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A novel multiplex PCR for virus detection by melting curve analysis

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ABSTRACT

Background: Rapid and accurate laboratory diagnoses of viral infections are crucial for the management and treatment of patients with viral infections. Conventional methods for virus detection are labourious, time consuming, and only a single virus can be analysed in one assay.

Objectives: The objective of this study was to develop a novel real-time PCR method for multiple virus detection by melting curve analysis using Taqman probes in a single reaction.

Study design: As a model, six respiratory viruses were detected in one tube using three fluorophores. The specificity was assessed by cross-reaction tests with other common respiratory pathogens. The analytical sensitivity was assessed by testing the limit of detection of the assay using artificial plasmids as the positive template. The clinical evaluation of the established assay was evaluated for the detection of respiratory viruses in clinical samples, and the results were compared with direct fluorescent antibody testing (DFA).

Results: The six respiratory viruses were clearly distinguished by their respective melting temperature values in the corresponding fluorescence detection channels. No cross reactions were observed by cross reaction tests. The detection limits of this assay were 2 to 2×10^3 copies per reaction for each virus. The clinical evaluation of this assay was demonstrated by analysing 352 clinical samples, and 67(19.0%) samples were positive for at least one virus. The accordance rate between the established PCR and DFA testing was high, and ranged from 94.57% to 100%.

Conclusions: Taqman probe-based melting curve analysis is well suited for detection of multiple viruses in clinical and research laboratories because of its high throughput, reliability, and cost savings.

1. Background

Multiple virus detection can considerably improve the correct management of clinical viral infections and reduce unnecessary antibiotic prescriptions (Mayer et al., 2017). A large number of different methods are currently available for the detection of viral infections in clinical laboratories. Traditional laboratory diagnostic methods include virus culture, rapid antigen tests and the direct fluorescent-antibody assay (DFA), but they are labourious, time consuming, and usually only a single virus can be analysed in one assay (Storch, 2000).

With the advances of molecular techniques, real-time PCR has been developed and applied in clinical virology laboratories because of the high sensitivity, specificity and reproducibility. Real-time PCR is also easy to automate and relatively inexpensive. We can use different fluorophore-labeled probes to detect multiple viruses simultaneously in one tube. The combinatorial use of the fluorophore and melting temperature (Tm) as a 2D-label enables a magnitude increase in the number of targets in the real-time PCR platform.

TaqMan probes are widely used to monitor real-time PCR in clinical laboratories with the advantages of cost savings, multiplexing, and high flexibility in probe design. Recent studies have shown that the TaqMan probes can be used not only for monitoring PCR in real time but also for melting curve analysis under asymmetric PCR conditions (Huang et al., 2011). Multicolor melting curve analysis using the TaqMan probe has been successfully applied in mutant gene detection and genotyping (Botezatu et al., 2017; Mou et al., 2016; Wang et al., 2017; Xia et al., 2016; Huang et al., 2016; Gao et al., 2015; Huang et al., 2017). Nevertheless, the application to multiple virus detection has not yet been reported.

In this study, we sought to use this approach to establish a novel real-time PCR for multiple virus detection. As a model, six respiratory viruses, including respiratory syncytial virus (RSV), influenza A (FluA),

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influenza B (FluB), parainfluenza virus type 1 (PIV1), parainfluenza virus type 2 (PIV2) and parainfluenza virus type 3 (PIV3), were detected in one tube using three fluorophores.

2. Study design

2.1. Viruses and synthetic plasmids

Viral isolates for RSV, FluA, FluB and PIV3 were maintained in our laboratory. PIV1 (ATCC-VR94) and PIV2 (ATCC-VR92) were obtained from the Shanghai Medical College of Fudan University.

Plasmids containing specific viral target fragment sequences were synthesized by Sangon (Shanghai, China) on the basis of the sequences provided by the Genome Sequencing Project in GenBank. The concentration of each plasmid was quantified by the NanoVue spectrophotometer (GE, USA).

