A mismatch-tolerant RT-quantitative PCR: application to broad-spectrum detection of respiratory syncytial virus

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ABSTRACT

Quantitative PCR (qPCR) is widely used to detect viruses. However, mismatches occurring in the 3'-end of the primers reduce amplification efficiency of qPCR and limit its capacity in detection of highly variable viruses. Here, we reported a mismatch-tolerant RT-qPCR with a small amount of additional high-fidelity DNA polymerase for simultaneous detection of RSV-A and RSV-B. The novel assay had higher amplification efficiency for various variants forming mismatches with the primers than the conventional RT-qPCR, and showed good specificity and sensitivity. It demonstrated a good correlation coefficient with a commercial RSV detection kit and had relatively lower Ct values than the kit for 16 of 20 RSV-positive samples. The mismatch-tolerant qPCR technique is a promising approach for sensitive detection of highly variable viruses.

METHOD SUMMARY

We developed a mismatch-tolerant RT-quantitative PCR by simply adding a small amount of additional high-fidelity DNA polymerase to the standard reaction mixtures. The new method well tolerates mismatches between primers and templates and is especially suited to the detection of highly variable viruses.

KEYWORDS:

detection • high-fidelity DNA polymerase • mismatch-tolerant qPCR • respiratory syncytial virus • virus variant

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Emerging and re-emerging infectious diseases seriously threaten global public health [1]. Most infectious diseases are caused by viruses, especially RNA viruses such as HIV-1, HCV, MERS-CoV, Ebola, Influenza A (H7N9) and Zika virus [2,3]. Rapid and accurate diagnosis of involved viruses is a pivotal step for the prevention and control of infectious diseases [1]. TaqMan probe-based real-time PCR was the most widely used and most robust method for qualitative and quantitative detection of viruses because of its high-sensitivity, close-tube operation and real-time monitoring [4]. However, viruses are more genetically variable than prokaryotic and eukaryotic organisms [5,6]. In particular, RNA viruses (e.g., HIV-1 and HCV) have higher mutation rates than DNA viruses, and often evolve into various genotypes and subtypes [7,8]. It is relatively difficult to find a completely conserved genomic region shared by different genotypes and subtypes for PCR primer design. Therefore, degenerate primers were often used for pan-genotype/subtype detection of RNA viruses [9,10]. Despite this, the presence of various variants may cause mismatch between the primers and the targets. One or more mismatches occurring in the 3' end of the primers will largely reduce amplification efficiency of qPCR [11], limiting the capacity of qPCR in the sensitive detection of highly variable viruses. Therefore, development of accurate, sensitive and broad-spectrum detection methods for highly variable viruses is still a big challenge.

Respiratory syncytial virus (RSV), an enveloped negative-strand RNA virus belonging to the genus *Pneumovirus* of the Paramyxoviridae family [12], is the main viral pathogen causing acute

respiratory tract infections (ARTIs) in infants and young children [13–15]. RSV infection is associated with bronchiolitis, pneumonia, otitis media, rhinitis and other reactive airway diseases [16]. It has been estimated that there are approximately 33.1 million RSV-related cases worldwide per year in children younger than 5 years, and the mortality was 118,200 [17,18]. Early diagnosis and monitoring of RSV infection play a crucial role in the treatment and management of RSV patients.

RSV is genetically divided into two major distinct groups, RSV-A and RSV-B [19]. Although some detection methods, including serological and various nucleic acid amplification assays, have been developed [20–28], there still lacks an efficient RT-qPCR assay for simultaneous detection of all RSV-A and RSV-B variants. In this study, we developed a mismatch-tolerant RT-qPCR technique with a small amount of additional high-fidelity DNA polymerase and used it to simultaneously detect various RSV-A and RSV-B variants.

MATERIALS & METHODS

Reaction system of the mismatch-tolerant RT-qPCR

Quant One Step RT-qPCR Kit (Tiangen Biotechnology Co., Ltd, Beijing, China) and Q5 high-fidelity DNA polymerase (New England Biolabs, USA) were used to establish the reaction system of the mismatch-tolerant RT-qPCR assay. The difference between the novel assay and the conventional TaqMan probe-based method is the addition of a small amount of high-fidelity DNA polymerase into the reaction system. The amount of Taq DNA polymerase and Q5 high-fidelity DNA polymerase was optimized at 0.75 U and 0.15 U per 25 µl reaction mix, respec- ►

Figure 1. The principle of the mismatch-tolerant qPCR. (A) The principle of the conventional qPCR. The presence of a mismatch at the 3' end of the primer will largely reduce or stop the amplification. (B) The principle of the mismatch-tolerant qPCR. When there is a mismatch between primer and target at the 3' end of the primer, the small amount of high-fidelity DNA will remove the mismatched base from the primer by 3'-5' exonuclease activity, and then Taq DNA polymerase will initiate primer extension. In theory, mismatches between primer and target have less influence on DNA amplification by the mismatch-tolerant qPCR. M: Copy numbers of templates; n: PCR cycle number.

