ORIGINAL ARTICLE

STANDARDIZATION AND VALIDATION OF AN IN HOUSE RT-LAMP MOLECULAR TEST FOR THE DIAGNOSIS OF SARS-CoV-2

Oscar Escalante-Maldonado[1,a], Margot Vidal-Anzardo[1,b], Fernando Donaires[1,c], Gilmer Solis-Sanchez[2,d], Italo Gallesi[1,e], Luis Pampa-Espinoza[1,c], Maribel Huaringa[1,e], Nancy Rojas-Serrano[1,e], Coralith García[3,c], Eddie Angles-Yanqui[4,5,c], Ronnie Gustavo Gavilán[1,f], Ricardo Durães-Carvalho[6,g], Jairo Mendez-Rico[7,h], César Cabezas[1,c], Paulo Vitor Marques-Simas[6,8,i]

[1] Centro Nacional de Salud Pública, Instituto Nacional de Salud, Lima, Perú.
[2] Oficina General de Investigación y Transferencia Tecnológica, Instituto Nacional de Salud, Lima, Perú.
[3] Hospital Nacional Cayetano Heredia, Lima, Perú.
[4] Hospital Nacional Arzobispo Loayza, Lima, Perú.
[5] Universidad Peruana Cayetano Heredia, Lima, Perú.
[6] Universidad de Campinas, Sao Paulo, Brasil.
[7] Pan American Health Organization, World Health Organization., Washington DC, United States of America.
[8] Universidad Nacional Mayor de San Marcos, Lima, Perú.
[a] Biologist, Doctor of Medical Sciences; [b] physician; [c] physician, specialist in Infectious Diseases; [d] dental surgeon; e Biologist; [f] Biologist, Doctor in Biochemistry and Molecular Biology; [g] Pharmacist, Doctor in Genetics and Molecular Biology; [h] Doctor in Biological Sciences; [i] Biologist, Doctor in Genetics and Molecular Biology.

ABSTRACT

Objectives: To standardize an in-house RT-LAMP test for the detection of SARS-CoV-2 and to validate it with laboratory and field samples in patients with clinical suspicion of COVID-19. **Materials and methods**: An in-house RT-LAMP molecular test was standardized for the detection of SARS-CoV-2, establishing the detection limit with Vero cells of isolated Peruvian strains of SARS-CoV-2, the robustness to different concentrations of primers, and in silico presence of cross-reactions. The laboratory test was validated with 384 nasal and pharyngeal swab samples (UFH) obtained between March and July 2020. For field validation, UFH samples were obtained from 383 suspected symptomatic cases of COVID-19 consecutively enrolled during activities For discard, all samples were evaluated by RT-LAMP and RT-qPCR. For laboratory and field validation, the RT-qPCR was considered as the reference standard, concordance measures and diagnostic performance were calculated. **Results**: The detection limit was consistent in cases with Ct <30 in both tests, showing efficiency to detect up to 1000 copies / µL of the target gene. Robustness was evidenced with half of the primer concentrations and 20 µL of final volume. Absence of amplification was identified for other HCoVs. Concordance in the laboratory obtained a kappa of 0.880 (95% CI: 0.831 - 0.930), in the east field it was 0.886 (0.838 - 0.935); the sensitivity in the laboratory was 87.4% (95% CI: 80.8 - 92.4) and 88.1% in the field (95% CI: 81.6 - 92.9), the specificity in both scenarios was 98.8% (95% CI: 96.4-99.7). **Conclusions**: The RT-LAMP in-house test is validated for its adequate robustness, no cross-reactions, good concordance, and diagnostic performance compared to RT-qPCR.

Keywords: COVID-19; Diagnosis; Molecular Diagnostic Techniques; Polymerase Chain Reaction; Validation Study; Sensitivity and Specificity; ROC Curve; Predictive Value of Tests; Cross Reactions (source: MeSH NLM).

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Correspondence: Oscar Escalante-Maldonado; Jirón Capac Yupanqui 1400, Jesús María, Perú; oscar.escmal@gmail.com

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INTRODUCTION

COVID-19 is a serious public health problem, with more than 98 million confirmed cases and more than two million deaths worldwide as of January 24, 2021 [(1)]. The large spread of the disease represents a major challenge for developing countries, which must deal with healthcare gaps regarding diagnosis. These limitations are evident in rural areas, where health technologies for early case diagnosis need to be developed [(2)].

Molecular testing requires considerable financial and logistical investment compared to other diagnostic tools. The standard test suggested by the World Health Organization (WHO) for the detection of SARS-CoV-2 is the real-time reverse-transcription polymerase chain reaction (RT-qPCR) test, which requires a molecular laboratory with expensive infrastructure, equipment and reagents, as well as specialized personnel; resources that are not always available in countries such as Peru [3,4].

