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Original article

Reverse transcriptase loop-mediated isothermal amplification (RT-LAMP)-based diagnosis: A potential alternative to quantitative real-time PCR based detection of the novel SARS-COV-2 virus

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ABSTRACT

The sudden outbreak of the novel Coronavirus infectious disease (COVID-19) resulted in significant challenges to global health systems. One of the primary challenges is rapid, reliable, and accurate detection of the severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) virus among the suspected COVID-19-infected individuals. At present, quantitative real-time PCR (qRT-PCR) is a widely used diagnostic method. However, it requires expensive instruments and expertise in the interpretation of results. These constraints reflect the significant need for the development of alternative diagnostic options.

This study will validate the use and efficiency of the reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay as a potential alternative for the detection of COVID-19. For this purpose, a cohort of 297 suspected COVID-19 patients was tested using both the RT-LAMP assay and the conventional RT-PCR method. For the RT-LAMP assay, three genes (orf-1ab, N, and S) were identified as the target sites for the detection of COVID-19. Based on a comparative assessment, 117 out of 124 positive COVID-19 cases were observed using the RT-LAMP technique with an overall 91.45% sensitivity. Interestingly, where a consensus on 163 individuals free of SARS-Cov-2 was observed, RT-LAMP specificity was 90%. Based on these findings, the robustness of the technique, and the reduced dependency on expensive instrumentation, RT-LAMP-based COVID-19 detection is strongly recommended as a potential alternative assay.

1. Introduction

The coronavirus infectious disease (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) is an aggressive human malignancy affecting millions globally (Wu et al., 2020). The global mortality rate of the disease is high at approximately 4%. The World Health Organization (WHO) declared COVID-19 a pandemic because of its high-risk factors and the multiple challenges to global health care systems (Spinelli and Pellino, 2020). Because the spread of the virus is very rapid, it is causing havoc in countries worldwide, especially in

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developing countries (Li et al., 2020, Saqlain et al., 2020). Further, because of the sudden infective outbreak, efficient and timely diagnosis of the disease remains a major challenge (Satyanarayana, 2020). To address this challenge, extensive screening and effective testing are recommended (Wang et al., 2020). The quantitative real-time polymerase chain reaction PCR (qRT-PCR)-based diagnostic method is widely used for COVID-19 detection (Xiao et al., 2020). However, based on a massive influx of patients, studies have reported several cases in which false negatives are being detected using the qRT-PCR method (Guan et al., 2020).

Similarly, the qRT-PCR method requires trained staff and proper laboratory conditions to detect the RNA virus accurately (Tang et al., 2020). To overcome these issues, some studies have suggested using qRT-PCR in combination with Chest computerized tomography (CT) scans (Bernheim et al., 2020). Although the diagnostic performance of the chest CT is good in terms of sensitivity, its specificity is still very low (He et al., 2020). Similarly, the results obtained through the combination of both of the assays were also largely inconsistent in one-time testing (Xie et al., 2020). Hence, there is an urgent need for an alternative assay for early COVID-19 screening. Before the global impact of COVID-19 and its underlying challenges, several molecular assays were in the process of development and optimization. They included the loop-mediated isothermal amplification-based method (La Marca et al., 2020).

In this study, a user-friendly technique for reverse transcription-loop mediated isothermal amplification (RT-LAMP) was designed and validated on clinical specimens. To determine the RT-LAMP results, blind testing of the same specimens using commercially available qRT-PCR kits was conducted. The RT-LAMP-based assay was specifically designed and validated because of its advantages over the qRT-PCR assay. Those advantages include limited dependency on expensive instruments, fast processing time, and user-friendly interpretation of results (Huang et al., 2020).

2. Materials and methods

2.1. Study cohort

A total of 297 suspected COVID-19 individuals enrolled at National Institutes of Health (NIH). Pakistani patients were included in the study cohort. Ethical and biosafety approvals were obtained from the referred patients according to the international standards recommended for COVID-19 sampling.

