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Coronavirus Pandemic

Development of nucleic acid extraction-free one-step real-time RT-PCR for diagnosis of SARS-CoV-2 infection

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Abstract

Introduction: The aim of this study is to develop a one-step real-time PCR assay for SARS-CoV-2 detection. The study was designed to circumvent the routine RNA isolation step and to optimize a lysis buffer and parameters for direct quantitative PCR.

Methodology: A lysis solution was prepared using Tween-20, Triton X-100, EDTA, and Tris buffer (pH 7.4). Various parameters including the use of detergent combinations, V/V ratios and usage of carrier molecules were standardized to achieve the optimal amplification curve and Ct values of SARS-CoV-2 gene for improving the routine diagnostics procedures.

Results: Adding carrier molecules [Poly(A), glycogen, and linear polyacrylamide] to the lysis solution significantly improved real-time reverse-transcription PCR (rtRT-PCR) efficacy. Poly(A) was the most effective of all carriers. The diagnostic potential of this Poly(A) solution was demonstrated using 150 patient swabs infected with SARS-CoV-2 and 200 uninfected swab samples, and the sensitivity of the rtRT-PCR diagnostic test was estimated to be 98.6 (95% CI: 96.0, 101.17, $p < 0.001$) for group 1; Ct ≤ 25 and 87.2 (95% CI: 80.2, 94.0, $p < 0.001$) for group 2; Ct ≥ 26–30, with excellent accuracy (0.9 < AUC < 1.0), and 100% specificity.

Conclusions: Our finding imply that this strategy is feasible, and it may contribute to the development of a rapid, less laborious, and economical rtRT-PCR test for dealing with the SARS-COV-2 disease in the pandemic area.

Key words: SARS-CoV-2; COVID-19; diagnostics; rtRT-PCR; lysis; carrier molecules.

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Introduction

The first SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2) epidemic was reported in China in late 2019 and became a pandemic in 2020 [1,2]. Many drugs and vaccines have been given emergency approval by the WHO (World Health Organization) and FDA (Food and Drug Administration), USA, for the early eradication of COVID-19 (coronavirus disease 2019). Despite strong vaccination effectiveness and adoption in many countries, COVID-19 hospitalizations will persist owing to the SARS-CoV-2 virus's proclivity for mutation. We are still facing negative repercussions due to recent COVID-19 waves. In 2021, the second wave of COVID-19 in India resulted in a high fatality rate, with approximately half a million deaths reported so far [3]. The gold standard method for detecting the global spread of COVID-19 illness has been real-time reverse transcription polymerase chain reaction (rtRT-PCR) targeting specific genes of SARS-CoV-2 [4]. Among all testing techniques, it is the most sensitive and specific assay for detecting COVID-19 infected people. For the detection of respiratory virus infection, a nasal swab is typically used for sampling and then applied to determine the infection and epidemic stages. Identification of virus in swabs through rtRT-PCR depends on the identification of specific biomarker genes of SARS-CoV-2 in infected patient samples, and it generally takes 3–4 hours [5]. The available rtRT-PCR kits have probe sets for a gene to first detect all beta coronaviruses, which are considered screening genes, and another set of probes to specifically identify the SARS-CoV-2 virus, which is considered a confirmatory gene, which are *RdRp* gene, *ORF-1ab* gene, and specific part of *N* gene [5, 6]. The current outbreak of COVID-19 has placed a heavy burden on health facilities, resulting in delayed testing, and increased chances of the epidemic spreading. There is a need to screen infected patients for minimizing the transmission of the virus and initiating early curative interventions to reduce the risk of serious complications due to COVID-19 illness, preferably during the outbreak [5]. Therefore, in the present scenario, a rapid, inexpensive rtRT-PCR test is the need of the hour. In routine diagnostics, the swabs are preferably collected by clinicians in viral transport medium, followed by the isolation of genetic materials and the detection of specific 2019-nCoV (2019-novel coronavirus) genomic biomarkers by rtRT-PCR. So, existing rtRT-PCR methods typically rely on RNA extraction steps. Skipping the isolation of genetic materials step can reduce time and cost for rtRT-PCR testing [7]. Many ionic detergents [sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB)] and neutral detergents, such as Tween and Triton series detergents, are routinely used for the isolation of genetic materials from different sources [8]. However, ionic detergents were reported to inhibit the enzymes used in cDNA synthesis and real-time-PCR [9]. In the last few years, many researchers have used Tween-20 for direct detection of genes of interest in the recombinant host through colony PCR [10]. Many neutral detergents are also used in molecular techniques used for the isolation of nucleic acids and proteins from host cells. However, recovering a tiny amount of nucleic acid from patient samples is a big challenge. In this circumstance, RNA carriers play a vital role in enhancing the yield of nucleic acid [11]. Various RNA carriers have been employed in nucleic acids recovery. We contend that adopting RNA-extraction-free methods that are employed directly in the existing rtRT-PCR-based testing pipeline can save significant time and also be economical when dealing with pandemic viruses (Figure 1). Therefore, in this study, attempts were made to develop a direct lysis method for the rapid detection of the SARS-CoV-2 virus in infected patients. Various parameters have been optimized, and the role of carrier RNA in the recovery of RNA from the patient's swab was also determined. Our findings suggest that direct rtRT-PCR is a viable alternative to routine extraction-based COVID-19 diagnostics, based on comparisons with commercial RNA extraction kit diagnosed patient samples.

