Rapid diagnostics of novel coronavirus infection by loop-mediated isothermal amplification

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This review presents the basic principles of application of the loop-mediated isothermal amplification (LAMP) reaction for the rapid diagnosis of coronavirus infection caused by SARS-CoV-2. The basic technical details of the method, and the most popular approaches of specific and non-specific detection of amplification products are briefly described. We also discuss the first published works on the use of the method for the detection of the nucleic acid of the SARS-CoV-2 virus, including those being developed in the Russian Federation. For commercially available and published LAMP-based assays, the main analytical characteristics of the tests are listed, which are often comparable to those based on the method of reverse transcription polymerase chain reaction (RT-PCR), and in some cases are even superior. The advantages and limitations of this promising methodology in comparison to other methods of molecular diagnostics, primarily RT-PCR, are discussed, as well as the prospects for the development of technology for the detection of other infectious agents.

Key words: *diagnostics; coronavirus infection; loop-mediated isothermal amplification; polymerase chain reaction; SARS-CoV-2*

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Экспресс-диагностика новой коронавирусной инфекции с помощью реакции петлевой изотермической амплификации

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В настоящем обзоре представлены основные принципы применения реакции петлевой изотермической амплификации (loop-mediated isothermal amplification, LAMP) для экспресс-диагностики коронавирусной инфекции, вызванной SARS-CoV-2. Кратко описаны базовые технические детали метода, наиболее популярные способы специфической и неспецифической детекции продуктов амплификации, обсуждены первые опубликованные работы по использованию рассматриваемой технологии для выявления фрагментов молекулы нуклеиновой кислоты вируса SARS-CoV-2, в том числе разрабатываемые в Российской Федерации. Для доступных тестов на базе LAMP перечислены основные аналитические характеристики наборов, которые нередко сравнимы с параметрами тест-систем на основе метода полимеразной цепной реакции с обратной транскрипцией (ОТ-ПЦР), а в ряде случаев превосходят их. Обсуждены преимущества и ограничения этого подхода в сравнении с другими способами молекулярной диагностики (в первую очередь ОТ-ПЦР), а также перспективы развития технологии для выявления возбудителей других инфекций.

Ключевые слова: *диагностика; коронавирусная инфекция; петлевая изотермическая амплификация; полимеразная цепная реакция; SARS-CoV-2*

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1. Introduction

In December 2019, in Wuhan (People's Republic of China, PRC) there was an outbreak of a new disease caused by the virus later named the severe acute respiratory syndrome coronavirus 2 (severe acute respiratory syndrome CoV-2, SARS-CoV-2, 2019-nCoV). The virus causes the so-called novel coronavirus disease 2019 (COVID-19) that was declared a global pandemic by the World Health Organization (WHO) in March 2020 (https://www.who.int/ru/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020). The SARS-CoV-2 virus belongs to the genus *Betacoronavirus*, the family *Coronaviridae*, representatives of which are mainly associated with infections in mammals [1]. The viral genome is represented by a single-stranded positive-sense RNA, approximately 30 thousand base pairs (bp) long, with 14 open reading frames encoding 27 proteins [2]. The infection causes symptoms similar to those of some other respiratory diseases, though a significant portion of patients may remain asymptomatic for quite a long time [3]. Therefore, monitoring of the novel virus spread is an extremely challenging task, considering that it is rather easily transmitted from a sick person to those who are not infected. As of January 2021, according to the John Hopkins Coronavirus Resource Center (https://coronavirus.jhu.edu/), there were more than 100 million confirmed COVID-19 cases and more than 2 million deaths worldwide, the numbers being illustrative of the speed of the virus spread and urgency of containment measures to halt the spread. Currently there are no effective treatments for the novel coronavirus infection. Many countries have just started their vaccination campaigns, while the exact durability of the immune response is still unknown. There is, therefore, a pressing need for a sensitive and, possibly, inexpensive rapid diagnosis method with robust reproducibility of results. A rapid test, which is efficient and easy to use, would be critical for solving the problem. Such tests would make it possible to identify infected patients at early stages, to limit their social contacts and to monitor their condition without putting other people's life and health at risk.

At present, molecular techniques used for diagnosis of the infection caused by SARS-CoV-2 are based on the reverse transcription-polymerase chain reaction (RT-PCR) [4, 5]. The RT-PCR tests detect viral nucleic acids in nasopharyngeal swab samples, demonstrating remarkably high levels of sensitivity and specificity. WHO and U.S. Centers for Disease Control and Prevention (CDC) approved RT-PCR tests as the «gold standard» for detection of the virus causing COVID-19 [6–8]. At the moment, in the Russian Federation, the Federal Service for Surveillance in Healthcare (Roszdravnadzor) has registered more than 40 RT-PCR-based tests from Russian manufacturers. Despite its obvious advantages, the method has some shortcomings. For example, the RT-PCR test requires expensive laboratory equipment: most of the thermal cyclers for real-time detection cost more than 2 million rubles and can barely be afforded by small diagnostic centers. Besides, the test must be performed by trained and competent laboratory personnel; the entire testing process takes several hours, not counting time needed to deliver biomaterials to the laboratory. These limitations are aggravated by the fast-moving pandemic, as RT-PCR does not have the screening capacity to keep pace [9]. Other commonly used methods of COVID-19 diagnosis include serological tests aimed at detection of antibodies or antigens associated with coronavirus infection. They are easy to use, produce quick results and are not exceedingly costly. On the other hand, because of their low sensitivity and specificity, such