In Peru, at the beginning of the pandemic, the RT-qPCR test could only be performed in a standardized manner in Lima at the National Referral Laboratory for Respiratory Viruses of the Instituto Nacional de Salud (INS). Progressively, its processing was extended to regional laboratories in a decentralized manner. Currently, there are more than 50 laboratories nationwide, but the demand for these tests has not been fully covered [5].

The pressing need for other acute phase diagnostic alternatives for SARS-CoV-2 infection has been evidenced by the development of tests based on the CRISPR/Cas system [6], or the reverse transcription loop-mediated isothermal amplification method (RT-LAMP) [7]. Other tests based on the loop-mediated isothermal amplification method (LAMP) have been applied in Peru for diseases such as zika [8], tuberculosis [9], malaria [10], and dengue [11]; showing adequate performance. Its implementation is more feasible and viable since more affordable equipment is used, employing four to six primers, two/three forward and two/three reverse to identify DNA targets for the amplification [12].

The RT-LAMP test is presented as a fast and efficient alternative for the identification of suspicious cases, since the sample processing time in the laboratory is approximately 50 minutes, compared to the four to eight hours required for the RT-qPCR test [7].

This study aims to standardize, in the laboratory, an RT-LAMP test developed in-house for the detection of SARS-CoV-2; as well as to validate it in the field in patients suspicious for COVID-19, obtaining diagnostic performance measures, and using the results of the RT-qPCR test as reference.

KEY MESSAGES

Motivation for the study: COVID-19 is a serious public health problem in Peru; it is important to develop new diagnostic methodologies for SARS-CoV-2.

Main findings: The RT-LAMP test developed in-house demonstrated adequate diagnostic performance and concordance when compared to the RT-qPCR test, both in the laboratory and in the field. No cross-reactivity with other coronaviruses was identified in silico. In the field, a 5.5% reduction in sensitivity, and a 0.4% increase in specificity, was found in subjects who were between the first and second week of illness.

Implications: The in-house RT-LAMP test evaluated is an effective alternative for molecular detection of SARS-CoV-2.

MATERIALS AND METHODS

Type of study

A cross-sectional study was carried out to evaluate an RT-LAMP test for the detection of SARS-CoV-2 in three stages: a) standardization, b) laboratory validation, and c) field validation. The reference standard considered in the study was RT-qPCR. The design of the standardization was descriptive cross-sectional, and the design of the laboratory and field validation was analytical cross-sectional.

Standardization

This stage was carried out at the INS National Referral Laboratory for Respiratory Viruses between June and July 2020. To evaluate the performance of the RT-LAMP test according to WHO standards, the SARS-CoV-2 strain isolated in VERO81 cells from a nasopharyngeal swab sample (NPS) with positive molecular diagnosis by RT-qPCR and NPS samples that were previously obtained during routine epidemiological surveillance of COVID-19 discard were used. Using the strain, we evaluated: a) detection limit (under serial dilution in base 10), b) robustness to changes in primer concentration (original and half - 0.5P) and percentage reduction of the final reaction volume (20% - 0.8V; 40% - 0.6V; 50% - 0.5V and 60% - 0.4V), and c) reproducibility.

Laboratory validation

Cross-reaction analysis was performed *in silico* by aligning the RT-LAMP external primer sequences with known human coronavirus (HCoV) reference sequences (NC_005831.2, HCoV-NL63; NC_002645.1, HCoV- 229E; NC_006213. 1, HCoV-OC43 ATCC strain VR-759; NC_006577.2, HCoV-HKU1; NC_004718.3, SARS-CoV-1; NC_019843.3, MERS-

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CoV Middle East respiratory syndrome-related coronavirus; FJ415324.1, HECoV 4408; NC_045512.2, Chinese SARS-CoV-2) [13]. In addition, external primers were aligned with 194 Peruvian strains available from GISAID (https://www.gisaid.org/). All *in vitro* experiments were performed in triplicate by the same operator under the same environmental and equipment conditions [13].

To establish the diagnostic validity of the test in the laboratory, a sample size was calculated using the formula for estimating the diagnostic performance by means of the Epidat program version 4.2, considering a sensitivity value of 91.489% and a specificity of 99.531%, figures calculated according to that reported by Jiang *et al*, in their total sample [14]. A significance level of 95%, absolute error of 5% and a positivity probability of 39.5% were considered (based on the proportion of positive results usually obtained in diagnostic activities of the rapid field response teams by the INS between July 6 and 8, 2020 in the jurisdiction of the Directorate of Integrated Health Networks (DIRIS) Center).

A loss rate of 20% was assumed in order to anticipate logistical problems that could arise during sample handling and/or analysis. With these parameters, we established the need for at least 379 samples. For this stage, samples of NPS were used, which were previously obtained during routine epidemiological COVID-19 evaluations between the beginning of the pandemic and July 2020; the vials were stored in INS laboratories. All samples evaluated were anonymized, and corresponded to positive and negative subjects identified by RT-qPCR for whom no additional information was available.

