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A comparative study of isothermal nucleic acid amplification methods for SARS-CoV-2 detection at point-of-care

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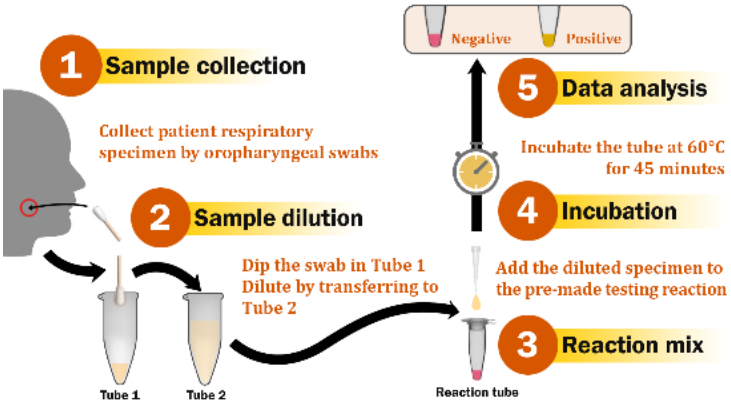
ABSTRACT

COVID-19, caused by the novel coronavirus SARS-CoV-2, has put most of the world under lockdown. Despite approved vaccines, COVID-19 cases, hospitalizations, and deaths have remained on the rise. Rapid diagnosis and necessary public health measures are still key parts to contain the pandemic. Here, the colorimetric isothermal nucleic acid amplification tests (iNAATs) for SARS-CoV-2 detection based on loop-mediated isothermal amplification (LAMP), cross-priming amplification (CPA), and polymerase spiral reaction (PSR) were designed and compared in performance for the first time. The findings showed that, for the detection of SARS-CoV-2 genomic-RNA, LAMP outperformed both CPA and PSR, exhibiting the limit of detection (LOD) of roughly 43.14 copies/reaction. The results can be read with the naked eye within 45 minutes, without cross-reactivity to closely related coronaviruses. The direct detection of SARS-CoV-2 RNA in simulated specimens by iNAATs was also successful. Additionally, the lyophilized reagents for LAMP reactions maintained the sensitivity and LOD of the liquid assays. The colorimetric LAMP assay was validated using clinical samples, showing 98.1% sensitivity and 100% specificity upon using extracted samples and 82.4% sensitivity and 86.2% specificity upon using unextracted specimens. The results indicate that the direct colorimetric LAMP assay developed is highly suitable for detecting SARS-CoV-2 at point-of-care.



***Keywords**: SARS-CoV-2; nucleic acid amplification test; LAMP; CPA; PSR; colorimetric; lyophilized reagents; direct; crude specimens*

INTRODUCTION

Coronavirus is a large family of RNA viruses, including human coronaviruses, which often cause respiratory illnesses with mild cold symptoms. The two exceptions that cause severe diseases include the fatal Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)[1] and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV).[2] By February 2020, the mortal pneumonia disease caused by a novel coronavirus called SARS-CoV-2 was named COVID-19 by the World Health Organization (WHO). In March 2020, the WHO classified the COVID-19 outbreak as a "Global Pandemic". As of Sep. 10th, 2021, COVID-19 has spread to over 220 countries and regions worldwide with over 220 million confirmed cases and more than 4.5 million casualties.

The genome of SARS-CoV-2 is a single-stranded positive-sense RNA molecule that is around 29.9 kb in length.[3] The viral genome is composed of 11 Open reading frames (ORFs), namely *ORF1ab*, *ORF2* (Spike protein or S gene), *ORF3a*,

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ORF4 (Envelope protein or E gene), *ORF5* (Membrane protein or M gene), *ORF6*, *ORF7a*, *ORF7b*, *ORF8*, *ORF9* (Nucleocapsid protein or N gene), and *ORF10*.[4] The *ORF1ab* gene expresses a polyprotein comprising of 16 nonstructural proteins.[4] The sequences of four structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, share high similarity to those of SARS-CoV and MERS-CoV.[3]

The diagnostic standard of SARS-CoV-2 involves clinical symptoms and molecular methods. WHO quickly introduced quantitative reverse transcription PCR (qRT-PCR) for accurate SARS-CoV-2 diagnosis.[5] Currently, the qRT-PCR test, which is carried out using respiratory samples (nasopharyngeal or oropharyngeal swabs), has been widely used as the gold standard method for SARS-CoV-2 diagnosis. Nevertheless, qRT-PCR requires high-cost equipment, and results are only available within a few hours to 2 days, limiting its application in resource-limited settings.

Distinctive virological and serological assays for rapidly detecting SARS-CoV-2 at point-of-care (POC) have been introduced. Virological diagnosis detects viral nucleic acids directly using isothermal nucleic acid amplification tests (iNAATs)[6-8] and the CRISPR-Cas12 based method,[9,10] whereas serological tests detect rising antibody titers between the acute and convalescent stages of infection or detect IgM in primary infection.[11-13] However, serological diagnosis usually shows lower sensitivity, especially in the early stages of infection.[14] Meanwhile, like PCR, iNAATs that amplify the viral nucleic acids at a constant temperature are expected to determine the presence of infectious viruses even in asymptomatic patients. Loop-mediated isothermal amplification (LAMP) was invented in 2000 and is broadly utilized nowadays.[15] LAMP was shown to be rapid, specific, and remarkably sensitive compared to conventional PCR.[16] Different findings revealed that the performance of LAMP assays was in a good correlation with qRT-PCR results when evaluated with clinical samples.[17,18] Standard LAMP uses only a *Bst* DNA polymerase possessing strand displacement activity and no modified/labelled DNA probes are required, simplifying the preparation procedure and significantly reducing the cost. Afterward, other isothermal DNA amplification methods developed later, which also depend on the strand displacement activity of the *Bst* DNA polymerase, include cross-priming amplification (CPA)[19] and polymerase spiral reaction (PSR).[20] While LAMP requires two to three primer pairs, PSR needs only one primer pair, and CPA uses six to eight cross-linked primers. Various studies indicated that PSR and CPA performance regarding sensitivity and specificity was comparable to that of LAMP.[21,22] Simple and fast detection methods for the amplified nucleic acids, such as using SyBr Green dye or pH-sensitive indicators, have been well developed and readily available to visualize the outcome of the three assays.[23] Also, with mutual advantages, including easy operation, fast reaction, low-cost requirements, these methods are suitable diagnostic tools for resource-restricted settings.

