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Original Article

Simultaneous detection of 15 respiratory pathogens with a fluorescence probe melting curve analysis-based multiplex real-time PCR assay

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Abstract: Acute respiratory tract infections are common worldwide and caused by a great diversity of pathogens. A rapid and accurate diagnosis method of respiratory infection is crucial for timely clinical intervention. Here, by combining fluorescence melting curve analysis and multiplex real-time assay, we developed a novel method which can simultaneously detect 15 respiratory viruses. The specificity for target genes was 100%, as assessed with a panel of 47 respiratory pathogens, which indicated no cross-reactions. The assay's limits of detection at the nucleic acid level ranged from 5 copies/μL to 500 copies/μL nucleic acids. Compared with conventional culture method, our assay showed more than 75% sensitivity and 100% specificity for each respiratory pathogen in 384 clinical samples. Even more, the kappa correlation for all the pathogens ranged from 0.86 to 1.00. Overall, this method has the characteristics of high throughput, low cost and high sensitivity and precision, which demonstrated our method is well suited for routine clinical testing in respiratory infection.

Keywords: Respiratory pathogen, multiplex real-time PCR assay, probe melting curve analysis

Introduction

Acute respiratory tract infections are the leading cause of morbidity and mortality worldwide, particularly in infants and young children [1]. Pathogens cause acute respiratory infections are highly variable, including most predominantly viruses (26 identified thus far [2]), as well as fungi and phylogenetically diverse bacteria, such as Gram-negative bacteria (e.g. streptococci), *Chlamydia* species, and mycoplasma (distinct for their lack of a cell well). The similar clinical symptoms of respiratory infections caused by various pathogens render differential diagnosis based on clinical parameters difficult. Thus, rapid and accurate detection of respiratory pathogens is important for timely clinical management, reduction of improper antibiotic abuse, and prevention of outbreaks and epidemics.

Advancements in molecular biology have improved clinical pathogen detection dramatically. Notably, polymerase chain reaction (PCR) and reverse-transcription PCR are now widely used to detect respiratory viruses, exhibiting superior sensitivity for respiratory virus detection over the more conventional methods of virus culture and rapid direct antigen detection tests [3-6]. The development of multiplex PCR technology from prior conventional PCR techniques has enabled simultaneous detection of multiple respiratory pathogens from a single patient sample, thus saving time, effort, and costs [7-10]. In recent years, a variety of novel multiplex PCR techniques based on common multiplex PCR have emerged, including dual priming oligonucleotide technology [11, 12], multiplex ligation-dependent probe amplification [13], and a target-specific extension technique, which combines multiplex PCR with liquid phase chip technology [14, 15]. The improvement of various aspects of common PCR conditions in these technologies has enabled high-throughput detection to be achieved with high sensitivity and specificity. Although use of these technologies has the advantages of increasing detection sensitivity and automation, they are associated with an increased number of operating steps together with increased operating time, risk of contamination, and cost.

Real-time (RT)-polymerase chain reaction has been the gold standard for analyzing disease-related genes for decades [16-18]. Current RT-PCR methods enable the simultaneous detection of only two or three pathogens in a single reaction [19, 20] due to the instruments' limited discriminatory capacity. Multicolor probe-based fluorescence melting curve analysis was developed to overcome these limitations [21, 22]. The principle of the fluorescent probe melting curve is to distinguish among PCR template sequences based on differences in the melting point temperature (*Tm*) when probes hybridize with PCR products. This differentiation increases the detection flux of each fluorescent detection channel greatly.

Based on the aforementioned principles, multiplex PCR technology was combined with fluorescence probe melting curve analysis in this study to establish a method for the simultaneous detection of 15 respiratory pathogens. The aim of this study was to assess the utility, sensitivity, and specificity of multiplex PCR to determine whether it is suitable for development into a cost-saving routine clinical detection approach.

