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Use of a rapid reverse-transcription recombinase aided amplification assay for respiratory syncytial virus detection

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ABSTRACT

In this study, a rapid reverse-transcription recombinase aided amplification (RT-RAA) assay was developed to detect respiratory syncytial virus (RSV) subgroups A and B, respectively. The reaction was performed at 39°C in less than 30 min. The analytical sensitivities of RSVA and RSVB at 95% probability by probit regression analysis were 38copies per reaction and 35 copies per reaction, respectively, and no cross reactions with other related respiratory viruses were observed. The RT-RAA assay was further utilized to detect and subgroup 306 clinical specimens and the results showed that 79(25.82%, 79/306) samples were positive for RSV, of those 16(20.25%, 16/79) were identified as RSVA and 63(79.75%, 63/79) were RSVB, which is completely consistent with the results obtained by RSV RT-qPCR assay. In conclusion, the developed RAA assay will be of benefit as a faster, sensitive and specific alternative tool for detection of RSV.

1. Introduction

Human respiratory syncytial virus (RSV) is the most common respiratory pathogen causing acute respiratory infection (ARI), particularly in infants and young children (Holberg et al., 1991; Sato et al., 2005; Simoes, 1999). Severe RSV disease can also occur in frail elderly persons and the severely immunocompromised (Agius et al., 1990; Dowell et al., 1996; Englund et al., 1988; Falsey et al., 1995; Han et al., 1999). Therefore, RSV infection will result in a substantial medical and economic burden.

RSV is an enveloped, single-stranded, negative-sense RNA virus belonging to the genus Pneumovirus of the Paramyxoviridae family. On the basis of its reactivities of the viruses with monoclonal antibodies and the genetic characteristics of the attachment protein (the protein G), RSV can be classified into the subgroups A and B (Anderson et al., 1985; Mufson et al., 1985). Epidemiologic studies showed that group A and B viruses can cocirculate in the community, the prevalence of each group can also predominate within annual epidemics and disease severity can vary in RSVA and RSVB infections (Coggins et al., 1998; Hendry et al., 1989; Martinello et al., 2002; Peret et al., 2000; Xian et al., 2013; Xiang et al., 2013). The epidemiological patterns of both subtypes provide important information about the strain diversity and clinical features of RSV. For appropriate treatment of RSV infection, it is crucial to have an accurate and timely diagnostic method for detection of RSV.

Molecular based diagnostic methods have been widely adopted for detection and identification of RSV, including conventional RT-PCR, real-time RT-PCR and multiplex RT-PCR (Bonroy et al., 2007; Do et al., 2012; Falsey et al., 2002, 2003; Hu et al., 2003; Li et al., 2013; Yamada et al., 2004). However, these methods are costly, time-consuming and require highly specialized equipment, which are not suitable for widely application in the field or in poorly resource-limited lab. Isothermal amplification has recently been introduced for the detection of respiratory viruses (Deiman et al., 2007; Hart and Van Zyl, 2014; Moore et al., 2006; Shirato et al., 2007; Ushio et al., 2005). These isothermal amplification assays using a designated primer set and the reaction mixture is simply incubated at a single temperature for less than 1 h with high sensitivity, specificity and efficiency. Recombinase aided amplification (RAA) is a novel isothermal amplification and detection assay, which utilizes specific enzymes and protein for rapid detection of pathogens at 39 °C in less than 30 min. The enzymatic mixture of RAA includes single strand DNA binding protein (SSB), recombinase UvsX and DNA polymerase. The recombinase UvsX is extracted from the *Escherichia coli*. RAA use

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UvsX to anneal primers to template DNA and SSB to form a D-loop structure to maintain the state of single strand of template DNA with the help of DNA polymerase for amplification and extension. The RAA assay can also use reverse transcriptase and a fluorescent probe system for real time detection of RNA amplicons (Bei et al., 2010; Zhang et al., 2017). The M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (RT) is a recombinant DNA polymerase that is necessary for the reverse transcription step. Detection of RNA amplicons relies on the use of an oligonucleotide probe with an internal abasic site mimic (tetrahydrofuran, THF) flanked by a dT-fluorophore and a corresponding dT-quencher group. The THF position is recognized and cleaved by an Exonuclease III only when the probe is bound to its complementary target sequence, thereby separating the fluorophore and the quencher and accumulating the fluorescence signal.

