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Coronavirus Pandemic

Development of a colorimetric RT-LAMP assay for the detection of SARS-COV-2 isolated from Oman

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Abstract
Introduction: A rapid and sensitive COVID-19 diagnostic test is required to aid in the prevention and control of the current COVID-19 pandemic spread. We developed a colorimetric, rapid, and sensitive RT-LAMP assay for the diagnosis of COVID-19 viral infection.
Methodology: Complete genome sequences of 41 SARS-CoV-2 isolates from Oman were used in this study. Three primer sets (CoV_S1, CoV_S2, CoV_M1) were developed from all Omani SARS-CoV-2 genome sequences available at the time, targeting the spike protein gene and the M gene. The primer set (CoV_S1) was found to be the most sensitive and specific among the three designed sets. The sensitivity and specificity of the assay were compared to that of qRT-PCR. Direct testing of SARS-CoV-2 spiked saliva with the developed assay was evaluated. Lyophilized colorimetric assays were stored at room temperature and 4 °C and their ability to detect positive samples were tested for a period of 8 weeks.
Results: The RT-LAMP assay was validated by testing 145 COVID-19 clinical samples with a sensitivity of 96.9% and specificity of 94.7% when compared to the validated qRT-PCR assay. The assay specificity was tested against SARS-CoV Frankfurt 1 RNA virus and avian coronaviruses as they tested negative with the developed assay. The assay was lyophilized and managed to detect the positive samples colorimetrically when stored at 4 ˚C for up to 8 weeks.
Conclusions: The assay can be utilized in its current form as a screening assay with the advantages of being simpler, quicker, and cheaper than the qRT-PCR.

Key words: SARS-CoV-2; LAMP assay; colorimetric; lyophilized.

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Introduction

Current testing for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) relies on real-time polymerase chain reaction (qRT-PCR) diagnostic assays. While PCR is sensitive, it requires purification of RNA from samples, expensive equipment, and it takes hours to get a result. Loop-mediated isothermal amplification (LAMP) is an alternative technique with applications in the diagnosis of infectious diseases. LAMP assays can be completed in less than an hour and can be performed using inexpensive equipment. LAMP provides a highly sensitive, highly specific, cost-effective, and less technically demanding alternative to PCR in detecting infectious agents [1]. The assay sensitivity is comparable to qRT-PCR but the sample requirements are less stringent and the readout can be as simple as observing a color change in the reaction mix. A colorimetric LAMP assay facilitates the instant readout of the amplification results, further reducing the time and need for sophisticated equipment for the interpretation of the results.

LAMP technique amplifies nucleic acids with high efficiency under isothermal conditions without significant influence of the co-presence of non-target nucleic acid [1]. The detection limit can be as low as a few copies, being comparable to that of polymerase chain reaction. LAMP is easy to perform once the appropriate primers are designed. It requires six primers, a DNA polymerase, and a regular laboratory water bath or heat block for the completion of the reaction. RT-LAMP combines reverse-transcription and target DNA amplification within the same reaction and can amplify RNA sequences with high efficiency. With the current situation of SARS-CoV-2, there is a need for a rapid, sensitive, and cost-effective technique

that can practically be adapted for a mass screening program or point-of-care testing.

Whilst some COVID-19 LAMP assays have been published, we have designed new LAMP assays to account for the evolution of viral sequences since the initial outbreak of the pandemic. Lamb *et al.* developed a LAMP assay for COVID-19 based on the sequence of 23 different strains [2]. However, this assay required more complex fluorescent detection and was only tested on single-stranded DNA templates, not RNA. Park *et al.* developed both a colorimetric and a fluorescence-based LAMP assay, but their assay required two separate reactions (reverse transcription followed by LAMP) [3]. Zhang *et al.* designed five sets of LAMP primers based on the ORF1a and Gene N of the COVID-19 sequence and tested these using a colorimetric LAMP assay [4]. This assay was as accurate as a commercial COVID-19 RT-PCR kit, but the sensitivity of both was not compared.