systems are not accurate enough to be used reliably in detection of SARS-CoV-2 (https://open.fda.gov/apis/device/covid19serology/). Furthermore, such tests are almost useless at very early stages of the disease [10], when identification and isolation of an infected person or people are exceptionally important for prevention of mass infection. Finally, diagnostic tests based on high-throughput sequencing techniques [11], capable of detecting all the known viral pathogens and, therefore, highly sought-after during outbreaks of infectious diseases of unknown etiology are still being developed and cannot be used for actual purposes during pandemics.

Therefore, there is a pressing need for new, effective solutions in the diagnosis of novel coronavirus infection. One of the approaches represents a group of isothermal amplification techniques, such as rolling circle replication (RCA) [12], nucleic acid sequence-based amplification (NASBA) [13], recombinase polymerase amplification (RPA) [14], helicase-dependent DNA amplification (HDA) [15], and loop-mediated isothermal amplification (LAMP) [16]. The latter is rightfully seen as the most promising type of isothermal amplification, being much more frequently referred to in scientific publications as compared to the other types. The LAMP technique is similar to conventional PCR, except that the amplification process occurs at constant temperatures; therefore, it does not require expensive thermal cyclers, which can be replaced with conventional thermostats. This amplification technique makes detection of viral DNA/RNA in clinical materials quicker and easier as compared to PCR tests, which was discussed in the recent review [17]. Other advantages of the LAMP method include a wide pH and temperature ranges that are acceptable for tests [18], the theoretical possibility to use non-processed samples [19], and flexibility of detection procedures, while maintaining specificity and sensitivity, which are almost equal to those demonstrated by PCR assays or sometimes even surpass them [20, 21]. For example, the specificity of LAMP-based tests can be much higher than with PCR assays, as the concurrent annealing of several pairs of primers to the DNA template in a specified order (see the detailed description below) results in exceptionally low probability of accidental amplification of the off-target genome fragment.

This review is aimed to outline the basic principles underlying the detection of nucleic acids by using loop-mediated isothermal amplification and to give a brief description of different LAMP techniques that were developed to be further used in COVID-19 diagnostics. We hope the review can provide general information to researchers who are interested in developing LAMP-based methods for detection of SARS-CoV-2 and other pathogens; it also can give a better picture regarding the potential of the discussed amplification type for battling the current pandemic and for preventing any possible threats in future.

2. Description of the loop-mediated isothermal amplification (LAMP) method

LAMP is a nucleic acid amplification method, which was developed in 2000 [16]; currently, it is playing an increasing role as a diagnostic method for infectious diseases, largely due to the COVID-19 pandemic and the need for large-scale screening of population within the shortest possible time. The process occurs at a constant temperature (normally 60–65 °C), thus eliminating the need for a thermal cycler (required for conventional PCR); besides, the technique is stable against most PCR inhibitors [18]. Initially, 4 primers were used for amplification of the target genome sequence [16]; later, the technique was optimized by using an additional pair of primers [22]. The reaction mix needs only one enzyme – the DNA polymerase with DNA strand displacement activity (for example, *Bst*-polymerase from *Geobacillus stearothermophilus* or *Bsm*-polymerase from *Bacillus smithii*).