2.2. Clinical samples

A total of 352 nasopharyngeal aspirates (NPA) were collected from children diagnosed with respiratory infection in the children's hospital of fudan university during January 2017 to February 2017. These samples were used to evaluate the multiplex PCR for its diagnostic capacity.

2.3. Nucleic acid extraction and reverse transcription

Viral nucleic acid was extracted from viral isolates or clinical samples using a kit (Mole, China) according to the protocol suggested by the manufacturer on the automated extraction platform (Tianlong, China).

Reverse transcription was performed in a reaction mixture consisting of 8 µl of extracted viral nucleic acid, 4 µl of 5 × PrimeScritBuffer, 2 µl of Random 6mers, 1 µl of PrimeScript RT Enzyme Mix I, and 5 µl of nuclease-free water (TaKaRa, China). Reverse transcription was performed using a model 2720 thermal cycler (Applied Biosystems, USA) with amplification parameters as follows: incubation at 37 °C for 30 min, followed by 85 °C for 5 s.

2.4. Primers and probes

The primers and probes for RSV, PIV1, PIV2, PIV3, FluA and FluB were designed using Primer Premier 5.0 (Primer Biosoft International, USA) based on the conserved regions of target genes and their specificity was confirmed by the Nucleotide BLAST search. The N gene of RSV, HN genes of PIV1, PIV2 and PIV3, M gene of FluA, and NS gene of FluB were selected as target genes for primer design. Three fluorophores were selected and probes with the same fluorophores were designed to have different Tm values. RSV and PIV2 probes were labelled with FAM; PIV1 and PIV3 probes were labelled with HEX; FluA and FluB probes were labelled with ROX. All primers and probes were synthesized and purified by Sangon, China. The sequences of the primers and probes are given in Table 1.

2.5. Multiplex PCR assay development

In order to generate excess single-stranded amplicon for probe hybridization to generate sufficient melting curve signals, asymmetric PCR and 5′-nuclease-deficient mTaq DNA Polymerase (Kangwei Biotech, China) were introduced in this assay. The lack of the 5′-3′ exonuclease activity of mTaq DNA polymerase can avoid hydrolysing probes in the reaction. After optimization, PCR and melting curve analysis were performed on a LightCycler 480II real-time system (Roche Diagnostics, Switzerland) in a 25-µL reaction containing 1 × PCR buffer, 0.2 mM dNTPs, 3.0 mM $MgCl_2$, 2.5 U mTaq DNA polymerase, primers and probes as listed in Table 1, and 2 µL cDNA template. The final amplification program started with denaturation for 5 min at 95 °C, followed by 50 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Melting curve analysis started with a denaturation step of 1 min at 95 °C, a hybridization step of 5 min at 35 °C, and an incremental temperature rise from 35 °C to 85 °C at a ramp rate of 0.01 °C/s with fluorescence acquired from the FAM, HEX, and ROX channels 10 times per °C.

2.6. Specificity and analytical sensitivity of the multiplex PCR

The specificity of the multiplex PCR assay was assessed by cross reaction tests with other common respiratory pathogens including respiratory viruses and bacteria. Clinical Samples positive for adenovirus (ADV), human rhinovirus/enterovirus (RV/EV), and human corona virus OC43 (hCoV-OC43) were confirmed by Filmarray, an automated nested multiplex PCR System (bioMérieux, France). Standard strains for influenza A/H1N1, influenza A/H3N2, influenza A/H7N9, influenza B/Victoria, influenza B/Yamagata, measles virus, mumps virus, rubella virus, varicella zoster virus, *E. coli*, *P. aeruginosa* and *S.aureus* were obtained from the National Institutes for Food and Drug Control, China.

The analytical sensitivity of the multiplex PCR was assessed by testing the limit of detection (LOD) of the assay. The plasmids containing specific viral target fragment sequences were serially 10-fold diluted from 10^5 copies/µl to 10° copy/µl and analysed using the multiplex PCR. Each concentration of plasmid was analysed in triplicate. Water was used as a negative control.