The objective of the current study was to develop a reverse-transcription recombinase aided amplification(RT-RAA) assay for the rapid detection of RSV -A and -B, respectively, and evaluate its performance in clinical specimens.

2. Materials and methods

2.1. Clinical samples collection

Nasopharyngeal aspirates (NPA) were collected from 306 hospitalized patients who had acute respiratory infection (ARI) and admitted to the children's hospital of Hebei, China between January and March, 2017. Of those 141 (46.08%) were female and 165 (53.92%) were male. Ages ranged from 21 days to 6 years old, and 276 (90.20%) were under 3 years old. A total volume of 0.5 ml of nasopharyngeal aspirate was collected in 3.5 ml of transport medium and stored at −80 °C.

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. Children's parents were apprised of the study's purpose and of their right to keep information confidential. Written informed consent was obtained from parents or caregivers.

2.2. RNA extraction

Total RNA was extracted from 200 μL of clinical sample using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). All of the RNA extractions were performed according to the manufacturer's protocol. The RNA was eluted in 50 μL of nuclease-free water and stored at −80 °C until needed.

2.3. Preparation of plasmid standard

A 320 bp (nt6623–6942, GenBank accession no.KY982517) and 648 bp fragment (nt5980–6627, GenBank accession no.KY249683) of the F gene of RSVA and RSVB were respectively cloned into the pClone007 Vector for DNA copy number quantification.

The recombinant plasmid DNA was prepared with a mini plasmid DNA extraction kit(Beijing Com Win Biotech Co., Ltd., China). The plasmid DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Dreieich, Germany). DNA copy number was calculated using the following formula: DNA copy number = (copy number / μL) = {[6.02 × 10^{14}× plasmid concentration (ng/μL) × 10^{-9}]} / [DNA in length × 660]. A dilution series with the standard recombinant plasmids ranging from 10^4 to 10^0copies DNA/reaction was prepared and used in the real-time fluorescence detection assay to evaluate the sensitivity of the RT-RAA.

2.4. Primers and exo-probes for real-time RT-RAA

The F gene of RSV was chosen as the target because it is one of the most conserved genes in the RSV genome. All the available F gene segments of RSVA and RSVB from the National Center for Biotechnology Information database (NCBI) were aligned and highly conserved regions were identified using Vector NTI version 11.5.1. Primers were designed with the aid of Oligo7 software and showed no major nonspecific sequence similarity on BLAST analysis. The primer sequences and the probe sequences for RT-RAA and RT-qPCR and the amplicon sizes are listed in Table 1. All of the primers and probes were synthesized and purified by high-performance liquid chromatography (HPLC) by Sangon Biotech (Shanghai, China).

2.5. Real-time RT-RAA protocol

The real time RT-RAA reaction was performed in a 50 μL volume using the RT exo kit(Jiangsu Qitian Bio-Tech Co. Ltd., China) which provides all enzymes(SSB,800 ng/μL, UvsX, 120 ng/μL, DNA polymerase, 30 ng/μL) and reagents necessary for the reverse transcription step and DNA amplification in lyophilized pellets according to the manufacturer's instruction. The reaction mixture containing the following components:2 μL of RNA Template, 25 μL of rehydration buffer, 15.7 μL of ddH_2O, 2.1 μL of each primer(10 μM) and 0.6 μL of target specific RAA exo-probe. Finally, 47.5 μL of master mix/template solution was transferred to each lyophilized RAA pellet provided in the kit.2.5 μL of 280 mM magnesium acetate was pipetted into the tube lids for each reaction. The tube lids were carefully closed and the tubes were vortexed briefly and centrifuged. The magnesium acetate was dropped into each reaction mixture through centrifugation and

Table 1
Primer and probe sequences used for RT-RAA and RT-qPCR assays.

