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BRIEF REPORT

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-acquired pneumonia

Dan Zhang[1,2] · Haiyan Mao[2] · Xiuyu Lou[2] · Junhang Pan[2] · Hao Yan[2] · Hongfeng Tang[3] · Yan Shu[3] · Yun Zhao[3] · Xiaoli Cheng[4] · Hong Tao[5] · Yanjun Zhang[2] · Xuejun Ma[1]

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Abstract

We developed a panel of multiplex quantitative real-time reverse transcription polymerase chain reaction (mqRT-PCR) assay consisting of seven internally controlled qRT-PCR assays to detect 16 different respiratory viruses. We compared the new mqRT-PCR with a previously reported two-tube mRT-PCR assay using 363 clinical sputum specimens. The mqRT-PCR assay performed comparably with the two-tube assay for most viruses, offering the advantages of quantitative analysis, easier performance, lower susceptibility to contamination, and shorter turnaround time in laboratories equipped with conventional real-time PCR instrumentation, and it could therefore be a valuable tool for routine surveillance of respiratory virus infections in China.

Community-acquired pneumonia (CAP) is a substantial public-health problem and a leading cause of illness and death in people of all ages [1–5]. Respiratory viruses are the most commonly detected causes of pneumonia [6, 7]. Rapid identification and accurate laboratory diagnosis are therefore crucial to guide effective treatment and prevention decisions. Diagnosis of CAP in both clinical-care and public-health settings has greatly advanced in recent years. Many commercial multiplex PCR assays for CAP-causing viruses have been reported, including ePlex Respiratory Pathogen Panel (GenMark Diagnostics, Carlsbad, CA, USA) [8]; xTAG RVP, RVP fast (Luminex Molecular Diagnostics, Toronto, Canada); Resplex II (QIAGEN, Mississauga, Canada); FilmArray Respiratory Panel (Idaho Technology Inc., Salt Lake City, UT, USA) [9, 10]; Anyplex™ II RV16 and Seeplex RV assays (Seegene, Seoul, Korea) [11]; and AdvanSure™ real-time RT-PCR (LG Life Science, Seoul, Korea) [12]. However, these assays are often costly and require dedicated laboratory equipment, which is unsuitable for routine surveillance of respiratory virus infections.

We previously reported a two-tube multiplex reverse transcription polymerase chain reaction (mRT-PCR) assay (two-tube assay) to detect 16 respiratory viruses based on amplicon size differences, using an automated electrophoresis system [13, 14]. Although the overall detection rate of the two-tube assay for each virus is comparable to that of the Luminex xTAG RVP Fast and Seeplex RV15 ACE assays, demonstrating the high sensitivity and specificity in the analysis of clinical samples, it has a few drawbacks that limit its

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✉ Yanjun Zhang
yjzhang@cdc.zj.cn

✉ Xuejun Ma
maxj@ivdc.chinacdc.cn

[1] Key Laboratory for Medical Virology, National Health and Family Planning Commission, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, No. 155 Changbai Road, Changping district, Beijing 102206, China

[2] Institute of Microbiology, Zhejiang Provincial Center for Disease Control and Prevention, Hangzhou 310051, China

[3] Department of Pathology, Children's Hospital, Zhejiang University School of Medicine, Hangzhou 310013, China

[4] Department of Pathology, School of Basic Medicine, Hubei University of Medicine, Shiyan, Hubei 442000, China

[5] Department of Rheumatology and Immunology Renmin Hospital, Hubei University of Medicine, Shiyan 442000, Hubei, China

wider application. First, an automated capillary electrophoresis device is needed, and many laboratories in the Centers for Disease Control and Prevention (CDC) in China do not have this specialized laboratory equipment. Second, the size differences of some amplicons are not large enough for reliable judging of results by untrained staff. Third, there is a need to open tubes after PCR amplification to analyze the PCR products by QIAxcel automatic electrophoresis, which significantly increases the risk of cross-contamination. Given that the laboratories from all of the provincial and most municipal CDC laboratories in China have access to conventional real-time PCR instrumentation, in this study, we aimed to develop a multiplex quantitative real-time RT-PCR (mqRT-PCR) consisting of a panel of seven internally controlled qRT-PCR assays to detect 16 different respiratory viruses: human coronavirus (CoV) 229E, CoV NL63, CoV OC43, CoVHKU1, parainfluenza virus (PIV)1, PIV2, PIV3, PIV4, influenza virus (IV) types A and B, human respiratory syncytial virus (RSV) types A and B, human rhinovirus (HRV), human metapneumovirus (HMPV), human adenovirus (ADV), and human bocavirus (HBoV). Clinical evaluation of the mqRT-PCR assay panel was conducted and compared with the two-tube assay in parallel.

