

## ORIGINAL RESEARCHES



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# Molecular methods for diagnosing novel coronavirus infection: comparison of loop-mediated isothermal amplification and polymerase chain reaction

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**Introduction.** Currently, the basis for molecular diagnostics of most infections is the use of reverse transcription polymerase chain reaction (RT-PCR). Technologies based on reverse transcription isothermal loop amplification (RT-LAMP) can be used as an alternative to RT-PCR for diagnostic purposes. In this study, we compared the RT-LAMP and RT-PCR methods in order to analyze both the advantages and disadvantages of the two approaches.

**Material and methods.** For the study, we used reagent kits based on RT-PCR and RT-LAMP. The biological material obtained by taking swabs from the mucous membrane of the oropharynx and nasopharynx in patients with symptoms of a new coronavirus infection was used.

**Results.** We tested 381 RNA samples of the SARS-CoV-2 virus (*Coronaviridae: Coronavirinae: Betacoronavirus; Sarbecovirus*) from various patients. The obtained values of the threshold cycle (Ct) for RT-PCR averaged  $20.0 \pm 3.7$  ( $1530 \pm 300$  s), and for RT-LAMP  $12.8 \pm 3.7$  ( $550 \pm 160$  s). Proceeding from the theoretical assumptions, a linear relationship between values obtained in two kits was proposed as a hypothesis; the correlation coefficient was approximately 0.827. At the same time, for samples with a low viral load (VL), the higher Ct values in RT-LAMP did not always correlated with those obtained in RT-PCR.

**Discussion.** We noted a significant gain in time for analysis using RT-LAMP compared to RT-PCR, which can be important in the context of testing a large number of samples. Being easy to use and boasting short turnaround time, RT-LAMP-based test systems can be used for mass screening in order to identify persons with medium and high VLs who pose the greatest threat of the spread of SARS-CoV-2, while RT-PCR-based diagnostic methods are also suitable for estimation of VL and its dynamics in patients with COVID-19.

**Key words:** *coronavirus infection; coronavirus SARS-CoV-2; polymerase chain reaction (PCR); reverse transcription loop-mediated isothermal amplification (RT-LAMP)*

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# Молекулярные методы диагностики новой коронавирусной инфекции: сравнение петлевой изотермической амплификации и полимеразной цепной реакции

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**Введение.** В настоящее время основой молекулярной диагностики большинства инфекционных заболеваний является использование полимеразной цепной реакции с обратной транскрипцией (ОТ-ПЦР; reverse transcription polymerase chain reaction, RT-PCR). Альтернативой этому методу при решении диагностических задач могут выступать технологии, основанные на петлевой изотермической амплификации с обратной транскрипцией (ОТ-ИТ; reverse transcription loop-mediated isothermal amplification, RT-LAMP). В данном исследовании нами выполнено сравнение ОТ-ИТ и ОТ-ПЦР с целью анализа как преимуществ, так и недостатков обоих подходов.

**Материал и методы.** При проведении экспериментов использованы наборы реагентов, предназначенные для анализа на основе ОТ-ПЦР и ОТ-ИТ. В работе использовался биологический материал, полученный из мазков со слизистой оболочки рото- и носоглотки у лиц с симптомами новой коронавирусной инфекции.

**Результаты.** В ходе исследования протестирован 381 образец РНК вируса SARS-CoV-2 (*Coronaviridae: Coronavirinae: Betacoronavirus; Sarbecovirus*) от различных пациентов. Полученные значения порогового числа циклов (cycle threshold, Ct) для ОТ-ПЦР составили в среднем  $20,0 \pm 3,7$  (диапазон  $1530 \pm 300$  с), для ОТ-ИТ –  $12,8 \pm 3,7$  (диапазон  $550 \pm 160$  с). Исходя из теоретических предпосылок, в качестве гипотетической была предложена линейная зависимость представленных величин; коэффициент корреляции составил  $\approx 0,827$ . При этом для проб с низкой вирусной нагрузкой (ВН) более высокие значения Ct при ОТ-ИТ не всегда соответствовали таковым в случае ОТ-ПЦР.