Primer	Sequence (5′–3′)	Gene	Size (bp)	Source
RSVA-F	CTTGTTGGAAACTACACACATCCCCTCTAT	F	227	This paper
RSVA-R	TGAATATGTCAATGTTGCAGAGATTTACCTCAC			
RSVA-P[a]	GTACTGTGACAATGCAGGATCAGTATCCTT[FAM-dT]T (THF)[BHQ-dT]CCCACAAGCTGAAAC[C_3-spacer]			
RSVB-F	AAAGCTGTAGTCAGTCTATCAAATGGGGTCA	F	189	This paper
RSVB-R	ACTAAATTCTCTGGTGATTTCCAACAATCTGC			
RSVB-P[a]	AGAGTTGTCGCATTTCCAACATTGAAACAGT[FAM-dT]A(THF) [BHQ-dT]AGAATTCCAGCAGAAG[C_3-spacer]			
A-F[b]	GCTCTTAGCAAAGTCAAGTTGAATGA	N	84	(Hu et al., 2003)
A-R[b]	TGCTCCGTTGGATGGTGTATT			
A-P[b]	FAM-ACACTCAACAAAGATCAACTTCTGTCATCCAGC -BHQ1			
B-F[c]	GATGGCTCTTAGCAAAGTCAAGTTAA	N	105	(Hu et al., 2003)
B-R[c]	TGTCAATATTATCTCCTGTACTACGTTGAA			
B-P[c]	VIC-TGATACATTAAATAAGGATCAGCTGCTGTCATCCA-BHQ1			

[a] For probe modifications: FAM, 6-Carboxyfluorescein; HEX,5-hexachlorofluorescein;THF, tetrahydrofuran; BHQ, black hole quencher; C_3-spacer, 3′ phosphate blocker.
[b] RSVA primers and probe used for RT-qPCR.
[c] RSVB primers and probe used for RT-qPCR.

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simultaneously triggered the RT-RAA reaction. The tube was then transferred to the tube holder on a RAA fluorescence detection device QT-RAA-F7200 (Jiangsu Qitian Bio-Tech Co. Ltd., China) set at 39 °C for 30 min for amplification. A negative control (nuclease-free water) was included in each run.

2.6. Analytical sensitivity and specificity

The standard recombinant plasmids with a dilution range between 10^4 and 10^0 were prepared in Diethyl pyrocarbonate (DEPC) treated water to determine the sensitivity of real time RT-RAA assay for RSVA and RSVB, respectively.

The specificity of the RT-RAA assay for RSVA and RSVB, was respectively evaluated using 227 RSV-negative (out of 306) clinical specimens in this study. These clinical samples were retrospectively tested by a GeXP-based multiplex RT-PCR assay (GeXP assay) (Li et al., 2013) previously reported in our laboratory, which enabled the simultaneous detection of sixteen common respiratory virus types/subtypes including RSVA and RSVB.

2.7. Detection of clinical samples

The clinical performance of the RT-RAA assay for the detection of RSVA and RSVB was evaluated using a total of 306 respiratory specimens selected from children hospitalized with acute respiratory illness. An RT-qPCR assay listed in Table 1 for the detection of RSVA and RSVB, respectively, was chosen as a parallel test as well as the reference method.

2.8. Statistical analysis

All of the statistical analyses were performed using IBM SPSS Statistics, version 13 (IBM Corporation, NY, USA). A probit analysis was performed to calculate the detection limit of the RT-RAA assay at a 95% probability level. The degrees of agreement among the

RT-RAA and RT-qPCR test results were measured with the kappa value (κ). A linear regression analysis of RT-RAA threshold time and RT-qPCR cycle threshold (CT) value was performed and R squared was measured to compare the degree of agreement among the RT-RAA and RT-qPCR test results.

