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Short communication

New method for the visual detection of human respiratory syncytial virus using reverse transcription loop-mediated amplification[☆]



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Human respiratory syncytial virus (HRSV) is a seasonal respiratory pathogen that causes respiratory infection in children and the elderly. A new, reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay was developed for the rapid (within 1 h), simultaneous detection of A and B group HRSV. Primers specific for groups A and B were designed to amplify the *N* and *L* genes of HRSV, respectively. A fluorescent dye, calcein, was used as an indicator for the endpoint visual detection and/or real-time amplification of HRSV RNA. The detection limit of the new method was 281.17 50% tissue culture infective doses (TCID₅₀)/ml for HRSV A and 1.58 TCID₅₀/ml for HRSV B. To evaluate the validity of this method, a comparison with RT-PCR was performed using 77 nasopharyngeal swabs as samples. Both RT-LAMP and RT-PCR detected HRSV in 38 HRSV samples, yielding a positive rate of 49%. Of the RT-LAMP positive samples, 36 (95%) were also positive by RT-PCR, while two were negative by RT-PCR. Among the 36 RT-LAMP and RT-PCR positive samples, 11 belonged to HRSV group A, while 25 belonged to group B. The results show that the new RT-LAMP is simple, rapid and well suited for HRSV diagnosis, especially in a limited-resource setting.

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Human respiratory syncytial virus (HRSV) is an enveloped, non-segmented, negative sense RNA virus belonging to the genus *Pneumovirus*, subfamily *Pneumovirinae*, and family *Paramyxoviridae*. The approximately 15.2 kb genome of HRSV contains 10 genes that encode 11 proteins (Collins and Melero, 2011). There are two distinct groups (A and B) of HRSV, which are distinguished by antigenic and genetic variation (Mufson et al., 1985; Cane and Pringle, 1991). These groups can be further subdivided into 11 A and 20 B genotypes (Arnott et al., 2011; Eshaghi et al., 2012). HRSV is the single most important viral cause of acute respiratory tract infections in children (Simoes, 1999). Approximately 90% of infants are infected by HRSV one or more times within 2 years of age, and the rate of hospitalization for primary infection is approximately

0.5% (Karron et al., 1999). The morbidity and mortality of HRSV are most significant in young children, the elderly and people who are immunocompromised or suffer from cardiopulmonary diseases (Hall et al., 1986; Falsey et al., 2005). In a report from the First Hospital of Lanzhou University, China, HRSV was responsible for 38.14% of the hospitalizations of children under the age of 14 (Zhang et al., 2010a). The mean cost of each HRSV-related hospitalization is US \$571.80, which is a heavy burden for the average family in a developing country (Zhang et al., 2013). Severe diseases associated with lower respiratory tract HRSV infection are considered to have long-term impacts on individual health, including higher risk of asthma or wheezing and lower forced expiratory volume (Stein et al., 1999; Everard, 2006). Rapid diagnosis could enable medical intervention at the early stage of HRSV infection, thereby providing an effective means by which to reduce treatment costs and prevent long-term lung damage. Thus, fast and accurate diagnosis of HRSV infection is necessary for the surveillance and control of HRSV epidemics.

Several methods, such as virus isolation, antibody-based tests and molecular tests, have been developed since the discovery of HRSV. Loop-mediated isothermal amplification (LAMP) is a simpler and faster pathogen diagnostic method compared to these other

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Table 1
Primer sequences of RT-LAMP for specific amplification of HRSV A and B groups.

	A group	B group
F3	TGTTCAATTTGGTATAGACAAT	GTAACACAGTTAGACTTATAGAGG
B3	CACCCAATTTTGGGCATA	GGACTTTTTGATACTGGCTG
FIP	CACTTGCCTGCACCATAGCTTACCAGAGGTGGCA	TGGCCTATACCTGCATACTTTATGTGACACCCATGCTCAAG
BIP	GGTGGGAGTCTTAGCAAAATCCCACAATTGTTCATTCTG	AAGGGAACAGAGACCTATATATCCCGATAGTACACTCCATTGTGCT
LF	CCTGCAAAAATCCCTTCAACTCTAC	GCAATTTAAGGCTATTTAATGCTAAACAAA
LB	AATATTATGCTAGGACACGCTAGTG	GAGATATGCAGTTTCATGAGCAAAA