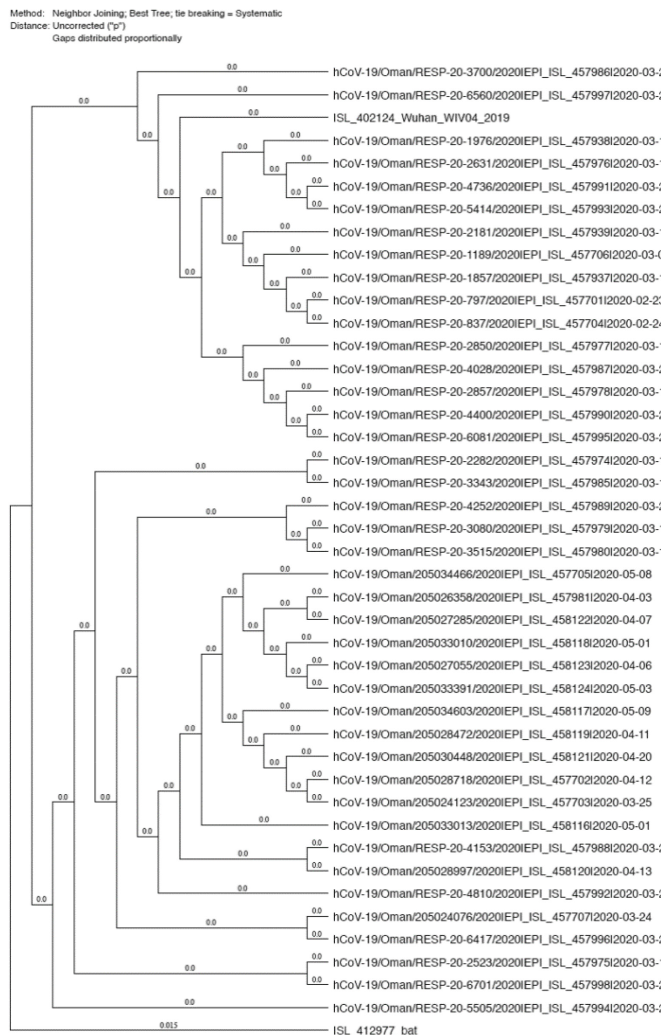


Figure 1. A phylogenetic tree showing the relatedness of the Oman genome sequences, Wuhan sequence and a closely related bat coronavirus sequence.

Since the early COVID-19 LAMP papers were published the virus has spread throughout the world and evolved into new sequence variants. The China National Center for Bioinformation, 2019 Novel Coronavirus Resource [5] contains a repository of annotated COVID-19 genome sequences. As of 23 April 2020, 11,320 sequences were available and 4,494 genome sequence variations had been identified. The evolution of the virus makes it imperative that diagnostic assays are confirmed to be able to detect all known variants, and may require the design of new primer sets for more highly conserved regions of the virus genome.

We have developed a rapid, ready-to-use assay that can be applied within a hospital setting or less sophisticated clinics, increasing the availability of accurate and rapid COVID-19 testing within Oman. The colorimetric RT-LAMP assay proved to be accurate and sensitive and will allow the testing to be done on samples without the need for the expensive equipment and reagent costs required for qRT-PCR testing.

Methodology

Primer design

Complete genome sequences of 41 SARS-CoV-2 isolates from Oman were used in this study. A phylogenetic tree showing the relatedness of the Oman genome sequences, Wuhan sequence, and a closely related bat coronavirus sequence, was created using (MacVector version 6.5.3, Oxford Molecular Ltd. Oxford, England) (Figure 1). The bat coronavirus is very closely related to SARS-CoV-2 throughout most of the genome, but the sequence diverges more in the spike protein.

Published LAMP primer sequences [2–4,6–9] were each individually compared with the Oman SARS-CoV-2 genome sequences using MacVector to look for any mismatches between the primer sequences and the genome sequences. There was a mismatch in at least one primer from all of the LAMP assays designed by [2,6,8], most LAMP assays of [4], and many of the assays of [7]. For those assays in which there was no sequence mismatch with the Omani virus sequences, further analysis indicated a high sequence similarity with a bat coronavirus sequence in some primers.

Due to the problems identified with published LAMP assay sequences we decided to design new LAMP assays that were better targeted to the SARS-CoV-2 genome sequences that had been identified in

Oman, focusing on the region of the genome from the spike protein gene through to the nucleocapsid protein gene (N gene). This region of the virus genome was broken down into smaller, overlapping sequences and analyzed by PrimerExplorer v5 (Eiken Chemical Co., Ltd., Tokyo, Japan) to design new LAMP assays. Three primer sets that contained no mismatches with the Omani SARS-CoV-2 genome sequences were selected for LAMP assay development (Table 1). T-linker sequences were added within the FIP and BIP primers.

