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RESEARCH ARTICLE

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Development and evaluation of a panel of multiplex one-tube nested real time PCR assay for simultaneous detection of 14 respiratory viruses in five reactions

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Abstract

Multiplex real-time quantitative polymerase chain reaction (mRT-qPCR) assay is commonly used to detect respiratory viruses, however, the sensitivity is limited for most reports. A panel of locked nucleic acid based multiplex closed one-tube nested real-time PCR (mOTNRT-PCR) assay consisting of five separate internally controlled RT-qPCR assays was developed for detection of 14 respiratory viruses. The sensitivity and reproducibility of mOTNRT-PCR panel were evaluated using plasmid standards and the specificity was evaluated using clinical samples. The clinical performance of mOTNRT-PCR panel was further evaluated with 468 samples collected from patients with an acute respiratory infection and compared with individual real-time PCR (RT-qPCR) assay. The analytical sensitivities of mOTNRT-PCR panel ranged from 2 to 20 copies/reaction, and no cross-reaction with common respiratory viruses was observed. The coefficients of variation of intra-assay and inter-assay were between 0.35% and 8.29%. Totally 35 clinical samples detected by mOTNRT-PCR assay panel were missed by RT-qPCR and confirmed true positive by sequencing of nested PCR products. The mOTNRT-PCR assay panel provides a more sensitive and high-throughput method for the detection of 14 respiratory viruses.

KEYWORDS

locked nucleic acid (LNA), multiplex one-tube nested real-time PCR (mOTNRT-PCR), respiratory virus

1 | INTRODUCTION

Respiratory viral infection causes widespread hospitalization rates and mortality rates in children and especially causes more deaths worldwide in children less than 5 years.[1,2] The common respiratory virus are respiratory syncytial virus (RSV), human rhinovirus (HRV), human parainfluenza virus (PIV), influenza A virus (FluA), influenza B virus (FluB), adenovirus (ADV), human coronavirus (HCoV), human metapneumovirus (HMPV), human bocavirus (HBoV), and enterovirus (EV).[3] In recent years, many mono real-time quantitative PCR (RT-qPCR), multiplex reverse transcription PCR and multiplex real-time quantitative PCR (mRT-qPCR) were widely applied in well-equipped laboratories for detection of respiratory virus. The mono RT-qPCR[4] has acceptable sensitivity and specificity for detection of respiratory virus, but only detects one virus per tube. The multiplex reverse transcription PCR[1] enables the detection of virus coinfection but is labor-intensive and susceptible to cross-contamination as it requires post PCR analysis. The mRT-qPCR[5] has the advantages of

TABLE 1 Primers and probes used in the multiplex closed one-tube nested real-time polymerase chain reaction assay panel