The mqRT-PCR assay panel consisted of seven separate assays with primer/probe sets covering 16 human respiratory viruses: assay 1, IVA and IVB; assay 2, CoV OC43 and CoV 229E; assay 3, CoV NL63 and CoVHKU1; assay 4, PIV1, PIV2, PIV3, and PIV4; assay 5, HMPV and HBoV; assay 6, HRV and AdV; assay 7, RSVA and RSVB. Primers and probes were designed based on conserved target regions of the viral genomes, using Primer Premier software version 5.0 [15]. For all primer and probe sequences, BLAST analysis was performed *in silico* to ensure specificity, and no cross-reactivity was observed. Subsequently, in the initial development of the mqRT-PCR assay panel, the sensitivity of each assay for each virus type/subtype was thoroughly evaluated individually using serial tenfold dilutions ranging from 10 to 10^5 copies of *in vitro* RNA transcripts (for RNA viruses) or cloned plasmids (for DNA viruses). The specificity of each assay was also extensively examined using *in vitro* RNA transcripts and cloned plasmids as well as archived samples. No obvious cross-reaction was observed in the multiplex assay panel. The limit of detection was 20 copies per reaction for PIV2, PIV3, RSVA, HBoV and Adv, and 200 copies per reaction for the other 11 virus type/subtypes. The individual reactions within each assay were distinguished using probes with different fluorophores. The sequences of the primers and probes and the target genes are listed in Table 1. Each assay also contained an endogenous RNase P (human genome ribonuclease P) gene as an internal control.

Each mqRT-PCR assay was carried out individually in a 25-µl reaction volume. RT-PCR buffer mix (7.5 µl) was combined with 7.5 µl of each primer/probe set and 5 µl of 5 × enzyme mix (AgPath-IDTM One-Step RT-PCR Kit; Applied Biosystems, Waltham, Massachusetts, USA). Nucleic acid extracts (5 µl) were added to seven wells of each primer/probe set. The following cycling conditions were used on a 7500 Real-Time PCR Instrument (Applied Biosystems): 30 min at 50 °C, 5 min at 95 °C, 40 cycles of 10 s at 95 °C and 45 s at 55 °C. Threshold cycle (Ct) values were determined by manually adjusting the fluorescence baseline to fall within the exponential phase of the amplification curves, and above any background signal. A test result was considered positive if a well-defined curve was obtained that crossed the threshold cycle within 40 cycles. A mixture of plasmids was included accordingly as external positive controls in all runs to monitor assay performance. For comparison, the two-tube assay was performed using a One-Step RT-PCR Kit (QIAGEN, Hilden, Germany) in a 25-µl volume according to previously described protocols, and the products were analyzed using QIAxcel automatic electrophoresis equipment with a QIAxcel DNA High Resolution Kit. If results were discordant between the mqRT-PCR assay panel and the two-tube assay, both tests were repeated concurrently to evaluate any problems relating to sample degradation or potential hands-on error. Assignment of samples as having concordant or discordant results was based on the results of duplicate testing by both methods. If the results were still discordant, RT-PCR was then performed, followed by sequencing using a pair of primers from other literature (data not shown) [16, 17].

A total of 363 sputum specimens were obtained from patients with CAP who were admitted to the Children's Hospital, Zhejiang University School of Medicine, China, between February and July 2017. Ages ranged from 1 month to 18 years, and 350 (96.4%) were under 3 years old. Trained staff collected sputum by adding 2 ml of transport medium and pipeted the samples into separate aliquots, which were then stored at − 80 °C. Total RNA/DNA was extracted from 200 µl of clinical sample using a QIAamp Viral RNA Mini Kit (QIAGEN). The extracts were eluted into 50 µl of DNase- and RNase-free water and stored at − 80 °C. All of the extracted nucleic acids were tested using both the mqRT-PCR assay panel and the two-tube assay. Of 363 samples tested, 340 (93.66%) and 332 (91.46%) were positive for one or more viruses by the mqRT-PCR assay panel and two-tube assay, respectively. PIV2, 229E and HKU1 were not detected by either of methods. The overall agreement in detection of each pathogen between the two-tube assay and mqRT-PCR assay panel is shown in Table 2. All the positive specimens with discordant results that were confirmed by mono-PCR and sequencing to be true positives are shown in Table 3.

