

Validation of an economical Reverse Transcription Loop mediated Isothermal Amplification based diagnostic testing for COVID19 related SARS-CoV2 surveillance in resource limited regions

*¹Dr. Mayur Tamhane, ^{2a}^{2b}Dr. Palak Agrawal, ^{3a}^{3b}Dr. Amol Ghule, ⁴Mr. Kalpesh Khutade,

^{5a}^{5b}Dr. Ramesh Chaturvedi

¹Scientific Officer, Head, Molecular Testing Laboratory, Department of Microbiology, ^{2a}Associate Professor, Department of Pharmacology, ^{2b}Chief Research Officer, ^{3a}Associate Professor, Department of Medicine, ^{3b}Assistant Medical Superintendent, ⁴Senior Laboratory Technician, Department of Microbiology, ^{5a}Professor, Department of Community Medicine, ^{5b}Dean, Vedantaa Institute of Medical Sciences, Vedantaa Hospital and Research Centre, Saswand, Dhundalwadi, Taluka Dahanu, District Palghar, Maharashtra-401606, India

Abstract: We have successfully validated the use of isothermal nucleic acid amplification testing method for rapid, economical surveillance of severe acute respiratory syndrome causing coronavirus type 2 strains (SARS-CoV2) and its potential spread of coronavirus related disease (COVID). A total of 102 patients were tested with colorimetric reverse transcription loop mediated isothermal amplification based nucleic acid amplification (RT-LAMP) testing and results validated with real time reverse transcription polymerase chain reaction (rRT-PCR) testing. The colorimetric RT-LAMP detection indicated a sensitivity of 98.87% (95% CI, 0.96 - 1.01), specificity of 100% and represents a viable alternative to cost-demanding thermal cycling technologies. The testing was performed in duration of 2 hours, within which RNA extraction was done in 90 minutes and SARS-CoV-2 detection was visually indicated in just 30 minutes by colour change of incorporated pH indicator. The sample cohort belonged to rural settings of various regions in Thane district of Maharashtra, India. Using the isothermal testing, we report the SARS-CoV2 detection in 32 of 42 female patients (76%) and in 48 of 60 male patients (80%). Through our results we propose employment of a rapid and economical Point of Care testing diagnostics based on an isothermal nucleic acid detection technology that does not require expensive thermal cycling equipment, and can be conducted and recorded economically with smartphone enabled color detection application, at the site of patient location. This would aid in limiting the spread of infectious SARS-CoV-2 strains, if present, in the symptomatic as well as asymptomatic human carriers or zoonotic reservoirs of severely pathogenic virus.

Index terms: SARS-CoV-2, COVID, Isothermal amplification, RT-LAMP, Molecular testing.

I. INTRODUCTION:

SARS-CoV-2 infection amongst human populations has become a severe obstacle in the economic progress of the global community (1–3). Besides significant knowledge and awareness about severe acute respiratory syndrome and its causative agents, identification of such reservoir harbouring this dreaded viral strain is necessary to adopt appropriate eradication methods (4,5). Numerous diagnostics platforms have erupted in the field of infectious disease detection, many of them based on molecular testing devices (6–12). Although immunological detection is commonly used in monitoring viral pathogenesis, the available antigen-antibody based diagnostics for SARS-CoV-2 cannot cover underlying infections that have a late onset of host immune response (13–17). Nucleic acid testing (NAT) and Nucleic acid amplification testing (NAAT) platforms are more sensitive in indicating presence of viral genomes in peripheral secretion by affected tissues (6,18–21). These tests are also useful in monitoring the progress or regress of eradication programs aimed at anti-coronavirus treatment. Given the mutating nature of RNA genome containing coronavirus family, it is essential to obtain the nucleotide sequences of NAAT amplified viral genetic material, using traditional as well as next generation sequencing platforms. The presence of unique and specific conserved regions of viral genomes, especially those that represent antigenic epitopes, can be achieved through NAAT combined with sequencing platforms (1,22–25).

Conventionally employed thermal cyclers play a central role in molecular diagnostics of infectious disease pathogens. Polymerase chain reaction and its modified assays have evolved the field of NAAT, through the use of Reverse Transcription Polymerase Chain Reaction (RT-PCR) (2,6,26,27). It has become the gold standard for diagnosis of SARS-CoV-2 and its related contiguous types. The cost of a single molecular test based on RT-PCR involves consideration of reagents and components used in obtaining the end point result. Typically the quantitative RT-PCR (QT-PCR) can measure the viral load in infected cells and tissue samples (28,29). Real time PCR technology allows detection of specific regions of the target viral genomes, through its

probe sequence hybridization principle. Both qualitative and quantitative detection can be carried out with Real Time PCR platforms for SARS-CoV-2 diagnosis (30,31).

However, to depict the spread of specific infectious SARS-CoV-2 strains, it is necessary to perform whole genome analysis of isolated SARS-CoV-2 strains from primary biopsies or cultured samples, along with sequence alignment with existing genome maps using bioinformatics platforms and algorithms (23,25,30). Considering the magnitude of declared SARS-CoV-2 pandemic, the consumables and equipment investment expense is often overlooked while calculating the test cost per sample. The economics of molecular detection of infectious disease pathogens, however, gets adversely affected through such negligence of cost-to-benefit analysis (32,33).

