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1. Introduction

The current pandemic COVID-19 caused by the coronavirus SARS-CoV-2, has generated different economic, social and public health problems. Moreover, wastewater-based epidemiology could be a predictor of the virus rate of spread to alert on new outbreaks. To assist in epidemiological surveillance, this work introduces a simple, low-cost and affordable electrochemical sensor to specifically detect SARS-CoV-2 in wastewater samples in a fast and sensitive manner [19]. The nucleic acid testing of wastewater for the presence of SARS-CoV-2 nucleic acid as a surveillance and management tool is essential to slow the spread of the virus [11]. SARS-CoV-2 may enter wastewater systems from pathogen shedding in human waste, resulting in a potentially fecal-oral transmission with a serious health consequence [12–14]. Recently, several groups in different countries isolated and detected the genetic material of SARS-CoV-2 in wastewater using reverse transcription quantitative polymerase chain reaction (RT-qPCR), as a gold standard technique [15–17]. However, one important problem is that RT-qPCR still requires expensive laboratory infrastructure and skilled technicians or scientists to complete the assay. Furthermore, more efforts are needed to develop rapid and accurate detection tools for wastewater surveillance and management of the SARS-CoV-2 spread using molecular diagnostics in limited-resources settings [18].

In this context, it results imperative the application of versatile and affordable tools to detect viral or microbiological pathogens in environmental samples in a fast and sensitive manner [19]. The nucleic acid

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**ABSTRACT**

The current pandemic COVID-19 caused by the coronavirus SARS-CoV-2, has generated different economic, social and public health problems. Moreover, wastewater-based epidemiology could be a predictor of the virus rate of spread to alert on new outbreaks. To assist in epidemiological surveillance, this work introduces a simple, low-cost and affordable electrochemical sensor to specifically detect SARS-CoV-2 in wastewater samples in a fast and sensitive manner [19]. The nucleic acid testing of wastewater for the presence of SARS-CoV-2 nucleic acid as a surveillance and management tool is essential to slow the spread of the virus [11]. SARS-CoV-2 may enter wastewater systems from pathogen shedding in human waste, resulting in a potentially fecal-oral transmission with a serious health consequence [12–14]. Recently, several groups in different countries isolated and detected the genetic material of SARS-CoV-2 in wastewater using reverse transcription quantitative polymerase chain reaction (RT-qPCR), as a gold standard technique [15–17]. However, one important problem is that RT-qPCR still requires expensive laboratory infrastructure and skilled technicians or scientists to complete the assay. Furthermore, more efforts are needed to develop rapid and accurate detection tools for wastewater surveillance and management of the SARS-CoV-2 spread using molecular diagnostics in limited-resources settings [18].

In this context, it results imperative the application of versatile and affordable tools to detect viral or microbiological pathogens in environmental samples in a fast and sensitive manner [19]. The nucleic acid
based isothermal amplification methods have been extensively deployed as a sensitive and straightforward techniques [20]. Specifically, loop-mediated isothermal amplification with simultaneous reverse-transcription (RT-LAMP) allows for rapid and analytically sensitive detection of nucleic acids within one hour that requires only a heat source [21]. Several groups are currently developing LAMP-based protocols for the detection of SARS-CoV-2 in clinical samples [22–25]. However, scarce information is reported about RT-LAMP technique as a cheaper and faster option for monitoring the genetic material of SARS-CoV-2 in wastewater-based epidemiology [26], and its integration with attractive sensing schemes.

Among the wide variety of transduction mechanisms for detecting nucleic acid amplification, one can find electrochemical-based sensors [27,28], optical devices [29], colorimetric assays [30], luminescence-based sensors [31], and surface plasmon resonance [32], to mention only a few. Particularly, electrochemical transduction has demonstrated its ability to provide a cost-effective alternative to circumvent manufacturing and integration processes to robust devices. Moreover, electrochemical sensors exhibit several advantages such as, low-cost, portability, miniaturization and high reliability, ideal for in-situ measurements [33,34]. Nevertheless, common electrochemical biosensors need a labeled receptor to be immobilized on the sensitive element or electrode [35,36]. Due to the COVID-19 pandemic, several investigations have been devoted to simplify the experimental processes and methods to provide affordable and versatile platforms suitable for a easy-to-develop sensors in resource-limited or field settings [37]. For instance, the work in [38] shows the trends in electrochemical sensors for rapid detection of SARS-CoV-2 from human samples focusing on viral nucleic acid, immunoglobulin, antigen, and the entire viral particles.

