Reverse Transcription-Loop-Mediated Isothermal Amplification-CRISPR-Cas13a Technology as a Promising Diagnostic Tool for SARS-CoV-2

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ABSTRACT
At the end of 2019, a new coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), caused a pandemic that persists to date and has resulted in more than 6.2 million deaths. In the last couple of years, researchers have made great efforts to develop a diagnostic technique that maintains high levels of sensitivity and specificity, since an accurate and early diagnosis is required to minimize the prevalence of SARS-CoV-2 infection. In this context, CRISPR-Cas systems are proposed as promising tools for development as diagnostic techniques due to their high specificity, highlighting that Cas13 endonuclease discriminates single nucleotide changes and displays collateral activity against single-stranded RNA molecules. With the aim of improving the sensitivity of diagnosis, this technology is usually combined with isothermal preamplification reactions (SHERLOCK, DETECTR). Based on this, we developed a reverse transcription-loop-mediated isothermal amplification (RT-LAMP)-CRISPR-Cas13a method for SARS-CoV-2 virus detection in nasopharyngeal samples without using RNA extraction that exhibits 100% specificity and 83% sensitivity, as well as a positive predictive value (PPV) of 100% and negative predictive values (NPVs) of 100%, 81%, 79.1%, and 66.7% for cycle threshold (Ct) values of <20, 20 to 30, >30 and overall, respectively.

IMPORTANCE
The coronavirus disease 2019 (COVID-19) crisis has driven the development of innovative molecular diagnosis methods, including CRISPR-Cas technology. In this work, we performed a protocol, working with RNA extraction kit-free samples and using RT-LAMP-CRISPR-Cas13a technology; our results place this method at the forefront of rapid and specific diagnostic methods for COVID-19 due to the high specificity (100%), sensitivity (83%), PPVs (100%), and NPVs (81% for high viral loads) obtained with clinical samples.

KEYWORDS
COVID-19, SARS-CoV-2, RT-LAMP, CRISPR-Cas13, CRISPR-Cas, diagnosis

Since their emergence at the beginning of the 21st century, coronaviruses have been recognized as a health concern because of their ability to cause severe respiratory infections in humans. At the end of 2019, a new coronavirus appeared, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), producing a novel illness, coronavirus disease 2019 (COVID-19), and showing two remarkable characteristics: the virus causes the development of an unusual viral pneumonia, and it is highly transmissible and thus spreads rapidly (1–3). This led to the SARS-CoV-2 pandemic, which persists to date and has caused more than 6.2 million deaths (WHO COVID-19 Dashboard [https://covid19.who.int/]).

Fortunately, vaccination campaigns have decreased the incidence of COVID-19 (4). However, specialists claim that this virus is likely to coexist with us for a long time, as...
the price of vaccines and the necessary cold-chain stability make it difficult for the vaccine to reach the most remote places in the world, as SARS-CoV-2 does. Together with the fact that no efficient therapy has been developed for COVID-19, this indicates that accurate and early diagnosis in point-of-care (POC) testing is required to minimize the prevalence of SARS-CoV-2 infection (1–3).

In the last couple of years, researchers have made great efforts to develop a diagnostic technique that maintains high levels of sensitivity and specificity, without the need for expensive equipment or highly trained personnel for its implementation. Such a diagnostic technique would allow the detection of SARS-CoV-2 infection in health centers, as well as at home or in the field, which would accelerate the identification of infected patients, enabling prompt treatment and halting the spread of SARS-CoV-2 worldwide (5).

The use of nucleic acids as biomarkers has become the diagnostic gold standard, because of the species specificity of the technique and because DNA and RNA can be amplified (6).

