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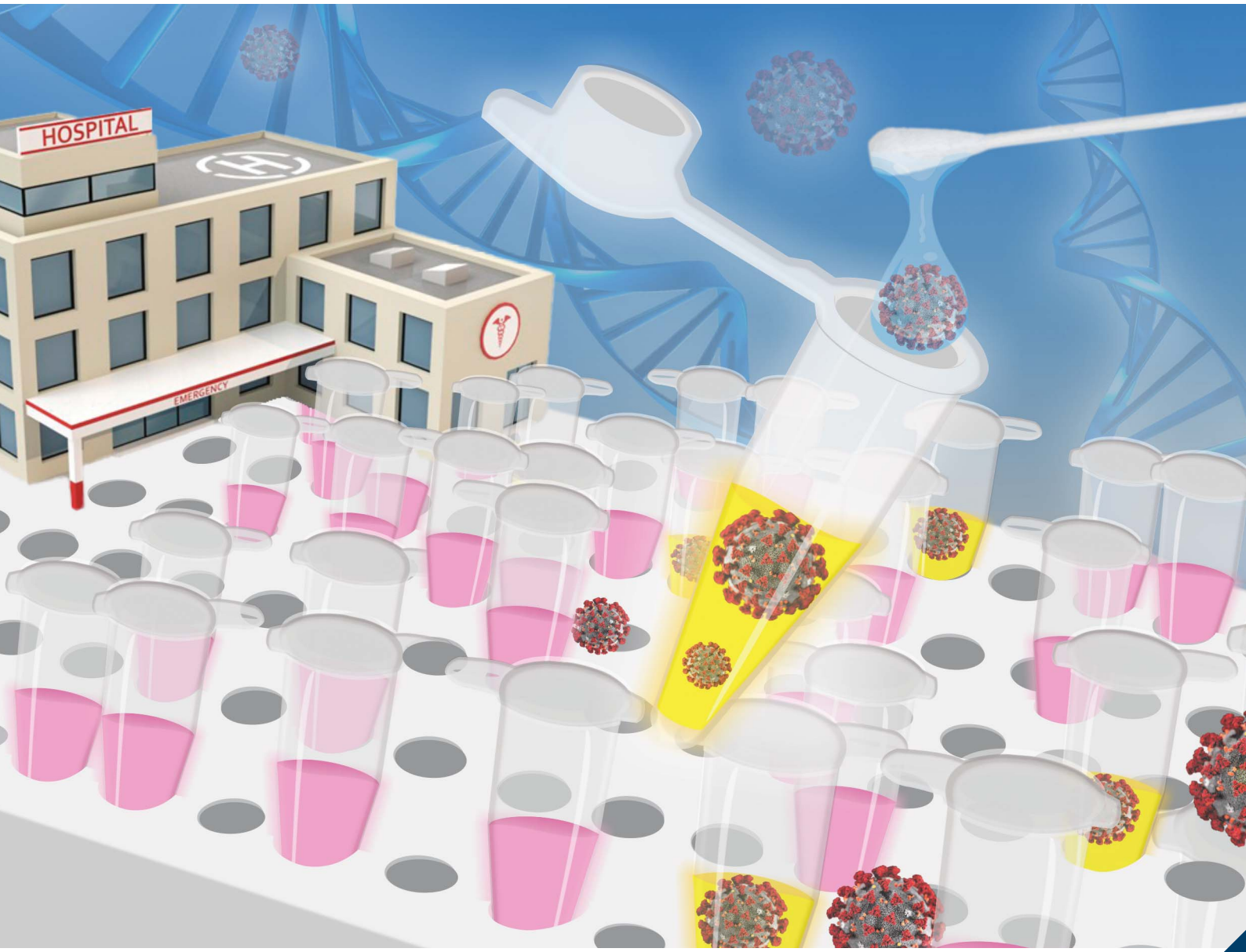

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PAPER

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Can a field molecular diagnosis be accurate? A performance evaluation of colorimetric RT-LAMP for the detection of SARS-CoV-2 in a hospital setting†

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SARS-CoV-2 currently represents a serious global public health problem. Non-pharmaceutical intervention measures (NPIs) have been widely adopted, and the testing strategy since the beginning of the infection is the most effective tool for tracking, isolating, and minimizing transmission. The high operating costs and the need for sophisticated instrumentation related to gold standard diagnostic for COVID-19, Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR), have highlighted the urgency and importance of developing and applying new diagnostic techniques, especially in places with scarce resources. Thus, alternative molecular tests, such as Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP), based on isothermal amplification have been used to detect SARS-CoV-2 using different protocols. The potential for field application of RT-LAMP is due to the lower cost and time and not requiring high-cost instrumentation. Here, we evaluate the colorimetric RT-LAMP to detect SARS-CoV-2 in a hospital environment and correlate its performance with tests performed in a reference laboratory. The analysis performed at the hospital showed high sensitivity (88.89%), specificity (98.55%), accuracy (95.83%), and a Cohen's kappa of 0.895. However, we achieved 100% of agreement when comparing the RT-LAMP results with the gold standard (qRT-PCR) results for samples with Ct < 30 in the hospital-based test. In addition, a similar performance was found in the field compared to the reference laboratory, corroborating the proposal to apply the test directly at point-of-care.