Monitoring and detecting SARS-CoV-2, however, remain a challenging task, even more when testing environmental samples due to its complex structure. To overcome the difficulties encountered in classical benchtop equipment and methods, herein we report the development and potentiality of an electrochemical sensor for detecting SARS-CoV-2 in wastewater samples. To the best of our knowledge, the sensor innovates in the following aspects:

- By working around a RT-LAMP reaction, it is a cost-effective and less-time consuming alternative to the classical RT-PCR amplification, without losing specificity.
- It works around screen-printed electrodes (SPEs) and minimal instrumentation, which is a current trend for field-deployable and low-cost detection systems.
- The sensor is primarily devoted to retrieve end-point results for detecting RT-LAMP amplicons, and additionally, shows promising results for real-time quantification.
- Its potentiality is demonstrated by measuring real wastewater samples for a current sanitary problem, which is an underestimated measure of the RT-LAMP reaction. The amplification and monitoring code the concentration of the nucleic acids according to the sensor model. Jointly, the turnaround time for a complete experiment is 2 h. To validate the electrochemical monitoring performance, a colorimetric assay was simultaneously performed on the evaluated wastewater samples.

2. Materials and methods

Fig. 1 shows the device workflow comprising four main stages: i) wastewater sampling, ii) RNA concentration, iii) RT-LAMP mixture, and iv) the electrochemical monitoring of RT-LAMP reaction. First, the samples are collected and then the nucleic acids are extracted and concentrated using a custom-developed method [18]. Together these methodologies take a time of 1 h 30 min in the laboratory with minimum infrastructure. Afterwards, the RNA is mixed with the RT-LAMP primers and methylene blue (MB) as a redox intercalator for the electrochemical transduction. Hence, a micro-volume sample is drop cast over the surface of custom fabricated screen-printed electrodes (SPEs), wherein the RT-LAMP reaction takes place by controlling the local temperature at 63 °C. Thereby, the resultant diffusion-controlled current, promoted by the redox process, is monitored by a portable potentiostat to provide a measure of the RT-LAMP reaction. The amplification and monitoring take approximately 30 min. Finally, the peak current change %ΔIp encodes the concentration of the nucleic acids according to the sensor model. Jointly, the turnaround time for a complete experiment is 2 h. To validate the electrochemical monitoring performance, a colorimetric assay was simultaneously performed on the evaluated wastewater samples.

2.1. Wastewater samples

The sampling was carried out at two wastewater treatment plants (WTTP) in the metropolitan area of the City of Queretaro, Mexico (see Table 1). In South and Santa Rosa plants, the influent was sampled. Samples were collected from the period between May 31 and June 7, 2021. The influent samples (500 mL) were collected during the morning (9–11 am) and kept at 4 °C until their use.

2.2. Concentration and extraction of RNA

Samples were concentrated the same day of sampling, using the electronegative membrane method owing its detection limit for SARS-CoV-2 genes [18]. Briefly, the pH of samples was adjusted to 3.5 with 2 N HCl and then were filtered through a negatively charged nitrocellulose membrane (0.45 μm pore diameter, Millipore, Netherlands). According to the manufacturer’s instructions, the membranes were cut and used directly in the RNeasy Power Microbiome extraction kit (Qiagen, Germany) for RNA extraction. RNA was stored at – 20 °C until its use.