2.7. Evaluation of the multiplex PCR using clinical samples

A total of 352 paediatric NPA samples described above were analysed using both the multiplex PCR assay and direct fluorescent antibody testing (DFA). DFA was performed for seven respiratory viruses: respiratory syncytial virus (RSV), influenza A (FluA), influenza B (FluB), parainfluenza viruses 1–3 (PIV 1–3) and adenovirus (ADV) using a panel of DFA assays (Diagnostic Hybrids, Athens, OH), a Food and Drug Administration (FDA) approved assay for respiratory virus detection. We were blinded to the DFA results while evaluating the NPA samples using the multiplex PCR assay. The results were compared for those viruses identified by both testing methods. Positive percent agreement is the percent of time that the multiplex PCR detected a virus when DFA detected it. Similarly, negative percent agreement is the percent of time that the multiplex PCR did not detect a virus when DFA did not detect it. The degree of agreement was measured by Cohen's kappa, which was performed as described previously (Watson and Petrie, 2010). Statistical analyses were carried out using STATA 10 (College Station, TX, USA).

The samples detected positive only by the multiplex PCR were confirmed by independent PCR and sequencing. The sequencing was performed by Sangon (Shanghai, China) and compared with the sequences in GenBank for pathogen identification using BLAST. The samples detected positive only by DFA were confirmed by conventional real-time PCR assays (Mole, China).

3. Results

3.1. Development of the multiplex PCR

To develop a melting curve-based multiplex PCR assay for the simultaneous detection of six human respiratory viruses in one tube, we designed 6 sets of primers and probes using three colour fluorophores. The actual Tm values of the probes ranged from 55.9 °C to 72.6 °C (Fig. 1). Six viruses can be clearly distinguished from each other by Tm value and fluorophore. The corresponding Tm values were 63.2 °C and 71.4 °C for PIV2 and RSV, respectively, in the FAM channel (Fig. 1A); 55.9 °C and 67.2 °C for PIV1 and PIV3, respectively, in the HEX channel (Fig. 1B); 69.0 °C and 72.6 °C for FluA and FluB, respectively, in the

Table 1
Primers and probes used for the multiplex PCR.

Virus	Primer or probe	Sequence (5'-3')	Final concentration
RSV	Probe	FAM-CTTCACGAAGGCTCCACATACACAGCTG-BHQ1	0.2 μM
	Forward primer	GCAAATATGGAAACATACGTGAACA	0.1 μM
	Reverse primer	GCACCCATATTGTWAGTGATGCA	1.5 μM
PIV2	Probe	FAM-ATTTACCTAAGTGATGGAATCAATCG-BHQ1	0.2 μM
	Forward primer	GCATTTCCAATCTTCAGGACTATGA	0.1 μM
	Reverse primer	ACCTCCTGGTATAGCAGTGACTGAAC	1.0 μM
PIV1	Probe	HEX-AACTTAATCACTCAAGGATG-BHQ2	0.2 μM
	Forward primer	CCTGATATGACTTCCCTA	0.1 μM
	Reverse primer	CCTTCATTATCAATTGGTG	1.5 μM
PIV3	Probe	HEX-TTTCCCAGGACACCCAGTTGTGTT-BHQ2	0.2 μM
	Forward primer	CATGGACTATGAGAYGCYTGA	0.1 μM
	Reverse primer	GGRTATGGAGGTCTTGAACA	2.0 μM
FluA	Probe	ROX-CAGTCCTCGCTCACTGGGCA-BHQ2	0.2 μM
	Forward primer	CAAANCGTCTACGYTGCAGTCC	0.1 μM
	Reverse primer	AAGACCRATYYTGTCACCTCTRACTAAG	1.0 μM
FluB	Probe	ROX-CCAATTCGAGCAGCTGAAACTGCGGTG-BHQ2	0.2 μM
	Forward primer	TCCTCAAYTCACTCTTCGAGCG	0.1 μM
	Reverse primer	CGGTGCTCTTGACCAAATTGG	1.5 μM

ROX channel (Fig. 1C). To verify the ability of established multiplex PCR to detect co-infection, a mixed template containing all six virus cDNAs was added to the reaction. Two distinguishable melting peaks with specific Tm values were generated in the FAM and HEX channels (Fig. 1A and 1B, black line), which indicate the presence of PIV2, RSV, PIV1 and PIV3 target amplicons. However, the melting peaks of FluA and FluB were fused, and generated a wide melting peak with a Tm value between 69.0 °C and 72.6 °C (Fig. 1C, black line).