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Introduction

Since 2020, the world has experienced a serious public health crisis caused by the rapid spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Once infected with SARS-CoV-2, patients can develop COVID-19, a respiratory syndrome that is, in mild cases, characterized mainly by cough, fever, tiredness, and loss of taste or smell. In severe cases, symptoms can evolve to shortness of breath and pneumonia, and medical assistance is required.[1,2] Therefore, it is essential to correctly diagnose infected patients so that they can be isolated and properly monitored. This measure can decrease the infection rate, thus avoiding hospital overload and reducing the number of new infections and deaths.[3]

The current SARS-CoV-2 diagnostic tools available are based on the detection of human antibodies,[4] viral antigens,[5] and viral nucleic acid.[6] The tests based on the identification of viral nucleic acid, such as RT-qPCR, are considered the "gold standard" and offer a sensitive and early detection of SARS-CoV-2.[7] The RT-qPCR is able to quantify the viral load that is determined by the value of the cycle threshold (Ct). The Ct is a parameter that determines the start of the exponential amplification, when the signal of the samples differs from the NTC (no template control) signal. Thus, the higher the viral load, the faster the amplification will occur and, therefore, the lower the Ct value, allowing the amplification time to be correlated with the viral RNA quantity. Although a very sensitive technique, RT-qPCR requires high-cost instrumentation and highly trained personnel.[8] In developing countries, these requirements are fatal drawbacks for mass testing, as laboratories suitable for performing RT-qPCR are usually located in larger

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cities. As a consequence of the lack of testing, there is under-reporting in the number of cases, and the real scenario is unknown.[9]

Alternative protocols for fast, inexpensive, easy to implement, and reliable molecular diagnostics are a current need. Among the new proposals, isothermal amplification methods such as LAMP (loop-mediated isothermal amplification) have emerged as suitable options that overcome the difficulties found in performing RT-qPCR.[10] Mautner *et al.* (2020) described the detection of SARS-CoV-2 applicable in point-of-care situations using a RT-LAMP methodology.[11] The authors reported that their assay is 12 times faster and 10 times cheaper than routine reverse transcription real-time polymerase chain reaction, depending on the assay used. Rodriguez-Manzano and co-workers (2021) reported the development of a diagnostic test applicable in the point of care, detecting SARS-CoV-2 RNA under 20 min using semiconductor technology.[12] Wei *et al.* (2021) recently described an RT-LAMP colorimetric test directly on clinical samples of nasopharyngeal swab in viral transport medium with 85% positive percentage agreement and 100% negative percentage agreement. The authors highlight the good cost-effectiveness and short time (30 min) without the need for specialized or proprietary equipment or reagents.[13]

Since it is not strongly affected by inhibitors, a variety of biological samples, such as serum,[14] whole blood,[15] urine,[16] oral fluid,[17] and semen,[18] can be used in the LAMP assay without the prior need for nucleic acid extraction. For these reasons, it can be applied in situations and places with scarce resources since it does not require sophisticated equipment or laborious stages of sample preparation.[19]

During the pandemic, due to the increase in demand that exceeded hospital capacity, significant delays between hospital admission, sample collection, and final diagnosis have been evidenced. This delay is likely influenced by the logistic difficulties in transporting patient samples to centralized laboratories for RT-qPCR testing. Point-of-care testing significantly lowers time to result, and it is an interesting approach that improves patient flow.[20] A rapid, sensitive, and low-cost test to detect positive cases that is applicable at point-of-care could assist in improving the response measures to halt the spread of the virus. In addition, the test performed in a hospital setting would be able to quickly guide treatment for infected patients, ensure that non-COVID-19 patients are not infected within the hospital facilities due to delay in diagnosis, and shorten the time between admission and diagnosis.

Although several studies have demonstrated the potential applicability of the RT-LAMP technique in the field,[10,21,22] such application to SARS-CoV-2 field detection is still poorly documented. Thus, in this present study, we evaluate the performance of RT-LAMP applied in a reference laboratory and the hospital to detect SARS-CoV-2. The colorimetric RT-LAMP was evaluated against RT-qPCR by testing 206 clinical specimens provided by the Hospital do Policial Militar, Goiás-Brazil. The successful performance of the colorimetric RT-LAMP for detecting SARS-CoV-2 in both environments demonstrated a great potential for point-of-care applications.

Experimental

Biological samples and thermal treatment after swab collection

Nasopharyngeal samples of 206 patients suspected of COVID-19 admitted at the Hospital do Policial Militar during December 2020 and January 2021 were collected using swabs and stored in a cryotube containing 1 mL 0.9% NaCl solution. To inactivate the virus and lyse some of the sample content, all samples were heat-treated in a thermoblock at 95 °C for 10 min before testing. A total of 110 samples were analyzed at the reference laboratory, and 96 samples were analyzed directly at the hospital.

The study was conducted after approval by the Research Ethics Committee of the Federal University of Goiás, with protocol number no. 4.111.485. All experiments were performed in compliance with nationally required guidelines, following the resolutions CNS 466/12 and CNS 441/11, and in compliance with institutional guidelines. All patients agreed to participate in the study with a written statement, according to local regulations.

Colorimetric detection RT-LAMP for SARS-CoV-2 and RNase P

RT-LAMP was performed using the WarmStart Colorimetric LAMP 2× Master Mix (New England Biolabs, Hitchin, UK), according to the manufacturer's instructions with minor modifications. The final reaction volume was 15 μL, consisting of 7.5 μL LAMP master mix, 1.5 μL 10× primer mix (2 μM of each outer primer [F3 and B3], 16 μM of each inner primer [FIP and BIP], and 8 μM of each loop primer [LF and LB]), 4.5 μL nuclease-free water [Sigma Aldrich, Missouri, EUA], and 1.5 μL of the heat-treated sample. The primers used to detect SARS-CoV-2 (Table 1) were previously described by Lamb *et al.*[23] and correspond to the region of the open reading frame (ORF) 1Ab.