A range of isothermal platforms have evolved in the due course, in order to reduce nucleic acid amplification based detection costs and improve the benefits (10,12,18,19,34). Loop mediated isothermal amplification is amongst the highly preferred method proposed for amplification of nucleic acids without the requirement of thermal cycling (18,19,35–40). Nucleic acid based sequence amplification (NASBA), Rolling circle amplification (RCA), Helicase dependant amplification (HDA) are some of similar technologies that are developed for NAAT in absence of thermal cycling (41–43).

In order to assess the employability of an economical, rapid and reliable Point-of-Care (PoC) based surveillance of SARS-CoV-2 caused COVID status in regions with resource limitations, we have validated clinical application of a color producing RT-LAMP assay in comparison with conventional real time RT-PCR testing. The cohort was tested and characterized for different types of assay read-out and correlated for agreement in their outcomes (38,39,44,45).

II. RESEARCH METHODOLOGY:

2.1 Sample collection and handling

The reported study was conducted in order to establish a rapid and economical alternative for surveillance of ongoing SARS-CoV2 pandemic in the surrounding regions of Mumbai, Maharashtra, India. A total of 102 nasopharyngeal samples were collected from patients with sterile nylon swabs in sterile 3ml viral transport medium (GC Life Science, India) contained in 15 ml conical tubes. Collected VTM samples were stored at -75°C until viral RNA extraction. The study cohort was randomly selected and belonged to various localities of Thane and Palghar districts of Maharashtra state in India. Collected samples were labelled with sample ID generated by a double blind de-identification protocol. An informed consent was obtained from each of the study participant along with ethical approval from the Institutional Review Board.

2.2 Extraction of Viral RNA

Total RNA was extracted from a 140 μl of uniformly suspended patient sample using TruPCR viral RNA extraction kit (Cat No.3B213V 3B Blackbio Biotech India Ltd.) as instructed by the manufacturer. Briefly, 140 μl of patient sample was mixed with 560 μl of lysis buffer and 5.6 μl of carrier RNA. After incubation at 25°C for 10 minutes, 560 μl of absolute ethanol was added and invert mixed. The suspension was passed through the kit provided viral RNA binding cationic exchange column and then washed twice with 500 μl wash buffer. After a dry spin of the column at 12000 RPM, the viral RNA was eluted in 50 μl of Elution buffer in a new microfuge tube. All individual patient RNA samples were stored at -75°C until further use.

2.3 SARS-CoV-2 detection by real time RT-PCR

SARS-CoV-2 RNA was detected using TRUPCR SARS CoV-2 PCR kit (Cat No.3B304 3B Blackbio Biotech India Ltd) as per the manufacturer's instructions. Briefly, 5 μl patient RNA sample was combined with RT-PCR master mix containing 1x reaction buffer, dNTPs, MgCl_2 , SARS-CoV-2 specific Primer Probe mix, Reverse Transcriptase, Taq DNA polymerase in a total reaction volume of 25 μl in 96 well format PCR plate (Cat No.PR2, HiMedia Laboratories, India) sealed with optical film (Cat. No.PR18, HiMedia Laboratories, India). For every real time RT-PCR run, a kit provided negative and positive control was used. A real time reverse transcription based polymerase chain reaction (real time RT-PCR) test specific for SARS-CoV-2 viral genes (Early region, E gene, RNA dependant RNA polymerase, RdRp and Nucleoprotein, N gene) and internal control of human RNaseP gene (Ribonuclease P) was performed using a thermal cycler (Insta Q96 Real time PCR detection system, LA1012, HiMedia Laboratories, India) with heated lid. Analysis of amplified viral genes was done by detection of specific probe hybridization confirmed by fluorescence output. The E gene represented the 5' region, while the RdRp and N gene represented the 3' regions of the SARS-CoV2 viral RNA genome. The threshold cut-off Ct values generated by the software (Insta Q96 Real time PCR detection system software, LA1012, HiMedia Laboratories, India) for negative control was used to identify negative and positive samples for SARS-CoV-2. A Ct value equal to or below that of the negative control was regarded as SARS-CoV-2 negative sample provided the human RNaseP gene was showed a non-zero Ct value.

2.4 SARS-CoV-2 detection by Colour indicating RT-LAMP

For each patient sample, 5 μl of extracted viral RNA was combined with kit provided WarmStart Colorimetric LAMP 2X master mix (Cat. No. M1800S, New England Biolabs, USA) containing 1x reaction buffer, dNTPs, MgSO_4 , Reverse Transcriptase, Bst DNA polymerase and Cresol red pH indicator. Six SARS-CoV-2 N gene (Nucleoprotein) specific loop mediated isothermal amplification primers were employed from previously published data (39) and purchased from IDT DNA (Integrated DNA Technologies, USA). These primers targeted the N gene of SARS-CoV2 present at the 3' end of the viral RNA genome. These oligonucleotides were also added to this reaction at a final concentration of 2 μM F3, 2 μM B3, 16 μM MFIP, 16 μM BIP, 4 μM LoopF and 4 μM LoopB with total volume of 25 μl . The RT-LAMP amplification was performed in 1.5ml microfuge tubes using a heated block (myBlock Mini Dry Bath, Benchmark, USA) maintained at a single temperature of 65°C . Patient samples were analysed along with similar negative and positive controls as used for rRT-PCR reactions.