3.2. Analytical sensitivity and specificity of the multiplex PCR

The LOD of the multiplex PCR for RSV, PIV1, PIV2, PIV3, FluA and FluB were 2×10^3, 2, 2×10^3, 2, 2, 2×10^2, and 2×10^3 copies per reaction, respectively (Fig. 2).

The specificity of the multiplex PCR was evaluated by cross reaction tests with other common respiratory pathogens as described above. Three common subtypes of influenza A (H1N1, H3N2, and H7N2) virus and two subtypes of influenza B (Yamagata and Victoria) virus can all be well detected using the multiplex PCR. There were no cross reactions observed against other common respiratory pathogens that were not involved in the detection profile. These results indicated that the multiplex PCR had good specificity for the detection of respiratory viruses.

3.3. Clinical evaluation of the multiplex PCR

Of the 352 clinical samples tested, 67 (19.0%) samples were positive by the multiplex PCR, which included 4 co-infections. DFA detected 46 (13.1%) positive samples of which 2 were co-infections.

The accordance rate between the multiplex PCR and DFA testing was high and ranged from 94.57% to 100% (Table 2). Most of the

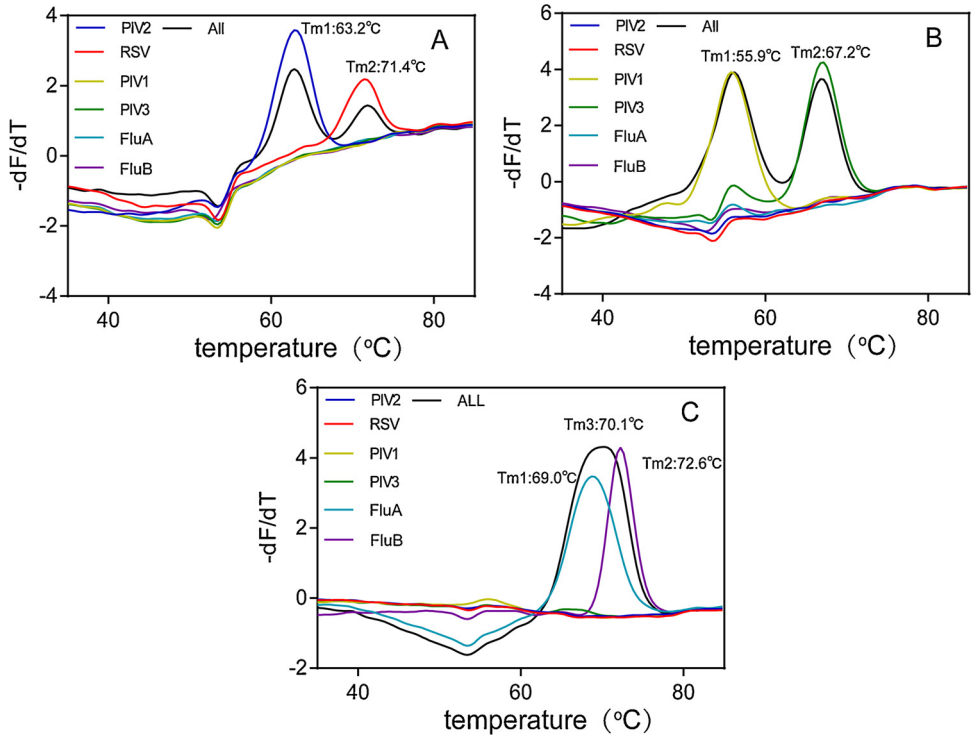

Fig. 1. Melting curves of six respiratory viruses using multiplex PCR. (A) The melting curves generated in the FAM channel; (B) the melting curves generated in the HEX channel; and (C)the melting curves generated in the ROX channel. -dF/dT, negative derivative of fluorescence with respect to temperature versus temperature.