Table 1 Primer sequence used in study

SARS-CoV-2 [23]		RNAseP [24]	
Primers	Sequence (5′ a 3′)	Primers	Sequence (5′ a 3′)
F3	TCCAGATGAGGATGAAGAAGA	F3	TTGATGAGCTGGAGCCA
B3	AGTCTGAACAACTGGTGTAAG	B3	CACCCTCAATGCAGAGTC
FIP	AGAGCAGCAGAAGTGGCACAGGTGATTGTGAAGAAGAAGAG	FIP	GTGTGACCCTGAAGACTCGGTTTTAGCCACTGACTCGGATC
BIP	TCAACCTGAAGAAGAGCAAGAACTGATTGTCCTCACTGCC	BIP	CCTCCGTGATATGGCTCTTCGTTTTTTTCTTACATGGCTCTGGTC
LoopF	CTCATATTGAGTTGATGGCTCA	LoopF	ATGTGGATGGCTGAGTTGTT
LoopB	ACAAACTGTTGGTCAACAAGAC	LoopB	CATGCTGAGTACTGGACCTC

The RT-LAMP reactions for SARS-CoV-2 detection were incubated at 65 °C for 30 min in a thermoblock (Kasvi, Paraná, BR). The primers used to detect the human RNAse P gene as an internal control were described previously by Curtis *et al.*[24] The reactions for endogenous control were incubated at 60 °C for 50 min. The temperatures for each test have been optimized and changes in the incubation temperature can result in reduced efficiency.

The primers were synthesized by Integrated DNA Technologies (IDT, Iowa, USA), and sequences are available in Table 1. At the end of the amplification time for each primer set, all tubes were removed from the thermoblock and placed over sheets of white paper for image acquisition using a smartphone camera (Redmi Note 8, Xiaomi). The samples' positivity was determined by observing the change in color from pink (negative sample) to yellow (positive sample). In all tests, negative (blank) and positive (SARS-CoV-2 synthetic target) controls were used.

Sensitivity of the SARS-CoV-2 RT-LAMP assay

To evaluate the limit of detection (LoD) of the colorimetric SARS-CoV-2 RT-LAMP, a synthetic DNA (gBlock) containing the target sequence was homogenized in RNA-free water and inserted in the master mixture to obtain dilutions of 10 000, 1000, 500, 250, 125, 100, and 75 copies per reaction. The LoD in matrix presence was determined considering eight independent replicates, and probit analysis was performed with gBlock spiked in negative swab samples to obtain dilutions of 10 000, 1000, 500, 250, 125, 100, and 75 copies per reaction. In the real samples, the RT-LAMP assay successfully amplified the target directly in the nasopharyngeal swab sample without the RNA purification step. The cycle threshold (Ct) values of swab samples considered positive by the RT-qPCR assay (≤40) were compared to the performance of RT-LAMP. The RT-LAMP-amplified products were determined by visual observation and gel electrophoresis (2% agarose and 0.5% Tris–EDTA–borate (TEB) buffer), revealed with GelRed-Biotium® in a UV trans-illuminator coupled to a photo documentation system.

RT-qPCR assay

The RNA of all samples was extracted using a QuickExtract RNA Extraction Kit (Lucigem, Wisconsin, EUA) according to the manufacturer's instructions and analyzed by RT-qPCR using the GoTaq probe 1-step RT-qPCR system (Promega, Wisconsin, USA) performed in an AriaMx Real-Time PCR System (Agilent, Califórnia, EUA). The total reaction volume was 10 μL (5 μL GoTaq Probe qPCR Master Mix, 0.2 μL Go Script RT Mix for 1-Step RT-qPCR, 1.55 μL nuclease-free water, 0.75 primers/probe, and 2.5 μL of RNA). The primers/probes used for detection targeted two coronavirus regions (N1 and N2) and human RNase P (internal control) were provided by Integrated DNA Technologies (IDT, Iowa, USA). The amplification program consisted of one cycle at 45 °C for 15 min for reverse transcription, one cycle at 95 °C for 2 min for reverse transcriptase denaturation and DNA polymerase activation, followed by 40 cycles at 95 °C for 3 s, and 55 °C for 30 s for denaturation and amplification.

RT-LAMP for the detection of SARS-CoV-2 in the hospital setting

The tests carried out in a hospital setting were performed with the support of a central laboratory, which was used to prepare the master mix. This mixture was then transported in a cooler support to the hospital facility. For the second stage, two different rooms were used inside the hospital. In the first room, one thermoblock was set at 95 °C for sample inactivation and release the RNA. After this processing, the samples were pipetted into both the SARS-CoV-2 and RNaseP RT-LAMP reagent mixtures. In the second room, one thermoblock was set at 65 °C for the SARS-CoV-2 reaction and the other was set at 60 °C for the RnaseP reaction.

Statistical validation

To calculate the limit of detection of RT-LAMP for SARS-CoV-2, we performed a probit regression using MedCalc software (Version 19.6.4, MedCalc Software, Ostend, Belgium). Eight independent replicates were performed for each RNA concentration, the data (concentration, number of replicates, number of positive results and hit rate) were input to the MedCalc software and the C95 value was obtained (concentration detectable 95% of the time), indicating the limit of detection.

To evaluate sensitivity, specificity, positive and negative predictive values, and test accuracy, the results obtained by RT-LAMP were compared with the results obtained by RT-qPCR. For simplicity, we used a MedCalc's Diagnostic Test Evaluation Calculator (available online at https://www.medcalc.org/calc/) to calculate specificity, sensitivity, negative predictive values, positive predictive values and accuracy. In addition, the mathematical equations are found in the ESI.† The four variables used for statistical analysis are (a) the number of samples positive by both RT-LAMP and RT-qPCR, therefore referred to as "True Positive", (b) the number of samples that were negative by RT-LAMP and positive by RT-qPCR, therefore referred to as "False Negative", (c) the number of samples that were positive by RT-LAMP and negative by RT-qPCR, therefore referred to as "False Positive" and (d) the number of samples that were negative by both methods, therefore referred to as "True Negative". The sensitivity of the test is determined by: $a/(a + b)$. Prevalence is the ratio between the number of samples positive by RT-qPCR $(a + b)$ and the total number of samples $(a + b + c + d)$. The specificity is determined by $d/(c + d)$. Accuracy is calculated by: sensitivity × prevalence + specificity × (1 − prevalence).

In order to assess the agreement between the two alternative techniques employed in this study, we calculated Cohen's kappa and p values using RStudio version 3.4.4 and the IRR library (available at: https://www.R-project.org/).