	Forward primer (5'-3')	Probe (5'-3')	Reverse primer (5'-3')
Genome sequence	TGATACACTCAACAAAGATCACTTCTG (363)	CATCCAGCAAAATACAC (378)	TCTCCTGTGCTCGTTGGAT (426)
	C.....T..... (55)	T..... (48)	T..... (6)
	T..... (4)	T..... (7)	C..... (2)
	C..... (5)	G..... (2)	T..... (1)
	C.....A (2)		
RSV-A	A.....A (1)		
	T.....A (1)		
	C.....T..... (1)		
	C.....A (1)		
	C.....CA (1)		
	T.....A (1)		
	T.....C..... (179)	T..... (199)	A.....A..... (189)
	T.....A.....T.....G.....C.....G.....A..... (11)	T.....C..... (1)	A.....A.....A..... (5)
RSV-B	T.....G.....G.....G..... (7)	T..... (1)	A.....A..... (4)
	A.....G.....A.....G..... (3)		G.....AGA..... (1)
	A.....A.....A..... (1)		C..... (1)
			T..... (1)
Standard strains			
RSV-A	T.....		
RSV-B	T.....G.....G.....		A.....A.....A.....

Figure 2. Sequence alignments of primer and probe regions of all available RSV sequences. A total of 636 sequences were downloaded from GenBank on June 8, 2018. The black small dots indicate identical bases to the topmost sequence. The numbers in the parenthesis show the number of identical sequences.

► tively. The optimized reaction mix of the novel assay included 12.5 μ l 2 $\times$ Quant One Step Probe RT-qPCR Master Mix, 0.75 U HotMaster Taq polymerase, 0.15 U Q5[®] High-fidelity DNA Polymerase (New England Biolabs, USA), 0.1 μ l Quant RT, 400 nM each forward and reverse primers, and 100 nM RSV probe. The cycling condition was reverse transcription at 50°C for 30 min,

enzyme activation and denaturation at 92°C for 3 min, then by 40 cycles of denaturation at 92°C for 10 s, annealing and extension at 55°C for 40 s. Fluorescence signal was collected at the annealing and extension step of each cycle. The threshold value was automatically set to 0.05 by the Light Cycler 96 Real-Time PCR System (Roche Diagnostics, Germany).

Virus strains & patient samples

To determine the sensitivity and limit of detection (LOD) of the novel RSV RT-qPCR assay, two RSV standard strains, RSV-A (VR-1540) and RSV-B (VR-1400) were used. Eight standard respiratory virus strains, including adenovirus (VR-930), enterovirus (VR-1076), influenza A and B viruses (VR-333 and VR-789), parainfluenza viruses 3 (VR-93), HCoV-229E (VR-740), HCoV-OC43 (VR-1558), and human rhinovirus (VR-489) were used as control viruses to test the assay specificity. All the above strains were purchased from the American Type Culture Collection (ATCC) and stored in the Lab at Institut Pasteur of Shanghai, Chinese Academy of Sciences.

To evaluate the performance of the novel assay, 40 nasopharyngeal swabs collected from children with ARTIs in our previous studies were used [29,30]. Among these samples, half were previously detected as RSV-positive and half as RSV-negative using our in-house multiplex RT-qPCR method. The positive samples included 14 RSV-A and six RSV-B.

RNA extraction & preparation of RNA standards

RNA was extracted from 140 μ l virus culture supernatants or clinical samples and eluted in 50 μ l nuclease-free H₂O by High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instruction. The RNA from $3.75 \times 10^{5.75}$ PFU/ml of RSV strain VR-1540 was used as a standard template to determine the LOD of the developed assay by serial tenfold dilution from 10^5 to 10^0 PFU/ml.

Construction of various mutants

To test the performance of the novel assay for mismatched targets, we constructed a series of mutants that form mismatches with the RSV-specific primers. The targeted RSV fragment was obtained by amplification of the standard strain with a T7 promoter-containing primer and cloned into pMD18-T plasmid vector (TaKaRa, Dalian, China). A series of mutants were constructed using a rapid mutagenesis system of transgenic biotechnology (TransGen, Beijing, China), and confirmed by Sanger sequencing. Mutant RNA templates were prepared by *in vitro* transcription using T7 RNA polymerase.

