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PROBLEMS AND PROSPECTS

Loop-Mediated Isothermal Amplification as a Promising Method for Mass COVID-19 Diagnostics

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Abstract—Real-time reverse-transcription polymerase chain reaction (RT-PCR) is currently the most popular method for early COVID-19 diagnostics. However, loop-mediated isothermal amplification (LAMP) is superior to real-time RT-PCR in rapidity and simplicity, since it does not require expensive laboratory equipment and trained personnel. LAMP-based diagnostic kits for COVID-19 testing already exist, but corresponding tests are not yet widely available. The method has great potential for mass application. Here, we discuss the technical and methodological aspects of its widespread adoption.

Keywords: COVID-19, SARS-CoV-2, LAMP, loop-mediated isothermal amplification, coronavirus, diagnostics

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INTRODUCTION

The SARS-CoV-2 virus belongs to the *Betacoronavirus* genus of the Coronaviridae family. The representatives of this family mainly contaminate mammals [1]. The viruses SARS-CoV and MERS-CoV, which cause atypical pneumonia and Middle East respiratory syndrome, respectively, also belong to this genus; their genomes have ~79% and ~50% homology with the SARS-CoV-2 genome, respectively [2]. The bat coronavirus RaTG13 genome demonstrates the greatest similarity (~96%) with the SARS-CoV-2 genome [3].

The SARS-CoV-2 consists of a single-strand (+) RNA containing ~29 800 nucleotides and 14 open reading frames; it encodes 27 proteins [4]. The entire cycle of virus reproduction occurs in the cytoplasm, although the location of the nucleocapsid N protein in the nucleolus was previously shown for betacoronavirus, and it was found that this protein disrupts cell division [5]. Signs of the nuclear location of the SARS-CoV-2 N protein were recently detected in the structure of this protein [6]. Interestingly, ivermectin, an antiparasitic and antiviral drug that inhibits the interrelation of viral proteins with importin α/β_1 (involved in nuclear import), has been proposed for the treatment of COVID-19 [7]. The addition of ivermectin to Vero-hSLAM cells 2 h after their contamination with SARS-CoV-2 reduced the amount of viral RNA by 5000 times as compared to the control 38 h after its introduction [8]. It is possible that this drug also affects other stages of SARS-CoV-2 interaction with the cell [9]; however, it is highly likely that some stages of the virus life cycle that are important for the successful virus reproduction are associated with the nucleus.

The virus enters the cell due to the function of the surface structural protein S (spike protein), which binds to angiotensin converting enzyme-2 (ACE-2), a peptidase located on the plasma cell membrane [3, 10]. The processing of S protein by serine proteases, membrane-associated TMPRSS2 [11], and furin, which processes proproteins, is also necessary for viral penetration of the cell [12]. It is assumed that, as with other viruses, this penetration occurs in two ways: the fusion of membranes near the cell surface and absorption via endocytosis, followed by its release from endosomes [13, 14]. These routes of entry into the cell are characteristic of not only viruses but also of a number of pro-

Abbreviations: ACE-2—angiotensin converting enzyme-2; LAMP—loop-mediated isothermal amplification; LBP—loop backward primer; LFP—loop forward primer; RT PCR—reverse-transcription polymerase chain reaction.

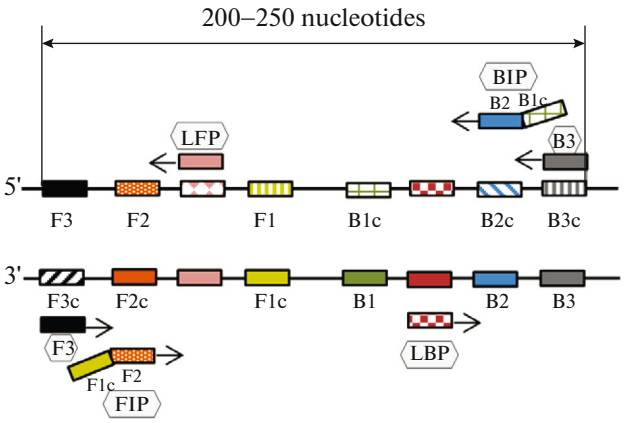

Fig. 1. Scheme of LAMP primers: F3—forward outer primer; B3—backward outer primer; FIP—forward inner primer; BIP—backward inner primer; LFP—loop forward primer, LBP—loop backward primer

tein toxins [15–17]. The highest level of ACE-2 expression was found in the lung epithelium and in the small intestine [18]. The latter is consistent with the data that ~10% of patients with COVID-19 have diarrhea, and SARS-CoV-2 was found in their feces [19]. ACE-2 is also expressed in other organs and tissues, in particular, in the nasal epithelium [20], the tong mucous membrane [21], the bronchi [22] and the central nervous system [23].

