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Reverse-Transcription Loop-Mediated Isothermal Amplification and alternative protocols for lower cost, large-scale COVID-19 testing: lessons from an emerging economy

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ABSTRACT

Introduction: Most successful cases of COVID-19 pandemic mitigation and handling have relied on extensive reverse-transcription quantitative polymerase chain reaction (RT-qPCR). However, many emerging economies have struggled with current molecular testing demands due to economic, technical and technological constraints.
Objective: To define a potential diagnostic protocol to increase testing capacity in current and post-pandemic conditions.
Methods: We reviewed the literature, patents and commercial applications, for alternatives.
Results: We found a good potential in saliva samples, viral inactivation and quick RNA extraction by heating; the use of an isothermal technology such as loop mediated isothermal amplification (LAMP) and naked eye test-result visualization by in-tube colorimetry or turbidity.
Conclusions: Saliva samples with quick RNA extraction by heating and colorimetric LAMP are promising options for countries with economic and infrastructure limitations.

Key words: emerging economies; molecular testing; SARS-CoV-2; saliva; loop mediated isothermal amplification.

The advent of SARS-CoV-2 and our collective failure in pandemic handling: In late 2019 a SARS-like novel disease was discovered in the city of Wuhan, China. By January 2020, the novel infection had been denominated COVID-19 and its causal pathogen was identified as a new coronavirus named SARS-CoV-2. The high infection rate of SARS-CoV-2 led to a rapid worldwide spread, forcing the World Health Organization (WHO) to declare COVID-19 a pandemic in April 2020. As of today, early June 2021, there have been nearly

176 million official COVID-19 cases and more than 3.8 million deaths worldwide (Johns Hopkins University & Medicine, 2020).

After many months of attempting to achieve epidemiological management through general containment measures (e.g. provisional lockdowns, social distancing, mandatory use of face masks) it has become apparent that such measures alone do not suffice to keep the virus in check and prevent its spread among large swaths of the population. As it has been the case with previous outbreaks, evidence indicates that the most effective non-pharmacological strategies to contain the spread of the virus include some combination of early detection, contact tracing and isolation, like the so called Find, Test, Trace, Isolate, and Support (FTTIS) approach (Rajan et al., 2020). This type of epidemiological management has traditionally relied on a clear clinical diagnosis that is confirmed by a reliable test. However, due to the large proportion of asymptomatic COVID-19 cases and the transmissibility of the SARS-CoV-2 from those asymptomatic individuals, the only alternative to detect enough cases to stop the spread of the virus is by using large-scale or random testing (Gandhi et al., 2020); doing so eliminates future pools of newly exposed individuals that replicate and amplify the contagion cycle. Countries that have used large-scale testing, like South Korea, appear to have been most successful at flattening the curve of cases, especially early during the pandemic (Chang et al., 2020). Even under the current situation of active mass COVID-19 immunizations or in a post-pandemic world, a FTTIS approach will continue to be a necessary tool in our epidemiological arsenal.

Unfortunately, any successful FTTIS approach for COVID-19 epidemiological management is resource intensive and costly. As evidence of this, scarcity or high cost of some components necessary for a FTTIS approach has prevented large-scale deployment of this type of strategies across several countries. Most prominently, scaling-up of testing capacity using real-time quantitative polymerase chain reaction (RT-qPCR), the current gold standard technique for COVID-19 diagnosis, stands as one of the most difficult bottlenecks to overcome in order to implement the large-scale random testing necessary for epidemiological management. Due to the many intrinsic technical difficulties and requirements of RT-qPCR, it has been challenging if not impossible for many countries around the world to scale up the RT-qPCR testing capacity for COVID-19 to meet the current needs. The struggle to ramp up RT-qPCR testing capacity has been especially obvious during periods with high numbers of cases and in emerging economies with more limited healthcare systems. Even when pool-based sampling methods can increase the population-level sensitivity of detection for at-large strategic public health responses (Mutesa et al., 2020), these remain constrained by the same technical bottlenecks and do not exclude intensive individual testing after a critical positivity rate has been reached.

Costa Rica is a middle-income emerging economy with an internationally praised universal healthcare system which has nonetheless struggled to meet several of the unexpected demands imposed by the COVID-19 pandemic. After a substantial investment in consumables, facilities, training, personnel and equipment the official capacity for RT-qPCR COVID-19 tests has plateaued at 4 500 tests per day. However, this number does not reflect the real number of daily tests –a fraction of daily tests corresponds to certification of known cases for nosocomial purposes– and the level at which COVID-19 testing is performed in Costa Rica yields very high positivity rates (i.e. percentage of positive cases over total tested individuals), around 36 %. This positivity rate, however, may increase during epidemiological peaks (Barquero, 2020) or decrease when public policy measures have a significant effect. The current testing strategy mainly targets either symptomatic individuals or those with a known immediate epidemiologic linkage, excluding most of the asymptomatic cases and leaving plenty of loose ends to maintain effective epidemiological tracing and management (Ministerio de Salud, 2020). In point of fact, the high positivity rate correlates

with complete contact tracing being infeasible since July 2020. Furthermore, it is unlikely that Costa Rica will be able to further increase RT-qPCR-based COVID-19 testing in the short term to reach a positivity rate of less than 5 %, which is the threshold initially recommended by the WHO for proper epidemiological surveillance (World Health Organization, 2020). This concern led our interdisciplinary research group to look for effective and efficient alternatives that may help Costa Rica to drastically improve the handling of the ongoing health crisis. The current review and opinion article describes our conclusion that alternatives to the traditional RT-qPCR-based COVID-19 testing are necessary for Costa Rica to improve the outcome of this crisis, and that this lesson can be generalized to other developing nations and emerging economies. This idea is beginning to materialize in a clinical validation of some of these testing alternatives in an effort coordinated by several researchers and healthcare officials from different Costa Rican institutions. We also recount here some of the lessons learned that may prove to be valuable for other countries and healthcare systems battling with similar conditions.

