Ciencia aplicada

Detección del virus SARS-CoV-2

a través de la prueba de amplificación isotérmica mediada por bucle con transcripción reversa (RT-LAMP)

Detection of SARS-CoV-2 virus by reverse transcription Loop-mediated isothermal amplification (RT-LAMP) assay

Elizabeth Ruiz Márvez[1],
Ana Luisa Muñoz Ramírez[2],
Orlando Alfredo Torres García[3],
Francisco Javier Vargas Ortíz[3],
Juana Patricia Sánchez Villamil[4*].

*juanipsan3@uan.edu.co

DOI: 10.54104/saywa.v5n6.1506

[1]Facultad de Ciencias Básicas, Investigadora postdoctoral Minciencias, Universidad Antonio Nariño, Bucaramanga, Colombia.

[2]Facultad de Ciencias, Grupo de Investigación en Biología Celular y funcional e Ingeniería de biomoléculas Universidad Antonio Nariño, Bogotá, Colombia.

[3]Facultad de Medicina Veterinaria y Zootecnia, Universidad Antonio Nariño, Bogotá, Colombia.

[4]Facultad de Ciencias Básicas, Grupo de Investigación en Salud Oral, Universidad Antonio Nariño, Bucaramanga, Colombia.

Palabras clave:
COVID-19, RT-LAMP, SARS-CoV-2.

Keywords:
COVID-19, RT-LAMP, SARS-CoV-2.

Resumen

El propósito del presente estudio fue el de estandarizar una metodología rápida y de simple ejecución para la detección de SARS-CoV-2, usando la técnica de amplificación de ácidos nucleicos mediada por bucle con transcripción inversa en condiciones isotérmicas (RT-LAMP, del inglés *Reverse Transcription Loop-mediated isothermal amplification*), para el diagnóstico de la COVID-19. La estandarización se realizó en muestras de ARN previamente confirmadas por RT-qPCR usando el protocolo Charité, Berlín, Alemania. Detallamos los pasos de la técnica y destacamos el uso del cebador S-123. En conclusión, la prueba RT-LAMP con el set de cebadores S-123 para SARS-CoV-2 en muestras de ARN, mostró un alto desempeño con relación a la técnica *gold standard* RT-qPCR.

Introduction

Severe Acute Respiratory Syndrome Coronavirus type 2 (SARS-CoV-2) belongs to the family *Coronaviridae*, genus Betacoronavirus, subgenus Sarbecovirus and is the causative agent of COVID-19 (Boni et al., 2020; Lamers & Haagmans. 2022).

Early diagnosis is relevant to control new cases of COVID-19 infection and to activate public health measures. In addition to reverse transcription quantitative polymerase chain reaction (RT-qPCR), there are other tests such as rapid antigen tests and antibody tests, which have different advantages at this time of pandemic control due to the constant emergence of SARS-CoV-2 variants. Molecular tests are more sensitive and remain necessary, considering the mutation rate of SARS-CoV-2 virus and, consequently, the diversity of lineages recorded, as well as the mixed infections detected with other viruses such as the one causing Dengue serotype 1 (DENV-1) (Moreno et al., 2021). Due to the high investment costs and laboriousness involved in the diagnosis of COVID-19 by RT-qPCR, the implementation of new non-invasive, inexpensive, rapid, and accessible diagnostic tests for the entire population is always welcome.