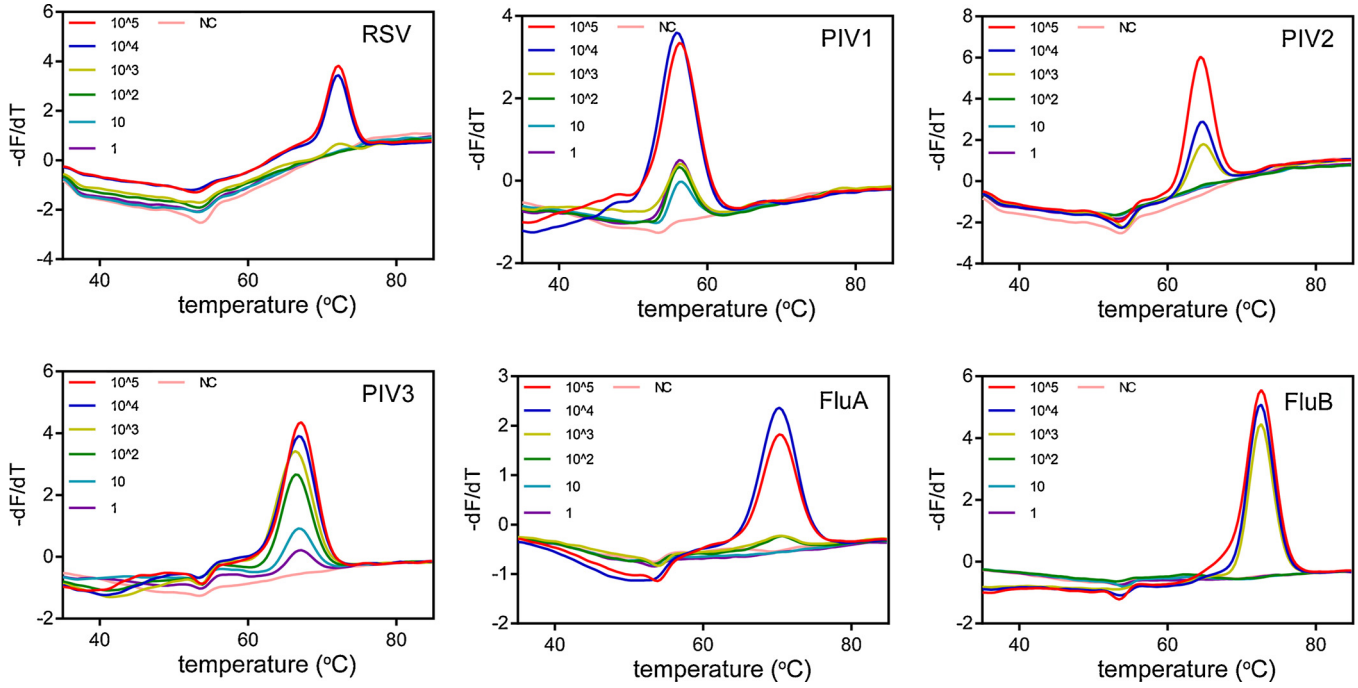

Fig. 2. Sensitivity of the multiplex PCR using serially diluted templates (10^5, 10^4, 10^3, 10^2, 10, and 1 copies/μl). NC, negative control.

Table 2
Performance of the multiplex PCR for clinical samples compared with the direct fluorescent antibody testing.

