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Medical Hypotheses 141 (2020) 109786

Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: www.elsevier.com/locate/mehy

Loop mediated isothermal amplification (LAMP) assays as a rapid diagnostic for COVID-19

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ARTICLE INFO

Keywords:
COVID-19
Polymerase chain reaction (PCR)
Loop mediated isothermal amplification (LAMP)
Diagnostic
Coronavirus

ABSTRACT

Recently, a novel coronavirus (SARS-CoV-2; coronavirus disease 2019, COVID-19) has emerged, rapidly spreading and severely straining the capacity of the global health community. Many nations are employing combinations of containment and mitigation strategies, where early diagnosis of COVID-19 is vital in controlling illness progression and limiting viral spread within the population. Thus, rapid and accurate methods of early detection are vital to contain COVID-19 and prevent further spread and predicted subsequent infectious waves of viral recurrence in future. Immediately after its initial characterization, Chinese and American Centers for Disease Control and Prevention (CDCs) rapidly employed molecular assays for detection of COVID-19, mostly employing real-time polymerase chain reaction (RT-PCR) methods. However, such methods require specific expensive items of equipment and highly trained analysts, requiring upwards of 4–8 h to process. These requirements coupled with associated financial pressures may prevent effective deployment of such diagnostic tests. Loop mediated isothermal amplification (LAMP) is method of nucleic acid amplification which exhibits increased sensitivity and specificity are significantly rapid, and do not require expensive reagents or instruments, which aids in cost reduction for coronavirus detection. Studies have shown the successful application of LAMP assays in various forms to detect coronavirus RNA in patient samples, demonstrating that 1–10 copies of viral RNA template per reaction are sufficient for successful detection, ~100-fold more sensitive than conventional RT-PCR methods. Importantly, studies have also now demonstrated the effectiveness of LAMP methodology in the detection of SARS-CoV-2 RNA at significantly low levels, particularly following numerous improvements to LAMP assay protocols. We hypothesise that recent advancements in enhanced LAMP protocols assay perhaps represent the best chance for a rapid and robust assay for field diagnosis of COVID-19, without the requirement of specialized equipment and highly trained professionals to interpret results. Herein, we present our arguments with a view to disseminate such findings, to assist the combat of this virus that is proving so devastating. We hope that this strategy could be applied rapidly, and confirmed for viability with clinical samples, before being rolled out for mass-diagnostic testing in these current times.

Introduction

Viral disease emergence represents a serious threat to global public health. Indeed, several viral epidemics over the past few decades have emerged with increasing frequency, including the severe acute respiratory syndrome coronavirus (SARS-CoV), H1N1 influenza, and more recently the Middle East respiratory syndrome coronavirus (MERS-CoV) [1]. Recently, a novel coronavirus (SARS-CoV-2; coronavirus disease 2019, COVID-19) emerged from its epicentre in Wuhan province in China, and its subsequent rapid spread has pushed the capacity of the global public health community to its sheer limits. As of 25 April 2020, there were over 2.8 million confirmed cases of COVID-19 and ~200,000 deaths recorded worldwide (https://www.worldometers.info/coronavirus/).

Many nations have employed combinations of containment and mitigation activities, intended to delay major surges of patients and levelling demand for care, while protecting the most vulnerable from infection [2]. However, early diagnosis of COVID-19 is vital in controlling illness progression and limiting viral spread within the population, employed as part of such mitigation strategies to stop onward transmission and decrease load on healthcare systems [3,4].