For now, a large number of test systems have been developed for the detection of SARS-CoV-2; they measure either the virus content or the amount of antibodies to this pathogen. However, the antibodies only appear by the second week of the disease; therefore, SARS-CoV-2 detection tests play a critical role in the identification of new pathogens. Most of these tests are based on reverse transcription combined with polymerase chain reaction PCR (RT-PCR); this method is at the moment the golden standard in the COVID-19 diagnostics for viral genetic material. However, other methods exist, in particular, those based on the isothermal amplification of nucleic acids, which have considerable advantages over RT-PCR in our current conditions, which require fast and massive COVID-19 diagnosis.

The most popular of these methods is loop-mediated isothermal amplification (LAMP) [24]. It is more rapid than PCR and requires a conventional thermostat instead of the expensive equipment used for amplification. *Taq*-polymerase, a PCR element, is replaced in LAMP by *Bst*-polymerase from the thermophilic bacteria *Bacillus stearothermophilus*. The latter is an enzyme that is capable of strand-displacement DNA synthesis, which renders unnecessary the DNA melting step used in PCR. The temperature maximum for the *Bst*-polymerase enzyme is ~65°C, and the rate of product formation is so high that it can already be detected within 10–20 min after the beginning of the reaction [25, 26].

Unlike PCR, which involves two primers, four primers (even six in a later modification) are involved in LAMP (Fig. 1) [25]. An increased number of primers improve the reaction specificity [24]. Let us suppose that the probability of a false positive interaction of the primer with a sequence identical to (or very similar to) the tested genomic DNA fragment is equal to p. Then, this value will become p^2, when two primers are used in PCR. Given that the primers should be in a correct order, this probability will be $p^2/2$. In a similar case with LAMP, the appearance of a false positive result requires the false functioning of six sequences, which must be arranged in the only possible order, an event with a probability of $p^6/6! = p^6/720$. Even if p is extremely large (close to 1), the difference between the p^2 and p^6 values is not high, but the resulting $p^2/2$ and $p^6/720$ values differ strongly. Thus, the probability of a false positive event is much lower with six primers instead of two.

The LAMP primers have the following structure: the outer primers F3 (forward outer primer) and B3 (backward outer primer) with a total length of 18–20 nucleotides are located at the ends of the amplicon, which usually contains ~200–250 nucleotides. The inner primers are commonly referred to as the forward inner primer (FIP) and backward inner primer (BIP). They are more complex and consist of two segments, each of which corresponds to a region of the target sequence, and these regions are 20–40 nucleotides apart from (Fig. 1).

The primers were chosen in such a way as to ensure the formation of a hairpin structure at the beginning of amplification, based on which numerous concatemers containing the primary amplicon subsequently form (for detail, see review [27]). The only function of the outer primers is the formation of the hairpin structure, and its further amplification is carried out with the involvement of FIP and BIP primers. Therefore, the content of the latter in the reaction mixture is several

times higher. As a result, the yield of the reaction products is quite high: the amount of DNA during the reaction increases by ~10^9 times [24]. The yield grew even more when the reaction was modified so that six primers were used instead of four [25]. The two new primers were called the loop forward primer (LFP) and loop backward primer (LBP). They were selected to correspond to the amplicon regions between the segment that are complementary to the FIP and BIP primers (Fig. 1). With six primers, the reaction products can already be detected within ~10 min after the start of the reaction, which is much faster than PCR [25, 26].

The selection of LAMP primers is a rather labor-consuming task. They should have the same properties as PCR primers, the most important of which is the absence of homo- and heterodimers, since these structures inhibit completely the target product formation during nontemplate amplification and provide a false positive result [28]. Typically, several sets of primers are selected and compared in order to choose the best one. The manual selection of primers is a laborious process; therefore, programs automating this task have currently been developed. The Primer Explorer software (https://primerexplorer.jp/e/) is now used more often.