Virus	No. of specimens by multiplex PCR *VS.* direct fluorescent antibody testing				Accordance rate (%)	Kappa	PPA (%)	NPA (%)
	+/+	+/-	-/+	-/-				
RSV	14	1	4	333	98.58	0.841	77.78	99.70
PIV3	24	18	0	310	94.57	0.701	100	94.51
PIV1	2	6	0	344	98.30	0.394	100	98.29
PIV2	1	0	0	351	100	1.000	100	100
FluA	2	2	0	348	99.43	0.664	100	99.43
FluB	1	0	0	351	100	1.000	100	100

PPA, positive percent agreement; NPA, negative percent agreement.

discordant results were samples that were positive for virus by multiplex PCR but negative by DFA (+/-).

Twenty-seven clinical samples positive by multiplex PCR but negative by DFA (+/-) were confirmed by sequencing. The sequencing results were in accordance with the multiplex PCR, except for one sample that was positive for PIV1 in the multiplex PCR but tested negative by sequencing.

Four clinical samples negative by multiplex PCR, but positive for RSV by DFA (-/+) were confirmed by the conventional PCR assay. Three samples were positive for RSV by the conventional PCR assay, and the other sample was negative (data not shown).

4. Discussion

An increasing number of recent studies have focused on the development of molecular methods to solve the problem of multiple virus detection (Kishimoto et al., 2017; Boyd et al., 2015; Pripuzova et al., 2012; Choudhary et al., 2013). In this study, we successfully developed a novel multiplex real-time PCR assay for the simultaneous identification of 6 respiratory viruses using probe-based melting curve analysis in a single reaction tube. The multiplex PCR developed and described here combined asymmetric PCR and multi-colour melting curve analysis techniques to achieve multiplex nucleic acid detection. This strategy uses the fluorophore and Tm as two measurable parameters (2D-label), which enables a magnitude increase in the number of targets that can be distinguished on the real-time PCR platform (Liao et al., 2013).

In our study, three fluorescence detection channels (FAM, HEX, and ROX) were introduced and two probes with discriminable Tm values were detected in each channel, thus six respiratory viruses could be detected simultaneously in a single reaction tube. This multiplex PCR accurately detected and discriminated multiple pathogens in one sample at the same time, which means this method has a good ability to detected co-infections. Because the Tm values of FluA and FluB are too close, their melting peaks are fused and generate a wide melting peak with a Tm value between them when they are positive at the same time. However, the melting peak can be well distinguished from single infection of FluA or FluB. Moreover, there are few co-infections of FluA and FluB (Ren et al., 2009).

To evaluate the clinical utility of the multiplex PCR, 352 paediatric NPA samples were analysed, and the results demonstrate a successful real-world application of this method. When compared to DFA, the multiplex PCR showed a high percent agreement, which ranged from 94.57% to 100%. The most common reason for discordance was the detection of pathogens by multiplex PCR in DFA-negative samples. These discordant NPA samples were confirmed by sequencing, and most of the sequencing results were consistent with the multiplex PCR. We believe this is due to the increased sensitivity of PCR when compared to DFA.

Compared with other virus detection techniques, this novel multiplex PCR has many advantages. First, the number of detectable targets is significantly increased in our 2D-label multiplex PCR when compared with those multiplex PCR assays using a label that involves either Tm or fluorophore alone (Wan et al., 2016; Simmons et al., 2016). In this study, 2 probes with discriminable Tm values were designed in each channel. It might be possible to design more probes in one channel, and then the number of targets detected in a single tube could be further enhanced. Second, this novel multiplex PCR is cost-effective. The cost of a probe-based real-time PCR assay primarily depends on the consumption of the fluorescence probe, and the TaqMan probe used in our study was easy to design and synthesize. The cost was lower than

probes with special modifications such as PNA and FRET. One peculiar requirement is asymmetric amplification, which is easy to perform without extra cost (Huang et al., 2011). Third, this multiplex PCR can be performed with almost any real-time PCR machine, making it possible for wide application in the clinical laboratory for multiple virus detection. Similar to most other PCR methods, this novel multiplex PCR is fast and can be completed within 3 h. Therefore enabling results, including sample processing, nucleic acid extraction and analysis to be obtained within a single working day.