Methodology

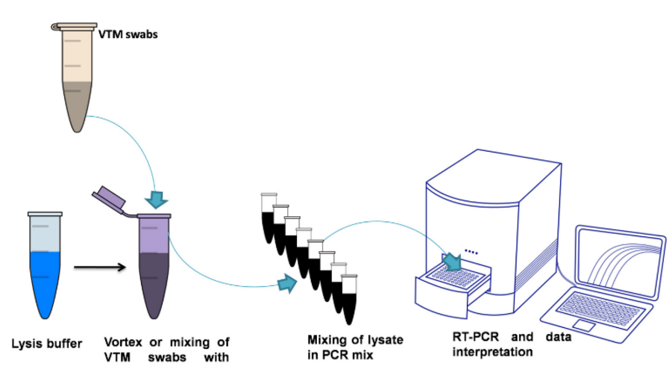
Sample characterization

Nasopharyngeal and oropharyngeal swabs were collected from different blocks and Civil Hospital, Palampur, India, as a part of routine COVID-19 testing. The swabs are transported to CSIR-IHBT testing facility in 3-mL Viral Transport Media (VTM) (TRIVITRON Healthcare System, Chennai, India) tubes. Safety measurements were properly taken, and all experiments were performed in a B2 cabinet (Esco Labculture Class II Type B2 biosafety cabinet) in the BSL-2+ facility at CSIR-IHBT, Palampur. RNA from VTM swabs was isolated using a commercial RNA isolation kit according to the manufacturer's protocol, and reverse-transcriptase real-time PCR was performed. Infected patients were classified based on Ct values of the SARS-CoV-2 gene obtained from routine rtRT-PCR testing. Furthermore, group 1 defined with a Ct value ≤ 25 and group 2 defined with ≥ 26–30 were characterized and experiments were performed to check the sensitivity and specificity of the method design.

Preparation of lysis buffer, swabs/lysis (V/V), and detergent ratio in lysis solution

Lysis buffers were prepared using 5 mM Tris-Cl (pH 7.2) and 1 mM EDTA (pH 8.0) and supplemented with different concentrations of detergents in preheated nuclease-free water (about 45°C) and vortex gently for a moment. For initial optimization, in determining the appropriate volume of VTM swabs in lysis solutions, the lysis solution was augmented with 0.1% Triton X-100 and 0.1% Tween-20, and various sample/lysis volume ratios (100:100, 100:300, 100:400, 200:100,200:400, 400:100, 400:200 in µL) were taken and incubated at room temperature for 15 minutes. For optimizing the concentration of detergents in lysis solutions, the lysis solutions were supplemented with different final concentrations of detergents in Triton X-100/Tween-20 ratios (0.1:0.1, 0.1:0.2, 0.1:0.4, 0.2:0.1, 0.2:0.3, 0.3:0.2; 0.4:0.1 in %), and incubated for 15 min at room temperature. To assess the effect of carrier molecules on rtRT-PCR efficacy, the lysis buffers were supplemented with final concentrations of glycogen (50, 100, 150, 200 µg/mL), LPA (linear

Figure 1. Schematic representation of Direct-lysis VTM-swabs rtRT-PCR method.



polyacrylamide) molecules (10, 15, 20, 25, 30, 40, 50 µg/mL) and Poly(A) carrier (5, 10, 15, 20 µg/mL) concentration as indicated in individual experiments.

Preparation of carrier molecule (linear polyacrylamide, LPA)

Linear polyacrylamide solution was prepared according to the protocol of Gaillard and Strauss, 1990 [12]. Briefly, 0.25 g acrylamide (Sigma Aldrich, USA) dissolved in 5 mL of 40 mM Tris-HCl (pH 8) (HiMedia Labs, Bangalore, India), 1 mM EDTA (Ethylenediaminetetraacetic acid) (HiMedia Labs, Banglore, India) and 20mM Sodium acetate (pH 7.5) (HiMedia Labs, Bangalore, India) followed by the addition of ammonium per sulphate (HiMedia Labs, Banglore, India) (0.1% final conc.) and 5 µL of TEMED (tetramethylethylenediamine) (HiMedia Labs, Bangalore, India) and allowed solution to polymerize for 30 min at RT. The viscous solution was further precipitated with 2.5 volumes of absolute ethanol. A large precipitated pellet was obtained after centrifugation, followed by washing with 70% ethanol and drying for 10 minutes at RT. The pellet was dissolved in 50 mL of Tris-EDTA buffer, pH 8. The final concentration was now to be 5 mg/mL.