2.5 Agarose Gel Electrophoresis

A volume of 5µl amplified nucleic acid products from RT-LAMP or RT-PCR reactions were resolved by 1.0% agarose gel electrophoresis containing ethidium bromide in 1x Tris Borate EDTA buffer and visualized on UV transilluminator (EPS Biosolutions, India) for presence or absence of amplified nucleic acid product along with a reference 1 Kb DNA marker (MBT051, Himedia Laboratories, India).

2.6 Result interpretation for RT-PCR and RT-LAMP testing

Result interpretation for RT-PCR testing was done by differentiating positive and negative samples based on a threshold Ct value obtained in comparison with control reactions for the confirmatory N gene. Result interpretation for RT-LAMP testing was done visually with naked eye to determine color change. Detection of positive viral gene amplification resulted in a pH drop that was indicated by colour change of incorporated Cresol red dye from pink to yellow. Negative samples were indicated by absence of change in the pink colour due to lack of viral gene amplification. Color change was also recorded by two methods, a) CYMK color analysis using smart phone camera (Samsung Model A20S) along with RGB color analysis android application (RGB Color Detector, Version 1.2.8, The Programmer) and b) Colorimetric plate reader (SK201-078, ELISA Reader, Sinthinker, China). Each of the 25µl RT-LAMP reaction volume was tested firstly by image acquisition with RGB Color Detector application using the smart phone android camera. Next, 5µl of the amplified product was tested with 1.0% agarose gel electrophoresis analysis. The volume of remaining 20µl reaction was increased to 100µl by adding deionized water and tested by colorimetric analysis in a 96 well microtiter plate (EP-7, Himedia Laboratories, India) using ELISA plate reader and recording absorbance at 405nm. Each patient sample was analysed similarly along with negative and positive controls.

2.7 Calculation of Sensitivity and Specificity

RT-PCR results were considered as reference gold standard to identify true and false negative and positive samples respectively. Assay sensitivity and specificity was calculated by using an online tool (Diagnostic Method, <https://www2.ccrb.cuhk.edu.hk/stat/ConfidenceInterval.htm>)

2.8 Ethical Statement

This study was conducted after obtaining ethical approval from the institutional review board of Vedantaa Institute of Medical Sciences, Dahanu, Maharashtra, India. Informed consent in written format were obtained from all study participants.

III. RESULTS

3.1 Sample cohort statistics

The cohort of patients that was tested for SARS-CoV-2 by real time RT-PCR and colorimetric RT-LAMP comprised of randomly selected patients. The cohort of 102 patients comprised of 42 females (41.17%, 15 years to 78 years) and 60 males (58.82%, 18 to 77 years). The clinical characteristics of patient are enlisted in Table 2 and represented in Figure 1.

3.2 Detection of SARS-CoV-2 by RT-PCR

From the total 102 RNA samples, 81 positive (79.41%) and 21 negative (20.58%) patients were determined on the basis of real time RT-PCR outcome. An internal control of human RNase P transcript was positively detected in all samples tested, thereby confirming reliability of sample collection and consistency of the tested genetic material. This was based on the fluorescence capture read-out of the SARS-CoV-2 specific DNA probe hybridizing with the RT-PCR amplified DNA. The distribution of positive RT-PCR results was further categorized based on cohort characteristics in comparison with colorimetric RT-LAMP read-out (Table 2).

3.3 Detection of SARS-CoV-2 across cohort by age and gender

The 81 positive samples belonged to 33 female (40.74%) and 48 male (59.25%) patients (Table 2). Similarly out of 21 negative samples, 9 (42.85%) belonged to females and 12 (57.14%) were from males, respectively. The distribution of SARS-CoV-2 status amongst the patient cohort is shown in table 2. In the female patients, maximum number of positive cases was observed in 51-60 years group, followed by groups of 61-70 and 21-30 years. In the male patients, the age group of 41-50 years showed high count of SARS-CoV-2 detection, followed by the group of 31-40 years. The age groups of 51-60 years in both female and male patients showed low frequency of SARS-CoV-2 positive status, although the sample size within these groups was very low. In female subjects of age groups 31-40 years and 61-70 years, all patients were found to be positive for SARS-CoV2. While in male subjects, age groups of 11-20 years, 21-30 years and 71-80 years showed all patients positive for SARS-CoV2.

3.4 Validation of RT-LAMP for detection of SARS-CoV-2

Colorimetric RT-LAMP test employing visual color change observation showed 80 positive (78.43%) and 22 negative (21.56%) out of the 102 total samples. Agarose gel electrophoresis of the amplified RT-LAMP nucleic acid products showed typical DNA pattern of LAMP amplification for all positive samples identified by color change visualization (Figure 2). This pattern of DNA amplification was not seen for negative samples. The colorimetric analysis done to quantify the color change showed 80 number of positive and 22 number of negative samples based on cut-off threshold OD comparison with that of the negative control. Out of the 81 true positive cases, colorimetric RT-LAMP showed positive result for 80 cases with 1 negative result. All 21 true negative samples as tested previously by real time RT-PCR, were determined as negative by colorimetric RT-LAMP (Table 2, Figure 3). The correlation of colorimetric RT-LAMP OD out-put with RT-PCR N gene Ct values is shown in

Figure 3. The samples showing high OD values had low corresponding Ct count. The color read-out as captured by the android phone camera and analysed by color detector software showed a distribution of 80 positive and 22 negative samples out of the 102 RT-LAMP reactions (Figure 4). This was based on the values generated by the CYMK analysis of the color recordings. Assay performance of colorimetric RT-LAMP CYMK read out is compared with either N or E gene RT-PCR test output.