However, there are some limitations of the multiplex PCR based on probe melting curve analysis. First, the TaqMan probes were designed based on the known sequence of conserved genes. As new variants of the virus continue to emerge, it might be possible that some variants could lead to mismatch of the probe against the target sequence, which could cause a Tm shift from the predefined values. In this regard, the established assay should be updated on an as needed basis. Second, as an end-point detection assay, melting curve analysis-based multiplex PCR is non-quantitative in nature and therefore restricts its application in virus quantitative detection. However, as shown in Fig. 2, the signal of the melting curves increased with increasing template concentrations within a certain range. In some cases, we observed that when the concentration of template exceeds a certain standard, an increase of concentration does not result in the expected increase in the melt peaks, and sometimes they are even reduced (Fig. 2, PIV1). The reason is that after reaching the amplification plateau, complementary DNA strands rapidly anneal to one another and fall out of the reaction, whereas the excessive single-stranded DNA continues to be available to the primer and be converted into the double stranded form. As a result, the concentration of the single-stranded DNA in asymmetric PCR may be decreased after reaching plateau (Botezatu et al., 2015). Third, the application of melting curve analysis increases the time required for analysis compared with standard real-time PCR. Another limitation is that the analytical sensitivity of the multiplex PCR is not high enough, especially for RSV, PIV2, and FluB. Further optimization of the reaction is needed.

In conclusion, we developed a novel multiplex PCR for the detection of six common respiratory viruses by melting curve analysis. This method is expected to be widely used in clinical and research laboratories due to its high throughput nature and easy application when compared with traditional methods such as DFA. Furthermore, this strategy can be modified and extended to detect additional pathogens responsible for many other infectious diseases, such as meningitis, gastrointestinal infections and bloodstream infections.

Competing interests

None declared.

Ethical approval

Not required.

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References

Botezatu, I.V., Nechaeva, I.O., Stroganova, A.M., Senderovich, A.I., Kondratova, V.N., Shelepov, V.P., Lichtenstein, A.V., 2015. Optimization of melting analysis with TaqMan probes for detection of KRAS, NRAS, and BRAF mutations. Anal. Biochem. 491, 75–83.

Botezatu, I.V., Panchuk, I.O., Stroganova, A.M., Senderovich, A.I., Kondratova, V.N., Shelepov, V.P., Lichtenstein, A.V., 2017. Scanning for KRAS, NRAS, BRAF, and PIK3CA mutations by DNA melting analysis with TaqMan probes. Mol. Biol. (Mosk) 51, 50–58.

Boyd, V., Smith, I., Crameri, G., Burroughs, A.L., Durr, P.A., White, J., Cowled, C., Marsh, G.A., Wang, L.F., 2015. Development of multiplexed bead arrays for the simultaneous detection of nucleic acid from multiple viruses in bat samples. J. Virol. Methods 223, 5–12.

Choudhary, M.L., Anand, S.P., Heydari, M., Rane, G., Potdar, V.A., Chadha, M.S., Mishra, A.C., 2013. Development of a multiplex one step RT-PCR that detects eighteen respiratory viruses in clinical specimens and comparison with real time RT-PCR. J. Virol. Methods 189, 15–19.

Gao, L., Liu, Y., Sun, M., Zhao, Y., Xie, R., He, Y., Xu, W., Liu, J., Lin, Y., Lou, J., 2015. Rapid detection of α-thalassaemia alleles of – SEA /, -α 3.7 / and -α 4.2 / using a dual labelling, self-quenching hybridization probe/melting curve analysis. Mol. Cell. Probes 29, 438–441.

Huang, Q., Liu, Z., Liao, Y., Chen, X., Zhang, Y., Li, Q., 2011. Multiplex fluorescence melting curve analysis for mutation detection with dual-labeled, self-quenched probes. PLoS One 6 e19206.

Huang, Q., Wang, X., Tang, N., Zhu, C., Yan, T., Chen, P., Li, Q., 2016. Rapid detection of non-deletional mutations causing alpha-thalassemia by multicolor melting curve analysis. Clin. Chem. Lab. Med. 54, 397–402.