Thus, rapid and accurate methods of early detection are vital to contain COVID-19, and prevent further spread and predicted subsequent infectious waves of viral recurrence in future (Fig. 1). Although

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https://doi.org/10.1016/j.mehy.2020.109786
Received 30 March 2020; Accepted 23 April 2020

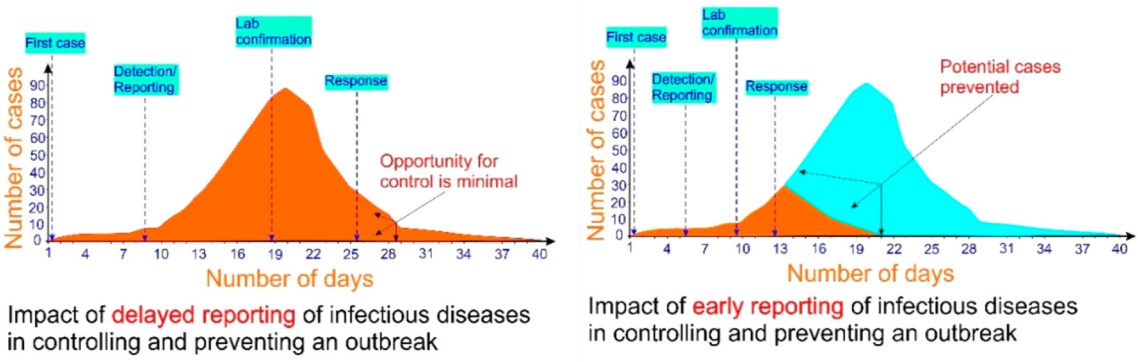

Fig. 1. The impact of rapid detection of infectious diseases in controlling and preventing an outbreak. Figure adapted from Nguyen *et al* 2020 [36].

Table 1
Comparison between PCR and LAMP based methods of viral RNA detection. Table adapted from Nguyen *et al* (2020).

PCR	LAMP
Bulky and cumbersome	Smaller, simpler, portable.
Specialised thermal cyclers required	Only a heat block is required
4–8 h until result	1 h until result
Requires skilled technicians	Requires no specific skill
Requires an additional reverse transcription step	Can be performed directly on RNA
Unstable reactions prone to inhibitors requiring purification steps	Stable and inhibitors tolerated, and thus purification steps not required
Detects DNA	Detects DNA and RNA
Tested on patient samples	Less tested on patient samples

COVID-19 is generally thought to be milder compared to SARS and MERS-CoV, the full extent of the impact from COVID-19 remains pending. However, this outbreak represents an opportunity for multiple governmental and health sector stakeholders to partner and develop rapid diagnostic tests for infectious agents of global health concern [5].

Hypothesis: Loop mediated isothermal amplification (LAMP) assays are a robust and rapid diagnostic method to detect viral RNA

To enable effective identification and isolation strategies, a rapid and robust diagnostic test is essential, conductible in the field and at local point-of-care (POC) centres, without the requirement of specialized equipment and highly trained professionals to interpret results. Herein, we propose from a collection of recently published articles a potential protocol based on loop mediated isothermal amplification (LAMP).

Following its initial characterization, Chinese and American Centers for Disease Control and Prevention (CDCs) rapidly employed molecular assays for detection of COVID-19 in clinical samples [5–7]. These, and methods by other groups, mostly employed development of real-time polymerase chain reaction (RT-PCR) methods to diagnose COVID-19, mainly targeting various combinations of the open reading frame (ORF), envelope (E), nucleocapsid (N), and RNA-dependent RNA polymerase (RdRp) genes [5–10]. Indeed, improved methods of quantitative RT-PCR characterized by rapid detection, high sensitivity and specificity, and are often prescribed as a gold standard for virus detection [3]. However, further novel PCR-based methods also present enhanced specificity and assay sensitivity.

PCR based methods of viral detection

PCR produces numerous copies (amplification) of a gene or series of genetic sequences by using a primer sequence and DNA polymerase enzymes to exponentially increase the amount of DNA required. PCR is widely used to amplify minute quantities of DNA to enable adequate requisite amounts for laboratory analysis of diagnosis. Owing to its simplicity, high sensitivity, and high sequence specificity, PCR-based methods are routinely and reliably capable of detecting coronavirus infection in patients [3,11,12]. In principle, such assays are employed following conversion of coronavirus RNA into complementary DNA by reverse transcription, following which PCR is performed and resultant amplification of DNA subject to specific detection or analytical methods such as electrophoresis or sequencing [3,13,14].