Preparation of glycogen and Poly(A) carrier molecules

Ten micrograms per microlitre of glycogen were purchased from ThermoFisher Scientific, Massachusetts, USA. Poly(A) carrier RNA (Qiagen, Hilden, Germany) was dissolved in nuclease-free water to make final concentration 5 µg/mL.

rtRT-PCR

Reactions were carried out using a commercial COVID Sure kit (Lab system diagnostics, Trivitorn Healthcare, Bangalore, India), which contains both reverse transcription and DNA-polymerase enzyme activities. All reactions were carried out on 96-well plates in a CFX-96 Real-Time System (BioRad, Hercules, USA) and each plate included samples RNA, positive controls (synthetic viral RNA, supplied in kit, and a non-template negative control). Each 15 µL reaction consisted of 10 µL of 2× hydrolysis mixes, 2.0 µL of primer probe mix [*ORF-1ab (open reading frame-1ab)* -5′-FAM, E (envelope)- HEX, and Internal Control (*RNase P*)-ROX] and 3 µL of water as needed, 6.5 µL of lysate was added in the final reaction mix. The cycling profile PCR was performed on an automated system that involved reverse transcription at 46°C for 15 minutes, initial activation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 58°C for 30 seconds. Fluorescence was measured at the combined annealing–extension step, and results were interpreted using Bio-Rad CFX Maestro software (supplied with thermocycler). Cases were dichotomized as either positive (infected) or negative (uninfected) for SARS-CoV-2, based on Ct (cycle threshold) values and RFU (relative fluorescence units) curves of the confirmatory gene, *ORF-1ab*.

Determining the sensitivity and specificity of the nucleic acid extraction-free rtRT-PCR method:

The appropriateness of diagnostic methods is determined by measuring the sensitivity and specificity of testing procedures for the diagnosis of the disease. The sensitivity and specificity were calculated through a procedure defined by Genders *et al*., 2012 [13,14]. Briefly, test positivity (test+) and test negativity (test−) are defined based on the Ct values of routinely validated PCR protocol for the diagnostic of SARS-CoV-2. A positive or negative test result might be based either on one observation randomly picked from each patient or on a summary measure of all the observations. When it comes to COVID-19 disease, a patient's test result is normally classified as true-positive, if the validated real-time-PCR showed a substantial Ct value and RFU curve for a patient sample. If the validated real-time PCR showed no significant Ct value or RFU curve at all, but the method used showed significant values, the patient's test result is frequently counted as false-positive. If the validated real-time-PCR indicated any significant Ct value or RFU curve at all, but the method used yielded no significant results, the patient's test result is usually considered false-negative. If the number of patients with a true-positive, true-negative, false-negative and false-positive test and the result are respectively true positive, true negative, false negative and false positive, then the calculation for sensitivity and specificity is done as follows:

$$\text{Sensitivity} = \frac{\text{True Positive (TP)}}{\text{True Positive (TP)} + \text{False Negative (FN)}}$$

$$\text{Standard errors (SE)} = \sqrt{\frac{\text{Sensitivity (1-sensitivity)}}{\text{TP} + \text{FN}}}$$

$$\text{95\% CIs : sensitivity} \pm 1.96 \cdot \text{SE (sensitivity)}$$

$$\text{Specificity} = \frac{\text{True Negative (TN)}}{\text{False Positive (FP)} + \text{True Negative (TN)}}$$

$$\text{Standards errors (SE)} = \sqrt{\frac{\text{specificity (1-specificity)}}{\text{FP} + \text{TN}}}$$

$$\text{95\% CIs : specificity} \pm 1.96 \cdot \text{SE (specificity)}$$

Calculation and statistical analysis

The results were statistically analyzed using the GraphPad PRISM software. The student's t-test was used to determine statistical significance (p-value). p-values of less than 0.05 were considered statistically significant. *p value 0.05, **p value 0.01 and ***p value 0.001 were deemed statistically significant. DeLong methodology was used for Receiver Operator Characteristic (ROC) curve analysis using MedCalc software [15]. The diagnostic test's accuracy can be assessed using the AUC (area under the curve), and its values range from 0 to 1. A value of one (1) represents the highest level of test accuracy, whereas a value of zero (0) represents an erroneous test.

ROC curve was used to measure the AUC of diagnostic test with the following equation;

$$\text{AUC} = \int_0^1 \text{ROC}\ (t)\text{d}t$$

Where: ROC (t) represents sensitivity and t represents, 1-specificity. The area under ROC determined the accuracy of the test, a larger area representing a more accurate diagnostic test.

Ethics approval

Swab samples of patients were collected for routine COVID-19 testing purposes in Kangra district, Himachal Pradesh, India. The Institute Ethics Committee of CSIR-Institute of Himalayan Bioresource and Technology, Palampur, India, approved the collection of human swabs. All the experiments were done according to the Ethical Guidelines for Biomedical Research on Human Subjects, Indian Council of Medical Research and Government of India.

Results

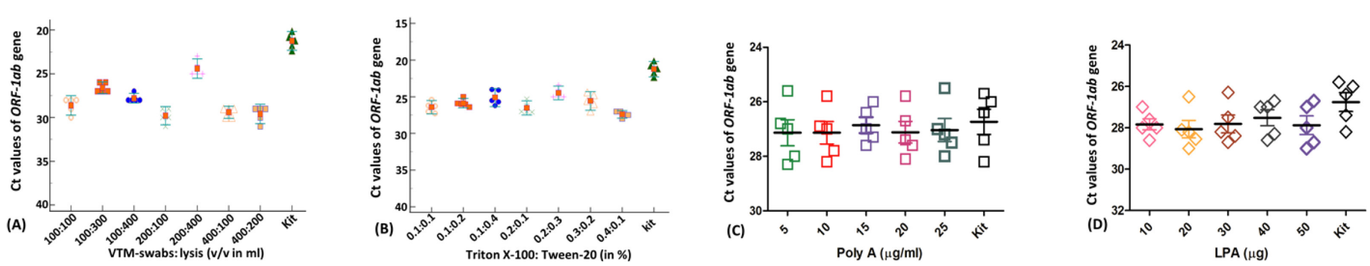
Effect of sample volume ratio and different concentrations of detergent on amplification of viral gene from infected VTM swabs