Although the reverse transcription-PCR (RT-PCR) assay is routinely used as the gold standard diagnostic test for COVID-19 (5, 7–10), throughout the pandemic period, it has shown sensitivity levels of 45% to 60% (10) and even lower than 40%, according to some authors (7), and worrying false-negative rates of 2% to 29% (10, 11). Additional downsides of this amplification method are the elevated costs (expensive equipment for implementation and readout of results), the need for specialized personnel in laboratories and the time required (4 to 6 h) (5, 8, 10). Consequently, isothermal amplification reactions are becoming especially important in the diagnosis of COVID-19 (5). Although different methods of isothermal amplification are available, recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) reactions are the methods most commonly used in research. The LAMP-based technique has displayed greater specificity than RPA (5, 12). LAMP has previously been used to detect several microorganisms, and the aforementioned advantages led to its optimization for COVID-19 diagnosis, and it has been applied in association with other techniques which increase diagnostic specificity, such as clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein (CRISPR-Cas) systems (5, 13–15).

Naturally, CRISPR-Cas systems provide adaptive immunity for bacteria and archaea, as they collect genomic fragments (spacers) from foreign elements (bacteriophages, plasmids, and other mobile genetic elements) that are expressed in an RNA molecule form (crRNA) that guides an endonuclease protein (Cas) to the pathogen for the final degradation of its nucleic acid material (16, 17).

Since their discovery, CRISPR-Cas systems have revolutionized the field of molecular biology. Initially, they were presented as highly specific tools for genome editing. However, they are also applicable for the diagnosis and treatment of infectious diseases and are now considered key for development in these areas (16, 17).

Class 2 CRISPR-Cas systems have a simpler effector structure, which makes them more attractive for use in genome editing, diagnosis, and treatment. In this class, Cas12 and Cas13 proteins display nonspecific endonuclease activity when activated (collateral activity) against single-stranded DNA (ssDNA) and RNA (ssRNA), respectively. This feature could be applied in clinical diagnosis, taking advantage of the reporter molecule target of this activity (collateral-based detection), which acts by amplifying the detection signal. Therefore, Cas12 and Cas13 are proposed as the most promising tools for use in diagnostic techniques, with the latter being particularly important in terms of specificity, as it has the ability to discriminate single nucleotide changes (16).

Researchers recently developed two novel assays for detecting SARS-CoV-2 based on CRISPR-Cas technology: DETECTR and SHERLOCK. The DETECTR technique uses reverse transcription-LAMP (RT-LAMP) for amplification and Cas12 as an endonuclease, while SHERLOCK uses RT-RPA for amplification and Cas13 (18, 19). On the basis of these works, in this study, we describe the development and optimization of a LAMP-CRISPR-Cas13a technique for the diagnosis of SARS-CoV-2 infection in clinical samples in a
process that does not require RNA extraction or purification (Fig. 1). With this technique, high levels of sensitivity and specificity, comparable to those associated with RT-PCR, were obtained.

RESULTS

Analysis of the state of the art. We obtained an output of more than 7,000 articles as a result of a search using the keywords “RT-PCR diagnosis COVID-19”, of which almost 4,000 were published in 2021 alone. This result was compared with the findings of Bhatt et al. (20) concerning papers related to RT-LAMP and CRISPR for SARS-CoV-2 diagnosis. Of these, we analyzed 10 articles on the RT-LAMP technique and 10 articles related to RT-LAMP-CRISPR-Cas technology, finding that only 1 applied the endonuclease Cas13 for SARS-CoV-2 diagnosis, but always on samples treated with an RNA extraction kit (21) (Table 1).

Data collected from the RT-LAMP articles were used to determine the range of values of the parameters considered: sensitivity, 81% to 98%; specificity, 36% to 100%; positive predictive value (PPV), 86% to 100%; and negative predictive value (NPV), 78% to 99% (Table 1). The results showed that major efforts have been made to detect SARS-CoV-2 in RNA-purified samples (8/10), although RNA extraction-free research has also yielded potentially useful results (sensitivity, >94%; specificity and PPV, 100%; NPV, >92%). However, the highest levels of sensitivity and specificity were obtained in projects involving extracted viral RNA (Table 1).
TABLE 1 Data and parameters collected from 10 articles applying different methods to detect SARS-CoV-2 infection