Materials and methods

Respiratory specimens

Throat swabs collected between December 2017 and April 2018 at Shenzhen Children's Hospital were used in this study. Of 431 such clinical samples collected for this study, 47 from cases of respiratory infection confirmed by immunofluorescence or a commercial fluorescent RT-PCR kit were used as positive controls. The control samples were positive for influenza virus A and B (IFV-A and -B) (n = 3 each virus), human respiratory syncytial virus (hRSV) (n = 8), human metapneumovirus (hMPV) (n = 2), parainfluenza virus types 1 and 2 (PIV-1 and -2; n = 2 each virus), parainfluenza virus type 3 (PIV-3) (n = 3), human coronavirus 229E (hCov 229E) (n = 1), human rhinovirus (hRV) (n = 5), adenovirus (ADV) (n = 5), human bocavirus (hBov) (n = 2), *Bordetella pertussis* (BP) (n = 4), *Legionella pneumophila* (LP) (n = 2), *Mycoplasma pneumoniae* (MP) (n = 3), and *Chlamydia pneumoniae* (CP) (n = 2). The remaining 384 throat swabs from patients with respiratory infections identified by culture and immunofluorescence were used for methodological evaluation.

Nucleic acid extraction

Pathogen RNA/DNA molecules were extracted with a commercial reagent (TIANamp virus DNA/RNA kit; Tiangen, China) according to the manufacturer's protocol. Two hundred microliters of each clinical sample were used for nucleic acid extraction and eluted in 30 μL elution buffer. Prepared nucleic acid samples were tested immediately after extraction when possible, or stored at -70°C until testing.

Primer and probe design and synthesis

Conserved regions of target genes were chosen to inform the design of oligonucleotide primers and molecular beacon probes. Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA), TmUtility (version 1.3; Idaho Technologies Inc., Salt Lake City, UT), and Oligo 6.0 (AVG Technologies Inc., Chelmsford, MA) software were used to aid design and to predict possible secondary structures and *Tm*s of primers and probes. All probes used for the assays were designed to be complementary to conserved regions within their target amplicons. To enable simultaneous detection of multiple viruses, each beacon was labeled with a different fluorophore at the 5' end and a quencher at the 3' end; the probe sequences were adjusted to create a unique *Tm*. Single-probe reactions were conducted to determine each probe's specificity and sensitivity for its target. Selected probes were then combined and tested in a multiplex format. The primers and probes were synthesized and purified by polyacrylamide gel electrophoresis by Sangon (Shanghai, China). The sequences of the molecular beacons and primers used are listed in **Table 1**.

Multiplex PCR and melting curve analysis

Two reactions were set up to detect the 15 respiratory pathogens; each reaction was carried out in a total volume of 25 μL with 5 μL of template. Reaction I (primers and probes for