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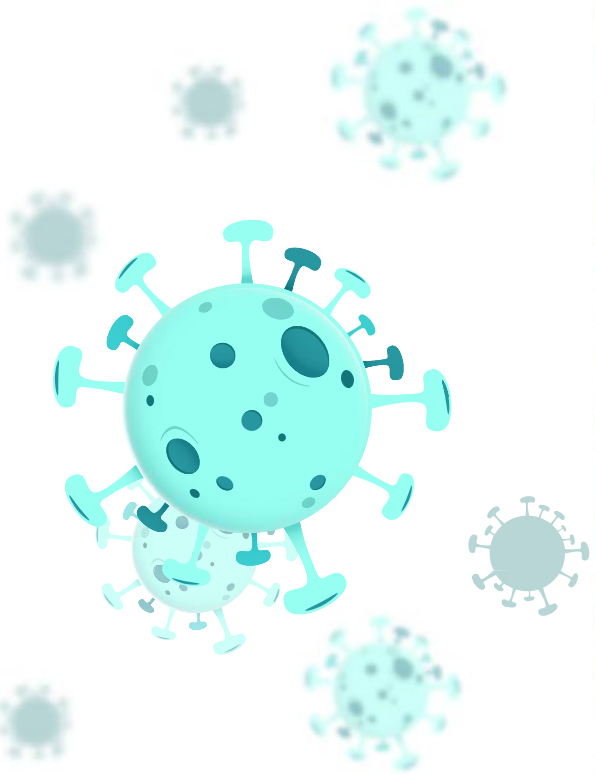
A nucleic acid amplification test that is gaining momentum in Colombia and worldwide is the loop-mediated isothermal amplification technique (LAMP) developed by Notomi and collaborators in 2000, which has been widely used for the detection of pathogens such as

Plasmodium spp., *Salmonella* spp., influenza virus, dengue virus, Chikungunya virus, Zika virus, among others (Artik et al., 2021; El-Tholoth et al., 2020; Huang et al., 2022).

The advantage of LAMP assays lies mainly in their fast-processing time of a maximum of 45 minutes and the simplicity of the assay setup. In addition, LAMP does not require high-end or complex instruments, making it widely accessible in field conditions, including in developing countries. In addition, LAMP works well even with a variety of unpurified samples, which is important for easy and fast assay preparation (El-Tholoth et al., 2020). Another important advantage is that the LAMP reading can be colorimetric or turbidity, easily displayed and recorded on a cell phone (Heithoff et al., 2022; Londoño-Avendaño et al., 2022). Moreover, being performed at lower constant temperatures than PCR, the LAMP technique can be easily combined with the reverse transcription loop-mediated isothermal amplification (RT-LAMP) reaction by directly detecting the target RNA without the need for a separate retrotranscription step, shortening the overall reaction time, making LAMP a powerful tool for the diagnosis of COVID-19 and other infectious diseases that could be applied in the field in hard-to-reach urban and rural areas (Huang et al., 2022; Kashir & Yaqinuddin. 2020).

Therefore, the LAMP technique, due to its performance conditions, would facilitate the diagnosis of COVID-19 in our country in terms of time and resources, as reported by (Hurtado et al., 2022; Londoño-Avendaño et al., 2022; Méndez et al., 2021). For the RT-LAMP technique to achieve acceptance as a diagnostic test for COVID-19 in Colombia, it is desirable to provide more information about it; therefore, the present study sought to standardize RT-LAMP under laboratory conditions for the detection of SARS-CoV-2 from nasopharyngeal swab specimens.



Materials and Methods

Molecular marker for SARS-CoV-2 detection

First, four primer sets previously described in the literature were tested for the detection of SARS-CoV-2 by RT-LAMP, named ORF1ab-4, S-123 (Yan et al., 2020), O117 and N15 (Huang et al., 2020). Primers ORF1ab and O117 amplified different regions of the constitutive ORF1ab genes; meanwhile, primers N15 and S-123 amplified regions of the genes that give rise to the nucleocapsid (N) and spike (S) structural proteins respectively, whose genes are registered in GenBank (Wu et al., 2020) and include a Forward outer primer (F3), a Backward outer primer (B3), a Forward Inner Primer (FIP), a Backward Inner Primer (BIP), a Forward Loop primer (LF), and a Backward Loop primer (LB) characteristic of LAMP amplifications. The complete sequence of each primer tested was recorded in Table 1.