Results

Protocol for RT-LAMP application under laboratory and field setup

In this study, we established guidelines executing RT-LAMP for the correct diagnosis. The schematic overview of the SARS-CoV-2 RT-LAMP procedure is shown in Fig. 1.

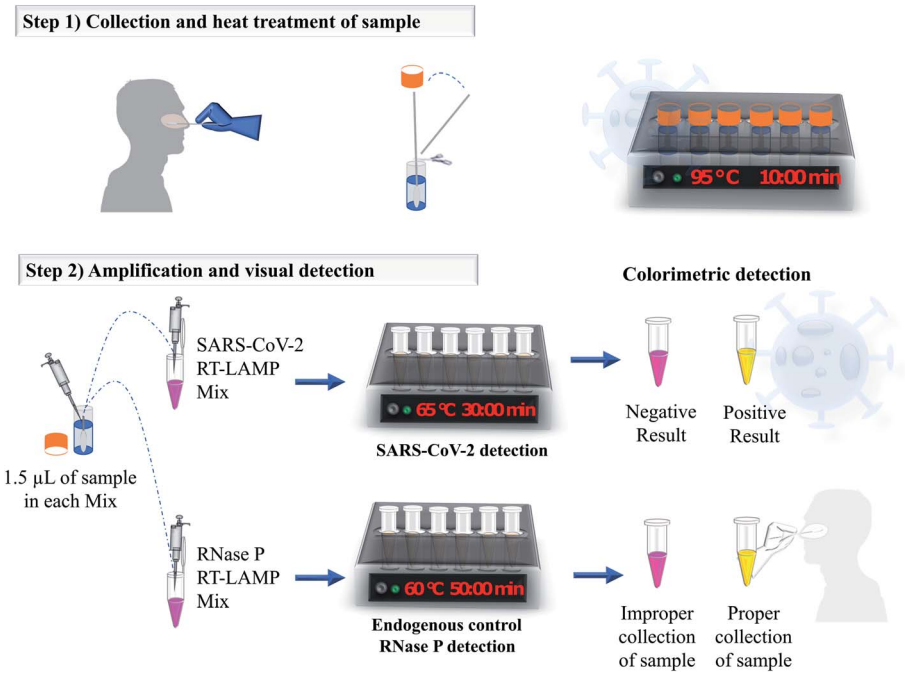


Fig. 1 Schematic overview of the RT-LAMP procedure. Step 1: swab collection and heat treatment of the samples. The nasopharyngeal sample was collected using a swab and stored in a cryotube in 1 mL 0.9% saline solution. The samples were submitted to 95 °C for 10 min for heat extraction and viral inactivation. Step 2: amplification and visual detection; 1.5 µL of sample was added to two mixtures, each containing either the SARS-CoV-2 or RNAse P primers, water, and WarmStart Colorimetric LAMP 2× Master Mix and then incubated in two thermoblocks; one for 30 min at 65 °C for SARS-CoV-2 detection, and the other for 50 min at 60 °C for RNase P detection in collected samples. The positivity of the samples was determined by observing the change in color from pink (negative sample) to yellow (positive sample). Changes in incubation temperatures for the detection of RNase P and SARS-CoV-2 can result in low efficiency in amplification so they should be maintained as previously described.

For the laboratory tests, the RT-LAMP mix, sample processing, addition, and amplification were performed in different rooms. The access to the amplification room is limited, and individuals involved in the pre-amplification workflow did not access the area due to the high risk of carryover contamination of LAMP amplicons.

The room set up for the RT-LAMP application at the hospital was also optimized in two rooms. Since the mix was previously prepared in a laboratory, the first was destined for viral inactivation of the samples and addition of the target in the reaction, and the second room was used only for the amplification process. Even though the amplification of RT-LAMP products is done in a separate and closed room, the reaction tubes should never be opened. This was to help mitigate false-positive results in the target-free reactions due to carryover contamination. The RT-LAMP reagents were prepared on the day of the experiment in a support laboratory (the same laboratory used for carrying the tests in this validation study), placed in a cooler rack, and then taken to the hospital to perform the test.

It is important to note that all samples were subjected to the inactivation process for 10 min at 95 °C to ensure safety during manipulation. Other biosafety procedures, such as the use of masks, lab coats, hair caps, facial protectors, and the constant disinfection of countertops, were also implemented and considered essential steps in this study's execution.

To assess the integrity and presence of human genomic material in the swab samples, we assayed a LAMP reaction targeting the human RNase P gene as an endogenous control, and the positive diagnostic for COVID-19 was determined using a combination of RNase P and SARS-CoV-2 detection. Failure to amplify the RNase P reaction in any circumstance would invalidate the test, and recollection would be advised. All 206 nasopharyngeal swab samples showed positive results for endogenous control, not rendering any of them useless.

Limit of detection of RT-LAMP for SARS-CoV-2 colorimetric detection

In reactions containing the SARS-CoV-2 synthetic target, the detection limit found by colorimetric RT-LAMP was 125 copies per reaction for visual detection, with the possibility of detecting up to 100 copies per reaction using the gel electrophoresis (Fig. 2). However, the additional step for gel electrophoresis detection is not worthwhile for rapid tests since both methods have very close detection limits of SARS-CoV-2 RNA.

The probit regression analysis (Fig. 3A) showed that the detection limit within 95% reliability was 255 copies per reaction of the SARS-CoV-2 RNA, with a confidence interval of 221-338 copies per reaction (Fig. 3B). The detection rate in simulated infected clinical samples of nasopharyngeal swabs reduced to 50% success in samples with 200 copies per reaction in the presence of the biological matrix. However, a 100% detection rate was found when the reaction had an initial concentration of 250 copies per reaction, directly in the nasopharyngeal swab (Fig. 3C).

