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Development of real-time fluorescent reverse transcription loop-mediated isothermal amplification assay with quenching primer for influenza virus and respiratory syncytial virus

Ikuyo Takayama[a], Mina Nakauchi[a], Hitoshi Takahashi[a], Kunihiro Oba[b], Shohei Semba[c], Atsushi Kaida[d], Hideyuki Kubo[d], Shinji Saito[a], Shiho Nagata[a], Takato Odagiri[a], Tsutomu Kageyama[a,*]

[a] Influenza Virus Research Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan
[b] Department of Pediatrics, Showa General Hospital, 8-1-1 Hanakoganei, Kodaira-shi, Tokyo 187-0002, Japan
[c] Eiken Chemical Co. Ltd., 4-19-9 Taito, Taito-ku, Tokyo 110-8408, Japan
[d] Division of Microbiology, Osaka Institute of Public Health, 8-34 Tojo-cho, Tennoji-ku, Osaka 543-0026, Japan

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ABSTRACT

Influenza virus and respiratory syncytial virus cause acute upper and lower respiratory tract infections, especially in children and the elderly. Early treatment for these infections is thought to be important, so simple and sensitive detection methods are needed for use at clinical sites. Therefore, in this study, real-time reverse transcription loop-mediated isothermal amplification assays with quenching primer for influenza virus and respiratory syncytial virus were developed. Evaluation of a total of 113 clinical specimens compared to real-time RT-PCR assays showed that the novel assays could distinguish between the types and subtypes of influenza virus and respiratory syncytial virus and had 100% diagnostic specificity. The diagnostic sensitivity of each assay exceeded 85.0% and the assays showed sufficient clinical accuracy. Furthermore, positive results could be obtained in around 15 min using the novel assays in cases with high concentrations of virus. The developed assays should be useful for identifying influenza virus and respiratory syncytial virus cases not only in experimental laboratories but also in hospital and quarantine laboratories.

1. Introduction

Influenza virus (IV) and respiratory syncytial virus (RSV) infections are common causes of acute upper and lower respiratory tract infections such as pneumonia and bronchiolitis and lead to high rates of hospitalization, especially in children and the elderly (Falsey and Walsh, 2000; Jain et al., 2015; Sugaya et al., 2000; Zhou et al., 2012). Antiviral drugs for IV, such as oseltamivir and zanamivir, reduce the duration, frequency of symptoms, and hospitalization if administered within 48 h of the onset of symptoms (Aoki et al., 2003; Hayden et al., 1997). Moreover, rapid detection of these viruses is important in the clinical management of patients and for the reduction of healthcare costs (Bonner et al., 2003). However, the clinical signs and symptoms of these viruses are sometimes similar, and it can be difficult to distinguish between causative viruses especially in the incipient stage of disease (Zambon et al., 2001).

In addition to the widely used detection methods for these diseases, including viral cultures, serology, real-time reverse transcription PCR (rRT-PCR), and rapid antigen detection tests (RADTs), several new methods that are easy-to-use and sensitive are currently being developed (Guatelli et al., 1990; Ishiguro et al., 2003; Kouguchi et al., 2010). One newly developed test is the loop-mediated isothermal amplification (LAMP) method, a rapid and sensitive nucleic acid amplification method that is performed under isothermal conditions and requires less complicated equipment than PCR (Nagamine et al., 2002; Notomi et al., 2000). This method can yield results in less than 1 h and can be utilized for the detection of many kinds of viral genomes (Kurosaki et al., 2016; Shirato et al., 2014; Yamazaki et al., 2013). A LAMP reaction can be monitored in real time by measuring the progressive increase in sample turbidity due to the precipitation of magnesium pyrophosphate (Mori et al., 2001), but this technology may compromise the specificity of the test because of the exponential amplification of primer dimers (Njiru, 2012) and, in some cases, the detection of non-specific LAMP products made by host-derived DNA. DNA intercalators or fluorescent dyes, such

* Corresponding author.
E-mail address: tkage@nih.go.jp (T. Kageyama).

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Table 1
rRT-LAMP primers designed for IV and RSV.