Approximately 10% of COVID-19 vaccine candidates have undergone Phase 3 clinical trials.[24] Among them, preliminary results from several candidates developed by companies such as Pfizer and AstraZeneca have shown efficacy of or higher than 90% against the development of symptomatic COVID-19.[25,26] Although all vaccine candidates were considered as still in the early testing stages, many governments have granted emergency authorization of the COVID-19 vaccines. Nevertheless, COVID-19 daily cases and deaths worldwide have kept surging even after vaccine programs have been rolled out across many countries. Additionally, there has been a recent emergence of new COVID-19 variants that are more transmissible, such as 501Y.V1 (B.1.1.7)[27] in the UK, 501Y.V2 (B.1.351)[28] in South Africa, P. 1[29] in Brazil, and B. 1. 617.2 in India.[30] Together with a global shortage of COVID-19 vaccine supply, the outbreak could even worsen until effective vaccines are widely distributed. Thus, basic preventative measures such as physical separation, mask use, handwashing, and mass, rapid testing remain the most effective tools for combating SARS-CoV-2, particularly in low- and middle-income countries where access to COVID-19 vaccines is still very limited.

In this study, we designed and for the first time compared colorimetric LAMP, CPA, and PSR assays for SARS-CoV-2 nucleic acid detection utilizing a pH-sensitive dye for readout visualization. In particular, the options of direct detection of SARS-CoV-2 from clinical samples and the use of ready-to-use lyophilized LAMP were also examined and discussed. Consequently, the colorimetric LAMP assay for SARS-CoV-2 diagnosis was evaluated using clinical samples.

RESULTS

Colorimetric iNAATs for SARS-CoV-2 detection

The extracted genomic RNA of SARS-CoV-2 was utilized as the template to perform the LAMP, CPA, and PSR reactions in the presence of the pH-sensitive indicator. The results indicated that the color change from red to yellow of the iNAAT reactions corresponded to the amplified products generated only when the template was present (Figure S1). Next, the optimal temperature and required time of iNAAT for the detection of SARS-CoV-2 were defined. For both the two primer sets of LAMP, 30 min was the minimum time required for the readout of positive amplification judged by the naked eye (Figure 1A, left panel), and the amplicons were produced from 60 to 70 °C (Figure 1A, right panel). Regarding CPA (the primer pair targeting the *ORF1ab* gene) and PSR (two primer pairs targeting *ORF1ab* and *N* sequences, respectively), amplified products could be well visualized after 40 min (Figure 1B and C, left panels). Meanwhile, 61 and 63 °C were the minimal temperatures for observing CPA and PSR amplified products (Figure 1B and C, right panels). Therefore, the incubation temperatures of the iNAAT reaction were set at 60 °C for LAMP and 63 °C for CPA and PSR. The incubation time was selected as 45 min for the most significant color shift. Note that the color of the negative control occasionally starts to change to yellow only after 60 min of incubation due to the spurious amplification. This is much longer than the 30 min reported in the other study.[8] We reasoned that the difference resulted from the differences in the sequences and amounts of primers used. Given that the

majority of false-positive LAMP results are caused by the formation of primer-dimers,[8] this result suggests that the primer conditions we developed can reduce the reaction's false-positive rate in practice.

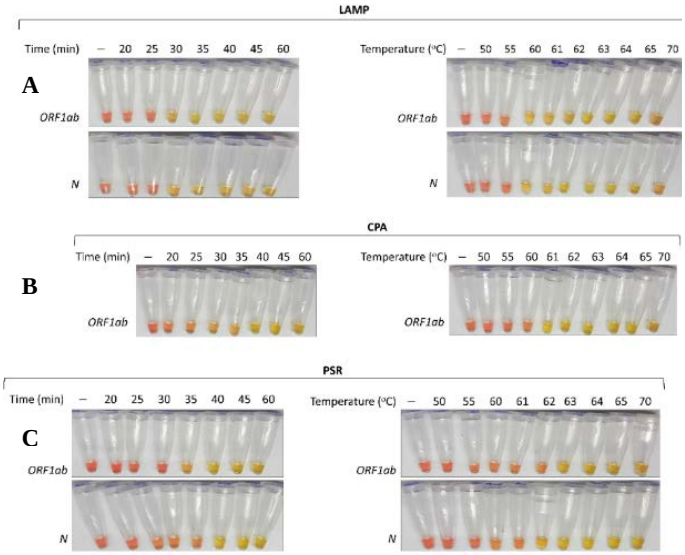

Figure 1. Optimization of the incubation time and temperature for iNAATs. The LAMP (in A), CPA (in B) and PSR (in C) reactions were incubated for 20 to 60 min at 60 °C (LAMP) and 63 °C (CPA and PSR) (left panels) and at different temperatures ranging from 50 to 70 °C for 30 min (right panels).

Specificity of SARS-CoV-2 colorimetric iNAATs

The iNAATs primer sequences were aligned to genome sequences of various coronavirus strains, including 13 SARS-CoV-2 strains, MERS-CoV, SARS-CoV, human coronaviruses related to the common cold (HKU1, OC43, NL63, and 229E), bat SARS-like-CoV, Murine hepatitis virus (*Murine coronavirus*), and *Betacoronavirus England* 1. The results showed that 0% mismatch with all tested SARS-CoV-2 variants (MN938384, MN975262, MN985325, MN988668, MN988669, MN988713, MN994467, MN994468, MN997409, MT007544, MT121215, MT123292 and NC045512) was observed, suggesting that the developed iNAATs with all sets of primer designed could detect different variants of SARS-CoV-2 (Table 1). In contrast, except for bat SARS-like-CoV 2015 and 2017 strains, most of the genomic RNA sequences of other coronaviruses showed nucleotide mismatches higher than 20% with the designed primers. Thus, it is likely that all designed primer sets would not amplify those sequences, ensuring the specificity of iNAATs for SARS-CoV-2. The results of *in silico* PCR and a virtual LAMP tool (Electric LAMP)[31] also supported the high specificity of the primer sets used (Table S1, S2 and S3). Further data demonstrated that all of the iNAATs primer pairs designed selectively detected the presence of SARS-CoV-2 DNA while no cross-reactivity was observed with SARS-CoV, MERS-CoV, bat SARS-like-CoV (Figure 2) and some other coronaviruses (Figure S2), confirming the absolute specificity of the assays.