A broad spectrum of DNA- and RNA-containing pathogens is detected by LAMP. In the case of RNA, LAMP is combined with reverse transcription (RT) right in the same tube (RT-LAMP). RT-LAMP has successfully been used in oncology to detect micrometastases in sentinel lymph nodes [30]. We have tested this method to detect single prostate cancer tumor cells in sentinel lymph nodes. It was shown that it is not inferior in sensitivity or specificity to existing immunochemical methods, and, at the same time, it is simpler and faster than them [31]. This allows the use of RT-LAMP for intraoperational diagnostics, which can significantly improve the quality of therapy.

To date, *Bst* polymerase (*Bst* 3.0) has been modified to combine the activities of DNA-polymerase I and reverse transcriptase. Nevertheless, both the previous *Bst* variants and the new *Bst* 3.0 are constantly used with reverse transcriptase (e.g., [26, 32, 33]). We showed a *Bst* 3.0 can be used in vitro without reverse transcriptase to identify markers of prostate cancer [34]. The reaction products in this case are detected 10 min later [34]; however, this slowdown is insignificant compared to the facilitation due to the use of fewer enzymes in LAMP.

The reaction products can be visualized in different ways. Pyrophosphate accumulates during the reaction: it is released during DNA synthesis due to the hydrolysis of nucleoside triphosphates. Pyrophosphate generates a white precipitate with the magnesium ions present in the buffer; therefore, the reaction mixture becomes turbid, which can be measured with the turbidimetric method [35, 36]. Another frequently used approach is the addition of fluorescent dyes, e.g., SYBR GREEN I, which makes it possible to plot amplification curves with a real-time PCR device. Some methods allow you to assess the reaction with the naked eye. As a rule, indicators that change color during the reaction are added to the mixture. Some indicators (e.g., cresol red or neutral red) react to pH changes during the reaction, from a set value of 8.8 at the beginning to ~6.0–6.6 [37]. Other indicators do not require special preparation of the initial reaction mixture (hydronaphtol blue or Quant-iT PicoGreen), and the change in their color depending on the reaction course can also be fixed with the naked eye [38]. When SYBR GREEN I is used, tubes are illuminated with a UV lamp to induce the contrast staining of positive samples. In this case, a UV lamp is only required, which can be replaced with a light-emitting diode [39].

CONCLUSIONS

The use of LAMP for the COVID-19 diagnostics has attracted a predictable attention amid the current pandemic. Although LAMP is more laborious at the stage of primer selection, it is much simpler in diagnosis. Primer kits have already been developed, prototypes have been created, and several test systems for SARS-CoV-2 detection by LAMP have been registered [26, 32, 40, 41], including those in Russia (http://rspp.ru/events/news/perechen-test-sistem-dlya-vyyavleniya-koronavirusnoy-infektsii). An advantage of LAMP is its low sensibility to impurities. For instance, tissue lysate and blood or respiratory secretions (1–2 μL) can be added to the reaction mixture (25 μL, as a rule) and analyzed [30, 42, 43]. This can decrease the sensitivity of the reaction as compared to the use of prepurified RNA (up to 86% in the case of SARS-CoV-2 RNA from a respiratory tract swab [43]); however, this significantly reduces the duration and simplifies analysis, which is ultimately important in mass diagnostics.

As a rule, the analytical sensitivity of the method (the minimal copy number of amplicon in a sample, which can be detected in at least 95% of all samples) is determined with artificially synthesized target DNA or RNA fragments. This characteristic is three copies per sample [49] for PCR and it is 10–50 copies per reaction for existing PCR tests for SARS-CoV-2 (test systems DNK-tekhnologii (ten copies), CerTest Biotec (20 copies), and R-Biopharm AG (50 copies)).

LAMP is comparable with PCR in terms of analytical sensitivity [32, 44–48]; however, the LAMP sensitivity strongly depends on the primer efficiency [26, 32, 48]. SARS-CoV-2 can be detected at a sensitivity of ten copies per reaction mixture (25 μL), while visual detection can reduce this sensitivity to 100 copies per reaction [50]. LAMP testing on clinical samples showed that its efficiency was comparable or slightly lower than that of PCR [32, 33].

All of the above indicates that RT-LAMP is promising in the diagnosis of COVID-19.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest.

This article does not contain any studies involving animals performed by any of the authors.

This article does not contain any studies involving human participants performed by any of the authors.

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