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A novel RdRp-based colorimetric RT-LAMP assay for rapid and sensitive detection of SARS-CoV-2 in clinical and sewage samples from Pakistan

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ARTICLE INFO

ABSTRACT

Novel corona virus SARS-CoV-2, causing coronavirus disease 2019 (COVID-19), has become a global health challenge particularly for developing countries like Pakistan where overcrowded cities, inadequate sanitation, little health awareness and poor socioeconomic conditions exist. The SARS-CoV-2 has been known to spread primarily through direct contact and respiratory droplets. However, detection of SARS-CoV-2 in stool and sewage have raised the possibility of fecal-oral mode of transmission. Currently, quantitative reverse-transcriptase PCR (qRT-PCR) is the only method being used for SARS-CoV-2 detection, which requires expensive instrumentation, dedicated laboratory setup, highly skilled staff, and several hours to report results. Considering the high trans-missibility and rapid spread, a robust, sensitive, specific and cheaper assay for rapid SARS-CoV-2 detection is highly needed. Herein, we report a novel colorimetric RT-LAMP assay for naked-eye detection of SARS-COV-2 in clinical as well as sewage samples. Our SARS-CoV-2 RdRp-based LAMP assay could successfully detect the virus RNA in 26/28 (93%) of RT-PCR positive COVID-19 clinical samples with 100% specificity (n = 7) within 20 min. We also tested the effect of various additives on the performance of LAMP assay and found that addition of 1 mg/ml bovine serum albumin (BSA) could increase the sensitivity of assay up to 10^1 copies of target sequence. Moreover, we also successfully applied this assay to detect SARS-CoV-2 in sewage waters collected from those areas of Lahore, a city of Punjab province of Pakistan, declared as virus hotspots by local government. Our optimized LAMP assay could provide a sensitive first tier strategy for SARS-CoV-2 screening and can potentially help diagnostic laboratories in better handling of high sample turnout during pandemic situation. By providing rapid naked-eye SARS-CoV-2 detection in sewage samples, this assay may support pandemic readiness and emergency response to any possible virus outbreaks in future.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the associated disease referred to as coronavirus disease 2019 (COVID-19) were first described during an unusual outbreak of pneumonia in Wuhan city of China in December 2019 and has since become a global health challenge. According to the latest tally from WHO, there are more than 120 million confirmed cases of COVID-19 and over 2.6 million fatalities globally (WHO, 2021). SARS-CoV-2 is a member of the enveloped single-stranded, positive-sense RNA virus family Coronaviridae which are known to infect birds, amphibians, and mammals (Pal et al., 2020). Genetic analysis showed that SARS-CoV-2 shares 96.2% homology with bat-derived SARS-like coronaviruses (Zhu et al., 2020) and 80% identity with SARS-CoV of 2003 outbreak (Lu et al., 2020). The >29 kb long genome of SARS-CoV-2 has a 5′ methylated cap and 3′ polyadenylated tail, and is structurally divided into 14 open reading frames (ORFs) that encode for a variety of structural, non-structural and accessory proteins responsible for virus structure, replication, survival in host, and pathogenesis (Astuti, & Yasrafil, 2020; Romano et al., 2020).

SARS-CoV-2 infection was initially thought to cause mild to severe respiratory problems only. However, numerous studies subsequently described the induction of gastrointestinal symptoms including diarrhea, nausea and vomiting (Chen et al., 2020; Gao et al., 2020; Wang

et al., 2020). A meta-analysis, based on 29 published studies, concluded that approximately 12% of COVID-19 patients manifest gastrointestinal symptoms (Parasa et al., 2020). The secretion of both viral genome and live virus have recently been reported in the feces/anal or rectal swabs and urine of mild to severely affected as well as asymptomatic COVID-19 patients (Gu et al., 2020; Wu et al., 2020; Guan et al., 2020; Sun et al., 2020; Song et al., 2020; Zhang et al., 2020). Continuous fecal shedding of SARS-CoV-2 was reported even after clearance of the respiratory tract with negative throat swab results (Ling et al., 2020 & Wu et al., 2020). Similarly, viral load was reported to be higher in feces when compared with respiratory samples (Xiao et al., 2020). These evidences not only suggest the possibility of fecal–oral or fecal–respiratory SARS-CoV-2 transmission but also emphasize the need and importance of SARS-CoV-2 monitoring in wastewater.

The surveillance of sewage water has become an extremely important tool to trace the circulation of viruses in a population, to describe their prevalence and genetic diversity and to study molecular epidemiology and geographical distribution (Sinclair et al., 2008; La Rosa & Muscillo, 2013). Sewage water monitoring may be more useful for viruses that do not manifest clinical symptoms or present at a very low level (Asghar et al., 2014). Earlier studies during previous coronavirus outbreaks reported the detection of SARS-CoV RNA in sewage samples (Wang et al., 2015). Likewise, during the current outbreak, molecular detection of SARS-CoV-2 in wastewater has been reported in several studies conducted in Australia, Netherlands, USA, France, and China. These studies successfully detected SARS-CoV-2 RNA in untreated wastewater with high viral loads (Ahmed et al., 2020; Lodder et al., 2020; Medema et al., 2020, La Rosa et al., 2020; Nemudryi et al., 2020; Wu et al., 2020 & Wurtzer et al., 2020). Findings of these studies have opened new horizons in the ongoing battle against this deadly pandemic. Sewage and wastewater surveillance may help in development of early warning systems for a feared second wave, rapid implementation of data driven non-pharmaceutical interventions for curtailing the virus spread, as well as real time monitoring of the efficacy of implemented interventions (Nemudryi et al., 2020; Wang et al., 2020). Numerous such studies are underway in different parts of the world. However, in Pakistan, not much has been done in this regard. To the best of our knowledge, currently only one non-peer reviewed pre-print has reported sewage water testing for SARS-CoV-2. The said study has a major limitation that it relies on conventional Real-time reverse transcriptase (rRT-PCR) method. Moreover, the authors have tested sewage water from only a single source (Yaqub et al., 2020).