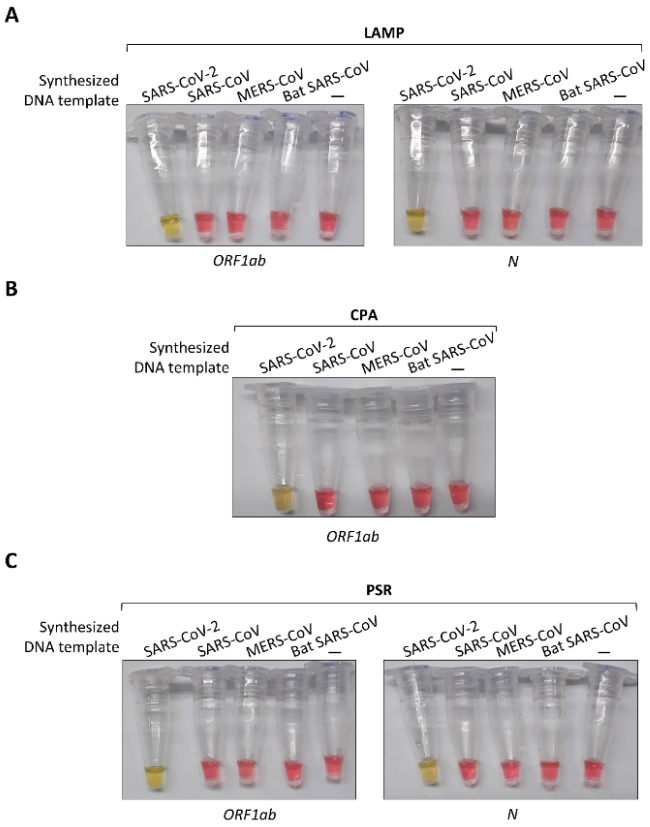

Figure 2. The specificity of the colorimetric iNAATs. The specificity of LAMP (in A), CPA (in B) and PSR (in C) assay was evaluated with the synthesized DNA (1 ng) of SARS-CoV-2, SARS-CoV, MERS-CoV, and bat SARS-like-CoV.

Limit of detection (LOD) of colorimetric iNAATs

The LOD values of iNAAT reactions were determined using synthesized DNA templates serially diluted in nuclease-free water. As shown in Figure 3, roughly a single copy of the synthesized targeted gene per reaction was the lowest template amount that iNAAT reactions could detect. In particular, both primer sets of LAMP, the primer pair of CPA amplifying *ORF1ab* sequence and the primer set of PSR targeting the *N* sequence can detect 1 copy/reaction of the synthesized target sequence. In contrast, the LOD of the PSR reaction targeting the *ORF1ab* sequence was 10^3 copies/reaction (Figure 3C). The obtained results indicated that the iNAAT reactions containing primer pairs designed for LAMP targeting *ORF1ab* and *N* genes, for CPA targeting the *ORF1ab* region and for PSR targeting *N* sequence were sensitive enough for practical diagnosis. The primer pair for PSR amplifying the *ORF1ab* sequence was thus eliminated from further analysis.

The genomic RNA of SARS-CoV-2 was extracted and quantitated using the standard curve based on qRT-PCR Ct-value as described in the previous study.[32] The LODs of the colorimetric iNAATs on this extracted SARS-CoV-2 genomic RNA were also evaluated, revealing the LOD values of LAMP reactions were around 21.57 (*ORF1ab*) and 43.14 (*N*) viral-RNA copies/reaction (Table 2). The obtained values were outstanding compared to 431.47 (*ORF1ab*) and 862.9 (*N*) geno-

Table 1. The percent mismatch of newly designed primers between SARS-CoV-2 and related taxa

Name	Accession number	LAMP-*ORF1ab*	LAMP-*N*	CPA-*ORF1ab*	PSR-*ORF1ab*	PSR-*N*
Murine hepatitis virus	NC001846	25.97	31.93	21.74	30.19	34.78
Betacoronavirus England 1	NC038294	29.83	29.52	25.36	35.85	30.43
Human Coronavirus 229E	NC002645	31.49	31.93	26.09	33.96	39.13
Human Coronavirus NL63	NC005831	28.73	31.33	25.36	32.08	34.78
Human Coronavirus OC43	NC006213	28.73	30.72	23.91	32.08	36.96
Human Coronavirus HKU1	NC006657	27.62	22.29	29.71	37.74	17.39
Middle East Respiratory CoV	NC019843	29.83	29.52	25.36	35.85	30.43
Civet SARS CoV SZ16/2003	AY304488	19.34	12.65	13.77	35.85	26.09
SARS CoV ZS-C	AY395003	19.34	12.65	13.77	35.85	26.09
SARS CoV MA15	FJ882957	19.34	12.65	13.77	35.85	26.09
SARS CoV	NC004718	19.34	12.65	13.77	35.85	17.39
Bat SARS CoV RM1/2004	KY417144	21.55	13.86	16.67	32.08	15.22
Bat SARS-like CoV 2015	MG772933	8.84	12.05	7.25	20.75	10.87
Bat SARS-like CoV 2017	MG772934	8.84	11.45	7.97	26.42	10.87
13 SARS-CoV-2 strains		0	0	0	0	0

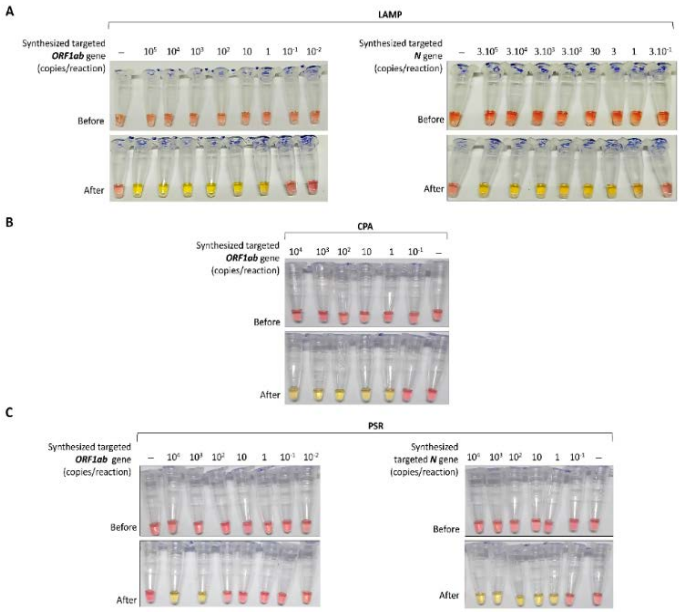

Figure 3. The LOD values for the targeted genes of colorimetric iNAATs. LOD of LAMP (in A), CPA (in B) and PSR (in C) assays were evaluated. The synthesized DNA template was serially diluted in nuclease-free water to the indicated concentrations and 1 µl of the diluted DNA templates was added to the reactions. The experiments were replicated at least 3 times.