methods. LAMP was first reported in 2000 (Notomi et al., 2000). This method employed three pairs of primers to efficiently amplify targets under isothermal conditions within 1 h. The amplification efficiency of LAMP is reported to be higher than PCR (Nagamine et al., 2002). The products of LAMP could be detected by visual observation of turbidity or changes in the color of calcein. Furthermore, the amplification plots of LAMP can also be obtained using a real-time turbidity monitor or fluorescence-detecting PCR instrument (Mori et al., 2001; Tomita et al., 2008). In combination with reverse transcriptase, LAMP is also able to detect RNA templates (Li et al., 2012; Wang et al., 2012). Its sensitivity is comparable to RT-PCR, and the reaction can be completed within 1 h (Hong et al., 2004; Parida et al., 2004). LAMP is conducted under isothermal conditions, thus a water bath or heat block can easily maintain the temperatures needed for LAMP (Savan et al., 2005). Therefore, LAMP can be widely used for the diagnosis of various pathogens, especially in resource-limited settings (Haridas et al., 2010; Li et al., 2012; Wang et al., 2012).

Two previous reports used RT-LAMP to detect HRSV (Ushio et al., 2005; Shirato et al., 2007). In these studies, the primer sets for HRSV groups A and B were all located in the same region. In addition, a limited number of HRSV genome sequences were used to design the primers, which may limit the ability of RT-LAMP to detect all HRSV genotypes. In the present study, 39 group A and 10 group B HRSV complete genome sequences, which do not contain degenerate nucleotides, were downloaded from GenBank. These sequences were aligned by Muscle in MEGA v5.05 (Tamura et al., 2011). Conserved regions in the A and B groups were identified by searching the entropy plot using BioEdit v7.1.3 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) and verified by a whole genome searching strategy, which searched for the region with the most invariable sites in the consensus multiple alignment (Text S1). It was observed in the whole genome search that HRSV A and B groups do not share the same conserved region. Therefore, group A- and B-specific primer sets were designed in different parts of the HRSV genome.

The conserved regions of A and B group HRSV were realigned by Clustal W on the EBI server (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), and then uploaded to the online LAMP primer design software (PrimerExplorer, <http://primerexplorer.jp/elamp4.0.0/index.html>). The positions of the primer regions generated in PrimerExplorer were further adjusted to avoid variable sites. The final primer sets were located in the *L* gene and *N* gene of HRSV genome (Fig. S1). The primer sequences are listed in Table 1. Primer sets A and B were designed to detect all groups of A and B HRSV, respectively. Accession numbers of complete genomes used in primer design are listed in Table S1.

The primer sets designed above could not amplify to the plateau stage within 60 min (data not shown) using the reaction system reported previously. To obtain optimal RT-LAMP reaction conditions for these primer sets, an orthogonal experiment was used to optimize reagent concentrations and reaction temperatures, as previously reported (Cobb and Clarkson, 1994). Briefly, four optimizing factors were fixed at three different levels (Table 2). These levels were combined into nine sets according to the orthogonal table (Table 3). These nine reactions were carried out using a SmartCycler

Table 2
Condition levels of each factor under optimization.

Level	Betaine (M)	dNTP (mM)	Magnesium sulfate (mM)	Temperature
1	0.3	1.0	6	60
2	0.5	1.4	8	62
3	0.8	1.6	10	65

Table 3
Reaction condition sets compiled from the orthogonal table.

Number	Betaine	dNTP	Magnesium sulfate	Temperature
1	0.3	1.0	6	60
2	0.5	1.4	8	60
3	0.8	1.6	10	60
4	0.3	1.4	10	62
5	0.5	1.6	6	62
6	0.8	1.0	8	62
7	0.3	1.6	8	65
8	0.5	1.0	10	65
9	0.8	1.4	6	65

(Cepheid, Sunnyvale, CA, USA) and fluorescent emissions between 565 and 590 nm were collected. Amplification curves were plotted (Fig. 1A). After calculating the mean positive time for each level of each factor, the optimal levels of the four factors were determined and the combination of the optimal level of each factor is listed in Table 4.

When judging the results by the naked eye, reaction mixtures containing 30 μ M calcein were incubated at 60 °C for 60 min. Calcein is a fluorescent metal indicator that emits green fluorescence when conjugated with divalent metal ions and the fluorescent intensity corresponds to the degree of amplification (Tomita et al., 2008). As shown in Fig. 2, the negative reaction mixture remained orange, while positive reactions turned green. Each reaction result was read by three individuals to confirm the color change. For real-time monitoring, reaction mixtures containing 15 μ M calcein were incubated in a SmartCycler (Cepheid) at 60 °C for 60 min, and the emission fluorescence with wavelengths between 565 and 590 nm was collected and reaction curves were plotted. When fluorescence, relative to that at the start of each assay, had risen from 10 to 100

Table 4
Optimized reaction conditions for HRSV-specific RT-LAMP.