Real-time reverse transcriptase PCR (rRT-PCR) is currently the gold standard method for detection of SARS-CoV-2 in clinical as well as in environmental samples. However, rRT-PCR is expensive, cumbersome, time consuming, and requires sophisticated instrumentation, dedicated laboratory setup and highly skilled staff (Corman et al., 2020). Rapid, robust, cost effective and simple diagnostic methods, which can be performed without the need of advanced instruments and highly trained technicians, may be a better diagnostic and surveillance approach during the times of emergency like COVID-19 outbreak. Loop-mediated isothermal amplification (LAMP) was originally developed as a rapid and reliable method to amplify small amounts of target sequences at a single reaction temperature, preventing the need of thermocycler (Notomi et al., 2000). Having the advantage of simplicity to perform and isothermal amplification, this method gained immense popularity for the effective diagnosis of pathogens. Herein, we report a novel colorimetric LAMP assay targeting RNA-dependent RNA polymerase (RdRp) gene of SARS-CoV-2 genome for sensitive and specific detection of the virus in clinical and sewage water samples from Pakistan.

2. Materials and methods

2.1. LAMP assay primer designing and analysis

A set of six sequence specific LAMP primers targeting a 225 nt long

sequence spanning nucleotide positions 15319 to 15543 of Pakistani SARS-CoV-2 isolate (MT240479.1) within the RdRp gene of viral genome were designed using an open access online software Primer Explorer V5. The sequences of these primers were Cor-RdRp-F3: 5'-GCTCGCAAACATACAACGT-3', Cor-RdRp-B3: 5'-GTTACCATCAGTA-GATAAAAGTGCA-3', Cor-RdRp-FIP: 5'-CGCCACACATGACCATTTCAC TCAATTTTGTTGTAGCTTGTCACACCGT-3', Cor-RdRp-BIP: 5'-AGGTGGAACCTCATCAGGAGATGTTTTAACATTGGCCGTGACAGC-3', Cor-RdRp-LF: 5'-CTTGAGCACACTCATTAGCTAATC-3' and Cor-RdRp-LB: 5'-CCACAACTGCTTATGCTAATAGTGT-3'.

2.2. Optimization of LAMP assay

For optimization of LAMP assay, a target DNA fragment (225 nt) corresponding to SARS-CoV-2 RdRp gene region cloned into pTOP Blunt V2 vector was obtained from Clonetech. The said fragment was amplified by PCR using outer primers (Cor-RdRp-F3 and Cor-RdRp-B3). The resulting PCR product was purified using GeneJet purification column and stored at -20 °C until further use. For optimization of the LAMP assay, we used this purified DNA fragment as a template and LAMP assay was performed using previously described reaction conditions (Zhang et al., 2020). Since, addition of certain additives in LAMP reaction has been described to influence the efficiency of LAMP assay (Xu et al., 2016; Syafirah et al., 2018), we tested three of the additives: $MgSO_4$ (8 mM), DMSO (5 %) and/or BSA (1 mg/ml) in the reaction mix. We performed the LAMP assay over a wide range of temperatures (50 to 65 °C) and color change was observed after every 10 min till 60 min. We also performed the LAMP assay reactions in 10 and 20 μl volumes and compared efficiencies. For a 10 μl total reaction volume, components were added as, 5 μl of WarmStart® Colorimetric Lamp 2X Master Mix (Cat # M1800S, NEB), 1 μl of 10X primer mix (16 μM each of forward inner primer (Cor-RdRp-FIP) and backward inner primer (Cor-RdRp--BIP), 2 μM each of forward outer primer (Cor-RdRp-F3) and backward outer primer (Cor-RdRp-B3) and 4 μM each of forward loop primer (Cor-RdRp-LF) and backward loop primer (Cor-RdRp-LB), 1 μl of 10 mg/ml BSA and 3 μl of the template. The assay was performed at an optimized incubation temperature (65 °C) and time (20 min). The assay was performed using an Applied Bio system thermocycler (Version 2.08). Color change from pink (at the start of the reaction) to yellow was interpreted as positive result while no change in color after 20 min at 65 °C was considered as negative.

2.3. Collection of clinical samples and RNA isolation

Mughal Diagnostics and Research Lab (MDRL) (Johar Town, Lahore, Pakistan) is among the approved Laboratories by Government of Pakistan for the detection of SARS-CoV-2 in clinical samples. Nasopharyngeal swabs were collected from suspected individuals at the designated laboratory and placed in vials containing viral transport media (VTM). Briefly, samples were inactivated at 56°C for 30 min in BSL-3 laboratory as described previously (Zhang et al., 2020). These samples were processed for total RNA isolation at above mentioned laboratory using GF1 viral DNA/RNA extraction kit (Cat # GF-RD-300, Vivantis), following manufacturers protocol. All these processes were carried out by wearing proper personal protective equipments (PPEs) and protocols recommended by WHO.[1] The qRT-PCR was performed at MDRL by using COVID-19 detection kit (Cat # Z-Path-COVID-19-CE, Genesig). In total, 35 RNA samples (28 positive and 7 negative) were retrieved from MDRL along with patients' history and Ct values. These RNA samples were transported to School of Biological Sciences, University of the Punjab Lahore, and stored at -80 °C until further processing. The present study was approved by Ethical Review Board of School of Biological Sciences, University of the Punjab Lahore, Pakistan. A written consent was obtained from each participant to use their samples for the validation of LAMP assay.

2.4. Sewage water sample collection and concentration of viruses

Raw sewage (1 L) samples were collected from different drain localities in Lahore in sterile collection jars. Adequate PPEs were used by personnel collecting sewage samples. Collected sample was mixed properly before aliquoting and stored at -20 °C. The viruses were concentrated by PEG method described previously (Hjelmsø et al., 2017). Briefly, glycine buffer (glycine 0.05 M, beef extract 3%, pH 9.6) was added to sewage water to extricate virions from organic materials. The samples were centrifuged at 7000 rpm for 30 min at 4 °C. Supernatants were collected and filtered through 0.45 μm polyethersulfone membrane (PES) to eliminate eukaryotic and bacterial cells. pH of filtrate was adjusted to 3.7 and filtrate was incubated with PEG-8000 (80 g/L) and NaCl (17.5 g/L) in shaker (100 rpm) overnight at 4 °C. The incubated filtrate was centrifuged at 7000 rpm for 2 h to precipitate viruses in a pellet. The resulting pellet was then dissolved in 1X phosphate buffered saline (PBS) and stored at -80 °C until further processing.