The bottlenecks and limitations of the traditional RT-qPCR-based COVID-19 testing: Our current dependence on RT-qPCR originates from the well-known flexibility and reliability of this technology. The basic form of PCR was developed 37 years ago. Since then, many different forms of PCR have been developed to detect all sorts of pathogens and remains especially useful for diagnosis of emerging infectious diseases. Theoretically, PCR could be used to detect any pathogen present in a biological sample and, to this day, RT-qPCR remains the primary diagnostic option for several viral and bacterial infections. Due to its intrinsic amplifying properties, all forms of PCR have very high sensitivity and its targeting of pre-defined and carefully selected genetic sequences secures a very high –and sometimes nearly perfect– specificity. However, nucleic acid amplification technologies, including RT-qPCR, tend to be more expensive and technically more complex than other types of diagnostic testing options. In the case of COVID-19 testing, it is unfortunate that simple and inexpensive alternatives (e.g. antibody and antigen testing) either miss the early infectivity window or lack the high sensitivity levels required to become an effective tool for epidemiological surveillance (Benzigar et al., 2021; Nagura-Ikeda et al., 2020).

As mentioned previously, RT-qPCR is a complicated test to perform. Most of the handling steps of this test must be carried out by either a highly trained laboratory technician or "pipetting" robots (Fig. 1).

Under normal circumstances, total processing time in the laboratory (not including sample collection, transportation and storage) for this type of RT-qPCR test is 3 to 6 h depending on whether steps have been automated by the use of robots or carried out by technicians. Furthermore, the most prevalent workflow with RT-qPCR must be carried-out in a biosafety level 2 (BSL-2) laboratory in order to protect the personnel from contagion due to aerosols arising from the samples, which means that every COVID-19 testing laboratory must have at least one BSL-2 cabinet. Additionally, RT-qPCR requires a real-time thermal cycler to be performed, which limits the rate of sample processing in a facility to the combined number of sample spots or "wells" available among all the real-time thermal cyclers in that facility. It should be noted that the most common configuration of a real-time thermal cycler is 96 wells and the turnaround time is typically 2 h for a 2-in-1 reaction encompassing both retrotranscription and amplification. Thus, the total sample processing rate using the most prevalent RT-qPCR protocol is limited by a composition of different bottlenecks that are difficult to overcome individually in any healthcare system, let alone all of them together. These bottlenecks include i) the number of BSL-2 laboratories available, ii) the processing or "pipetting" capacity of the technicians or robots and iii) the availability of "wells" per real-time thermal cycler to carry

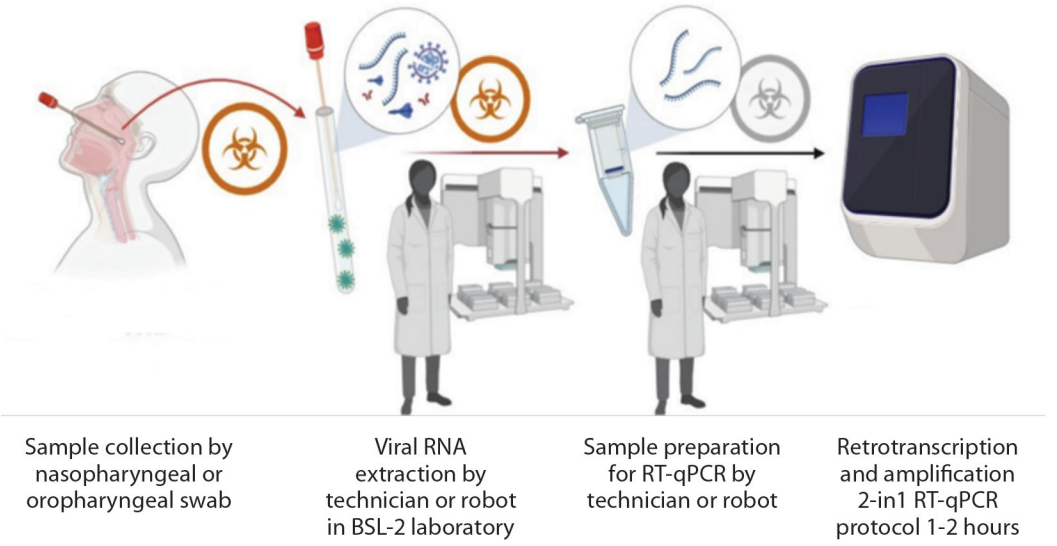

Fig. 1. Schematic workflow of COVID-19 testing using the standard RT-qPCR protocol. Two separate steps require sample handling and pipetting by a technician or robot. Red biohazard symbols illustrate steps with potentially bio-contagious samples, while gray biohazard symbols represent steps that no longer have contagious potential. Created with BioRender.

out the amplification and detection. In order to scale-up testing capacity by RT-qPCR, all of these bottlenecks must be overcome together in every single testing facility since a weak link in the chain may hamper the investments and improvements in other steps of the workflow or even render them useless.

MATERIALS AND METHODS

We reviewed the corresponding literature, patents and commercial applications for alternative technologies and protocols to detect SARS-CoV-2 in human samples that do not rely on RT-qPCR. Likewise, we reviewed the corresponding literature, patents and commercial applications for evidence of alternative sample collection and genetic material extraction that does not rely on nasopharyngeal swabs and traditional RNA purification. From this information we elaborated an argument for the use of alternative technologies that could effectively overcome the current bottlenecks of RT-qPCR-based diagnosis.