**Обсуждение.** Мы отметили существенное преимущество во времени при выполнении анализа с помощью ОТ-ИТ по сравнению с ОТ-ПЦР, что может быть важно в условиях тестирования большого количества образцов. Разработанные на основе методики ОТ-ИТ тест-системы в силу простоты в использовании и относительной быстроты получения результата могут быть применены в процессе массового скрининга с целью выявления лиц со средней и высокой ВН, представляющих наибольшую угрозу распространения SARS-CoV-2. В свою очередь, диагностические методы на базе ОТ-ПЦР подходят в том числе для оценки ВН и её динамики у пациентов с COVID-19.

**Ключевые слова:** *коронавирусная инфекция; коронавирус SARS-CoV-2; полимеразная цепная реакция (ПЦР); петлевая изотермическая амплификация с обратной транскрипцией (ОТ-ИТ)*

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## Introduction

The novel coronavirus infection (COVID-19) caused by the virus known as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in late 2019 in Wuhan, Hubei Province, the People's Republic of China, and then rapidly spread across the world [1, 2]. In 2.5 months, on March 11, 2020, the World Health Organization (WHO) declared the beginning of COVID-19 pandemic. Despite numerous epidemic control measures, including long quarantine periods all over the world, as of November 2021, more than 260 million confirmed cases of COVID-19, and more than 5 million deaths had been reported globally (<https://www.worldometers.info/coronavirus/>). Another serious problem aggravating the situation was the overloaded diagnostic infrastructure; as a result, the existing laboratory facilities were not always able to keep up with a large number of laboratory tests and assessments, which had to be completed within short timeframes, especially during spikes in the incidence. There was a pressing need for new, fast-to-perform and accurate test systems for detection of the COVID-19 virus and being of critical importance for the control over the infection spread among the population.

Molecular and genetic methods have a special place in the system of infectious-disease diagnostics and epidemiological surveillance, being overall notable for their high specificity/sensitivity, reproducibility, and short turnaround time. Most significantly, *in vitro* tests, which are deployed using this diagnostic approach, tend to minimize medical involvement. Solutions based on nucleic acid amplification techniques (NAATs), which include the widely used technique of polymerase chain reaction (PCR), come as better and viable options when the target pathogen belongs to the group of difficult-to-culture or non-culturable types or is present in small amounts.

Today, molecular diagnostics used for most infectious diseases, including novel coronavirus infection is based

on reverse transcription polymerase chain reaction (RT-PCR) [3, 4]. PCR reagent kits are very efficient, showing high sensitivity/specificity in detecting viral nucleic acid in samples from nasal and oropharyngeal swabs. Note that WHO and U.S. Centers for Disease Control and Prevention (CDC) approved using RT-PCR tests as gold standard tests for diagnosing COVID-19 [2, 5]. Yet, despite all their advantages underlying their immense popularity and their significant role in laboratory assays and diagnostic analytics, PCR tests have a number of drawbacks and limitations. First of all, they require expensive equipment and a relatively long time for amplification (often from 1 to 2 hrs) [6, 7], and, consequently, a longer turnaround time, considering the sample preparation. In turn, all the above adds to the existing problem of highly overloaded laboratories.

A viable alternative to using RT-PCR for solving diagnostic problems can be offered by techniques based on reverse transcription loop-mediated amplification (RT-LAMP) [8–10]. Generally, compared to PCR techniques, loop-mediated amplification does not require thermal cycling and, consequently, expensive special equipment; it is easy to perform and has shorter time to result. RT-LAMP technique requires a reverse transcriptase, thermostable DNA polymerase with displacement activity (a large fragment of *Bst* polymerase from *Geobacillus stearothermophilus* or *Bsm* polymerase from *Bacillus smithii*), and at least 4 primers. RT-LAMP is performed at a constant temperature of around 65 °C [11] and normally takes maximum 30 min, including the reverse transcription process. Furthermore, as has been pointed out in many studies, the RNA extraction, the process common to RT-PCR and RT-LAMP, can be omitted in the latter test; while slightly affecting the sensitivity, such omission can save a lot of time [12–14]. Therefore, RT-LAMP can be seen as an efficient diagnostic tool with the limit of detection ranging from 2 to 100 viral RNA copies per reaction. This method is characterized by variability depending on