3. Results

3.1. Sensitivity and specificity of RSV RT-RAA assay

To ascertain the detection limit of the RT-RAA assay for RSVA and RSVB, the assay was performed using the prepared recombinant plasmid standard with a dilution range from 10^4 to 10^0 copies per reaction with eight replicates. An example of the output is shown in Fig. 1A and B. The time to onset of a rise in fluorescence increased as the amount of target DNA in the sample decreased. As shown in Table 2, standard DNA ranging from 10^4 to 10^2 copies per reaction produced positive results for all eight replicates within 30 min. Results indicated that both RSVA and RSVB RT-RAA assays were capable of detecting 10 copies per reaction. The detection limits of RSVA and RSVB RT-RAA at 95% probability were 38 copies per reaction and 35 copies per reaction respectively (probit analysis, $p \leq 0.05$).

Among the 306 respiratory specimens selected from children hospitalized with acute respiratory illness, RT-RAA detected 79 positive. All

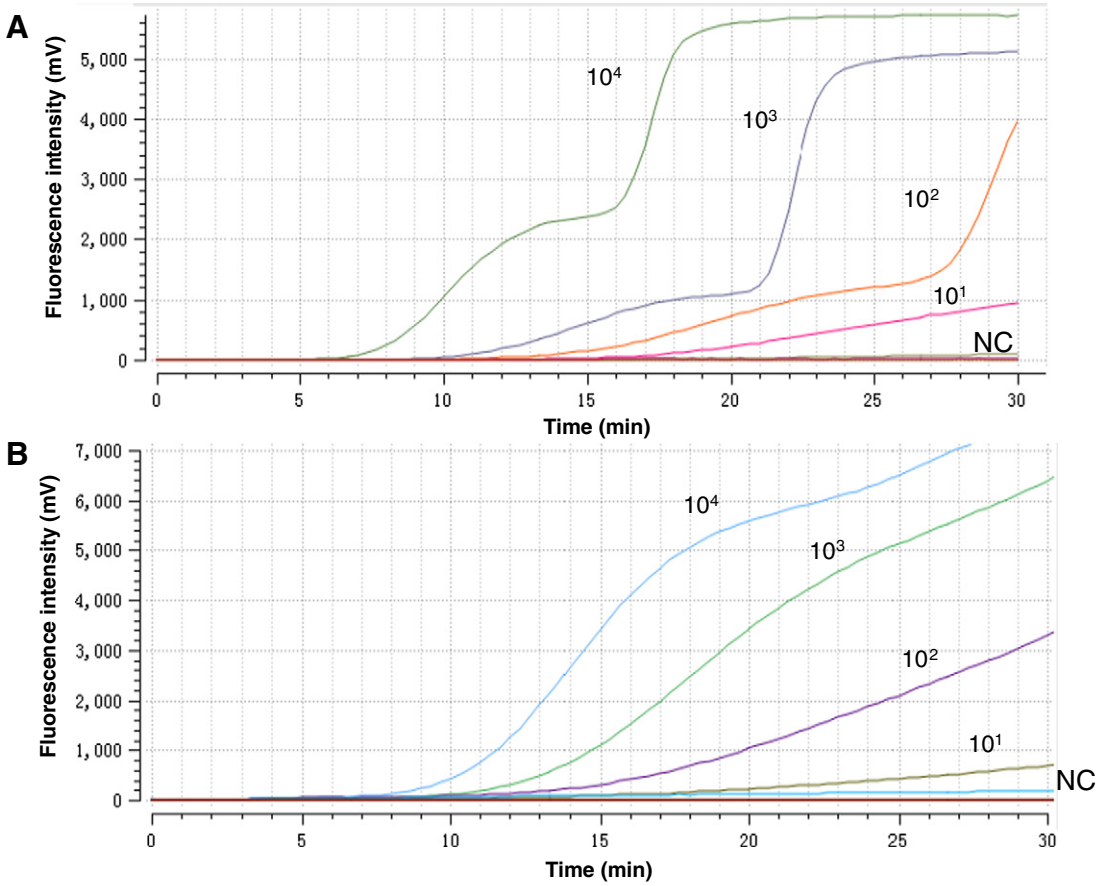

Fig. 1. Representative RSVA and RSVB RT-RAA reaction Curve. A dilution range from 104 to 100 copies/reaction of recombinant plasmid standard of RSVA and RSVB were respectively used to evaluate the detection limit of RSVA (A) and RSVB (B) RT-RAA assay, NC represents negative control.

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Table 2
Assay data used for probit analysis to calculate the detection limit of RSVA and RSVB RT-RAA.

Copies/reaction	No. of positive sample/no. of samples tested by the RT-RAA assay for detection of RSVA and RSVB[a]	
	RSVA RT-RAA	RSVB RT-RAA
10^4	8	8
10^3	8	8
10^2	8	8
10^1	3	4
1	0	0

[a] Each dilution was tested in a total of 8 replicates.