2.3. RT-LAMP reactions

In a two step RT-LAMP assay, RNA (5 μL) was reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen, Germany) following manufacturer’s instructions. 50 μL LAMP reaction contained: 5 μL Buffer Bst (NEB), 3 μL MgSO4 (NEB), 5 μL of 2 mM dNTPs (Thermo Scientific), and 5 μL of 2 mM dNTPs (Thermo Scientific), 2.3. RT-LAMP reactions

In a two step RT-LAMP assay, RNA (5μL) was reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen, Germany) following manufacturer’s instructions. 50 μL LAMP reaction contained: 5 μL Buffer Bst (NEB), 3 μL MgSO4 (NEB), 5 μL of 2 mM dNTPs (Thermo Scientific),
2 mL of 10X primer’s core mix, 2 mL of 10X primer’s loop mix, 10 U of Bst 2.0 DNA polymerase (NEB), 4 mL cDNA and nuclease free water to make up 50 mL of reaction volume. The specific primers were designed by [39] for the SARS-CoV-2 N and ORF1ab genes, and were validated using PrimerExplorer V5 (https://primerexplorer.jp/e/) as shown in Table 2.

RT-LAMP reactions were monitored by the electrochemical sensor using MB at 6 μM in uniform temperature for 30 min. Also, the reactions of RT-LAMP assays were checked on 1% agarose gel stained with SYBR Safe DNA Gel Stain for the presence of ladder pattern, and the products were also verified by sequencing. Afterwards, the concentration of the RT-LAMP amplified products was estimated using a Nanodrop™ spectrophotometer. Finally, to test the specificity of the amplification, the products of the RT-LAMP reactions were verified by sequencing (Genbank OM522662).

### 2.4. Electrochemical monitoring

For electrochemical monitoring, MB was added to the RT-LAMP mixture as an electroactive intercalator [40], exhibiting strong and specific binding ability to dsDNA amplex without inhibiting the RT-LAMP process. A negative control template (NTC) was composed by performing a cyclic voltammetry (CV) in the range from 1.0 to 2.0 V and a scan rate of 100 mV/s scan rate using a Tris acetate buffer prepared by performing a CV.

The assay was performed in a 50 μL reaction mixture containing 4 μL of 10X primer mix of 16 μM (each) of Forward Inner Primer (FIP) and Backward Inner Primer (BIP), 2 μM (each) of F3 and B3 primers, 4 μM (each) of Forward Loop (LoopF) and Backward Loop (LoopB) primers, 20 μL of WarmStart™ Colorimetric Lamp 2X Master Mix (M1800, New England BioLabs INC.) 10 μL of DNAse, RNAse free water, and 5 μL of RNA template. The reaction mixture was heating at 63 °C for 30 min on a dry bath. Finally, the concentration of the RT-LAMP amplified products were estimated using a Nanodrop™ spectrophotometer.

### 3. Results and discussion

#### 3.1. Performance and specificity

As the first step, we characterized the electrochemical sensor by using well-know concentrations of double-stranded DNA. Fig. 2(a) shows the square-wave voltammograms retrieved for a NTC, as the base signal and, for eight concentrations (c1 = 0.001, c2 = 0.01, c3 = 0.1, c4 = 1, c5 = 10, c6 = 100, c7 = 1000 and c8 = 10000 × 10⁻³ ng/μL). Therein, one can see how the peak current is located around the MB formal potential, −0.25 V. Moreover, it is worth to notice a decrease in the peak current as the concentration grows up. This situation is due to the MB acting as a redox intercalating probe. For the NTC sample, there are no amplicons, and hence, the electroactive molecules tend to diffuse onto the surface of the working electrode, thus giving a high peak current signal. On the other hand, as the concentration increases due to the RT-LAMP reaction, more dsDNA was synthesized and the MB intercalated, and hence, the electroactive molecules tend to diffuse onto the surface of the working electrode, thus giving a high peak current signal. The concentration of the RT-LAMP amplified products was estimated using a Nanodrop™ spectrophotometer.