3.5 Sensitivity and Specificity of SARS-CoV-2 detection by colorimetric RT-LAMP

Considering the rRT-PCR data as reference, the number of true positive, true negative, false positive and false negative samples was enumerated as shown in table. Using an online statistics analysing software, the assay sensitivity for colorimetric RT-LAMP test was calculated to be 98.87% (95% CI, 0.96 - 1.01) with specificity value of 100%.

IV. DISCUSSION

We have performed a qualitative assessment of SARS-CoV-2 detecting reverse transcription loop mediated isothermal amplification assay on randomly collected 102 patient samples. The RT-LAMP assay performance was validated using the conventionally used reference RT-PCR testing. The validation was successful in diagnosing negative patients reliably as indicated by the specificity of 100%. Of the 82 positive patients, RT-LAMP could detect 80 patients as seen by the sensitivity of 98.87% (95% CI, 0.96 - 1.01). RT-LAMP assay performance characteristics reported for detection of SARS-CoV-2 in other studies have shown sensitivity and specificity range of 82% -100% and 85% - 100%, respectively (19,27,46-49).

The cohort distribution analysis of SARS-CoV-2 status in this study indicated age group of 51-60 years to be more susceptible in contracting the infection, in both female and male counterparts. Gender based comparison for the presence of SARS-CoV-2 indicated no significant difference in susceptibility to infection, as seen by SARS-CoV2 detection in 32 of 42 female patients (76%) and in 48 of 60 male patients (80%). Inter gender analysis of SARS-CoV-2 infection status showed high risk in the age groups 51-60 years and 41-60 years for females and males, respectively.

The read-out method used to measure RT-LAMP output correlated in direct proportion with that of rRT-PCR data as indicated by our data (Figure 3 and 4). Although the naked eye visualization is a rapid way to identify positive tests, measurement by color detection provides quantifiable assay output. Colorimetric analysis of RT-LAMP has been shown in many studies using laboratory stationed microtiter plate reader (12,24,37,39,40,46,50). However, from the viewpoint of developing RT-LAMP based point of care (PoC) testing platforms, heavy laboratory equipments pose difficulty in transportation to the site of field testing.

Through this communication, we also report the application of smart phone and color detector software to characterize the assay output of SARS-CoV-2 diagnostic colorimetric RT-LAMP, for the first time. The use of such economical, rapid and reliable isothermal testing of SARS-CoV-2 disposing the requirement for expensive thermal cycling technology approves our approach of Point of Care (PoC) SARS-CoV-2 testing. We aim to employ PoC testing for rapid surveillance of COVID19 (Coronavirus caused Disease of 2019) and also monitoring of candidate vaccines and associated genetic therapies attempting eradication of COVID19 pandemic.

V. CONCLUSION

The current study has successfully evaluated clinical application of an isothermal NAAT assay available as colorimetric RT-LAMP test. Based on this study, we intend to develop an economical and reliable molecular testing protocol for rapid screening mass populations exposed to infectious SARS-CoV-2 strains in resource limited global locations. Our results indicate the colorimetric RT-LAMP test to be specific and sensitive for clinical application focussed on monitoring global COVID status, especially in resource restrained regions. The isothermal RT-LAMP testing has exhibited acceptable assay performance characteristics in comparison to real time RT-PCR, especially, in terms of sensitivity, specificity and faster read-out duration with simple equipment requirements. Using such approach for Point-of-Care (PoC) on-site testing, our proposed protocol could potentially be employed to monitor existing viral reservoir and the progress of vaccination programs targeted at eradication of this contiguous viral agent.

VI. DECLARATION OF COMPETING INTERESTS

There is no conflict of interest to be declared by the authors

FIGURES AND TABLES:

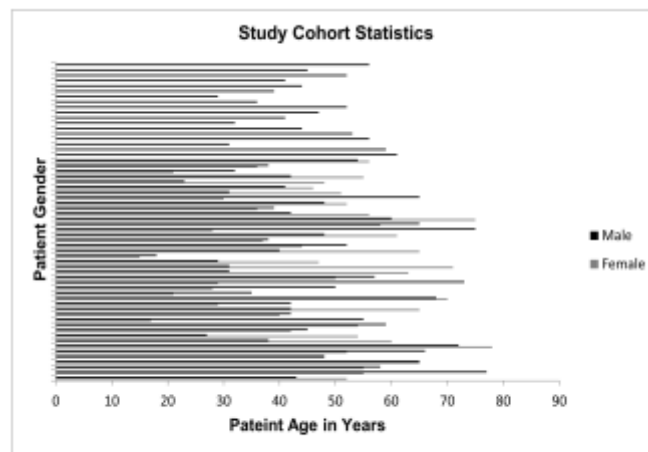


Figure 1. Age and Gender characteristics of the Patient Cohort

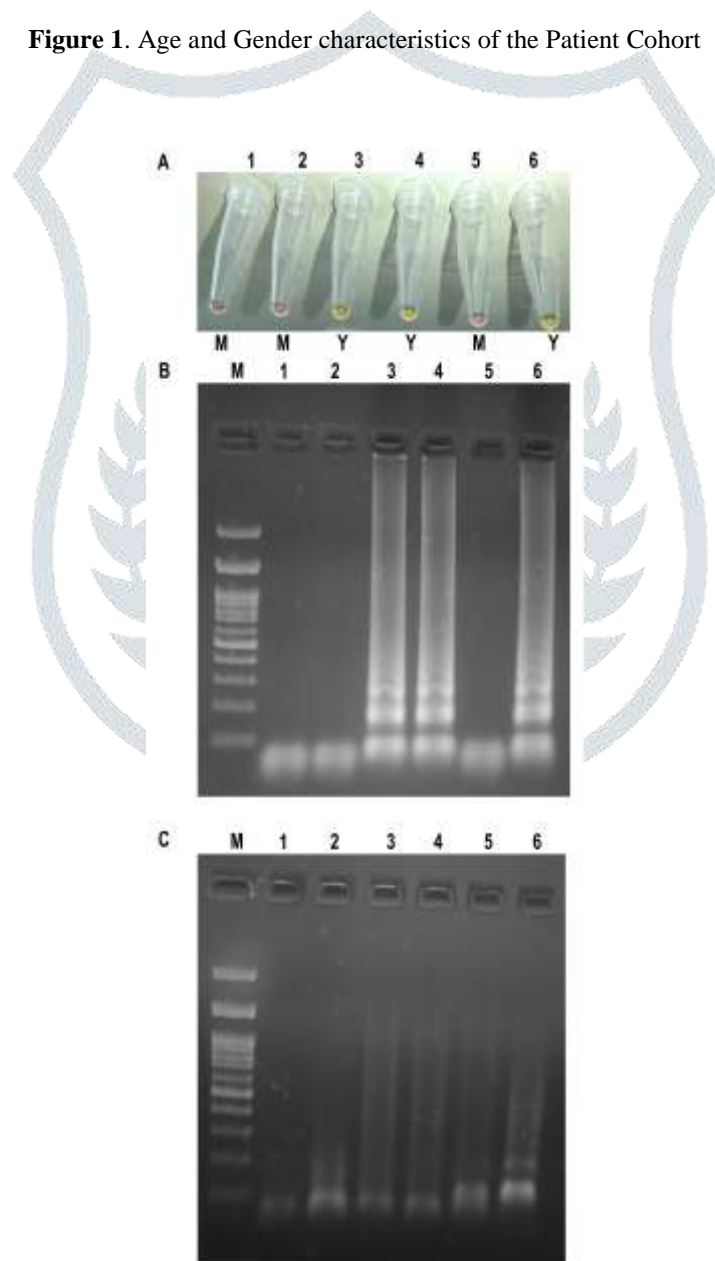


Figure 2. (A) Visual color detection of RT-LAMP amplified patient viral RNA samples for SARS-CoV2. Lane 1 – Negative Control, Lane 2 to 5 – Patient Viral RNA, Lane 6 - Positive Control, M – Magenta, Y - Yellow. Gel electrophoresis results for colorimetric RT-LAMP (B) or real time RT-PCR (C) amplified patient viral RNA samples for SARS-CoV2. Lane M – 1Kb DNA ladder, Lane 1 – Negative Control, Lane 2 to 5 – Patient Viral RNA, Lane 6 - Positive Control

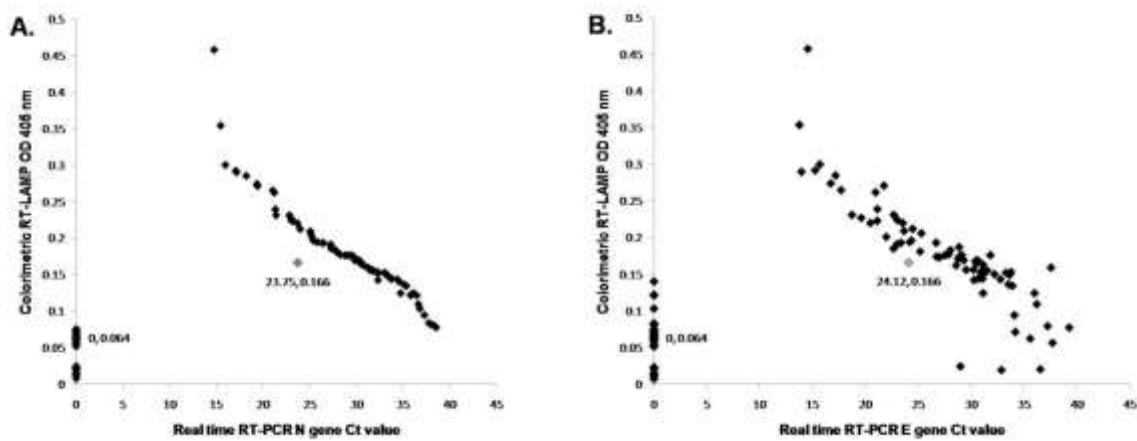


Figure 3. Comparison of SARS-CoV2 detection with real time RT-PCR and colorimetric RT-LAMP. Correlation of Real time RT-PCR generated N gene (A) or E gene (B) Ct values with Absorbance output (OD at 405nm)of colorimetric RT-LAMP color analysis. Negative and Positive Controls are indicated with co-ordinate values.