Table 1. Primer and probe information

Pathogen	Primer/probe sequences (5'→3')
hCov 229E	DP: FAM-CCGTTGCTGTTGATGGTGCTAACGG-Dabcy1
	F: ACTAGAAAGGGCAAACGGGTG/R: AATTCTTGCGCCTAACACCG
hMPV	DP: FAM-CGCCAGCAGTCACAGCAGGCATTGCGGCG-Dabcy1
	F: TCCCAGACAATCAAGATTTGTCC/R: ACTCTCAAGCCTTATGGTTTTGG
PIV-1	DP: FAM-CCGCACACATCTGGCTACTGATTGCGG-Dabcy1
	F: TTGGTCTACAACCCGAAATGAC/R: GCATAGGATCATGATAATGAAGGAC
PIV-2	DP: Cy5-CGCCTCTTGGTGGTCTGCATCGGCG-Dabcy1
	F: GTCATGATGGGTGCAGAAGGTAG/R: AGGACGGTACCCATTGAGCC
PIV-3	DP: Cy5-CGGGAGAAACAAGGCAGTCAACCCG-Dabcy1
	F: GGACCGAGCAAGCTACAGAATC/R: CGTCCTGGTTCGTTCTGTTTG
hRV	DP: Cy5-CCAGCTGAATGTGGCTAACCTTAAGCTGG-Dabcy1
	F: TAGACCTGGCAGATGAGGC/R: CAAAGTAGTTGGTCCCGTCC
IFV-A	DP: ROX-CCAGCCCCTCAAAGCCGAGATCGCTGG-Dabcy1
	F: TTCTAACCGAGGTCGAAACG/R: CCAGCAAAGACATCTTCAAGTCT
IFV-B	DP: ROX-CCGCTGAAGCCATTCGATTTATAGGGCGG-Dabcy1
	F: TGGTGTTGCAATCAAAGGAGG/R: TTGGCTTTGATGTCTCTCAATAGC
hRSV	DP: ROX-CCGCTACCAGAGGTGGCAGTAGAGTTGAAGCGG-Dabcy1
	F: GTTCATTTTGGTATAGCACAATCTTC/R: ACCATAGGCATTCATAAACAATCCT
ADV	DP: FAM-CGGCAATACCGCAGCTGGTACCTTGCCG-Dabcy1
	F: GACAGAGGACAGCAAGAAACGCAG/R: GAGTGCAAAGGAGGGTCCATGA
hBov	DP: FAM-CCGAAGCTGCTGCACTTCGG-Dabcy1
	F: CCATAACCACTCCCAGGAAATGACG/R: TCACCACAAGCGTGGAGCT
BP	DP: Cy5-CTAGGCGCCGACTACATGGCCTAG-Dabcy1
	F: CTGTTCTGGCTGCGCGAG/R: TCCGGTTCGGATGAACCATGC
CP	DP: Cy5-GGCAGCACTGCAAACTATACTACTGCTGCC-Dabcy1
	F: TCTATGGGAGCCAAACCTACTGGA/R: AGGCAATGAAGCCTGCATTAGTGAAC
MP	DP:ROX-CTGGCTTCGTGTAGTTCAAGATTGCCAG-Dabcy1
	F: GTATGGTGGCGGGGGTCA/R: CCAAGTGGACTTGGACAAGGCAG
LP	DP: ROX-CTGGCGGACCTATTGGCCCAAATGCCAG-Dabcy1
	F: TGATGCCCGCTGGATCAACTTG/R: AGCCTTTACAAAGAGAGCATCCCTCTC

DP, detection probe; F, forward primer; R, reverse primer. Underlined sequences are complementary to targets. Pathogen abbreviations are defined in the Methods.

IFV-A, IFV-B, hRSV, hMPV, PIV-1-3, hCov 229E, and hRV) was performed with 2.5 μL 10 × RT-PCR reaction buffer, 0.25 mmol/L dNTP, 3.5 mmol/L $MgCl_2$, 2 U Taq enzyme, 40 U Moloney murine leukemia virus reverse transcriptase, 10 U RNase inhibitor, 0.06 μmol/L of each forward primer, 0.3 μmol/L of each reverse primer, and 0.045 μmol/L of each probe. Reaction II (primers and probes for ADV, hBov, BP, LP, MP, and CP) was performed with 2.5 μL 10 × PCR reaction buffer, 0.25 mmol/L dNTP, 3 mmol/L $MgCl_2$, 1.5 U Taq enzyme, 0.1 μmol/L of each forward primer, 0.5 μmol/L of each reverse primer, and 0.04 μmol/L of each probe.

Multiplex PCR, fluorescence signal collection, and melting curve analysis were performed in an ABI 7500 RT-PCR system (Applied Biosystems, Carlsbad, CA) with the following cycling parameters: a 30-min contamination control procedure at 50°C for reverse transcription, then a 15-min hold at 95°C, followed by 45 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. Melting curve analysis was started with a 2-min denaturation at 95°C, 2-min hybridization at 40°C, and a stepwise temperature increase from 40°C to 85°C at a thermal transition rate of 0.5°C/s. Fluorescence was recorded on 6-carboxyfluorescein and indodicarbocyanine-5, carboxy-X-rhodamine, and hexachlorofluorescein channels. Melting curves were obtained by plotting the negative derivative of fluorescence with respect to temperature versus temperature (-Rn'). *Tm* values were determined by identifying the peaks of the melting curves.

