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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Funding information
Hebei Key Project Plan for Medical Science Research, Grant/Award Number: 20180616; China Mega-Projects for Infectious Disease, Grant/Award Numbers: 2017ZX10104001 and 2018ZX10713-002, 2018ZX10711001

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-qPCR1 has acceptable sensitivity and specificity for detection of respiratory virus, but only detects one virus per tube. The multiplex reverse transcription PCR1 enables the detection of virus co-infection but is labor-intensive and susceptible to cross-contamination as it requires post PCR analysis. The mRT-qPCR3 has the advantages of...
| Assay | Primer/Probe | Sequence (5’-3’) | Product size, bp | Gene | References |
|-------|-------------|------------------|-----------------|------|------------|
| 1     | RSV outer-F* | CA+C+GA+GA+TG+C+CAAAT+CTATAAATTCA | 374 | N | Kim et al; Sanghavi et al |
|       | RSV outer-R* | CW+GA+TC+TR+CT+CCT+GCTGTCTA | | | |
|       | RSV inner-F  | CACWGAAGATGCGWATCATAAATTTCA | 89 | | |
|       | RSV inner-R  | GTATYTTTATGTCTCTTCTTCAAACC | | | |
|       | RSV probe    | FAM-TAACAGTTATTTATATGCAATG-BHQ1 | | | |
|       | HRV outer-F* | HC+AA+GYA+CTCTCT+GTYWCCCSG | 397 | 5’UTR | Wisdom et al; Hammitt et al |
|       | HRV outer-R* | GA+AA+CAC+GGA+CA+C+CGCAGTAGT | | | |
|       | HRV inner-F  | TGGACAGGCTGTGAAGAGCG | 144 | | |
|       | HRV inner-R  | CAAAGTGCTGCTCCATCC | | | |
|       | HRV probe    | CYS-TCCTCCGCCCCTGAAATG-BHQ3 | | | |
|       | HMPV outer-F* | CATATAAG+CA+TA+GA+TTAAAAGA+GCTTC | 475 | N | Maertzdorf et al; Dare et al |
|       | HMPV outer-R* | GT+GAATATTAA+G+G+GCA+GACATACATAAAAAA | | | |
|       | HMPV inner-F | CATATAAGCACTGTCTATTAAAAGAAGTTC | 163 | | |
|       | HMPV inner-R | CCTATTCTTCAGCATATTGTTAATCAG | | | |
|       | HMPV probe   | VIC-TGGAATGGAGGAGGCCACTGCCGTTG-BHQ1 | | | |
| 2     | PIV1 outer-F* | A+GGA+TGT+GA+GAATAGGGAA | 195 | HN | Perrott et al |
|       | PIV1 outer-R* | GT+CT+CT+TT+CA+CAGTGGCCA | | | |
|       | PIV1 inner-F  | TTTAACCCCGTAATTTCCTATACCT | 81 | | |
|       | PIV1 inner-R  | CCCCTTGTTCCTCGACTTATT | | | |
|       | PIV1 probe    | FAM-TGCAATACAGCAAGACAGAGAAATCTTTG-BHQ1 | | | |
|       | PIV2 outer-F* | AA+C AA+CT+CT+GCTG CAGCAT T | 158 | HN | Sanghavi et al; Bellau-Pujol et al |
|       | PIV2 outer-R* | C+GT+GG+CA+TA+AT+CTTCTTTT | | | |
|       | PIV2 inner-F  | CCATTCCACTAAGTGGAA | 116 | | |
|       | PIV2 inner-R  | CGTGCGCATATTCTTTTT | | | |
|       | PIV2 probe    | CYS-ATCCGAAAAGCTGTCAGTCAC-BHQ3 | | | |
|       | PIV3 outer-F* | T+TA+CARA+TA+GG+GATAAACTGT | 151 | HN | Sanghavi et al; Bellau-Pujol et al |
|       | PIV3 outer-R* | CTT+TG+GA+GT+TA+CGTGGG | | | |
|       | PIV3 inner-F  | TTAACATAGGGTAATATCTGT | 115 | | |
|       | PIV3 inner-R  | TTGGAGTGTAGAAGACAT | | | |
|       | PIV3 probe    | VIC-AAAATCAGTCCTGTGCCATTAAAT-BHQ1 | | | |
| 3     | EV outer-F*   | HC+AA+GYA+CTTCT+GTYWCCCSG | 402 | 5’UTR | Wisdom et al; Brittain-Long et al |
|       | EV outer-R*   | GA+AA+CAC+GGA+CA+C+CGCAGTAGT | | | |
|       | EV inner-F    | GTGYYAGATGTCATATTGAGCTA | 141 | | |
|       | EV inner-R    | ACACCCAAAGTGATCGGT | | | |
|       | EV probe      | FAM-CGCGCCCTGAAATGGGCTCAATC-BHQ1 | | | |
|       | FluA outer-F* | TCA+AGAG+CAGATCCGCGCAG | 189 | M | This study |
|       | FluA outer-R* | G+CATT+TTG+GA+CAAGAGCTTAC | | | |
|       | FluA inner-F  | GAATGGCTAAAGACAAGCAAT | 118 | | |
|       | FluA inner-R  | GCATTCTTGGCAAACAGGCTTAC | | | |
|       | FluA probe    | CYS-AGTCCCTGCTCACTGGGACGCTG-BHQ3 | | | |
|       | FluB outer-F* | TG+CCT+CCAC+AAATTACGG | 218 | HA | Perrott et al |
|       | FluB outer-R* | C+C+TG+CAAT+CATTCTCTCCA | | | |
|       | FluB inner-F  | AAATACGGTGGTAATAAATAAAGCATAA | 170 | | |
|       | FluB inner-R  | CCA GCA ATA GCT CCG AAG AAA | | | |
|       | FluB probe    | VIC-CACCCCATATTGGGCAATTCTTATGGC-BHQ1 | | | |
| 4     | HCoV229E outer-F* | CA+GT+CAAAT+GGGCTGATGCA | 638 | N | Hammitt et al; Li et al |
|       | HCoV229E outer-R* | A+CGA+GAA+GG+CTTAGGAGTAC | | | |
|       | HCoV229E inner-F | CAGTCACAATGGGCTGATGCA | 76 | | |
|       | HCoV229E inner-R | AAAGGCTATAAAGAGAATAAGGATGATTC | | | |
|       | HCoV229E probe | FAM-CCCTCGACGACACGTTGTCGTA-BHQ1 | | | |
TABLE 1 (Continued)

