Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of SARS-CoV2

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Abstract

Background. Highly sensitive real-time reverse transcription polymerase chain reaction (RT-PCR) methods have been developed for the detection of SARS-CoV2. However, they are costly. Loop-mediated isothermal amplification (LAMP) assay has emerged as a novel alternative isothermal amplification method for the detection of nucleic acid. **Methods.** A rapid, sensitive, and specific real-time reverse transcription LAMP (RT-LAMP) assay was developed for COVID-19 detection. **Results.** This assay detected 10 copies of SARS-CoV2 RNA in 30 mins. Both the clinical sensitivity and specificity of this assay were 100%. The RT-LAMP showed comparable performance with real-time RT-PCR. Combining simplicity and cost-effectiveness, this assay is therefore recommended for use in resource limiting settings.

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Abstract

Background. Highly sensitive real-time reverse transcription polymerase chain reaction (RT-PCR) methods have been developed for the detection of SARS-CoV2. However, they are costly. Loop-mediated isothermal amplification (LAMP) assay has emerged as a novel alternative isothermal amplification method for the detection of nucleic acid.

Methods. A rapid, sensitive, and specific real-time reverse transcription LAMP (RT-LAMP) assay was developed for COVID-19 detection.

Results. This assay detected 1 copy of SARS-CoV2 RNA in 30 mins. Both the clinical sensitivity and specificity of this assay were 100%. The RT-LAMP showed comparable performance with real-time RT-PCR. Combining simplicity and cost-effectiveness, this assay is therefore recommended for use in resource limiting settings.

Introduction

A novel coronavirus, SARS-CoV2, was recently identified causing pneumonia in humans, termed coronavirus disease 2019 (COVID-19). Cases of this new infection were first reported in Wuhan, China, and the outbreak spread to more than 155 other countries in a short time [1]. The World Health Organization declared the COVID-19 outbreak as a pandemic on 11 March 2020. Several real-time reverse transcription polymerase chain reaction (RT-PCR) methods have been developed and recommended by Centers for Disease Control [2] and WHO [3,4] for detection of SARS-like coronaviruses and specific detection of SARS-CoV2. These methods are highly sensitive and specific but are too expensive to be widely made available in many developing countries. Real-time RT-PCR also requires experienced personnel, maintenance of reagents in cold storage facility, and use of a high-precision thermal cycler. Loop-mediated isothermal amplification

(LAMP) has emerged as a novel alternative isothermal amplification method for the detection of nucleic acid [5]. The LAMP assay was reported to take <1 hour to perform at a constant temperature. Since LAMP does not require any major equipment and is simple to perform, it represents an ideal diagnostic tool for use in areas with limited resources. LAMP assays have been described for the detection of various other infectious agents including dengue viruses [6,7] and malaria parasites [8]. In order to further reduce costs and enable detection by the naked eye, we used hydroxynaphthol blue (HNB) dye for the colorimetric detection of the amplification reaction. This is the first report of the detection of SARS-CoV2 by RT-LAMP assay with HNB

Materials & Methods

RT-LAMP assay

Primers were designed using the Primer-Explorer V4 software (Eiken Chemical Co., Ltd., Tokyo, Japan) based on SARS-CoV2 N1 marker (GenBank accession no MN988713.1, LC528233.1 and MT123293.1). Reverse transcription LAMP (RT-LAMP) was carried out using Loopamp RNA amplification kit (Eiken Chemical Co., Ltd., Japan). The amplification was carried out in a Loopamp real-time turbidimeter (LA-320; Teramecs, Co., Ltd., Tochigi, Japan) with 2X reaction mixture, 5 µL RNA template, and species-specific primers. The primers sequences are as follows: F3, 5′-GTTGTTCGTTCTATGAAGACT-3′; B3, 5′-GACGTTGTTTTGATCGCG-3′; FIP, 5'-TGGGGTCCATTATCAGACATTTTAGTTTTAGAGTATCATGACGTTCG-3'; BIP, 5'-CGAAATGCACCCCGCATTACCCACTGCGTTCTCCATTC-3'; FLP,5'-TGTTCGTTTAGATGAAATC-3'; and BLP, 5'-TGGTGGACCCTCAGATTCAA-3' (In-progress of patent application). Endpoint assessment was done by visual inspection following the

addition of 1 µl HNB (Sigma, USA); a positive amplification was indicated by a colour change from violet to sky blue.

Analytical sensitivity and specificity

To determine the analytical sensitivity of the SARS-CoV2 RT-LAMP assay, the limit of detection (LODs) were determined using previously published method [9], using 10-fold serially diluted *in vitro* transcript RNA with known numbers of nucleic acid copies (10 cp/µL, 5 cp/µL, 2 cp/µL, 1 cp/µL and 0.1 cp/µL) and comparing the assay with RT-PCR. The reactions were carried out in duplicates.