Table 1. List of primers used for the detection of SARS-CoV-2 through RT-LAMP assay

ORF1*ab*	*Sequence* (5´-3´)
orf1ab-4F3	GGTATGATTTTGTAGAAAACCCA
orf1ab-4B3	CAACAGGAACTCCACTACC
orf1ab-4FIP	GGCATCACAGAATTGTACTGTTTTTGCGTATACGCCAACTTAGG
orf1ab-4BIP	AATGCTGGTATTGTTGGTGTACTGAGGTTTGTATGAAATCAC-CGAA
orf1ab-4LF	AACAAAGCTTGGCGTACACGTTCA
O117	*Sequence* (5´-3´)
F3	CCCCAAAATGCTGTTGTT
B3	TAGCACGTGGAACCCAAT
FIP	GGTTTTCAAGCCAGATTCATTATGGATGTCACAATTCAGAAG-TAGGA
BIP	TCTTCGTAAGGGTGGTCGCAGCACACTTGTTATGGCAAC
LF	TCGGCAAGACTATGCTCAGG
LB	TTGCCTTTGGAGGCTGTGT
N15	*Sequence* (5´-3´)
F3	AGATCACATTGGCACCCG
B3	CCATTGCCAGCCATTCTAGC

FIP	TGCTCCCTTCTGCGTAGAAGCCAATGCTGCAATCGTGCTAC
BIP	GGCGGCAGTCAAGCCTCTTCCCTACTGCTGCCTGGAGTT
LF	GCAATGTTGTTCCTTGAGGAAGTT
LB	GTTCCTCATCACGTAGTCGCAACA
S-123	*Sequence* (5´-3´)
S-123F3	TCTATTGCCATACCCACAA
S-123B3	GGTGTTTTGTAAATTTGTTTGAC
S-123FIP	CATTCAGTTGAATCACCACAAATGTGTGTTACCACAGAAATTC-TACC
S-123BIP	GTTGCAATATGGCAGTTTTTGTACATTGGGTGTTTTTGTC-TTGTT
S-123LF	ACTGATGTCTTGGTCATAGACACT
S-123LB	TAAACCGTGCTTTAACTGGAATAGC

Source: Own elaboration of the authors.

RNA sample processing of nasopharyngeal swabs

For the detection of SARS-CoV-2 by RT-LAMP assay, 20 RNA samples isolated with the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) from nasopharyngeal swabs of patients previously diagnosed with COVID-19 by the gold standard RT-qPCR technique were used, 50% of which were negative (samples number 11 to 20). All RNA samples were diluted 1:10 in ultrapure water (Promega, Inc., Madison, WI, USA), then heated at 56°C for 30 minutes and incubated for another 10 minutes on ice. Finally, samples were incubated at 95°C for 5 minutes and allowed to cool to room temperature (RT) (In-House protocol).

RT-LAMP assay

RT-LAMP reactions were assembled in a 96-well plate (Applied Biosystems). Each 10 µL reaction contained 5 µL of 2X Warm Start Colorimetric Master Mix M1800L (New Englands Biolabs, Ipswich, MA, USA), 1 µL of primer set mix (final concentrations of 1.6 µM for FIP or BIP primer,

0.4 µM for FL or BL primer, and 0.2 µM for F3 or B3 primer), 1 µL of inactivated target RNA and 3 µL of DNase/RNase-free water (Table 2). All RT-LAMP reactions were performed in a thermal cycler C1000 Bio-Rad, under the amplification profile 65°C for 10 min and 63°C for 35 min, adopting the temperature of 63°C from Yan et al., 2020 (In-House protocol). The colorimetric change of the reaction was recorded by one camera both before and at the end of the reaction following the manufactureŕs instructions (New Englands Biolabs, Ipswich, MA, USA), where yellow samples were positive and pink samples were negative.

Table 2. *RT-LAMP standard reaction mix for one sample*

Reactive	*Volume (µL)*
Warm Start Colorimetric LAMP 2X Master Mix	5
LAMP primer mix (10X)	1
Sample of inactivated RNA	1
Ultra-Pure water	3
Final volume	10

Source: Own elaboration of the authors.