Evaluation of the assay using clinical samples

To assess the performance of the novel assay for RSV-A and RSV-B RNA detection, we evaluated it using 40 clinical samples and compared it with the conventional assay and a commercial kit (Huayin RSV RNA kit, Huayin Pharmaceutical Technology Co., Ltd, Guangdong, China). The kit was designed for detection of RSV-A and -B RNA and is often used in clinical RSV diagnosis in China. A Bland–Altman analysis was used to assess the level of agreement between the novel assay and the commercial kit. A range of agreement was defined as the mean of difference (bias) ± 1.96 SD.

RESULTS & DISCUSSION

The principle of the mismatch-tolerant RT-qPCR

Taq DNA polymerase that has 5'–3' exonuclease activity is widely used in conventional qPCR to emit a fluorescent signal by 5'–3' cleavage of the TaqMan probe. Because of the lack of 3'–5' exonuclease activity of the Taq DNA polymerase, mismatches between primers and templates, especially at the 3' end of the primers, prevent primer extension or reduce the extension efficiency of PCR in the conventional method that uses Taq enzyme (Figure 1A). High-fidelity DNA polymerase that has a 3'–5' exonuclease activity can repair misincorporated bases and was previously used to detect mutation and quantify HIV-1 viral load, as well as gene expression [31–33]. To improve the applicability of the qPCR to variable templates, we developed a mismatch-tolerant RT-qPCR method by adding a small amount of high-fidelity DNA polymerase into the reaction. When mismatches are formed between primers and templates, the small amount of high-fidelity DNA polymerase is triggered to recognize and remove the mismatched bases from the primers, and then Taq DNA polymerase is responsible for primer extension (Figure 1B). The addition of high-fidelity DNA polymerase does not affect the release of fluorescent signal mediated by Taq DNA polymerase.

Our previous study demonstrated that 0.15 U high-fidelity DNA polymerase per 25 μ l reaction was the optimal amount for the proof-reading function to remove mismatched bases, but not to compete

Figure 3. Flexibility of the new RSV RT-qPCR assay. WT: Wild-type; Mu: Mutant; NTC: No template control.

with Taq DNA polymerase for primer extension [32]. To determine the optimal amount of high-fidelity DNA polymerase in the mismatch-tolerant RT-qPCR, different amounts (from 0 to 1 U) of Q5 high-fidelity DNA polymerase were added into the 25 μ l qPCR reaction system with 1 U of Taq DNA polymerase to amplify mutant templates. The best amplification results were obtained when 0.15 U Q5 high-fidelity DNA polymerase was added (data not shown). Increased amount of high-fidelity DNA polymerase reduced the PCR amplification efficiency possibly due to the competition between both high-fidelity DNA polymerase and Taq DNA polymerase. With 0.15 U Q5 high-fidelity DNA polymerase, the amount of Taq DNA polymerase was further optimized at 0.75 U per 25 μ l reaction. The optimal 25 μ l reaction mix with 0.75 U of Taq and 0.15 U of Q5 DNA polymerases was used in subsequent experiments.

Sequence analysis of RSV-A & -B strains

To analyze the inter- and intra-variability of two RSV groups, we downloaded all available RSV-A and -B sequences from the GenBank database. A total of 636 sequences were obtained, including 435 RSV-A and 201 RSV-B genomic sequences. Sequence analyses showed that RSV-A and -B shared low genetic similarity (approximately 47.8%). In particular, RSV-A had lower intra-similarity

(73.5%) than RSV-B (97.5%), indicating RSV-B has more genetic variation than RSV-A. Therefore, to develop a new RSV RT-qPCR assay for simultaneous detection of RSV-A and RSV-B, degenerate primers are needed. In this study, we used a pair of degenerate primers and a minor groove binder probe that were previously described [23]. To evaluate the primers and probe, we analyzed the sequence features of all RSV strains in the positions of the primers and probe. The results showed that RSV-A and -B have very different sequence features in those positions (Figure 2). The main sequence difference between RSV-B and RSV-A occurs near the 3' end of the forward primer (Figure 2), which provides an explanation for why most methods had low detection efficiency for RSV-B strains. Furthermore, there are some RSV-A variants carrying one or more mutations at the 3' end of the forward primer, implying a possibility of missed detection and false negative results.