-me copies/reaction of CPA and PSR assays, respectively (Table 2).

Performance of direct SARS-CoV-2 colorimetric iNAATs with simulated clinical specimens

The activity of the isothermal polymerase utilized in LAMP, CPA and PSR is more tolerant to various PCR inhibitors such as trace quantities of whole blood, hemin, urine or stools.[33]

Table 2. LODs of colorimetric iNAATs for extracted SARS-CoV-2 genomic RNA

Viral RNA copies (per reaction)	**Ratio of positive tests to the total test number**					
	LAMP		Lyophilized LAMP		CPA	PSR
	ORF1ab	*N*	*ORF1ab*	*N*	*ORF1ab*	*N*
1725.89	3/3	3/3	3/3	3/3	3/3	3/3
862.95	-	-	-	-	3/3	3/3
431.47	-	-	-	-	3/3	1/3
215.74	-	-	-	-	0/3	0/3
172.59	3/3	3/3	3/3	3/3	0/3	0/3
86.30	3/3	3/3	3/3	3/3	0/3	0/3
43.15	3/3	3/3	3/3	3/3	0/3	0/3
21.57	3/3	0/3	0/3	0/3	0/3	0/3
17.26	0/3	0/3	0/3	0/3	0/3	0/3
1.73	0/3	0/3	0/3	0/3	0/3	0/3
LOD	21.57	43.14	43.14	43.14	431.47	862.95

Therefore, we attempted to use these iNAATs to directly detect SARS-CoV-2 nucleic acid from nasopharyngeal and oropharyngeal swab specimens. Synthesized DNA of SARS-CoV-2 was spiked into the crude samples to mimic the clinical specimens. Unfortunately, the direct use of undiluted crude samples interfered with the color indicator of the iNAATs; the reaction color changed to yellow immediately when samples were added. As expected, sample dilution was required to clearly establish the colorimetric reactions between positive and negative signals (data not shown). Accordingly, a 10-fold dilution of nasopharyngeal and oropharyngeal swab specimens,

which was sufficient to distinguish the colors between positive and negative samples, was selected to examine the simulated clinical specimens prepared.

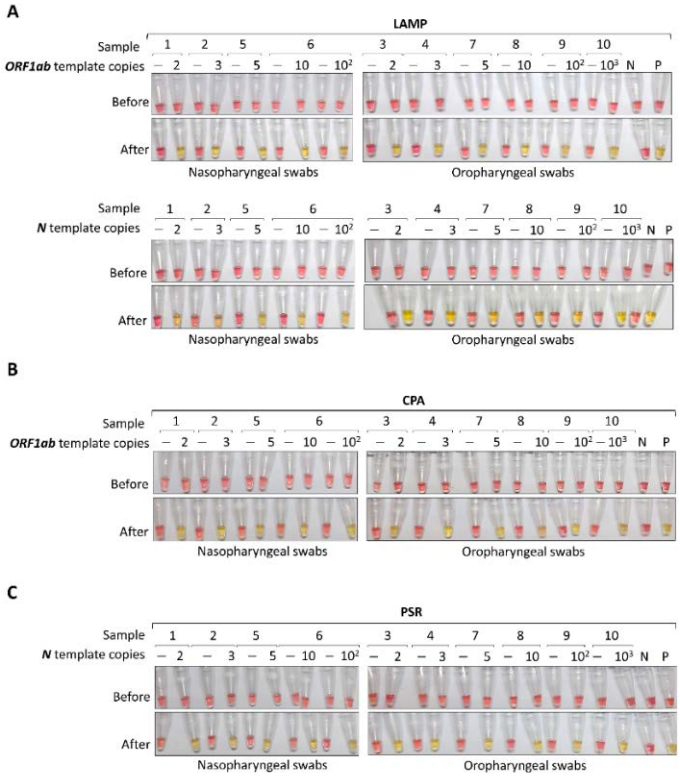

Figure 4. The direct iNAATs for detecting SARS-CoV-2 in simulated samples. Different amounts of the synthetic-DNA template were spiked into the nasopharyngeal and oropharyngeal swab samples to simulate the clinical samples containing SARS-CoV-2, and 1 µl of the 10-fold diluted specimens were added to the LAMP (in A), CPA (in B) and PSR (in C) reactions. The number indicates the template copy per reaction. Abbreviation, N: negative control; P: positive control.

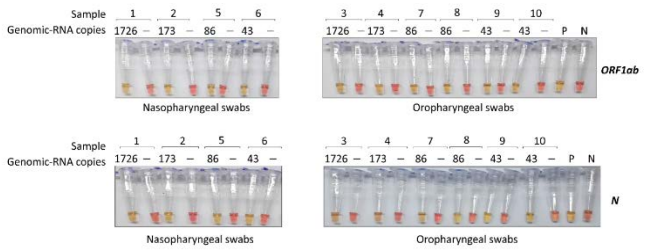

Figure 5. Colorimetric LAMP assays for detection of SARS-CoV-2 genomic RNA in simulated samples. Various concentrations of genomic RNA of SARS-CoV-2 were spiked into the nasopharyngeal and oropharyngeal swab samples to simulate the clinical specimens containing SARS-CoV-2. The mimicked samples were 50-fold diluted and 5 µl of the diluted specimens were added to the LAMP reactions. The number indicates the copy of viral RNA per reaction. Abbreviation, N: negative control; P: positive control.

As a result, all nasopharyngeal and oropharyngeal swab samples without spiked DNA produced negative signals while the samples containing a various number of spiked DNAs produced positive signals (Figure 4), indicating that iNAATs successfully detected the nucleic acid of SARS-CoV-2 in crude specimens. Among the three methods, based on the best performance on detecting genomic RNA of SARS-CoV-2, LAMP assays using both primer sets designed were used for further evaluation with simulated clinical samples varied in the amounts of spiked viral-RNAs. Note that instead of transferring 1 µl of the 10-fold diluted samples to the reaction, 5 µl of the spiked viral-RNA specimens were added to the reaction. Thus, a 50-fold dilution of the simulated specimens was prepared proportionally. The results indicated that the LAMP assays can directly detect SARS-CoV-2 genomic-RNA in crude samples (Figure 5), supporting the success of the direct SARS-CoV-2 colorimetric LAMP assay designed.