Assay	Primer/Probe	Sequence (5′-3′)	Product size, bp	Gene	References
1	RSV-outer-F[a]	CA+CW+GAA+GA+TG+CWAAT+CATAAATTCA	374	N	Kim et al[8]; Sanghavi et al[9]
	RSV-outer-R[a]	CW+GA+TC+TRT+CT+CCT+GCTGCTA			
	RSV-inner-F	CACWGAAGATGCWAATCATAAATTCA	89		
	RSV-inner-R	GTATYTTTATRGTGTCTTCYCTTCCTAACC			
	RSV-Probe	FAM-TAATAGGTATGTTATATGCKATGTC-BHQ1			
	HRV-outer-F[a]	HC+AA+GYA+CTTCT+GTYWCCCCSG	397	5′UTR	Wisdom et al[10]; Hammitt et al[11]
	HRV-outer-R[a]	GA+AA+CAC+GGA+CA+CCCAAAGTAGT			
	HRV-inner-F	TGGACAGGGTGTGAAGAGC	144		
	HRV-inner-R	CAAAGTAGTCGGTCCCATCC			
	HRV-Probe	CY5-TCCTCCGGCCCCTGAATG-BHQ3			
	HMPV-outer-F[a]	CATATAAG+CA+T+G+C+TA+TATTAAAA+GAGTCTC	475	N	Maertzdorf et al[4]; Dare et al[12]
	HMPV-outer-R[a]	GT+GAATATTAA+G+G+CA+C+CTACACATAATAARA			
	HMPV-inner-F	CATATAAGCATGCTATATTAAAAGAGTCTC	163		
	HMPV-inner-R	CCTATTTCTGCAGCATATTTGTAATCAG			
	HMPV-Probe	VIC-TGYAATGATGAGGGTGTCACTGCGGTTG-BHQ1			
2	PIV1-outer-F[a]	A+GGA+TGT+G+CA+GATATAGGGAA	195	HN	Perrott et al[13]
	PIV1-outer-R[a]	GT+CT+CA+TT+CA+CAGTGGGCAA			
	PIV1-inner-F	TTTAAACCCGGTAATTTCTCATACCT	81		
	PIV1-inner-R	CCCCTTGTTCCTGCAGCTATT			
	PIV1-Probe	FAM-TGACATCAACGACAACAGGAAATCATGTTCTG-BHQ1			
	PIV2-outer-F[a]	AA+C AA+T+CT+G+CTG CAGCAT TT	158	HN	Sanghavi et al[9]; Bellau-Pujol et al[14]
	PIV2-outer-R[a]	C+GT+GG+CA+TA+AT+CTTCTTTTT			
	PIV2-inner-F	CCATTTACCTAAGTGATGGAA	116		
	PIV2-inner-R	CGTGGCATAATCTTCTTTTT			
	PIV2-Probe	CY5-AATCGCAAAAGCTGTTCAGTCAC-BHQ3			
	PIV3-outer-F[a]	T+TA+CARA+TA+GG+GATAATAACTGT	151	HN	Sanghavi et al[9]; Bellau-Pujol et al[14]
	PIV3-outer-R[a]	CTTT+GG+GA+GTT+GAACACAGTT			
	PIV3-inner-F	TTACARATAGGGATAATAACTGT	115		
	PIV3-inner-R	TTAGGAGTGCTAGAGAACAT			
	PIV3-Probe	VIC-AAACTCAGACTTGGTACCTGACTTAAAT-BHQ1			
3	EV-outer-F[a]	HC+AA+GYA+CTTCT+GTYWCCCCSG	402	5′UTR	Wisdom et al[10]; Brittain-Long et al[15modified]
	EV-outer-R[a]	GA+AA+CAC+GGA+CA+CCCAAAGTAGT			
	EV-inner-F	GTGYGAAGAGTCTATTGAGCTA	141		
	EV-inner-R	ACACCCAAAGTAGTCGGTT			
	EV-Probe	FAM-CGGCCCCTGAATGCGGCTAATC-BHQ1			
	FluA-outer-F[a]	TCA+AAGC+CGAGATCGCGCAG	189	M	This study
	FluA-outer-R[a]	G+CATT+TTG+GA+CAAAGCGTCTAC			Lee et al[16modified]
	FluA-inner-F	GAATGGCTAAAGACAAGACCAAT	118		
	FluA-inner-R	GCATTTTGGACAAAGCGTCTAC			
	FluA-Probe	CY5-AGTCCTCGCTCACTGGGCACGGTG-BHQ3			
	FluB-outer-F[a]	TG+CCT+CCAC+AAAAATACGG	218	HA	Perrott et al[13]
	FluB-outer-R[a]	C+C+TG+CAAT+CATTCCTTCCCA			
	FluB-inner-F	AAATACGGTGGATTAAATAAAAGCAA	170		
	FluB-inner-R	CCA GCA ATA GCT CCG AAG AAA			
	FluB-Probe	VIC-CACCCATATTGGGCAATTTCCTATGGC-BHQ1			
4	HCoV229E-outer-F[a]	CA+GT+CAAAT+GGGCTGATGCA	638	N	Hammitt et al[11]; Li et al[17modified]
	HCoV229E-outer-R[a]	A+CGA+GAA+GG+CTTAGGAGTAC			
	HCoV229E-inner-F	CAGTCAAATGGGCTGATGCA	76		
	HCoV229E-inner-R	AAAGGGCTATAAAGAGAATAAGGTATTCT			
	HCoV229E-Probe	FAM-CCCTGACGACCACGTTGTGGTTCA-BHQ1			

TABLE 1 (Continued)