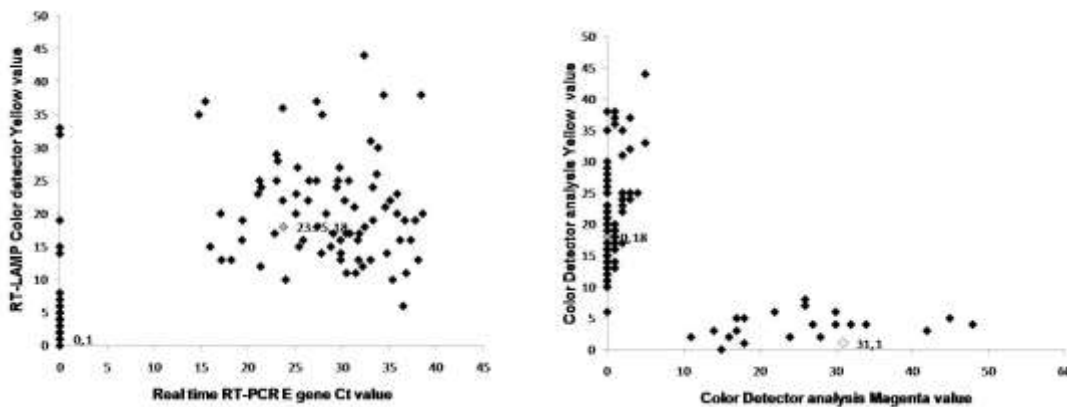


Figure 4. Color detector application performance for detection of SARS-CoV2 with colorimetric RT-LAMP. A. Correlation of Real time RT-PCR generated N gene Ct values with the Yellow value read-out recorded by CYMK color detector application for patient RNA colorimetric RT-LAMP testing. B. Inverse proportion of corresponding Magenta and Yellow values recorded for colorimetric RT-LAMP amplified patient samples. Negative and Positive Controls are indicated with co-ordinate values.

Table 1. Sensitivity and Specificity of Colorimetric RT-LAMP SARS-CoV2 diagnosis of 102 patient samples

| NAAT | True Positive | False Negative | True Negative | False Positive | Clinical Sensitivity | Clinical Specificity |
|----------------------|---------------|----------------|---------------|----------------|------------------------------|----------------------|
| Colorimetric RT-LAMP | 80 | 1 | 21 | 0 | 98.87% (95% CI, 0.96 - 1.01) | 100% |
| Real time RT-PCR | 81 | 0 | 21 | 0 | 100% | 100% |

Table 2. Age and Gender based distribution of SARS-CoV2 positive patients

| Female Age Group (Years) | Age Frequency (Number of Patients per Group) | | Real time RT-PCR Positive | | Colorimetric RT-LAMP Positive | |
|--------------------------|---|------|---------------------------|------|-------------------------------|------|
| | Female | Male | Female | Male | Female | Male |
| 11 to 20 | 2 | 1 | 1 | 1 | 1 | 1 |
| 21 to 30 | 7 | 4 | 6 | 4 | 6 | 4 |
| 31 to 40 | 4 | 14 | 4 | 13 | 4 | 13 |
| 41 to 50 | 7 | 18 | 5 | 14 | 5 | 14 |
| 51 to 60 | 13 | 13 | 9 | 8 | 8 | 8 |
| 61 to 70 | 6 | 6 | 6 | 4 | 6 | 4 |
| 71 to 80 | 3 | 4 | 2 | 4 | 2 | 4 |

VII. ACKNOWLEDGEMENT

The research work was supported by funds and facilities provided by Vedantaa Institute of Medical Sciences, Dahanu, India.

VIII. REFERENCES

1. Wang H, Li X, Li T, Zhang S, Wang L, Wu X, et al. The genetic sequence, origin, and diagnosis of SARS-CoV-2. *Eur J Clin Microbiol Infect Dis*. 2020 Sep;39(9):1629–35.
2. Li C, Ren L. Recent progress on the diagnosis of 2019 Novel Coronavirus. *Transbound Emerg Dis*. 2020 Jul;67(4):1485–91.
3. Venter M, Richter K. Towards effective diagnostic assays for COVID-19: a review. *J Clin Pathol*. 2020 Jul;73(7):370–7.
4. Siam MHB, Nishat NH, Ahmed A, Hossain MS. Stopping the COVID-19 Pandemic: A Review on the Advances of Diagnosis, Treatment, and Control Measures. *J Pathog*. 2020;2020:9121429.
5. Shih H-I, Wu C-J, Tu Y-F, Chi C-Y. Fighting COVID-19: A quick review of diagnoses, therapies, and vaccines. *Biomed J*. 2020 Aug;43(4):341–54.
6. Arena F, Pollini S, Rossolini GM, Margaglione M. Summary of the Available Molecular Methods for Detection of SARS-CoV-2 during the Ongoing Pandemic. *Int J Mol Sci*. 2021 Jan;22(3).
7. Machado BAS, Hodel KVS, Barbosa-Júnior VG, Soares MBP, Badaró R. The Main Molecular and Serological Methods for Diagnosing COVID-19: An Overview Based on the Literature. *Viruses*. 2020 Dec;13(1).
8. Andryukov BG, Besednova NN, Kuznetsova TA, Fedyanina LN. Laboratory-Based Resources for COVID-19 Diagnostics: Traditional Tools and Novel Technologies. A Perspective of Personalized Medicine. *J Pers Med*. 2021 Jan;11(1).