2.5. RNA extraction from sewage samples and cDNA synthesis

RNA was isolated from concentrated sewage water samples by Trizol method. Briefly, 400 μl of each sample concentrate was mixed with 1 ml Trizol reagent following addition of 300 μl of chloroform in sterile micro centrifuge tubes. Mixture was vortexed for 10 sec, incubated at room temperature for 3 min and subjected to centrifugation at 12000 rpm for 10 min at 4 °C. After centrifugation, upper layer was transferred to a new micro centrifuge tube, mixed with equal volume of chilled 100% isopropanol and incubated at room temperature for 10 min following centrifugation. Obtained pellet was washed with 70% ethanol, air dried and dissolved in 50 μl DEPC treated water. Isolated RNA was quantified using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). To synthesize cDNA, the reverse transcription was performed. Eleven microliters of extracted RNA were mixed with 1μl of RdRp reverse outer primer Cor-RdRp-B3 (10 μmol/ml) and incubated at 65 °C for 5 min. After incubation, 8 μl of second mixture containing 4 μl of 5X reaction buffer, 1 μl of ribolock RNase inhibitor, 2 μl of 10 mM dNTPs and 1 μl of RevertAid reverse transcriptase was added and incubated at 25 °C for 5 min followed by an incubation of 60 min at 42 °C and final incubation at 70 °C for 5 min.

2.6. RT-PCR amplification

After cDNA synthesis, RT-PCR was performed using RdRp outer primer set. In total, 10 μl reaction mixture was prepared using 5 μl of PCR MasterMix (LUNA), 0.25 μM of each forward and reverse outer primer and 2 μl of template cDNA and volume was made up to 10 μl. Amplification was performed using initial denaturation at 95 °C for 5 min followed 40 cycles of denaturation of 30 sec at 95 °C, annealing for 30 sec at 58 °C and extension for 30 sec at 72 °C with final extension at 72 °C for 5 min. Each PCR contained RdRp synthetic fragment as positive control and nuclease free sterile water as negative control. PCR products were analyzed on 1.5% agarose gel.

2.7. LAMP assay for clinical and sewage samples

The LAMP assay was performed on the clinical samples (n =35) as well as the sewage water samples in 10 μl reaction volume using optimized reaction conditions described above. Each sample was assayed three times. The assay results for clinical samples were compared with the Ct values retrieved from the data bank of MDRL. For sewage samples, assay results were compared with the results of in-house RT-PCR.

2.8. In-silico analysis of primer specificity

Sequences of the 4 primers (F3, B3, LF, LB) as well as F1c, F2, B1c, and B2 regions of primers FIP and BIP, respectively, were individually aligned with SARS-CoV-2 sequences and sequences of various closely related coronaviruses. Primer sequences were aligned with genome sequences of related coronaviruses Human coronavirus OC43 (NC_006213.1), Middle East respiratory syndrome-related coronavirus (MERS) (NC_019843.3), SARS coronavirus Tor2 (NC_004718.3), Human coronavirus 229E (NC_002645.1), Human coronavirus NL63 (NC_005831.2), and Human coronavirus HKU1 (NC_006577.2). In order to check the specificity of primers for emerging SARS-CoV-2 variants, genome sequences of the three most prevalent variants, namely: UK variant (GISAID Accession ID: EPI_ISL_601443), South African variant (GISAID Accession ID: EPI_ISL_678597), and Brazilian variant (GISAID Accession ID: EPI_ISL_804814) as well as sequence of SARS-CoV-2 Wuhan-Hu-1 isolate (NCBI Reference Sequence NC_045512) and Pakistani isolate SARS-CoV-2/human/PAK/Gilgit1/2020 (GenBank: MT240479.1) were also included in the analysis.

Primers were aligned with the subject sequences using NCBI program BLASTN 2.11.0 (Altschul et al., 1997) using following parameters: Word size 7, Expect value 1000, Match/Mismatch scores 1,-3, Gapcosts 5,2. Specificity of each primer was determined by analyzing the E-values obtained.

3. Results

## 3.1. Optimization and validation of RdRp-based LAMP assay

We used synthetic RdRp gene fragment as template for the optimization of our newly developed LAMP assay. Genomic location of the target region within the SARS-CoV-2 genome and the map of plasmid having cloned target fragment is shown as Fig. 1 and Supplementary Fig. 1, respectively. Initially, no amplification was obtained after 30 min of incubation when LAMP assay was performed according to the previously described procedure (Zhang et al., 2020). However, LAMP assay produced yellow color when we further incubated the reaction for 60 min at 65 °C (Supplementary Fig. 2). With the aim to improve the sensitivity of the assay as well as to reduce the reaction time, we optimized several parameters. We first tested the effect of various additives including BSA, $MgSO_4$, and/or DMSO, on the amplification efficiency and sensitivity. Our results showed that presence of BSA in the reaction mixture significantly increased the efficiency and sensitivity of LAMP assay (Fig. 2A). Addition of BSA to a final concentration of 1mg/ml in LAMP reaction could achieve amplification within 20 min. While no visible color change was observed in the absence of BSA. The other two additives *i.e.* DMSO and $MgSO_4$ did not show any significant effect on the assay efficiency.

We further tested the effect of incubation time and temperature on efficiency of the assay. Our findings showed that the time to obtain amplification signal in LAMP assay reduced with the increase in the incubation temperature. There was a complete colour change from pink to yellow at 65 °C in 20 min. On the other hand, incubation time also seemed to be having an impact on the assay. For instance, assay produced color even at temperature as low as 55 °C when incubated for 60 min (Fig. 2B). Based on these observations, we slightly modified the reaction conditions for further testing. In contrast to the procedure reported by Zhang et al. (2020) and also the protocol recommended by the manufacturer, where a 30 min incubation at 65 °C is suggested, we used only 20 min incubation for a reaction volume of 10 μl, in the presence of BSA at a final concentration of 1 mg/ml.