The samples were selected in a non-probabilistic way by convenience, the evaluation by RT-LAMP was blinded (the evaluators were unaware of the previous result by RT-qPCR). The use of the samples was authorized by head resolution No. 00006918, health emergency decree No. 0064-2020-OGA/INS (April 7, 2020) and informative note No. 0055-2020 "Plan de Acción del Instituto Nacional de Salud para Prevención, Diagnóstico y Control de COVID-19, en el marco del Decreto SupremoNo. 008- 2020-SA".

RNA extraction

RNA extraction from all samples was performed using the GenElute™ total RNA purification kit (Sigma-Aldrich - Merck), according to the manufacturer's instructions (https://www.sigmaaldrich.com/technical-documents/protocols/biology/viral-rna-purification.html), then frozen at -80 °C until further processing.

RT-qPCR reaction

Reactions were standardized in Rotor-Gene Multiplex RT-PCR Kit (Qiagen, Germany) using primers and probes for SARS-CoV-2 detection (RdRP) and an internal control (human GAPDH) (green channel, FAM: 470-510nm and orange, ROX: 585-610nm, respectively). Reactions were considered positive when cycle threshold (Ct) values < 37 (FAM) and Ct < 40 (ROX) were obtained concomitantly. Primer sequences, probes and reaction conditions are available in the supplementary material. More information on the in-house RT-qPCR assay is available at: http://dx.doi.org/10.17504/protocols.io.bsm2nc8e.

RT-LAMP Reaction

RT-LAMP reactions were performed according to Lamb *et al*. [15], using WarmStart® Colorimetric LAMP 2X Master Mix DNA and RNA (Eiken Chemical Co., Ltd., Tokyo, Japan), which contains a pH indicator for colorimetric visualization. Robustness was tested from the standard primer concentration and the final reaction volume. Forty-four µM of the FIP primers (16 µM), BIP (16 µM), F3 (2 µM), B3 (2 µM), LOOP F (4 µM), BUCLE B (4 µM), and 56 µM of water were used; in addition, 20 µL of the reagents MIX-LAMP (12.5 µL), MIX-Primers (2.5 µL), RNA (5 µL), and 5 µL of water were used. Reactions were carried out at 65 °C for 45 min, and at 80 °C for 5 min.

Field validation

After laboratory validation, the field evaluation was carried out by selecting persons suspected of COVID-19 infection with up to 15 days of symptoms, who attended hospitals in Lima (Hospital Cayetano Heredia, Hospital Hipólito Unanue and Hospital Arzobispo Loayza), and persons who were evaluated by home care teams (rapid response teams) between August and September 2020. People over 18 years of age, with mild symptoms, without previous diagnosis of COVID-19 by molecular testing were included; pregnant women, and serious or critical patients were excluded. The sample size for this stage was based on the same calculation made for the laboratory validation because they shared the same objective (to identify diagnostic performance measures) although samples had different sources (stored and directly obtained); for the selection of subjects in the field a non-probabilistic consecutive sampling was followed.

The sex, age and clinical picture of each patient, and the time of illness in days, from symptom onset were registered. Each participant underwent NPS, using the Yocon Biology Technology Company sampling kit, which includes viral transport media and flocked dacron swabs. Samples were transported the same day to the INS using triple containers with cold accumulators, at temperatures between 2 °C and 8 °C. All samples were analyzed following the procedure previously described. The RT-qPCR and RT-LAMP results were obtained simultaneously from two different laboratories; the evaluators of each laboratory were unaware of the results of the opposite test they were analyzing.

Statistical analysis

Data analysis was performed using the Stata statistical package version 16.1 (Stata Corporation, College Station, Texas, USA). Summary measures of frequency and percentage were used for the clinical and epidemiological characteristics of the sample of subjects evaluated in the field validation. For both laboratory and field validation, the degree of concordance between RT-qPCR and RT-LAMP test results was determined using Cohen's Kappa index. Sensitivity, specificity, positive and negative predictive value, accuracy value and area under the curve for RT-LAMP were also calculated. Stratified analysis of field results was performed according to week of illness [16,17]; this was not feasible for the laboratory samples because information on time of illness was not available; subjects who did not have information regarding time of illness were excluded from the stratified evaluation. The measures were calculated by means of point estimators and 95% confidence intervals (95% CI); inferences were made considering a significance level of 0.05.

Ethical considerations

Standardization and validation in the laboratory did not require evaluation by the Institutional Research Ethics Committee (CIEI), since the samples used were obtained in routine activities established within the INS action plan, highlighting that they were anonymized. Field validation was carried out using a research protocol approved by the INS CIEI, as shown in RD No. 283-2020-OGITT-INS. All study subjects included in this phase provided informed consent to participate and their results were reported in less than 72 hours.