Assay	Primer/Probe	Sequence (5′-3′)	Product size, bp	Gene	References
	HCoVOC43-outer-F[a]	ATT+GCA+CCA+GGAGTCCCA	316	N	Hammitt et al[11]; Li et al[17]modified
	HCoVOC43-outer-R[a]	TT+CC+T+GA+GC+CTT+CAATATAGTAAC			
	HCoVOC43-inner-F	ATGAGGCTATTCCGACTAGG	72		
	HCoVOC43-inner-R	TTCCTGAGCCTTCAATATAGTAAC			
	HCoVOC43-Probe	CY5-TCCGCCTGGCACGGTACTCCCT-BHQ3			
	HCoVNL63-outer-F[a]	A+GATGA+GCA+GATT+GGTTATTGG	211	N	Bastein et al[18]; Esposito et al[19]modified
	HCoVNL63-outer-R[a]	ATTACGTTT+GC+GATTA+C+CAAGACT			
	HCoVNL63-inner-F	GACCTTAAATTCAGACAACGTTCT	96		
	HCoVNL63-inner-R	ATTACGTTTGCGATTACCAAGACT			
	HCoVNL63-Probe	VIC-TAACAGTTTTAGCACCTTCCTTAGCAACCCAAA CA-BHQ1			
5	ADV-outer-F[a]	TACATGCA+CATCKCSGGVCAGGA	113	Hexo	Sanghavi et al[9]; Lam et al[20]modified
	ADV-outer-R[a]	GT+GG+GGTTYCT+GAACTTGT			
	ADV-inner-F	GCY TCG GAG TAC CTG AG	89		
	ADV-inner-R	GTGGGGTTYCTGAACTTGT			
	ADV-Probe	FAM-CTGGTGCAGTTCGCCCGTGCCA-BHQ1			
	HBoV-outer-F[a]	GA+C+TAA+GCAA+GAG+GAATGCTA	214	NS1	Chieochansin et al[21]; Jansen et al[22]modified
	HBoV-outer-R[a]	TCT+GC+GAT+CT+CT+ATATT+GAAGG			
	HBoV-inner-F	AAATCTCTTCTGGCTACACG	136		
	HBoV-inner-R	TCTGCGATCTCTATATTGAAGG			
	HBoV-Probe	CY5-ATGTTGCCGCCAGTAACTCCACC-BHQ3			
	Rnasep-F	AGATTTGGACCTGCGAGCG	65	Rnasep	Zhang et al[5]
	Rnasep-R	GAGCGGCTGTCTCCACAAGT			
	Rnasep-Probe	VIC-TTCTGACCTGAAGGCTCTGCGCG-BHQ1			

Note: modified, the internal primers from the literature were modified by removing a few bases.
Abbreviations: ADV, adenovirus; BHQ, Blackblack hole quencher; CY5, Cyanine-5; EV, enterovirus; FAM, 6-carboxyfluorescein; FluA, influenza A virus; FluB, influenza B virus; HRV, human rhinovirus; HBoV, human bocavirus; HCoV, human coronavirus; HMPV, human metapneumovirus; LNA, locked nucleic acid; PIV, human parainfluenza virus; RSV, respiratory syncytial virus; VIC, 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein; 2′-chloro-7′-phenyl-1,4-dichloro.
[a]'+' denotes a nucleotide with LNA modification.

high throughput detection and labor-saving, however, the sensitivity is not yet adequate for assessing clinical specimens with a low viral load.

In our previous study, we successfully applied the locked nucleic acid (LNA) technology to develop the OTNRT-PCR assay to detect RSV[6] and the mOTNRT-PCR assay to detect RSV, HRV, and HMPV in one tube,[7] however, we only included 3 respiratory viruses. Aiming to detect more respiratory viruses and improve the sensitivity of the mRT-qPCR, in the present study, we adopted the design of locked nucleic acid-modified primers and developed a multiplex one-tube nested real-time PCR (mOTNRT-PCR) assay panel with the advantages of higher sensitivity, easier performance, and better cost-effectiveness.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

A total of 468 nasopharyngeal aspirates samples were collected from inpatients presenting with acute respiratory symptoms at the Children's Hospital of Hebei Province (China) from June to October, 2017 and from February to June, 2018. Of those 264 (56.41%) were female and 204 (43.59%) were male. Ages ranged from 34 days to 11 years old and 453 (96.79%) were under 5 years old. Briefly, 1.8 mL of nasopharyngeal aspirate was collected in 2 mL of transport medium containing sodium glycerophosphate, sodium thioglycolate, cysteine hydrochloride, $CaCl_2$, methylene blue, and agar and stored at −80°C. The study was conducted with the approval of the Ethics Committee of Children's Hospital of Hebei Province, and written informed consent was obtained from the children's parents.

2.2 | Nucleic acid extraction

Total RNA/DNA was extracted from 200 µL of clinical samples using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). The extracts were eluted in 50 µL of DNase-free and RNase-free diethylpyrocarbonate(DEPC)-treated water and stored at −80°C until use.

2.3 | Primers and probes design for mOTNRT-PCR assay panel

The mOTNRT-PCR assay panel consisted of five separate internally controlled RT-qPCR assays targeting 14 respiratory viruses. Assay 1, RSV, HRV, and HMPV; assay 2, PIV1, PIV2, and PIV3; assay 3, EV, FluA, and FluB; assay 4, HCoV229E, HCoVOC43 and HCoVNL63; assay 5, ADV, HBoV, and Rnasep. All the sequences of LNA-outer primer, inner primer, and probe were obtained either from the reported literature[4,8–22] or slightly modified by removing a few bases of reported inner primers. All the outer primers were modified by LNA. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was then performed to ensure the specificity of primers and probes. All the primers probes were tested using Oligo7 to minimize duplex formation and hairpin formation. The primers and probes were synthesized from Sangon Biotech (Shanghai, China). The detailed sequences of primer and probe are shown in Table 1.