The efficacy of real-timePCR was initially determined by varying the sample/lysis ratio with 0.1% Tween 20 and 0.1% Triton X-100. At all V/V ratios, the *ORF-1ab* gene was amplified. Any decrease or increase in VTM swabs volume (mL) in VTM:lysis mixture resulted in a decrease amplification of *ORF-1ab* gene (Figure 2A). As a result, the conditions were optimized so that the maximum volume of VTM swabs was added in lysis to achieve the best result or viral gene amplification via real-time-PCR. Interestingly, the Ct values of the *ORF-1ab* gene showed higher amplification (Figure 2A) when the ratio was 1:2 (200 µL sample: 400 µL lysis). As a result, the 1:2 (200 µL sample: 400 µL lysis) ratio was chosen for further experiments (Figure 2A). PCR efficacy was also tested by varying the detergent concentrations in the lysis buffer. The addition of 0.3% Tween-20 and 0.2% Triton X-100 resulted in higher *ORF-1ab* gene amplification. However, a slight decrease in amplification was noticed with increasing concentrations of Triton X-100 detergents in lysis mixture (Figure 2B). However, the average Ct value difference between kit and lysis was 5, and the larger difference represents low efficacy in lysis-treated rtRT-PCR. Additionally, heat shock was applied to the lysate for 3 minutes at 80°C, but no changes were observed in the amplification of viral genes at any of the detergent combinations, swabs/lysis ratios, or incubation time points (data not shown). The internal control *RNAase P* (Ribonuclease P) gene was detected in all samples tested (not shown).

Use of Poly(A) in lysis solution

To enhance the PCR efficacy, different carrier molecules were supplemented in lysis, and amplification of the *ORF-1ab* gene was measured. The most widely accepted carrier molecule for the extraction of low amounts of RNA is poly(A). Poly(A) carrier RNA molecules range in size from 200 to 10,000 adenine base pairs. In this study, different

Figure 2. (A) Direct lysis-VTM swabs rtRT-PCR method depicted the effect of different sample/lysis volume ratio in amplification of confirmatory gene, *ORF-1ab* from infected swabs lysed in 1:1 with detergent lysis buffer for 15 minutes at room temperature. (B) Effect of various detergents ratio on the amplification of SARS-CoV-2 gene. (C) Direct lysis-VTM swabs rtRT-PCR method depicted the effect of different concentrations of Poly(A) carrier molecules in amplification of confirmatory gene, *ORF-ab* in infected swabs. (D) Effect of various concentration of LPA molecules on amplification of *ORF-ab* gene from infected swabs.



concentrations of poly(A) (5, 10, 15, 20 and 25 µg/mL) were used in lysis buffer, and amplification was noted. Inclusion of poly(A) molecules in lysis solution resulted in a sharp increase in the amplification of *ORF-1ab* gene in real time-reverse transcriptase PCR. Our result corroborated that the amplification of viral genetic material was obtained at all concentrations of poly(A). However, significant amplification was obtained with poly(A) carrier molecules at a concentration of 15 µg/mL (Figure 2C).