Table 2

| Gene      | Primer            | Sequence (5’ to 3’) | Concentration (μM) |
|-----------|-------------------|--------------------|--------------------|
| ORF1ab    | Amplicon: 203 bp  | TGGTCTAGCTAGCTATG  | 0.2                |
|           | F3                | TTAATTGCTATCTGCTCTT | 0.2                |
|           | B3                | TCAGTACTAGTCTGCTGCC | 1.6                |
|           | FIP               | TCAGTACAGGCGTTTTTGCACTCTA | 1.6 |
|           | BIP               | CTGTTAGCGGGTGTGGACAAACAA | 0.8 |
|           | Loop F            | CTGCTTACAGCGCA     | 0.8                |
| N Amplicon| Loop B            | GTAGGTCTAGCTAGCTATGCA | 0.8 |
| ORF1ab    | Amplicon: 203 bp  | TGGTCTAGCTAGCTATG  | 0.2                |
|           | F3                | TTAATTGCTATCTGCTCTT | 0.2                |
|           | B3                | TCAGTACTAGTCTGCTGCC | 1.6                |
|           | FIP               | TCAGTACAGGCGTTTTTGCACTCTA | 1.6 |
|           | BIP               | CTGTTAGCGGGTGTGGACAAACAA | 0.8 |
| N Amplicon| Loop B            | GTAGGTCTAGCTAGCTATGCA | 0.8 |
amplicons and for the NTC, respectively. Following this rationale, Fig. 2(b) shows the calibration curve of the proposed sensor. The plot illustrates the experimental data (black dots), the uncertainty (vertical lines) and the linear model that best fits them (solid line). As expected, the output of the sensor, $\% \Delta I_p$, exhibits an increasing trend as the concentration $c$ grows up. Indeed, a large amount of amplicons implies less amount of free MB at the electrode surface, and hence, the current ratio $I_m/I_0$, in 1, diminishes. Thereby, the sensor sensitivity can be computed as $S = 8.42 \left( \frac{\% \Delta I_p}{\log[c]} \right)$; whereas, $r^2$ determines a highly linear behavior and a goodness of the fit $\sim 99\%$, thus leading to a limit-of-detection (LoD) of $0.038 \times 10^{-3}$ ng/μL in the concentration range from 0.001 to $10,000 \times 10^{-3}$ ng/μL, which is in the same order of magnitude as previous reports [43].

3.2. RT-LAMP monitoring

Once the performance of the sensor was assessed, we performed experiments in two scenarios to verify the ability of the sensor to monitor RT-LAMP reactions. First, we considered an end-point measurement to retrieve information about SARS-CoV-2 positive samples. Lastly, we monitored the time course of the RT-LAMP reaction for a positive sample at different concentrations to assess the dynamic performance of the sensor.

3.2.1. End-point measurements

To test the reliability of our sensor in the basis of MB/RT-LAMP reaction, we performed standard RT-LAMP (without MB) and MB-LAMP in eppendorf tubes while also measuring a similar MB/RT-LAMP reaction with our electrochemical test strip. To guarantee reproducible results, we used the same target concentrations, for three positive samples
labeled as L9, IPS and ISR (see Table 1) and verified the results using gel electrophoresis. Firstly, electrochemical measurements were performed following an end-point procedure. That is, the voltammograms were measured after 30 min of the RT-LAMP reaction. Subsequently, we computed the peak current change % ΔIp as in 1. Fig. 3(a) depicts the results retrieved by our sensor for these three samples. One can see, the peak current change decreased almost 55%, which reflects the success of the RT-LAMP reaction. It makes sense, the intercalation of MB to double-stranded amplicons significantly reduced the concentration of free MB at the electrode surface, and hence, diminished the peak current with respect to the negative control sample. To verify that the end-point measurements were reliable we performed an electrophoresis test on a 1% agarose gel for RT-LAMP N and ORF1ab reaction products, respectively. Fig. 3(b) shows the agarose gel for molecular weight markers (MW), negative control samples (NTC) and the three tested samples (L9, IPS and ISR). From Fig. 3(b), one can observe that only the positive reactions resulted in a ladder pattern, while the NTCs did not show any detectable amplicons. Ultimately, by using the calibration curve shown in Fig. 2(a), we computed the estimated concentrations by our sensor for two SARS-CoV-2 genes, N and ORF1ab, in three samples. To validate the electrochemical sensor, we concurrently performed a colorimetric assay as shown in Fig. 2(c). Therein, one can see the negative reactions indicated in pink, and how the positive reactions change the color to yellow. As expected, this effect is due to the presence of phenol red within the RT-LAMP reaction mix, which allows a straightforward differentiation among positive and negative samples. Finally, the concentration of both, electrochemical and colorimetric assays, was verified with the Nanodrop™ spectrophotometer. Table 3 summarizes the concentration results given by its mean value and uncertainty. These results thereby allow us to confirm that the proposed sensor reproduces well the Nanodrop™ measurements and agree with the colorimetric readings. Thus, the sensor accuracy is above 90%, with the largest error for the sample IPS, which is the one more concentrated. End-point measurements allowed to measure the concentration of the amplicons for the N and ORF1ab genes after the amplification process promoted by the RT-LAMP reaction. One should keep in mind that, those concentrations are not the number of copies in the total RNA isolated from the wastewater samples. Hence, this experiment was useful to validate the sensor to only detect the presence of SARS-CoV-2 genome, and to corroborate the concentration of the dsDNA products given by the redox current change due to the RT-LAMP amplification.

