SHORT REPORT

SIMPLE RNA EXTRACTION METHOD FOR REVERSE TRANSCRIPTION LOOP-MEDIATED AMPLIFICATION DETECTION OF SARS-COV-2

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Abstract. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a robust and cost-effective assay for rapid diagnosis of SARS-CoV-2 compared to reverse transcription quantitative (RT-q) PCR. The study evaluated the performance of RT-LAMP technique that incorporated a simple Chelex 100 resin-based RNA extraction step for SARS-CoV-2 detection targeting virus *E* (encoding envelope protein) and *RdRP* (encoding RNA-dependent RNA polymerase). Using primer sets for *E* and RdRP, the developed RT-LAMP assay had a limit of detection (LOD) of 1 copy/µl transcribed RNA. For nasopharyngeal and oropharyngeal swab samples (n = 58), in comparison to the gold standard RT-qPCR (amplifying *E* and RdRP) sensitivity, specificity, positive predictive value, and negative predictive value of SARS-CoV-2 RT-LAMP assay targeting *E* gene was 88% (95% confidence interval (CI): 75-96%), 87% (95% CI: 59-98%), 99% (95% CI: 97-100%), and 28% (95% CI: 14-48%), respectively and for *RdRP* gene was 67% (95% CI: 51-98%), 87% (95% CI: 59-98%), 100% (95% CI: 96-100%), and 12% (95% CI : 8-18%), respectively. The whole process of RT-LAMP assay was completed within ~60 minutes. This developed RT-LAMP method for on-site COVID-19 detection should be useful in resource limited settings.

Keywords: COVID-19, Chelex resin, rapid detection, LAMP assay, SARS-CoV-2

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INTRODUCTION

Since December 2019, the world has been affected by a pandemic coronavirus disease 2019 (COVID-19), caused by a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (WHO, 2020; Hui *et al*, 2020). This has resulted in a critical demand for an easy, non-invasive, rapid and sensitive detection method suitable for mass screening of suspected infected populations for treatment and control of the pandemic. As SARS-CoV-2 is an RNA virus, the current gold standard detection method is reverse-transcription quantitative (RT-q) PCR (Udugama *et al*, 2020). Although exquisitely sensitive, the technique is time consuming and costly. A recent innovation is a loop-mediated amplification (LAMP) method (Notomi *et al*, 2000), which is an isothermal reaction requiring a thermal stable *Bacillus stearothermophilus* (*Bst*) DNA polymerase and has a shorter turn-around time than PCR. LAMP amplification utilises four to six specifically designed primers that bind to six to eight regions of the target gene, thereby avoiding nonspecific binding and resulting in high specificity. It soon became obvious that by including a prior reverse-transcription step one could readily create a RT-LAMP technique. A commercially available mutant *Bst* 3.0 DNA polymerase that performs both the reverse transcription step and LAMP has allowed RT-LAMP to become a one-pot assay (Lee *et al*, 2016).

Here, we employed a simple and direct RNA extraction method for use in RT-LAMP assay of COVID-19 infection by amplifying SARS-CoV-2 *E* and *RdRP* (Ulloa *et al*, 2020; Guan *et al*, 2021). The modified extraction method presented in this study helps to reduce the cost of RNA extraction. As compared to the commercial kit (USD6.45/ reaction), our newly developed RNA extraction method is cheaper which costs only USD2.27/reaction. Hence, this simple RNA extraction method also can be used as an alternative for laboratories experiencing shortage of extraction kits.

MATERIALS AND METHODS

Source of samples

Nasopharyngeal and oropharyngeal swab samples (n = 58) in 250 µl of viral transport medium (VTM) (FC-BIOS, Selangor, Malaysia) collected from patients ≥18 years of age between 16-27 August 2021, with subjects' names redacted, were kindly provided by The Institute for Medical Research (IMR) in Kuala Lumpur, Malaysia. Samples were stored at -80°C.

Study protocols were approved by the UMMC Medical Ethics Committee (permit no. 202041-8418) and the Malaysian Ministry of Health Medical Research Ethics Committee (MREC) (permit no. NMRR-20-2344-56994). According to the ethics committee,

no written consent was required as samples used in this study were left over from routine diagnostics of patients with clinical concern for COVID-19. Only anonymised samples were tested and there was no direct interaction with patients.

RNA extraction

RNA was extracted using a modified Chelex 100 Resin extraction method as previously described (Janíková *et al*, 2021; Perez *et al*, (2021). A 30% (w/v) Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA) suspension in RNase-free water was vortexed vigorously for 3 minutes and kept at 4°C until use. A 15-µl aliquot of sample was mixed with 22.5 µl of the Chelex-water suspension under aseptic conditions, incubated at 95°C for 10 minutes, placed on ice for 2 minutes, then centrifuged at 12,000 g for 30 seconds, and the supernatant served as virus RNA template in the RT-LAMP assay.