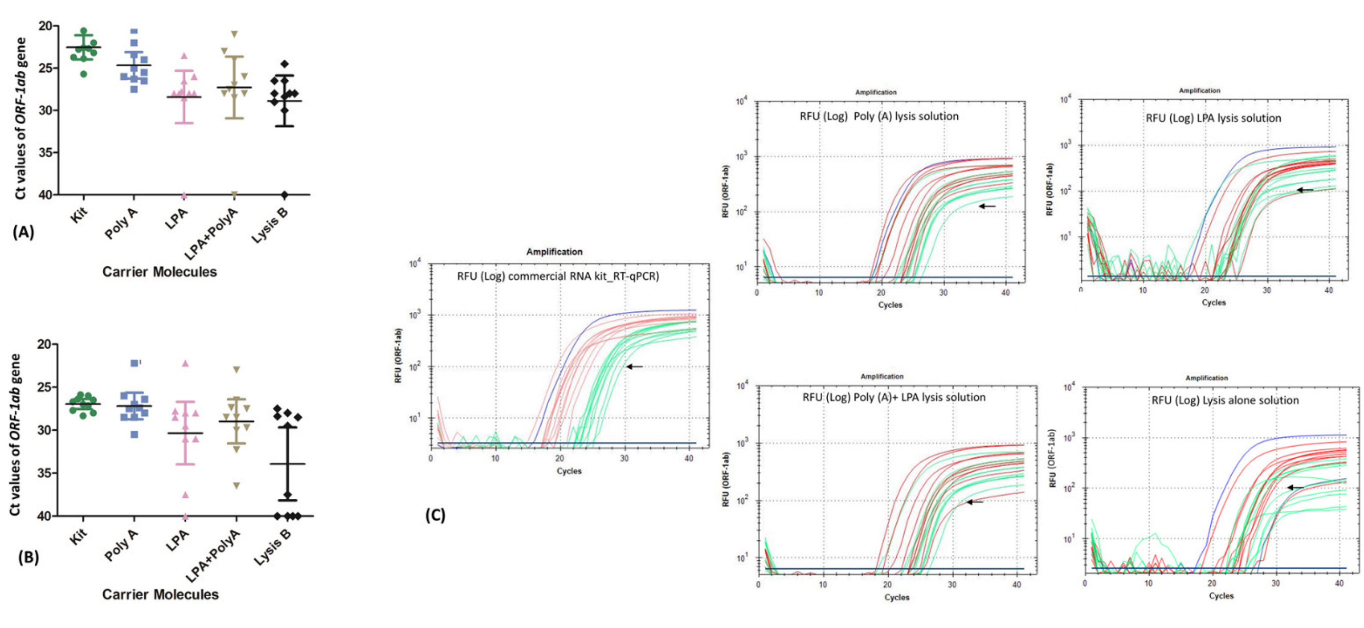
Use of LPA in lysis solution

Many studies have used linear polyacrylamide as a carrier molecule to improve the yield of low amounts of nucleic acids. The LPA molecules were synthesized, and their impact on viral gene amplification from VTM-swabs was noted in this study. Different concentrations of LPA molecules (10, 20, 30, 40 and 50 µg) were utilized in lysis, and the *ORF-1ab* gene was amplified at all concentrations of LPA. However, the maximum amplification was achieved with 40 µg of LPA molecule (Figure 2D).

Poly(A) and LPA enhanced the efficacy of real-time PCR for amplification of the ORF1-ab gene

Samples were classified based on Ct values obtained using a commercial RNA extraction kit-based routine diagnostic assay after optimizing the detergent concentration and V/V conditions. Group 1 is defined as having Ct values of *ORF-1ab* gene equal to or less than 25, indicating a high viral load in VTM, whereas group 2 is defined as having Ct values of *ORF-1ab* gene greater than 25 but not exceeding 30, indicating a relatively low viral load. A total 20 positive (infected) patients (10 each from groups 1 and 2) and 20 negative (uninfected) swabs were taken for the determination of specificity and sensitivity of the test. Our result corroborated that the carrier molecules improve PCR efficacy. In the case of group 1, the *ORF1-ab* gene was amplified in all cases, including lysis alone (with one false negative). The Ct values of the poly(A) lysis solution were comparable to those of the commercial RNA extraction kit, with noticeable RFU curves. The average Ct difference between LPA, poly(A)+LPA, and lysis treated samples was ±2 to ±3.5, indicating that the use of poly(A) in lysis solution is highly significant (Figure 3A and C). In group 2, all infected patient swabs were accurately detected in poly(A) solution, and the RFU curve and Ct values were comparable to those of the commercial RNA extraction kit, whereas 50% of swabs were identified as false negatives while using lysis buffer alone. In lysis containing LPA and poly(A)+LPA solutions only one false negative was observed (Figure 3B and C). The presence of clinical swabs in the VTM was validated by the detection of human gene (*RNaseP*/*IC*) amplification in all tested

Figure 3. Direct lysis-VTM swabs rtRT-PCR method depicted the effect of different carrier molecules in sensitivity of lysis PCR method. infected swabs samples were characterized on the basis on the Ct values for confirmatory gene *ORF1-ab* through routine diagnostic methods and experiments were performed (A) Effect of carrier molecules on sensitivity of PCR reactions on Ct value below 25 [group 1, Ct ≤ 25] (B) on Ct value above 25 [group 2, Ct ≥ 26-30]. (C) *ORF-1ab* gene amplification plots for the performed results (A) and (B); the back arrows indicate the threshold lines; the blue amplification plots correspond to positive controls (supplied in kits); the red amplification plots correspond to group 1 samples; and the cyan amplification plots for group 2 samples.

