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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

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A B S T R A C T

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 (Falsy 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
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

| Target | Name of primer | Sequence (5’ to 3’) | Reference or source |
|--------|----------------|---------------------|---------------------|
| IAV    | F3-1           | GACTTGAAGATGCTTTTG  | (Nakauchi et al., 2011b) |
|        | F3-2           | GACTGGAAAGTGCTTTTG  |                     |
|        | B3-1           | TTGGTTGGGTCCATT     |                     |
|        | B3-2           | TTGTTGGGTCCATT      |                     |
|        | FIP            | TTGGTCTACGCCTGCTGGAGCCAAGAGGATCATG |                     |
|        | BIP            | TTGGTCTACGCCTGCTGGAGCCAAGAGGATCATG |                     |
|        | LF             | CAGTTGAGGAGGCCAGCT    |                     |
|        | LH             | CMAGTGAGGGAGGACTG    |                     |
|        | QPrimer        | CMAGTGAGGGAGGACTG    |                     |
| IBV    | F3             | GCAACCAATGCACATATA  | This study          |
|        | B3             | TTTGGGTTTATGTAATGT   |                     |
|        | FIP            | AGAGCATCTATTAGTGTGCTTCCAYAGTGGAGAATATGCRAC |                     |
|        | BIP            | GAGACACCTATGTCGAGTGGTTGC |                     |
|        | LF             | CCATCAAGAATCTATGAG   |                     |
|        | LB             | AGAARTGGGAGAGTGGTTGAGA |                     |
|        | QPrimer        | CCATCAAGAATCTATGAG   |                     |
| A/H1pdm| F3             | AGCTAAGAGAGGATTTTGA  | (Nakauchi et al., 2011b) |
|        | B3             | TTTGGGTTTATGTAATGT   |                     |
|        | FIP            | AGAGCATCTATTAGTGTGCTTCCAYAGTGGAGAATATGCRAC |                     |
|        | BIP            | GAGACACCTATGTCGAGTGGTTGC |                     |
|        | LF             | CCATCAAGAATCTATGAG   |                     |
|        | LB             | AGAARTGGGAGAGTGGTTGAGA |                     |
|        | QPrimer        | CCATCAAGAATCTATGAG   |                     |
| A/H3   | F3-1           | AGCTGGATCAGATCTCTCT  | This study          |
|        | F3-2           | AATGGAGCTATAGTGCATT  |                     |
|        | B3             | CCGAACATCATRARGGGTAAC |                     |
|        | FIP            | AGAGCATCTATTAGTGTGCTTCCAYAGTGGAGAATATGCRAC |                     |
|        | BIP            | GAGACACCTATGTCGAGTGGTTGC |                     |
|        | LF             | CCATCAAGAATCTATGAG   |                     |
|        | LB             | AGAARTGGGAGAGTGGTTGAGA |                     |
|        | QPrimer        | CCATCAAGAATCTATGAG   |                     |
| RSV A  | F3             | GAGTTGAAGAGGATTTTGA  | This study          |
|        | B3             | TTTGGGTTTATGTAATGT   |                     |
|        | FIP            | AGAGCATCTATTAGTGTGCTTCCAYAGTGGAGAATATGCRAC |                     |
|        | BIP            | GAGACACCTATGTCGAGTGGTTGC |                     |
|        | LF             | CCATCAAGAATCTATGAG   |                     |
|        | LB             | AGAARTGGGAGAGTGGTTGAGA |                     |
|        | QPrimer        | CCATCAAGAATCTATGAG   |                     |
| RSV B  | F3             | TGGTTTGATTGTCAGTTAT  | This study          |
|        | B3             | CTTGGTTTATGTAATGT    |                     |
|        | FIP            | CACCCATGACCTGAGAGGATTTAATGTCATT  |                     |
|        | BIP            | GAGACACCTATGTCGAGTGGTTGC |                     |
|        | LF             | CCATCAAGAATCTATGAG   |                     |
|        | LB             | AGAARTGGGAGAGTGGTTGAGA |                     |
|        | QPrimer        | CAGCCACTTCTCCACATC   |                     |
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 Uni1 (5′-AGCAGG-3′) or Uni9 (5′-AGCAGAACAG-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 from the full-length of M and HA genes of A/Narita/1/2009, 1.4 mM of each dNTP, 0.8 M betaine, 20 mM Tris – HCl (pH 8.8), 70 mM KCl, 8 mM MgSO4, 15 mM (NH4)2SO4, 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 time 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, 2015). 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 adenosivirus.

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

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/Hygase/4/2011 |
| H5N1    | A/whooper swan/Hokkaido/4/2011 |
| H5N1    | A/whoop swan/Kyoto/93/2004 |
| H5N2    | A/chicken/Ibarani/1/2005 |
| H6N2    | A/turkey/Massachusetts/3740/65 |
| H7N1    | A/duck/Hong Kong/301/1978 |
| H7N9    | A/Asahi/1/2013 |
| H8N4    | A/turkey/Ontario/611/68 |
| H8N4    | A/duck/Shizuoka/45/2011 |
| H9N2    | A/turkey/Wisconsin/1/66 |
| H10N6   | 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   | A/B/Massachusetts/2/2012 |
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

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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.
Table 4

| Reaction times (min) of novel and conventional rRT-LAMP assays for detecting I. Takayama, et al. Journal of Virological Methods 267 (2019) 53–58 |
|--------------------------------------------------|
| The way of detection | Concentration of RNA (copies/reaction) |
|----------------------|--------------------------------------|
|                      | 5000 | 500 | 250 | 50 | 5 | 0.5 | N.C. |
| Q Primer (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 | – | – | – | – | – |

* The assays were carried out in triplicate at each concentration.

Discussion

The authors declare that they have no competing interests.

Funding

This research was supported by a Grant-in-Aid (Grant Number...
Table 5

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

JPD18k0108030) from Japan Agency for Medical Research and Development (AMED).

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