RT-LAMP assay

SARS-CoV-2 *E* (encoding envelope protein) and *RdRP* (encoding RNA-dependent RNA polymerase) were amplified using primers previously reported (Table 1) (Lai *et al*, 2021). RT-LAMP assay was carried out in a 25-µl reaction mixture comprising of 9.3 µl of RNase-free water, 2.5 µl of 10X isothermal amplification buffer II (New England Biolabs, Ipswich, MA), 1.5 µl of 100 mM $MgSO_4$, 1.4 µl of 100 mM dNTPs, 3.8 µl of primer mix (consisting of 40 pmol FIP and BIP each, 10 pmol of FLP and BLP each, 5 pmol of F3 and B3 each), 1 µl of *Bst* 3.0 DNA polymerase I (New England Biolabs, Ipswich, MA), 1 µl of RNaseOUT recombinant ribonuclease inhibitor (Thermo Fisher Scientific, Waltham, MA), 2 µl of 1 mM hydroxynaphthol blue (HNB) (Sigma, St Louis, MO), and 2.5 µl of RNA template. Reaction was performed at 65°C for 45 minutes using a Loopamp Real-Time Turbidimeter LA 500 (Eiken Chemical Co Ltd, Taito-ku, Japan). Time (minutes) is recorded when sample exhibits a sky blue color (positive sample), while a negative sample has no change in violet color at 45 minutes.

Sensitivity, specificity, positive predictive value, and negative predictive value of RT-LAMP assay

SARS-CoV-2 *E* and *RdRP* were inserted into a recombinant plasmid (pGEMT-T vector system, Promega Corporation, Madison, WI) and used to transform TOP10F' *E. coli*-competent cells (Thermo Fisher Scientific, Waltham, MA). Target regions in recombinant plasmid were amplified using F3 and B3 primers of both *E* and *RdRP* (Table 1), and amplicons were inserted into a pGEM-T vector (Promega Corporation, Madison, WI) for transformation of TOP10F' *E. coli*-competent cells (Thermo Fisher Scientific, Waltham, MA). Qiagen Spin Miniprep kit (Qiagen, Hilden, Germany) was used to extract recombinant plasmids, which were sequenced (Apical Scientific Sdn Bhd, Selangor, Malaysia) for confirmation of

Table 1

SARS-CoV-2 *E* and *RdRp* primers used for RT-LAMP assay in the study

Primer*	Sequence (5'→3')
E	
FIP	CGCAGTAAGGATGGCTAGTGTAGCGTACTTCTTTTTCTTGCTT
BIP	TCGATTGTGTGCGTACTGCTGTTTTTAACACGAGAGTAAACGT
FLP	CTAGCAAGAATACCACG
BLP	GTTAACGTGAGTCTTG
F3	TTTCGGAAGAGACAGGTAC
B3	AGGAACTCTAGAAGAATTCAGA
RdRp	
FIP	TGAGCACACTCATTAGCTAATCTCGCAAACATACAACGTGTTG
BIP	GAGTGAAATGGTCATGTGTGGAAGCAGTTGTGGCATCTC
FLP	GAAACGGTGTGACAAGCT
BLP	GTTAAACCAGGTGGAACCTC
F3	TGGCCTCACTTGTTCTTG
B3	AGCTTGACAAATGTTAAAAACAC

*Primers used in this study followed those of Lai *et al* (2021)

B3: backward primer; BIP: backward inner primer; BLP: backward loop primer; F3: forward primer; FIP: forward inner primer; FLP: forward loop primer; RT-LAMP: reverse transcription loop-mediated isothermal amplification; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

correct inserts. Recombinant plasmids were linearized with *Sal*I (New England Biolabs, Ipswich, MA) and transcribed into RNA using a RiboMAX™ Large Scale RNA Production System (Promega Corporation, Madison, WI). RNA copy number was determined according to the formula:

$$\text{Copy}/\mu\text{l} = \frac{6.02 \times 10^{23} \times 10^{-9} \times \text{concentration (ng}/\mu\text{l)}}{\text{fragment length} \times 340}$$

Then, RNA solutions were prepared in 10-fold serial dilutions, ranging $1x10^6$-1 copy/µl.