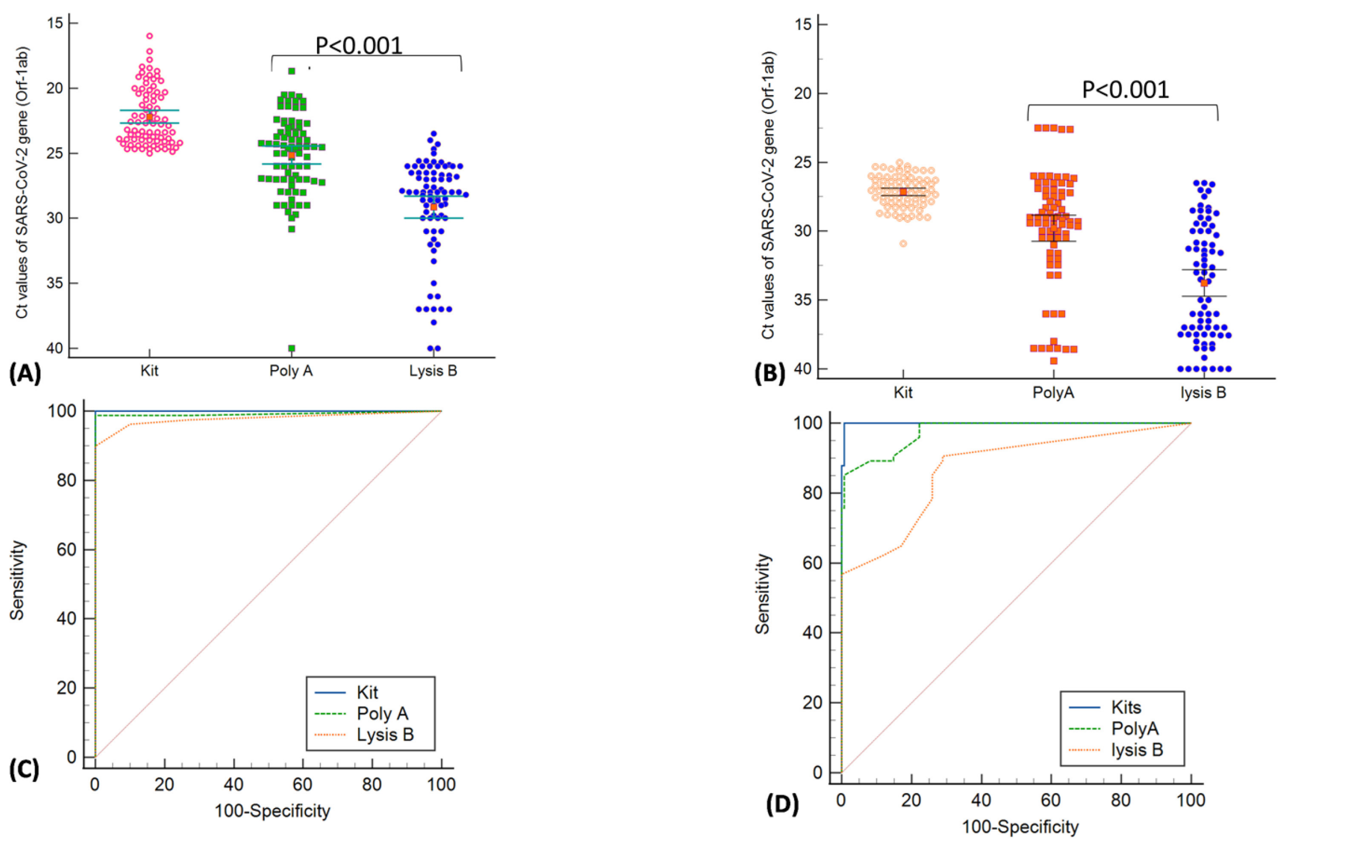


samples, regardless of whether the swabs were positive (infected) or negative (uninfected); otherwise, samples were deemed ineligible for method testing if no amplification (*RNaseP/IC*) was detected (data not shown).

Role of Poly(A) molecule in efficacy of real time-PCR for diagnostic purpose

In total, 150 infected patient swabs (75 each from groups 1 and 2) and 200 uninfected swabs (tested separately in each group) were taken for the determination of specificity and sensitivity of the method. All clinically diagnosed samples with Ct values ≤ 25 (group 1) were correctly identified when swabs were treated with the poly(A) lysis solution (Figure 4A). Our findings showed that the diagnosis of *ORF-1ab* genes in swab samples through the clinically diagnosed and tested poly(A) lysis solution method was comparable in group 1, apart from one sample detected as negative in poly(A) lysis direct rtRT-PCR. In group 1, the mean Ct differences of clinically diagnosed samples with poly(A) solution and lysis alone were ± 2.5 and ± 5.0, respectively. The poly(A) solution performed well on both high and low viral load samples with clinical values of Ct ≤ 25 (74/75 positives detected), and those with Ct ≥ 26–30 (64/75 positives detected). The lysis alone also executed well on high viral load samples with clinical values of Ct ≤ 25 (62/75 positives detected), but not on those with Ct ≥ 26–30, only 42 positives detected out of 75 (Figure 4 A and B). Both groups 1 and 2 demonstrated 100% specificity in poly(A) and lysis alone solution, the sensitivity was

Figure 4. Direct lysis-VTM swabs rtRT-PCR method described the diagnostic significance of poly(A) lysis method (A) Determination of diagnostic ability of poly(A) solution in amplification of confirmatory gene of SARS-CoV-2 in group 1 swab samples (Ct ≤ 25) (B) group 2 swab samples (Ct ≥ 26-30). ROC curve analyses were showing the sensitivity and specificity of the direct VTM swab lysis method for all infected and uninfected samples (C) group-1 swab samples (Ct ≤ 25), (D) group-2 swab samples. Statistical analysis was assessed for using student's *t*-test.