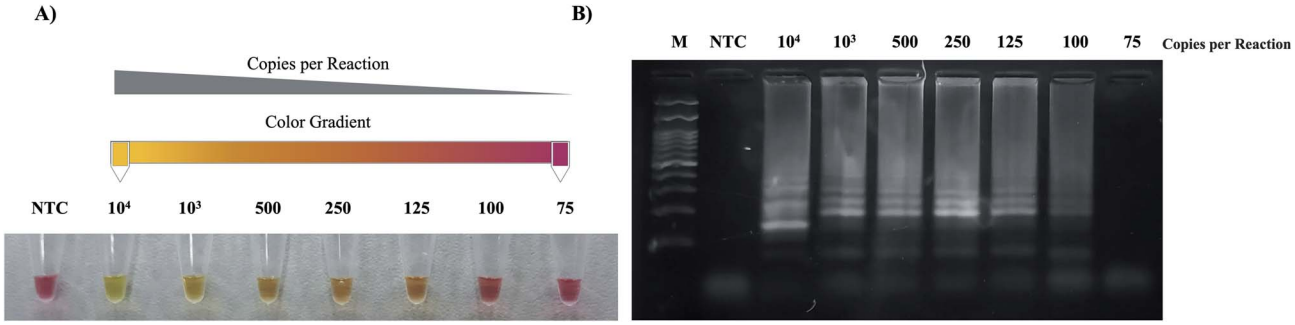
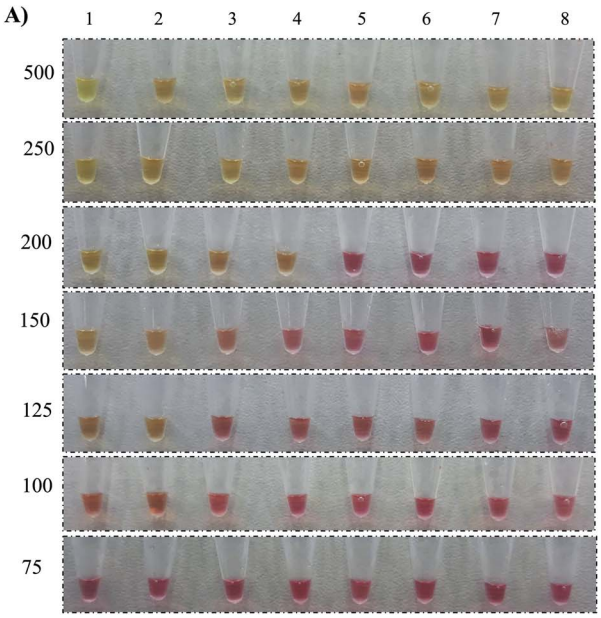
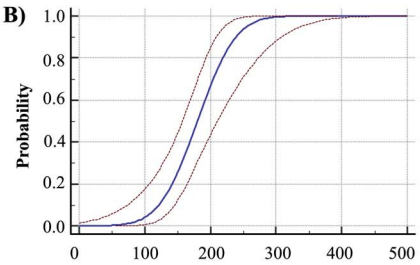


Fig. 2 Analytical sensitivity of the RT-LAMP assay using a purified gene fragment. (A) Visual detection. (B) Amplified products separated on 2% agarose gel. M – marker and NTC – Negative Template Control.





C)

SARS-CoV-2 Concentration (copies per reaction)	No. of Replicates	No. of Positive Results	Hit Rate in %
500	8	8	100
250	8	8	100
200	8	4	50
150	8	2	25
125	8	2	25
100	8	0	0
75	8	0	0

Fig. 3 Limit of detection of the RT-LAMP assay for SARS-CoV-2 detection spiked in a biological sample. (A) Sensitivity of the colorimetric RT-LAMP assay for SARS-CoV-2 detection. (B) Probit regression analysis curve considering eight independent replicates with different initial copies per reaction. (C) Table with the hit rate (%) reached in all eight replication replicates.

We checked the correlation between the detection limit and Ct value of eight samples with different Sars-CoV-2 viral loads (no Cq and $18 \leq Ct \leq 32$) previously confirmed by RT-qPCR. The detection of the human RNase P gene for all samples analyzed is shown in Fig. 4A. In the colorimetric RT-LAMP assay in real samples, all positive samples with a $Ct < 30$ (~250 copies per reaction) changed the color of the reaction mixture (pink to yellow) within 30 min of the reaction (Fig. 4B), as confirmed by gel electrophoresis.

Performance of the colorimetric RT-LAMP test for the detection of SARS-CoV-2

We performed an RT-LAMP-based molecular detection for SARS-CoV-2 in clinical samples at a laboratory with a total of 110 samples. Samples were also analyzed by RT-qPCR. Of these 110 samples, 27 were positive, and 83 were negative by RT-qPCR. Among the 27 positive samples by RT-qPCR, 23 were also positive by RT-LAMP, and among the 83 negative samples by RT-qPCR, 83 were also negative by RT-LAMP. Four false-negative results were observed. The Ct values, the number of SARS-CoV-2 RNA copies obtained by RT-qPCR, and the results of the RT-LAMP assay for each sample are shown in Table S1.†

As shown in Table 2, the colorimetric RT-LAMP performed in the laboratory resulted in a sensitivity of 85.19% (95% CI: 66.27–95.81%), a specificity of 100% (95% CI: 95.65–100%), a negative predictive value of 95.40% (95% CI: 89.36–98.09%), and a positive predictive value of 100.00% (95% CI: 77.34–99.41%), with an accuracy of 96.36% (95% CI: 89.67–98.85%). The RT-LAMP showed high concordance with RT-qPCR with a kappa coefficient of 0.897 (95% CI: 0.798–0.966) and p value < 0.05.