Reagent	Final concentration
Betaine	0.5 M
dNTP	1.6 mM
Thermopol RB	1*
Magnesium sulfate	6 mM
Tween-20	0.2%
AMV RTase	0.5 U/25 μ l
Bst polymerase	8 U/25 μ l
Primer (FIP/BIP)	1.6 μ M
Primer (LF/LB)	0.8 μ M
Primer (F3/B3)	0.2 μ M
Calcein	30 μ M (color change)
dH ₂ O	Add to 25 μ l

* Refer to product sign.

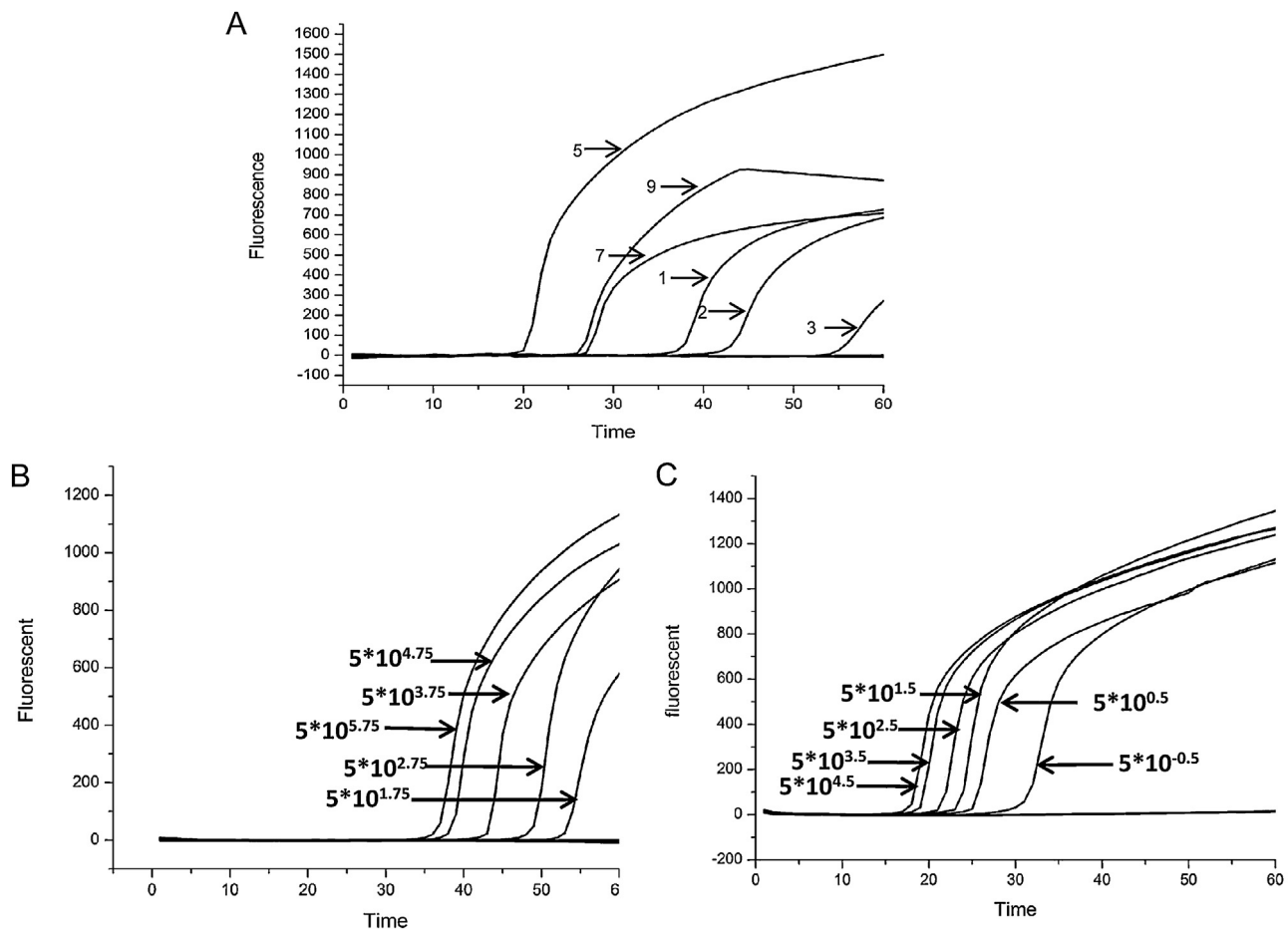


Fig. 1. Reaction curves of the orthogonal experiment and detection limits. Panel A: real-time reaction curves of orthogonal experiments in which the x-axis refers to reaction time (min) and the y-axis refers to relative fluorescent intensity. Reactions were conducted in SmartCycler (Cepheid). Reactions in which relative fluorescence rose from 10 to 100 within 5 min were considered positive. The numbers of the arrows are identical to the coding numbers in Table 3. Panels B and C: detection limit of RT-LAMP assay for A and B groups of HRSV. RT-LAMP was performed using mixed group A- and B-specific primer sets. RNA templates were extracted from culture supernatants of strain VR-1540 ($5 \times 10^{5.75}$ TCID₅₀/ml) and strain VR-1400 ($5 \times 10^{4.5}$ TCID₅₀/ml). RNA was 10 times serially diluted and used as templates. Numbered arrows refer to the TCID₅₀ value of each reaction.

within 5 min, the reaction was considered positive. The relative fluorescence values of positive results were between 700 and 1600 when the amplification process reached the plateau stage, and a logarithmic increase of the relative fluorescence was observed in all positive results. Real-time detection of HRSV using calcein was more sensitive than detection by the naked eye. Other dyes, such as SYTO-9, are compatible with a real-time fluorometer, but they cannot be observed visually (Njiru et al., 2008; Mahony et al., 2013).

The reaction specificity of the primers was assessed by testing the primers with RNA prepared from standard strains of HRSV and related viruses in a 30 μ M calcein reaction mixture. Eleven standard virus strains, including HRSV A and B, Influenza A and B, Rhinovirus, Reovirus, Measles virus, and Coronavirus, were used. These virus strains were all purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultivated according to ATCC recommended conditions. RNA was extracted from 140 μ l of standard virus strains and clinical specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. RT-LAMP tests were conducted as mentioned previously. According to results of the RT-LAMP (Fig. 2), primer set A reacted only with A group templates and primer set B reacted only with B group templates (Fig. 2A and B). A mixture of primer sets A and B reacted with both A and B group templates (Fig. 2C) and did not react with other viruses in related families (Fig. 2D).

The detection limit of HRSV RT-LAMP was determined by testing with serial dilutions of RNA templates in the 15 μ M calcein reaction mixture. A VR-1540 (A group) culture supernatant with $5 \times 10^{5.75}$ TCID₅₀/ml and a VR-1400 (B group) culture supernatant with $5 \times 10^{4.5}$ TCID₅₀/ml were used for RNA extraction. The TCID₅₀ of VR-1400 and VR-1540 were determined using the Reed–Muench method in HEP-2 and Vero cells, respectively (Reed and Muench, 1938), and mixed primers were used to determine the detection limit. A serial, 10-fold dilution of extracted RNA was used as template. Amplification curves based on relative fluorescent intensity were plotted. RT-LAMP detected group B HRSV templates at a concentration of 1.58 TCID₅₀/ml and group A templates at a concentration of 281.17 TCID₅₀/ml, which were both lower than the viral loads in clinical samples of infected patients (Hall et al., 1976). The RT-LAMP assay for group B had a lower detection limit and B group primers generated positive results faster than A group primers (Fig. 1B and C).

To determine the validity of the RT-LAMP assay, RT-PCR was used as the gold standard. A one-step RT-PCR targeting the HRSV G gene was designed to detect HRSV in clinical samples, as reported previously (Zhang et al., 2010b). Primer GA480 or GB496 (400 nM) was mixed with 400 nM of primer F164, 1 μ l dNTP mix (containing 10 mM of each dNTP), 5 μ l of 5 \times Qiagen OneStep RT-PCR buffer, 1 μ l of enzyme mix (containing Omniscript reverse transcriptase, Sensiscript reverse transcriptase, HotStarTaq DNA Polymerase), 2.5 μ l