Target	Name of primer	Sequence (5' to 3')	Reference or source
IAV	F3-1	GACTTGAAGATGTCTTTGC	(Nakauchi et al., 2011b)
	F3-2	GACTGGAAAGTGTCTTTGC	
	B3-1	TRTTATTTGGGTCTCCATT	
	B3-2	TRTTGTTTGGGTCCCCATT	
	FIP	TTAGTCAGAGGTGACARRATTGCAGATCTTGAGGCTCTC	
	BIP	TTGTKTTCACGCTCACCGTGTTTGGACAAAGCGTCTACG	
	LF	GTCTTGTCTTTAGCCA	
	LB	CMAGTGAGCGAGGACTG	
	QPrimer	CMAGTGAGCGAGGACTG	
IBV	F3	GCAACCAATGCCACCATA	This study
	B3	TTCTCTCTTCAAGRGACATC	
	FIP	TAGTCAAGGGCYCTTTGCCACTTTGAAGCAGGAATTCTGGA	
	BIP	CAAGACCGCCTAAACAGACTAAACTTTTACTTTCAGGCTCACTT	
	LF	TGAAAGYCTTTCATAGCAC	
	LB	CAAGAATAAAGACTCACAAC	
	QPrimer	CAAGAATAAAGACTCACAAC	
A/H1pdm	F3	AGCTAAGAGAGCAATT	(Nakauchi et al., 2011b)
	B3	TTTCCCTTTATCATTAATGTAGGATTTG	
	FIP	ACCTTTGTTCGAGTCATGATTGGTCTCAGTGTCATCATTTGAAAGGTTT	
	BIP	TAACGGCAGCATGTCCTCAGTATGAATTTCCTTTTTTAACTAGCCA	
	LF	CCATGAACTTGTCTTGGGGAATA	
	LB	GCTGGAGCAAAAAGCTTCTACA	
	QPrimer	CCATGAACTTGTCTTGGGGAATA	
A/H3	F3-1	AGCTGGTTCAGARTTCCT	This study
	F3-2	AATTGAAGTTACTAATGCTACTG	
	B3	CGGCACATCATARGGGTAAC	
	FIP	AGAGCATCTATTAGTGTGCAGTTTCAAYAGGTGAAATATGCRAC	
	BIP	GGAGACCCTCAGTGTGATGGTGCTGTRGGCTTTGC	
	LF	CCATCAAGGATCTGATGAGGAC	
	LB	AGAARTGGGACCTTTTTGTTGAAC	
	QPrimer	CCATCAAGGATCTGATGAGGAC	
RSV A	F3	GAGTTGAAGGGATTTTTGCA	This study
	B3	TGGGTTGTTCAATATATGGTAGA	
	FIP	TAACTGATTTTGCTAAGACCCCCGGATTGTTTATGAATGCCTATGG	
	BIP	CAAGCAGAAATGGAACAAGTTGTGCTGCTTCTCCACCCAATT	
	LF	CACCGTAACATCACTTG	
	LB	GAGGTGTATGAGTATGCTCAGA	
	QPrimer	CACCGTAACATCACTTG	
RSV B	F3	TGACATCAGAAATACAAGTCAAT	This study
	B3	CGTTTTTTAAGACATTGTTTGCC	
	FIP	CATCCCACAGTCTGGAGAATCAAGAAAGTCCTACAAAAAAATGC	
	BIP	GCTGCCCTTGTAATAACCAAATTAGCTCCTAATTACTGCAGTAAGACC	
	LF	GAGCCACTTCTCCCATC	
	LB	CAGCAGGAGATAGATCA	
	QPrimer	CGAGCCACTTCTCCCATC	

as calcein, can also be used for real-time monitoring of the LAMP reaction (Seyrig et al., 2015; Tomita et al., 2008). Although these methods yield higher analytical sensitivity and shortened reaction times compared with turbidity-based real-time LAMP, the detection principles of the methods are the same.

In this study, quenching primer (QPrimer) was utilized for the detection of LAMP products by targeting an internal sequence of the amplicon. QPrimer has a cytosine labeled with a fluorescent dye such as BODIPY® FL at the 5' end. When QPrimer hybridizes to its target nucleotide sequence, the fluorescence is quenched by photoinduced electron transfer between the fluorescent dye and a guanine residue in the target (Crockett and Wittwer, 2001; Kurata et al., 2001; Torimura et al., 2001). The establishment of a novel real-time reverse transcription LAMP (rRT-LAMP) assay for the detection of IV and RSV using QPrimer was reported here.