| Method            | Sample        | RNA extraction kit | Cas     | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Ref. |
|-------------------|---------------|--------------------|---------|-----------------|-----------------|---------|---------|------|
| RT-LAMP           | Saliva        | No                 | Cas     | 94.3            | 100             | 100     | 92.6    | 22   |
|                   | NPS/OPS       | No                 | Cas     | 95.2            | 100             | 100     | 92.6    | 23   |
|                   | NPS           | Yes                | Cas     | 81.8            | 100             | 100     | 95.2    | 32   |
|                   | NPS           | Yes                |         | 94.5            | 99              | 98.8    | 95.2    | 25   |
|                   | NPS/Saliva    | Yes                |         | 85.9            | 99.5            | 96.8    | 97.4    | 26   |
|                   | NPS           | Yes                |         | 98.1            | 36.4            | 93.7    | 66.7    | 27   |
|                   | NPS           | Yes                |         | 98              | 90.9            | 87.5    | 98.6    | 28   |
|                   | NPS/OPS/Saliva| Yes                |         | 97.8            | 99.9            | 99.8    | 99.9    | 29   |
|                   | NPS/Saliva    | Yes                |         | 94.1            | 60.5            | 86.7    | 78.8    | 30   |
|                   |               |                    |         | 87              | 98.5            | 97.9    | 90.2    | 31   |
| RT-LAMP-CRISPR    | NPS/OPS       | Yes                | Cas13   | 97.4            | 100             | 100     | 66.7    | 34   |
|                   | NPS/OPS       | No                 | Cas12   | 89.7            | 100             | 100     | 78.6    | 24   |
|                   | Respiratory   | Yes                | Cas12   | 94              | 100             | 100     | 94.3    | 35   |
|                   | Saliva        | Yes                | Cas12   | 87.7            | 100             | 100     | 73.6    | 36   |
|                   | NPS/OPS       | Yes                | Cas12   | 85.7            | 100             | 100     | 50      | 37   |
|                   | NPS           | Yes                | Cas12   | 93.1            | 98.5            | 98.4    | 93.4    | 38   |
|                   | Respiratory   | Yes                | Cas12   | 92.6            | 100             | 100     | 93.1    | 39   |
|                   | NPS           | Yes                | Csm complex | 73.9          | 100             | 100     | 45.5    | 21   |
|                   | NPS           | Yes                | Cmr complex | 77.5          | 100             | 100     | 52.6    | 33   |
|                   | NPS           | Yes                | Cas3    | 90              | 95.2            | 90      | 95.2    | 40   |

*NPS, nasopharyngeal swab; OPS, oropharyngeal swab.

Most of the reviewed papers (8/10) related to RT-LAMP-CRISPR-Cas technology used samples treated with extraction kits. Moreover, only 1 study applied the Cas13 enzyme as an effector protein and used RNA extracted using a kit. In this case, the values for the calculated data were 73% to 97% for sensitivity, 95% to 100% for specificity, 90% to 100% for PPV, and 50% to 95% for NPV (Table 1).

**SARS-CoV-2 detection.** The best results for collateral-based detection reaction were achieved with 50 nM Cas13a enzyme and a Cas13a/crRNA molar ratio of 2:1. On the other hand, the HybriDetect lateral flow kit showed higher sensitivity when reporter 2 was used at a final concentration of 1,000 nM and the assay buffer was supplemented with 5% polyethylene glycol (PEG).

Determination of the limit of detection (LOD) of the CRISPR-Cas13a-based technology revealed that this technique detects as few as 1 to 10 SARS-CoV-2 particles (Fig. 2). After proteinase K-heat inactivation (PK-HID) treatment, the LAMP-CRISPR-Cas13a technique correctly detected samples with a cycle threshold ($CT$) value of <20 as positive. From samples with $CT$ values of 20 to 30 and >30, the technique identified coronavirus as present in 83.3% and 62.5% of the samples, respectively (Fig. 3C). Finally, the CRISPR-Cas13a technology did not detect SARS-CoV-2 infection in negative samples (Fig. 3A). Based on the results obtained (Fig. 3B), we estimated that the RT-LAMP-CRISPR-Cas13a method for COVID-19 detection exhibits 100% specificity and 83% sensitivity, as well as a PPV of 100% and NPVs of 100%, 81%, 79.1%, and 66.7% for $CT$ values of <20, 20 to 30, >30 and overall, respectively (Fig. 3C). The statistical analysis yielded a receiver operating characteristic (ROC) curve with an area under the curve (AUC) of 0.84 (95% confidence interval [CI], 0.73 to 0.93) (Fig. 4A); in addition, examination of the scatterplot revealed that diagnostic results could be confused in nasopharyngeal samples with a $CT$ value of >30 (Fig. 4B).