3.2. Estimation of assay sensitivity

For the determination of sensitivity and detection limit of the assay, LAMP assay was performed with 10-fold serial dilutions of synthetic DNA fragment. We tested a range of starting copies (10^6 to 10^0) of the target RdRp synthetic DNA fragment. Our results showed that using optimized parameters the assay was able to detect as low as 10^1 copies of the target region (Fig. 3A). Moreover, the sensitivity range of our LAMP
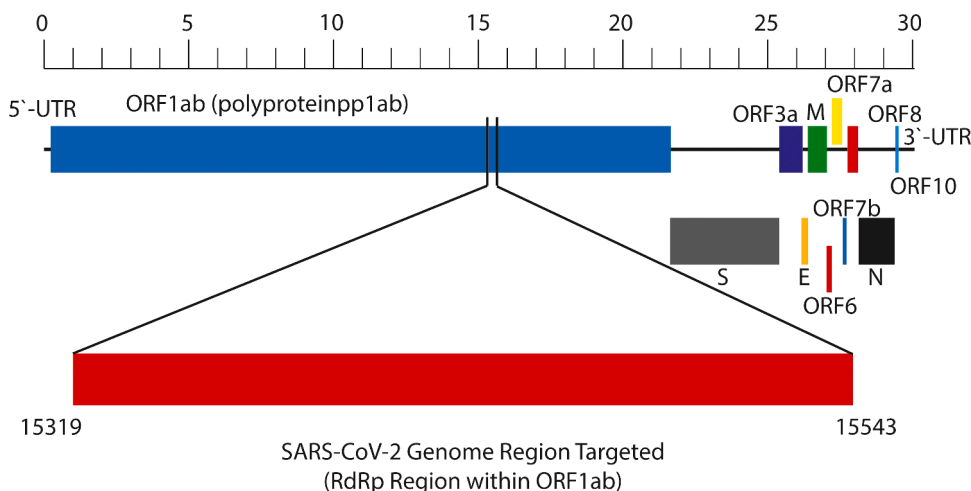
Fig. 1. Genomic location of the target region within the SARS-CoV-2 genome. A set of specific LAMP primers targeting a 225 bp long sequence spanning nucleotide positions 15319 to 15543 of Pakistani SARS-CoV-2 isolate (MT240479.1) within the RdRp gene region (RNA dependent RNA polymerase) of viral genome, were designed. S, E, M and N represent the structural proteins Spike, Envelope, Membrane and Nucleocapsid.

assay spanned over several orders of magnitude for serial dilutions of RNA, obtained from a qRT-PCR confirmed clinical sample of COVID-19 (Fig. 3B).

3.3. SARS-CoV-2 detection in the COVID- 19 clinical samples

In order to test the capability of our optimized assay in detecting clinically relevant SARS-CoV-2 in real samples, we performed LAMP assay on 35 RNA samples obtained from designated COVID-19 diagnostic laboratory. These samples had already been tested via qRT-PCR at the diagnostic laboratory and included confirmed SARS-CoV-2 positive (n=28) and negative (n=7) cases. We performed LAMP assay on these samples in an operator blind fashion and compared the results subsequently with data obtained from qRT-PCR. In total, 26 out of the 35 samples analyzed showed color change at the end of LAMP reaction, while no color change was observed in the remaining 9 samples. A comparison with the qRT-PCR revealed that our assay showed efficiency comparable to the qRT-PCR as 26 out of the 28 qRT-PCR positive samples also tested positive with our LAMP assay. A comparison of Ct values and LAMP assay results is given in Supplementary Table 1. Out of the 28 qRT-PCR positive samples, only samples P1 and P2 tested negative with our LAMP assay. The Ct values for samples P1 and P2 were 36.84 and 36.35, respectively. These Ct values are close to the cutoff value of 38 indicating low viral concentration. It must be noted that none of the remaining 26 samples which tested positive for both qRT-PCR and LAMP had comparably high Ct values. Highest Ct value observed among these 26 samples was 30.77 for P18. On the other hand, our assay showed 100% specificity as all the 7 qRT-PCR negative samples did not show any color change in LAMP reactions. Results from some of the representative clinical samples are shown in Fig. 4.

3.4. Detection of SARS-CoV-2 in sewage water

One of the primary objectives of this study was to apply the LAMP assay for the detection of SARS-CoV-2 in the environmental samples, particularly the sewage water that receives feces and urine from COVID-19 affected communities. For this purpose, samples were collected from 6 major drains of Lahore (Supplementary Fig. 3). The sample collection sites were chosen to cover especially the areas declared as infection hotspots by local government. These samples were concentrated and processed for RNA isolation. SARS-CoV-2 RNA was detected using our optimized LAMP assay in all of the sewage water samples. Tap water and water collected from Lahore canal were used as controls. The control samples were subjected to the same RNA isolation procedure and LAMP

assay. No amplification (change in color) was observed in any of the controls (Fig. 5A). We further verified the results of our LAMP assay by carrying out standard qRT-PCR analysis of sewage and control samples. qRT-PCR results substantiated the findings of our LAMP assay (Fig. 5B and Supplementary Table 2).

3.5. Specificity of LAMP assay

In order to check the specificity of LAMP assay for SARS-CoV-2, all the eight regions targeted by primers were individually aligned with the reference genome sequences of six related coronaviruses. Additionally, similarity of primers with three of the most prevalent newly emerging SARS-CoV-2 variants commonly known as UK (B.1.1.7), South African (B.1.351), and Brazilian (P.1) variants was also analyzed. The results of alignments are presented in Supplementary Tables 3-10. Our *in-silico* analysis showed no significant similarity of primers F3, B3, and LF as well as B1c and B2 regions of primer BIP and F2 region of primer FIP. F1c region of FIP primer showed significant similarity with SARS Tor2 genome (E-value 6e-05), however closer look at the alignment revealed a similarity of nucleotides 1-22 of F1c region (total length 25 bp) with positions 15381-15360 of Tor2 genome, with 1 mismatch. Similarly, F1c region showed high similarity with the Human coronavirus HKU1 (E-value 2e-04). F1c sequence showed similarity of nucleotides 1-25 with positions 15574-15550 of HKU1 genome, with two mismatches. On the other hand, LB primer also showed high similarity with Tor2 genome (E-value 6e-08). Nucleotides 3-25 of LB primer (total length 25bp) were identical to positions 15426-15448 of Tor2 genome.