2. Material and methods

2.1. Primer design for the rRT-LAMP assay

Primers for detecting influenza A (IAV) and influenza A subtype H1pdm09 (A/H1pdm) viruses were modified for the circulating strains from those originally described by Nakauchi et al. (Nakauchi et al., 2011b). Primers for detecting influenza B virus (IBV), influenza A subtype H3 (A/H3) virus, respiratory syncytial virus type A (RSV A), and respiratory syncytial virus type B (RSV B) were designed using conserved regions of the NS gene of IBV, the HA gene of influenza A/H3 virus, and the N genes of RSVs (Table 1). LAMP primers were designed from candidate conserved regions using Primer Explorer V4 software (Eiken Chemical, Tokyo, Japan). All primers were synthesized by Life Technologies Japan (Tokyo, Japan) and cartridge-purified.

2.2. Clinical specimens

From November 2014 through May 2015 and from November 2015 through March 2016, 113 nasal aspirates, secretions, or swabs were collected from patients presenting with influenza-like illnesses at the outpatient department of Showa General Hospital. Participants or the parents of participants provided written informed consent. This study was approved by the institutional medical ethical committees of the National Institute of Infectious Diseases and Showa General Hospital. Nasal aspirates, secretions, or swabs were collected in 1 mL of universal

Table 2
Panel of 24 IVs used to determine the analytical specificity of the rRT-LAMP.

Subtype	Virus
H1N1	A/duck/Alberta/35/76
H1N1	A/Brisbane/59/2007
H1N1pdm09	A/Narita/1/2009
H2N3	A/duck/Germany/1215/73
H3N8	A/duck/Ukraine/1/63
H3N2	A/Uruguay/716/2007
H4N6	A/duck/Czechoslovakia/56
H4N6	A/duck/Hyogo/1/2010
H5N1	A/whooper swan/Hokkaido/4/2011
H5N1	A/blow fly/Kyoto/93/2004
H5N2	A/chicken/Ibaraki/1/2005
H6N2	A/turkey/Massachusetts/3740/65
H7N1	A/duck/Hong Kong/301/1978
H7N9	A/Anhui/1/2013
H8N4	A/turkey/Ontario/6118/68
H8N4	A/duck/Shizuoka/45/2011
H9N2	A/turkey/Wisconsin/1/66
H10N7	A/chicken/Germany/N/49
H11N6	A/duck/England/56
H12N5	A/duck/Alberta/60/76
H13N6	A/gull/Maryland/704/77
H14N5	A/mallard/Gurjev/263/82
H15N8	A/duck/Australia/341/83
TypeB	B/Massachusetts/2/2012

transport medium (UTM; Copan, Brescia, Italy) and frozen at −80 °C until use.

2.3. In vitro-transcribed RNA

In vitro-transcribed RNA was used as a standard for the rRT-LAMP assay. RNA transcripts for the rRT-LAMP assay for IV were prepared from the full-length of M and HA genes of A/Narita/1/2009 (H1N1) pdm09 (GISAID accession nos. EPI180038 and EPI179437), HA gene of A/Texas/50/2012 (H3N2) (EPI391247), and NS gene of B/Massachusetts/02/2012 (EPI439259). The primers Uni12 (5′-AGCAAAAGCAGG-3′) or Uni9 (5′-AGCAGAAGC-3′) (Zou, 1997) were used for reverse transcription using a SuperScript® III Reverse Transcriptase Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The entire coding region of each gene was amplified by PCR using Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) with paired primers, with the reverse primer containing the T7 promoter sequence. RNA was transcribed using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Madison, WI) and treated with TURBO® DNase (Thermo Fisher Scientific) to degrade the template DNA. The dNTPs and NTPs were removed using MicroSpin G-25 Columns (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. The transcribed RNAs were quantified using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific), and the absorbance value was used to calculate the copy numbers of the transcribed RNAs. The integrity of each transcribed RNA was assessed with a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA). RNA transcripts for the rRT-LAMP assay for RSV were prepared from the full-length of N gene of RSV/OsakaC.JPN/16.2012 (Genbank accession no. LC415429) and RSV/OsakaC.JPN/38.2011 (LC415430) and produced as described above.