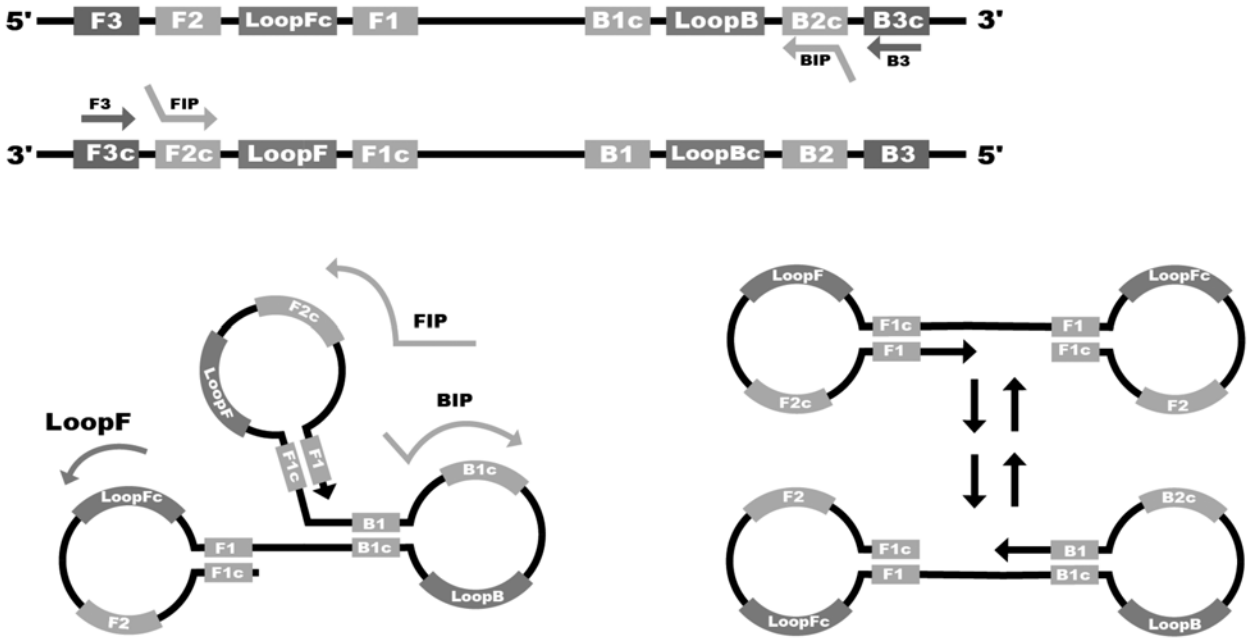
As shown in the **figure**, the LAMP primer set for a standard test consists of 2 outer (F3 and B3) and 2 inner (FIP and BIP) primers, which recognize 6 distinct regions of the target DNA sequence (F3c, B3c, F2c, B2c, F1 and B1). The forward inner primer (FIP) in the 5'→3' direction consists of the F1c region, the complementary sequence of the F1 target, and the F2 region that is complementary to the F2c region. Likewise, the backward inner primer (BIP) consists of the B1c and B2 regions that are complementary to the B1 and B2c sequences in the target DNA. The outer F3 and B3 primers have sequences complementary to the F3c and B3c regions, respectively. LAMP primers must be optimized to meet a number of criteria, including the melting temperature, GC content, the length and distance between regions in the target DNA, stability at the 5' end and the 3' end, etc. [23].

The amplification process starts with FIP hybridizing to the target DNA in the F2 region to form double-stranded DNA, in equilibrium at around 65 °C [23]. Then, the *Bst*-DNA-polymerase with strand displacement activity initiates the DNA synthesis with FIP, simultaneously displacing one strand of DNA, if any. When the initiation stage is over, the F3 primer binds to its complementary F3c region and displaces the FIP-complementary strand. Because of the presence of the sequence F1c in FIP, the latter can self-anneal and form a loop structure at one end

of DNA. This strand then serves as the target for BIP-initiated DNA synthesis and subsequent displacement of the strand from the B3-primed DNA synthesis. This allows the other end of the single-stranded DNA to form a loop, thus resulting in a dumbbell-like DNA structure, which serves as a template for the next amplification. The initial idea about the LAMP mechanism is well presented in the video recorded by New England Biolabs, a major manufacturer of *Bst*-polymerase (USA) (https://youtu.be/9Kv_hSDEX7M; https://youtu.be/cnXDTjCXGjE). Primers are frequently designed by using the specialized PrimerExplorer software (http://primerexplorer.jp/lampv5e/index.html). It is important to avoid homo- and heterodimers, which can inhibit the production of the target product during non-template amplification, thus leading to false results.

After a dumbbell-like structure is formed, exponential amplification of similar structures is initiated, with the DNA-polymerase triggering the DNA synthesis in the F1 region. FIP also hybridizes with the one-loop structure from the F2 region, and the DNA synthesis of that primer causes the F1-primed strand to be displaced and to self-bind into a loop structure. Finally, the self-primed DNA synthesis amplifying the current template and creating a new one from the displaced FIP-complementary strand starts on the other side of the DNA fragment, in the new loop in the B1 region. This repeatedly occurring process produces large amounts of nucleic acids – DNA can be amplified as much as 10^9 times within an hour [24]. The six-primer (loop primer) LAMP technique was developed to further improve the amplification efficiency [22]. This technique employs 3 pairs of primers instead of 2 pairs; forward and backward loop primers (LoopF and LoopB) are annealed to regions between F1/F2 and B1/B2 respectively [23]. Such primers are known to enhance the reaction, as the amplification takes less time due to the increased number of starting points for DNA synthesis [24].

