.33	19.54				3.79		1.15	
6	No	24.33	21.37				2.97		0.90	
7	Yes	24.63	20.61				4.02		1.22	
8	Yes	25.09	21.25				3.84		1.16	
9	No	31.31	24.33				6.98		2.11	
10	No	34.91	25.85				9.06		2.74	
11	No	37.59	21.33	ND	25.87	26.00	16.26	14.13	4.93	4.28
12	No	20.17	16.09	37.56	ND	ND	4.08	–	1.24	–
13	Yes	19.08	11.01	33.02	ND	ND	8.07	–	2.44	–
14	No	30.97	21.07	27.54	ND	ND	9.90	–	3.00	–
15	No	30.93	22.50	34.53	ND	ND	8.43	–	2.56	–
16	No	30.94	22.18	33.73	ND	ND	8.76	–	2.66	–
17	No	25.93	18.20	27.57	ND	ND	7.73	–	2.34	–
18	No			16.31	11.89			4.42		1.34
19	No			18.45	14.99			3.46		1.05
20	No			19.90	15.42			4.49		1.36
21	No			21.52	18.91			2.61		0.79
22	No			21.55	18.81			2.74		0.83
23	No			21.76	18.77			2.99		0.90
24	No			22.17	19.67			2.50		0.76
25	No			23.68	19.79			3.90		1.18
26	No			24.84	21.56			3.28		0.99
27	No			24.99	22.24			2.75		0.83
28	No			27.18	23.25			3.93		1.19
29	No			27.31	22.52			4.79		1.45
Average							6.76	4.31	2.05	1.30
Standard deviation							3.34	3.05	1.01	0.92

[a] All Ct values have been corrected for loss of RNA with IEC-B Ct 22.85 ± 0.53.
[b] Sputum-like consistency.
[c] Not diagnosed as RSV positive in privious clinical evaluation.

different respiratory samples, including sputum. This generic nucleic acid extraction method should be readily applicable for any other respiratory pathogen (viruses and/or bacteria). Cumbersome sputum manipulations were circumvented using ultrasone waves at 1 °C (AFA, Covaris). The ultrasone method proved quick (about 30 s per sample) and required only the addition of PBS/10% DTT, without the need for glass beads or filtration. Additionally, no direct contact is made with the samples, avoiding the risk of cross-contamination. This is also true for the nucleic acid extraction, due to the simplified back- and forth movements of the extraction robot and the dedicated sample needle.

Initially TaqMan monoplex real-time assays were used due to decreasing sensitivity observed during multiplexing (data not shown). An alternative real-time PCR technology called MNAzymes seemed promising in that respect since intra-assay competition effects seemed to be minimal. The structure of the MNAzymes allows easy design of new targets by changing only the sensor arms of the partzymes. The reporter arms stay fixed for a set of five pre-designed and multiplex-optimized reporter probes, completely independent of the desired target sequence. Undoubtedly, future developments in probes and real-time detection will allow expanding this range. Additionally, MNAzyme reagents have a higher fidelity compared to TaqMan reagents (as observed with the false positive signals for RSV-B with the TaqMan assay): a functional MNAzyme requires target-specific binding of two partzymes and two PCR primers (four levels of specificity) while TaqMan assays have only three levels of specificity (two primers and one target-specific probe). Although only a limited amount of samples with co-infections or non-RSV pathogens were processed, no cross reactivity of the RSV reagents (TaqMan or MNAzymes) was observed.

In conclusion, the ultrasone/EasyMAG-MNAzyme method should allow an average increase in sensitivity around 2 logs compared to currently available methods with a higher specificity compared to TaqMan-based assays. The dynamic range of the assay,

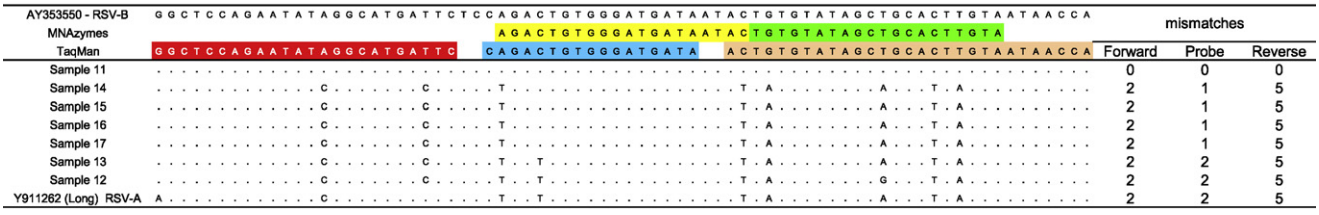

Fig. 2. Alignment of the TaqMan RSV-B primers and probes and the MNAzyme RSV-B partzymes with the corresponding N-gene nucleotide sequence obtained from sequencing the N-gene regions from specimens 11–17. Yellow: Sensor arm partzyme A; Green: Sensor arm partzyme B; Red: TaqMan forward primer RSV-B; Blue: TaqMan RSV-B probe; Brown: TaqMan reverse primer RSV-B.

monitored by External Quantification Controls, spanned on average 7.2 logs for RSV-A and 6.9 logs for RSV-B. Additionally, an Internal Extraction Control was developed allowing for correction of RNA loss during extraction.

Conflict of interest

The authors declare no conflict of interest.

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