Since RT-LAMP has excellent reliability and accuracy in a controlled environment, such as a laboratory, we implemented the test in a hospital environment to serve as proof of its potential application in the field. A total of 96 samples were analyzed (Table S1†). Among the 96 samples, 27 were positive, and 69 were negative by RT-qPCR. RT-LAMP identified 24 of 27

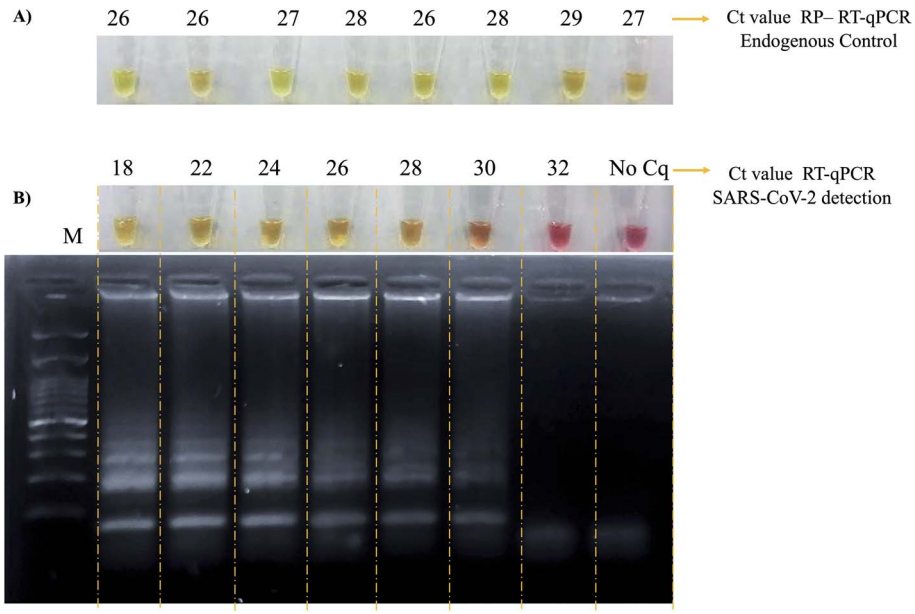


Fig. 4 Detection of the RT-LAMP amplified product. (A) Evaluation of human RNase P (RP) endogenous control quality in swab samples; eight (8) positive samples by RT-qPCR. (B) Detection of SARS-CoV-2 RT-LAMP in eight (8) samples with different viral loads visualized by visual detection and electrophoretic separation. No. Cq:no. cycle quantification.

Table 2 Statistical evaluation of the RT-LAMP in the laboratory and hospital setting[a]

Laboratory			Hospital		
Statistic	Value	95% CI	Statistic	Value	95% CI
Sensitivity	85.19%	66.27% to 95.81%	Sensitivity	88.89%	70.84% to 97.65%
Specificity	100.00%	95.65% to 100.00%	Specificity	98.55%	92.19% to 99.96%
Disease prevalence	24.55%	16.84% to 33.67%	Disease prevalence	28.12%	19.42% to 38.22%
Positive predictive value	100.00%	—	Positive predictive value	96.00%	77.34% to 99.41%
Negative predictive value	95.40%	89.36% to 98.09%	Negative predictive value	95.77%	89.67% to 98.85%
Accuracy	96.36%	90.95% to 99.00%	Accuracy	95.83%	89.67% to 98.85%
Cohen's kappa	0.897	0.798 to 0.996	Cohen's kappa	0.895	0.794 to 0.995

[a] CI-confidence interval.

positive samples by RT-qPCR and 68 of 69 samples negative by RT-qPCR. Of all the samples analyzed in the hospital, only four had non-concordant results between the two tests; one false-positive and three false-negatives were associated with RT-LAMP. However, it is important to note that false-negative results are associated with the limit of the test itself.

RT-LAMP in the field presented a sensitivity of 88.89% (95% CI: 70.84–97.65%), a specificity of 98.55% (95% CI: 92.19–99.96%), a negative predictive value of 95.77% (95% CI: 88.83–98.51%), and a positive predictive value of 96% (95% CI: 77.34–99.41%), with an accuracy of 95.83% (95% CI: 89.67–98.85%). The kappa value was 0.895 (95% CI: 0.794–0.995) (Table 2) with a p value < 0.05.

Correlation between the percentage of hit rate of RT-LAMP and Ct values

The correlation between the hit rate of the RT-LAMP and Ct of the samples was analyzed. After separating the samples by Ct ranges (≤19, 20–24, 25–29, and ≥30), we observed that the hit rate of RT-LAMP to samples analyzed in the laboratory with Ct values ≤ 19, 20–24, and 25–29 was 100%. However, with Ct ≥ 30, the reaction did not show positivity (Fig. 5A). Among the four positive samples determined to be false-negatives by RT-LAMP, three samples presented Ct values of Ct ≥ 30 and one presented Ct values in the range 25–29 (Fig. 5B).

This same correlation was observed for the samples analyzed at the hospital. The hit rate of RT-LAMP for samples with Ct ≤ 19, 20-25, and 25–30 was 100% (Fig. 5C). The correlation of Ct values with RT-LAMP results showed that all false-negative samples presented Ct > 30 (Fig. 5D). These data demonstrate that the RT-LAMP assay was able to effectively identify SARS-CoV-2 in samples up to Ct 30.