Performance of SARS-CoV-2 colorimetric LAMP using lyophilized reagents

Concerning the practice of developing countries and rural areas, extending the assay's shelf-life time and eliminating the dependence on the cold chain by using lyophilized reagents would be advantageous. Thus, we also evaluated the LAMP assay performance using the dried reagents. The lyophilized reagents exhibited the same LOD value of 1 DNA copy/reaction, which is equivalent to 10 DNA copies/µl in simulated specimens (Figure 6A). Subsequently, the LOD values of LAMP lyophilized reagents for the detection of SARS-CoV-2 genomic RNA were also evaluated. Both primer sets could identify approximately 43.14 copies of viral RNA per reaction (Table 2). Note that the defined LOD values correspond to a Ct value of roughly 36.5 when tested by qRT-PCR for the *E* gene.[32] This high Ct value (>35) strongly indicates that the designed LAMP lyophilized reagents could identify the specimens with low-level infection of SARS-CoV-2 found during early infection or asymptomatic carriage. Importantly, all mimicked samples containing different amounts of synthesized DNA or viral RNA were detected by the LAMP lyophilized reagents (Figure 6B and C). The direct addition of unextracted clinical specimens to the reaction can markedly reduce the time required for sample preparation and thus, simplify the operation procedure. When all those features are considered, the LAMP lyophilized reagents are highly suitable for POC diagnosis.

Validation of SARS-CoV-2 colorimetric RT-LAMP using clinical samples

The direct colorimetric RT-LAMP method established here was validated using clinical samples which were already diagnosed by qRT-PCR. First, the viral RNA extraction samples isolated from 210 clinical specimens were tested by the SARS-CoV-2 colorimetric RT-LAMP assay. The results revealed that the suitability of the SARS-CoV-2 colorimetric RT-LAMP kit with qRT-PCR was 98.1% (103/105) sensitivity and 100% (105/105) specificity (Table 3). Second, 63 nasopharyngeal and oropharyngeal swab specimens collected from suspected patients who had just been diagnosed by qRT-PCR were examined directly by the SARS-CoV-2 colorimetric RT-LAMP assay without viral RNA extraction. The obtained data showed

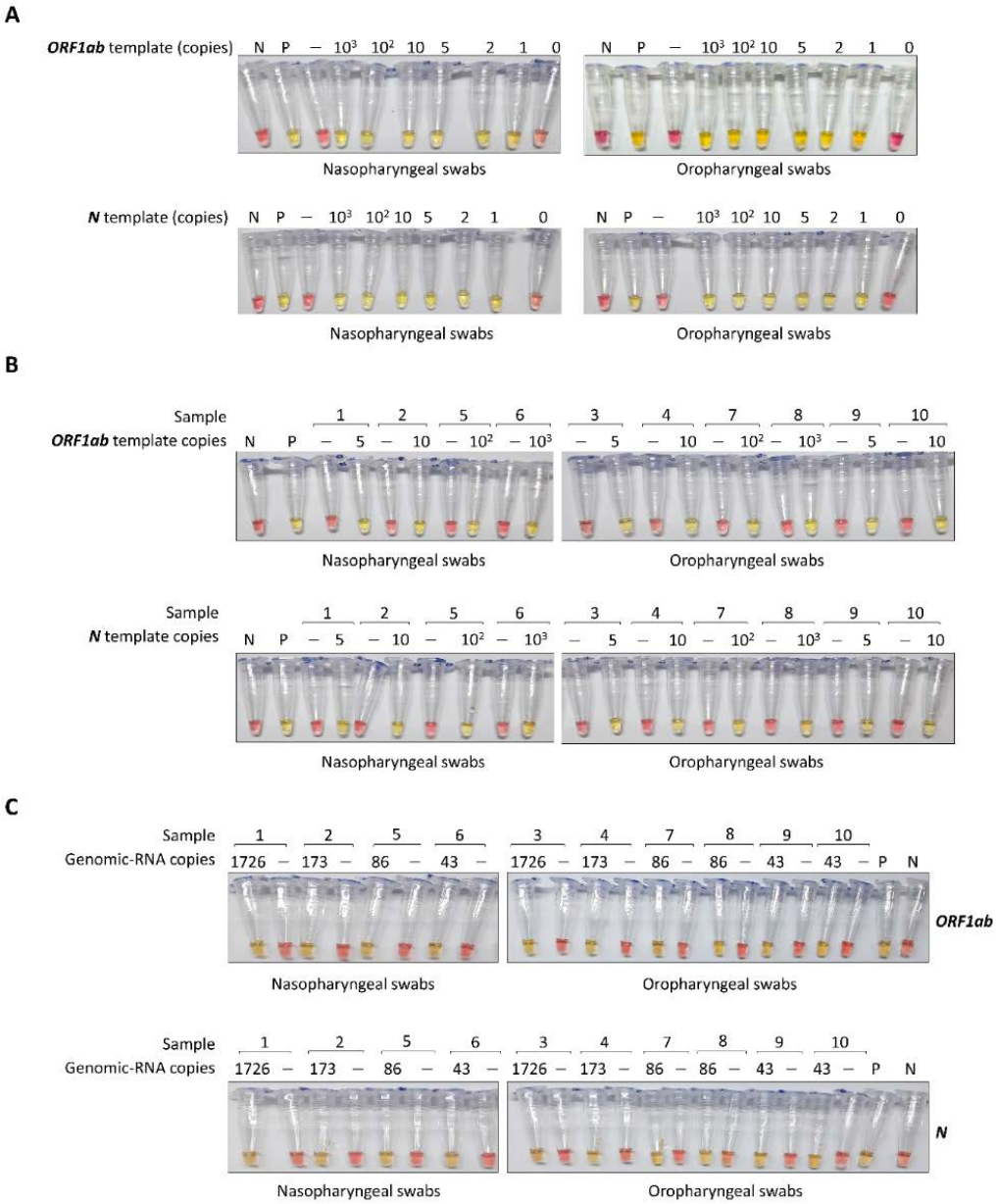

Figure 6. The performance of SARS-CoV-2 colorimetric LAMP lyophilized reagents. (A) LODs of LAMP lyophilized reagents evaluated in the simulated samples. Defined amounts of synthetic DNA templates were spiked into the nasopharyngeal and oropharyngeal swab samples, followed by the 10-fold dilution of simulated specimens into water and 1 µl of the diluted samples were added to the reaction. The sensitivity of LAMP lyophilized reagents was examined with mimicked clinical specimens containing different amounts of spiked synthetic DNA (in B) and SARS-CoV-2 genomic RNA (in C). The number indicates the copy number of synthetic DNA or viral RNA in reaction. Abbreviation, N: negative control; P: positive control.

that suitability of the SARS-CoV-2 direct colorimetric RT-LAMP assay with qRT-PCR was 82.4% (28/34) sensitivity and 86.2% (25/29) specificity (Table 3), adamantly supporting that the direct RT-LAMP kit developed in this study is suitable for practical use as a rapid screening method for COVID-19 patients.