RESULTS AND DISCUSSION

Testing technology requirements for effective public health responses: In terms of public policy making, the role of testing technologies is to inform strategic and tactical recommendations. These are devised using test outcomes obtained from a limited number of COVID-19 samples that must be both sufficient and representative (Hilborne et al., 2020). Most generally, recommendations aim to address the most relevant and current stage of an epidemiological crisis. At the onset and early stages, FTTIS aims to contain and remove infective individuals to prevent further spread, particularly of asymptomatic cases, as a way to delay or even prevent reaching a rapid growth phase; simulation of COVID-19 spread under various systematic testing regimes suggests that remotion of asymptomatic individuals is one of the main mechanisms behind the effectiveness of scalable testing technologies (Núñez-Corrales & Jakobsson, 2020). If a rapid growth phase is reached, testing must intensify proactively as a way to understand how the

underlying population structure fuels the rise in cases, and then systematically craft measures capable of preventing the overload of healthcare systems and irreversible economic damage. When mitigation efforts are successful and the situation is stable, testing can be optimized to detect and control new outbreaks and drive the epidemic process to a manageable level until pharmacological alternatives are applied and herd immunity is reached. The ability to use testing as an anticipatory tool depends on the existence of testing technology capable of scaling rapidly and reliably across several orders of magnitude depending on the stage of the epidemic process. As evidenced by the reasoning above, RT-qPCR cannot particularly provide such flexibility.

COVID-19 testing technologies must strive to minimize false negatives by increasing their sensitivity (West et al., 2020) since these represent individuals that can restart and amplify even more the contagion cycle. In addition, testing must also provide the flexibility to address intrinsic uncertainties in the process (Gray et al., 2020). Both can be resolved by re-testing individuals. Essentially, an adequate testing technology not only should scale to accommodate sudden increases in new cases but also allow re-testing of known cases whose outcome was uncertain or whose epidemiological link is updated. In a situation such as the Costa Rican one where RT-qPCR testing is extremely limited, this forces a compromise between discovering new cases and certifying the recovery of existing ones; testing people before they are released from hospitalization takes priority. Statistically, we start by looking at the disjoint sensitivity between two consecutive tests and $\sigma_{A \vee B}$ given by

$$\sigma_{A \vee B} = \sigma_A + (1 - \sigma_A)\sigma_B \ ,$$

and since the same test is applied twice (i.e. $A=B$)

$$\sigma_{2A} = \sigma_A + (1 - \sigma_A)\sigma_A = 2\sigma_A + {\sigma_A}^2$$

which is a monotonically increasing convex function in the domain $\sigma_A \in [0,1]$ with range $\sigma_{2A} \in [0,1]$. Succinctly, applying two consecutive disjoint tests reduces the probability of false negatives. Conversely, specificity $\tau_{A \vee B}$ decreases with each test quadratically for $A=B$ as

$$\tau_{2A} = {\tau_A}^2 \ .$$

Arguably, lower specificity of two disjoint tests is not a significant concern as long as the specificity of a single test is high enough. For instance, $\tau_A = 0.95$ for a single test produces a combined disjoint specificity of $\tau_{2A} \approx 0.9$. False positives are in this case benevolent since the protocol for a new positive case entails mandatory isolation for two weeks, a time beyond the 12 days required for more than 97.5 % of individuals to have ended their incubation period (Lauer et al., 2020). Worst case scenario, excess false positives lead to a limited number of individuals being isolated, decreasing the pool of available susceptible individuals slightly. In consequence, any scalable testing technology for COVID-19 must ensure high specificity geared towards repeated testing of individuals for rapid disambiguation. Saliva-based technologies such as the alternative reported in this article overcomes this limitation thanks to having a specificity at least comparable to that of traditional nasopharyngeal swabs samples (Chen et al., 2020; Takeuchi et al., 2020; Williams et al., 2020; Wyllie et al., 2020).

Finally, time-to-outcome is a critical variable for COVID-19 testing technologies. The ability to perform contact tracing to stop contagion depends on rapidly acquiring and processing samples from suspected individuals or from proactive measures. The complexity RT-qPCR entails prevents having certified test outcomes within the first 24-48 h in most cases. This time is critical for contact tracing to identify potentially exposed individuals and extend the search promptly; simulation results suggest that performing the testing-contact tracing cycle within the same day can significantly reduce the effective reproductive number regardless of contact tracing technology (Kretzschmar et al., 2020). Since performing the test depends on specialized equipment and trained personnel, it cannot usually be geographically decentralized.

Hence, a scalable technology should also aim to be time and geographically scalable, particularly in entry-level health attention centers.

Simplified sample collection by using saliva: Nasopharyngeal and oropharyngeal swabs are the main method of sample collection for COVID-19 testing (Fig. 1). However, the invasive nature of this procedure can be uncomfortable for many patients and may even cause aversion to COVID-19 testing, potentially reducing public compliance with health authorities. Nonetheless, the presence of SARS-CoV-2 in saliva is indisputable and saliva samples for COVID-19 testing have been proven to be effective in the detection of the virus in a non-invasive manner using a RT-qPCR-based protocol (Chen et al., 2020; Takeuchi et al., 2020; Williams et al., 2020; Wyllie et al., 2020).

When sensitivity for COVID-19 testing has been compared between nasopharyngeal swabs and saliva samples using traditional viral RNA extraction methods and amplification by RT-qPCR, the saliva sample has shown only a slightly lower sensitivity than its comparison standard, thus, both types of samples should be regarded as equally useful from a clinical standpoint. In the case of specificity, both technologies have shown near perfect scores (Jamal et al., 2021; Pasomsub et al., 2020; Procop et al., 2020; Teo et al.,2021; Uwamino et al., 2020).