sample preparation techniques, enzymes and master mixes in use, as well as detection methods [15]. Besides, the application of RT-LAMP reagent kits can offer a fast and affordable solution for increasing the laboratory testing throughput without additional equipment.

Considering a large number of articles discussing the pros and cons of both NAATs without references to publications containing direct comparison results, we compared RT-LAMP and RT-PCR tests using 2 reagent kits developed by the Central Research Institute of Epidemiology of Rospotrebnadzor during 2020–2021. The aim of our experimental study was to analyze advantages and disadvantages of both approaches. Primary attention was given to the assessment of the performance speed, convenience, ease of use, and consistency of results for each method. The estimation of exact parameters of sensitivity/specificity for the kits was beyond the scope of our study, as these parameters are specified in the related documentation.

### Material and methods

The study was conducted using biological materials from nasal and oropharyngeal swabs taken from patients having symptoms of novel coronavirus infection. The presence of SARS-CoV-2 RNA in the samples was confirmed through the RT-PCR test using an AmpliSens COVID-19-FL reagent kit (AmpliSens, Central Research Institute of Epidemiology) for detection and quantification of COVID-19 virus genetic material (Registration Certificate RZN 2021/14026). The study was conducted with the informed consent of the patients; the research protocol was approved by the Ethics Committee of the Central Research Institute of Epidemiology (Protocol No. 111 of December 22, 2020). The samples were placed in transport medium (Registration Certificate FSR 2009/05011). The RIBO-prep reagent kit (InterLabService LLC., Russia) (Registration Certificate FSR 2008/03147) was used to extract RNA from clinical samples. The study included a total of 381 samples, though preference was given to the clinical samples with the threshold cycle (Ct) not exceeding 20 (245 samples, 64% of the total number) in the RT-PCR test. Such selection preference was important as these samples were also intended for high throughput sequencing or next generation sequencing (NGS) followed by bioinformatic analysis and by uploading of the obtained data on genomic sequences into the VGARus database (<https://genome.crie.ru>). The impact of high viral load (VL) and low RNA degradation on the quality of genome reads was also taken in consideration. The NGS-produced data were used for checking primer annealing sites for any impact of the existing mutations on the amplification results. In addition, samples with RT-PCR Ct ranging from 25 to 35 were also included in the tests to assess potential deployment of RT-LAMP in analysis of samples with the medium and low VL compared to samples with high Ct values in RT-PCR.

In our tests, we used 2 reagent kits: an AmpliSens COVID-19-FL (Registration Certificate RZN 2021/14026) kit designed for RT-PCR tests and an AmpliSens SARS-CoV-2-IT (AmpliSens, Central Research Institute of Ep-