To extend the LAMP capability range from DNA detection to RNA detection and to implement multiplex detection, the original LAMP protocol was upgraded to include several improvements aimed at development of different methods, including reverse transcription LAMP (RT-LAMP) and multiplex LAMP. In the first case, reverse transcriptase is added to the reaction mix to cause conversion of viral RNA into complementary DNA (cDNA), which will be used for amplification. This procedure proved its worth in detection of a multitude of RNA viruses [24], including SARS-CoV-2. According to the records of 2020, the reagent kit for synthesis of cDNA from an RNA template (developed by the Central Research Institute of Epidemiology, Russia) was included in more than 25% kits for diagnosis of the novel coronavirus infection in Russia. Multiplexed LAMP assays were developed to detect multiple pathogens in



Schematic representation of the loop-mediated isothermal amplification. External primers are labeled as F3/B3, internal – FIP/BIP, consisting of F2/B2 regions and complementary regions of the F1/B1 sequences, loop primers – LoopF/B. During the amplification reaction a cyclic transition between two forms and their subsequent elongation into dumbbell-like structures with multiple loops occur.

the same test tube by using a larger number of primers or unique fluorescent signals [25, 26]. The above description of LAMP tests shows the high potential of the method for rapid diagnosis of viral infections. Consequently, this method has great potential in SARS-CoV-2 detection, which will be discussed in the subsequent section.

3. Detection of LAMP amplification products

3.1. Non-specific detection methods

LAMP amplification involves accumulation of pyrophosphate, which is released during the DNA synthesis following the nucleoside triphosphate hydrolysis. In its turn, it interacts with magnesium ions (Mg^{2+}) present in the buffer, producing a white precipitate. It causes turbidity of the solution, which can be measured, for example, with a turbidity meter (a haze meter) [27]. Another frequently used method of non-specific detection involves intercalating dyes such as SYBR™ Green I, EvaGreen™ or SYTO added to the reaction mix, thus making it possible to obtain amplification curves by using a PCR amplifier and real-time detection. However, the disadvantage of these (though not all) dyes is their reaction-inhibiting effect that may affect the sensitivity of LAMP-based tests [28]. At the same time, it has been found that the LAMP method is tolerant to PCR inhibitors, and such tolerance is its significant advantage [18]. When using some dyes (for example, SYBR™ Green I or EvaGreen™), test tubes can be exposed to ultraviolet light emitted by a UV lamp or light-emitting diodes, thus resulting in color change in positive specimens [29].

In addition, a number of techniques for field applications have been developed for naked-eye visualization of final products. Normally, the reaction mix is used with indicator dyes, which change their color during the reaction. For some of them (including cresol red or neutral red), pH of the original reaction mix must have a specified value (~8.8). During the reaction, its value decreases to ~6.0–6.5 along with the color change in the dye [30]. Other indicator dyes do not require a specially prepared mix: for example, hydroxy naphthol blue or fluorescent dye Quant-iT™ PicoGreen™, color changes in which can be visible to the naked eye [31]. There is another simple-to-use detection method based on gel electrophoresis [32]. Besides, amplification results can be quantified by measuring concentrations of manganese ions (Mn^{2+}). It was suggested that before the reaction, calcein (fluorexon), a fluorescent metal indicator, should be added to the solution. In the absence of Mn^{2+}, it emits fluorescence when exposed to light at a specific wavelength (495 nm) [33]. If the above ions were present in the original mix, calcein will bind to them, forming an insoluble manganese-pyrophosphate salt complex. Subsequently, fluorescence is further intensified when free calcein binds to magnesium ions (Mg^{2+} from the LAMP reaction mix) and could be easily observed under UV light at the wavelength of 365 nm [24]. Finally, other common non-specific methods include melting and annealing curve analysis, bioluminescence through pyrophosphate conversion [34] and electrochemiluminescence [35].

It should be noted that even though the probability of accidental amplification of similar sequences of genomic fragments belonging to other organisms is fairly low and, to a large extent, can be controlled through elaborately designed primers, the above listed DNA sequence-independent detection methods can indicate extended primer dimers as false positive results [36]. Therefore, recent advancements brought to the fore methods of specific (dependent on nucleic acid sequence, i.e. sequence-specific) detection of LAMP amplification products. Some of such methods are described in the next section.

3.2. Specific detection methods

Presently, there are more than 30 published detection methods and techniques for DNA LAMP-based amplification; all of them have been extensively discussed in the recent review [37]. Here, we will give a brief summary of those that are most promising, in our opinion, for practical diagnostics of COVID-19.

3.2.1. Molecular beacon

The DNA probe specific to the target fragment is modified with fluorophore and a quencher at each end. Its 5’ end is complementary to its 3’ end through 5–7 nucleotides, allowing the probe to form a hairpin or frying pan-like structure [38, 39]. In the absence of amplification products, the molecular beacon exists in a closed loop form due to intramolecular hybridization between fragments at the 5’ and 3’ ends. Consequently, the fluorophore and the quencher are in close proximity, thus leading to fluorescence quenching. In the presence of amplification products, the probe specifically hybridizes with an amplicon, causing the loop to open. This causes separation of the fluorophore and the quencher as well as the subsequent release of fluorescence. Previously, molecular beacons were used for LAMP detection of viral nucleic acids of human immunodeficiency virus (HIV), hepatitis B, C, and E viruses (HBV, HCV, HEV), dengue virus (DENV) and West Nile virus (WNV) [38]. The analytical sensitivity was between 50–100

viral particles per reaction when HCV and HBV were detected. The high analytical specificity was confirmed by testing with various viral nucleic acids. In addition, clinical studies were conducted with biological materials obtained from patients. The outcome diagnostic sensitivity and specificity reached 97 and 100%, respectively. Molecular beacons were also used for detection of a part of the *ompW* gene, 1,031 bp long, from *Vibrio cholerae* [39]. The instability of hairpins in the LAMP reaction may result in fairly high signal-to-noise ratios. Locked nucleic acids (LNA) generating higher melting temperatures can be used to improve the thermal stability of molecular beacons by decreasing the background fluorescence signal [40].