2.2. Specimen collection and RNA isolation

Throat swabs of the suspected individuals were collected at a designated specimen collection facility. The swabs were immediately immersed in a virus transport medium and kept at 4 °C before further processing. The commercially available RNA isolation kit recommended for the (Taiwan Advanced Nanotech Inc., Taiwan) (TANBead) Nucleic Acid Extractor (model SLA-16/32) was used according to the manufacturer's instructions. The kit used silicon dioxide layer coated magnetic beads to isolate the nucleic acid in 40 min.

2.3. Primer design for the RT-Lamp assay

Primer sequences specifically targeting three genes—namely, the open reading frame (ORF-1ab), nucleocapsid (N), and Spike (S) genes were extracted from the literature (Park et al., 2020, Yan et al., 2020) are shown in Table 1. The primer sequences were then synthesized from Macrogen, Korea. A parent stock of 100 μM for each primer vial was prepared using water under the standard protocol. Three tubes containing 10X primer mix working stock were prepared for each gene from the parent stock vials of the respective genes.

2.4. Development and validation of the RT-LAMP assay

WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs) vials were used for the RT-LAMP assay. Each reaction tube used for the assay contained 2X master mix (12.5 μl), 10X primer mix (1X; 2.5 μl), RNA (2 μl), and water (8 μl). The tubes were incubated at 65 °C with variable times ranging from 45 to 75 min for optimization. The optimum duration set for the RT-LAMP assay using all three genes was 60 min at 65 °C.

2.5. Estimation of assay sensitivity and specificity

The RT-LAMP is a colorimetric assay in which the generation of concatemers reduces pH from a basic to acidic state, subsequently resulting in altered color patterns. In this study, color change in the

Table 1
RT-LAMP specific primers used in the study.

Genes	Primer sequence	Reference
orf1ab gene		Yan et al., 2020
orf1ab-4F3	GGTATGATTTTGTAGAAAACCCA	
orf1ab-4B3	CAACAGGAACTCCACTACC	
orf1ab-4FIP	GGCATCACAGAATTGTACTGTTTTTGCGTATACGCCAACTTAGG	
orf1ab-4BIP	AATGCTGGTATTGTTGGTGTACTGAGGTTTGTATGAAATCACCGAA	
orf1ab-4LF	AACAAAGCTTGGCGTACACGTTCA	
S gene		
S-123F3	TCTATTGCCATACCCACAA	
S-123B3	GGTGTTTTGTAAATTTGTTTGAC	
S-123FIP	CATTCAGTTGAATCACCACAAATGTGTGTTACCACAGAAATTCTACC	
S-123BIP	GTTGCAATATGGCAGTTTTTGTACATTGGGTGTTTTTGTCTTGTT	
S-123LF	ACTGATGTCTTGGTCATAGACACT	
S-123LB	TAAACCGTGCTTTAACTGGAATAGC	
N gene		Park et al., 2020
F3	GCCAAAAGGCTTCTACGCA	
B3	TTGCTCTCAAGCTGGTTCAA	
FIP	TCCCCTACTGCTGCCTGGAGGCAGTCAAGCCTCTTCTCG	
BIP	TCTCCTGCTAGAATGGCTGGCATCTGTCAAGCAGCAGCAAAG	
LF	TGTTGCGACTACGTGATGAGGA	
LB	ATGGCGGTGATGCTGCTCT	

F3 = outer forward primer; B3 = outer backward primer; FIP = forward inner primer; BIP = backward inner primer; LF = loop forward primer; LB = loop backward primer.

reaction mix from pink to yellow was used as a marker to detect the virus's presence. In addition, samples were validated using gel electrophoresis and further compared with the qPCR findings.