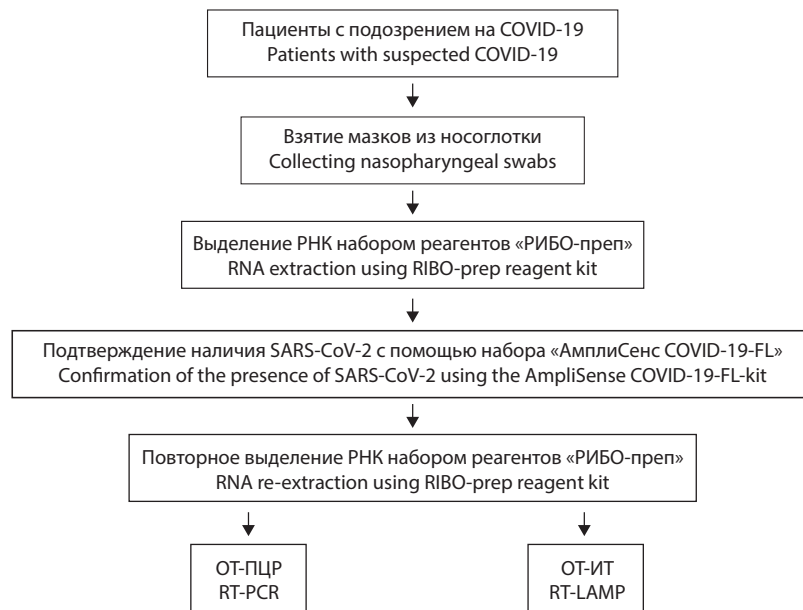
idemiology) (Registration Certificate RZN 2021/14599) kit based on the RT-LAMP technique. The SARS-CoV-2 RNA samples were tested using the above kits in accordance with the manufacturer manuals and the CFX96 Real-Time System (BioRad, United States). The obtained data were analyzed using the Bio-Rad CFX Manager v3.1 software in accordance with the manufacturer manual. **Fig. 1** presents a schematic diagram of stages of the study.

### Results

We have tested 381 SARS-CoV-2 RNA samples from different (by gender, age, type, and severity of the disease) patients with COVID-19 symptoms. The pre-test stage included thawing reagents, preparation, and placing of the reaction mix into tubes, adding RNA samples to it, and, when 96-well PCR plate was used, took  $\approx 40$  min both for RT-PCR and for RT-LAMP. The amplification with the CFX96 Real-Time System took 93 min in RT-PCR and 40 min in RT-LAMP. Besides, the time before the detection of the fluorescence signal intensity, including reverse transcription and, in RT-PCR, polymerase activation at 95 °C, was equal to 40 and 5 min for RT-PCR and RT-LAMP, respectively. The Ct values obtained after the amplification averaged  $20.0 \pm 3.7$  (the  $1,530 \pm 300$  s range) for RT-PCR and  $12.8 \pm 3.7$  (the  $550 \pm 160$  s range) for RT-LAMP; note that the specified Ct values for the AmpliSens COVID-19-FL reagent kit do not include 5 “blind” cycles. Thus, the amplification time in two techniques demonstrated the 2.7-fold difference; however, it should be remembered that samples with relatively low Ct values accounted for quite a large proportion and could affect the results.

**Fig. 2** presents Ct values for different techniques. It should be noted that the cycles differ in their duration. For RT-PCR and RT-LAMP, 1 cycle is equal to 78 sec and 43 sec, respectively, though, according to the manuals for AmpliSens COVID-19-FL and AmpliSens SARS-CoV-2-IT reagent kits, the duration of 1 cycle, net of the fluorescence signal detection time and thermal block temperature change time, is 30 sec. Proceeding from the theoretical assumptions, the linear relationship between Ct values for RT-PCR and RT-LAMP was offered as an alternative hypothesis. The correlation coefficient was  $\approx 0.827$ , being consistent with the determination coefficient  $R^2$  equal to 0.684. Thus, the offered linear regression provides satisfactory explanation of the experimental data and the relationship between RT-PCR Cts and RT-LAMP Cts for both kits.

As stated above, since these samples were analyzed through high throughput sequencing, most of the biological samples had relatively high VL. To assess the range of applicability of loop-mediated amplification in studies of samples with low VL, we also used the samples where the Ct values estimated by RT-PCR reached 35. With lower VL, the RT-LAMP results are less accurate in showing the content of viral genetic material in the sample due to the number and specificity of the primers as well as due to stochastic annealing processes and concatemers formed in the amplified region. Therefore, for samples with low VL, higher Ct values in RT-LAMP do not always cor-