**DISCUSSION**

Study of the state of the art revealed that greater efforts must be made to innovate in diagnostic methods; Bhatt et al. (20) found 1,286 papers related to RT-LAMP and CRISPR for SARS-CoV-2 diagnosis (surprisingly, only 98 of these applied RT-LAMP integrated with CRISPR-Cas technology), in contrast with the 7,000 studies involving RT-PCR. This indicates that efforts should also be focused on developing more efficient RT-LAMP-CRISPR-Cas protocols without RNA purification, which would reduce the cost of the testing and also produce results faster. Only 3 of the 20 papers reviewed did not use an RNA extraction kit (22–24). In addition, there are several advantages to the
application of Cas13 endonuclease, as it has been reported to be more specific than other effector proteins (16).

In this work, our research group developed an RT-LAMP-CRISPR-Cas13a protocol for diagnosing SARS-CoV-2 infection with an LOD of 10 viral copies, which is similar to the LOD of the RT-PCR method, considered the gold standard for diagnosis of COVID-19 (5, 7–10, 41). However, it has been reported that the RT-PCR for SARS-CoV-2 detection has a limited sensitivity of 45% to 60% (10), while the RT-LAMP-CRISPR-Cas13a technology increases this value significantly, up to 83%. As previously mentioned, the gold standard
shows downsides in terms of costs, implementation, and time consumption (5, 8, 10) that are surpassed by the RT-LAMP-CRISPR-Cas13a technique. (i) RT-PCR requires a high-quality RNA extraction method, while our technology is applied on samples processed using the simple PK-HID protocol. (ii) The gold standard depends on expensive equipment and specialized personnel, which raise the price per reaction and difficulty of use outside of laboratories; by contrast, the RT-LAMP-CRISPR-Cas13a protocol eliminates the need for a thermocycler and sophisticated readout equipment, allowing easier implementation. (iii) The RT-PCR protocol takes at least 4 to 6 h, in contrast with the RT-LAMP-CRISPR-Cas13a method, which takes less than 2 h. For all these reasons, this RT-LAMP-CRISPR-Cas13a-based assay is proposed as a strong option to replace the current molecular gold standard diagnostic test.

Furthermore, considering the criteria recommended by the WHO (42), this novel technique fulfills the three key features of accuracy, accessibility, and affordability. This is because on the one hand, it showed an accuracy \((\text{true positive } \frac{TP}{\text{total}} + \text{true negative } \frac{TN}{\text{total}})\) of 87.2%, and on the other hand, it is both accessible and affordable.

Comparing our results on sensitivity, specificity, PPV, and NPV with those obtained in previous studies, we found that the specificity and PPV values of the RT-LAMP-CRISPR-Cas13a technology were higher than those in 7 of the 10 RT-LAMP papers reviewed (25–31), and in one case, the sensitivity of this novel technique was even higher (32). Moreover, this technique showed higher sensitivity and NPV values than those in 2 of the 10 RT-LAMP-CRISPR papers reviewed which applied an RNA extraction kit to the clinical samples (21, 33). Among the others, 7 of 8 studies used DNA target-endonuclease effectors, and thus, a higher sensitivity could be obtained due to the intrinsic stability of DNA in contrast to that of RNA molecules. The lower sensitivity of the RT-LAMP-CRISPR-Cas13a protocol (83%) than that described in a previous study (97.4%) could be explained by the fact that the researchers used an RNA extraction kit (Direct-zol), so that the RNA was purified and concentrated, and also that the results were revealed by fluorescence (34).