Despite slight similarity of certain primer sequences with unrelated coronavirus sequences, it is highly unlikely that this similarity will result in any significant non-specific amplifications. Firstly, as F2, and B2 regions do not have any significant similarity with any of the closely related coronaviruses initial binding of FIP and BIP primers with the template is not possible. Additionally, F3 and B3 sequences are also not identical to any other coronaviruses except SARS-CoV-2 therefore strand displacement also cannot ensue. Despite slight similarity of F1c region with Tor2 genome or being completely identical to HKU1 genome, it will not lead to any loop formation and amplification from this loop seems highly unlikely due to non-complementarity at the F2 region of FIP. Similarly, high similarity of LB primer with Tor2 genome alone in the absence of any significant similarity at B1c and B2 regions will not affect the overall specificity of the assay.

On the other hand, all the eight regions targeted by LAMP primers showed 100% similarity with various SARS-CoV-2 sequences. In addition to the sequence of Pakistani isolate, which was used to design the

A

B
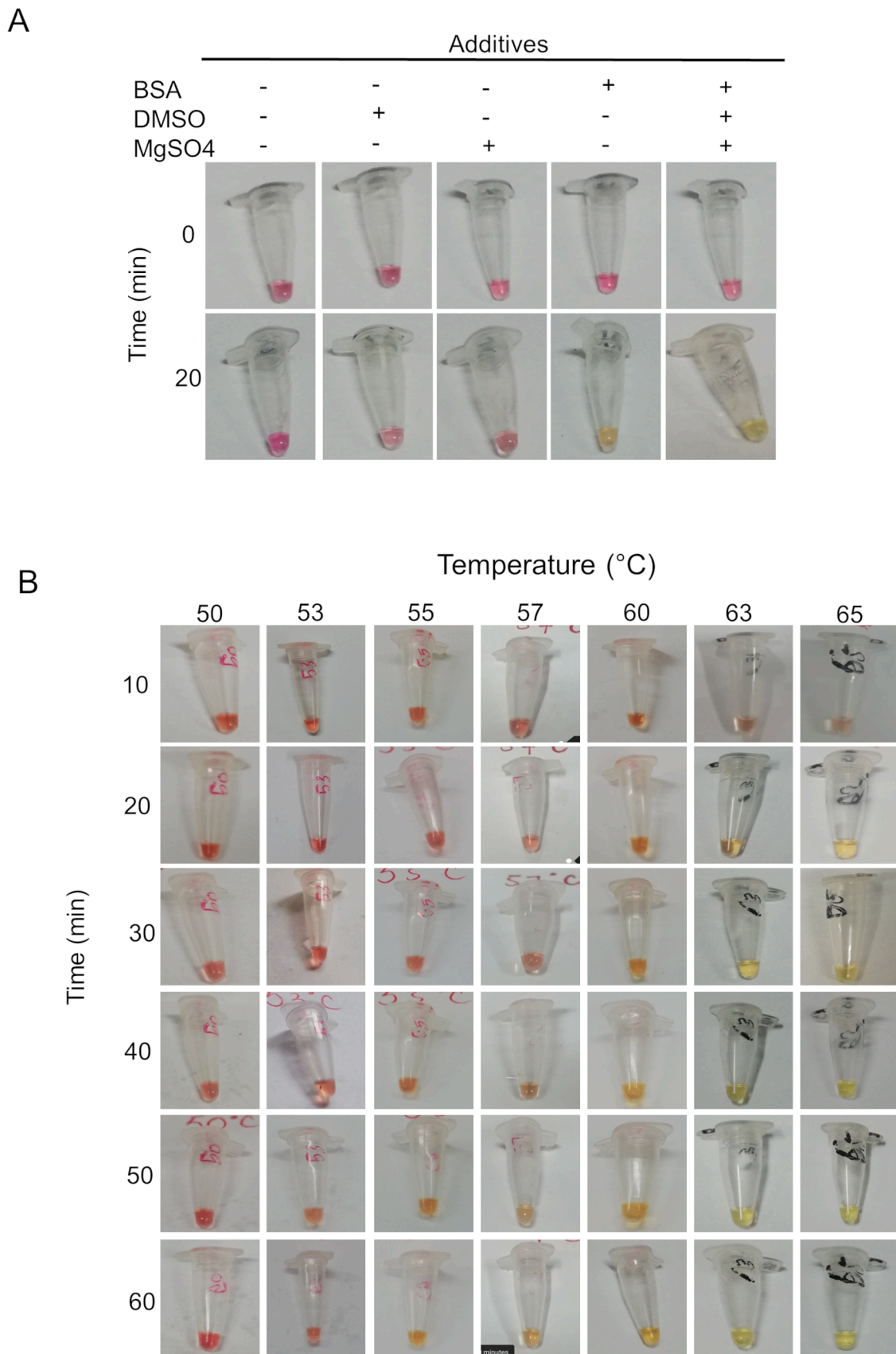
Fig. 2. Optimization of LAMP Assay. The LAMP assay was performed using 225 nt long synthetic RdRp gene fragment. **A)** The figure shows results of RdRp-gene based LAMP assay in the presence and absence of amplification-enhancing additives *i.e.* BSA, MgSO4, and/or DMSO. **B)** Images show RdRp-gene based LAMP assay results at various temperature and time points. In both figures, pink and yellow colors show negative and positive results, respectively.