3.2.2. Alternatively binding quenching probe competitive LAMP

The alternately binding quenching probe competitive LAMP (ABC-LAMP), which was originally developed for quantification of DNA, uses guanine quenching and is based on competitive hybridization [41]. The reaction mix includes 2 targets: the amplicon of interest and an internal amplification standard (known as the competitor). Both targets include an identical sequence in the loop region, which is complimentary to the special fluorescent-labeled alternately binding quenching probe (AB-QProbe). Binding of the probe to the target amplicon reduces the fluorescence intensity through guanine quenching. And, on the contrary, binding to the competitor tends to maintain the fluorescence of the probe label, as guanine in the competitor is replaced with cytosine. The ratio of the amplification products from the target to the products from the competitor can be calculated by the fluorescence intensity. The ABC-LAMP procedure is described for a model target, the *amoA* gene, which encodes the ammonia-oxidizing enzyme in environmental bacteria. Although analytical parameters were not assessed, the accurate quantification was successfully demonstrated. The advantage of ABC-LAMP for the quantification of the target DNA is that any inhibitors that are present in the LAMP mix have an equal impact on the amplification of the real target and of the competitor. The latter serves as an internal standard and makes it possible to quantify amplification products by using measurements of the fluorescence at the beginning and at the end of the amplification process, and no real-time monitoring is required. Yet, the approach has its limitations, and one of them is that the amplification efficiencies of the target and the competitor must be balanced, which implies the building of a standard curve.

3.2.3. Fluorescence of loop primer upon self-quenching

The inner or loop primer can be modified with fluorophore (at positions 2 or 3 of thymine at the 3' end) capable of self-quenching. It is a non-FRET (fluorescence resonance energy transfer) based quenching mechanism, as no further fluorogenic molecules are required. The details of re-occurrence of the previously quenched fluorescence of the conjugated label are still unclear; however, fluorescence quenching may involve the guanine donor capability, which facilitates the transfer of the charge between the nucleobase and the nearest dye [42]. Initially, the fluorophore-modified primer is not bound to the DNA template, and as such, it is unbound and free in the solution, being capable of self-quenching. During amplification, the modified primer is incorporated into a double-stranded amplicon, causing dequenching and, consequently, increased fluorescence. The increasing number of such amplicons results in increasing fluorescence intensity, thus providing the evidence of successful LAMP-based amplification. The analytical and clinical validation of the fluorescence of loop primer upon self-quenching (FLOS-LAMP) was conducted for the human, highly infectious, DNA-containing *Varicella zoster* virus [43]. The researchers used 2 types of materials: crude sample material (without pre-extraction of nucleic acids) and extracted DNA, with the latter showing a more robust signal. The analytical sensitivity was 500 copies per reaction, while the diagnostic sensitivity and specificity were 96.8 and 100%, respectively. A technical challenge in the discussed experiment is to find fluorophores capable of high self-quenching and a primer suitable for modification with fluorophore, which is not an easy task and imposes certain restrictions on design requirements for oligonucleotide structures. Visual detection can be possible by adding polyethylenimine (PEI), which would lead to the formation of an insoluble amplicon-PEI complex. Fluorescence-modified probes are incorporated into the precipitate and can be detected with a standard UV lamp. Differentiation between 2 targets is also possible, thus, providing the format similar to the multiplex when only one of the targets is present in the mix.

3.2.4. Universal QProbe

A universal QProbe [44] is used together with the single-stranded joint DNA, which contains the sequence of one of the main primers, which is complementary to the target DNA, and the 5' end universal sequence complementary to the additional universal oligonucleotide with the quencher attached to its 3' end. The joint DNA operates

as a coupling between the QProbe and the target by hybridizing with the template during the amplification. The electron transfer between the fluorophore and the guanine in the target sequence results in fluorescence quenching. The advantage of the method is that the nucleotide sequence of the QProbe is fixed and does not contain any target-specific (variable) regions; besides, it offers the possibility of using internal control. Yet, this method implies a target sequence-specific mechanism of detection of amplification products, as the fluorophore attached to the 3' end of the universal sequence is quenched by the guanine in the target strand when the latter is in close proximity. This works only if guanine bases are present at the required position in the target sequence; therefore, to a large extent, this method can be seen as semi-universal.