From a practical standpoint, using saliva samples for COVID-19 testing offers several advantages over traditional swabs that could be very beneficial during a large-scale testing initiative. Such advantages include better public acceptance and compliance, shorter sample collection times, reductions in frontline personnel in charge of sample collection and even the development of sample self-collection to be shipped to or dropped-off at a testing facility. Another advantage of using saliva samples is the availability of many types of low-cost sterilized screw-capped containers that can be used to collect liquid samples, including small urine and stool sample cups, falcon tubes and an assortment of other laboratory containers such as cryogenic tubes. The use of this readily available variety of containers would make the saliva sample collection process less susceptible to the common market shortages experienced during the last few months for most COVID-19 testing consumables, including medical swabs.

Quick viral RNA extraction and virions inactivation by heating: Traditional RNA extraction is a lengthy and costly process requiring highly trained technicians or specialized equipment like “pipetting” robots in order to isolate RNA from other biochemical components like DNA, proteins and lipids. Even though purified RNA is the ideal genetic material to perform retrotranscription and subsequent amplification in RT-qPCR or any isothermal amplification technology, it is not necessary to have this material completely isolated from other biochemical components present in some biological samples. In fact, most forms of PCR and almost any nucleic acid amplification technology allow for some level of flexibility regarding DNA, protein and lipid carryover. In most samples containing trace amounts of these types of biochemical components such as saliva and swabs, it is perfectly possible to perform a direct sample-to-amplification protocol bypassing traditional RNA extraction (Esbin et al., 2020). Quick viral RNA extraction by means of a heating step has been developed as an alternative method of viral RNA extraction. This quick heating step allows viral RNA to be accessible for the retrotranscriptases and polymerases used in PCR or isothermal amplification. Alternatively, some protocols may either replace or complement the rapid high temperature heating step with a long mid-temperature heating step, such as 65 °C for 30 min after mixing the sample with a lysis buffer that contains detergents and a highly active protease like Proteinase K (Esbin et al., 2020; L’Helgouach et al., 2020). This long mid temperature heating step with a lysis buffer seems to achieve similar results to the quick high temperature heating step in

regard to facilitating the release of the viral RNA and the two methods might work interchangeably. However, the fact that the rapid high temperature heating step achieves good results without the need for a lysis buffer makes it more attractive to our goals of simplifying test development. Furthermore, one more point must be taken into account when it comes to this simplified method of viral RNA extraction, and that is RNA degradation. Because the viral RNA is released into a biological sample with other components, it immediately becomes a target for endogenous RNAses in that sample. This problem can be disregarded if the sample is tested immediately, but sample storage for any length of time might have an effect on the detectable viral RNA in it. For that reason, some protocols may also include in the sample buffer or the reaction mix RNAses inhibitors or carrier RNA as protectors of the integrity of the viral RNA (Wei et al., 2021).

Quick viral RNA extraction using a heating step has one more important advantage over the traditional RNA extraction. During a heating step at 95-98 °C for 10 to 15 min, any virions potentially contained in a saliva sample are inactivated and all samples can be regarded as of low biosecurity risk after that point (Fig. 2). If sample processing protocols are devised in which sample containers are properly disinfected at the point of collection and never opened inside the laboratory before the heat inactivation step, processing of all these samples could be carried out in any BSL-1 facility.

This reduces the biosecurity equipment necessary to process samples, most notably the biosafety cabinet. It also opens the possibility of establishing temporary testing facilities in entry level healthcare attention centers that normally don't have BSL-2 laboratories and other places of interest that could not support a traditional clinical laboratory (e.g. schools, airports, factories, etc.). It must be mentioned that the protocol developed by Wei et al (2021) uses no heating step and takes the saliva sample directly into a single step RT-LAMP reaction. This alternative protocol seems advantageous for currently functioning testing laboratories as it greatly simplifies most of the testing workflow; however, we consider that this method still does not fit well with our interests of

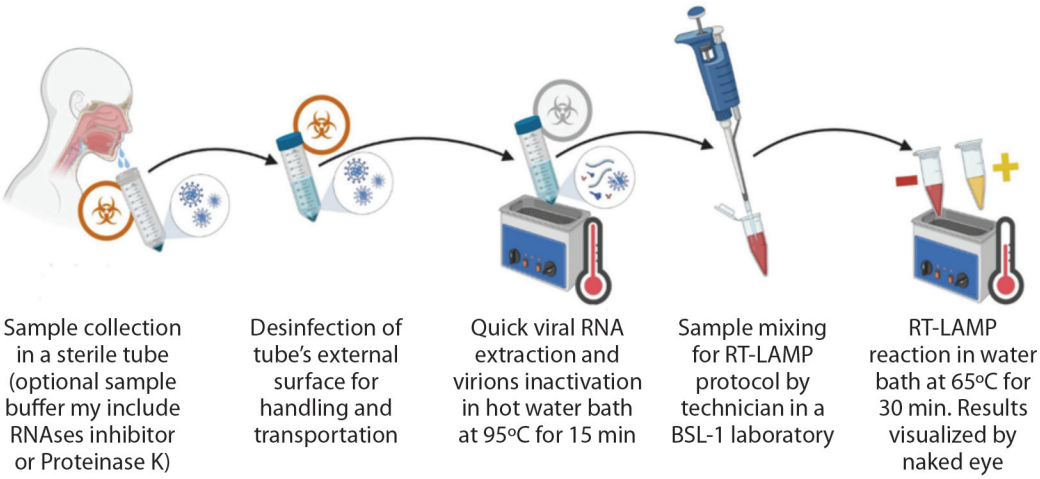

Fig. 2. Schematic workflow of simplified COVID-19 testing combining saliva sample, quick viral RNA extraction, RT-LAMP and results visualization by in-tube colorimetry. Red biohazard symbols illustrate steps with potentially bio contagious samples, while gray biohazard symbols represent steps that no longer have contagious potential. Since the first step inside the laboratory is the sample heating to release the viral RNA extraction and inactivate the virions, and this step is performed without opening the sample container, the entire process can be carried out in a BSL-1 laboratory. Created with BioRender.

completely simplifying COVID-19 testing since bypassing of all heating steps would mean that the sample must still be handled in a BSL-2 laboratory.