2.4 | Preparation of plasmid standards

PCR products of outer primers of mOTNRT-PCR assay panel targeting 14 respiratory viruses were cloned respectively by TsingKe Biotech Corp (Beijing, China) and confirmed by sequencing. The recombinant plasmids were quantified using a Qubit dsDNA HS Assay Kits (Life technologies Invitrogen). For mOTNRT-PCR assay panel, each standard plasmid was adjusted to a concentration of 10^7 copies/μL and equally mixed in each assay. The mixed plasmids were used to prepare 10-fold serial dilutions for sensitivity analysis.

2.5 | mOTNRT-PCR assay panel

Each assay of mOTNRT-PCR panel was carried out in a 10 μL reaction volume using a One-Step RT-PCR kit (Qiagen, Hilden, Germany), containing 2 μL of extracted sample, 2 μL of the 5× buffer, 0.4 μL of the dNTP mix, 0.8 μL of the enzyme mix, 0.05 μL of RRI (Takara, Dalian, China). One microliter of 10× Primers and probes mix, and 3.75 μL of RNase-free water. PCR amplification was performed on the CFX96 Real-Time PCR System (Bio-Rad) and the conditions were: 50°C for 30 minutes, a 15 minutes denaturation step at 95°C, and 10 cycles at 94°C for 30 seconds, 64°C for 40 seconds and 72°C for 40 seconds, followed by 40 cycles at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds, with fluorescent readings taken at the annealing phase of the last 40 cycles. Cycle threshold (C_t) values were calculated using the software at the automatic threshold setting. Positive and negative controls were included in each run. The results were defined as positive if the C_t value was not higher than 35 for all the reactions.

2.6 | Analytical sensitivity, reproducibility, and specificity of the mOTNRT-PCR assay panel

Ten-fold dilutions of mixed recombinant plasmids ranging from 10^7 to 10^0 copies/μL were used to analyze the sensitivity of the mOTNRT-PCR assay panel in five reactions. In a 10 μL reaction system, the reaction mixture was prepared to contain 5 μL of 2× Qiagen Multiplex PCR Master Mix (Qiagen), 2 μL of mixed plasmid, 1 μL of 10× Primers and probes mix, and 2 μL of RNase-free water. PCR amplification was performed on the CFX96 Real-Time PCR System (Bio-Rad) and the conditions were: a 15 minutes denaturation step at 95°C, and 10 cycles at 94°C for 30 seconds, 64°C for 40 seconds and 72°C for 40 seconds, followed by 40 cycles at 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds, with fluorescent readings taken at the annealing phase of the last 40 cycles. The reproducibility of the mOTNRT-PCR was evaluated using three concentrations of mixed recombinant plasmids (10^6, 10^4, and 10^2 copies/μL). Intra-assay reproducibility and inter-assay reproducibility of mOTNRT-PCR were tested using three plasmids in three replicates and in three different days within a week.The specificity was retrospectively evaluated by using archived common respiratory viruses-positive samples previously tested by respiratory pathogen 13 detection kit (13 × kit) in our lab.[23]

2.7 | Comparison of clinical performance between the mOTNRT-PCR assay panel and the RT-qPCR assay

A total of 468 clinical samples were detected by mOTNRT-PCR. For comparison, the previously published RT-qPCR assays[9,11–13,15,16,19,22] were also performed in parallel. Sequencing of traditional two-step nested PCR[14,20,24] products were performed to resolve discrepant results among the two assays.

2.8 | Statistical analysis

IBM SPSS Statistics, version 21 (IBM Corporation, NY) was used to perform statistical analysis. The results were analyzed using χ^2 tests, and value of $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Sensitivity, reproducibility, and specificity of the mOTNRT-PCR assay panel

The sensitivity was analyzed using 10-fold dilutions of mixed recombinant plasmids ranging from 10^7 to 10^0 copies/μL, yielding 20 copies/reaction for PIV1, PIV2, and PIV3, and 2 copies/reaction for other 11 viruses type/subtype. The coefficient of variation

TABLE 2 The intra-assay and inter-assay reproducibility of the multiplex closed one-tube nested real-time polymerase chain reaction assay panel