3.2.5. LightCycler probes

LightCycler probes were used in the LAMP reaction by Chou P.H. et al. [36] and are currently commercially available for PCR. These probes include an acceptor probe modified with a quencher at the 5' end and a donor probe labelled with fluorophore at the 3' end. The distance between the 5' end of the acceptor part and the 3' end of the donor part of the target sequence is usually 2 bp, thus making it possible to guarantee close proximity between fluorophore and the quencher. During the LAMP process, amplicons are generated, providing probe-complementary sequences, with which the probe system hybridizes, bringing the fluorophore and the quencher into close proximity. The analytical sensitivity was measured for the white spot syndrome virus (WSSV) of penaeid shrimps (*Penaeidae*) and was equal to approximately 100 copies per reaction. The specificity was confirmed by testing on the host DNA and other DNAs of viral pathogens found in shrimps. The clinical significance was demonstrated by testing of the genomic DNA of WSSV-infected whiteleg shrimps (*Penaeus vannamei*) [36].

3.2.6. Cas12 and thin-layer chromatography (TLC) based detection

Broughton J.P. et al. [8] reported their development and initial validation of a CRISPR–Cas12-based assay for detection of SARS-CoV-2 RNA in patient specimens. The assay was named DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR). The assay performed simultaneous reverse transcription and isothermal amplification using loop-mediated amplification (RT-LAMP), followed by Cas12 detection of predefined coronavirus sequences, after which cleavage of a reporter molecule confirms detection of viral RNA. Visualization of the Cas12 detection reaction is achieved by using a FAM-biotin reporter molecule and lateral flow strips designed to capture labeled nucleic acids. Uncleaved reporter molecules are captured at the first detection (control) line, whereas indiscriminate Cas12 cleavage activity generates a signal at the second detection (test) line.

3.3. Other detection methods

Undoubtedly, the list of techniques for specific detection of LAMP-amplified products can go on; however, their detailed description is beyond the scope of this review. Other potential methods include loss-of-signal guanine quenching [45], fluorescence using labeled primers and intercalator – ethidium bromide ($C_{21}H_{20}BrN$) [46], detection of amplification by release of quenching (DARQ) [47], assimilating probe [48], one-step strand displacement (OSD) [49].

4. LAMP limitations

One of the main disadvantages of LAMP-based tests is the complex design of primers, as the test requires 6–8 sites within a short DNA fragment. Amplification of its other (unwanted) sequences, especially in the host genome must be avoided, which considerably limits the choice of target sites. The large number of primers increases the risk of generation of concatemers [50]. As the total primer coverage area is larger than in classical PCR, the probability of point mutations is high, especially in RNA-viruses, including SARS-CoV-2. It can lead to lower efficiency of primer annealing and false negative results [51]. Therefore, any new mutative changes in genomes of studied pathogens must be constantly monitored to be able to correct the situation by replacing oligonucleotides or inclusion of the so-called degenerate bases. Besides, two and more genome fragments can be used at the same time in one test, especially for non-specific detection of amplified products, thus reducing the effect of replacements in the genome and providing additional time for redesigning of primers, if necessary.

Another problem, which is partially described above, is associated with challenges posed by specific detection of amplification products, though they are addressed by different approaches (see Section 3) [37]. Thus, while multiplexed LAMP assays are designed for detecting multiple pathogens in the same test tube, which is important in diagnosis of acute respiratory and intestinal infections caused by a multitude of bacteria and viruses, their application still poses a problem. This fact, in a certain way, complicates the process of introducing an internal control (IC), which gradually becomes an almost mandatory step in preparation of commercial diagnostic assays.

Finally, we cannot but mention the continuing limited supply on the Russian market for reagents required for loop-mediated isothermal amplification (for example, *Bst*-polymerase), though the situation is definitely going to improve.

5. Progress in RT-LAMP-enabled SARS-CoV-2 detection

Shortly after the COVID-19 pandemic had started (2020), Lamb L.E. et al. [21] reported the development of a rapid LAMP-based screening test for detection of SARS-CoV-2 RNA with the assay time less than 30 min, without the extraction process. To achieve this goal, the consensus sequences of 23 identified virus strains were used. The LAMP Designer software (Premier Biosoft™, USA) was used to design RT-LAMP primers for targeting consensus sequences that were shared among all known coronavirus strains, but differed in closely related viruses (such as the bat SARS-like coronavirus (BtCoV)). To verify the test performance characteristics, samples from healthy people were used as simulated samples after synthetized oligonucleotides based on the GenBank MN908947.3 sequence were added to them. The readout of the RT-LAMP assay was monitored through 3 methods: color change in the reaction mix, the fluorescence level, and gel electrophoresis. The test was found to have high specificity towards SARS-CoV-2, as no signal was observed in multiple tests using other viruses such as MERS (Middle East respiratory syndrome), BtCoV and MHV (mouse hepatitis virus). The sensitivity was also evaluated with the limit of detection as low as 1.02 fg per reaction, using simulated samples. Finally, the test was confirmed to have high clinical utility, as it correctly detected SARS-CoV-2 nucleic acid molecules, regardless of the sample type (serum, urine, saliva, oropharyngeal swabs).