The specificity of the LAMP-LF assay was determined by using genomic RNA of coronaviruses (HCoV-OC43 and SARS-CoV), adenovirus, human metapneumovirus, influenza A (A/H1pdm2009 and A/H3) viruses, influenza B virus, parainfluenza virus 3, rhinovirus A, respiratory syncytial virus B and enterovirus D68.

Clinical sensitivity and specificity

Forty-seven RT-PCR positive and 42 RT-PCR negative nasopharyngeal swabs samples from a recent COVID-19 outbreak in Malaysia (2020) were collected by Hospital Sungai Buloh, Malaysia. Total RNA was extracted using QIAamp viral RNA minikit (Qiagen, Germany) according to the manufacturer's instructions and 50 µL elution was obtained for each sample. The RNA samples were analysed by RT-PCR, as previously described [3,4]. SARS-CoV2 RT-LAMP reactions were run at 65°C for 30 min. Clinical sensitivity was calculated as (number of true positives)/(number of true positives + number of false negatives) and clinical specificity was calculated as (number of true negatives)/(number of true negatives + number of false positives)

comparing to RT-PCR. Ethical approval for this study was obtained from Medical Research Ethics Committee (MREC) Ministry of Health Malaysia (NMRR-20-535-53855).

Results

The SARS-CoV2 RT-LAMP assay was able to detect 1 copy per reaction of SARS-CoV2 RNA in 30 mins while the LOD for RT-PCR was 5 copies per reaction. No cross-reactivity with other viruses was found in either assay. Addition of HNB to the LAMP reaction solution did not affect the time taken for amplification (data not shown).

The RT-LAMP assay demonstrated a 100% sensitivity as all the RNA samples that were positive by RT-PCR were tested positive with RT-LAMP. None of the 42 RT-PCR negative samples were positive for SARS-CoV2 using this assay. No false-positive reactions were noted (Supplementary Table 1).

Discussion

The COVID-19 RT-LAMP reaction was sensitive enough to detect 1 copy of RNA per reaction, 5-fold better than real-time PCR. Several studies have found that LAMP out-performs RT-PCR for other viral infections [10,11], which is consistent with our results.

The analysis showed that the RT-LAMP developed is 100% specific and sensitive for the detection of COVID-19 with no false positives detected. The specificity and sensitivity levels of RT-LAMP are comparable to real-time RT-PCR methods as reported in other studies [12,13]. Encouragingly, the time required for confirmation of results by the RT-LAMP assay was less than 30 mins, 1.5-fold faster than the time required by real-time PCR. Even including the RNA isolation step, RT-

LAMP assay can be completed in less than 1 hour, which is very short when compared to that for real time RT-PCR (2-2.5 hours).

Due to its high sensitivity, RT-LAMP is prone to aerosol contamination. LAMP assays can be analysed by running an agarose gel or adding SYBR Green I. SYBR Green I has to be added after the completion of LAMP reaction, which can be inhibited by high concentrations of SYBR Green I. Tubes used for RT-LAMP reactions have to be opened for gel electrophoresis for addition of SYBR Green I, which can contaminate the air and subsequent reactions. Therefore, in our study, to avoid contamination, HNB dye was used to enable interpretation of the results by the naked eye, without requiring the tubes to be opened. Addition of HNB to the LAMP reaction solution did not affect amplification efficiency. This approach has been shown to be sensitive and simple for visual detection of turkey coronavirus RNA in tissues and faeces [14].

Conclusion

There are several limitations in this study. First, the LODs of the SARS-CoV2 RT-LAMP assay were not determined using serial dilutions of purified cell culture supernatant of SARS-CoV2 due to the unavailability of BSL3 laboratory. Secondly, we were unable to obtain culture materials such as HCoV-229E, HCoV-NL63, MERS-CoV and influenza C virus for specificity analysis of SARS-CoV2 RT-LAMP assay during the study period. These experiments should be included in the future to enhance the stringency of the RT-LAMP assay. In order to improve the efficiency of the RT-LAMP reaction, parameters such as heating temperature, dNTP concentration, and RT-LAMP reaction time can be further optimized. Lastly, the sensitivity and specificity of the RT-LAMP assay can only be compared with RT-PCR as there is no true gold standard for SARS-

CoV2 detection. It is possible that either test may have misclassified the true result of some of the samples.

To conclude, a cheap, rapid, sensitive, and specific RT-LAMP assay was successfully designed for SARS-CoV2 detection. The simplicity of RT-LAMP combined with rapid turnaround time has shown it to be a valuable and applicable tool for the diagnosis of infectious diseases, particularly in poor countries where resources are limited. In addition, RT-LAMP can be easily adapted to point-of-care diagnosis of COVID-19 as supported by Nguyen et al., (2020) [15]. An early and accurate diagnosis of SARS-CoV2 infection can assist in patient management and reduce health care utilization costs. Early diagnosis is crucial to identify patients with SARS-CoV2 infection for prompt institutionalization of infection control and public health measures, and when available, treatment.

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