The advantages of isothermal amplification technologies: Different nucleic acid isothermal amplification technologies have been developed over the last few decades. These include Nicking Endonuclease Sequence Amplification Reaction (NESA), Ligase Chain Reaction (LCR), Recombinase Polymerase Amplification (RPA), Strand Displacement Amplification (SDA) and Loop Mediated Isothermal Amplification (LAMP), among others (Fakruddin et al., 2013; Kiesling et al., 2007; Zhao et al., 2015). All of these technologies have the capacity to detect specific genetic sequences in the same way that RT-qPCR does; however, all forms of isothermal amplification have one important advantage over the different forms of PCR; as the term isothermal implies, these technologies perform the entire amplification reaction at a single temperature and therefore, there is no need for a thermal cycler. Thus, the reaction for the different forms of isothermal amplification can be carried out using any stable heating source such as a water bath, a heating block, a hoven or even a water container on top of a heating plate. This particularity allows the development of isothermal amplification protocols using only basic laboratory tools like pipettes and opens the possibility of creating equipment-free diagnostic tests. Furthermore, the number of reactions or samples that can be performed with isothermal amplification does not rely on the number of spots or "wells" available in a device but rather the number of tubes with samples that can be fitted into a heat source like a water bath.

In addition, isothermal amplification technologies perform the entire reaction faster than PCR since it is not necessary to change the temperature of the reaction. Likewise, the reaction master mix for isothermal amplification can be developed to contain both a retrotranscriptase and a polymerase that work in parallel and at the same temperature, thus performing both reactions at the same time and reducing the total time required.

For example, this combination of two steps into one reaction creates what is known as retrotranscription-LAMP (RT-LAMP). These key differences reduce the total reaction time of isothermal technologies when compared to standard RT-qPCR protocols. As an example, most RT-LAMP protocols for SARS-CoV-2 detection take 30 min to complete a single step reaction while the standard RT-qPCR diagnostic protocol usually takes 1 to 2 h to complete both retrotranscription and amplification.

Despite all of these advantages, isothermal amplification technologies in general still suffer from limitations compared to the more traditional PCR. In our experience, probably the greatest drawback of developing tests based on isothermal technologies is the lack of a well-developed manufacturing network with ready-to-use consumables such as recombinant enzymes, master mixes and additives. In our search for this type of products to use in RT-LAMP, we found a limited number of manufacturing companies that commercialize them but the number of providers around the world and the variety of products is but a small fraction of what can be found in the market for PCR consumables. Another limitation compared to PCR is the lack of options to perform tests using primers with differential affinity by temperature such as touchdown, gradient or nested PCR. In the particular case of LAMP, it also suffers of other drawbacks. It requires a complex system of 6 different primers, which is far more than the 2 primers used for PCR (Fakruddin et al., 2013; Li et al., 2017; Notomi et al., 2015). Furthermore, at least 2 of these primers are hybrid sequences are not found in the targeted DNA. Therefore, this complex set of primers must be designed and validated *in silico* using bespoken programs. However, a few different tools are available for free (such as Primer Explorer https://www.primerexplorer.jp/e/) and some others are offered under a commercial license. Because of this complex design, the primers sets required for RT-LAMP may not be suitable for some genomic areas,

especially repetition-rich loci. However, those particular obstacles are unlikely to affect the capacity of RT-LAMP to detect most pathogens since these types of repetitions are not common in the genome of prokaryotes and viruses.

In this article we are not describing in detail the complex interaction of RT-LAMP primers or its mechanism of amplification. However, Eiken Chemical Co., the original inventors of LAMP, offer a detail description of its principle in their website both in the form of an illustration (http://www.loopamp.eiken.co.jp/e/lamp/principle.html), as well as an animation (http://www.loopamp.eiken.co.jp/e/lamp/anim.html).

In this balance between pros and cons, isothermal amplification technologies have not been considered as an alternative that could challenge the predominance of traditional forms of PCR either for clinical or research use until recently. In the case of LAMP, it has mainly remained in the fringes of the clinical field as a simple and low-cost alternative for the development of field deployable diagnostic tests for animal and agricultural diseases for which RT-qPCR-based testing in a laboratory is not cost-effective. RT-LAMP also seems to have gathered momentum as an inexpensive alternative diagnostic technology for pathologies that occur in areas that lack the healthcare infrastructure to provide an expensive option like RT-qPCR. This latter case is clearly illustrated by the different triple testing protocols that have been independently developed to differentially diagnose dengue, zika and chinkungunya; three mosquito transmitted viral diseases with similar clinical presentations and commonly found in the same tropical areas of emerging economies (Ganguli et al., 2020; Priye et al., 2017; Yaren et al., 2017; Yaren et al., 2018). However, the particular needs created by COVID-19 could change the vision of RT-LAMP from fringe alternative to a secure position within the mainstream of the technological spectrum for clinical diagnosis (Khan et al., 2020). In our case, we chose RT-LAMP over other isothermal amplification technologies because of its several advantages, including the fact that it is the most maturely developed and most used of these alternatives. Another reason to choose LAMP as the isothermal amplification technology for massive testing is the current lack of intellectual property protections in most countries. The LAMP technology was one of the first isothermal amplification technologies to be developed. As such, its initial intellectual property protections have recently expired or are close to. LAMP was first patented in Japan by Eiken Chemical Co. in November 1998 (Japan patent No. JP2000283862) and suffered an anticipated expiration in 2019; however, at least the protection granted for this invention in the United States is anticipated to expire in November 2021 (US patent No. US7494790B2). In the case of Costa Rica, we have found no applications or patents granted that protect the original or any subsequent inventions by Eiken Chemical Co., or by other inventors regarding LAMP. We believe this lack of intellectual property protections includes most emerging economies in Latin America, Africa and most of Asia and Eastern Europe. This lack of legal protections allows the development of testing protocols faster and at a lower cost compared to technologies that must be acquired from a licensed manufacturer or licensed directly from a patent's owner.