| Assay                  | Primer/Probe         | Sequence (5’-3’)                                                                 | Product size, bp | Gene | References          |
|------------------------|----------------------|---------------------------------------------------------------------------------|------------------|------|---------------------|
| HCoVOC43-outer-F      | ATT+GCA+CCA+GGAGTCCCA | 316                                                                             | N                | Hammitt et al; Li et al modified |
| HCoVOC43-outer-R      | TT+CC+T+GA+GC+CTT+CAATATGTAAC | 72                                                                          | NS1              | Bastein et al; Esposito et al modified |
| HCoVOC43-inner-F      | ATGAGGCTATTCCGACTAGG |                                                                                | NS1              | Bastein et al; Esposito et al modified |
| HCoVOC43-inner-R      | TTCCGTAGCCCTTCAATATGTAAC | 211                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| HCoVOC43-Probe        | CY5-TCCGCTGGCGCAGGCTACTCCCT-BHQ3 | 211                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| HCoVNL63-outer-F      | A+GATGA+GCA+GATT+GGTTATTGG | 211                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| HCoVNL63-outer-R      | ATTACGTTT+GC+GATTA+C+CAAGACT | 96                                                                           | Hammitt et al     | Hammitt et al; Li et al modified |
| HCoVNL63-inner-F      | GACCTTAATCAGACAACTGCTTCT | 211                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| HCoVNL63-inner-R      | ATTACGTTTGGCGATTACCAAGACT | 89                                                                           | Hammitt et al     | Hammitt et al; Li et al modified |
| HCoVNL63-Probe        | VIC-TCAGGTTTACAGCACCTCTTCCTAGCAAGCCAAA CA-BHQ1 | 211                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| ADV-outer-F           | TACATGCA+CATCKCG5GGVGAGGA | 113                                                                         | NS1              | Chieochansin et al; Jansen et al modified |
| ADV-outer-R           | GT+GG+GGTTYCT+GAACTTG | 113                                                                         | NS1              | Chieochansin et al; Jansen et al modified |
| ADV-inner-F           | GYC TCG GAG TAC CTG AG | 5                                                                             | Hexo             | Sanghavi et al; Lam et al modified |
| ADV-inner-R           | GTGGGTTYCTGAACTTGT | 5                                                                             | Hexo             | Sanghavi et al; Lam et al modified |
| ADV-Probe             | FAM-CTGGTGGCAGTCCGCGTGCCA-BHQ1 | 5                                                                             | Hexo             | Sanghavi et al; Lam et al modified |
| HBoV-outer-F          | GA+C+TAA+GCAA+GAAG+GAATGG | 214                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| HBoV-outer-R          | TCT+GC+GAT+CT+CT+ATATT+GAAG | 214                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| HBoV-inner-F          | AAATCTCTCTGCTCAGACG | 136                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| HBoV-inner-R          | TCTGCGATCTCTATATTGGAAG | 136                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| HBoV-Probe            | CY5-ATGGTCGCGCGAGATACCTTCCACC-BHQ3 | 136                                                                         | Hammitt et al     | Hammitt et al; Li et al modified |
| Rnasep-F              | AGATTGGACCTGCGAGG | 65                                                                           | Hammitt et al     | Hammitt et al; Li et al modified |
| Rnasep-R              | GAGCGGCTGTCTCCCAAGAT | 65                                                                           | Hammitt et al     | Hammitt et al; Li et al modified |
| Rnasep-Probe          | VIC-TCAGGTTTACAGCACCTCTTCCTAGCAAGCCAAA CA-BHQ1 | 65                                                                           | Hammitt et al     | Hammitt et al; Li et al modified |