9. D'Cruz RJ, Currier AW, Sampson VB. Laboratory Testing Methods for Novel Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2). *Front cell Dev Biol.* 2020;8:468.
10. Javalkote VS, Kancharla N, Bhadra B, Shukla M, Soni B, Goodin M, et al. CRISPR-based assays for rapid detection of SARS-CoV-2. *Methods.* 2020 Oct;
11. Jamshaid H, Zahid F, Din IU, Zeb A, Choi HG, Khan GM, et al. Diagnostic and Treatment Strategies for COVID-19. *AAPS PharmSciTech.* 2020 Aug;21(6):222.
12. Ali Z, Aman R, Mahas A, Rao GS, Tehseen M, Marsic T, et al. iSCAN: An RT-LAMP-coupled CRISPR-Cas12 module for rapid, sensitive detection of SARS-CoV-2. *Virus Res.* 2020 Oct;288:198129.
13. Dinnes J, Deeks JJ, Berhane S, Taylor M, Adriano A, Davenport C, et al. Rapid, point-of-care antigen and molecular-based tests for diagnosis of SARS-CoV-2 infection. *Cochrane database Syst Rev.* 2021 Mar;3:CD013705.
14. Deeks JJ, Dinnes J, Takwoingi Y, Davenport C, Spijker R, Taylor-Phillips S, et al. Antibody tests for identification of current and past infection with SARS-CoV-2. *Cochrane database Syst Rev.* 2020 Jun;6(6):CD013652.
15. Porte L, Legarraga P, Vollrath V, Aguilera X, Munita JM, Araos R, et al. Evaluation of a novel antigen-based rapid detection test for the diagnosis of SARS-CoV-2 in respiratory samples. *Int J Infect Dis IJID Off Publ Int Soc Infect Dis.* 2020 Oct;99:328–33.
16. Chia WN, Tan CW, Foo R, Kang AEZ, Peng Y, Sivalingam V, et al. Serological differentiation between COVID-19 and SARS infections. *Emerg Microbes Infect.* 2020 Dec;9(1):1497–505.
17. Mak GC, Cheng PK, Lau SS, Wong KK, Lau CS, Lam ET, et al. Evaluation of rapid antigen test for detection of SARS-CoV-2 virus. *J Clin Virol Off Publ Pan Am Soc Clin Virol.* 2020 Aug;129:104500.
18. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 2000 Jun;28(12):E63.
19. Subsoontorn P, Lohitnavy M, Kongkaew C. The diagnostic accuracy of isothermal nucleic acid point-of-care tests for human coronaviruses: A systematic review and meta-analysis. *Sci Rep.* 2020 Dec;10(1):22349.
20. Khan MI, Khan ZA, Baig MH, Ahmad I, Farouk A-E, Song YG, et al. Comparative genome analysis of novel coronavirus (SARS-CoV-2) from different geographical locations and the effect of mutations on major target proteins: An in silico insight. *PLoS One.* 2020;15(9):e0238344.
21. Mehrotra D V, Janes HE, Fleming TR, Annunziato PW, Neuzil KM, Carpp LN, et al. Clinical Endpoints for Evaluating Efficacy in COVID-19 Vaccine Trials. *Ann Intern Med.* 2021 Feb;174(2):221–8.
22. Omotoso OE, Babalola AD, Matareek A. Mutational hotspots and conserved domains of SARS-CoV-2 genome in African population. *Beni-Suef Univ J basic Appl Sci.* 2021;10(1):11.
23. Kandeel M, Ibrahim A, Fayez M, Al-Nazawi M. From SARS and MERS CoVs to SARS-CoV-2: Moving toward more biased codon usage in viral structural and nonstructural genes. *J Med Virol.* 2020 Jun;92(6):660–6.
24. Zhou Y, Wan Z, Yang S, Li Y, Li M, Wang B, et al. A Mismatch-Tolerant Reverse Transcription Loop-Mediated Isothermal Amplification Method and Its Application on Simultaneous Detection of All Four Serotype of Dengue Viruses. *Front Microbiol.* 2019;10:1056.
25. Kaushal N, Gupta Y, Goyal M, Khaiboullina SF, Baranwal M, Verma SC. Mutational Frequencies of SARS-CoV-2 Genome during the Beginning Months of the Outbreak in USA. *Pathog (Basel, Switzerland).* 2020 Jul;9(7).
26. Liu X, Liu C, Liu G, Luo W, Xia N. COVID-19: Progress in diagnostics, therapy and vaccination. *Theranostics.* 2020;10(17):7821–35.
27. Lamb LE, Bartolone SN, Ward E, Chancellor MB. Rapid detection of novel coronavirus/Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) by reverse transcription-loop-mediated isothermal amplification. *PLoS One.* 2020;15(6):e0234682.
28. Drosten C, Günther S, Preiser W, van der Werf S, Brodt H-R, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med.* 2003 May;348(20):1967–76.
29. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, et al. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. *Euro Surveill Bull Eur sur les Mal Transm = Eur Commun Dis Bull.* 2012 Sep;17(39).
30. Drexler JF, Gloza-Rausch F, Glende J, Corman VM, Muth D, Goettsche M, et al. Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. *J Virol.* 2010 Nov;84(21):11336–49.
31. Muth D, Corman VM, Roth H, Binger T, Dijkman R, Gottula LT, et al. Attenuation of replication by a 29 nucleotide deletion in SARS-coronavirus acquired during the early stages of human-to-human transmission. *Sci Rep.* 2018 Oct;8(1):15177.