Results

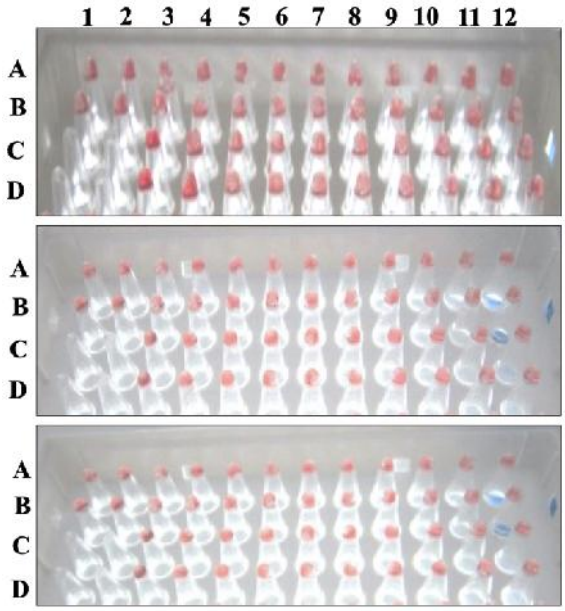
Detection of SARS-CoV-2 in clinical samples

First, in standardization assays, the four primer sets listed in Table 1 were tested to amplify different regions of the SARS-CoV-2 genome using the 20 RNA samples described above. A first amplification assay with the four primer sets listed in Table 1 evidenced 100% concordance with those of RT-qPCR with primers S-123 and O117. Meanwhile, a false positive rate for the N15 primer set of 30% and an inconclusive result for the ORF1ab-4 primer set were observed (data not shown).

To establish the repeatability of SARS-CoV-2 detection with the S-123 and O117 primers, the RT-LAMP protocol was applied with the 20 nasopharyngeal swab RNA samples; ultrapure water was included as a negative control and all reagents, without RNA and without additional water, as a control for the PCR-LAMP reaction. The color of the pH indicator in each sample was recorded prior to amplification (Figure 1a). Detection of SARS-CoV-2 was consistent across all three biological replicates obtaining 100% concordance with the diagnostic results for COVID-19 by RT-qPCR solely with the S-123 primers set (Figure 1b). The sensitivity and specificity data recorded for the best performing primer sets are shown in Table 3.

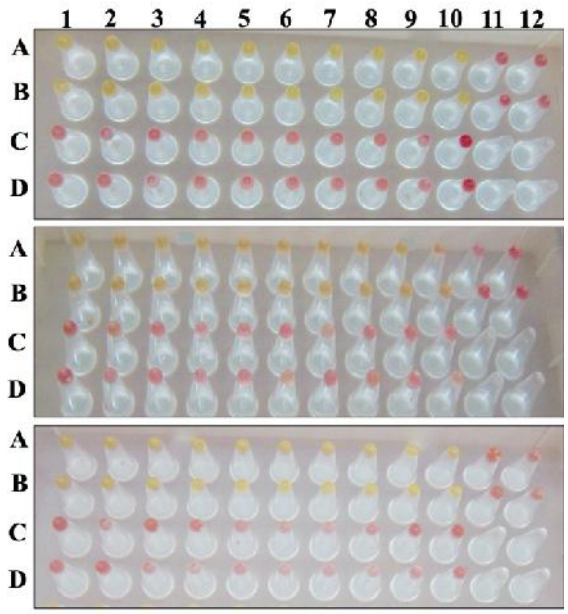
Figure 1. Detection of SARS-CoV-2 with S-123 primers, using RT-LAMP.

a). Recording of RT-LAMP reactions in each replicate before their incubation at 65°C/10 min and 63°C/35 min.



a). Samples before incubation

b). Rows A and B numbers 1-10 contain duplicate incubated samples, which were positive for RT-LAMP. Meanwhile, rows A and B numbers 11-12, along with rows C and D numbers 1-8 were negative samples for RT-LAMP. C9-D9 correspond to the negative control and C10-D10 to the RT-LAMP control in the three biological replicates.



b). Samples after incubation

Source: Own elaboration of the authors.