RESULTS

Performance evaluation of RT-LAMP compared to RT-qPCR

The detection limit of SARS-CoV-2 by RT-LAMP was 1000 copies/µL; which indicated that all cases with Ct values < 30 were concordant between RT-qPCR and RT-LAMP (Figure 1, Table 1). Regarding robustness evaluations, high-throu-

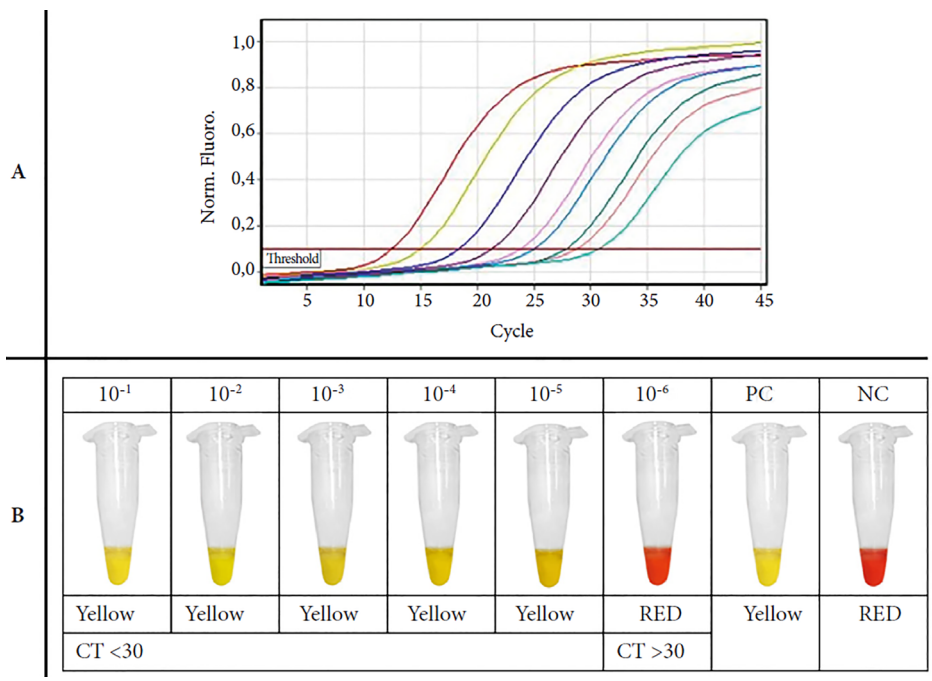

Figure 1. Standard curve of RT-qPCR reactions (panel A) and limit of detection by RT-LAMP (panel B) for the detection of SARS-CoV-2.

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Table 1. Comparison of the detection limit between RT-qPCR and RT-LAMP reactions for detecting the presence of SARS-CoV-2.

Serial Dilution	Concentration (Number of copies/µL)	Ct Value (RT-qPCR)	Change of color (RT-LAMP)
10^{-1}	10^{7}	13.6	Yes
10^{-2}	10^{6}	16.7	Yes
10^{-3}	10^{5}	20.4	Yes
10^{-4}	10^{4}	25.0	Yes
10^{-5}	10^{3}	29.2	Yes
10^{-6}	10^{2}	35.1	No
10^{-7}	10^{1}	-	No

Ct: cycle threshold, RT-qPCR: Real-Time Reverse-Transcription Polymerase Chain Reaction, RT-LAMP: Reverse Transcription Loop-Mediated Isothermal Amplification.

ghput reactions were obtained with half the primer concentrations (0.5P) and with 20 µL final volume (0.8V of the final standard reaction volume).

Laboratory validation

Cross-reaction analysis performed *in silico*, did not identify consistent similarity with reference sequences of other human coronaviruses (NL-63, HKU1, OC43, 229E, SARS-CoV-1, MERS and HECoV, Figure 2). Furthermore, when these same primers were aligned with 194 Peruvian strains available in the GISAID initiative there was no exclusion of conserved regions, which exhibit high similarity and specificity, this can be designated as absence of concomitant detection of other human coronaviruses other than SARS-CoV-2.

For the evaluation of laboratory diagnostic performance, 384 samples were included, of which 37.2% (n=143) had previously positive results by RT-qPCR; when the results were obtained by RT-LAMP, a statistically significant concordance ($p < 0.001$) of 0.88 (95% CI: 0.83-0.93) was identified by Kappa test. Three false positives (FP) were found with a false positive rate (FPR) of 1.2%; in addition, there were 18 false negatives (FN) with a false negative rate (FNR) of 12.6% (Table 2).

The sensitivity obtained from the evaluated samples was 87.4% (95% CI: 80.8-92.4), the specificity was 98.8% (95% CI: 96.4-99.7), the positive predictive value was 97.7% (95% CI: 93.3-99.5), and negative 93.0% (95% CI: 89.1-95.8); the accuracy value of the test was 94.5% (95% CI: 91.8-96.6), and the area under the ROC curve was 93.1% (95% CI: 90.3-95.9) (Table 3).