RT-LAMP assay

Master mixes were prepared at room temperature for each reaction immediately before use with 5 µL of the WarmStart Colorimetric RT-LAMP 2X Master Mix (M1800, New England Biolabs, Ltd, Hitchin, UK), 1 µL of the 10x primer mix, 3 µL with nuclease-free water, with the addition of 1 µL of the sample just before starting the reaction. Tubes were kept on ice during RNA template addition. Master mix preparation and RNA template addition were done in separate rooms to minimize contamination. Reaction tubes were incubated in a heat block with a lid (BSH5001, myBlock I Dry Bath, Benchmark Scientific, USA) at 65 °C for 30 minutes. Photographs were taken with a high-quality cell phone camera for color change documentation.

Detection limits

The detection limit of the three primers was assessed using the WarmStart Colorimetric RT-LAMP 2X Master Mix and Quantitative RT-PCR (qRT-PCR) Control RNA from Heat-Inactivated SARS-related Coronavirus 2, Isolate USA-WA1/2020 (NR-52347, BEI Resources, Manassas, Virginia, USA) as a positive control (5.0×10^7 genome equivalents/mL). A 5-fold dilution series of the positive control was prepared and tested using the 3 primer sets and color change of each dilution was evaluated and photographed. To determine the amplification curves of the 5-fold serial dilution for the promising primer set, a non-colorimetric WarmStart® LAMP Kit (E1700S, New England Biolabs, Ltd, Hitchin, UK) with the addition of fluorescent dye (B1700, New England Biolabs, Ltd, Hitchin, UK) was used in a qRT-PCR (Applied Biosystems™ 7500 Real-Time PCR, Thermo Fisher Scientific, USA) and samples were incubated for at 65 °C for 30 minutes. Samples were tested in triplicates with 5 µL of the WarmStart RT-LAMP 2X Master Mix) and 1 µL of the 10x primer mix, 0.2 µL fluorescent dye, and filled up to 2.8 µL with nuclease-free water with the addition of 1 µL of the sample just before starting the reaction in a 96 well PCR plate then sealed with an optically clear adhesive seal. The FAM channel was used to read the dye.

RT-LAMP assay validation using clinical sample

Extracted RNA from 145 COVID-19 clinical samples was kindly provided by the Department of Virology, Sultan Qaboos University Hospital. All the samples have already been tested for SARS-CoV-2 by qRT-PCR using a screening gene (E gene) and a

Table 1. Developed primers for SARS-COV-2 RT-LAMP assay.

Primer	Sequence (5’-3’)
CoV_S1*	
F3:	GGCAGAGACATTGCTGAC
B3:	AGAACCTGTAGAATAAACACGC
FIP:	GACACCACCAAAAGAACATGGTTTTTACTACTGATGCTGTCCGTG
BIP:	CCAGGTTGCTGTTCTTTATCAGGTTTTGGAGTAAGTTGATCTGCATGA
LF:	TCAAGAATCTCAAGTGTCTGTGGAT
LB:	GCACAGAAGTCCCTGTTGCTAT
CoV_S2	
F3:	TTGCTGCTAGAGACCTCA
B3:	GAACATTCTGTGTAACTCCAAT
FIP:	ACAGTGCAGAAGTGTATTGAGCAATTTTTGTGCACAAAAGTTTAACG
BIP:	GCGGGTACAATCACTTCTGGTTTTTCCTATAAGCCATTTGCATAGC
LF:	TGAGCAAAGGTGGCAAAACAGT
LB:	TGGTGCAGGTGCTGCATT
CoV_M1	
F3:	TGTGGCTCAGCTACTTCA
B3:	TCACAGCGTCCTAGATGG
FIP:	GTGGCACGTTGAGAAGAATGTTAGTTTTTGCTTCTTTCAGACTGTTTG
BIP:	CATGGCACTATTCTGACCAGACTTTTCCAGCAATACGAAGATGTCC
LF:	ATGACCACATGGAACGCGTAC
LB:	ACTCGTAATCGGAGCTGTGATCC

*The chosen primer set for the RT-LAMP assay.

COVID-19 specific (N gene). Samples were tested using the developed colorimetric RT-LAMP assay in a 9 µL master mix using the primer set (CoV_S1) with the addition of 1 µL of the clinical sample RNA template and heated at 65 °C for 30 minutes. To avoid any RNA or DNA contamination, DNAZap^{TM} (Thermo Fisher Scientific, USA) was used to spray the working bench, pipettes, and the heat block between each run.