DISCUSSION

The pneumonia outbreak COVID-19 has recently become a global pandemic. While there has not been efficient chemotherapeutics successfully developed, rapid diagnosis and necessary biosecurity procedures are the most essential actions to control the disease. POC such as airports or stations should be strictly managed to avoid spreading COVID-19. However, the prevention measures, including body heat monitoring, checking personal travel history, and clinical symptoms, have been proven to be insufficient as many asymptomatic people are infectious. Self-isolation and group quarantine of suspected cases can temporarily limit the transmission, but it will be more difficult to control when the number of suspects is high. While the gold standard for identifying patients is qRT-PCR, the standard operating procedure for diagnosis, including

preparation of the master mix, extraction of nucleic acid template, and the requirement of expensive qRT-PCR devices to examine multiple specimens simultaneously, is not highly portable. Moreover, the testing time required is at least a few hours, if not including the time needed for transferring the sample to the labs, thus, slowing down the quarantine process of the infected patient. Consequently, the qRT-PCR tests only serve to verify a small number of cases while the actions cannot be made immediately. Therefore, POC diagnostic detection methods that are rapid and accurate can help the authority to effectively monitor the spreading of the virus.

The three isothermal amplification methods interested, including LAMP, CPA and PSR, all require DNA polymerase strand displacement activity. Among them, LAMP was first introduced and has been widely utilized. Meanwhile, CPA and PSR were later developed and studies have shown that their performance was comparable to the LAMP assay.[21,22] Moreover, since the robust amplified process of LAMP, CPA and PSR techniques induces proton release, which significantly drops the pH of reactions, allowing the reaction outcome to be easily read by the naked eye with the use of an economic pH-sensitive indicator, thus simplifying the handling process and reducing the cost of assays. In contrast, detection based on the pH change is more limited for PCR or other isothermal methods such as RPA (Recombinase polymerase amplification) or HDA (Helicase-dependent amplification). In this work, for the first time, the three methods, including LAMP, CPA and PSR were applied to detect SARS-CoV-2 and directly compared. Surprisingly, despite showing similar performance regarding the identification of the synthetic DNA template of SARS-CoV-2, LAMP was superior to CPA and PSR in the detection of genomic RNA of SARS-CoV-2. The finding indicates that LAMP is the best among the three isothermal techniques evaluated to be used as the diagnostic method for the identification of SARS-CoV-2 in practice.

Table 3. Clinical performance of SARS-CoV-2 colorimetric RT-LAMP assay

Type of samples	Method		qRT-PCR		Total
			Positive	Negative	
RNA extraction	SARS-CoV-2 colorimetric RT-LAMP	**Positive**	103	0	103
		Negative	2	105	107
		Total	105	105	
	Sensitivity		98.1%		
	Specificity		100%		
Unextracted specimen	SARS-CoV-2 direct colorimetric RT-LAMP	**Positive**	28	4	32
		Negative	6	25	31
		Total	34	29	
	Sensitivity		82.4%		
	Specificity		86.2%		

Under the burden of the COVID-19 pandemic,[44] multiple RT-LAMP assays have been developed to rapidly detect SARS-CoV-2 and their findings were well summarized in different review articles.[34,35] Most of the published studies also targeted the conserved regions of *N* and *ORF1ab* genes due to the high homology and divergence from the other coronaviruses. In fact, the *N* gene has already been shown to possess the top read coverage of all coronavirus genes when RNAs extracted from cell cultures infected with coronavirus HCoV-229E were sequenced.[34] Most of the RT-LAMP studies for COVID-19 diagnosis reported clinical sensitivity ranging from 75-100% and specificity ranging from 80 to 100%, applying repeated RT-PCR as reference.[34,35] These results show that the sensitivity and specificity obtained by the developed RT-LAMP test in this study using extracted RNA samples remain among the highest of SARS-CoV-2 RT-LAMP assays reported. One of the most distinctive features of the SARS-CoV-2 colorimetric LAMP kit developed herein is the direct use of minimally processed clinical samples. The only step required to prepare samples before adding to the reaction is nothing more than dilution. The study was inspired by different previous results showing that LAMP was successfully conducted in crude clinical specimens without sample extraction. We speculate that the crude samples already contained some free viral RNA and that the moderately high temperature (60 °C) of the incubation process could help to break the virus envelop, thereby releasing more genomic RNA into the solution. Clearly, sample dilution can reduce the chance to detect SARS-CoV-2 if the virus concentration in the original sample is considerably low. Also, the detection limit of the assay will depend on the dilution factor. Therefore, it is important to choose the proper dilution factor that can meet the balance point between the pros of not hindering the reaction color shift and the cons of causing the viral concentration to be too diluted below the detection threshold. Nevertheless, here, the volume of water used to collect the patient swab was only 400 µl, which is approximately 7.5-10 times smaller than the volume of VTM often used following the standard sample collection protocol. Consequently, the virus concentration in the specimen collected in the water was significantly higher than in the typical VTM. Note that when a swab specimen stored in VTM is subjected to extraction, there is a considerably high amount of RNA lost during the procedure.

To our knowledge, initially, this work was one of the first reports attempting to develop the RT-LAMP assay for SARS-CoV-2 detection using unextracted nasopharyngeal and oropharyngeal swab specimens. To date, only a few studies have evaluated RT-LAMP tests in the direct identification of the SARS-CoV-2 presence in unextracted clinical samples.[8,36,37] It should be noted that the Variplex™ test system has previously been reported to fail to reliably detect SARSCoV2 without RNA extraction,[38] implying that successful implementation of the COVID-19 diagnosis method based on the LAMP technique using unextracted clinical specimens has been challenging. Without the requirement for viral RNA extraction, the colorimetric RT-LAMP kit developed is very suitable for POC diagnosis, not only because it is much less time-consuming and laborious but also does not depend on RNA extraction kits that have been in shortage due to overwhelming global demand.

Regarding LOD evaluation, data from articles revealed that the LOD values of different SARS-CoV-2 RT-LAMP assays fell in the range of 1-304 RNA template copies per reaction.[35] Nevertheless, it is worthwhile to note that some studies reported the LOD to be lower than 50 copies/reaction because the authors either used *in vitro* transcript RNA[39] or the cloned vector system[6,40] as the template or combined the LAMP method with innovative techniques[10,41] that can increase the testing cost to some extent. Furthermore, even fewer papers have investigated the lyophilized reagents for the LAMP assay to rapidly detect SARS-CoV-2. In particular, a commercial kit called Loopamp® 2019-SARSCoV-2 Detection Reagent Kit, which uses dried RNA amplification reagent, has exhibited a LOD of 10 copies/µl equivalent to 100 copies/reaction,[42] which is less sensitive than the assay we developed in this study.