Field validation

For this stage of the study, 383 subjects were included according to the aspects foreseen in the research protocol, of which 51.7% (n=198) were women; the most frequent age group was young adults (n=236, 61.6%). The most frequent symptoms found were cough (n=268, 70.0%) and pharyngeal pain (n=262, 68.4%); the mean time of illness was 7.1 (SD: 3.3) days; 56.1% were in the first week of illness and 43.6% in the second week, one subject was identified (0.3%) who did not remember the date of onset of the symptoms, and was excluded in the stratified evaluation by time of illness (Table 4).

Of the subjects evaluated, 37.3% (n=143) were positive by RT-qPCR, while positivity by RT-LAMP was 33.7% (n=129). The concordance of the results obtained between both tests had a Kappa of 0.88 (95% CI: 0.84-0.94); when stratified by week of symptoms it was found that during the first week this value was 0.92 (95% CI: 0.86-0.97), while for the second week there was a Kappa of 0.85 (95% CI: 0.76-0.93), in all scenarios the concordance was statistically significant ($p < 0.001$). In the overall sample, 20 (5.2%) cases were found with discordant results between both tests, three FP with a FPR of 1.3%, and 17 FN with a FNR of 11.9%; The FPR was 1.4% for the first week and 1.0% for the second week, while the FNR for the first week of symptoms was 7.9% and for the second week 16.4% (Table 2).

We found that the sensitivity of the overall sample was 88.1% (95% CI: 81.6-92.9). For the first week of symptoms group sensitivity was 92.1% (95% CI: 83.6-97.0) and for the second week group it was 86.6% (95% CI: 72.5-91.5). As for specificity, the value for the overall sample was 98.8% (95% CI: 96.4-99.7), for the first week 98.6 (95% CI: 94.9-99.8) and for the second week 99.0% (95% CI: 94.6-100). The positive predictive value (PPV) and negative predictive value (NPV) for the overall sample were 97.7 (95% CI: 93.4- 99.5) and 93.3 (95% CI: 89.5-96.1), respectively; in the evaluation by week of symptoms the PPV in the first week was 97.2% (95% CI: 90.3 - 99.7) and 98.2% (95% CI: 90.6 -100) for the second