2.4. RNA extraction and rRT-LAMP assay

Viral RNA was extracted by QIAamp® Viral RNA Mini kit (Qiagen, Dusseldorf, Germany) using 140 µL of UTM mixed with clinical specimens according to the manufacturer's instructions. RT-LAMP was performed using a 25 µL volume reaction mix that contained 5 µL template RNA, 1.4 mM of each dNTP, 0.8 M betaine, 20 mM Tris − HCl (pH 8.8), 70 mM KCl, 8 mM $MgSO_4$, 15 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 8 U Bst 2.0 polymerase (New England Biolabs), and 0.25 U AMV reverse transcriptase (Nippon Gene, Tokyo, Japan). The reaction mixture also contained 0.2 µM each of F3 and B3 primers, 1.6 µM each of FIP and BIP primers, and 0.8 µM each of LF and LB primers for each assay. In the IAV and A/H3 assay, the final concentration of each F3 and B3 primer was 0.2 µM, although more than 2 primers were used as F3 and B3 primers. For the novel rRT-LAMP assay, 5% of the LB or LF primer of each assay was substituted by the QPrimer-5 G (Nippon Steel and Sumikin Eco-Tech, Tokyo, Japan). The rRT-LAMP reaction was performed at 63 °C for 30 min using LightCycler® 480 II (Roche, Basel, Switzerland). Fluorescence was measured at wavelengths of 465 nm (excitation) and 510 nm (emission) after 4 min of reaction and again every 1 min thereafter. The results were determined by observation in real time and considered positive following fluorescence quenching. To compare with a turbidity-based rRT-LAMP assay, the time to positivity was considered as the first time the fluorescence quench rate increased by more than 3% within 3 min. To obtain the relative fluorescence rate at each detection point, the fluorescence intensity measured at each time point was divided by that measured at the beginning. The turbidity-based rRT-LAMP assay was conducted at 63 °C for 30 min using Loopamp Realtime Turbidimeter LA-320C (Eiken Chemical). Turbidity readings of the optimal density at 650 nm (OD650) were obtained every 6 s, and the reaction was considered positive when the turbidity values were over 0.05. To obtain corrected absorbance, the average turbidity from 2 to 5 min after the initiation of the LAMP reaction was used as the correction base line. GraphPad Prism 5.0 software (Graph Pad Software, La Jolla, CA) was used to generate the figures.

2.5. rRT-PCR assay

All viral RNA extracted from the 113 specimens was tested using the one-step rRT-PCR assays for detection of the types and subtypes of IV as the reference test, as described previously (Nakauchi et al., 2014, 2011a). In addition, another 15 viral respiratory pathogens were identified by rRT-PCR assays developed by Do et al. (2010) and Kaida et al. (2014), namely, RSV A and B; human parainfluenza virus type 1, 2, 3, and 4; influenza C virus; human rhinoviruses; human metapneumovirus; human coronavirus OC43, 229E, NL63, and HKU1; human bocavirus; and human adenovirus.

2.6. Validation and evaluation of QPrimer-based rRT-LAMP assay

The sensitivity of the QPrimer-based rRT-LAMP assays was assessed using various concentrations of quantified *in vitro*-transcribed RNA in triplicate at each concentration. The type/subtype specificity of the QPrimer-based rRT-LAMP assays for IV was validated using 24 representative subtypes of IAV and IBV (Table 2). All statistical analyses were performed with the MedCalc free statistical calculator (http://www.medcalc.org, MedCalc Software bvba, Ostend, Belgium).

3. Results

The principle of guanine quenching was used to detect the amplification process in the novel rRT-LAMP. Fluorescence quenching is detectable in real time because QPrimer reduces the fluorescence once it is incorporated within the LAMP product. Thus, the fluorescence signal is at a maximum at the beginning of the amplification reaction and is quenched progressively throughout the amplification process down to a stable plateau, where it remains until the end of the reaction. On the other hand, the precipitation of magnesium pyrophosphate increases as the LAMP reaction proceeds in the turbidity-based rRT-LAMP assay (Fig. 1). The reaction times of rRT-LAMP for IAV in the two types of LAMP detection, namely, turbidity and QPrimer, were examined. To compare the reaction times in the two formats, various concentrations of *in vitro*-transcribed RNA were used. The RNA detection time at each