To develop a fast and simple-to-use COVID-19 test kit, Huang W.E. et al. designed 4 sets of LAMP primers targeting different regions of SARS-CoV-2 [52]. Out of them, 2 primer sets target the *N* gene of the virus, while the other 2 sets target the *S* gene and the *ORF1ab* gene. The latter is close to the 5' end of the viral RNA, while the *N* gene is close to the 3' end. It was assumed that the detection performance for different genome regions can be different, as degradation of RNA occurs from the 5' end to the 3' end during the sampling and RNA extraction. FIP primers conjugated with FAM at the 5' end, making the technique effective and reliable both for fluorescent and for colorimetric detection of amplification products. The high specificity of all primers for SARS-CoV-2 detection was confirmed, as they do not amplify human DNA and, therefore, can be used for assays of clinical samples. The accuracy of the assay also was acceptable: the test results were in good agreement with those obtained with the conventional RT-PCR. In detecting viral RNA, the primer sets performed with a sensitivity ranging from 2 to 20 RNA copies per reaction (25 μl) within 30 min of the amplification, depending on the target. An additional one-step test was performed to check if the RNA extraction step can be omitted from the RT-LAMP process. As observed, 40 min of the RT-LAMP process caused a color change visible to the naked eye, thus confirming that the RT-LAMP test can be performed without extraction of nucleic acids. The primer sets detected accurately all 8 positive and all 8 negative clinical samples, proving that the test can be used to replace conventional RT-PCR tests for SARS-CoV-2 detection, especially when a specialized laboratory is not available.

Since the beginning of 2020 a large number of LAMP-based test systems for rapid diagnosis SARS-CoV-2 have been developed and reported in publications. The PubMed search gives more than 80 publications (starting from March 2020) that address testing the novel coronavirus infection by using loop-mediated isothermal amplification. Note, these do not include preprints as well as commercial solutions unavailable in public sources. For example, several sets of primers specific for different genes of the novel coronavirus have been designed [53] and have demonstrated high sensitivity and specificity. Another example is a portable smartphone-based instrument to perform end-point fluorescence detection of LAMP assays on a microfluidic chip [54]. It was found that in LAMP-based diagnostics, specificity could reach almost 100% [55] and sensitivity could exceed 90%. Dao Thi V.L. et al. [56] described the RT-LAMP assay that reliably detected SARS-CoV-2 RNA with an RT-PCR cycle threshold (Ct) value of up to 30, with a sensitivity of 97.5% and a specificity of 99.7%. They also developed a swab-to-RT-LAMP assay that did not require a prior RNA isolation step, which retained excellent specificity (99.5%), but showed lower sensitivity (86% for Ct <30) than the RT-LAMP assay with prior RNA isolation. Another LAMP innovation was a microfluidic disk-based LAMP chip integrating sample preparation and detection [57]; the solution mixing was achieved by modulating the rotation rate of the disk; the chip was kept at the required temperature, and the results were detected as fluorescence change. The assumed detection limit for viral RNA was 2 copies per reaction, the throughput capacity – 21 tests per disk, the time to result – ~70 min. Another RT-LAMP detection

technique for SARS-CoV-2 employed a custom-made cartridge and a smartphone-based instrument, making it possible to conduct tests directly at a sampling site [58]. The technique has a limit of detection (LOD) of 50 RNA copies per 1 μl reaction mix within ~30 min. The authors of the technique claim that it was the first mobile isothermal amplification-based device for detection of novel coronavirus RNA, which had a disposable cartridge and did not require an RNA isolation step. Another similar technique from Color Genomics (USA) demonstrated high throughput capacity and sensitivity (LOD of 0.75 copies per 1 μl); however, it requires a prior RNA isolation step.

To fulfill the need for a standalone colorimetric isothermal nucleic acid amplification platform, an ultra-low-cost diagnostic device has been developed. It consists of an integrated computer, imaging camera, and artificial intelligence-based image processing algorithm [59]. A total of 200 samples from COVID-19 suspected cases were tested to assess the performance of the assay. The levels of reliability, specificity and sensitivity were higher than those demonstrated by current «gold standard» PCR. Seven types of coronavirus (HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV and SARS-CoV-2) were detected by using a special microfluidic device and isothermal amplification within 40 min [60]. The fast and easy-to-use RT-LAMP technique specifically amplifying regions of *ORF8* and *N* genes employed low-cost laboratory equipment, thus being a perfect candidate for railway stations, airports, and hospitals [61]. Another RT-LAMP test system is designed to detect simultaneously 2 SARS-CoV-2 genes (*S* and *RdRP*); its analytical sensitivity and specificity, limit of detection and accuracy are comparable to those of RT-PCR [62]. The assay is designed for 2 targets and has promising potential, considering a never ceasing flow of new mutations of the virus, which can adversely affect the effectiveness of some tests (as it happened to a number of diagnostic tests due to the «British mutation») [51]. Finally, there is another approach involving RT-LAMP multiplexing and validated sensitivity [63]. Multiplexing of primers in RT-LAMP reactions is a high-potential method capable of increasing sensitivity through detection of multiple genomic fragments. The PCR and LAMP combination was used to increase the sensitivity level of the test [64]. At present, an assay from New England Biolabs (USA) is commercially available for rapid detection of SARS-CoV-2 by using colorimetric loop-mediated isothermal amplification; it has reagents for LAMP-amplification of novel coronavirus *N* and *E* gene fragments and assay performance control. The reaction is performed at 65 °C and takes only 30 min. The reaction mix includes a pH-sensitive dye to simplify visual colorimetric readouts.