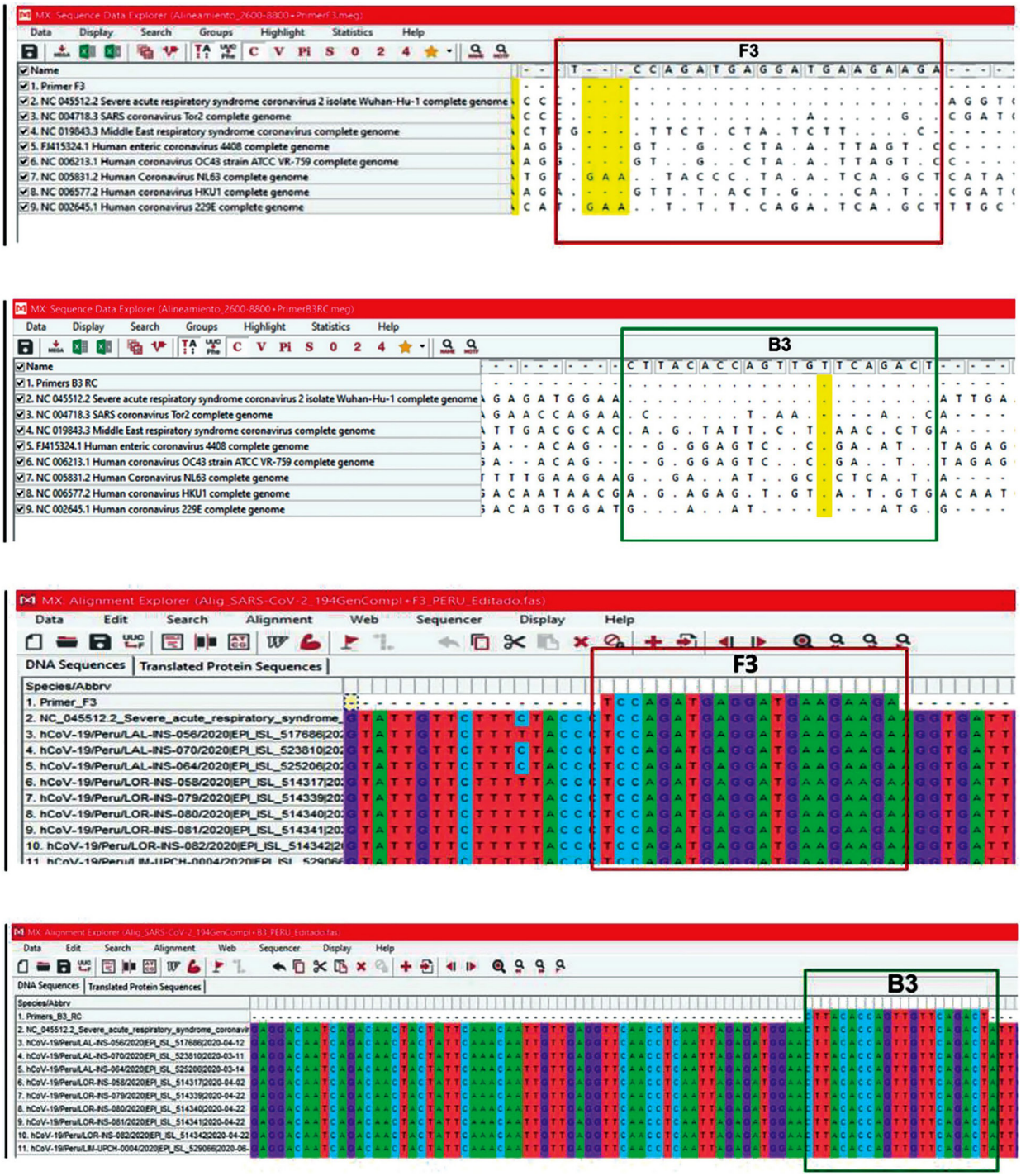

Figure 2: Multiple sequence alignment between the F3 and B3 primers for RT-LAMP and the reference sequences of all known human coronaviruses, as well as with all available Peruvian SARS-CoV-2 strains in the GISAID initiative. Alignment was performed in ClustalW using MEGA. The sequence primers (panel A - F3, panel B - B3) were aligned with all known human coronavirus reference sequences (NC_005831.2, HCoV-NL63; NC_002645.1, HCoV-229E; NC_006213.1, HCoV-OC43 ATCC strain VR-759; NC_006577. 2, HCoV-HKU1; NC_004718.3, SARS-CoV-1; NC_019843.3, MERS; FJ415324.1, HCoV-4408 and NC_045512.2, SARS-CoV-2 Wuhan-Hu-1 isolate) and all 194 Peruvian SARS-CoV-2 strains (panel C - F3, panel D - B3). The yellow column in panels A and B, and the asterisks in panels C and D, represent conservative regions in the *nsp3* gene fragment among all known human coronaviruses and all genome-wide Peruvian SARS-CoV-2 strains, respectively.

week, the NPV was 95.8% (95% CI: 91.0-98.4) for the first week and 90.0 (95% CI: 82.8-94.9) for the second week. The area under the ROC curve was 93.4% (95% CI: 90.7-96.2) in the overall sample, while for those in the first week of symptoms it was 95.3% (95% CI: 92.1-98.5), and 91.3% (95% CI: 86.7-95.9) for those in the second week.

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Table 2. Concordance analysis between the results of laboratory and field evaluations obtained by RT-qPCR and RT-LAMP tests.

RT-LAMP	RT-q CR		Total	Kappa (95% CI)	p Value	Results			
	Positive	Negative				Correct (TP+TN)	Incorrect (FP+FN)	FP (FPR)	FN (FNR)
Laboratory evaluation									
Positive	125	3	128	0.880 (0.831 – 0.930)	<0.001	363 (94.5%)	21 (5.5%)	3 (1.2%)	18 (12.6%)
Negative	18	238	256						
Total	143	241	384						
Field evaluation									
General									
Positive	126	3	129	0.886 (0.838 – 0.935)	<0.001	363 (94.8%)	20 (5.2%)	3 (1.3%)	17 (11.9%)
Negative	17	237	254						
Total	143	240	383						
First week of symptoms									
Positive	70	2	72	0.918 (0.862 – 0.974)	<0.001	207 (96.3%)	8 (3.7%)	2 (1.4%)	6 (7.9%)
Negative	6	137	143						
Total	76	139	215						
Second week of symptoms									
Positive	56	1	57	0.847 (0.764 – 0.930)	<0.001	155 (92.8%)	12 (7.2%)	1 (1.0%)	11 (16.4%)
Negative	11	99	110						
Total	67	100	167						

95% CI: 95% confidence interval, TP: true positive, TN: true negative, FP: false positive, FN: false negative, FPR: false positive ratio, FNR: false negative ratio, RT-qPCR: Real-Time Reverse-Transcription Polymerase Chain Reaction, RT-LAMP: Reverse-Transcription Loop-Mediated Isothermal Amplification.

DISCUSSION

The RT-LAMP test developed in-house demonstrated adequate diagnostic performance and concordance when compared to the RT-qPCR test, both in the laboratory and in the field. There is a need for diagnostic tools with adequate diagnostic performance, high feasibility in the field, requiring less logistics to obtain results and comparable to RT-qPCR; all this in order to improve coverage and meet the existing demand. Taking all these points into account, RT-LAMP is shown to be a viable alternative for all these requirements [18].