Virus	Intra-ssay CV (%)			Inter-ssay CV (%)		
	2×10^6 (copies/reaction)	2×10^4 (copies/reaction)	2×10^2 (copies/reaction)	2×10^6 (copies/reaction)	2×10^4 (copies/reaction)	2×10^2 (copies/reaction)
RSV	3.66	0.64	1.57	3.67	3.19	0.83
HRV	0.74	1.36	0.51	1.87	1.91	2.13
HMPV	3.39	1.33	2.01	3.45	1.91	1.20
EV	0.35	0.62	2.18	2.93	3.75	2.05
FluA	1.12	0.62	1.35	8.29	3.57	2.58
FluB	2.09	1.87	1.59	5.48	2.96	2.10
PIV1	1.61	1.03	1.12	4.26	2.09	1.05
PIV2	1.02	1.23	1.05	1.90	2.04	1.93
PIV3	0.79	1.07	0.99	1.46	1.55	1.55
HCoV229E	2.49	1.13	0.91	2.06	1.97	1.12
HCoVOC43	1.50	1.75	0.97	1.50	2.54	1.99
HCoVNL63	3.23	1.07	1.50	2.05	1.81	1.21
ADV	2.66	1.75	0.58	3.92	2.05	2.61
HBoV	3.25	1.39	0.89	3.60	4.76	2.36

Abbreviations: ADV, adenovirus; CV, coefficient of variations; EV, enterovirus; FluA, influenza A virus; FluB, influenza B virus; HCoV, human coronavirus; HMPV, human metapneumovirus;
HRV, human rhinovirus; HCoV, human coronavirus; PIV, human parainfluenza virus; RSV, respiratory syncytial virus.

(CV) for intra-assay and inter-assay ranged from 0.35% to 3.66% and 0.83% to 8.29% (Table 2). A total of 407 archived positive clinical samples retrospectively detected by 13 kit[23] were used to assess the specificity of the mOTNRT-PCR. No cross-reaction was obtained (data not shown), indicating high specificity for the mOTNRT-PCR panel.

3.2 | Comparison of clinical performance between the mOTNRT-PCR assay panel and the RT-qPCR assay

All of the 468 clinical samples were tested by the mOTNRT-PCR assay panel and the RT-qPCR assay in parallel. Totally, 427 of 468 (91.24%) specimens were positive by mOTNRT-PCR, including 262 (55.98%), 135 (28.85%), 29 (6.20%), and 1(0.21%) samples were positive for a single virus, 2 viruses, 3 viruses, and 4 viruses, respectively. As shown in Table 3 and Figure 1, HRV, RSV, and PIV3 were the most frequently viruses detected by mOTNRT-PCR assay with 179 (38.25%), 97 (20.73%), and 42 (8.97%), respectively. RT-qPCR assay detected HRV 174 (37.18%), RSV 94 (20.09%), and PIV3 38 (8.12%), respectively. HCoVNL63 was not detected by either assay. A total of 35 clinical samples were positive by the mOTNTR-PCR assay but negative by the RT-qPCR assay using the cutoff of the RT-qPCR assay with Ct > 40. Sequencing of traditional two-step nested PCR[14,20,24] products confirmed that 35 samples were true positives. The comparison of percentages of detection of specimens with $C_t \leq 40$ between the mOTNRT-PCR and mono RT-qPCR for a total of 468 clinical samples is displayed in Figure 1. A total of 69 clinical samples detected by mOTNRT-PCR assay were missed by RT-qPCR using the positivity cutoff (Ct = 35) of the RT-qPCR assay. Among them, 34 samples had a CT range of 35 to 40 detected by RT-qPCR assay. The sensitivities of detection of the different viruses were 100%, and the specificities were more than 98% between the mOTNTR-PCR assay and the RT-qPCR assay. Concordance between the two assays for all viruses was more than 98%, and the kappa correlation ranged from 0.66 to 0.98.

4 | DISCUSSION

LNA has been reported to modify primers and probes in many studies, thus increasing the maximum annealing temperature of primers/probes and improving amplification sensitivity and specificity.[25,26] In the present study, LNA-modified outer primers (without changing the sequences) were used to develop an mOTNRT-PCR assay panel for simultaneous detection of 14 respiratory viruses in five reactions. The purpose of this design was to maximize the difference in the annealing temperatures (64°C vs 54°C) between the outer primer

TABLE 3 Clinical performance of the multiplex closed one-tube nested real-time polymerase chain reaction panel compared with the real-time quantitative polymerase chain reaction