RT-LAMP Specificity

To determine the specificity of the RT-LAMP assay, it was tested against SARS-CoV Frankfurt 1 RNA virus (Ref-SKU: 004N-02005, European Virus Archive Global, UK), Avian Coronavirus, Massachusetts (formerly Avian Infectious Bronchitis Virus) (NR-49096, BEI Resources, USA), Porcine Respiratory Coronavirus, ISU-1 (NR-48572, BEI Resources, USA), and Alphacoronavirus 1, Purdue P115 (attenuated) (formerly Porcine Transmissible Gastroenteritis Virus) (NR-48571, BEI Resources, USA).

Testing saliva spiked with SARS- Coronavirus 2 RNA

Mock samples were prepared from the saliva of SARS-CoV-2 qRT-PCR negative individuals. The saliva was collected in RNAse free tube and a 1:1 dilution was prepared with SARS-CoV-2 RNA (NR-52347, BEI Resources, Manassas, Virginia, USA) to achieve a final concentration of (2.5×10^7 genome equivalents/mL) of which a 2-fold dilution series was prepared and tested directly without RNA extraction using our developed colorimetric LAMP assay as previously described by Williams *et al.* [10].

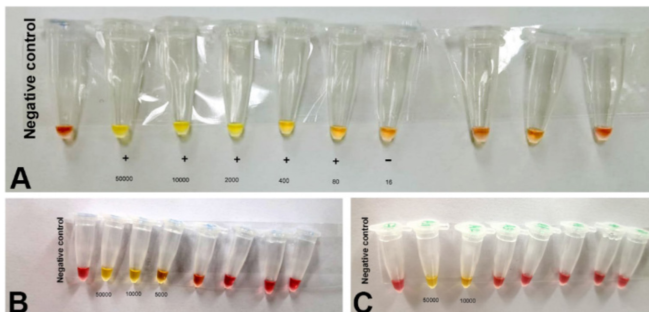


Figure 2. Detection limits of the three designed primer sets using WarmStart Colorimetric LAMP 10X kit. Red indicates negative and yellow indicates positive amplification. Primer sets; A) CoV_S1/ detection limit of 80 genome equivalents; B) CoV_S2/ detection limit of 5000 genome equivalents; C) CoV_M1/ detection limit of 10000 genome equivalents.

Freeze-drying the assay

The master mix was prepared for a 25 µL reaction using 1x primers, Warmstart master mix 12.5 µL, 2.5 µL 10x buffer, and 9 µL water. The mix was frozen for 30 minutes, then subjected to vacuum for 1 hour followed by heating for 1 hour using (LyoQuest -55 Plus, Telstar, Spain). The tubes containing the dried master mix were divided into two sets where one was kept at room temperature and the other was stored at 4 °C. The activity of the dried master mix and primers were tested weekly for eight consecutive weeks. Dried LAMP reactions were carried out by adding 24 µL of water and 1 µL of the template followed by incubation of the tubes in a heat block at 65 °C for 30 minutes and observing the color change.

Statistical analysis

Sensitivity (the fraction of those with the disease correctly identified as positive by the qRT-PCR test) and specificity (The fraction of those without the disease correctly identified as negative by the qRT-PCR test) of the assay were calculated using the hybrid Wilson/Brown method using GraphPad Prism 6 (Graphpad Software, San Diego, CA, USA)).

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Medical Research Ethics Committee (MREC), College of Medicine and Health Sciences, Sultan Qaboos University (Ref. NO. SQU-EC/067/2020).

Results

Detection limits

The detection limits of the 3 designed primer sets were evaluated using a 5-fold dilution of a SARS COVID-19 as a positive control (5.0 × 107 genome equivalents/mL). The primer set CoV_S1 was found to be the most sensitive set when compared to the other two primer sets (CoV_S2 and CoV_M1). The detection limit of the CoV_S1 primer was 80 genome equivalents compared to 5000 genome equivalent for the CoV_S2 primer set and 10,000 genome equivalent for the CoV_M1 primer set (Figure 2). The detection limit of the CoV_S21 set was tested using fivefold dilution with the non-colorimetric WarmStart LAMP with a fluorescent dye through a qRT-PCR using the FAM channel in triplicates where the assay showed similar results to the colorimetric assay with 80 genome equivalents detection limit as shown in (Figure 3).