The RT-LAMP test is based on a rapid colorimetric reaction by pH change in the presence of a specific amplification, and can provide results in less than two hours after RNA extraction. The Peruvian INS research team selected the protocol described by Lamb *et al.* [15] to compare its diagnostic performance with the RT-qPCR test recommended by WHO to detect the RdRp gene, described by Corman *et al.* [19].

This study had two phases in which 767 clinical samples were processed. Our results showed that the RT-LAMP test has a diagnostic performance similar to that of RT-qPCR, where the detection limit was 1000 copies/µL, ten times lower than the standardized RT-qPCR implemented in routine molecular diagnostics by the INS. However, this difference may be associated with the replication of coronaviruses in general, and their corresponding subgenomic RNAs; this characteristic may cause genes closer to the 3' end to have more copies during replication than those closer to the 5' end [20]. Primers for RT-LAMP were designed to align to the ORF1a region to detect a fragment of the SARS-CoV-2 nsp3 gene, and primers for RT-qPCR were designed to align to the ORF1b region for the RdRp gene fragment.

Based on these replication characteristics of the *Coronaviridae* family, the WHO has suggested that diagnosis should be made using primers for the Nucleocapsid (N) gene or for the ORF1ab genes. However, since ORF1ab represents two thirds of the entire genome (reference sequence NC_045512.2), it should be considered that genes located in the 5' genome have less copies during the replication cycle. Therefore, the nsp3 gene may have a lower amount of RNA

Table 3. Diagnostic performance measures of RT-LAMP in laboratory and field validation, considering RT-qPCR results as reference standard.

RT-LAMP	Laboratory validation (n=384)		Laboratory validation (n=384)					
			General (n=383)		First week of symptoms (n=215)		Second week of symptoms (n=167)	
	%	95% CI	%	95% CI	%	95% CI	%	95% CI
Sensitivity	87.4	80.8 – 92.4	88.1	81.6 – 92.9	92.1	83.6 – 97.0	86.6	72.5 – 91.5
Specificity	98.8	96.4 – 99.7	98.8	96.4 – 99.7	98.6	94.9 – 99.8	99.0	94.6 - 100
Positive predictive value	977	93.3 – 99.5	97.7	93.4 – 99.5	97.2	90.3 – 99.7	98.2	90.6 - 100
Negative predictive value	93.0	89.1 – 95.8	93.3	89.5 – 96.1	95.8	91.0 – 98.4	90.0	82.8 – 94.9
Accuracy	94.5	91.8 – 96.6	94.8	92.1 – 96.8	96.3	92.8 – 98.4	92.8	87.8 – 96.2
Area under the curve	93.1	90.3 – 95.9	93.4	90.7 – 96.2	95.3	92.1 – 98.5	91.3	86.7 – 95.9

95% CI: 95% confidence interval, RT-qPCR: Real-Time Reverse-Transcription Polymerase Chain Reaction, RT-LAMP: Reverse-Transcription Loop-Mediated Isothermal Amplification.

during replication compared to the amount of RNA for the RdRp gene, which would justify the lower sensitivity of the RT-LAMP assay. To overcome these difficulties, we designed a new set of primers for other regions of the genome, especially for RdRp. to properly compare the diagnostic performance considering the same genomic region and the assays are in final validation phase according to the parameters presented in this work.

It was also demonstrated by *in silico* analysis that the set of primers used for RT-LAMP was indeed specific for detecting the Peruvian SARS-CoV-2 strains and did not cross-react with other human coronaviruses in molecular testing. This is considered as a limitation for this study because the analysis should be performed *in vitro* using clinical samples, which was not possible because the INS does not have clinical samples positive for other human coronaviruses. Due to the need to quickly evaluate the performance of this diagnostic method and eventually begin to transfer this technology to the point of care, the alternative of verifying the occurrence of cross-reactivity measured by *in silico* analysis was the most appropriate and scientifically feasible at the time.

Perfect identity in the alignment region of primers F3 and B3 with all available Peruvian SARS-CoV-2 strains also indicated specific detection and possibly no false negative results due to the specificity of the primers.

The evaluation of the robustness of this protocol included variables such as primer concentration and final reaction volume. This strategy considered the possibility that the reactions were performed by individuals who have no routine contact with molecular biology techniques. Since the performance of the reactions was not compromised when using half the primer concentrations and 80% of the final reaction volume, technical errors can be made during small volume pipetting without compromising the results.