Flexibility of the mismatch-tolerant RT-qPCR

To assess the performance of the novel mismatch-tolerant RT-qPCR assay, we constructed four RSV mutants that form one to two mismatches with the forward primer at the 3' end (Figure 3). For the wild-type, the novel method had a smaller threshold cycle (Ct) value compared with the conventional ►

Figure 4. Specificity of the new RSV RT-qPCR assay. (A) Using standard virus strains. (B) Using clinical samples. A total of 14 positive clinical samples for RSV-A, RSV-B, adenovirus, enterovirus, influenza-A, -B, and -C viruses, parainfluenza virus 3, HCoV-OC43, HCoV-NL63, human rhinovirus, bocavirus and human metapneumovirus, were used. NTC: No template control. (n): The number of positive clinical samples.

► method, and generated substantially higher fluorescent intensity (1.31 vs 0.46; $p < 0.01$, paired T-Test). Importantly, we found that the novel mismatch-tolerant RT-qPCR assay could amplify all four mutants (Ct: 17.07 to 21.62), while the conventional method failed to amplify almost all mutants except mutant 2 (RSV-Mu2) (Ct: 25.97) (Figure 3). Slightly higher amplification efficiency was observed for the templates forming one mismatch at the 3' end of the primer than for those forming two mismatches by the novel assay, indicating that more mismatches at the 3' end of the primer had stronger inhibitory effect on amplification. In spite of this, compared with the conventional RT-qPCR assay, the novel

mismatch-tolerant RT-qPCR assay generated not only lower Ct values, but also higher fluorescent intensity, indicating that the novel assay largely improves the amplification efficiency of qPCR for templates carrying mismatches.

We then asked whether the qPCR assay with only high-fidelity DNA polymerase can also tolerate the mismatches between primer and template well, and whether the novel mismatch-tolerant RT-qPCR is also valid for other viruses. To answer the two questions, we performed an assay with only high-fidelity DNA polymerase, and compared it with the novel mismatch-tolerant and the conventional qPCR assays. We tested two viruses (RSV and

HCoV-229E), and found that the assay with only high-fidelity DNA polymerase had similar performance to the novel mismatch-tolerant RT-qPCR (with a mix of Taq and high-fidelity DNA polymerases) for RSV, both of which are better than the conventional qPCR, but with worse performance than the novel RT-qPCR assay and the conventional qPCR for HCoV 229E (Supplementary Figure S1). The results suggested the assay with only high-fidelity DNA polymerase has low amplification stability for mutants forming mismatches, and is not suitable for wide application. Tests for bocavirus further demonstrated the wide applicability of the novel mismatch-tolerant RT-qPCR for virus detection (Data not shown).

Specificity & sensitivity

To determine the specificity of the novel assay, a total of eight other respiratory virus reference strains were tested together with RSV-A and -B strains. Except for the two RSV strains, no positive results were observed for any other non-RSV respiratory virus (Figure 4A). We further used 14 positive clinical samples for RSV-A, RSV-B and 11 other respiratory viruses to test the specificity of the novel assay. No amplification was observed for all 12 positive clinical samples for non-RSV respiratory viruses (Figure 4B). The results indicated that the new assay is highly specific to RSV.

Sensitivity testing showed that when the template input was more than 10 PFU/ml RSV RNA, all 10 reactions (100%) had positive amplification. When the template input was 1 PFU/ml RSV RNA, five of the 10 reactions were positive (Table 1). The LOD of the new RSV RT-qPCR assay was calculated to be 1.8 PFU/ml.

Evaluation of the novel RSV assay using 40 clinical samples

The standard curve shows a strong linear relationship ($R^2 = 0.99$) between log RSV RNA PFU/ml and Ct values with a dynamic scope from 1×10^0 to 1×10^5 PFU/ml (Figure 5A). To evaluate the novel RSV RT-qPCR method, we used 40 clinical samples from children with ARTIs and compared the novel assay with the conventional method. 20 samples were detected as positive, and the others as negative by both methods. Although linear correlation

Table 1. Limit of detection of the novel mismatch-tolerant qPCR.

RSV standard (PFU/ml)	Positive results/total tested	Positive percentage (%)
1×10^4	10/10	100
1×10^3	10/10	100
1×10^2	10/10	100
1×10^1	10/10	100
5	10/10	100
2.5	10/10	100
1	5/10	50

for 20 positive samples was observed between the novel assay and the conventional method ($R^2 = 0.99$), Ct values generated by the novel assay appeared to be slightly lower (implying higher copy number) than those by the conventional method (Figure 5B), suggesting that the novel assay was slightly more sensitive than the conventional method.

To further evaluate the performance of the new method for RSV detection, we compared it with a commercial kit, the Huayin RSV RNA kit, using the same 40 clinical samples. 20 positive samples as shown by the novel assay were also positive according to the commercial kit, demonstrating a 100% consistency (Table 2). The correlation coefficient (R^2) between both methods was 0.69 (Figure 5C). The 95% limits of agreement between the two methods was estimated to be within the range of -0.7 to $+1.34$ log (PFU/ml) by Bland–Altman analysis (Figure 5D).