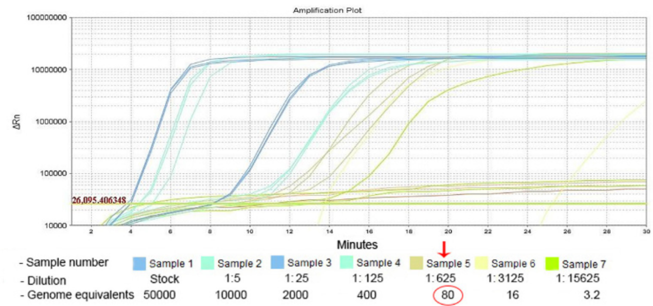
RT-LAMP assay validation

RNA extracted from 145 COVID-19 clinical samples were tested using the developed RT-LAMP assay and compared to the results obtained from Sultan Qaboos University Hospital qRT-PCR results. The RT-LAMP assay successfully detected 122 out of 127 positive samples and one false-positive sample where the color change was not sufficient to determine if it was positive or negative. The LAMP assay successfully detected 18 true negative samples and four false-negative samples. The four false-negative samples had a qRT-PCR Ct value above 31, which is below the detection limits of the RT-LAMP assay. The RT-LAMP sensitivity was 96.9% and the specificity was 94.7%. There is still room for improvement of assay sensitivity that needs to be tested by optimizing primer concentrations. Based on this data, the colorimetric RT-LAMP would be more suitable as a screening test that can identify patients with a moderate to high viral load but perhaps not detect an individual with a low viral load. Moreover, the cost of the assay was calculated to be 0.39 Riyal Omani (RO) (excluding the nasal swap and the RNA extraction) when using the master mix at 10 µL compared to 25 RO (Ministry of Health). The results can be visually detected without any further within 30 minutes and without the need for sophisticated equipment which further reduces the costs dramatically. Based on the results shown in Figure 2, it may be possible to reduce the colorimetric RT-LAMP assay time to 20 minutes with little loss in sensitivity.

RT-LAMP Specificity

The RT-LAMP assay was highly specific and did not detect the SARS-CoV Frankfurt 1 RNA virus or the Avian Coronavirus. On the other hand, it was able to amplify both the Porcine Respiratory Coronavirus and the Porcine Transmissible Gastroenteritis Virus as there is a similarity in the spike protein gene that is amplified by the primer set.

Figure 3. Amplification curves of SRARS COVID- 19 positive control; sample 1 (Stock) and the further 5-fold dilutions with successful amplification of the triplicates up to the 5th sample (Equivalent to 80 genomes) using the primer set CoV_S1.



Testing saliva spiked with SARS- Coronavirus 2 RNA

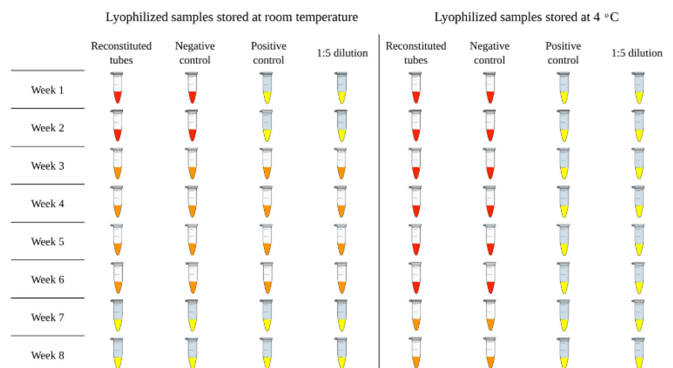
The developed RT-LAMP assay was successfully able to amplify the RNA target in the mock saliva spiked samples without prior nucleic acid extraction and detected 6,250 genome equivalent of the SARS-Cov-2 while the negative control sample remained negative (Figure 4).

Figure 4. Detection limits of the RT-LAMP assay using the CoV_S1 primer set and the mock saliva samples spiked with SARS-Cov-2 RNA without RNA extraction. Red indicates negative and yellow indicates positive amplification.

Lyophilized RT-LAMP assay

The results of testing the lyophilized RT-LAMP assay were summarized in Figure 5. Tubes that were stored at room temperature gave a bright red colour after reconstitution with water for up to 2 weeks and were able to amplify the target successfully with a clear change in colour from red (negative) to yellow (positive). On week three, the reconstituted tubes showed a faint orange colour rather than the bright red, and they become completely yellow in the 7th week, which made it impossible to determine the negative and

Figure 5. The results of testing the lyophilized RT-LAMP assay for 8 weeks.