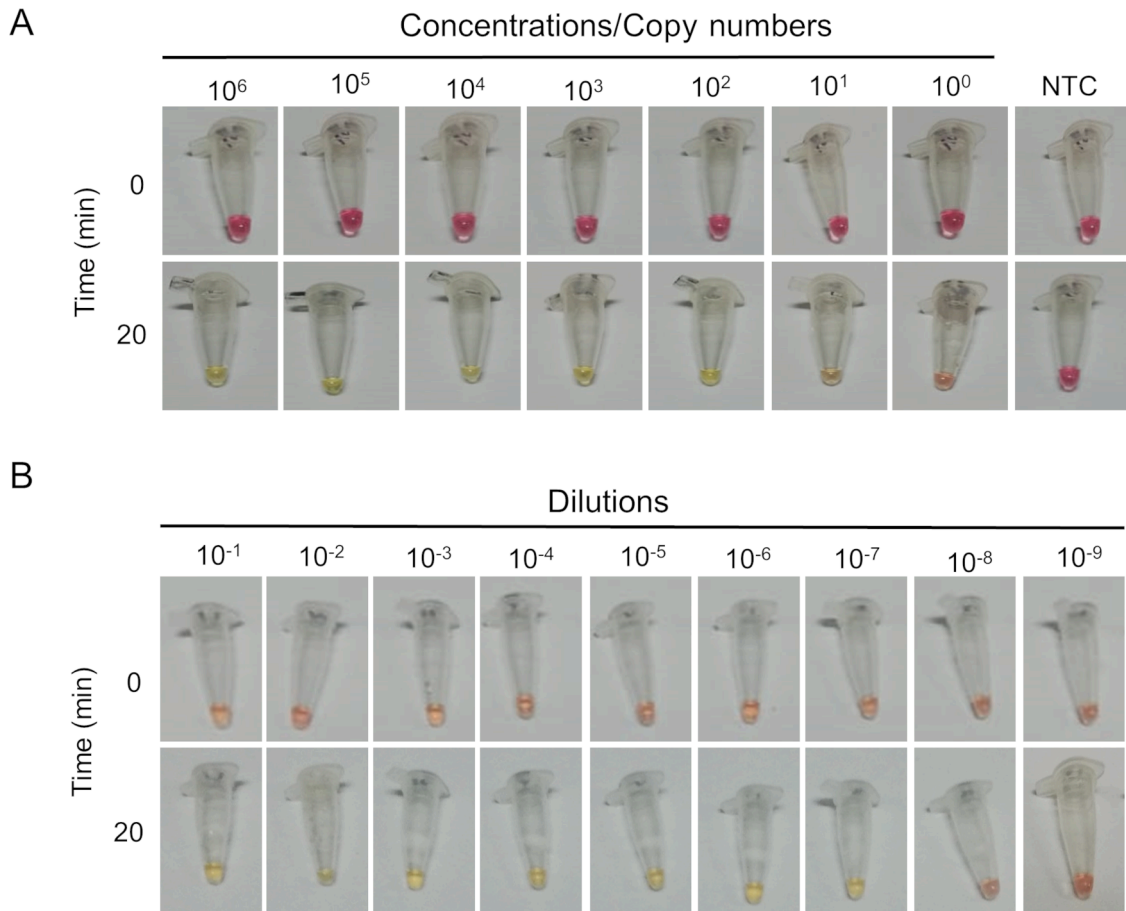
Fig. 3. Assessment of LAMP assay sensitivity. **A)** Figures show results of LAMP assay performed by using serial dilutions (copy number 10^6 to 10^0) of synthetic fragment of RdRp-gene of SARS-CoV-2 as template. NTC: no template control. **B)** Images are results of LAMP assay performed by using dilutions (10^{-1} to 10^{-9}) of RNA from a COVID-19 clinical sample.
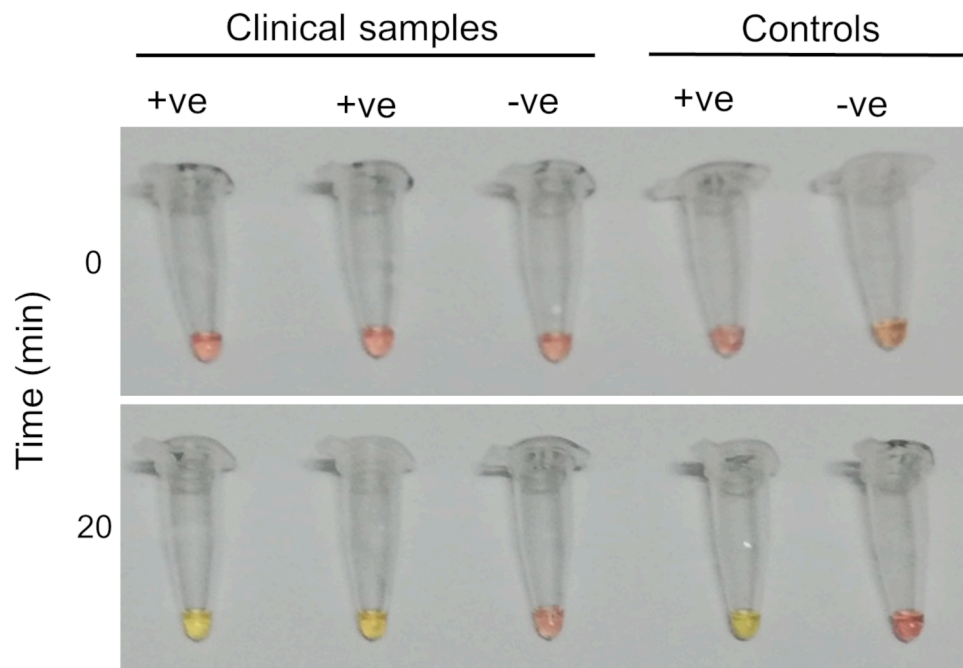
Fig. 4. Validation of RdRp-gene LAMP assay for COVID-19 clinical samples. Images are of LAMP assay performed on representative clinical samples obtained from COVID-19 patients (two +ve and one -ve). Synthetic RdRP gene was used as positive control while nuclease free water as negative control. Assay was repeated three times for each sample and the representative image is presented here.
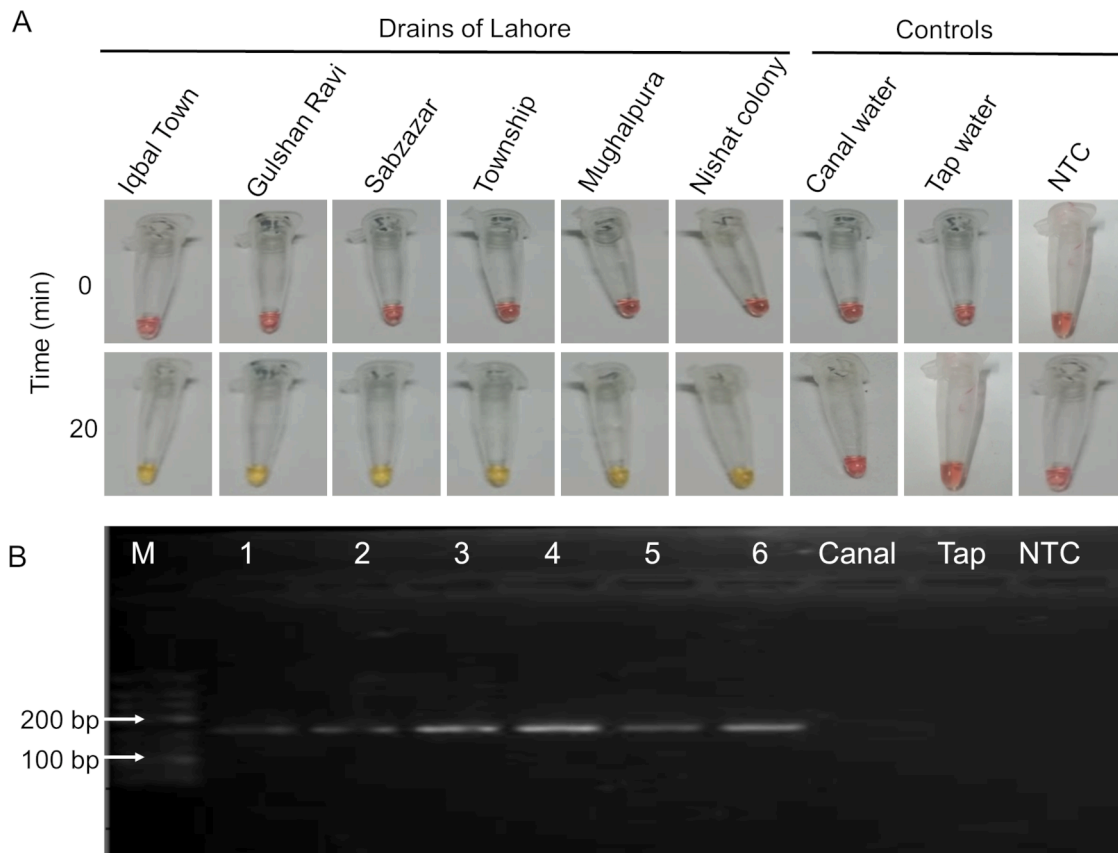
Fig. 5. Detection of SARS-CoV-2 in sewage samples. **A)** LAMP assay was performed on RNA samples obtained from different drains (Iqbal Town, Gulshan Ravi, Sabzazar, Township, Mughalpura and Nishat colony) of Lahore located in the areas of Lahore city where local Government had reported the largest number of COVID-19 positive cases. Canal and Tap water were used as negative controls. Assay was performed three times on each sample and representative images are shown here. **B)** Agarose gel shows the results of RT-PCR performed on cDNA of 6 selected drains samples (L2 - 7) of Lahore. L1: DNA marker (M), NTC: no template control. Assay was repeated at least two times and the representative images are presented here.