2.6. qRT-PCR-based detection of COVID-19 for respective specimens

All isolated RNA (297) were analyzed using an available COVID-19 diagnostic kit (BGI's Real-Time Fluorescent RT-PCR Kit). RNA samples of these specimens were processed for diagnosis using the RT-PCR kit according to the manufacturer's instructions. This was done to cross-validate the RT-LAMP assay's findings with the already utilized RT-PCR standard testing facility.

2.7. Calculations for sensitivity, specificity, and likelihood ratios

Sensitivity and specificity were calculated to assess the performance and diagnostic accuracy of the RT-LAMP assay. The RT-LAMP assay's diagnostic performance was calculated for comparison to the results of the reference standard (RT-PCR). Sensitivity was calculated to identify the proportion of individuals infected by COVID-19; the RT-LAMP assay correctly identified them as positive results.

$$\text{Sensitivity} = \text{truepositives}/(\text{truepositives} + \text{falsenegatives})$$

Similarly, specificity was calculated to estimate the proportion of individuals who were not affected by COVID-19 and remain negative in RT-LAMP assay.

$$\text{Specificity} = \text{truenegatives}/(\text{truenegatives} + \text{falsepositives})$$

To further validate the accuracy of the RT-LAMP-based test, likelihood ratios were computed. For this purpose, a positive likelihood ratio (LR+) was calculated to correctly establish how much more likely it was that a positive result would be obtained from RT-LAMP testing among COVID19-affected individuals than among individuals who are not affected by COVID-19.

$$\text{LR+} = \text{sensitivity}/(1 - \text{specificity})$$

A negative likelihood ratio (LR−) was also computed to estimate how much more likely it is that a negative result would be obtained from the RT-LAMP testing among truly COVID19-affected individuals than among the individuals who are not affected by COVID19 condition.

$$\text{LR−} = (1 - \text{sensitivity})/\text{specificity}$$

2.8. Statistical analysis

The R software version 3.6.1 was used for statistical analysis of the data. To assess the consistency between the qRT-PCR and RT-LAMP results, a Welch Two Sample *t*-test and McNemar's test were conducted. The probability value (p-value) of 0.05 was considered statistically significant. Furthermore, a Fagan Nomogram plot was produced to determine the post-test probability of COVID-19 based on the likelihood ratios of the RT-LAMP diagnostic method.

3. Results

3.1. Identification of COVID-19 in the cohort

Most of the individuals included in the study cohort were male expatriate (83%) Pakistanis returning home; the cohort's median age was 38 years (55%). According to the diagnostic results, 124 individuals were found to be positive for COVID-19, while 173 individuals tested negative.

3.2. Detection of the RT LAMP-based COVID-19 positive individuals

A total of 113 of the 124 infected individuals detected by the RT-PCR were also identified as positive by the RT-LAMP assay. Similarly, 163 of the 173 infected individuals were detected as negative by the RT-LAMP assay..

3.3. Comparative assessment of the RT-PCR and RT-LAMP-based COVID-19 assays

Based on the qRT-PCR-based testing, a comparison with the RT-LAMP assay was conducted in this study. The findings suggest that RT-LAMP-based testing had potential advantages over conventional qRT-PCR-based testing (Table 2). Amplification of COVID-19 positive samples showed a pronounced change in color from pink to yellow for Orf-1ab, N, and S genes (Fig. 1).

3.4. Sensitivity and specificity of the RT-LAMP assay

Sensitivity and specificity values for each gene-based screening for Orf-1ab, N, and S are shown in Table 3. The overall positive predictive value (PPV) calculated for the assay was 92% (Table 4). Significantly, 11 false-negative samples out of 123 positive cases showed late Ct values ≥ 31 in the qRT-PCR screening. Furthermore, McNemar's (p-value = 0.2482131) and *t*-test (p-value = 0.5594) results confirmed no significant difference between the results of standard qRT-PCR testing and the newly developed RT-LAMP assay (Fig. 2).