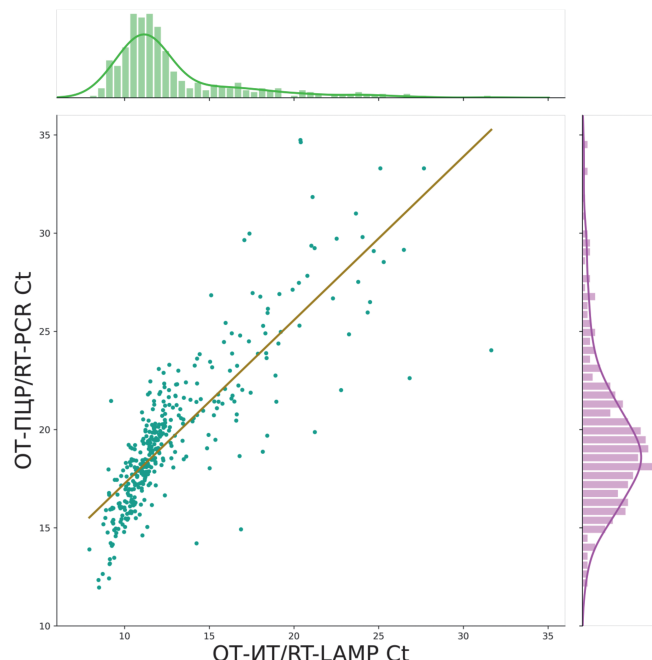


**Fig. 1.** General scheme of the study.

Initially, oropharyngeal and nasopharyngeal swabs were taken from patients with symptoms of the new coronavirus infection. The presence or absence of SARS-CoV-2 in the biological material was confirmed by RT-PCR using the AmpliSense COVID-19-FL reagent kit. Then, positive native samples with Ct values suitable for the experimental conditions were reused to isolate the virus RNA, and reverse transcription and amplification had been performed.

**Рис. 1.** Общая схема проведения исследования.

Первоначально у пациентов с симптомами новой коронавирусной инфекции забирали мазки из рото- и носоглотки. Наличие или отсутствие в биоматериале SARS-CoV-2 подтверждали методом ОТ-ПЦР с применением набора реагентов «АмплиСенс COVID-19-FL». Положительные нативные образцы со значениями Ct, удовлетворяющими условиям эксперимента, повторно использовали для выделения РНК вируса, проведения обратной транскрипции и амплификации.



**Fig. 2.** Scatter plot showing Ct values for samples analyzed by RT-LAMP and RT-PCR.

**Note.** Each sample was analyzed by two methods. The abscissa axis shows the Ct values for RT-LAMP, the ordinate axis shows the same values for RT-PCR. Histograms of Ct values distribution for LAMP and PCR (highlighted in green and purple, respectively) are also shown.

**Рис. 2.** Диаграмма рассеяния, демонстрирующая значения пороговых циклов Ct для образцов, исследованных методами ОТ-ИТ и ОТ-ПЦР.

**Примечание.** Каждый образец проанализирован двумя методами. По оси абсцисс – значения пороговых циклов Ct для ОТ-ИТ; по оси ординат – аналогичные показатели для ОТ-ПЦР. Приведены гистограммы распределения величин Ct для ЛАМР и ПЦР (выделены зелёным и лиловым цветом соответственно).

relate with the values demonstrated in RT-PCR. In addition, note that 2 samples with Ct of 31 and Ct of 35 in RT-PCR were negative in RT-LAMP. No cases when sample was positive in the RT-LAMP test, while being negative in RT-PCR, were observed.

### Discussion

During our study, we compared 2 leading NAATs – RT-PCR and RT-LAMP deployed for detection RNA of the novel coronavirus, SARS-CoV-2, using 2 reagent kits: AmpliSens COVID-19-FL (Registration Certificate RZN 2021/14026) and AmpliSens SARS-CoV-2-IT (Registration Certificate RZN 2021/1459914599). While PCR has remained the most popular method of molecular diagnostics for many years, having proved its efficiency and reliability, loop-mediated isothermal amplification has only recently found wide-scale application in diagnostic laboratories; previously, any related techniques were described mainly in scientific publications. However, the COVID-19 pandemic highlighted the pressing need for faster and more efficient methods of detection of nucleic acids of infectious pathogens, giving an incentive to deployment of non-mainstream, though promising amplification methods in diagnostic practice.