LAMP assay, the primers were also 100% identical to the reference sequence (Wuhan-1 isolate) as well as various newly emerging SARS-CoV-2 variants of concern. These results indicate that our novel LAMP assay is capable of highly specific detection of SARS-CoV-2 as no amplification of any of the related coronavirus sequences is possible. Moreover, it can possibly detect most of the common SARS-CoV-2 variants as the assay targets one of the most conserved viral regions i.e. RdRp.

4. Discussion

On 31st December 2019, the World Health Organization was informed of a cluster of cases of pneumonia of unknown etiology in Wuhan, China. Subsequent investigations identified a novel coronavirus named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causative agent of the ongoing pandemic. Considering the need for highly sensitive, specific, easy to perform laboratory diagnostics required for screening of population for COVID-19 at larger scale, we developed and compared the performance of a novel RdRp-based RT-LAMP assay for the rapid detection of SARS-CoV-2 in clinical as well as environmental samples. Although Loop-mediated isothermal amplification (LAMP) assay for SARS-CoV-2 has been reported targeting ORF and N-genes (Zhang et al., 2020), to the best of our knowledge none of the studies published so far have reported on the use of RNA dependent RNA polymerase (RdRp) gene for virus detection using LAMP assay. RdRp gene is an excellent choice for being highly conserved (Chana, Yip, & To, 2020; (Nawattanapaiboon et al., 2021). Remarkably, RT-LAMP is reported to be ~ 100-fold more sensitive than conventional RT-PCR

methods (Khan et al., 2018). Moreover, RT-LAMP assay is faster relative to qRT-PCR and therefore it could be used as a reliable method in laboratories to keep up with the increasing demand of screening the potential patients during the pandemic situations.

Primarily, our assay was meant to be qualitative. As limit of detection and reproducibility are the key performance matrices for a diagnostic assay, we used synthetic target fragment to demonstrate the performance of our assay. Our assay showed optimal reproducibility not only for synthetic fragment but also for clinical and environmental samples. Our assay demonstrated a reliable limit of detection of 10 copies per reaction in less than 20 min, which is better as compared to the previously reported RT-LAMP assays. For example, Lamb et al. (2020) were able to detect nearly 300 viral copies and Bharda et al (2020) reported the detection of 100 copies of SARS-CoV-2 genomic RNA. We successfully evaluated the performance of our assay in clinical settings, recruiting 35 RNA samples including 28 confirmed COVID-19 cases. The results of our assay for these clinical samples were largely consistent with those of qRT-PCR. Only two samples tested positive by qRT-PCR were negative by our RT-LAMP while none of the samples tested negative by qRT-PCR were tested positive by our assay. The observed difference in positive predictive value of RT-LAMP and qRT-PCR may be caused by less/more RNA inputs (Jiang et al., 2020). In addition to assay performance, cost per test is also an important consideration, particularly in rapidly spreading pandemic situations. Cheaper, yet reliable, tests may help reducing the financial burden of testing and the resources can be diverted to other healthcare facilities. We optimized our assay using 10 μl reaction volume compared to the volume recommended by the manufacturer (25 μl), thus reducing the

cost per test 2.5 times.