Limit of detection analysis

To analyze sensitivity, RNA standards for the nine viruses in reaction I (IFV-A, IFV-B, hRSV, hMPV, PIV-1-3, hCov 229E, and hRV) were constructed with a commercial *in vitro* transcription reagent (Large Scale RNA Production System-T7; Promega, USA), and recombinant plasmids containing the detection target genes for the six reaction II pathogens (ADV, hBov, BP, LP, MP, and CP) were constructed as standards by thymine-adenine cloning. The standards were quantitated spectrophotometrically at 260 nm by a Nanoplex nanophotometer. Then, RNA/DNA copy numbers were calculated and 10-fold serial dilutions of the standards from 5×10^7 to 5×10^0 copies/μL were performed with diethyl pyrocarbonate-treated water. These dilutions were tested, and the results were analyzed in terms of the melting curve *Tm* values.

Multiplex PCR assay validation

Blind testing of the 384 clinical samples from patients with previously identified respiratory infections was performed to validate the multiplex PCR assay.

Statistical analysis

The fluorescence probe melting curve assay results were compared with those obtained by conventional methods. A 2 × 2 table was used to estimate indices of sensitivity and specificity. Kappa correlation values were calculated.

Results

Multiplex PCR assay development

We set out to produce a two-tube multiplex real-time PCR assay to be used in combination with melting curve analysis. The assay was run with a temperature program that has two stages, namely amplification and melting, which were completed consecutively within 3.5 h. Being that it was a multiplex PCR, it was necessary to select primer sets and molecular beacons that were compatible with each other. Based on the rationally designed primers and beacons, all target genes could be identified accurately and specifically without any cross-reaction. We used primers that generate relatively short amplicons (100-150 base pairs), which produce brighter fluorescent signals because molecular beacons are better able to compete with complementary strands for target-strand binding when the amplicons are short.

Specificity of detection

A total of 47 positive clinical samples were used in specificity testing, the results of which are summarized in **Figure 1**. All pathogens [IFV-A and -B (*n* = 3 each virus), hRSV (*n* = 8), hMPV (*n* = 2), PIV-2, PIV-3 (*n* = 2 each virus), PIV-3 (*n* = 3), hCov 229E (*n* = 1), hRV (*n* = 5), ADV (*n* = 5), hBov (*n* = 2), BP (*n* = 4), LP (*n* = 2), MP (*n* = 3), and CP (*n* = 2)] were identified correctly, with no observed cross-reactivity.

Limits of detection

Sensitivity was evaluated with serial RNA transcript/plasmid DNA dilutions and analyzed in terms of melting curve *Tm* values, and the analysis results are shown in **Figure 2**. In reaction I, the limits of detection for IFV-A, IFV-B, and hCov 229E were 500 copies/μL; the limits of detection for hRSV, hMPV, PIV-1-3, and hRV were 50 copies/μL. In reaction II, the limits of detection for ADV and hBov were 500 copies/μL; the limits of detection for BP, LP, and MP were 50 copies/μL; and the limit of detection for CP was 5 copies/μL.

Performance in double blind testing

All 384 clinical specimens from patients with respiratory infections subjected to multiplex PCR assay testing were detected. Among them, 92 were detected as positive specimens (23.96%). As shown in **Table 2**, the pathogens with the highest frequencies of being positively detected were hRSV (n = 21, 5.47%), hRV (n = 11, 2.86%), PIV3 (n = 8, 2.08%), and ADV (n = 8, 2.08%); hCov 229E was not detected in these samples. In addition, 5 of 92 positive samples (5.43%) were shown to be co-infections (though no co-infection was detected by viral culture and immunofluorescence). Positive multiplex PCR assay results were obtained for 12 samples that had negative results by conventional methods. Sequencing indicated that these 12 divergent samples were indeed positive. Multiplex PCR assay testing was found to yield 75%