For evaluation of RT-LAMP assay specificity against other RNA viruses, genomic RNA from a variety of viruses [coronaviruses HCoV 229E and HCoV-OC43, influenza A H3N2, influenza A H1N1, influenza B, adenovirus 4, parainfluenza virus 1, parainfluenza virus 2, respiratory syncytial virus (subtype A), and respiratory syncytial virus (subtype B)] (Vircell, Granada, Spain) were employed. Sensitivity of the RT-LAMP assay is calculated using the following formula:

$$\text{Sensitivity} = \frac{\text{number of true positives}}{\text{number of true positives + number of false negatives}}$$

Specificity is calculated using the following formula:

$$\text{Specificity} = \frac{\text{number of true positives}}{\text{number of true negatives + number of false positives}}$$

Positive predictive value (PPV) is calculated using the following formula:

$$\text{Positive predictive value} = \frac{\text{sensitivity x prevalence}}{\text{[(sensitivity x prevalence) + ((1-specificity) x (1-prevalence))]}}$$

Negative predictive value (NPV) is calculated using the following formula:

$$\text{Negative predictive value} = \frac{\text{specificity x (1-1)}}{\text{[(specificity x (1-1)) + ((1-sensitivity) x 1)]}}$$

RESULTS

RT-LAMP assay employing SARS-CoV-2 *E* and *RdRP* primer sets had a limit of detection of transcribed virus RNA of 1 copy/µl and did not detect transcribed RNA of nine different RNA viruses (data not shown). Employing *E*, RT-LAMP assay detected 40/58 samples (time of color change

Table 2

Comparison of RT-LAMP and RT-qPCR assays of SARS-CoV-2 from nasopharyngeal and oropharyngeal swab samples of subjects ≥18 years of age from The Institute for Medical Research, Kuala Lumpur, Malaysia (16-27 August 2021)

Sample number	RT-LAMP		RT-qPCR gene (C_T value)[b]
	E (Minutes)[a]	*RdRP* (Minutes)[a]	
NPS001	25.3	Neg	22
NPS002	27.2	Neg	20
NPS003	Neg	Neg	18
NPS004	30.5	Neg	19
NPS005	31.5	36.5	18
NPS006	Neg	Neg	26
NPS007	41.2	Neg	24
NPS008	32.4	Neg	16
NPS009	34.2	Neg	28
NPS010	20.0	34.0	19
NPS011	32.0	36.0	17
NPS012	25.0	27.5	15
NPS013	30.4	33.5	14
NPS014	29.1	Neg	23
NPS015	27.1	38.3	22
NPS016	28.2	37.1	16
NPS017	28.5	32.5	17
NPS018	28.0	32.1	22
NPS019	23.4	29.4	15
NPS020	23.5	36.2	16
NPS021	27.1	30.5	18
NPS022	27.2	32.4	24
NPS023	32.0	36.0	20

Table 2

Sample number	RT-LAMP		RT-qPCR gene (C_T value)[b]
	E (Minutes)[a]	*RdRP* (Minutes)[a]	
NPS024	25.2	37.2	22
NPS025	29.5	36.4	22
NPS026	27.4	28.4	25
NPS027	26.2	26.2	25
NPS028	Neg	30.4	16
NPS029	28.4	26.0	15
NPS030	Neg	35.2	24
NPS031	31.0	35.5	29
NPS032	31.2	33.5	24
NPS033	31.3	39.5	28
NPS034	32.1	36.5	19
NPS035	21.0	38.5	24
NPS036	27.3	34.4	37
NPS037	32.1	Neg	36
NPS038	29.2	Neg	36
NPS039	26.3	30.1	37
NPS040	26.2	Neg	37
NPS041	32.5	37.3	39
NPS042	19.2	Neg	34
NPS043	Neg	Neg	35
NPS044	Neg	Neg	Neg
NPS045	Neg	Neg	Neg
NPS046	33.1	Neg	Neg
NPS047	Neg	Neg	Neg
NPS048	Neg	Neg	Neg
NPS049	Neg	Neg	Neg

Table 2

Sample number	RT-LAMP		RT-qPCR gene (C_T value)[b]
	E (Minutes)[a]	*RdRP* (Minutes)[a]	
NPS050	Neg	Neg	Neg
NPS051	Neg	Neg	Neg
NPS052	Neg	Neg	Neg
NPS053	Neg	Neg	Neg
NPS054	Neg	Neg	Neg
NPS055	Neg	Neg	Neg
NPS056	Neg	Neg	Neg
NPS057	28.2	29.0	Neg
NPS058	Neg	28.4	Neg

[a]Negative (Neg) if no change in color occurs within 45 minutes; [b]Negative (Neg) if C_T value >40 (Lai *et al*, 2022)

C_T value: threshold cycle value; *E*: SARS-CoV-2 envelope protein gene; RdRP: SARS-CoV-2 RNA-dependent RNA polymerase gene; RT-LAMP: reverse transcription loop-mediated isothermal amplification; RT-qPCR: reverse transcription quantitative PCR; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

ranging 19.2-41.2 minutes), while with *RdRP*, there were 31/58 positive samples (time of color change ranging 26.0-39.5 minutes) (Table 2). Among the same set of 58 samples, a previous study employing the gold standard RT-qPCR assay amplifying SARS-CoV-2 N gene reported 43 samples being positive (C_T value ranging 14-39) with the remaining negative (C_T value >40) (Table 2) (Lai *et al*, 2022). Three samples (NPS046, NPS057, and NPS058) were detected by RT-LAMP but not by RT-qPCR, most likely due to contamination as RT-LAMP was the more sensitive method.