the remaining 227 RSV-negative samples were retested by GeXP-based multiplex RT-PCR assay (GeXP assay) to determine the specificity of RT-RAA assay. The results showed that 35(11.44%,35/306) were identified as human rhinovirus (HRV), 24(7.84%,24/306) were identified as parainfluenza virus type 3(PIV-3), 12(3.92%,12/306) were identified as adenovirus(Adv), 7 (2.29%,7/306) were identified as human metapneumovirus (HMPV), 4(1.31%,4/306) were identified as influenza virus type A(Flu A), 3(0.98%,3/306) were identified as parainfluenza virus type 1(PIV-1) and 1(0.33%,1/306) was identified as influenza virus type B (FluB). A total of 141 out of 227 were negative detected by the GeXP assay. No unspecific amplification or detection of RT-RAA was observed on all positive specimens of other related respiratory viruses, suggesting the high specificity of RSV RT-RAA assay.

3.2. Performance of the RT-RAA Assay on clinical samples

To evaluate the clinical performance, a total of 306 respiratory specimens selected from children hospitalized with acute respiratory illness was diagnosed and differentiated the subgroups by RT-RAA and compared with RT-qPCR assay as the reference method.

A threshold cycle (CT) value of 38 was designated as the cut-off value for positive results. RT-qPCR detected 79 out of 306 sample (25.82%, 79/306) positive. Of these 79 RSV-positive specimens, 16(20.25%, 16/79) were identified as RSVA and 63(79.75%, 63/79) were identified as RSVB. The CT values obtained by RT-qPCR for positive samples ranged from16.13 to 37.71. (see supplementary material, Table S1). In comparison to RT-qPCR, RT-RAA correctly identified and differentiated all 79 positive samples with 100% sensitivity and specificity (Table 3). Clinical samples with a very low viral load (Ct values >35) were also scored positive by RT-RAA. A clear concentration-dependent correlation between threshold time (RT- RAA) and the CT values (RT-qPCR) (Fig. 2A and B) was observed. There was no significant difference between the detection results of RT-RAA and RT- qPCR. The kappa value (κ) of RT-RAA and RT- qPCR was 1.0 ($p < 0.001$).