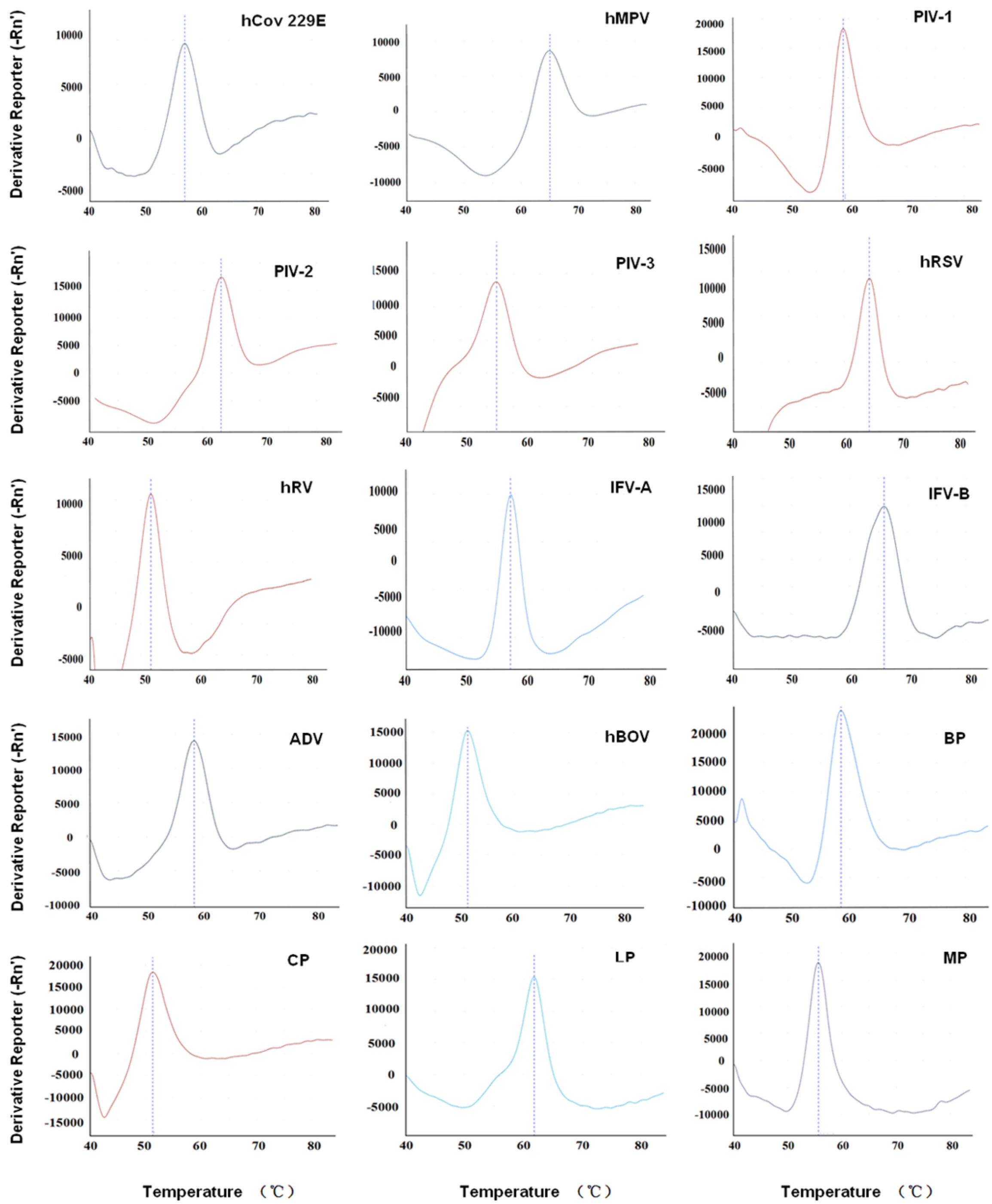

Figure 1. Melting curve analysis assay for the identification of 15 respiratory pathogens in two reactions. In reaction I, nine pairs of primers and nine probes were mixed. The probes targeting hCov 229E, hMPV, and PIV-1 were labeled with 6-carboxyfluorescein (FAM); probes targeting PIV-2, PIV-3, and hRSV were labeled with indodicarbocyanine-5 (Cy5); and probes targeting hRV, IFV-A, and IFV-B were labeled with carboxy-X-rhodamine (ROX). In reaction II, six pairs of primers and six probes were mixed. The probes targeting ADV and hBov were labeled with FAM; probes for BP and CP were labeled with Cy5; and probes for LP and MP were labeled with ROX. After PCR amplification, unique *Tm* values were obtained by melting curve analysis. Pathogen abbreviations are defined in the Methods.

sensitivity and 100% specificity for the respiratory pathogens tested. The kappa correlation values obtained for the pathogens ranged from 0.86 to 1.00 (**Table 2**).