Clinical parameters of RT-LAMP vs RT-qPCR for COVID-19 diagnosis: Several recent studies have published data on the detection of SARS-CoV-2 using RT-LAMP from the 2 main types of samples (nasopharyngeal swabs and saliva). These publications also describe a wide variety of RNA extraction methods and targeting primers. While an exhaustive review of all of these variables is beyond the scope of this article, Table 1 summarizes the articles published or pre-printed studies where SARS-CoV-2 is detected in clinical saliva samples or virions spiked saliva using RT-LAMP. Table 1 also summarizes the type or RNA extraction used (traditional RNA extraction vs heating step) and the gene or genes targeted by the RT-LAMP primers sets. It must be noted that

TABLE 1
RT-LAMP performance in the detection of SARS-CoV-2 presence in saliva sample

Reference	Type of RNA extraction	Genes targeted*	Specificity**	Sensitivity**	LOD
Ben-Assa et al., 2020	heating step	N	High	High	Medium to high viral loads
Bhadra et al., 2021	heating step	Orf1ab, N, E	Not determined	Not determined	3.3 x 106 copies/ml
Howson et al., 2021	traditional RNA extraction	E	High	High	Viral loads high in dilutions (1:40 to 1:640)
L'Helgouach et al., 2020	heating step	Not described	95.70 %	72.70 %	NA
Lalli et al., 2020	heating step	Orf1ab, N	High	High	102 viral particles per reaction
Lamb et al., 2020	traditional RNA extraction	Orf1ab	Not determined	Not determined	3.802 ×10^10 to 228 copies of virus
Nagura-Ikeda et al., 2020	traditional RNA extraction	Orf1ab, N	High	High	Viral loads vary with time
Wei et al., 2021	direct saliva-to-reaction	Orf1ab	100 %	97 %	2 copies of viral RNA per µL

*For simplicity, all primers targeting any of the different genes within the Orf1a or Orf1b loci are simply referred to as targeting Orf1ab.
**Sensitivity and specificity are relative to a RT-qPCR test.

while some detection methods use a single set of RT-LAMP primers, it is possible to use up to 2 sets of primers in some cases. The RT-LAMP kit developed by New England Biolabs to detect SARS-CoV-2 uses 2 different sets of RT-LAMP primers targeting the N and E genes, effectively multiplexing the detection reaction. However, mixing different RT-LAMP primers sets should always be evaluated on a case-by-case basis.

In the case of RNA extraction methods, it should be noted that traditional RNA extraction from saliva samples secures a sensitivity that closely matches the current gold standard with RT-qPCR. However, the quick viral RNA extraction from saliva by a heating step may not yield the same level of detection, but it still produces a sensitivity that is clinically useful for most cases. The highest clinical sensitivity (97 % compared to the gold standard) of a saliva RT-LAMP test for SARS-CoV-2 detection was achieved by Wei et al. (2021) in a direct saliva-to-reaction solution without any form of RNA extraction. This LAMP based test also has the lowest Limit of Detection (LOD), with 2 copies of viral RNA per µL, which is on par with the most sensitive RT-qPCR tests designed to date. However, the formulation developed by Wei et al. (2021) requires some specialized materials such as a buffer containing carrier RNA to protect the viral RNA in the sample. Whether this method could be mass produced at a cost that still makes it competitive to RT-qPCR has not been established. Nonetheless, the protocol created by Wei et al. (2021) is currently used at Columbia University to test students and faculty and the technology was licensed to Sorrento Therapeutics, in order to obtain FDA approval and commercialize it in the United States under the name COVI-TRACE.

With all this in mind, it is clear that RT-LAMP can achieve clinical values of sensitivity, sensibility and LOD that are close to those of the current gold standard. Nonetheless, these clinical parameters must be evaluated during the development of any RT-LAMP-based test, especially if this technology is coupled with detection from a saliva sample or using quick viral RNA extraction by a heating step.

Simplified options to detect amplification of genetic material by RT-LAMP and other isothermal amplification methods: Theoretically, RT-LAMP and all other forms of isothermal amplification can be quantified and detected in real time using fluorescence. For example, RT-LAMP can be performed and quantified in real time using any traditional double-strand DNA binding dye such as SYBR-Green in a real time thermal cycler (Da Silva et al., 2020; Fakruddin et al., 2013; Khan et al., 2020; Notomi et al., 2015). However, using an isothermal amplification technology in a thermal cycler defeat most of its advantages and thus, simpler methods of detection have been devised in order to visualize and even quantify RT-LAMP amplification results. These simplified methods of detection include:

UV detection shows in-tube presence of amplicons by naked eye using an UV light source and a marker that fluoresces under such light if nucleic acids amplification has occurred. These UV sensitive markers include traditional double-strand DNA binding dyes such as ethidium bromide, SYBR-Green, SYBR-Safe and ionic detection markers such as calcein. UV detection can also be semi-automated using devices that emit and detect light at the correct spectra, such as a fluorescence plate reader.