Discussion

In places with limited resources, several challenges have been associated with PCR based diagnostics, including the lack of

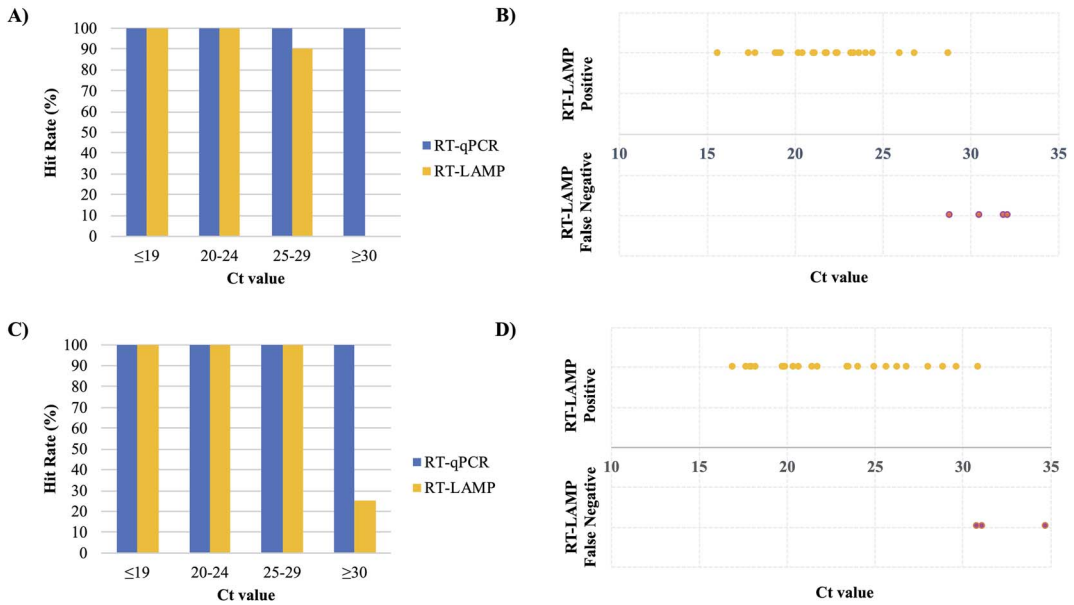


Fig. 5 The performance of colorimetric RT-LAMP analyzing clinical samples previously quantified by RT-qPCR. (A) Hit rate of RT-LAMP and (B) correlation of Ct with positive and false-negative samples analyzed in the laboratory. (C) Hit rate of RT-LAMP and (D) correlation of Ct with positive and false-negative samples analyzed in the hospital.

infrastructure, trained personnel, and expensive equipment. In this context, the demand for instrumentally simpler tests ("rapid tests"), usually based on lateral flow (LFA) detection, has emerged rapidly. Currently, lateral flow tests are available for both antibody and antigen detection. Krüttgen and co-workers[25] developed a study to compare the Real Star SARS-CoV-2 RT-PCR kit and the SARS-CoV-2 Rapid antigen test (lateral flow assay) using 75 swabs from patients previously tested positive and 75 swabs from patients previously tested negative by SARS-CoV-2 qPCR. The authors reported a specificity of 96% and a sensitivity according to different cycle threshold values. For Cts < 25, 25 – <30, 30 – <35, and ≥35 the sensitivity was 100%, 95%, 44.8% and 22.2% respectively. Although it is possible to read the final results of lateral flow assays within 30 min, as well as the test developed in this manuscript, the lateral flow still requires high-cost reagents such as antibodies and labels, as opposed to the detection method used here, based on the pH indicator. Regarding lateral flow tests for antibody detection, these tests are serological tests (IgG and IgM detection), which perform an indirect detection, that is, the detection of the immune response of the host to the SARS-CoV-2 infection.[26] As well, serological tests are not suitable for diagnosing patients at the beginning of the infection, unlike molecular tests, which are able to detect the infection even in the early days of symptoms. On the other hand, it would be possible to use a molecular test such as RT-LAMP and perform detection by lateral flow, however, we do not recommend it, as (i) in addition to the increased cost, (ii) it would require a step of opening the tube after the amplification stage, increasing the probability of cross-over contamination, thus compromising the reliability of the test.

During the Global Research and Innovation Forum, the World Health Organization warned about the need for research into diagnoses of point-of-care for use at the community level.[27] Several studies have proposed the use of the colorimetric RT-LAMP technique for the development of simple tests with potential for application at the point-of-care. For this, simple extraction, amplification, and detection methodologies similar to those addressed in this paper were developed. Diego *et al.* (2021), with samples of RNA extracted from nasopharyngeal swab inactivated with lysis buffer, carried out a vast study between primers and enzymes for colorimetric RT-LAMP.[28] The authors reported that primers based on the ORF1ab and N region with the enzyme Bst 2.0 DNA Polymerase resulted in better performance in the detection of SARS-CoV-2 RNA (amplification times and analytical sensitivity). Yamazaki *et al.* (2021) described a simple method to extract RNA from saliva samples using semi-alkaline proteinase, compatible with colorimetric RT-LAMP in a 45 minute test with 82.6% diagnostic sensitivity (19/23) and a limit of 250 copies per reaction.[29] Bokelmann *et al.* combined RNA purification by hybridization capture with colorimetric LAMP, and were able to correctly identify 100% of positive samples with high viral loads with Ct < 24, when using primers in the Orf1a region.[30] Therefore, our manuscript brings clear advantages, performing the detection of SARS-CoV-2 in 100% of the samples with Ct < 30 in the field, directly in nasopharyngeal swab samples employing only the heat treatment. The heat treatment of the sample, as described in our paper, is advantageous because in addition to being quick, it does not require steps to open sample tubes, corroborating biosafety in the performance of the tests. But in addition to the sample preparation process, in this paper, we use an endogenous control (RP), which determines the viability of the sample giving greater reliability to the test. Furthermore, in this study, we exceed the limits of "potential application of the method" for effective application in point-of-care (hospital

environment) settings with good results in terms of rapid, accurate and sensitivity diagnosis which can contribute to the control of the pandemic, especially in environments with scarce resources.

Here, we evaluated the performance of RT-LAMP in a laboratory and hospital environment. The results showed that the performance of RT-LAMP is similar in both environments, even with the high possibility of contamination within a hospital, confirming its feasibility of application in the field. However, the major challenge when planning to use molecular diagnostic tests is the correct structuring of the working environment and protocol to be used. The extreme capacity of RT-LAMP amplification is also accompanied by its great sensitivity to cross-contamination or its own amplified products.[23,24,31] The configuration of rooms used to optimize tests in the laboratory and hospital proved to be sufficient to minimize problems with contamination by amplicons. In places with limited resources, several challenges have been associated with PCR based diagnostics, including the lack of infrastructure, trained personnel, and expensive equipment.