Huang, Q., Wang, X., Tang, N., Yan, T., Chen, P., Li, Q., 2017. Simultaneous genotyping of α-Thalassemia deletional and nondeletional mutations by real-time PCR–Based multicolor melting curve analysis. J. Mol. Diagn. 19, 567–574.

Kishimoto, M., Tsuchiaka, S., Rahpaya, S.S., Hasebe, A., Otsu, K., Sugimura, S., Kobayashi, S., Komatsu, N., Nagai, M., Omatsu, T., Naoi, Y., Sano, K., Okazaki-Terashima, S., Oba, M., Katayama, Y., Sato, R., Asai, T., Mizutani, T., 2017. Development of a one-run real-time PCR detection system for pathogens associated with bovine respiratory disease complex. J. Vet. Med. Sci. 79, 517–523.

Liao, Y., Wang, X., Sha, C., Xia, Z., Huang, Q., Li, Q., 2013. Combination of fluorescence color and melting temperature as a two-dimensional label for homogeneous multiplex PCR detection. Nucleic Acids Res. 41, e76.

Mayer, L.M., Kahlert, C., Rassouli, F., Vernazza, P., Albrich, W.C., 2017. Impact of viral multiplex real-time PCR on management of respiratory tract infection: a retrospective cohort study. Pneumonia 9, 4.

Mou, Y., Athar, M.A., Wu, Y., Xu, Y., Wu, J., Xu, Z., Hayder, Z., Khan, S., Idrees, M., Nasir, M.I., Liao, Y., Li, Q., 2016. Detection of anti-hepatitis B virus drug resistance mutations based on multicolor melting curve analysis. J. Clin. Microbiol. 54, 2661–2668.

Pripuzova, N., Wang, R., Tsai, S., Li, B., Hung, G.C., Ptak, R.G., Lo, S.C., 2012. Development of real-time PCR array for simultaneous detection of eight human blood-borne viral pathogens. PLoS One 7 e43246.

Ren, L., Gonzalez, R., Wang, Z., Xiang, Z., Wang, Y., Zhou, H., Li, J., Xiao, Y., Yang, Q., Zhang, J., Chen, L., Wang, W., Li, Y., Li, T., Meng, X., Zhang, Y., Vernet, G., Paranhos-Baccalà, G., Chen, J., Jin, Q., Wang, J., 2009. Prevalence of human respiratory viruses in adults with acute respiratory tract infections in Beijing, 2005–2007. Clin. Microbiol. Infect. 15, 1146–1153.

Simmons, M., Myers, T., Guevara, C., Jungkind, D., Williams, M., Houng, H.S., 2016. Development and validation of a quantitative, one-step, multiplex, real-time reverse transcriptase PCR assay for detection of dengue and Chikungunya viruses. J. Clin. Microbiol. 54, 1766–1773.

Storch, G.A., 2000. Diagnostic virology. Clin. Infect. Dis. 31, 739–751.

Wan, Z., Zhang, Y., He, Z., Liu, J., Lan, K., Hu, Y., Zhang, C., 2016. A melting curve-based multiplex RT-qPCR assay for simultaneous detection of four human coronaviruses. Int. J. Mol. Sci. 17, 1880.

Wang, X., Hong, Y., Cai, P., Tang, N., Chen, Y., Yan, T., Liu, Y., Huang, Q., Li, Q., 2017. Rapid and reliable detection of nonsyndromic hearing loss mutations by multicolor melting curve analysis. Sci. Rep. 7, 42894.

Watson, P.F., Petrie, A., 2010. Method agreement analysis: a review of correct methodology. Theriogenology 73, 1167–1179.

Xia, Z., Chen, P., Tang, N., Yan, T., Zhou, Y., Xiao, Q., Huang, Q., Li, Q., 2016. Rapid detection of G6PD mutations by multicolor melting curve analysis. Mol. Genet. Metab. 119, 168–173.