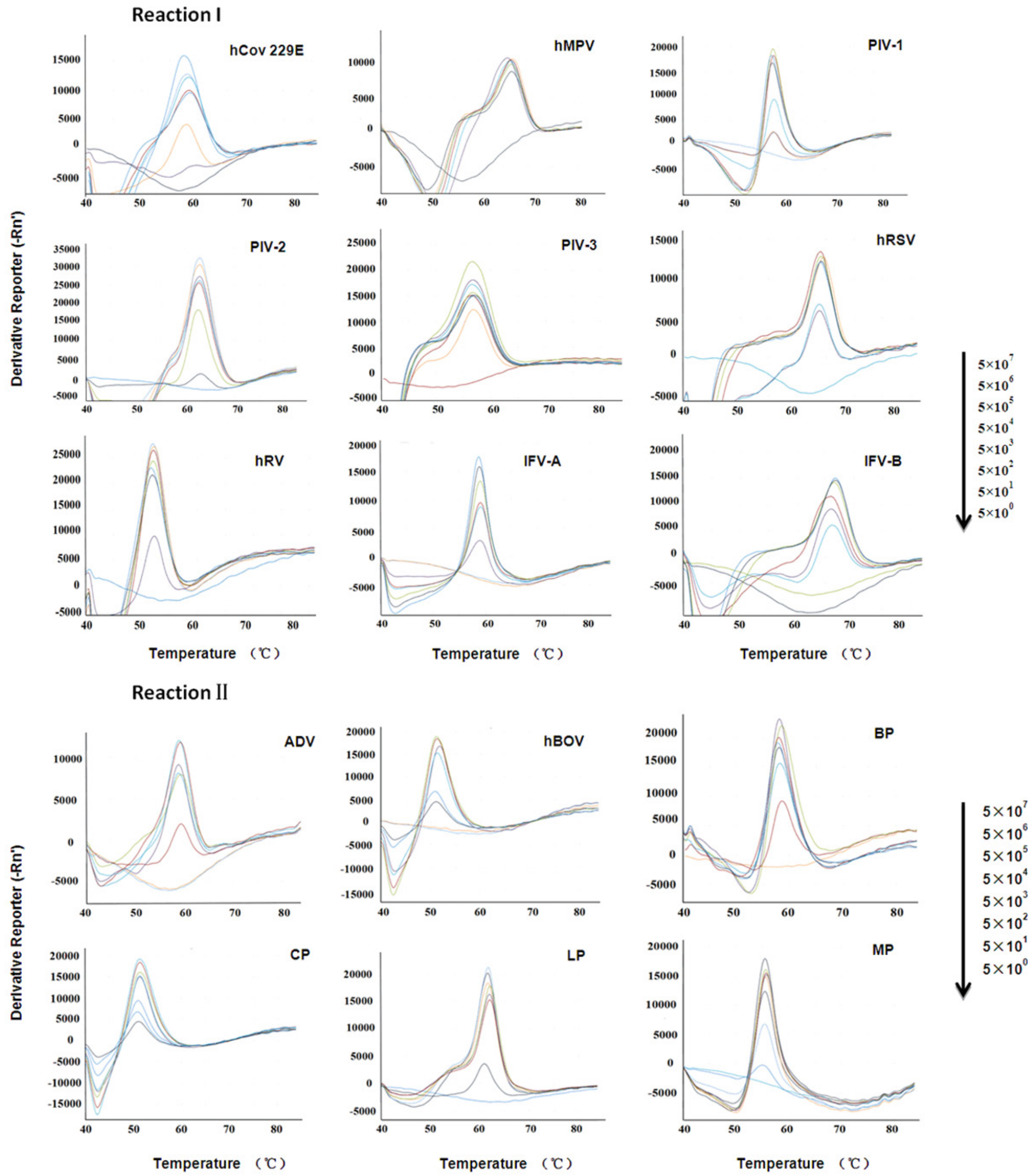

Figure 2. Detection limits for 15 respiratory pathogens assessed with 10-fold serial dilutions of standard templates (5.0×10^7-5.0 copies/μL). Pathogen abbreviations defined in the Methods.