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.

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 glyceraldehyde, sodium thioglycolate, cysteine hydrochloride, CaCl2, 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 | MATERIALS AND METHODS

2.1 | Clinical samples

A total of 468 nasopharyngeal aspirate samples were collected from inpatients presenting with acute respiratory symptoms at the Children's Hospital of Hebei Province.

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 \(^{1,8-22}\) 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 three plasmids in three replicates and in three different days within a week. The specificity was retrospectively evaluated by sequencing of traditional two-step nested PCR tested using three plasmids in three replicates and in three different days within a week. The specific primers 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\(^9\) 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 \(\chi^2\) 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\(^9\) 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
(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 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 products confirmed that 35 samples were true positives. The comparison of percentages of detection of specimens with Ct ≤ 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. In the present study, LNA-modified outer primers (without changing the sequences) were used to develop an mOTNTR-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

| Virus   | No. of samples | C\textsubscript{t} value | Clinical performance of the mOTNRT-PCR compared with the RT-qPCR | Kappa value |
|---------|----------------|---------------------------|-----------------------------------------------------------------|-------------|
|         | mOTNRT-PCR | RT-qPCR | mOTNRT-PCR vs RT-qPCR | Sensitivity, % | Specificity, % | Accordance rate, % | |
| 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.
To our best knowledge, this is the first report on and Figure 17| 3079
468 clinical samples. A total of 34 samples had a specificity, and reliable reproducibility (Table 3).

LNA is a sensitive, labor-saving, and cost-effective method for detecting 14 respiratory viruses in five reactions. It may have great potential for routine surveillance of respiratory virus infection in China.

FIGURE 1 The percentages of detection of specimens with C<sub>t</sub> ≤ 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<sub>t</sub> 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). 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. 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<sub>t</sub> of 35. When a C<sub>t</sub> 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<sub>t</sub> 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%, 150/468) were the most commonly found in co-infections, which is similar to the previous report.

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.

ACKNOWLEDGMENTS
This work was supported by grants from China Mega-Projects for Infectious Disease (2018ZX10711001, 2017ZX10104001, and 2018ZX10713-002) and Hebei Key Project Plan for Medical Science Research (20180616).
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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How to cite this article: Zhao L, Li G-x, Wang J, et al. 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. J Med Virol. 2020;92:3073-3080. https://doi.org/10.1002/jmv.25686