3.5. Diagnostic accuracy of the RT-Lamp test

Likelihood ratios were computed for the RT-Lamp diagnostic test results to assess their accuracy in relation to the gold standard qRT-PCR test. The positive likelihood ratio (LR+) of the qRT-LAMP assay was 9.6, indicating that the positive test was 9.6 times more likely among the individuals who tested positive for COVID-19 than those who tested negative. Similarly, the negative likelihood ratio (LR−) of 0.1 indicated that a negative test was 0.1 times more likely among individuals who are COVID-19 positive than among those who are COVID-19 negative. The likelihood ratios computed for the RT-LAMP assay in this study's cohort suggest that the RT-LAMP assay's diagnostic accuracy is high and can be the preferred alternative to the RT-PCR method. Furthermore, the Fagan nomogram depicted how the RT-LAMP diagnostic method changes the probability that an individual will have the disease (Fig. 3). The post-test probability was 90%, suggesting that if any individual tests positive for COVID-19 using the RT-LAMP diagnostic test,

Table 2
Comparison of RT-Lamp and qRT-PCR based testing.

Properties	RT-LAMP	qRT-PCR
Sensitivity	Highly sensitive	Low sensitivity
Amplification specificity	Very high	Lower than RT-LAMP
Testing duration	Rapid (requires 1 h at maximum)	Long duration (requires approximately 3 h)
Cost	Cost-effective	Expensive
Result interpretation	User-friendly result interpretation (does not require expertise)	Gel electrophoresis is required for result interpretation (Expertise in the field are required)
Temperature conditions	Constant temperature (60–65C)	Variable temperature for denaturation, annealing and polymerization
Detection limit	Very high detection power and limit	Very low detection limit

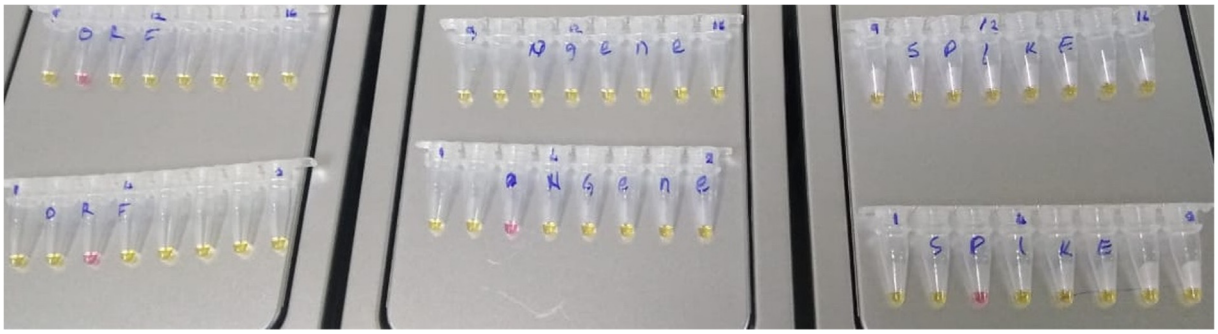

Fig. 1. Representative image of colorimetric RT-LAMP assay results of COVID-19 patients for open reading frame (ORF 1ab), nucleoprotein (N) and Spike (S) genes, respectively. Yellow is the indicator of presence of virus.

Table 3
Sensitivity and specificity of ORF-4ab, S and N gene.

Genes	True positive sensitivity	True negative specificity
Orf-4a	103 (83%)	170 (98%)
N gene	112 (88%)	169 (97.7%)
S- gene	109 (90%)	165 (95.3%)

Table 4
Calculation of sensitivity, specificity, predictive values, likelihood ratios, pre-test and post-test probabilities from the COVID19-detection results obtained from RT-PCR and RT-Lamp methods.