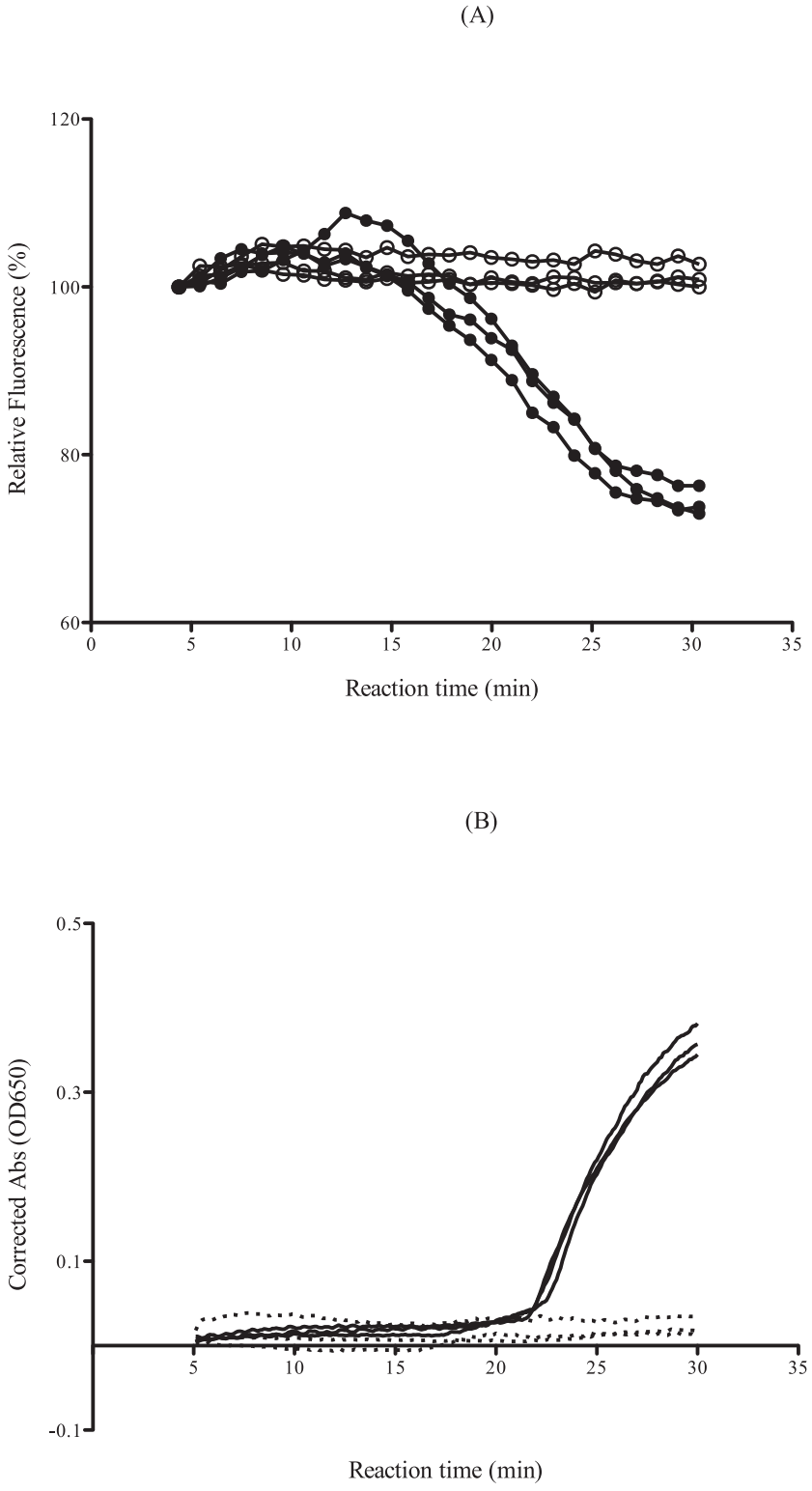

Fig. 1. Comparative reaction times of the rRT-LAMP assay for IAV. The assay was performed using *in vitro*-transcribed standard RNA. (A) LAMP products were detected by fluorescence quenching using QPrimer (novel QPrimer-based rRT-LAMP). The results of 5000 copies/reaction (filled circles) and negative control samples (open circles) are indicated. (B) LAMP products were detected by real-time turbidity (conventional turbidity-based rRT-LAMP). The results of 5000 copies/reaction (straight lines) and negative control samples (dotted lines) are indicated.

dilution point by QPrimer was earlier than that by turbidity measurements, and the detection rate of target RNAs was more stable in the novel QPrimer-based rRT-LAMP assay compared with the turbidity-based RT-LAMP assay (Table 3).