	No. of samples		C_t value	Clinical performance of the mOTNRT-PCR compared with the RT-qPCR			
Virus	mOTNRT-PCR	RT-qPCR	mOTNRT-PCR vs RT-qPCR	Sensitivity, %	Specificity, %	Accordance rate, %	Kappa value
RSV	97/468	94/468	12.37-35.00 vs 18.03-39.56	100	99.20	99.36	0.98
HRV	179/468	174/468	7.58-33.43 vs 21.01-39.66	100	98.30	98.93	0.98
HMPV	36/468	34/468	10.79-34.85 vs 18.62-38.84	100	99.54	99.57	0.97
EV	18/468	15/468	13.06-32.13 vs 19.20-37.65	100	99.33	99.36	0.91
FluA	34/468	32/468	10.01-29.53 vs 19.65-37.39	100	99.54	99.57	0.97
FluB	15/468	13/468	13.14-34.15 vs 21.21-39.18	100	99.56	99.57	0.93
PIV1	23/468	21/468	8.38-34.99 vs 18.31-39.69	100	99.55	99.57	0.95
PIV2	6/468	3/468	20.06-31.63 vs 36.14-39.89	100	99.35	99.35	0.66
PIV3	42/468	38/468	12.77-31.03 vs 24.00 −39.61	100	99.07	99.14	0.95
HCoV229E	5/468	3/468	19.98-25.69 vs 28.65-34.98	100	99.57	99.57	0.75
HCoVOC43	10/468	9/468	10.37-28.94 vs 19.04-36.62	100	99.78	99.79	0.95
HCoVNL63	0/468	0/468	NA	NA	100	100	NA
ADV	35/468	31/468	13.66-34.52 vs 22.90-38.63	100	99.08	99.15	0.93
HBoV	18/468	16/468	9.40-35.00 vs 16.02-39.01	100	99.56	99.57	0.94

Abbreviations: ADV, adenovirus; CV, coefficient of variations; FluA, influenza A virus; FluB, influenza B virus; HBoV, human bocavirus; HMPV, human metapneumovirus; HRV, human rhinovirus; HCoV, human coronavirus; mOTNRT-PCR, multiplex closed one-tube nested real-time polymerase chain reaction; PIV, human parainfluenza virus; RSV, respiratory syncytial virus; RT-qPCR, real-time quantitative polymerase chain reaction.

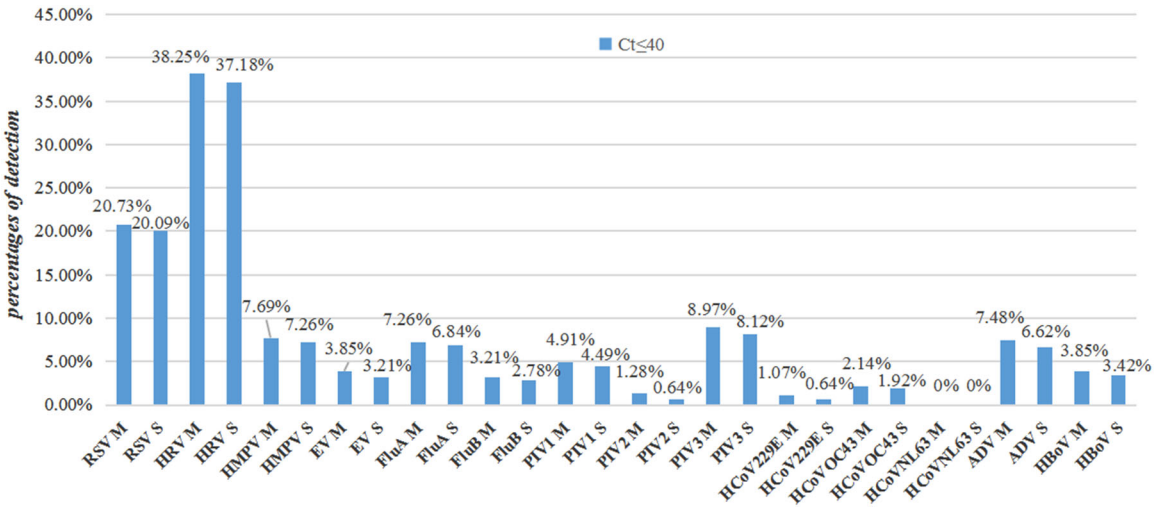

FIGURE 1 The percentages of detection of specimens with $C_t \leq 40$ analyzed by mOTNRT-PCR panel (M) and mono RT-qPCR (S) for a total of 468 clinical samples. A total of 34 samples had a C_t range of 35 to 40 detected by RT-qPCR assay. mOTNRT-PCR, multiplex closed one-tube nested real-time polymerase chain reaction; RT-qPCR, real-time quantitative polymerase chain reaction

modified by LNA and inner primer sets, allowing one-step nested amplification successfully to be carried out via temperature switch PCR (TSP).[27,28] To our best knowledge, this is the first report on the simultaneous detection of 14 respiratory viruses in five closed one-tube reactions using LNA.

For the mOTNRT-PCR assay panel, the working concentration of LNA-modified outer primers, inner primers, and probes in each reaction was carefully optimized, enabling minimize the competition between the outer primers and inner primers, as well as primers and probes of targeted viruses in each reaction. The reaction parameters and the running conditions of the mOTNRT-PCR assay were also optimized, allowing the five reactions to perform simultaneously. This study demonstrated that mOTNRT-PCR assay panel revealed high sensitivity range from 2 to 20 copies/reaction of 14 respiratory viruses using 10-fold dilutions of mixed recombinant plasmids, high specificity, and reliable reproducibility (Table 3).