RT-PCR is significantly more sensitive than conventional methods [15,16], and is routinely employed as the predominant method to detection most coronaviruses [17,18], including COVID-19 [19]. A concern, however, particularly in the current demanding times, is that such analysis requires various specialist and expensive items of equipment, alongside highly trained analysts. Furthermore, current PCR-based methods of analysis require (realistically speaking, particularly when dealing with increasing volumes of potential infected patients) upwards of 4–8 h to process. These requirements, with the addition of financial pressures dictating that samples to be tested be sent potentially hundreds of miles away where requisite facilities and resources are present to perform such diagnostic analysis, may result in further inflation of the required time and finances to perform such diagnostic tests.

Evaluation of the Hypothesis: LAMP-based methods of viral detection

Loop mediated isothermal amplification (LAMP) is a novel isothermal nucleic acid amplification method. LAMP exhibits increased sensitivity and specificity due to an exponential amplification feature that utilises 6 different target sequences simultaneously identified by separate distinct primers in the same reaction [20]. LAMP assays are significantly rapid, and do not require expensive reagents or instruments, which aids in cost reduction for coronavirus detection [3].

Current improved LAMP assays employ a total of six primers, recognising eight distinct sites of the target sequence. A strand-displacing DNA polymerase is employed to initiate synthesis, while two primers form loop structures to facilitate and accelerate subsequent rounds of amplification. Specifically, LAMP employs two inner primers (FIP and BIP, which in turn consist of two parts each) and two outer primers (F3 and B3 which can recognize a total of six distinct regions within the target DNA). Two loop primers are employed (Forward loop primer; LF, and backward loop primer; LB) to accelerate amplification and detection efficiency [21–23].

First, FIP anneals to the template, and extension occurs using a polymerase with displacement activity (such as *Bst* polymerase), which will displace the product obtained from FIP by the extension reaction associated with the F3 primer. Subsequently, an extension reaction occurs using BIP on the product of FIP, and not on the template DNA due to displacement by DNA synthesis associated with the B3 primer. These reactions result in a product with a dumbbell-like structure which

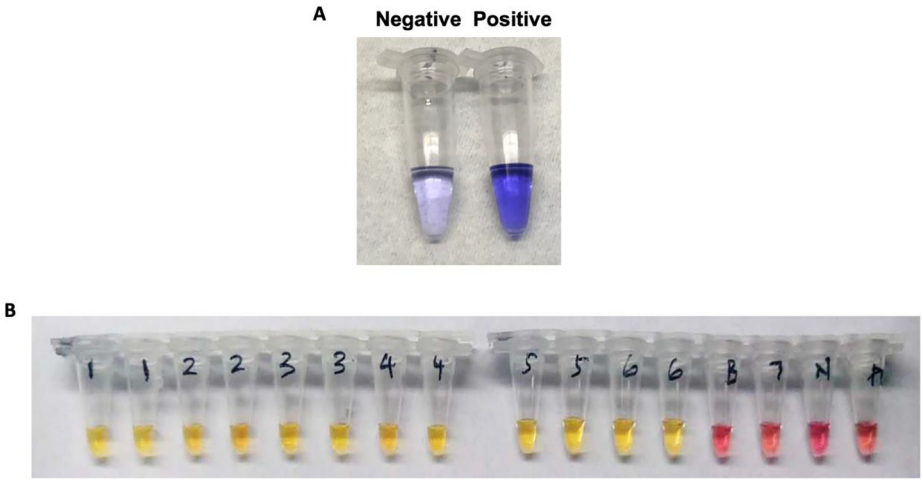

Fig. 2. Demonstrating the outcome of the LAMP assay using a colorimetric change to detect presence of COVID-19 viral DNA in A) simulated patient samples employed by El-Tholoth *et al.*, 2020 (darker colour represents a positive assay, while lighter colour represents negative assay result), and B) actual patient samples (n = 7) from Wuhan province in China, analysed by Zhang *et al.*, 2020 (yellow colour represents positive assays, while pink tubes represent negative assay results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