In general, the SARS-CoV-2 direct colorimetric RT-LAMP assay developed in this study possesses the following features: (i) fast detection of SARS-CoV-2 genomic-RNA directly from the nasopharyngeal and oropharyngeal specimens within 45 minutes; (ii) high sensitivity (roughly 43 copies of the viral RNA in the reaction is sufficient for detection); (iii) naked-eye readout of results; (iv) possible detection of the virus in the early stage of infection; (v) only a common thermal incubator is needed; (vi) suitable for both types of clinical samples, including viral RNA extraction and unextracted specimens; (vii) is amenable to high throughput testing; (viii) portable to use in any place and does not require specialized personnel to do the test; and (ix) particularly useful for resource-limited settings. This RT-LAMP kit is hence more scalable for mass testing and a promising candidate for POC diagnosis of COVID-19. The data from the initial clinical trial further strengthens the feasibility of the SARS-CoV-2 direct colorimetric RT-LAMP kit developed for practical usage. The positive cases can be further tested by qRT-PCR to verify the results. Meanwhile, the negative cases can be subsequently re-tested by the SARS-CoV-2 direct colorimetric RT-LAMP assay after a few days. Finally, the results gained in this study set a solid foundation for future extensive clinical trials of our kit.

Table 4. Primers sequences used in this study

Nº	Name	Nucleotide sequence (5′ - 3′, length in nucleotide)	Position (MN908947)
1	LAMP-Orf-F3 CPA-4s-Orf-F3	GATTTAGATGAGTGGAGTATGG (22)	2951-2972
2	LAMP-Orf-B3 CPA-5a-Orf-B3	GCACCAAATTCCAAAGGTT (19)	3138-3156
3	LAMP-Orf-FIP	CTGGAGGGTAGAAAGAACAATACATCATACTACTTATTTGATGAGTCTGG (50)	3023-3047, 2976-3000
4	LAMP-Orf-BIP	GAGGATGAAGAAGAAGGTGATTGTTTGGTAATCATCTTCAGTACCATA (48)	3050-3073, 3110-3133
5	LAMP-Orf-LoopF	GTGAAGCCAATTTAAACTC (19)	3002-3020
6	LAMP-Orf-LoopR	AGTTTGAGCCATCAACTCAATAT (23)	3084-3106
7	LAMP-N-F3	TGGACCCCAAAATCAGCG (18)	28285-28302
8	LAMP-N-B3	GCCTTGTCCTCGAGGGAAT (19)	28468-28486
9	LAMP-N-FIP	CCACTGCGTTCTCCATTCTGGTAAATGCACCCCGCATTACG (41)	28353-28374, 28303-28321
10	LAMP-N-BIP	CGCGATCAAAACAACGTCGGCCCTTGCCATGTTGAGTGAGA (41)	28377-28397, 28438-28457
11	LAMP-N-LoopF	TTGAATCTGAGGGTCCACCAAA (22)	28322-28343
12	LAMP-N-LoopR	GTTTACCCAATAATACTGCGTCTTG (25)	28404-28428
13	PSR-Orf-F	acgattcgtacatagaagtatagAGAAGATTGGTTAGATGATGATAGTCAA (51)	3193-3220
14	PSR-Orf-R	gatatgaagatacatgcttagcaTTCCATCTCTAATTGAGGTTGAACC (48)	3286-3310
15	PSR-N-F	acgattcgtacatagaagtatagTGATAATGGACCCCAAAATCAGCG (47)	28379-28302
16	PSR-N-R	gatatgaagatacatgcttagcaAACGCCTTGTCCTCGAGGGAAT (45)	28468-28489
17	CPA-2a-Orf-B2	TTGGTAATCATCTTCAGTACCATA (24)	3110-3133
18	CPA-3a-Orf-B1	ACAATCACCTTCTTCTTCATCCTC (24)	3050-3073
19	CPA-Orf-1s	TTGGTAATCATCTTCAGTACCATACTGGAGGGTAGAAAGAACAATACAT (49)	3110-3133, 3023-3047

MATERIALS AND METHODS

Primer design

Primers for LAMP, CPA, and PSR assays targeting the *N* and *ORF1ab* sequences of SARS-CoV-2 (GenBank accession number MN908947) were designed using the free online software Primer Explorer V5 (https://primerexplorer.jp/e/). Primer selection was carried out as instructed (https://primerexplorer.jp/e/v4_manual/). Two sets of primer pairs for LAMP (targeting the *N* and *ORF1ab* genes), two sets of primer pairs for PSR (targeting the *N* and *ORF1ab* genes), and one set of primer pairs for CPA (targeting the *ORF1ab* gene) were selected. Primers were synthesized by Phu Sa Biochem (Can Tho, Vietnam) and their sequences are listed in Table 4.

Synthesized DNA template preparation

The synthesized DNA templates were obtained from Phu Sa Biochem, (Can Tho, Vietnam). The sequences from 28274 to 28516 of the *N* gene and from 2853 to 3452 of the *ORF1ab* gene of SARS-CoV-2 (GenBank MN908947) were selected to serve as the positive control templates for iNAATs. The *N* and *ORF1ab* sequences (single-strand) of MERS-CoV (GenBank JX869059.2; 28567-28751 and 3075-3303), SARS-CoV (GenBank MK062184.1, 28278-28482 and 2954-3162), and bat SARS-like-CoV (GenBank MN996532.1, 28205-28504 and MG772933.1, 2851-3350) were also prepared for primer specificity testing.

Primer specificity analysis

The reference genomes of SARS-CoV-2 and related species were downloaded from NCBI (https://www.ncbi.nlm.nih.gov). The primer sequences were aligned to genomes of different coronaviruses to calculate the number of mismatches using Geneious Prime 2020.0.3 (https://www.geneious.com). The percentage of mismatch was calculated by dividing the total number of different bases between primers and genome sequences by the total length of the primers. The software FastPCR available at http://primerdigital.com/fastpcr.html was used for *in silico* PCR analysis. The software eLAMP downloaded at https://www.nybg.org/files/scientists/dlittle/eLAMP.html was utilized for virtual LAMP analysis.