The mOTNRT-PCR assay panel was further evaluated and compared with the RT-qPCR assay using 468 clinical samples. As shown in Table 3 and Figure 1, HRV, RSV, and PIV3 were the most commonly found respiratory viruses by the two assays which is consistent with the previous report.[17] The reported RT-qPCR thresholds typically ranged from 35 to 40. A total of 69 clinical samples detected by the mOTNRT-PCR assay were missed by RT-qPCR using the positivity cutoff of the RT-qPCR assay set at a C_t of 35. When a C_t cutoff of 40 for RT-qPCR was used, 35 samples were deemed to be positive by mOTNRT-PCR assay but negative by RT-qPCR (Table 3 and Figure 1). Moreover, the range of C_t values of the mOTNRT-PCR assay (7.58-35.00) was smaller than the RT-qPCR (16.02-39.89) in those positive samples using both assays (Table 3), suggesting that the mOTNRT-PCR assay is more sensitive than the reported RT-qPCR assay in detecting clinical specimens. Besides, the mOTNRT-PCR can detect 14 common respiratory viruses in five reactions in 2.7 hours and cost $7.5 (excluding nucleic acid extraction) for one sample. Compared with the mono RT-qPCR assay for detecting one virus per tube, the mOTNRT-PCR has the advantages of being highly sensitive, easy to operate, rapid, and cost-effective. In addition, the human RNasep gene as an internal control can validate the RNA extraction procedure and prevent errors in the process of sampling and RT-PCR.

As to the mixed infections, 165 of 468 (35.26%) specimens were detected by the mOTNRT-PCR, including 135 of 468 (28.85%), 29 of 468 (6.20%) and 1 of 468 (0.21%) samples involved in 2 viruses, 3 viruses, and 4 viruses, respectively. Virus coinfections were more frequently detected in children less than 5 years old (32.05%, 150/468) compared with the older children (3.21%; 15/468; $P < 0.05$). For children less than 5 years, RSV and HRV (7.05%, 33/468) were the most commonly found in co-infections, which is similar to the previous report.[29]

The mOTNRT-PCR assay panel has limitations. First, it requires five parallel assays with only moderate throughput in each assay. Second, we only evaluate the clinical specimens from children and the clinical data is incomplete. Future research will attempt to develop a mOTNRT-PCR assay to detect as many viruses as possible in one assay, evaluate a large number of samples from different populations and integrate clinical information for more comprehensive analysis.

In summary, the mOTNRT-PCR assay developed in this study using LNA is a sensitive, labor-saving, and cost-effectiveness method for detecting 14 respiratory viruses in five reactions. It may have great potential for routine surveillance of respiratory virus infection in China.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ETHICS STATEMENT

All aspects of the study were performed in accordance with national ethics regulations and approved by the Institutional Review Boards of National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China.