Discussion

In this study, we demonstrated the utility of a successfully developed multiplex real-time PCR assay for simultaneous detection of 15 respiratory pathogens in two reactions. Employing rationally designed primers and beacons, all target genes could be identified accurately and specifically without any cross-reactivity. The specificity for target genes was 100% with a panel of 42 respiratory pathogens, consistent with no cross-reactions. The detection limits of this assay ranged from 5 copies/μL to 5×10^2 copies/μL at the nucleic acid level. The method was used to detect 15 pathogen species in 384 clinical samples, and the results were validated by comparison with conventional culture method results, which indicated ≥ 75% sensi-

Table 2. Validation of melting curve analysis assay with clinical samples

Pathogen	CMs	Melting curve, n		Assay performance, %			Agreement, Kappa value
		+	-	Consistency	Sensitivity	Specificity	
IFV-A	+	4	0	100	100	100	1.000 (*P* < *0.001*)
	-	0	380				
IFV-B	+	3	0	100	100	100	1.000 (*P* < 0.001)
	-	0	381				
hRSV	+	21	0	98.4	77.8	100	0.867 (*P* < 0.001)
	-	6	357				
PIV-1	+	3	0	100	100	100	1.000 (*P* < 0.001)
	-	0	381				
PIV-2	+	1	0	100	100	100	1.000 (*P* < 0.001)
	-	0	383				
PIV-3	+	8	0	100	100	100	1.000 (*P* < *0.001*)
	-	0	376				
hCov229E	+	0	0	-	-	-	-
	-	0	384				
hRV	+	11	0	99.5	84.6	100	0.914 (*P* < 0.001)
	-	2	371				
hMPV	+	4	0	100	100	100	1.000 (*P* < 0.001)
	-	0	380				
Adv	+	8	0	99.7	88.9	100	0.940 (*P* < 0.001)
	-	1	375				
hBov	+	3	0	100	100	100	1.000 (*P* < 0.001)
	-	0	381				
BP	+	5	0	99.7	83.3	100	0.908 (*P* < 0.001)
	-	1	378				
CP	+	3	0	100	100	100	1.000 (*P* < 0.001)
	-	0	381				
LP	+	1	0	100	100	100	1.000 (*P* < 0.001)
	-	0	380				
MP	+	6	0	99.5	75.0	100	0.855 (*P* < 0.001)
	-	2	376				

CM, conventional method; +, positive; -, negative.

tivity and 100% specificity for each respiratory pathogen tested, with high kappa correlation values (range, 0.86-1.00).

To the best of our knowledge, this work presents the first attempt to use two measurable parameters (*Tm* tags and corresponding fluorogenic probes) to detect a multitude of respiratory pathogens by combining multicolor probes with unique *Tm* values in a single reaction. Compared with other multiplexing techniques, our novel assay presents several advantages. Firstly, amplification and detection are carried out within a consecutive procedure in a closed-tube that conserves the sample and reduces the risk of PCR product contamination. Secondly, because our approach relies on a RT-PCR-based method, the results can be obtained automatically and accurately. Thirdly, the melting curve analysis provides a fast and intuitive readout of results. In addition, the assay is extremely cost-effective and highly efficient.

Notably, our current assay has limitations that require improvement. The numbers of positive clinical samples were not sufficient to conclude significant evaluations regarding sensitivity and specificity in this study; more samples will be collected for further validation. Additionally, although the probes used for each virus were designed according to a highly conserved region, it is possible that variation in these conserved regions exists. Thus, a larger number of clinical samples should be examined to verify the assay and to guide further optimization.

In conclusion, the presently introduced assay provided accurate identification of 15 respiratory pathogens and was validated with a large number of clinical samples. The method has a number of advantages, including rapidity, sensitivity, specificity, and low cost, which should facilitate its acceptance in clinical and public health laboratories.

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Disclosure of conflict of interest

None.

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