The analytical sensitivity of the QPrimer-based rRT-LAMP assays was observed using testing various dilutions of quantified *in vitro*-transcribed RNA of each target gene in triplicate. As shown in Table 4, the assays enabled the detection of each target gene at 25–250 copies/reaction at the lowest concentration, and no false positive results were observed for any of the negative control samples in either assay. The

Table 3
Reaction times (min) of novel and conventional rRT-LAMP assays for detecting IAV in *in vitro*-transcribed standard RNA.[a]

The way of detection	Concentration of RNA (copies/reaction)							
	5000	500	250	50	25	5	0.5	N.C.
QPrimer (novel rRT-LAMP)	16.0	19.0	22.0	26.0	25.0	–	–	–
	17.0	23.0	19.0	–	–	–	–	–
	16.0	21.0	27.0	–	–	–	–	–
Turbidity (conventional rRT-LAMP)	23.2	27.2	26.8	–	–	–	–	–
	23.3	–	25.6	29.9	–	–	–	–
	23.8	26.1	29.9	–	–	–	–	–

[a] The assays were carried out in triplicate at each concentration.

type/subtype specificity of the assays for IV were validated using 24 representative IAVs and IBV (Table 2). The assay showed positive reactions only for each target type/subtype virus and no cross-reactivity with any other subtypes of IAV and IBV (data not shown).

To assess the utility of the QPrimer-based rRT-LAMP assay for the clinical diagnosis of IV and RSV, an evaluation of 113 clinical specimens collected from patients with influenza-like illness was conducted. Table 5 summarizes the comparison of results between QPrimer-based rRT-LAMP and rRT-PCR. The QPrimer-based rRT-LAMP assays had higher than 85.0% sensitivity and 100% specificity for all targets. The average threshold cycle (Ct) values in the reference rRT-PCR assay for samples, which were positive by both QPrimer-based rRT-LAMP and rRT-PCR, were 24.9 (range, 18.6–32.7) for IAV, 26.1 (range, 18.6–33.9) for IBV, 26.2 (range, 20.9–34.1) for A/H1pdm virus, 24.0 (range, 17.8–29.1) for A/H3 virus, 23.6 (range, 19.5–32.9) for RSV A, and 28.2 (range, 21.4–37.2) for RSV B. Among the samples, 1–3 showed false negative results in the QPrimer-based rRT-LAMP assay, with Ct values between 31.0 and 39.8 in the reference rRT-PCR assay. Four specimens were co-infected with IV and RSV in this evaluation. Of these, IBV and RSV B were not detected by QPrimer-based rRT-LAMP in 1 specimen co-infected with IBV and RSV A or in 1 specimen co-infected with RSV A and RSV B. On the other hand, both pathogens were detected by QPrimer-based rRT-LAMP in the other 2 specimens co-infected with RSV A and RSV B. Among the 113 specimens, 14 (12.4%) that were negative for all pathogens tested by rRT-PCR were also negative via the QPrimer-based rRT-LAMP assays. Furthermore, the QPrimer-based rRT-LAMP did not amplify RNA from specimens that contained other respiratory viruses not targeted by RT-LAMP in this study. That is, none of the samples showed false positive results via QPrimer-based rRT-LAMP assay.

4. Discussion

In this study, a rapid, specific, and sensitive detection assay for IV and RSV using a novel rRT-LAMP method was developed. Currently, RADTs are widely used and popular at clinical sites because it is considered important to provide early treatment for IV and RSV and avoid the unnecessary use of antibiotic therapy. However, the sensitivity and specificity of some RADTs remain relatively poor, especially those for RSV (sensitivity range, 71.15–80.77%) (Bell et al., 2014; Dunn et al., 2014; Kanwar et al., 2015). The novel QPrimer-based rRT-LAMP assays enabled the detection of each target gene at 25–250 copies/reaction at the lowest concentration (Table 4) and showed less sensitive than the reference rRT-PCR assays which limit of detection were 6–9 copies/reaction as determined in previous studies (Nakauchi et al., 2014,). However, the QPrimer-based rRT-LAMP assays showed high sensitivity (≥85.0%) and sufficient clinical accuracy in clinical specimens from patients with influenza-like illness compared with RADTs. Furthermore, the design of QPrimer in the novel method is simple and universally applicable; specifically, part of one of the loop primers with a cytosine at the 5′ end is substituted by the QPrimer, or it can add a cytosine to the 5′ end of the QPrimer if there are no suitable loop primers with a cytosine at the 5′ end, such as with the RSV B primer sets in this study (Table 1). The QPrimer-based rRT-LAMP assays can be conducted using general-purpose real-time PCR instruments and an isothermal nucleic acid amplification system with fluorescence measurements.