Colorimetric detection uses colorimetric dyes that change the color of the solution if amplification has occurred. These include pH sensitive dyes such as phenol red or metal ion sensitive dyes such as hydroxynaphthol blue (HNB) (Goto et al., 2009). These color changes can be determined in-tube by naked eye or using a light absorbance device equipped to measure the correct spectra (e.g. 650 nm for HNB). It should be noted that pH sensitive dyes such as phenol red may not be well suited as a detection method for samples with high or low pH as enough of these conditions may be carried over from the sample to the final reaction and cause interference with the results detection. This type of carryover can potentially happen with the saliva of some individuals, as well as in swab samples that were preserved in universal or viral transport media (UTM and VTM, respectively). In such cases, metal ion sensitive dyes such as HNB might be the preferred option.

Turbidity detection measures the change in turbidity of the LAMP solution due to the natural precipitation of magnesium pyrophosphate as byproduct of DNA synthesis (Mori et al., 2001). Turbidity change can be determined in-tube by naked eye or using a turbidometer. The use of some turbidometers such as the Loopamp Realtime Turbidimeter (LA-500, Eiken Chemical Co., Japan) also allows for real-time quantification of the RT-LAMP amplification.

CRISPR Cas12a/Cas13a detection relies on genetic material that has been pre-amplified by isothermal amplification, including LAMP or RPA, to detect specific genetic sequences using targeted digestion with a CRISPR system using either Cas12a, Cas12b or Cas13a enzymes. The genetic sequences specifically cleaved by the CRISPR Cas12/Cas13 enzymes can be detected using a lateral flow chromatography strip and visualized directly by naked eye (Khan et al., 2020).

We observe that none of these methods of amplification detection are exclusively designed or used for RT-LAMP. In fact, all of them can be used to detect amplicons produced by PCR or other forms of isothermal amplifications. However, these alternative methods for amplification detection will probably play a crucial role in the development of easily scalable diagnostic tools for COVID-19, especially those methods that do not require costly and specialized amplification or detection equipment such as thermal cyclers or plate readers. More specifically, our attention should be focused on detection methods that allow the visualization of the final result directly in the reaction tube, bypassing time-consuming extra steps. In general, the coupling of an isothermal technique such as RT-LAMP with a form of amplicon detection by the naked eye such as in-tube colorimetry promises to produce diagnostic tools for COVID-19 that are effective, simple, easily scalable and low-cost.

Public attitudes toward COVID-19 massive testing: While no official information has been gathered so far regarding public attitudes towards COVID-19 testing among the Costa Rican population, a preliminary statistical survey was performed by the School of Statistics at the University of Costa Rica to determine self-reported public knowledge and attitudes towards COVID-19 testing technologies, both available and potential (Ramírez-Hernández & Madrigal Pana, 2020). The survey was applied to a sample of 1 287 residents within the national territory in October 2020 with ages 18 years and over, who own a cell phone. An assessment of both the willingness of the population to undergo COVID-19 testing and of using a new test based on a saliva sample was included in the questionnaire.

Results suggest a mostly positive response of the general public to COVID-19 testing. In general, 84.1 % of the population would accept being tested were a massive diagnosis program implemented. A significant portion of the population (78.6 %) claims to have information about the type of sample required for COVID-19 testing, with most responders referring to the nasopharyngeal swab protocol. Belief in the effectiveness of testing as a tool to gain control over this pandemic, however, is much lower (66.8 %). Even when further statistical probing and analysis must occur, current disaggregate data from this survey appear to indicate that individual health and financial concerns drive public perception of COVID-19 despite perceived uncertainty about its effectiveness as a strategy to mitigate and control the spread of the virus. This latter point highlights the need to provide the general public with a clear picture of the mechanism by which massive testing operates at population levels (i.e. removing a large portion of asymptomatic carriers and exhausting large shares of the infective pool) in an effort to further improve compliance and public trust as part of official public health communication initiatives (Lazarus et al., 2020).

Most significantly, the situation appears to be even better for saliva-based COVID-19 testing. Willingness to receive a saliva-based tests ranks high (92.3 %), with a similar response for the prospect of a round of testing once per week (89.5 %). Disaggregate responses by sex, educational attainment, nationality or subjective income did not exhibit significant differences with respect to the sample mean. Willingness to receive the test, however, appears to decrease for individuals above 50 years or more (77.6 %), and increases in proportion to one's perception of being at risk (low risk: 75.8 %; medium risk: 85.2 %; high risk: 86.8 %). These data suggest that information about saliva-based testing technologies must be specifically tailored to age groups via different media platforms to ensure maximum coverage. Another determinant factor that must be studied at depth is trust in public health infrastructures regarding data privacy, in which perceived consequences of testing data mismanagement for individuals may vary per country.

Finally, out-of-pocket costs per test appears to be a major factor for public acceptance of saliva-based testing technologies in Costa Rica. The largest fraction of the sample in the study was that of individuals who would accept the test only if it is free for the population (37.1 %). As out-of-pocket cost increases, a smaller share of the population reports willingness to receive the test. A price point equal to or below US $10 appears to be required in order to ensure at least a substantial proportion of the population (62.9 %) are likely to perform testing at a scale sufficient to have a reasonable impact individually and collectively.