Importantly, we found that 16 (80%) of 20 positive samples had higher viral copy numbers according to the novel method compared with the Huayin RSV RNA kit, and another four samples showed opposite results (Figure 5C). In particular, there were two samples (Sp19 and Sp16) with obvious higher copy numbers according to the novel method compared with the commercial kit, and this was speculated to be the result of mismatches between primers and viral variants. To address this hypothesis, we sequenced the targeted RSV genomic regions from samples Sp19 and Sp16, as well as another two samples (Sp8 and Sp9) that had slightly lower copy numbers according to the novel method. As expected, Sp16, Sp19 and Sp8 carried G–A and C–T mutations in the primer and probe regions, respectively (Figure 5E), which formed a mismatch with the 3' end of the forward primer, and a mismatch with the third site of the 3' end of the probe (Figure 5E). An additional mismatch at the 3' end of the reverse primer was observed in sample Sp19. There were five mutations in the sample Sp9 to form mismatches with the forward primer. Further phylogenetic analysis showed that RSV strains from the samples Sp16, Sp19 and Sp8 belonged to RSV-A, and that in the sample Sp9 was RSV-B (Supplementary Figure S2). These results indicated that the novel

Figure 5. Comparison of the novel RSV assay with the conventional method and a commercial kit in RSV detection of 40 clinical samples. (A) The standard curve of the novel RSV assay. (B) Comparison between the novel and the conventional assays. The diagonal line is shown in the plot. (C) Comparison between the novel RSV assay and a commercial RSV detection kit (Huayin, China). (D) Bland–Altman plot to assess the agreement between the novel assay and the kit for 20 RSV-positive clinical samples. (E) Sequence analysis of primer and probe regions of several special RSV strains in clinical samples.

method has high capacity to amplify virus variants forming mismatches to primers or probe.

Viruses, especially RNA viruses, have higher mutation rates and a faster replication cycle than bacteria and fungi pathogens, and often exist in the form of quasispecies in the human body, implying a large viral population of intra-host variants [5,6]. Current qPCR methods have low power to efficiently detect various virus variants [33]. In this study, we developed a

mismatch-tolerant qPCR method by simply adding a small amount of high-fidelity DNA polymerase into the conventional TaqMan qPCR system. The novel method tolerates mismatches between primers and template well, and exhibits high efficiency to amplify virus variants. Therefore, the mismatch-tolerant qPCR represents a simple, sensitive and promising approach to replace conventional qPCR for accurate and sensitive detection of highly variable viruses. ►

Table 2. RSV detection of 40 clinical samples by the novel RSV assay and a commercial kit.

		The Novel RSV assay	
		Positive	Negative
The Commercial RSV	Positive	20	0
RNA detection kit	Negative	0	20

► FUTURE PERSPECTIVE

This work provides a mismatch-tolerant qPCR technique that can tolerate mismatches between primers and templates well, and is especially suited to detect various virus variants. Use of this method in multiple systems will be particularly helpful in the diagnostics of infectious diseases. Droplet digital PCR is a promising technique that enables precise and absolute quantitation of nucleic acids without requiring a calibration curve. However, the presence of virus variants forming mismatches with primers may result in failure in DNA amplification in some miniemulsion droplets, thereby causing an underestimate of actual nucleic acid copy number in a given sample. This new method can be of use in droplet digital PCR assays, and improve their accuracy in absolute nucleic acid quantification. Furthermore, loop-mediated isothermal amplification (LAMP) is a promising tool for diagnostics of infectious diseases in resource-limited settings. A similar principle to the mismatch-tolerant qPCR is recommended to be used in the LAMP assay by adding a small amount of high-fidelity DNA polymerase to improve the applicability of LAMP to highly variable viruses.

AUTHOR CONTRIBUTIONS

CZ conceived and designed the study. YL carried out the experiments. CZ, YL, ZW, YH, YZ and QC analyzed data. CZ, YL, ZW and YH interpreted the results. CZ and ZW contributed reagents and materials. CZ and YL wrote the manuscript. All authors read and approved the final manuscript.

COMPETING & FINANCIAL INTERESTS DISCLOSURE

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ETHICAL CONDUCT OF RESEARCH

The use of patients' samples in this research was approved by the Ethics Committees of Shanghai Public Health Clinical Center. Oral or written informed consent was obtained from childrens' parents or guardians before enrollment.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0184

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