32. Du Z, Pandey A, Bai Y, Fitzpatrick MC, Chinazzi M, Pastore Y Piontti A, et al. Comparative cost-effectiveness of SARS-CoV-2 testing strategies in the USA: a modelling study. *Lancet Public Heal*. 2021 Mar;6(3):e184–91.
33. Neilan AM, Losina E, Bangs AC, Flanagan C, Panella C, Eskibozkurt GE, et al. Clinical Impact, Costs, and Cost-Effectiveness of Expanded SARS-CoV-2 Testing in Massachusetts. *Clin Infect Dis an Off Publ Infect Dis Soc Am*. 2020 Sep;
34. Wu T, Ge Y, Zhao K, Zhu X, Chen Y, Wu B, et al. A reverse-transcription recombinase-aided amplification assay for the rapid detection of N gene of severe acute respiratory syndrome coronavirus 2(SARS-CoV-2). *Virology*. 2020 Oct;549:1–4.
35. Zhang Y, Tanner NA. Development of multiplexed reverse-transcription loop-mediated isothermal amplification for detection of SARS-CoV-2 and influenza viral RNA. *Biotechniques*. 2021 Mar;70(3):167–74.
36. Chaouch M. Loop-mediated isothermal amplification (LAMP): An effective molecular point-of-care technique for the rapid diagnosis of coronavirus SARS-CoV-2. *Rev Med Virol*. 2021 Jan;e2215.
37. Ahn SJ, Baek YH, Lloren KKS, Choi W-S, Jeong JH, Antigua KJC, et al. Rapid and simple colorimetric detection of multiple influenza viruses infecting humans using a reverse transcriptional loop-mediated isothermal amplification (RT-LAMP) diagnostic platform. *BMC Infect Dis*. 2019 Aug;19(1):676.
38. Park G-S, Ku K, Baek S-H, Kim S-J, Kim S Il, Kim B-T, et al. Development of Reverse Transcription Loop-Mediated Isothermal Amplification Assays Targeting Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). *J Mol Diagn*. 2020 Jun;22(6):729–35.
39. Huang WE, Lim B, Hsu C-C, Xiong D, Wu W, Yu Y, et al. RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. *Microb Biotechnol*. 2020 Jul;13(4):950–61.
40. Kim JH, Kang M, Park E, Chung DR, Kim J, Hwang ES. A Simple and Multiplex Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid Detection of SARS-CoV. *Biochip J*. 2019;13(4):341–51.
41. James AS, Alawneh JI. COVID-19 Infection Diagnosis: Potential Impact of Isothermal Amplification Technology to Reduce Community Transmission of SARS-CoV-2. *Diagnostics (Basel, Switzerland)*. 2020 Jun;10(6).
42. Zhao Y, Chen F, Li Q, Wang L, Fan C. Isothermal Amplification of Nucleic Acids. *Chem Rev*. 2015 Nov;115(22):12491–545.
43. Reid MS, Le XC, Zhang H. Exponential Isothermal Amplification of Nucleic Acids and Assays for Proteins, Cells, Small Molecules, and Enzyme Activities: An EXPAR Example. *Angew Chem Int Ed Engl*. 2018 Sep;57(37):11856–66.
44. Rabe BA, Cepko C. SARS-CoV-2 detection using isothermal amplification and a rapid, inexpensive protocol for sample inactivation and purification. *Proc Natl Acad Sci U S A*. 2020 Sep;117(39):24450–8.
45. Baek YH, Um J, Antigua KJC, Park J-H, Kim Y, Oh S, et al. Development of a reverse transcription-loop-mediated isothermal amplification as a rapid early-detection method for novel SARS-CoV-2. *Emerg Microbes Infect*. 2020 Dec;9(1):998–1007.
46. Lu R, Wu X, Wan Z, Li Y, Jin X, Zhang C. A Novel Reverse Transcription Loop-Mediated Isothermal Amplification Method for Rapid Detection of SARS-CoV-2. *Int J Mol Sci*. 2020 Apr;21(8).
47. Lalli MA, Langmade JS, Chen X, Fronick CC, Sawyer CS, Burcea LC, et al. Rapid and Extraction-Free Detection of SARS-CoV-2 from Saliva by Colorimetric Reverse-Transcription Loop-Mediated Isothermal Amplification. *Clin Chem*. 2021 Jan;67(2):415–24.
48. Hu X, Deng Q, Li J, Chen J, Wang Z, Zhang X, et al. Development and Clinical Application of a Rapid and Sensitive Loop-Mediated Isothermal Amplification Test for SARS-CoV-2 Infection. *mSphere*. 2020 Aug;5(4).
49. Lee JYH, Best N, McAuley J, Porter JL, Seemann T, Schultz MB, et al. Validation of a single-step, single-tube reverse transcription loop-mediated isothermal amplification assay for rapid detection of SARS-CoV-2 RNA. *J Med Microbiol*. 2020 Sep;69(9):1169–78.
50. Dao Thi VL, Herbst K, Boerner K, Meurer M, Kremer LP, Kirmaier D, et al. A colorimetric RT-LAMP assay and LAMP-sequencing for detecting SARS-CoV-2 RNA in clinical samples. *Sci Transl Med*. 2020 Aug;12(556).