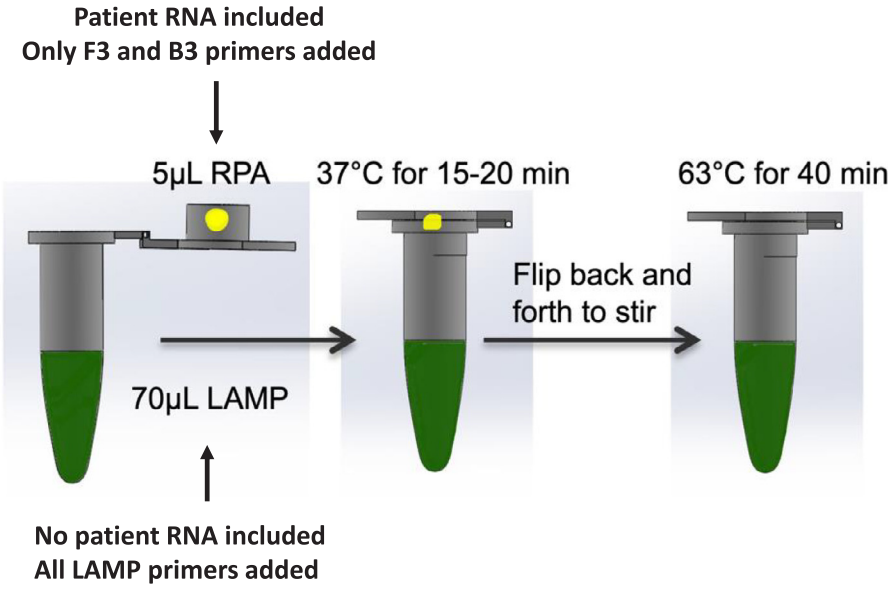

Fig. 3. Schematic representation of the experimental procedure of the Penn-RAMP procedure in the same tube. While the reactions could be performed in separate tubes and combined later, this envisaged procedure ensures rapid and simple flow-through and prevents potential for contamination. Figure adapted from El-Tholoth *et al.*, (2020).

Table 2
Sequences of COVID-19 Penn-RAMP primers used by El-Tholoth *et al.*, (2020).

Primer	Sequence (5′ – 3′)
F3	TGCTTCAGTCAGCTGATG
B3	TTAAATTGTCATCTTCGTCCTT
FIP	TCAGTACTAGTGCCTGTGCCCACAATCGTTTTTAAACGGGT
BIP	TCGTATACAGGGCTTTTGACATCTATCTTGGAAGCGACAACAA
Loop F	CTGCACTTACACCGCAA
Loop B	GTAGCTGGTTTTGCTAAATTCC

is essential for LAMP to establish isothermal amplification as the loop structures are always single stranded and can be annealed by FIP or BIP. This loop structure formation eliminates the denaturing step, which is otherwise essential in PCR for obtaining single-stranded DNA, and also establishes a cyclic reaction between the dumbbell-like structure and its complementary product, leading to elongated products with various copies of the target sequence produced (for reviews and detailed schematics, refer to [22,23]).

Numerous studies have now shown the successful application of LAMP assays in various forms to detect coronavirus RNA in patients samples [24–27], demonstrating that 1–10 copies of viral RNA template per reaction was sufficient for successful detection, which were ~ 100-fold more sensitive than conventional RT-PCR methods [26–31]. Of course, however, as with any emerging technology, there are some disadvantages associated with LAMP assays. Such methodology prevents inclusion of an internal PCR inhibition control, necessitating duplication of reactions while testing. Another disadvantage is the perceived complexity of the methodology, requiring a complex primer design system which can constrain target site selection and resolution or specificity. Furthermore, as the final product is a large fragment, downstream applications such as cloning are limited.