We have tested 381 samples from patients with novel coronavirus infection. The RT-LAMP test is much shorter in its turnaround time than the RT-PCR test, being highly beneficial when large amounts of biological material must be tested within a short time.

The main limitation of this study is that most of the samples were collected from the patients with high VL (low Ct values). One of the reasons is that the same samples were tested using NGS sequencing [16]. Nevertheless, we also included samples with higher (up to 35) RT-PCR Ct values in the study. On the whole, the RT-LAMP and RT-PCR results are consistent, though in 2 samples with Ct of 31 and 35 in RT-PCR, the viral RNA was not detected by the RT-LAMP test, which would mean a false-negative test result for the patient. The data obtained during our study show that RT-LAMP is a perfect tool for detection of SARS-CoV-2 in patients with medium and high VL ( $>5 \times 10^4$  copies/ml); however, its sensitivity can be insufficient in cases with VL  $<2 \times 10^3$  copies/ml (low and very low VL).

Concurrently, the unsolved question is what Ct value should be deemed significant with the positive RT-PCR result regarding the transmission of the virus from person to person. Although in long-lasting disease cases, VL values can be higher or lower, depending on the time of collection of biological material, the general tendency is that the highest threat in terms of virus transmission is posed by people with high VL. Based on the studies by different researchers, the chances for SARS-CoV-2 culturing tend to decrease for biomaterial samples where RT-PCR Ct values exceed 25 [17]. It has been found that the likelihood of successful culturing of the above virus can decrease to 8% at RT-PCR Ct  $>35$  [18]. There are published data confirming that the novel coronavirus can be potentially cultured from 70% of samples with Ct

$\leq 25$  (RT-PCR), while only  $<3\%$  samples can be used for successful culturing when Ct  $>35$  (RT-PCR) [19]. Other research groups reported that this virus could not be cultured in samples with Ct  $>24$  (RT-PCR) [20]. In addition, it has been found that RT-PCR tests can detect SARS-CoV-2 in patients during several weeks after the symptoms disappeared and there is no risk of virus transmission to another person [20].

Summarizing the above, we can conclude that RT-LAMP testing systems make it possible to detect novel coronavirus RNAs in biological samples from patients who can transmit the infectious agent to other people, though, in rare cases, they can produce false-negative results for convalescents having the “residual” VL. Another limitation of LAMP techniques is their need for thorough optimization of a number of parameters applicable to the reaction mix and their lack of quantification, as the amplification of concatemers is not identical to doubling of products during each cycle of amplification in the PCR test [21]. However, these limitations are outweighed by multiple advantages, first of all, the high speed of the analytical process and a wide range of detection methods for amplification products. The molecular LAMP diagnostics gains increasing popularity as a cost-effective tool for detection of novel coronavirus infection and for its epidemiological surveillance [22, 23]. Besides, point-of-care RT-LAMP tests for SARS-CoV-2 are currently developed for deployment in remote regions [23, 24]. Based on the above, it is obvious that isothermal amplification-based diagnostic systems need further studies with larger numbers of samples and better represented samples with low VL.

### Conclusion

The results obtained during our study lead to conclusion that loop-mediated isothermal amplification techniques can be used in laboratory diagnostics for detection of SARS-CoV-2 RNAs in clinical samples. Being easy to use and boasting short turnaround time, RT-LAMP testing systems can be used for mass screening to identify people who have relatively high VL and pose the highest threat of novel coronavirus transmission and spread, while RT-PCR-based diagnostic methods are more suitable for estimation of VL and its dynamics in patients with COVID-19, especially in cases with relatively low VL. With the need for testing systems for timely detection of SARS-CoV-2 remaining high, LAMP techniques expand the scope of molecular diagnostics, and, when combined with the PCR test and tests for detection of the respective antigen, add flexibility and efficiency to the epidemiological surveillance of the novel coronavirus spread.

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