Variable	AUC	SE [a]	95% CI [b]
Kit	1.000	0.000	0.980 to 1.000
Poly_A	0.992	0.00798	0.965 to 0.999
Lysis_B	0.978	0.0116	0.945 to 0.994

[a] DeLong et al., 1988
[b] Binomial exact

Variable	AUC	SE [a]	95% CI [b]
Kits	0.999	0.000944	0.981 to 1.000
PolyA	0.976	0.00819	0.946 to 0.992
lysis_B	0.870	0.0258	0.817 to 0.913

[a] DeLong et al., 1988
[b] Binomial exact

slightly higher in the case of group 1, and it was 98.6% (95% CI: 96.0, 101.17) vs 87.2 (95% CI: 80.2, 94.0) for poly(A) solution, while in lysis alone, it was 85% (95% CI: 77.8, 92.6) vs 67.2% (95% CI: 75.7, 58.7), indicating that the sensitivity in poly(A) solution was significant (Table 1). The internal control *RNAse P* gene was detected in all tested samples (not shown); Similarly, Receiver Operator Characteristic (ROC) curve analyses also revealed that the carrier molecules outperformed the lysis alone method ($p = 0.005$ for both groups, Figure 4). In group 1, the AUC values for carrier molecules and lysis alone samples were 0.992 and 0.978, respectively, which was highly significant. The AUC values between 0.9–1.0 and 0.8–0.9 were found to be in the range of excellent and good accuracy classifications for a diagnostic case. In case of low viral load, group 2, it was found to be 98.6% and 87.2% for poly(A) and lysis solution. The result clearly showed that the poly(A) solution had excellent accuracy for both groups. No signal of *ORF1-ab gene* was noted in negative samples (data not shown).

Discussion

Different detergents have been used to resolve different scientific issues for several decades. However, detergent characteristics can play a key role in their usage in various research. The most frequent detergents used for nucleic acid isolation from the prokaryotic to the eukaryotic system are SDS and CTAB [16]. Nevertheless, the use of SDS and CTAB detergents cannot be advised, as ionic detergents might bind to enzymes employed in PCR and impede their activity [17]. However, a low concentration of theses detergents has been recommended in a few studies [17-18]. Few studies have shown that SARS-CoV-2 can be directly detected from patient samples, albeit this is usually accompanied with decreased viral detection compared to that of RNA extracted realtime-PCR, which can be addressed at least in part by heat and/or detergent lysis [19-23]. In this present study, Triton X-100 and Tween 20 were used in lysis buffer and the RNA extraction process was circumvented. The sample swabs were thoroughly mixed with lysis buffer, and lysate was then subjected to rtRT-PCR for the confirmation of viral genetic materials. Our result corroborated that the 1:2 swab/lysis ratio resulted in optimal amplification, while increasing the VTM-swabs volume resulted in low amplification, indicating that the VTM swabs are deleterious for PCR. Our findings confirmed that increasing the concentration of Triton X-100 in the lysis solution resulted in low amplification for the viral gene. Our findings are consistent with the study of Craig *et al*., (2022), who used lysis buffers supplemented with IGEPAL CA-630, 1 or 10% Triton X-100, or 5% Tween-20 [21]. Virus lysis buffer was thoroughly mixed with VPM (containing viruses) by pipetting at a 1:1 ratio and incubated at room temperature for 20 minutes. Detergent lysis buffers containing Triton X-100 (1%), Tween-20 (5%), and IGEPAL CA--630 (0.25%) showed comparable sensitivity with the purified viral RNA (Ct 14.75–15.01) [21]. However, the qPCR reaction was hampered with 10% Triton X-100 and 5% Tween-20 buffers, which consistently resulted in lower fluorescence [21]. A study of Smyrlaki *et al*., 2020 showed that the Ct values were only slightly affected (+1–2 Ct) in the presence of high concentrations of detergents [Triton X-100 (5%) or Tween-20 (10%)]. However, at higher detergent concentrations, a lower level of fluorescence was observed [25].

Viral genetic material is diluted in VTM carrying swab samples, and subsequent dilution in lysis solution may further lower the amount, making it difficult to detect through direct rRT-PCR. Therefore, various RNA carrier molecules were utilized in this study to enhance the PCR efficacy. The presence of carrier molecules resulted in significantly increased in sensitivity. Molecular-grade glycogen is frequently employed for the isolation of genetic material to recover nanogram or picogram amounts of nucleic acid [26, 27]. It is an inert carrier that is preferably used to increase nucleic acid recovery from alcohol precipitation. In our investigation, glycogen was used as a carrier molecule in the lysis mixture and the sensitivity of the reaction was analyzed. The presence of glycogen in the lysis buffer resulted in increased efficacy of rtRT-PCR, compared to the lysis alone. However, the results were not statistically significant (data not shown). Linear

Table 1. Determination of sensitivity and specificity of the diagnostic method using infected patients' swabs.

	Sensitivity (% ± SE)	95% CI	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
Carrier (Poly A)						
Ct ≤ 25	98.6 ± 1.3	96.0-101.17	100	100	99.50	99.40
Ct ≥ 26-30	87.2 ± 3.6	80.2-94.0	100	100	94.78	94.00
Lysis alone						
Ct ≤ 25	85.2 ± 3.8	77.8-92.6	100	100	93.89	93.50
Ct ≥ 26-30	67.5 ± 4.3	75.7-58.7	100	100	89.68	82.90