4. Discussion

This study describes a highly sensitive and specific molecular diagnostic method that can detect and subgroup RSVA and RSVB genomes in clinical specimens. The assay established in this study revealed a satisfactory performance that the detection limits of RSVA and RSVB RT-RAA are as few as 38 copies per reaction and 35 copies per reaction, respectively(probit analysis, $p \leq 0.05$). RSV was also detected in nasopharyngeal aspirates from children with acute respiratory infection and the results were 100% consistent with RT-qPCR as a reference method, suggesting that the RSV RT-RAA developed in the present study may be used for diagnosing RSV infections in children. The RT-RAA assay could be completed in 30 min, while RT-qPCR generally requires about 2–3 hours. In addition to the shorter turn-around time, the requirement of a constant temperature of 39 °C provides RT-RAA advantages over RT-qPCR, which normally requires varying cycling temperatures under rigorous conditions. The RT-RAA detection system does not require a sophisticated laboratory setting or expensive equipment, which can be performed with a portable device. As for the running cost of RT-RAA, the cost for one sample by RT-RAA is approximately 60% of that by RT-qPCR. What's more, the results can be obtained by viewing fluorescence without opening the sample tubes, minimizing concern on contamination of amplified products. Therefore, the RT-RAA assay is useful for qualitative detection of RSV, and can be used to amplify quantitatively the virus genome in real-time (Zhang et al., 2017).

The application of the armored internal control (IC) RNA to monitor each step of nucleic acid amplification is critical to prevent false-negative or invalid results due to inhibition or human error. The RNA of bacteriophage MS2 coat protein derived from Virus-like particles(VLPs)was used as an internal control to monitor the detection process of each specimens (Wei et al., 2008; Zhan et al., 2009). In the present RT-RAA assay, we made an initial attempt to utilize the bacteriophage coat protein gene, which differs in detection format and amplicon size to monitor reverse transcription and amplification in each specimen by spiking IC RNA derived from MS2 phage core particle into the extracted RNA of clinical samples. The clinical samples are considered as positive when both the targeted pathogen and MS2 are present. The samples are considered to be negative if samples are negative for pathogen but positive for MS2. If both the target and MS2 are absent, the samples are classified as invalid. No invalid sample was found in the RT-RAA assay. As demonstrated in Figs. S1 and S2 of supplementary material, which displayed FAM fluorescence data corresponding to real-time RSV amplification, an increase in detectable amplification time and a decrease in fluorescence intensity of the RSV/MS2duplex assay were observed in clinical samples with low initial target concentration compared to that of the RSV RT-RAA without IC described previously. However, the time at which the amplification and fluorescence intensity are detectable was not obviously affected by the coamplification of MS2 RNA in clinical samples with high initial target concentration. In addition, no RSV specific fluorescence signal was detected with the implementation of MS2 IC in those clinical samples when the amplification appeared after 24 minutes in RT-RAA assay without IC described previously. HEX fluorescence data corresponding to real-time MS2 amplification and the concentration of RSV RNA had little effect on the amplification of MS2. Thus, the utilization of MS2 RNA as an internal control had an adverse effect on the detection efficiency of RSV-positive specimens with low concentration and had a little effect on RSV-positive specimens with high concentration. Here, the target and IC are co-amplified using a different primer set within one RSV/MS2duplex assay in which two reactions with different kinetics proceed simultaneously. Therefore, it is possible to infer the kinetics of each reaction might have mutual antagonistic effect or MS2 primer/probe-target amplicons might affect the

Table 3
The clinical performance of RT-RAA for the detection of respiratory specimens compared with RT-qPCR as the reference method.

RT-qPCR	RT-RAA		Total	Performance of RT-RAA compared with RT-qPCR			
	Positive	Negative		Sensitivity (%)	Specificity (%)	Accordance rate(%)	Kappa value (κ)
Positive	79	0	79	100	100	100	1.00
Negative	0	227	227				
Total	79	227	306				