We compared the performance of the mqRT-PCR assay panel with that of the two-tube assay and found that 19 viruses in 17 samples were detected only by the two-tube

Table 1 Primers and probes used in the mqRT-PCR assay panel

Assay	Target virus	Target gene	Primer	Sequence (5'-3')
1	IVA	Matrix	IVA-F	GACCRATCCTGTCACCTCTGA C
			IVA-R	GGGCATTYTGGACAAAKCGTCTACG
			IVA-P	FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ1
	IVB	Nucleoprotein	IVB-F	CCCACCRAGCAACAAACG
			IVB-R	CCTTCCGACATCAGCTTCACT
			IVB-P	VIC-CCCGGAACCCATCCCCGGA-BHQ1
2	CoV OC43	Nucleocapsid	OC43-F	CCCAAGTAGCGATGAGGCTA
			OC43-R	AGGAGCAGACCTTCCTGAGC
			OC43-P	FAM-ACTAGGTTTCCGCCTGGCACGGTA-BHQ1
	CoV 229E	Nucleocapsid	229E-F	CAGAAAACGAAAGATTGCTTCA
			229E-R	CAAGCAAAGGGCTATAAAGAGA
			229E-P	VIC-ATGGCTACAGTCAAATGGGCTGATGC-BHQ1
3	CoV NL63	Nucleocapsid	NL63-F	GTTCTTCTGGTACTTCCACTCCT
			NL63-R	TTCCAACGAGGTTTCTTCAA
			NL63-P	FAM-AGCCTCTTTCTCAACCCAGGGCTG-BHQ1
	CoV HKU1	Polyprotein	HKU1-F	TGAATTTTGTTGTTCACATGGT
			HKU1-R	ATAATAGCAACCGCCACACAT
			HKU1-P	VIC-ATCGCCTTGCGAATGAATGTGCTC-BHQ1
4	PIV1	HN	PIV1-F	CCTGATTTAAACCCGGTAATTTCTC
			PIV1-R	TTCCTGCAGCTATTACAGAACATGAT
			PIV1-P	FAM-CCTATGACATCAACGACAAC-BHQ1
	PIV2	HN	PIV2-F1	CAGGACTATGAAAACCATTTACCT
			PIV2-R1	CGTGGCATAATCTTCTTTTTCA
			PIV2-P1	ROX-TCGCAAAAGCTGTTCAGTCACTGCT-BHQ1
	PIV3	HN	HPIV3-F	TGATGGYTCAAACTCAACAACAAGAT
			HPIV3-R	CATACCCGAGAACTATTATTTTGCCTT
			HPIV3-P	VIC-TATATCCCTGGTCCAACAGATG-BHQ1
	PIV4	HN	HPIV4-F	ATTTTATTCCAACTGCTACAACTCC
			HPIV4-R	CCACTGARCAACTCTTTCGATTC
			HPIV4-P	CY5-ATGCATTCGAATTCCATCATTCTCC-BHQ1
5	HMPV	Fusion	HMPV-F	GTCAGCTTCAGTCAATTCAACAGA
			HMPV-R	ARGTCCAADGATATTGCTGGTGTT
			HMPV-P	FAM-CTGCATTGTCTGAAAAYTGCCGCA-BHQ1
	HBoV	NS1	HBoV-F	AGCTTTTGTTGATTCAAGGCTATAATC
			HBoV-R	TGTTTCCCGAATTGTTTGTTCA
			HBoV-P	VIC-TCTAGCCGTTGGTCACGCCCTGTG-BHQ1
6	HRV	5' UTR	HRV-F	GTGAAGAGCCSCRTGTGCT
			HRV-R	GCTSCAGGGTTAAGGTTAGCC
			HRV-P	FAM-TGAGTCCTCCGGCCCCTGAATG-BHQ1
	AdV	Hexon	AdV-F	AGGACGCCTCGGAGTACCT
			AdV-R	CCACCGTGGGRTTYCTAAA
			AdV-P	VIC-CTGGTGCAGTTYGCCCGYGC-BHQ1
7	RSVA	Fusion	RSVA-F	GATGTAAGCAGCTCCGTTATCACA
			RSVA-R	TTGGATGCTGTACATTTAGTTTTGC
			RSVA-P	FAM-CTCTAGGAGCCATTGTGTCATGCT-BHQ1
	RSVB	Nucleoprotein	RSVB-F	CTGTCATCYAGCAAATACACTATTCA
			RSVB-R	GCACATCATAATTGGGAGTGTCA
			RSVB-P	VIC-CGTAGTACAGGAGATAAT-BHQ1
	Rnasep	RNase P	Rnasep-F	AGATTTGGACCTGCGAGCG
			Rnasep-R	GAGCGGCTGTCTCCACAAGT
			Rnasep-P	NED-TTCTGACCTGAAGGCTCTGCGCG-BHQ1