ROC analysis has become a popular method for evaluating the accuracy of medical diagnostic systems, as it provides accurate indices for the techniques tested that are not distorted by fluctuations caused by the use of arbitrarily chosen decision criteria or cutoff points (43). The AUC determines the inherent ability of the test to correctly identify a person as infected or not, where an AUC value of 0.5 indicates an absence of capacity for discrimination between infected and healthy populations, a value of 0.5 to

**FIG 4** (A) ROC curve for RT-LAMP-CRISPR-Cas13a technology. (B) Scatterplot of two groups, false-negative and true-positive detections with RT-LAMP-CRISPR-Cas13a, versus the \(C\) values of the respective samples.
0.7 is related to unsatisfactory discrimination, and the discrimination power is acceptable when the AUC value is between 0.7 and 0.8, excellent for values contained in the range 0.8 to 0.9, and perfect when the AUC is close to 1 (44). The value of the area under the ROC curve, calculated by statistical analysis, validated our RT-LAMP-CRISPR-Cas13a technique as a reliable diagnostic method. Furthermore, the results shown in Fig. 4B indicate that this protocol provides less accurate diagnostics when viral loads are low. However, we should bear in mind that at this stage of infection, individuals present almost no risk of being contagious (45, 46).

In summary, the high levels of specificity, sensitivity, PPV, and NPV obtained using this promising protocol working with RNA extraction kit-free samples place the LAMP-CRISPR-Cas13a technology at the forefront of rapid and specific diagnostic methods for infectious diseases. Thus, this technique could be established as a diagnostic tool for detecting other viral (papillomavirus [47, 48], Zika virus [49, 50], dengue virus [50], African swine fever virus [51], Ebola virus [52]) and bacterial (53, 54) (tuberculosis [55]) diseases, as previously done by other authors for infections such as those caused by multiresistant pathogens (56, 57). However, Cas13 detection methods should be optimized to enable direct diagnosis without prior amplification of nucleic acids.

MATERIALS AND METHODS

Study of the state of the art. A study of the state of the art was conducted with the aim of comparing the use of different novel diagnostic techniques. First, we conducted a search in PubMed with the keywords “RT-PCR diagnosis COVID-19” and compared the output with the number of publications on RT-LAMP and RT-LAMP-CRISPR strategies for COVID-19 diagnosis (20). Then, we collected data on the different sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) from 10 papers related to RT-LAMP and 10 papers on the RT-LAMP-CRISPR-Cas COVID-19 diagnostic technique (21–40). We used the results to calculate the parameters needed for the comparison.

In silico analysis and design of RT-LAMP primers, crRNAs, and RNA reporters. The nucleocapsid gene (GenBank Gene ID: 43740575) of the SARS-CoV-2 virus was selected for study due to the fact that it shows a higher abundance of subgenomic mRNAs than other targets, which boosts the sensitivity of the diagnostic technique (58). Furthermore, the mutation rate found in this gene is lower than that in other targets, such as the spike gene and the ORF gene (59, 60). The target sequence was analyzed in silico with the aim of designing specific primers for amplification of a genetic region without any previously described mutation (N gene region, 12 to 213 bp [N2 gene]) (61). Three pairs of LAMP primers were designed using PrimerExplorer V5 software (F3-B3, FIP-BIP, and Floop-Bloop) to amplify the SARS-CoV-2 N2 gene. The FIP LAMP primer contained the T7 polymerase promoter in its sequences for the subsequent transcription step (Table 2).

Two different RNA reporters (reporters 1 and 2) were used to reveal the results in order to select the one with the best signal. Both contained a single isomer derivative of fluorescein modification (FAM) at the 5’ extreme and a biotin molecule at the 3’ extreme (Table 2).

Clinical samples. Clinical samples were supplied by the Microbiology Service of the Teresa Herrera Materno Infantil Hospital (A Coruña, Spain). The samples (n = 133) were obtained from nasopharyngeal swabs for SARS-CoV-2 detection (Table 3).
**TABLE 3** Positive and negative samples for SARS-CoV-2

| Result          | No. of samples | Origin       | $C_T$*  |
|-----------------|----------------|--------------|---------|
| Positive        | 27             | Nasopharyngeal | <20     |
|                 | 48             | Nasopharyngeal | 20–30   |
|                 | 24             | Nasopharyngeal | >30     |
| Total           | 99             |              |         |
| Negative        | 34             | Nasopharyngeal |         |

*C<sub>T</sub>* cycle threshold.