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REFERENCES

1. Li J, Qi S, Zhang C, et al. A two-tube multiplex reverse transcription PCR assay for simultaneous detection of sixteen human respiratory virus types/subtypes. *BioMed Res Int*. 2013;2013:327620.
2. Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. Estimates of worldwide distribution of child deaths from acute respiratory infections. *Lancet Infect Dis*. 2002;2(1):25-32.
3. Radko S, Ian Stuart J, Zahariadis G. Evaluation of three commercial multiplex assays for the detection of respiratory viral infections. *J Virol Methods*. 2017;248:39-43.
4. Maertzdorf J, Wang CK, Brown JB, et al. Real-time reverse transcriptase PCR assay for detection of human metapneumoviruses from all known genetic lineages. *J Clin Microbiol*. 2004;42(3):981-986.
5. Zhang D, Mao H, Lou X, et al. Clinical evaluation of a panel of multiplex quantitative real-time reverse transcription polymerase chain reaction assays for the detection of 16 respiratory viruses associated with community-aacquired pneumonia. *Arch Virol*. 2018; 163(10):2855-2860.
6. Zhao L, Wang J, Li G, et al. A highly sensitive 1-tube nested real time RT-PCR assay using LNA-modified primers for detection of respiratory syncytial virus. *Diagn Microbiol Infect Dis*. 2019;93(2):101-106.
7. Feng Z, Zhao L, Wang J, et al. A multiplex one-tube nested real time RT-PCR assay for simultaneous detection of respiratory syncytial virus, human rhinovirus and human metapneumovirus. *Virol J*. 2018;30(1):167.
8. Kim H, Kang N, An K, Kim D, Koo J, Kim MS. MRPrimerV: a database of PCR primers for RNA virus detection. *Nucleic Acids Res*. 2017; 45(D1):D475-D481.
9. Sanghavi SK, Bullotta A, Husain S, Rinaldo CR. Clinical evaluation of multiplex real-time PCR panels for rapid detection of respiratory viral infections. *J Med Virol*. 2012;84(1):162-169.
10. Wisdom A, Leitch ECM, Gaunt E, Harvala H, Simmonds P. Screening respiratory samples for detection of human rhinoviruses (HRVs) and enteroviruses: comprehensive VP4-VP2 typing reveals high incidence and genetic diversity of HRV species C. *J Clin Microbiol*. 2009;47(12): 3958-3967.
11. Hammitt LL, Kazungu S, Welch S, et al. Added value of an oropharyngeal swab in detection of viruses in children hospitalized with lower respiratory tract infection. *J Clin Microbiol*. 2011;49(6):2318-2320.
12. Dare R, Sanghavi S, Bullotta A, et al. Diagnosis of human metapneumovirus infection in immunosuppressed lung transplant recipients and children evaluated for pertussis. *J Clin Microbiol*. 2007;45(2):548-552.
13. Perrott P, Smith G, Ristovski Z, Harding R, Hargreaves M. A nested real-time PCR assay has an increased sensitivity suitable for detection of viruses in aerosol studies. *J Appl Microbiol*. 2009;106(5):1438-1447.
14. Bellau-Pujol S, Vabret A, Legrand L, et al. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. *J Virol Methods*. 2005;126(1-2):53-63.
15. Brittain-Long R, Nord S, Olofsson S, Westin J, Anderson LM, Lindh M. Multiplex real-time PCR for detection of respiratory tract infections. *J Clin Virol*. 2008;41(1):53-56.
16. Lee HK, Loh TP, Lee CK, Tang JWT, Chiu L, Koay ESC. A universal influenza A and B duplex real-time RT-PCR assay. *J Med Virol*. 2012; 84(10):1646-1651.
17. Li J, Mao NY, Zhang C, et al. The development of a GeXP-based multiplex reverse transcription-PCR assay for simultaneousdetection of sixteen human respiratory virus types/subtypes. *BMC Infect Dis*. 2012;12:189.
18. Bastien N, Robinson JL, Tse A, Lee BE, Hart L, Li Y. Human coronavirus NL-63 infections in children: a 1-year study. *J Clin Microbiol*. 2005;43(9):4567-4573.
19. Esposito S, Bosis S, Niesters HGM, et al. Impact of human coronavirus infections in otherwise healthy children who attended an emergency department. *J Med Virol*. 2006;78(12):1609-1615.
20. Lam WY, Yeung ACM, Tang JW, et al. Rapid multiplex nested PCR for detection of respiratory viruses. *J Clin Microbiol*. 2007;45(11): 3631-3640.
21. Chieochansin T, Chutinimitkul S, Payungporn S, et al. Complete coding sequences and phylogenetic analysis of human bocavirus (HBoV). *Virus Res*. 2007;129(1):54-57.
22. Jansen RR, Schinkel J, Koekkoek S, et al. Development and evaluation of a four-tube real time multiplex PCR assay covering fourteen respiratory viruses, and comparison to its corresponding single target counterparts. *J Clin Virol*. 2011;51(3):179-185.
23. Zhao M, Li G, Zhang D, et al. Clinical evaluation of a new single-tube multiplex reverse transcription PCR assay for simultaneous detection of 11 respiratory viruses, Mycoplasma pneumoniae and Chlamydia in hospitalized children with acute respiratory infections. *Diagn Micr Infec Dis*. 2017;88(2):115-119.
24. Manning A, Russell V, Eastick K, et al. Epidemiological profil and clinical associations of human bocavirus and other human parvoviruses. *J Infect Dis*. 2006;194(9):1283-1290.
25. Osterback R, Tevaluoto T, Ylinen T, et al. Simultaneous detection and differentiation of human rhino and enteroviruses in clinical specmens by real-time PCR with locked nucleic acid probes. *J Clin Microbiol*. 2013;51(12):3960-3967.
26. Ballantyne KN, van Oorschot RAH, Mitchell RJ. Locked nucleic acids in PCR primers increase sensitivity and performance. *Genomics*. 2008; 91(3):301-305.
27. Niu P, Qi S, Yu B, et al. Development of a highly sensitive real-time nested RT-PCR assay in a single closed tube for detection of enterovirus 71 in hand, foot and mouth disease. *Arch Virol*. 2016; 161(11):3003-3010.
28. Shen X, Qiu F, Zhao H, et al. A novel and highly sensitive real-time nested RT-PCR assay in a single closed tube for detection of enterovirus. *Diagn Microbiol Infect Dis*. 2018;90(3):181-185.
29. Jiang W, Wu M, Zhou J, et al. Etiologic spectrum and occurrence of coinfections in children hospitalized with community-acquired pneumonia. *BMC Infect Dis*. 2017;17(1):787.

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