### 3.2.2. Time course measurements

As the last experiment, we measured the time course of the RT-LAMP reaction. For this purpose, we used five different initial nucleic acid concentrations using the sample L9 (1.9–1 = 251.8, L9–2 = 25.18, L9–3 = 2.518, L9–4 = 0.02518 and L9–5 = 0.002518 × 10⁻³ ng/µL), to specifically detect the fragment N of SARS-CoV-2 genome. Electrochemical measurements were carried out every 5 min up to 60 min. That is, at each time instant, we collected the voltammogram retrieved by SWV, and computed the peak current change with respect to a negative control sample. Fig. 4(a) shows the mean value for a biological triplicate of the peak current change % ΔIp as a function of RT-LAMP reaction time. Therein, one can see the amplification time course described by sigmoidal-like curves as in classical genetic amplification processes [44]. From left to right, it is possible to see the effect of the concentration in the RT-LAMP time course. Interestingly, for concentrations L9–1, L9–2 and L9–3, the amplification traces show an exponential phase occurring starting from 15 up to 20 min. This situation could be attributed to the relatively high concentration of the samples, such that the RT-LAMP amplicons can be easily generated within a short-time period. On the other hand, for highly diluted concentrations, the RT-LAMP reaction takes more time to start generating amplicons, starting at approximately 30 and 50 min for samples L9–4 and L9–5, respectively. Therefore, MB remains free at the surface of the electrodes for a relatively long time until amplicons start generating.

As shown in Fig. 4(a), the dashed horizontal line indicates the threshold value of the peak current change at which RT-LAMP exhibits an exponential transition. Hence, for each concentration the time-to-threshold value tₜₐₜ indicates the needful time for the amplification to succeed. To quantitatively assess the RT-LAMP performance, Fig. 4(b) depicts the relationship among the time-to-threshold and the concentration in a logarithmic scale. From there, one can deduce that the sensor operates in two regimes.

- For concentrations below 10⁻³ ng/µL, the required amplification time is above to 30 min.
- For highly concentrated samples, greater than 10⁻³ ng/µL, the time-to-threshold of RT-LAMP reaction takes less than 25 min.

Though this behavior was to be expected, it is convenient to focus on the last three samples. Therefore, the inset of Fig. 4(b) confirms an inverse correlation between the time-to-threshold and the concentration, with a sensitivity of 1.17 min/log[c], and linearity of approximately 99%. This result shows how the electrochemical sensor could also serve as an alternative method for quantitative molecular tests with enough sensitivity. Also, the time course experiment confirmed that the electrochemical test strip was able to detect concentrations as low as 2.5 × 10⁻⁴ ng/µL. However, it is worth to notice that, for highly diluted samples, the isothermal reaction requires more time to reach the plateau. This could be acceptable for classical molecular tests; nonetheless, for our purposes, it could be problematic as we are interested in fast measurements for applications in limited-resources settings.