**Ethical approval.** Ethical approval was granted by the Galicia Drug Research Ethics Committee (CEIm-G), and internal ethical approval was received by the Institute of Research A Coruña (INIBIC) from Coruña Hospital (CHUAC) (2020/207).

**Sample processing.** For sample processing, a proteinase K-heat inactivation (PK-HID) protocol was applied to samples from swabs stored in viral transport medium (Gibco) (62) as follows. Aliquots (95 μL) of samples were treated for 15 min at 55°C with 5 μL of proteinase K (10 mg/mL; stock), prepared at 1 mg/mL in a final volume of 100 μL, and heat-inactivated at 98°C for 5 min. Finally, the extracted RNA samples were stored at −80°C.

**RT-LAMP reaction.** Amplification using the RT-LAMP (WarmStart LAMP kit [DNA and RNA]; NEB) reaction was performed following the manufacturer's protocol. Briefly, RNA samples (5 μL) were added to a reaction mixture containing 12.5 μL of WarmStart LAMP 2× master mix and 2.5 μL of 10× primer mix (FIP-BIP, 16 μM; BIP-BF, 2 μM; Floop-Bloop, 4 μM; stock) adjusted to a final volume of 25 μL with dH<sub>2</sub>O.

The reaction mixtures were incubated at 65°C for 1 h.

**Collateral-based detection.** Each Cas13a-based detection reaction mixture was incubated at 37°C for 30 min with the following reaction components: 2 μL of 10× cleavage buffer (200 mM HEPES, 90 mM magnesium chloride, 600 mM sodium chloride), 0.5 μL of deoxynucleoside triphosphates (dNTPs) (HiScript T7 quick high-yield RNA synthesis kit), 0.5 μL of T7 polymerase (HiScript T7 quick high-yield RNA synthesis kit), 20 U RNase murine inhibitor (NEB), 0.15 μL Cas13a endonuclease (25 nM; MCLAB), 0.5 μL crRNA (50 nM; IDT), 2 μL reporter (1,000 nM; IDT), and 5 μL of a cDNA sample, adjusted to a final volume of 20 μL with dH<sub>2</sub>O.

Different concentrations of Cas13a and crRNA (200, 100, and 50 nM) were tested, and two different enzyme/guide molar ratios were used (1:1 and 2:1).

**HybriDetect lateral flow assay.** Results were revealed using the HybriDetect lateral flow assay as described by the manufacturer (Milenia Biotech), with some modifications. Briefly, 20 μL of collateral-based detection product was mixed with 80 μL of assay buffer in a 96-well plate. Immediately, the gold extreme of the trip was submerged in the mix and held for 2 to 3 min.

Following the manufacturer’s instructions, the reactive strips required calibration before application for management of an optimal RNA reporter concentration, and as mentioned, reporters 1 and 2 were tested. The results obtained using two different assay buffers were also compared: the kit assay buffer and the same supplemented with 5% polyethylene glycol (PEG).

The results obtained, i.e., true positive (TP), false positive (FP), false negative (FN), and true negative (TN), were used to calculate the following parameters: sensitivity (TP/TP + FN), specificity (TN/TN + FP), PPV (TP/TP + FP), and NPV (TN/TN + FN).

**Limit of detection.** For estimating the number of initial SARS-CoV-2 viral particles that the CRISPR-Cas13a technology was able to detect, we serially diluted (1:10) the RNA extracted using hospital equipment from two clinical samples with $C_T$ values of 20 and 25. Finally, 5-μL aliquots of each dilution were used for calculation of the limit of detection (LOD). Here, we applied an estimated correlation between the $C_T$ value and the viral load.

**Statistical analysis.** Statistical analysis was conducted using the GraphPad Prism9 program to construct a receiver operating characteristic (ROC) curve with a confidence interval of 95% (Wilson/Brown method) and to construct a scatterplot of two groups (false-negative and true-positive samples) against the $C_T$ value of each sample.

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