Result	Positive	Negative	Row total
Positive	107 (TP)	10 (FP)	117
Negative	17 (FN)	163 (TN)	180
Column total	124	173	297

Sensitivity = 107/107 + 10 = 0.91.
Specificity = 163/180 = 0.905.
LR+ = 0.91/1–0.905 = 9.6.
LR− = 1–0.91/0.905 = 0.1.
Pre-test probability = 117/297 = 0.4.
Pre-test odds = 0.4/1–0.4 = 0.7.
Post-test probability (positive test result) = (0.7 × 9.6)/(1 + (0.7 × 9.6)) = 0.9.
Post-test probability (negative test result) = (0.7 × 0.1)/(1 + (0.7 × 0.1)) = 0.1.

the post-test probability that COVID-19 infects the individual is 97%. In contrast, if an individual tests negative for COVID-19 using the RT-LAMP assay, the post-test probability that COVID-19 infects the individual is approximately 17%.

4. Discussion

A comparative assessment showed that the RT-LAMP assay demonstrated strong sensitivity and specificity and can be an alternative strategy for rapid COVID-19 testing. Earlier, the LAMP assay had been widely used to screen for COVID-19 (Yu et al., 2020), the avian influenza virus, and gastric cancer metastasis (Horibe et al., 2007). Significantly, several modifications leading to the earliest identification of concatemers improved and significantly reduced the assay's dependency on expensive instruments. LAMP products could also be detected using agarose gel electrophoreses after one hour of incubation at a high temperature (Notomi et al., 2000). Later, the formation of a white precipitate (magnesium pyrophosphate) in the reaction tube could be traced with the naked eye or spectrophotometry (Mori et al., 2001). The binding of pyrophosphate with a divalent metallic magnesium ion (Mg++) was the principle underlying the development of turbidity directly correlated with synthesized DNA. Alternatively, the introduction of calcein (fluorexon) as a fluorescent metal indicator enhanced the visual detection of the LAMP reaction.

At the beginning of the reaction, the binding affinity of the manganese ion (Mn2+) was responsible for the quenching of calcein signals. During the amplification of the targeted reaction, pyrophosphate was generated, forming a complex with Mn2+. This facilitated the release of calcein, resulting in fluorescence detected under UV (Tomita et al., 2008). Similarly, ethidium bromide and Malachite Green dyes have been used to detect the products of LAMP amplification (Pham et al., 2005). In the WarmStart mastermix, a

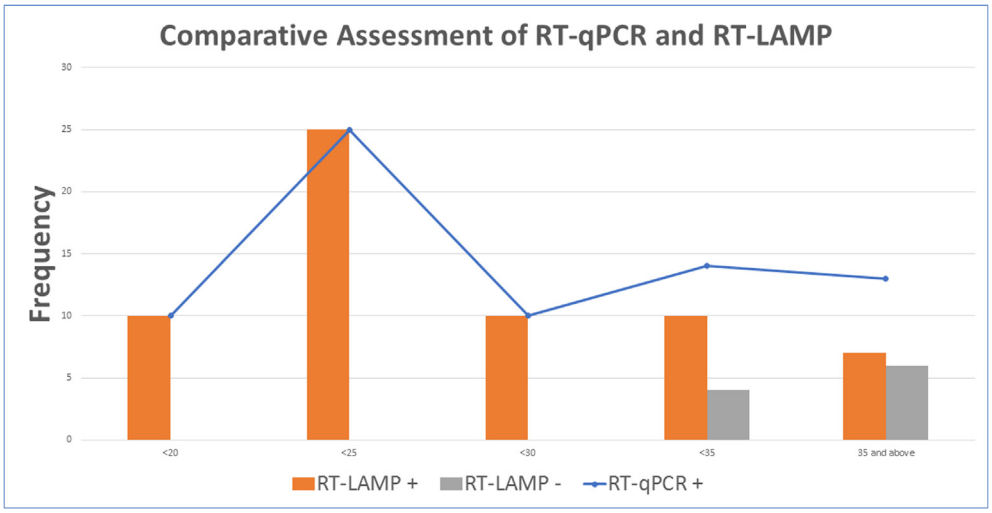

Fig. 2. Comparative assessment of qRT-PCR and RT-LAMP based COVID-19 detection.