The common real-time PCR probe was labeled at the 5' end with 6-carboxyfluorescein (FAM) and at the 3' end with Black Hole Quencher (BHQ)

NS, nonstructural; HN, hemagglutinin-neuraminidase; NP, nucleoprotein; UTR, untranslated region; NC, nucleocapsid

Table 2 Performance of the two-tube assay for individual pathogens compared with the mqRT-PCR assay panel

Virus	No. of specimens: two-tube assay /mqRT-PCR assay panel				Performance of the two-tube assay compared with the mqRT-PCR assay					
	+/+	+/−	−/+	−/−	Sensitivity %	Specificity %	PPV %	NPV %	Accordance rate %	Kappa
IVA	3	0	0	360	100	100	100	100	100	1.00
IVB	2	0	0	361	100	100	100	100	100	1.00
s09H1N1	0	3	0	358	NA	99.17	0	100	99.17	0.99
PIV1	11	0	0	352	100	100	100	100	100	1.00
PIV2	0	0	0	363	NA	100	NA	100	100	1.00
PIV3	100	3	1	259	99.01	98.85	97.09	99.62	98.90	0.99
PIV4	4	0	0	359	100	100	100	100	100	1.00
HRV	128	10	0	225	100	95.74	92.75	100	97.25	0.97
HMPV	39	4	0	320	100	98.77	90.70	100	98.90	0.99
Adv	16	0	0	347	100	100	100	100	100	1.00
RSVA	6	0	0	357	100	100	100	100	100	1.00
RSVB	46	0	0	317	100	100	100	100	100	1.00
OC43	12	2	0	349	100	99.43	85.71	100	99.45	0.99
229E	0	0	0	363	NA	100	NA	100	100	1.00
HKU1	11	0	0	352	100	100	100	100	100	1.00
NL63	0	0	0	363	NA	100	NA	100	100	1.00
HBoV	12	0	0	351	100	100	100	100	100	1.00

This table shows the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the kappa values for each target using the confirmed results as the reference for comparison. All the accordance rate values were above 97.07%, except for NA; all the kappa values were above 0.75

NA, not applicable

Table 3 The confirmed results for specimens with discordant results between mqRT-PCR assay panel and the two-tube assay

Case no.	Two-tube assay	mqRT-PCR assay panel	Validation results
P58	**HRV**[a], Hbov	Hbov	HRV, Hbov
P189	**HRV**	ND[b]	HRV
P201	ADV	ADV, **PIV3**	ADV, PIV3
P207	**HMPV**, HRV	HRV	HMPV, HRV
P208	ADV, RSVB, **HMPV**	ADV, RSVB	ADV, RSVB, HMPV
P210	**HMPV**	ND	HMPV
P222	**HRV**	ND	HRV
P223	**OC43**, **PIV3**	ND	OC43, PIV3
P227	**HRV**, PIV3	PIV3	HRV, PIV3
P241	HMPV, **HRV**	HMPV	HMPV, HRV
P243	**PIV3**	ND	PIV3
P268	**HMPV**, IVA, OC43	IVA, OC43	HMPV, IVA, OC43
P276	**HRV**, PIV3	PIV3	HRV, PIV3
P277	**HRV**	ND	HRV
P331	**OC43**, PIV3	ND	PIV3, OC43
P411	**HRV**	ND	HRV
P498	**HRV**	ND	HRV