Using RT-qPCR as standard method, RT-LAMP assay using *E* primer sets had a higher sensitivity compared to *RdRP* with 88% (95 % confidence interval (CI): 75-96%) and 67% (95 % CI: 51-98%) respectively, *p*-value = 0.023. However, both genes demonstrated the same specificity of 87% (95% CI: 59-98%). PPV and NPV for *E* RT-LAMP was 99% (95% CI: 97-100%) and 28% (95% CI: 14-48%) respectively, and for *RdRP* PPV and NPV was 100% (95% CI: 96-100%) and 12% (95% CI: 8-18%) respectively.

DISCUSSION

Different types of SARS-CoV-2 genes, such as those encoding structural proteins and species-specific genes encoding *E*, *N* or *RdRP* have been widely used as target genes for molecular diagnosis of COVID-19 (Cho *et al*, 2020). Apart from the highly conserved *N1*, *E* serves as the first-line screening of SARS-CoV-2 assay while *RdRP* is used in confirmatory test (Benrahma *et al*, 2020; Ishige *et al*, 2020). The most often chosen targets for SARS-CoV-2 RT-LAMP are *E*, *N1* and ORF1ab region (encoding *RdRP*) (Jang *et al*, 2021). Thus, *E* and *RdRP* were selected as target genes of interest in the present work. However, there is less published data on the use of *RdRP* compared to *E* and *N1* for SARS-CoV-2 detection (Benrahma *et al*, 2020). Alves *et al* (2021) reported RT-LAMP LOD targeting SARS-CoV-2 *E* is 19 copies/µl transcribed RNA and Jang *et al* (2021) was able to reduce RT-LAMP LOD of both *RdRP* and *E* of 10 copy/µl, whereas in the present study RT-LAMP LOD for both *E* and *RdRP* was 1 copy/µl.

Given the COVID-19 pandemic situation, it is crucial to have a reliable and rapid on-site diagnostic assay. Currently, RT-qPCR remains the gold standard in all diagnostic laboratories even though it is costly, time-consuming (at least 2 hours), and requires expensive reagents and equipment. Moreover, virus RNA has to be extracted (using available commercial extraction kits following by a series of spinning, changing of columns, washing, purification and elution), a manual process requiring more than 1 hour, prior to performing RT-qPCR assay. Here, we simplified the RNA extraction step by using Chelex 100 resin extraction procedure that requires about 15 minutes. In addition, a 15-µl volume of VTM samples was required. Together with RT-LAMP procedure, the whole process - from extraction to completion of diagnosis - takes only 1 hour. Chelex 100 resin prevents RNA from degrading during the heating step and as a metal ion chelating agent helps to minimize RNA degradation during storage (Ulloa *et al*, 2020; Guan *et al*, 2021; Perez *et al*, 2021). Another advantage of employing a Chelex resin in RNA extraction is that it improves detection of genome copy number and thereby sensitivity. Guan *et al* (2021) demonstrated that using the Chelex 100 resin method LOD of RT-qPCR and reverse transcription digital droplet PCR targeting SARS-CoV-2 *N* allows detection of 1 copy/µl transcribed RNA.

In the present study, HNB was employed as a dye for end point detection of RT-LAMP assay. HNB color change can be readily monitored by l eye without the need to open the tube as the dye is added during the preparation of RT-LAMP reaction mix. This reduces the chance of carry-over contamination and

minimize false-positive results. Although this can happen as evidenced by the false-positive RT-LAMP results observed in our study. In order to minimize this carry-over contamination, pre- and post-amplification steps should be carried out in separated areas.

In conclusion, this study shows that RT-LAMP assay coupled with Chelex 100 resin extraction method allows detection of SARS-CoV-2 virus from nasopharyngeal and oropharyngeal swab samples in a virus transfer medium to a level of 1 copy/µl transcribed RNA. The whole process could be completed within ~60 minutes. This method obviated the necessity of an expensive thermal cycler instrument and required relatively less costly reagents. The RT-LAMP method developed in the present study for on-site COVID-19 detection should be applicable in resource limited settings.

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CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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