Colorimetric iNAATs

WarmStart® Colorimetric LAMP 2X Master Mix (DNA & RNA) was purchased from NEB (MA, USA). The iNAAT reaction volume was 15 µl, consisting of 1 or 5 µl of template sample and 7.5 µl of the WarmStart® Colorimetric Mastermix. The LAMP reaction contains 0.8 µM each inner primer (FIP and BIP), 0.1 µM each outer primer (F3 and B3), 0.2 µM each loop primer (FLoop and Bloop). The CPA reaction contains 0.5 µM cross primer 1s, 0.3 µM each of primers 3a and 2a, 0.05 µM each of displacement primers 4s and 5a. The PSR reaction contains 1.6 µM each of primers (PSR-F and PSR-R). The iNAAT reactions were run for 30 to 45 minutes at 60 °C (LAMP reaction) or 63 °C (CPA and PSR reactions) in the BioSan Dry block thermostat Bio TDB-100. The amplification products were visualized by the color shifting from red to yellow of the test reaction, which is based on the use of phenol red, a pH-sensitive indicator as instructed by the manufacturer.

Evaluation of limit of detection of colorimetric iNAATs

The copy number of the synthetic DNA template was calculated using the Endmemo program (http://endmemo.com/bio/dnacopynum.php). The synthesized DNA template was serially diluted to various concentrations and 1 µl of the diluted DNA sample was added to the iNAAT reactions. The reactions were then incubated for 30 min at different temperatures according to each method.

SARS-CoV-2 was isolated from the clinically positive COVID-19 samples and cultured at the Pasteur Institute (Ho Chi Minh City, Vietnam). Extracted genomic-RNA of SARS-CoV-2 was quantified via a standard curve based on qRT-PCR Ct-value[32] and then serially diluted. Five µl of the diluted RNA samples were added to the iNAAT reactions. The reactions were then incubated for 45 min at the proper temperature.

Collection and preparation of mimicked clinical specimens

Ten nasopharyngeal and oropharyngeal swab specimens from volunteer nurses and doctors were collected at a local hospital (Ho Chi Minh City, Vietnam). All participants confirmed negative for SARS-CoV-2 by qRT-PCR provided their informed consent to participate in the trial. The oropharyngeal or nasopharyngeal specimen was collected using a sterile flocked plastic swab which was then soaked into 400 µl of nuclease-free water. Fresh samples were kept on ice until analysis or frozen for subsequent assays.

To prepare the simulated clinical specimens, various concentrations of SARS-CoV-2 synthesized DNA or extracted viral genomic-RNA were spiked into the collected nasopharyngeal and oropharyngeal swab samples. The simulated swab specimens containing the synthetic-DNA were 10-fold diluted in the nuclease-free water and 1 µl of the diluted sample was added to the iNAAT reactions. The samples containing the viral RNA were 50-fold diluted in the nuclease-free water and 5 µl of the diluted sample were added to the iNAAT reactions. Non-spiked specimens were used as negative samples.

Preparation of lyophilized reagents for LAMP assay

A 0.2 ml reaction tube containing 7.5 µl of WarmStart Colorimetric LAMP 2X Master Mix and primers (0.1 µM each F3 and B3, 0.8 µM each FIP and BIP, and 0.2 µM each FLoop and Bloop) was mixed well and subjected to lyophilization in a freeze-dryer (Operon, South Korea) using a protocol based on the method described by Saleki-Gerhardt and Zografi.[43]

Validation of SARS-CoV-2 colorimetric RT-LAMP assay

The RNA extraction samples (210) isolated from the nasopharyngeal and oropharyngeal swab specimens of the COVID-19 suspect patients and already diagnosed by qRT-PCR (Table S4) were utilized to evaluate the clinical performance of the SARS-CoV-2 colorimetric RT-LAMP assay. Note that the swab specimens were previously collected in Viral Transport Medium (VTM) in March 2021 and stored for less than two months until evaluated by the SARS-CoV-2 colorimetric RT-LAMP assay. Among them, 105 samples were confirmed positive and 105 samples were confirmed negative by qRT-PCR using the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany)

following the WHO recommendation protocol.[5] RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) except that the elution buffer was replaced by nuclease-free water. Five µl of the RNA extraction samples were sequentially added into the colorimetric RT-LAMP reactions, separately amplifying *ORF1ab* and *N* genes. The reactions were then incubated for 45 min at 60 °C. The sample was concluded as positive for SARS-CoV-2 by the SARS-CoV-2 colorimetric RT-LAMP assay if one or both RT-LAMP reactions succeeded. In contrast, if both RT-LAMP reactions failed, the sample was concluded as negative for SARS-CoV-2 by the SARS-CoV-2 colorimetric RT-LAMP assay.

Additionally, the nasopharyngeal and oropharyngeal swab specimens (63) of the COVID-19 suspect patients recently diagnosed by qRT-PCR (Table S5) were collected in nuclease-free water as described above. Among them, 34 positive and 29 negative specimens were concluded by qRT-PCR using the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) following the WHO recommendation protocol.[5] All participants provided their informed consent to participate in the trial. The clinical samples were then 50-fold diluted and 5 µl of the diluted samples were sequentially added into the colorimetric RT-LAMP reactions, separately amplifying *ORF1ab* and *N* genes. The reactions were then incubated for 45 min at 60 °C. The diagnosis results were read similarly as mentioned above.

CONCLUSION

In this study, the colorimetric iNAATs for SARS-CoV-2 detection based on three methods including LAMP, CPA, and PSR were developed and compared the testing performance for the first time. The results indicated that iNAATs could directly identify the genomic RNA of SARS-CoV-2 in unextracted patient specimens. The results can be easily observed with the naked eye within 45 minutes, without cross-reactivity to closely related coronaviruses. However, LAMP outperformed both CPA and PSR, exhibiting the best LOD value of approximately 43.14 viral-genome copies per reaction. Additionally, the lyophilized reagents for LAMP reactions maintained the testing performance of the liquid assays, showing that the lyophilized format of this colorimetric assay is very suitable for detecting SARS-CoV-2 nucleic acids in POC diagnosis settings. Last, the clinical performance of SARS-CoV-2 colorimetric RT-LAMP was evaluated using extracted and unextracted specimens, revealing high suitability with the diagnosis results acquired by qRT-PCR, further demonstrating the efficacy of the direct colorimetric RT-LAMP developed in this study.

DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials and are available from the corresponding author upon request.

ETHICAL STATEMENT

The sample collection was approved by Hospital Management. The internal use of samples was agreed upon under the medical and ethical rules of each participating individual. The study was approved by the Research Ethics Committee of the Pasteur Institute in Ho Chi Minh City, Vietnam.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPLEMENTARY INFORMATION

Supplementary data provides *in silico* analysis of the specificity of the primer pairs designed and the information about qRT-PCR results of clinical specimens.

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