Table 3. Sensitivity and Specificity of RT-LAMP for diagnosis of SARS-CoV-2

Reference	*Primers set*	*Sensitivity (%)*	*Specificity (%)*
Yan et al., 2020	S-123	100	100
Huang et al., 2020	O117	100	72.5
Huang et al., 2020	N15	100	70

Source: Own elaboration of the authors.

Discussion

Since its inception, the LAMP technology developed by Notomi et al. (2000), has been efficient in the identification of a broad spectrum of infectious disease causative agents (Silva et al., 2019). In the context of COVID-19, the detection of SARS-CoV-2 by RT-LAMP has been improving its sensitivity and specificity with respect to RT-qPCR (Amaral et al., 2021), largely due to the design of the primers used to amplify different regions of the virus genome (Zhang et al., 2022). Herein we undertook, to standardize RT-LAMP amplification conditions for three SARS-CoV-2 genes using four primer sets.

Considering the results obtained by us with S-123 primers, it would be interesting to develop a new study, expanding the number of clinical samples and applying the standardization conditions established both in laboratory and field conditions

The S-123 primers were the most efficient in terms of sensitivity and specificity under the established conditions (Table 3). Recently, Zhang et al. (2022), tested 18 primer sets to detect SARS-CoV-2 by RT-LAMP and included the S-123 primer set developed by Yan et al., 2020 which they named S11. Zhang et al. (2022), found that the S-123 primers at temperatures of 65°C and 60°C showed a high level of sensitivity, both individually and in combination with the S10 primers, which coincides with the results recorded in the present study. Therefore, the researchers corroborated that Yan et al., 2020 when they designed the S123 primers, selected a conserved region of the S gene (Spike protein) of SARS-CoV-2 without mutations, with approximately 216 bp and obtained, when they evaluated 130 clinical samples, 100% concordance in terms of sensitivity and specificity.

Considering the results obtained by us with S-123 primers, it would be interesting to develop a new study, expanding the number of clinical samples and applying the standardization conditions established both in laboratory and field conditions (Hurtado et al., 2022; Londoño-Avendaño et al., 2022; Méndez et al., 2021).

On the other hand, the literature specifies that primers that amplify regions such as the nucleocapsid (N) gene (Reynés et al., 2021) or the ORF1ab gene (Lalli et al., 2021) shows the best performance results. However, we obtained different results, because the primers N15 and O117 designed by Huang et al. (2020), presented false positive results (Table 3). In the first amplification assay with the N15 primers, a false positive rate of 30% was obtained and with the O117 primers, a false positive rate of 100% was obtained in the third biological replicate. As reported by Huang et al., 2020 one of the issues they observed with these two primer sets was carryover or aerosol contamination after performing RT-LAMP reactions several times in the laboratory and self-hybridization of the O117 primers in the amplification process as reported by Lim et al. (2021). Possibly, these are the causes for the observation of the problem of self-amplification and off-target amplification of the primers.

RT-LAMP as a technology in the process of improvement for the diagnosis of COVID-19 is emerging as an excellent diagnostic alternative for this purpose, not only because of the rationality of the technique but also because of the simplicity of its execution and the easy visualization of the results in a short period time. Taken together, our results corroborate those reported by Yan et al. (2020) and Zhang et al. (2022). Finally, the new perspective of multiplex RT-LAMP diagnostics raised by Zhang et al. (2022), based on the application of at least two sets of primers to amplify different regions of the SARS-CoV-2 genome could be adopted by us in the future.

Conclusion

The RT-LAMP assay using the S-123 primers under the established experimental conditions allowed the detection of SARS-CoV-2 in RNA samples, corroborating that it is a promising, powerful, and cost-effective molecular tool for the detection of SARS-CoV-2 in clinical samples which could be applied in rural regions of difficult access in Colombia.

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