SARS-CoV-2 is classified as a risk group 3 biological agent, and the correct compliance with the guidelines of laboratory safety procedures and sample handling is essential.[32] The protocol for viral inactivation by heat treatment is already well established[33,34] and shows advantages for field application and compatibility with the colorimetric kit used in this study. Studies have already shown that heating at 95 °C for five minutes could inactivate the virus.[35] Since we work with freshly collected samples, we decided to double the time to ensure safety in handling the samples.

Since the use of chemical reagents, such as lysis buffers, significantly changes the pH of the samples, making them unfeasible for testing using a pH-based colorimetric assay, the pre-heating protocol, besides promoting the inactivation process by denaturing proteins essential for attachment and replication of the virus in a host cell,[36–38] proved to be an ideal pretreatment of nasopharyngeal swabs to release RNA using heat excluding the need for nucleic acid extraction. This approach of pre-heating the samples was also used by Barza *et al.* as a strategy to expose the viral genome and denature possible inhibitors of the PCR reaction.[39]

In relation to the detection limit, our SARS-CoV-2 results feature a strong concordance with other studies that describe the decrease in sensitivity due to the presence of a biological matrix.[40] The performance of RT-LAMP reported in this study is also in agreement with those previously described in the literature.[21,40,41] Nawattanapaiboon *et al.*,[40] using viral RNA extracted from 2120 clinical samples of nasopharyngeal and throat swabs collected in Thailand, obtained an accuracy of 95.8% using the same colorimetric master mix (WarmStart® Colorimetric LAMP 2× Master Mix) and time (30 min). The other statistical values demonstrate the potential of the test to be used as a screening for SARS-CoV-2, mainly in places with low laboratory resources.

The Ct values of the RT-qPCR reaction are inversely correlated with the viral load, high Ct values represent low viral load, and low Ct values represent high viral load.[42] Some studies have correlated the potential of infectivity and Ct values.[43] Magleby *et al.* correlated the Ct of patients with the disease severity, and Ct > 30 is related to lower mortality.[44] Thus, considering the detection limit found in our analyses and that it is according with the limit reported in others studies,[45] the assay proposed here can be useful for screening patients at the beginning of the infection with high viral load and consequently with a greater potential for viral spread and progression to severe clinical conditions due to infection of SARS-CoV-2. Therefore, although the RT-LAMP test described here has a lower sensitivity than RT-PCR, the detection of Ct < 30 is adequate to detect likely contagious individuals and aid in the implementation of isolation measures.

An important factor to be considered in the management of COVID-19 is the waiting time for current tests, which is difficult for decision making related to the immediate care of patients with major problems and their correct isolation. In this context, decentralized tests, when compared to RT-PCR, are associated with a significant reduction in the time to release the results. Brendish *et al.*[20] showed that the application of point-of-care PCR reduced approximately 14 hours of waiting for results. In our study, we also observed an even greater reduction in time from the request of the test to the result, requiring less than 1 hour to perform all the RT-LAMP steps in the field. The nasopharyngeal sample collection step, using a swab, takes less than 5 minutes. Then, 10, 50 and 5 minutes are spent in the heat treatment, amplification and detection process, respectively. Counting the time needed to prepare the reaction mix, label and transporting the microtubes across the rooms, the total analysis time is less than 1.5 h. Thus, considering the simplicity of the test execution, the speed of results, the lowest aggregate cost, analyzing the scenario of overcrowding in emergency rooms due to the growing demand for care during the pandemic, and the need to isolate infected patients, the results presented here demonstrate that RT-LAMP has important clinical application, mainly in the hospital screening sector, to try to minimize the problem of overcrowding, allowing immediate care for the most urgent patients.

Conclusion

The colorimetric RT-LAMP assay applied in the hospital setting proved to be a powerful tool for detecting SARS-CoV-2 with a great cost-benefit in the context of point-of-care. The pre-heat treatment of samples is a simple strategy compatible with pH-based tests that promotes safety in handling and performing tests. Besides, it also assisted in the detection of SARS-CoV-2 nucleic acid directly in unextracted swab samples. The results show that the methodology was able to deliver reliable and accurate results, both in the field and the laboratory. Results can be obtained in less than 1.5 h, as long as the proposed workflow is respected. It is of uttermost importance to use the RT-LAMP endogenous control as it can help identify samples with low quantities of biological material as a consequence of failure in collection. Furthermore, the RT-LAMP carried out in the hospital exhibited a diagnostic performance comparable to RT-qPCR and the assay performed in the reference laboratory with high sensitivity (88.89%), specificity (94.20%), and

a detection limit of ~255 copies of RNA per reaction. Based on the data, the limitation of RT-LAMP in contrast to the gold standard is sensitivity. In a recently published study conducted by Alcoba-Florez and colleagues, a direct RT-qPCR assay performed on clinical samples for the detection of SARS-CoV-2 using a nasopharyngeal heat treatment swab yielded a sensitivity of 87.8%, 100% specificity and 99.9% accuracy. The detection limit found in this study is greater than that reported in the literature by Alcoba-Florez and collaborators who reached a detection limit of 120 copies of RNA per reaction with an average Ct value of 38.48 ± 0.57.[46] In fact, the RT-LAMP is less sensitive than the RT-qPCR assay, however, the direct RT-LAMP technique is easier to perform, thus eliminating the need for complex equipment, which can help increasing the frequency of tests. Advantages, such as directly detecting the target in clinical swab samples without the need for RNA extraction, the use of low-cost equipment, and the easy reading and differentiation of positive and negative results by the naked eye through the color change from pink to yellow, suggest that the RT-LAMP method can be broadly applied to many other kinds of places without great infrastructure. Our RT-LAMP assay has great potential to aid in on-field SARS-CoV-2 screening and enable rapid and accurate treatment of infected patients.

Conflicts of interest

There are no conflicts to declare.

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