### 3.3. Towards sensor-based wastewater surveillance

The use of wastewater surveillance as an epidemiological tool has raised more interest in detecting the increment of the viral load, which could be related to disease spread in the population. Another approach though, is the near-source tracking, applying the surveillance on a small spatial scale, in vulnerable or higher risk groups, like people in prisons, schools, hospitals and factories. Therein, the only detection of the virus in their wastewater or sewage system is a valuable tool to prevent a local outbreak, followed by targeted clinical tests [45]. For instance, using a conventional RT-qPCR approach monitoring the sewage system for a prison, Carrillo-Reyes et al. [18] were able to detect the presence of the SARS-CoV-2 previous the report of clinical cases by the local health authorities. Following the current trends, the proposed electrochemical sensor showed promising results for detecting SARS-CoV-2 in real wastewater samples. The main advantages of the device are that, it does not require sophisticated infrastructure to succeed; and the electrochemical detection preserve acceptable sensitivity compared with optical methods, but its instrumentation is cost-effective for field-deployable devices. Following this rationale, the described approach is at least 5000 USD cheaper than a classical setup by replacing the thermocycler and the optical detection apparatus. In further studies, the proposed sensor can be modified to integrate all the methods, such as concentration and extraction stages, in a single device. This

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Table 3

| Sample | Gene | Concentration (ng/µL) | Sensor | Nanodrop | Colorimetry |
|--------|------|-----------------------|--------|----------|-------------|
| L9     | N    | 2.42 ± 0.26           | 2.29 ± 0.17 | 2.15 ± 0.38 |
|        | ORF1ab | 2.60 ± 0.28          | 2.77 ± 0.13 | 2.84 ± 0.18 |
| IPS    | N    | 3.15 ± 0.42           | 3.47 ± 0.04 | 2.98 ± 0.32 |
|        | ORF1ab | 3.45 ± 0.86           | 3.69 ± 0.03 | 3.01 ± 0.14 |
| ISR    | N    | 2.71 ± 0.47           | 2.82 ± 0.13 | 2.90 ± 0.50 |
|        | ORF1ab | 1.95 ± 0.34           | 1.99 ± 0.05 | 2.03 ± 0.45 |
improvement could be a significant advance towards point-of-collection devices for automated analysis in wastewater surveillance for near-source tracking to rapidly identify SARS-CoV-2, as well as other pathogens.

4. Conclusions

In this work, a low-cost, affordable and accurate electrochemical sensor for the sensitive detection of SARS-CoV-2 nucleic acids was successfully developed and evaluated. The results demonstrated the ability of the sensor to perform measurements in real wastewater samples, and were validated with a colorimetric assay and a commercial apparatus. The proposed sensor was primarily devoted to detect the presence of SARS-CoV-2 genome by means of end-point measurements with a detection limit of \(38 \times 10^{-6} \text{ng/mL}\). Moreover, the sensing device was also able to track the time course of the RT-LAMP reaction for concentrations as low as \(2.5 \times 10^{-6} \text{ng/mL}\). Though is a promising result for quantitative assays, it requires further validation with RT-qPCR experiments. The versatility and features of the electrochemical RT-LAMP-based sensor make it as an attractive alternative to detect SARS-CoV-2 in low-resource settings for surveillance the COVID-19 spread in environmental scenarios. Finally, the device could be further improved to be an integrated system for stand-alone measurements, and can be extended for detecting other pathogens.

CRediT authorship contribution statement

R. G. Ramírez-Chavarría: Conceptualization, Methodology, Funding acquisition, Writing – original draft. E. Castillo-Vil-lanueva: Investigation, Methodology, Writing – original draft. B. Hervé: Investigation, Methodology, Writing – review & editing. J. Carrillo-Reyes: Investigation, Methodology, Validation, Writing – review & editing. R. M. Ramírez-Zamora: Writing – review & editing, Validation, Resources. G. Buitron: Writing – review & editing, Validation, Resources. L. Alvarez-Icaza: Writing – review & editing, Validation, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 4. RT-LAMP time course measurements for sensing SARS-CoV-2 in wastewater samples. (a) Retrieved peak current change \(\%\Delta I_p\) for five concentrations L-1-1 = 251.8, L-1-2 = 25.18, L-1-3 = 2.518, L-1-4 = 0.02518 and L-1-5 = 0.002518 \times 10^{-3} \text{ng/mL}\) as a function of the reaction time. (b) Correlation between the time-to-threshold \(t_h\) and concentration \(c\) in a logarithmic scale.

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