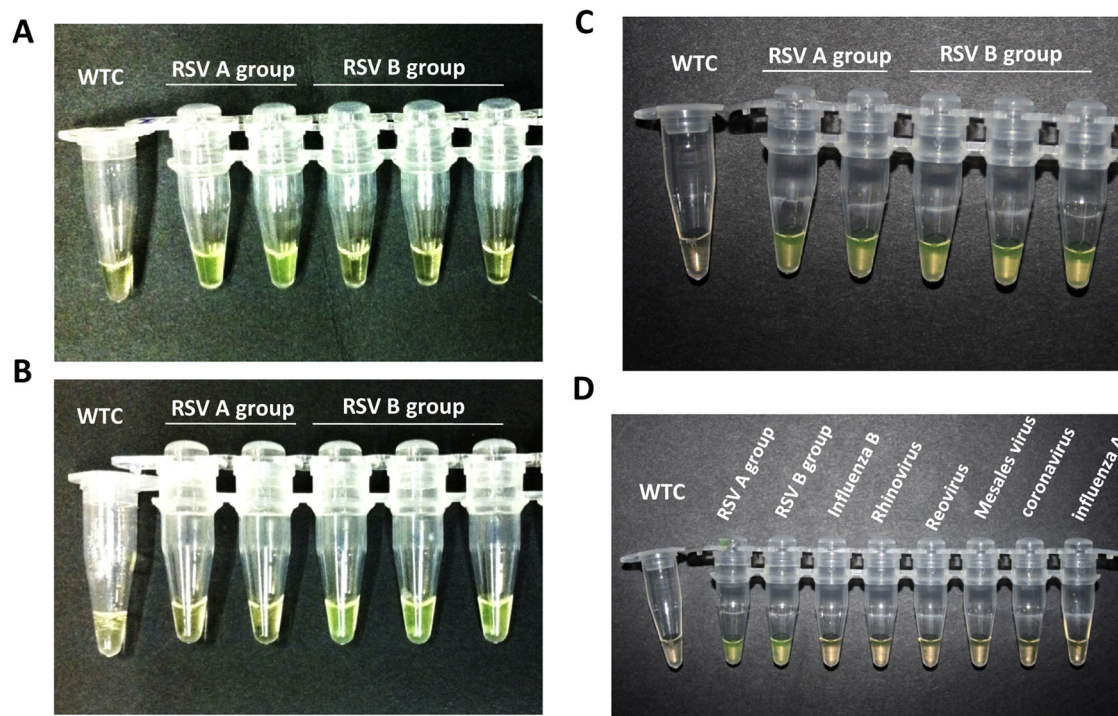


Fig. 2. Reaction specificity of HRSV group A and B specific primer sets. Panels A and B: reaction of group A (panel A) and group B (panel B) specific primers with RNA from five standard HRSV strains. Panel C: reaction of A and B group primer mixtures with RNA from five standard HRSV strains. Panel D: reaction of mixed primers with RNA from HRSV and related viruses. HRSV group A strains include VR-26 and VR-1540, while group B strains include VR-1580, VR-955 and VR-1400. Related viruses include Influenza A and B, Rhinovirus, Reovirus, Measles virus, and Coronavirus. WTC (without template control).

of extracted RNA and 13.5 μ l of RNase-free water. The amplification process was initiated with a reverse transcription step at 50 °C for 30 min, followed by denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, with a final extension of 72 °C for 10 min. The products were detected by electrophoresis in 1.5% agarose gels stained with GelRed (Biotium, Hayward, CA, USA).

Seventy-seven nasopharyngeal swab samples were collected from children with respiratory tract infections between 2009 and 2012 in Nanxiang Hospital, Shanghai, China. Nasopharyngeal swabs were immersed in virus transport media containing Hank's buffer, BSA, HEPES and antibiotics (gentamicin and anti-fungal agents). The supernatant was then aliquoted into three tubes and stored at –80 °C. One aliquot was used to extract RNA. Extracted RNA was used as template in RT-LAMP and RT-PCR. Reaction results of RT-LAMP were judged by color change with the naked eye. The positive RT-PCR results were sequenced and the sequencing results were aligned with the HRSV genotyping reference reported previously (Zhang et al., 2010b) by Muscle in MEGA v5.05. An evolutionary tree was constructed and the RT-PCR positive results were classified into A and B groups of HRSV.

The positive rate of RT-LAMP and RT-PCR were both 49% (38/77 samples), but there were two samples that were only positive using RT-LAMP and two samples that were only positive using RT-PCR (Table 5). All reactions were performed three times to confirm the results. Thirty-five of 37 RT-LAMP positive results were sequenced and genotyped, which revealed that 11 belonged to the HRSV A

group and 25 belonged to the B group. Therefore, this new RT-LAMP assay could detect all groups of HRSV virus in clinical samples accurately and quickly.

In summary, two sets of RT-LAMP primers were designed based on 49 HRSV complete genomes retrieved from GenBank, and the optimized reaction system was developed to establish a new RT-LAMP assay for fast and accurate detection of HRSV. The comparison with RT-PCR using 77 clinical samples suggested that the new RT-LAMP is simple, rapid and well suited for HRSV diagnosis, especially in limited-resource settings.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2014.06.005>.

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Table 5

Comparison of the performance of RT-LAMP with RT-PCR in detecting clinical samples.

N = 77	RT-PCR positive	RT-PCR negative
RT-LAMP positive	36	2
RT-LAMP negative	2	37

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