The conventional turbidity-based rRT-LAMP detection of a target by turbidity is simple. However, because it cannot exclude nonspecific reactions caused by primer dimers, the time taken to positivity can be delayed. In this study, QPrimer was utilized for detection by targeting an internal sequence of the amplicon. By using QPrimer, reaction time delay by turbidity-based rRT-LAMP assay was overcame (Table 3). In addition, higher diagnostic test specificity was achieved and all assays had 100% specificity (Table 5). These valuable results agree with current reports on QPrimer and quenching probe (QProbe), which are used for amplicon detection in some nucleotide amplification methods (Ayukawa et al., 2017; Hiramatsu et al., 2017; Toyama et al., 2015). An rRT-PCR assay usually takes 1–2 h after sample preparation, whereas the novel QPrimer-based rRT-LAMP assay provides positive results in around 15 min for samples with high concentrations of viral RNA, suggesting that this assay can be characterized by its rapid results. The results suggest that the QPrimer-based rRT-LAMP assay could be used as a fast and sensitive diagnostic test for detecting IV and RSV.

In conclusion, the newly developed rRT-LAMP assay with QPrimer for IV and RSV demonstrated high diagnostic sensitivity (≥85.0%) and high diagnostic specificity (100%) in clinical specimens from patients with influenza-like illness. The QPrimer-based rRT-LAMP assays can be performed without skilled personnel, and positive results can be achieved faster than they are by rRT-PCR assay. The QPrimer-based rRT-LAMP assay can be used to test for other pathogens and is a powerful tool for use not only in experimental laboratories but also in hospital and quarantine laboratories.

Competing interests

The authors declare that they have no competing interests.

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Table 4
Number of positive results of the QPrimer-based rRT-LAMP assay in the detection of each *in vitro*-transcribed standard RNA.[a]

Target	Standard RNA	Concentration of RNA (copies/reaction)							
		5000	500	250	50	25	5	0.5	N.C.
IAV	A/Narita/1/2009 (H1N1)pdm09 M gene	3	3	3	1	1	0	0	0
IBV	B/Massachusetts/2/2012 NS gene	3	3	3	2	1	0	0	0
A/H1pdm	A/Narita/1/2009 (H1N1)pdm09 HA gene	3	3	3	2	1	0	0	0
A/H3	A/Texas/50/2012 (H3N2) HA gene	3	2	2	0	0	0	0	0
RSV A	RSV/OsakaC.JPN/16.2012 N gene	3	3	2	2	0	0	0	0
RSV B	RSV/OsakaC.JPN/38.2011 N gene	3	2	2	0	0	0	0	0

[a] The assays were carried out in triplicate at each concentration.

Table 5
Performance of novel QPrimer-based rRT-LAMP assay for IV and RSV compared with the reference rRT-PCR assay.

Target	LAMP result	rRT-PCR result		LAMP sensitivity % (95% CI)	LAMP specificity % (95% CI)
		Positive	Negative		
IAV	Positive	26	0	89.7	100
	Negative	3	84	(72.7–97.8)	(95.7–100)
IBV	Positive	10	0	90.9	100
	Negative	1	102	(58.7–99.8)	(96.5–100)
A/H1pdm	Positive	16	0	88.9	100
	Negative	2	95	(65.3–98.6)	(96.2–100)
A/H3	Positive	10	0	90.9	100
	Negative	1	102	(58.7–99.8)	(96.5–100)
RSV A	Positive	17	0	85.0	100
	Negative	3	93	(62.1–96.8)	(96.1–100)
RSV B	Positive	13	0	92.9	100
	Negative	1	99	(66.1–99.8)	(96.3–100)

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