RT-LAMP showed high sensitivity and specificity both in the laboratory and in the field, obtaining results similar to those reported by Hu *et al.* (88.57% and 98.98%, respectively) [21], and lower than those described by Jiang *et al.* (91.4% and 99.5%, respectively) [14], as well as by Kitagawa et al. (100% and 97.6%, respectively) [22]. These differences could be associated with the Ct values used to establish positivity by RT-qPCR; moreover, only the positive samples that presented Ct values > 30 differed with those obtained by RT-LAMP in this study.

The concordance values obtained between both tests indicated the applicability of the RT-LAMP protocol as an alternative to RT-qPCR. RT-LAMP could be implemented at the first level of healthcare, becoming useful to identify infected patients in the active transmission phase.

We found that the RT-LAMP test had a PPV of 97.7%, similar to that reported by Jiang *et al.* [14], and much higher than that mentioned by Hu *et al.* (PPV: 91.18%) [21]; we must point out that the latter study evaluated 329 samples of asymptomatic cases, unlike our study in which samples were taken from symptomatic cases. Similarly, the RT-LAMP test had a NPV of 93.3%, which is lower than that reported by Jiang *et al.* [14], who found a NPV of 98.1%.

The degree of concordance in the identification of SARS-CoV-2 between RT-qPCR and RT-LAMP in the clinical assessment was 94.8%, a result similar to that described in other studies such as that of Lu *et al.* [23] and Kitagawa *et al.* [22], where it was over 90%. In our study we found 20 discordant results between RT-LAMP and RT-qPCR in the clinical assessment, 17 FN and three FP; Jiang *et al.* [14], found five discordant results, four FN and one FP. Kitagawa *et al.* [22] reported only

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Table 4. Clinical and epidemiological characteristics of the subjects evaluated in the field.

Characteristics	n	%
Sex		
Male	185	48.3
Female	198	51.7
Age group (years)		
Young (≤ 29)	62	16.2
Young adult (30-59)	236	61.6
Older adult (≥ 60)	85	22.2
Signs and Symptoms		
Ageusia	19	5.0
Anosmia	37	9.7
Headache	214	55.9
Nasal congestion	127	33.2
Diarrhea	80	20.9
Respiratory distress	90	23.5
Joint pain	27	7.0
Sore throat	262	68.4
Muscle Pain	113	29.5
Chest Pain	67	17.5
Fever/chills	179	46.7
Irritability/confusion	2	0.5
General malaise	232	60.6
Nausea/vomiting	46	12.0
Cough	268	70.0
Time of illness		
Not specified [a]	1	0.3
First week	215	56.1
Second week	167	43.6
RT-PCR result		
Negative	240	62.7
Positive	143	37.3
RT-LAMP result		
Negative	254	66.3
Positive	129	33.7

a Patient does not remember the onset of symptoms.

RT-qPCR: Reverse-Transcription Real-Time Polymerase Chain Reaction RT-LAMP: Reverse-Transcription Loop-Mediated Isothermal Amplification

two discordant cases, which were FP. Hu *et al.* [21] also identified four discordant samples (theoretically FP); however, these were confirmed as positive for SARS-CoV-2 by a genetic sequencing test.

When evaluating the performance of RT-LAMP by time of symptom onset, we found that sensitivity and NPV were higher in the first week, and although PPV and specificity showed an increase towards the second week, this increase was not significant. Furthermore, RT-qPCR has shown a higher performance in the first week of symptoms; these findings could be verified by the area under the curve, which decreased from 95.3% in the first week to 91.3% by the second week of symptom onset.

It is worth mentioning that the analysis of quantitative data from RT-qPCR reactions of lower respiratory tract samples is very reliable, especially in cases with small amounts of the virus [24]; however, this analysis could not be performed in our study; in which we only established a relation between Ct values and the time of onset of the disease, showing that samples collected from people in the first week of symptoms presented lower Ct values; this could indirectly cause the viral load to be higher in these people.

The laboratory had the limitation of not having the clinical and epidemiological data of the subjects from whom the samples were taken, for this reason it was not possible to carry out a stratified analysis by time of illness. In the field evaluation, the memory bias of the subjects included in the study was recognized, due to the fact that the data on the time of illness was obtained by self-reporting; only one subject stated that he could not remember the date of onset of his symptoms, and was therefore excluded from the stratified analysis. Our results showed robust confidence intervals with amplitudes in accordance with the estimators used to determine the sample size, as well as the discordant values obtained (FN and FP); studies in which sample sizes are planned with lower absolute error could help to increase precision and thus reduce confidence intervals.

Finally, our data allows us to conclude that the RT-LAMP test developed in-house has been validated as a convenient and acceptable alternative for the detection of SARS-CoV-2 in symptomatic patients, these results being limited to clinical pictures within the first two weeks of illness. This test is proposed as an additional alternative to existing tests, which helps to meet the diagnostic demand during the COVID-19 pandemic. The rapid detection of cases would allow the establishment of effective control measures that would result in the interruption of the chain of infection and a desirable reduction in incidence.

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