However, despite such drawbacks, LAMP is an ultrasensitive nucleic acid amplification method that can detect minute quantities of DNA or RNA templates within roughly an hour, far outstripping normally utilised RT-PCR methods, particularly with the current demands for rapid and sensitive testing. As the growing number of suspected COVID-19 cases exceeds the capacity of many hospitals, many patients remain untested impeding efforts to the control the disease. A rapid, point-of–care diagnostic for the COVID-19 is urgently needed, which we propose to be the LAMP method of detection (Table 1).

Consequences of the hypothesis and Discussion: LAMP-based assays for rapid detection of COVID-19

El-Tholoth *et al.*, [32] recently described design of a two stage LAMP (COVID-19 Penn-RAMP) strategy, which could be carried out in closed tubes with either fluorescence or colorimetric detection. Performance of

such assays were not only comparable with conventional RT-PCR assays, but also exhibited ~ 10-fold higher sensitivity when testing purified targets. Similarly, Lamb *et al.*, [33] also described successful and rapid detection of COVID-19 RNA within 30 min of experimentation. However, while significant advances, these assays and methods have not yet been applied to confirmed patient samples, with both these studies relying upon 'simulated' patient samples where swabs and blood samples were artificially 'spiked' with COVID-19 RNA.

Importantly, however, Zhang *et al.*, [34] have described the successful application of LAMP methodology to identify COVID-19 viral RNA from purified RNA or patient cell lysis using a visual, colorimetric detection. These results were further verified using RNA samples purified from respiratory swabs collected from COVID-19 patients in Wuhan, China with performance comparable to commercial RT-PCR tests, requiring only heating and visual inspection. Intriguingly, results obtained were also comparable between 'spiked' RNA samples and patient samples, suggesting that the improved methodology of El-Tholoth *et al.*, [32] should yield satisfactory results with patient samples (Fig. 2).

An enhanced protocol for COVID-19 diagnosis

Considering the success obtained by Zhang *et al.*, [34] using relatively less-sensitive LAMP, the enhanced sensitivity of the Penn-RAMP strategy employed by El-Tholoth *et al.*, [32] attributable to a modified two-step LAMP protocol could prove significantly successful as a diagnostic. Penn-RAMP involves a preliminary reaction with outer LAMP primers to amplify all targets through recombinase polymerase amplification (RPA), in which all targets are amplified concurrently. After this, a second highly specific LAMP reaction is initiated. Specifically, the first stage uses outer LAMP primers F3 and B3, while the second stage further combines the other 4 RAMP primers. This 'nested' principle significantly enhanced LAMP sensitivity by ~ 10–100-fold compared to normal LAMP, particularly when operating with purified and crude samples [35]. Indeed, the Penn-RAMP methodology when applied on mock trials by El-Tholoth *et al.*, [32] provided a success rate of 100% at 7–10 copies of viral RNA per reaction, compared to a 100% success rate at 700 viral RNA copies required for PCR methods [32,35]. To this degree, we hypothesise that the modified LAMP assay proposed by El-Tholoth *et al.*, [32] perhaps represent the best chance for a rapid and robust assay for field diagnosis of COVID-19 (Fig. 3), using the primer sequence strategies utilised (Table 2)

We hypothesise that this assay is will be a rapid, cost-effective, and simple method that could be applied within the field at short-notice and utilised by users with even limited training. All the equipment that would be required would be a hot-block or heater capable of differential heating. Reagent-wise, the costs would be similar to that of real time RT-PCR, but the real advantage of this would be the rapidity of this assay, yielding results within an hour of testing, compared to 4–8 h taken with RT-PCR methods. The aim is not necessarily a quantitative measure of infection, but rather a simple positive/negative assay for rapid detection/confirmation. We consider that this strategy should be applied rapidly, and confirmed for viability with clinical samples, before being rolled out for mass-diagnostic testing in these current times.

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.

Acknowledgements

The manuscript was conceived and written by both JK and AY, and submitted following approval of both authors.

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