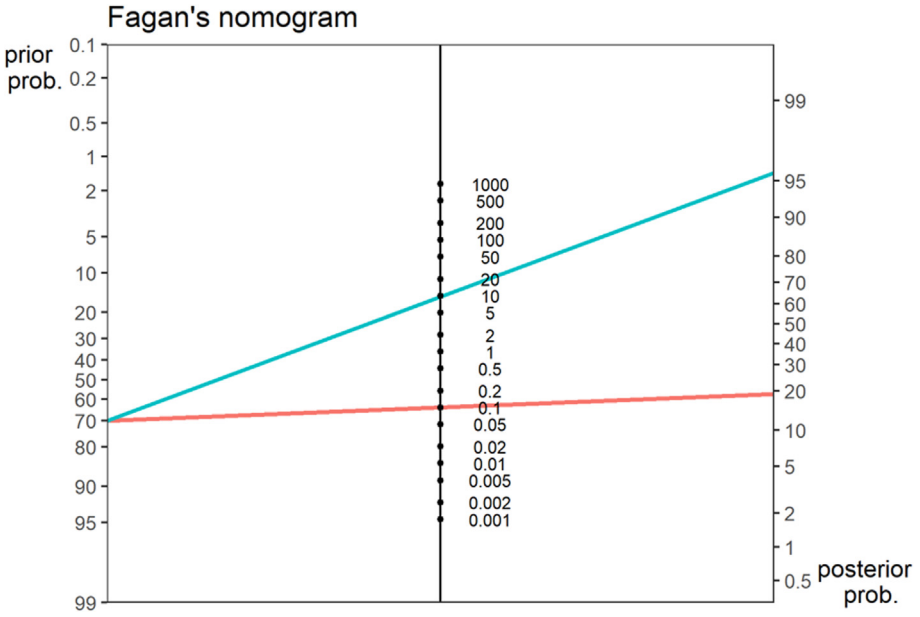

Fig. 3. Fagan's nomogram for calculation of post-test probabilities. Solid line in turquoise blue depicts the post-test probability of 97%, if the individual is truly COVID19-affected. On the other hand, the pinkish-red line indicates that if the individual in test negative, the post-test probability of individual being truly COVID19-infected would be around 17%.

pH-based approach was used in which the release of pyrophosphate resulted in lowering the pH from pink to yellow. This colorimetric assay is potentially the easiest method, reducing dependency on expensive instruments and highly trained technicians.

Currently, few LAMP-based kits like the ID NOW COVID-19 and iAMP COVID-19 have been approved by the FDA or are commercially available for SARS-Cov-2 detection. ID NOW COVID-19 (Abbott Diagnostics) targets isothermal amplification of a single gene (RdRP) with a fast processing time (~13 min). However, it can process only one sample per run with close dependency on reagents and consumables according to manufacturer instructions. iAMP COVID-19 (Atila BioSystems) targets isothermal amplification of the ORF1ab and N genes with a processing time of 1.5 h.

5. Conclusion

Based on the burden of global disease, the availability of these kits in developing countries like Pakistan would be extremely challenging, mostly when the export of certain kits is barred from their countries of manufacture (like the Eiken Loopamp 2019-nCOV Detection Kit exported from Japan). In this study, the detection of SARS-Cov-2 utilizing three genes (ORF1ab, N, and S) substantially improved the LAMP test detection threshold and the ability to rely on in-house resources. Based on the study findings, robustness, and less dependency on expensive instrumentation, the RT-LAMP based COVID-19 detection kit is highly recommended as a potential alternative to the conventional qRT-PCR-based assay. The kit has a fast processing time, a minimal risk of specimen transfer, and the ability to use available resources. The LAMP-based detection of COVID-19 is strongly recommended, especially for developing countries and regions where financial resources are scarce.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2020.10.064.

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