In Russia, there have been several attempts to put tests based on isothermal amplification into diagnostic use. For example, the reagent kit designed by Medical Biological Union, LLC (Russia) incorporated the LAMP method, thus leading to significant reduction in the time required for examination of samples, as compared to RT-PCR. The procedure is reduced to heating of samples in the lysis buffer, transferring them to a plate, adding the reaction mix, and performing amplification, while the claimed sensitivity equals 10^3 copies per 1 ml. Tests from Evotech Mirai Genomics (Russia – Japan) incorporate the modified method of loop-mediated isothermal amplification (SmartAmp), which is as good as the PCR method in terms of accuracy, while the testing time is 4 times shorter than in PCR. The sensitivity level claimed by the manufacturer equals 10^2 copies per 1 ml. The reagent kit for RT-LAMP detection of the novel coronavirus RNA, which is manufactured by the Lytech Research and Production Company (Russia), has no claimed analytical characteristics. Another reagent kit (Syntol, Russia) [64] was designed for an RT-LAMP reaction aimed at real-time detection of specific RNA fragments of the novel coronavirus through fluorescent detection. It makes it possible to detect 2 targets in one tube: a fragment of viral RNA and a fragment of internal positive control DNA; it excludes false-negative results; the assay takes not more than 40 min. The express test from Sistema Bio Tech, LLC (Russia) detects SARS-CoV-2 within 20 min. The system is designed for diagnosis at all stages of the disease; it can be used for screening at any medical facilities; the virus is detected by color change in the reaction mix in a tube. Besides, different organizations in Russia work on localization of manufacturing of reagents for LAMP tests to reduce their price and to facilitate their extensive clinical use. The SARS-CoV-2 RNA detection express-test announced at the beginning of 2021 by the Central Research Institute of Epidemiology (Russia) is based on the LAMP reaction and employs domestically manufactured enzymes; the development of this test is an important stage in the future transition from conventional PCR-based diagnostic assays to new-generation technology.

6. Conclusion

Rapid, low-cost, and user-friendly molecular diagnostic methods are an important prerequisite to address outbreaks of infectious diseases. The COVID-19 pandemic has shown that there is an urgent need for

increasing the testing capacity that should be many-fold above the level achievable with current standard methods and approaches [65]. LAMP is an innovative gene amplification technique that shows great promise as a detection tool for viral nucleic acids, especially during the novel coronavirus pandemic. This review summarized recent achievements in LAMP assays for rapid and accurate detection of SARS-CoV-2 RNA. Even though such assays may produce false results, especially those that are known as false negative, the advantages of using the LAMP method are still impressive. One of them is the high speed of the assay: the confirmation of results for the typical RT-LAMP procedure is much faster than that of RT-PCR [23]. The omission of a denaturing step and the integration of amplification and detection processes make it possible to perform the assay with faster time to results. Another advantage is the ease of use, as the isothermal nature of the amplification test suggests using low-cost equipment. The isothermal conditions also contribute to high amplification efficiency, as there are no losses of time that otherwise would be spent on thermal cycling (in conventional RT-PCR) [24]. Finally, the visualization of results is relatively simple, as either a pH dye or solution turbidity can be used as indicators visible to the naked eye. Therefore, the speed, simplicity and cost-effectiveness of the LAMP procedure make it a good candidate for monitoring of the SARS-CoV-2 spread.

Besides improvement of sensitivity and specificity in the design of LAMP primers, to further optimize the technique for large-screening of COVID-19, one of the potential trends is to develop a «true» one-step, closed-tube LAMP assay [17]. This procedure will require the end-user to load a sampling swab into the reaction mix for the further close-tube amplification at a constant temperature. Thus, the assay may be used for detection of SARS-CoV-2 and other pathogens at home, demonstrating high detection capacity, as it has been achieved in the test system from Lucira™ (USA). In the near future, molecular diagnostics at a patient's bedside may become truly affordable, and it will not only boost the virus detection capacity, but also significantly reduce the costs incurred by the healthcare system. The approach can play an important role in combating pandemic outbreaks today and in future.

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