Succinctly, the prospect of saliva-based testing in Costa Rica –an emerging economy with significant infrastructure and socioeconomic challenges– is overall positive and encouraging. Even when the mechanism by which massive testing helps mitigate and contain the pandemic appears not to be well understood, individual health and financial concerns seem to drive the perceived need for massive testing. Saliva-based testing appears to attain better public perception, possibly due to perceptions of being less intrusive than nasopharyngeal swabs, while still in need of

better public health campaigns if deployed. As expected, out-of-pocket costs drive public acceptance in the midst of a public health crisis with strong reverberations into the economic and social fabric of a mid-income country. Given the dominant magnitude of negative economic impacts of COVID-19 in comparison to testing costs and other forms of mitigation in Organization for Economic Co-operation and Development (OECD) member states (López-Valcárcel & Vallejo-Torres, 2021), RT-LAMP may become an ideal low-cost alternative for developing nations and emerging economies since its overall cost may be absorbed by public health organizations or international relief efforts as a means to approximate a situation of zero out-of-pocket expenses for the general public.

Could RT-LAMP replace RT-qPCR as the main option for large-scale COVID-19 testing?: Given the current situation in which it is infeasible for many healthcare systems to reach the necessary levels of COVID-19 testing to adequately manage the health crisis, it is unlikely that such a goal will be met unless we develop practical alternatives to the traditional RT-qPCR-based testing. Simplified alternative protocols for different steps of the process all promise to expand our diagnostic capacity and help bridge the testing gaps. These simplified alternatives include isothermal amplification technologies like RT-LAMP, use of saliva samples, quick viral RNA extraction by heating and results visualization by naked eye using in-tube colorimetry. While these alternative protocols have been proven to work together, they do not necessarily constitute an "all or none" package. In fact, some of these processes were originally designed to operate as part of a simplified testing protocol using RT-qPCR; for example, viral RNA directly extracted from saliva by a quick heating step has been successfully used as the start material for RT-qPCR, a substitution that dramatically reduces the cost and turnaround time for COVID-19 testing (Esbin et al., 2020). Conversely, others have developed a direct-swab-to-amplification protocols in which a traditional nasopharyngeal swab is used but the commonly used RNA extraction is replaced by a quick heating step, and the amplification is performed either by RT-qPCR or RT-LAMP (Bruce et al., 2020; Dao-Thi et al., 2020). Regardless of the plethora of potential protocol variations that can be developed by exchanging alternative options in each step, the coupling of all or most of the simplified alternative technologies into a single uncomplicated and low-cost testing protocol might be the only feasible option for many developing countries where equipment such as real-time thermal cyclers, pipetting robots and biosecurity cabinets might be difficult to obtain or economically burdensome. For that reason, we are not surprised that research groups from other emerging economies have arrived at the exact same conclusions (Ohilebo et al., 2020).

On the other hand, given the very high sensitivity and specificity of RT-qPCR, it is unlikely that RT-LAMP or other alternative technologies will replace the gold-standard for COVID-19 diagnosis in the short term. Also, the already installed RT-qPCR-based testing capacity will continue to be absolutely necessary during the pandemic. Furthermore, expansion of the RT-qPCR-based testing capacity should continue until the end of this crisis whenever and wherever it is feasible and cost-effective. Even so, it is likely that alternative COVID-19 diagnostic options will emerge in many parts of the world using RT-LAMP as well as parts of the simplified testing protocols for sample collection, RNA extraction and result detection. Some of these alternative protocols may also be tailored to function as part of a modified RT-qPCR diagnostic test in an attempt to increase sample processing rates in traditional laboratory settings. Nonetheless, healthcare systems that are already struggling to bridge the testing gaps are likely to continue suffering from some level of deficit during the rest of the pandemic, and the only sensible decision will be to make use of all available options. With this in mind it is unlikely that RT-LAMP and other alternative COVID-19 diagnostic protocols will replace the traditional

RT-qPCR test but rather that they will become complements that could strengthen each other's weaknesses and thus reciprocally fill their gaps. In summary, COVID-19 has unveiled the need for an ecosystem of affordable and effective testing technologies capable of scaling to different intensities, geographies and development possibilities of countries during a pandemic. RT-LAMP exemplifies such an alternative alongside RT-qPCR, with the potential of enabling the discovery of new methods capable of solving the hurdles of today as a way to more effectively anticipate and respond to the public health crises of tomorrow.

Ethical statement: the authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that we followed all pertinent ethical and legal procedures and requirements. All financial sources are fully and clearly stated in the acknowledgements section. A signed document has been filed in the journal archives.

RESUMEN

Amplificación isotérmica mediada por bucle de transcripción inversa y protocolos alternativos para pruebas COVID-19 a gran escala y de bajo costo: lecciones de una economía emergente.

Introducción: la mayoría de los casos exitosos de mitigación y manejo de la pandemia de COVID-19 se han dado mediante pruebas basadas en la reacción en cadena de la polimerasa cuantitativa (RT-qPCR por sus siglas en inglés). Sin embargo, muchas economías emergentes han tenido problemas con las demandas actuales de pruebas moleculares debido a limitaciones económicas, técnicas y tecnológicas.
Objetivo: Definir un protocolo de diagnóstico potencial para aumentar la capacidad de prueba en las condiciones actuales y posteriores a la pandemia.
Métodos: Revisamos la literatura, las patentes y las aplicaciones comerciales, en busca de alternativas.
Resultados: Encontramos un buen potencial en muestras de saliva, inactivación viral y extracción rápida de ARN por calentamiento; el uso de una tecnología isotérmica como la amplificación isotérmica mediada por horquillas (LAMP, por sus siglas en inglés) y la visualización del resultado de la prueba a simple vista mediante colorimetría o turbidez en el tubo.
Conclusiones: Las muestras de saliva con extracción rápida de ARN por calentamiento y LAMP colorimétrico son opciones prometedoras para países con limitaciones económicas y de infraestructura.

Palabras clave: economías emergentes; pruebas moleculares; SARS-CoV-2; saliva; amplificación isotérmica mediada por bucle.

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