In recent months, the emergence of novel SARS-CoV-2 variants has led to unexpected rise in number of cases of COVID-19 in various countries. UK, South Africa, and Brazil are among the countries which appear to be most severely hit by emergence of novel variants. Each of these countries has been suspected to be the place where a particular variant emerged. For example, the B.1.1.7 clade emerged in UK, while B.1.351 is known to have emerged in South Africa. Both of these variants harbor a mutation (N501Y) in the receptor binding domain of spike protein which confers high transmissibility to these variants. It has been estimated that these variants have 40-70% higher transmission rates compared to wild type. B.1.351 has at least two known additional mutations (E484K and K417N) in spike protein which can potentially make the variant less susceptible to neutralization by antibodies. The third variant P.1 which is thought to have emerged in Brazil harbors the N501Y mutation as well as two additional mutation E484K and K417T. While emergence of these variants is a major concern for vaccination efforts, performance of diagnostic assays may also be affected. Although based on the available data the effect of genome mutations in the newly emerging variants on performance of most commonly used diagnostic assays seems to be minimal, a cautious approach may be needed as several mutations have been reported around the regions targeted by available diagnostic assays. We carried out *in-silico* analysis of the specificity of primers used in our assay and demonstrated that all of them show 100% similarity to various SARS-CoV-2 isolates and more importantly three of the newly emerged variants of concern (Fontanet et al., 2021).

Recent discoveries about detection of SARS-CoV-2 RNA as well as viable virus in stools and sewage samples has suggested the possibility of fecal-oral transmission that warrants far-reaching consequences for public health and for pandemic control strategies (Heller et al., 2020). Furthermore, a few studies have reported the secretion of both viral genome and viable viruses in the feces of mild to severely affected as well as asymptomatic COVID-19 patients (Gu et al., 2020; Wu et al., 2020; Holshue et al., 2020) and this fecal shedding of SARS-CoV-2 were reported to be continued even after clearance of respiratory tract and negative throat swab results (Kitajima et al 2020). Considering the implications of these reports, we reasoned that detection of SARS-CoV-2 in sewage water may offer a good strategy to measure the true scale of coronavirus outbreak (Mallapaty, 2020). We selected 6 different drains located in various urban areas of the city of Lahore that received the sewage water from areas marked as hotspots by the local government. Recently, SARS-CoV-2 RNA has been detected in wastewater samples from various countries like Netherlands (Medema et al., 2020), Australia (Ahmed et al., 2020) and USA (Wu et al., 2020). However, all these testes were based on qRT-PCR. Here, we successfully report detection of SARS-CoV-2 in the sewage water samples based on our optimized RT-LAMP assay, and the results were further validated by conducting RT-PCR. To the best of our knowledge, this is the first study that reports the detection of SARS-CoV-2 in sewage samples using a highly sensitive LAMP assay. Although in this study we have demonstrated the applicability of LAMP assay on sewage sample isolated RNA, further work may be needed to develop a standardized assay. Currently one of the major issues being faced in this regard is that none of the reported studies have provided information on the percent recovery of SARS-CoV-2 from sewage samples due to the risk associated with handling SARS-CoV-2 and the requirements for a BSL-3 facility. To the best of our knowledge, only one study has to date reported the percent recovery of SARS-CoV-2 from sewage samples which was estimated to be only 1% using an electropositive membrane (Ahmed et al., 2020). Since the characteristics of SARS-CoV-2 are different from other enteric viruses, more research may be needed to streamline protocols for effective recovery and downstream LAMP based detection of SARS-CoV-2 from sewage and/or other environmental samples.

One of the biggest challenges, in using sewage water SARS-CoV-2 detection to assess the scale of virus spread in corresponding community, would be to establish correlation between quantities detected in sewage water and the actual number of cases in the community. Medema et al. (2020) reported RT-qPCR data, while another recent study reported ~250 copies/ml SARS-CoV-2 in sewage water in Massachusetts, USA (Wu et al., 2020). The authors acknowledged that the estimated concentration was much greater (5% of all fecal samples in the catchment) than the confirmed cases (0.026%). The authors listed several factors and assumptions for this discrepancy and considered their results conservative. Another challenge in using sewage water for SARS-CoV-2 detection is the possible contamination of sewage water with related coronaviruses. These related coronaviruses show high overall sequence similarity with the SARS-CoV-2 thus there is a possibility of false positive results due to the contamination of sewage water with related coronaviruses if the molecular assay being used is not highly specific. We carried out in silico analysis of specificity of our primers. Our analysis showed that most of the primer sequences were highly specific for SARS-CoV-2 with no significant similarity to any of the six related coronaviruses included in the analysis. Although F1c and LB regions showed similarity to Tor2 and HUK1, and Tor2 viruses, respectively, as discussed in section 3.5 these similarities cannot possibly lead to non-specific amplifications. Thus, due to inherent specificity of LAMP assay (as 6 primers are used which target 8 distinct regions in target), as well as careful design of primers, we demonstrate that our LAMP assay can potentially differentiate SARS-CoV-2 from various related coronaviruses, and at the same time can detect most common SARS-CoV-2 variants with equal efficiency.

For application of sewage-based epidemiology to assess SARS-CoV-2 spread, further systematic research is needed covering aspects from effective sampling and preservation through to data interpretation. Development of effective enveloped virus concentration methods and understanding the decay of SARS-CoV-2 in sewage are some of the major research avenues needing attention. Information on the composition of sewage and environmental factors such as stormflow and its impact on sewage may also be useful. Since application of such a rapid isothermal viral RNA detection platform may be particularly useful in point-of-need and off-laboratory settings, integration of LAMP based SARS-CoV-2 detection in novel handheld and miniaturized microfluidic devices is also one of the most important research area for development of rapid, simple and low-cost viral detection methods. These requirements are expected to be achievable and it is anticipated that LAMP based SARS-CoV-2 detection will not only support routine viral diagnostics but may also enable better approaches for rapid assessment of the disease burden in the community.

5. Conclusion

In this study, we report a novel RdRp based sensitive RT-LAMP assay for SARS-CoV-2 detection in clinical as well as sewage water samples. Rapid and low-cost detection ability may potentially help in controlling the outbreak and spread of the disease by enabling implementation of data-driven smart control interventions.

Disclosure Statement

There is no conflict of interest among all contributing authors.

Funding Sources

No funding was received for this study.

Author statement

All the authors have read the manuscript and are agreed with the current version.

Conflict of interest

The authors declare no conflict of interest

Acknowledgement

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2021.198485.

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