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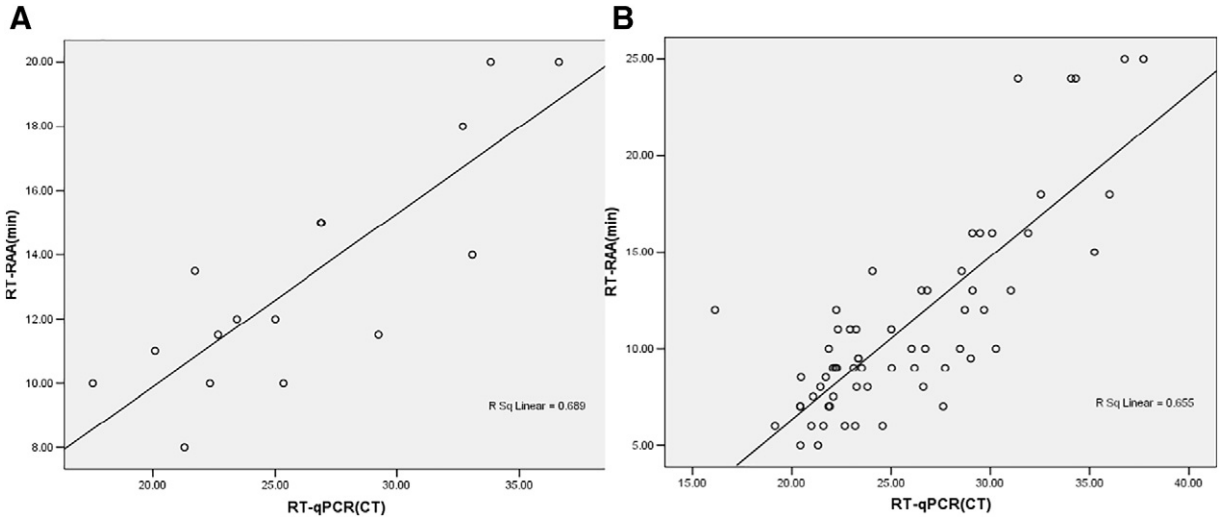

Fig. 2. Comparison between RT-RAA and RT-qPCR for the detection of RSVA and RSVB in clinical samples. Sixteen RSVA samples (A) and 63 RSVB samples (B) were positive in both assays with 100% agreement. Linear regression analysis of RT-RAA threshold time (Y axis) and RT-qPCR cycle threshold values (X axis) were determined by SPSS 11.0. R squared value was 0.689 and 0.655, respectively.

recombinase mediated insertion of the oligonucleotides thus impede the stretch of the localized secondary or tertiary DNA structures so as to inhibit the binding efficiency of RSV primer/probe to target.

It is possible that the performance of the RAA assay could be improved from that observed in this study. Evaluation of the performance of specific molecular tests for different specimen types is also critical since interference factors may vary from one specimen type to another and thereby a complete evaluation is required prior to use in the clinical laboratory (Mahony, 2008; Mahony et al., 2011). Herein, we only collected nasopharyngeal aspirates (NPA) from hospitalized children as specimen and a further evaluation of the performance of our assay for different specimen types is needed in the future work. The length of the primer and probe of the RAA assay, which require more than 30 base oligonucleotides for the formation of efficient recombinase filaments increases the specificity of RAA assay, but limits the efficiency of primers and probe complementary to the target which has highly variant genome sequence. This could be explained that the length of the primer or probe is correlated with the fact that the recombinase combines the ssDNA and forms into the nucleoprotein complex to make a conformational change that activates the filament for strand exchange. Further exploration of the enzymatic function and the enzyme-DNA interaction are needed in the future work. In addition, the use of recombinase enzymes can reduce the likelihood of false positive results due to their inherent proofreading capabilities (Chen et al., 2008). But the impact of the localized secondary or tertiary DNA structures on the recombinase remains unknown. However, this is beyond the scope of this investigation. In conclusion, as shown in the present study, the RAA assay was proven to be as good as or comparable to RT-qPCR assay. It is expected that this technique will attract more attention and further developed in the near future making it a new start in nucleic acid detection in more fields.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2017.10.005.

Conflicts of interest

None.

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Ethical approval

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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