[a]Discordant results are highlighted in boldface

[b]ND, not detected

assay and confirmed as true positives by sequencing [10, 18, 19]. These viruses included HRV (n = 10), PIV3 (n = 3), OC43 (n = 2) and HMPV (n = 4). The two-tube assay appeared to have superior sensitivity to the mqRT-PCR assay panel for detecting these four viruses. We further tested the performance of the mqRT-PCR panel using 25 archived clinical samples that were positive for four viruses (10 for HRV, and five each for PIV3, OC43 and HMPV). Complete agreement with the two-tube assay results was achieved in nearly half of the samples (five for HRV, three for PIV3 and OC43, and two for HMPV). These discrepancies are most likely due to primer design, since we selected different conserved regions of the same genes or different gene targets for these two methods. For example, two primers (HMPV-1 and HMPV-2) were designed to amplify the L and N genes of HMPV in the two-tube assay, while the mqRT-PCR assay panel was designed for amplifying the F gene. The 10 HRV-positive specimens that were positive in the two-tube assay but negative in the mqRT-PCR assay panel were from cases of HEV infection. HRV and HEV are closely related viruses. Using this method, it is difficult to distinguish the 5' untranslated regions of these groups of viruses, resulting in possible cross-reactivity. The primers of the mqRT-PCR assay panel were designed for the detection of the HN gene of PIV3, while the two-tube assay was designed for amplifying the HA gene. This discrepancy might lead to higher sensitivity of the two-tube assay for the detection of PIV3.

In this study, the samples were not collected in winter (February to July). The prevalence of viruses can vary by geography and season. PIV3 infections were most commonly detected in the spring and summer, although infections do occur year round. Only four specimens tested positive for PIV4, and lower respiratory tract infection with PIV2 and PIV4 occurred less frequently than with PIV1 and PIV3 in previous studies [20–23]. In the United States, 229E, OC43 and HKU1 have been shown to follow different seasonal patterns, with outbreaks of 229E occurring in winter, OC43 in spring and autumn, and HKU1 in summer [24, 25]. OC43 and HKU1 were detected in this study following the same seasonal patterns. Although PIV2, 229E and NL63 were not detected in this study, we detected a few stock clinical samples previously identified as PIV2, 229E and NL63 using both the mqRT-PCR panel and two-tube assays. This demonstrated that both assays worked well for detection of these three viruses (data not shown).

The two-tube assay requires that the tubes are opened after PCR amplification for QIAxcel automatic electrophoresis, whereas the mqRT-PCR assay panel is a closed system that effectively avoids contamination. By splitting a panel into seven multiplex reaction assays, individual mixes can be modified easily if needed without affecting the other mixes, which can allow for more-efficiently targeted testing based on epidemiological findings. Another advantage of the mqRT-PCR assay panel is the saving of hands-on time. The assay panel requires 2–3 h to complete 54 samples, including 30 min to prepare the PCR mixture, while the two-tube assay usually takes 3–4 h. Also, the mqRT-PCR assay panel is easily integrated into the workflow of laboratories using conventional real-time PCR platforms and can be performed after little training. In addition, the human RNaseP gene as an internal control is readily detected to validate the RNA extraction procedure and to prevent sampling and RT-PCR errors. Notably, the mqRT-PCR assay panel allows for quantitative analysis, which should become increasingly helpful for epidemiological studies, for assessing clinical outcome according to virus type or viral load, and for evaluating antiviral agents [17].

The mqRT-PCR assay had some limitations. First, the mqRT-PCR assay panel required seven parallel assays with only moderate throughput in each assay. It should be optimized to develop a single-tube mqRT-PCR to detect as many viruses as possible in one assay. Second, for the detection of HRV, PIV3, OC43 and HMPV, the sensitivity of the mqRT-PCR assay panel was lower than that of the two-tube assay. Competition for reagents might have decreased sensitivity for detection of these viruses in a multiplex format. Third, our study was limited by the failure to collect samples throughout the entire year. Increasing the sample size and year-round detection would be important improvements.

In summary, this study demonstrates the clinical performance of an mqRT-PCR assay panel for the detection of 16 viral respiratory pathogens. The mqRT-PCR assay panel is easy to use, and brings us closer to the mainstream adoption of molecular diagnostic testing. The further optimization, evaluation and widespread use of the mqRT-PCR assay will provide a valuable tool for routine surveillance of respiratory virus infection in China.

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Compliance with ethical standards

Conflict of interest All the authors approved the final manuscript, and they have no conflict of interest to declare.

Informed consent All aspects of this study were performed in accordance with national ethics regulations and approved by the Institutional Review Boards of the Center for Disease Control and Prevention of Zhejiang. Children's parents were apprised of the study's purpose and

of their right to keep information confidential. Written informed consent was obtained from parents or caregivers.

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