polyacrylamide (LPA) and poly(A) are commonly used for the co-precipitation of minute quantities of DNA, and we speculated that it might enforce the recovery of low amount levels of SARS-CoV-2 genetic material from the swab samples. Linear polyacrylamide (LPA) intermixes with the sample significantly enhances the recovery of nucleic acids [28]. Li *et al*., 2020 demonstrated that the incorporation of glycogen or LPA with either ethanol or isopropanol resulted in higher recovery rates of all types of nucleic acids than their counterparts without co-precipitator [29]. PCR product with ethanol was recovered at 72% with either glycogen or LPA, compared to 63%, when carriers were not present. Similarly, isopropanol recovered 65% with glycogen and 67% with LPA, and achieved higher recovery levels than PCR products containing no co-precipitators (54%) [26]. Our result corroborated that the addition of LPA molecules in lysis buffer resulted in improved efficiency of SARS-CoV-2 rtRT-PCR.To achieve higher amplification with low viral load samples, poly(A) was utilized in the lysis mixture. Our result corroborated that the presence of poly(A) in lysis buffer resulted in significant improvement in the Ct values of the *ORF-1ab* gene, and lysis buffer containing 15 µg/mL of poly(A) was found to be superior among all other concentrations and other carrier molecules . Our findings are consistent with the findings of Shaw *et al*., 2009, which showed that adding poly(A) carrier RNA to the chaotropic salt solution resulted in a noticeable increase in the recovery of low amounts of DNA, up to 25 ng, when compared to the absence of RNA carrier in a silica-based monolith [30]. The ratio of carrier molecules to DNA has a significant impact on yield, as demonstrated by the fact that a ratio of 50 ng carrier poly(A)/DNA (1 ng) yielded a higher DNA extraction ratio [30]. Our present study corroborated that the poly(A) solution correctly identified all clinically diagnosed infected patients with significant Ct values and an RFU curve. The poly(A) system demonstrated superior performance compared to the other carrier molecules. To validate the adopted method, a large number of patient swabs (clinically diagnosed) were used to test the method's efficacy. The real-time PCR efficacy in poly(A) lysis attained 98% sensitivity and 100 % specificity with high viral load samples, in contrast to the 87% sensitivity when carriers were not added. Similarly, real-time PCR efficacy achieved higher sensitivity levels in low Ct values groups when incubated with poly(A) lysis (85%) compared to lysis alone (67%). The specificity and PPA remained the same in all tested groups. However, the negative predictive values differed in the tested groups due to the confirmation of false negative test. Lysis alone resulted in lower NPA values than Poly(A)-lysis, indicating a higher likelihood of a negative screening test for diseased individuals. Receiver Operator Characteristic (ROC) curve analyses clearly showed that the AUC values for poly(A) solution were found to be in the range of excellent accuracy classification for a diagnostic case. Our finding showed that all clinically diagnosed infected patients with high virus loads were correctly detected by the poly(A) lysis solution. Poly(A) lysis was found to have much higher sensitivity and specificity than lysis alone with both high and low viral load swabs. Overall, we attempted to detect SARS-CoV-2 without the need for nucleic acid extraction. This method may lower the costs, increase throughput, and eliminate the need for RNA extraction systems, which may be in short supply during a pandemic or a COVID-19 disease wave. Even though the data is limited, our findings suggest that this method is feasible and can be employed for one-step real-time PCR detection of SARS-CoV-2 infection. The very low viral load samples with Ct values over 30 were excluded from this study, as the sensitivity of the method decreased with low viral load samples, The dilution factors can have a significant impact on this procedure, which can be further assessed by estimating the limit of detection. Swabs with very low viral loads can be more sensitive if added directly to the lysis solution rather than viral transport media.

Conclusions

Globally, there have been few attempts to develop a single-step PCR method for the detection of SARS-CoV-2 infection, with the goal of limiting the infection at the epidemic stage by massive screening in the shortest amount of time. This study also includes a cutting-edge method that can support the feasibility of one-step real-time reverse transcriptase PCR method for diagnosis of pathogens from the clinical samples. This is perhaps the first report in which SARS-CoV-2 viral genes were identified through rtRT-PCR directly from the VTM-swabs. Neutral detergents are the key components of lysis buffers that can be used directly in the development of single-step assays. The lysed swab samples could be immediately subjected to rtRT-PCR analysis, with no further intervening steps. Although VTM-swabs typically contain trace amounts of viral genetic material, adding RNA carrier molecules improves rtRT-PCR sensitivity even in low viral load samples. Poly(A) carrier molecules were among the most effective carriers for recovering a small amount of genetic material. This method saves a pre-PCR processing time of 1 to –1.5 hours or more, depending on the number of samples, this method could save time and enable large-scale massive screening of samples in a minimal time. The cost of pre-PCR processing would be also economical and can be valuable for the developing nations. Our study concluded that the direct rtRT-PCR pipeline for COVID-19 testing might be made even more efficient by sampling directly into a designed lysis buffer from this study.

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Authors' contributions
AK:; Conceptualization, study designed, performed experiments and wrote manuscript; AK and YP: scientific discussions, data analysis and manuscript editing; SS: characterization and and classification of samples, SK: main conceptual idea, scientific discussions and conceived this the study.

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Declarations
Research involving human participants and/or animal : The Institute Ethics Committee of CSIR-Institute of Himalayan Bioresource and Technology, Palampur, India, approved the work on human swab samples. All the experiments were done according to the Ethical Guidelines for Biomedical Research on Human Subjects, Indian Council of Medical Research, Government of India.
Informed consent: Not applicable; nasopharyngeal and oropharyngeal swabs samples were collected from different blocks and Civil Hospital, Palampur, as a part of routine COVID-19 testing.

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Conflict of interests
No conflict of interests is declared.

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