Red Tube: Negative; Yellow: positive; Orange: questionable.

positive samples based on the colour change. On the other hand, lyophilized assay stored at 4 °C showed a clear yellow colour for positive samples up to week 8, however, the reconstituted tubes with water and the negative control gave an orange colour starting from week 7.

Discussion

The developed RT-LAMP assay utilizes primers that target the spike protein S gene and not the highly conserved coronavirus N gene that has been used in other developed LAMP assays [2,4,6]. The detection limit of the developed RT-LAMP assay was 80 genome copies which is close to the sensitivity of the qRT-PCR. Lamb *et al.* described RT-LAMP-based assay with a higher sensitivity of 0.2 femtograms but, it requires ultraviolet light to interpret the results in contrast to our colorimetric assay [2]. We also demonstrated that the assay is highly specific for SARS-CoV-2 and no cross-reactivity with SARS-CoV-1 and Avian Coronavirus was detected, however, it was able to detect Enteric and Respiratory Porcine Coronaviruses. Cross-reactivity between SARS-CoV-2 and Bat SARS-like CoV was excluded since we targeted the more diverges sequence of the spike protein.

The RT-LAMP technique is a simple and rapid gene amplification technique that has promising diagnostic capabilities for the rapid diagnosis and early detection of various infectious pathogens [11].

Our RT-LAMP assay sensitivity was 96.9% and the specificity was 94.7% compared to the validated qRT-PCR results from the tested clinical samples. Close results were reported by other investigators who developed similar RT-LAMP assays for the diagnosis of SARS-CoV-2 [2,4,6]. The assay failed to detect four samples that were positive according to the qRT-PCR results as they had a low viral load below the detection limits of the developed RT-LAMP assay. We also tried to simulate the direct use of the assay without nucleic acid extraction by spiking the SARS-CoV-2 RNA to saliva samples from a healthy individual and despite the successful RT-LAMP amplification, there was a decrease in detection limits of the assay to 6,250 genome copies. Simple RNA extraction methods that only require heat were described by Garcia-Venzor *et al.* [12] where samples were stored at 42 °C for 20 min, followed by 64 °C for 5 minutes, and used for LAMP assay. It is now well established that the SARS-CoV-2 can be inactivated by heating the virus at 65 °C for 15 minutes [13]. Thus, the use of RT-LAMP provides a direct detection method that is not only sensitive but also safe to be used in underequipped laboratories or clinics. The test results can be obtained within 20 minutes from the start of the reaction and its interpretation does not require any sophisticated equipment as it can be easily detected by a change in color from red to yellow.

To provide greater ease of implementation of our assay, we also tested the development of a lyophilized version of the test that could be stored at room temperature or refrigerator. Tubes that were stored at room temperature were usable for up to 2 weeks after which it was difficult to interpret the results based on the colour change. Much better results were obtained with the refrigerated lyophilized tubes as we could obtain clear results for up to 2 months. García-Bernalt *et al.* developed an RT-LAMP lyophilized assay that was valid for 2 months when stored at room temperature and although they used the same master mix that has been used in our assay, different primers and lyophilizing conditions were applied [14].

Based on our data, the colorimetric RT-LAMP would be more suitable as a screening test that can identify patients with a moderate to high viral load but does not detect patients with a low viral load. Moreover, the cost of the assay was calculated to be 0.39 Riyal Omani (RO) (excluding the nasal swab and the RNA extraction) when using the master mix at 10 µL compared to 25 RO (Ministry of Health, Oman). The test described here has many advantages over PCR testing as more people can be screened at a lower cost and the results can be obtained much more quickly, potentially enabling earlier identification of infected individuals to help prevent outbreaks of disease.

Conclusions

The developed colorimetric RT-LAMP proved to be highly specific and highly sensitive when validated with clinical samples and compared to qRT-PCR results. Despite the inability to amplify the RNA from clinical samples with a very low viral load at its current state, the test is still suitable to be used as a screening test that can identify the majority of patients with COVID-19. The test could be implemented at workplaces, mobile labs, small clinics, and airports since there is no need for sophisticated equipment and the results can be interpreted immediately with the eye without the need for further analysis. The test proved to be quick (30 minutes) and reproducible with robust results as a screening test with reduced cost.

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