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Research Article

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Design of primers and probes for detection of influenza A and SARS-CoV-2 viruses RNA by real-time RT-PCR

Cost-effective, accurate, and rapid analysis is essential for testing and diagnosing both common and emerging viruses in clinical virology laboratories. In this study, we designed and selected oligonucleotides for the detection of influenza A virus and SARS-CoV-2 using real-time reverse transcription PCR (RT-qPCR). The development of domestic test systems for diagnosing influenza A virus and SARS-CoV-2 is an urgent task due to the need for early disease detection. The aim of this study is to select primers and probes for the diagnosis of influenza A and SARS-CoV-2 using reverse transcription PCR in real-time (RT-PCR RT). We present the results of designing primers and probes for the identification of influenza A and SARS-CoV-2 RNA. In our studies on the selection of specific primers and probes, the M gene was chosen as a target gene for detecting the influenza A virus, and the RdRp gene for the SARS-CoV-2 virus. A pair of oligonucleotide primers was selected and synthesized for influenza A InfM2 F and InfM2 R, as well as the InfM2 Probe, and for SARS-CoV-2 — RdRp-1 F and RdRp-2 R, the RdRp-2 Probe, which, when performing RT-PCR RT with a working concentration of 20 pmol showed high efficiency in detecting the influenza A virus and SARS-CoV-2. Primers were selected using the Primer Blast and Vector NTI computer programs. The designed primers and probes will be further used to create a domestic multiplex RT-PCR RT test system.

Keywords: influenza A, coronavirus, RT-PCR, diagnostics, test-system.

Introduction

Acute respiratory infections (ARIs) are a broad group of acute infectious diseases caused by various pathogens, such as viruses, bacteria, chlamydia, and mycoplasma [1]. ARIs pose a serious threat to humanity, directly affecting the daily lives of millions of people and negatively impacting the global economy [2, 3, 4]. Currently, respiratory infections account for up to 90 % of all infectious diseases [5]. Each year, over 1 billion cases of acute respiratory infections are recorded worldwide, which significantly exceeds the number of patients with serious diseases such as cancer, HIV, coronary heart disease, or malaria [1].

Influenza occupies a significant place among acute respiratory infections, remaining one of the most significant viral infections. Approximately 1 billion cases of seasonal influenza are registered annually, of which 3 to 5 million are severe. Respiratory diseases caused by influenza viruses kill between 290 000 and 650 000 people annually [6].

There are four types of seasonal influenza viruses: types A, B, C and D. Influenza A and B viruses circulate and cause seasonal epidemics. Influenza A viruses are divided into subtypes based on the protein combinations on the virus surface. Currently, influenza viruses of subtypes A(H1N1) and A(H3N2) circulate among humans. A(H1N1) is also referred to as A(H1N1)pdm09 because it caused the 2009 pandemic and replaced the seasonal influenza A(H1N1) virus that circulated before 2009. Only influenza type A viruses are known to cause pandemics.

Recently, the emergence and rapid spread of a novel coronavirus (SARS-CoV-2), which has become a significant acute respiratory disease, has raised serious concerns about global health [7]. Since its discovery, SARS-CoV-2 has rapidly spread to more than 230 countries. As of January 2025, more than 777 million confirmed cases of infection have been recorded worldwide, resulting in 7 079 925 deaths [8].

The clinical manifestations of infection caused by SARS-CoV-2 are similar to those that occur with influenza virus infection [9]. Highly effective and sensitive diagnostic tests are needed to accurately distinguish influenza viruses from SARS-CoV-2 [10]. Differential diagnosis of SARS-CoV-2 and influenza viruses is essential for the development of effective public health strategies and patient treatment. This is especially relevant for identifying suspected cases, severe forms of the disease, and assessing potential outbreak threats. Integrating influenza testing into existing COVID-19 assays significantly reduces the time required for diagnosis and improves the use of existing equipment, resources and personnel. This approach is cost-effective and contributes to a more effective fight against COVID-19 and influenza [11].

Research into the development and application of rapid diagnostic methods for influenza A and SARS-CoV-2 viruses based on real-time RT-PCR is ongoing and is being conducted in many countries worldwide [12–15]. The development of a domestic test-system for the diagnosis of influenza A and SARS-CoV-2 viruses is driven by the need for early detection. The advantages of real-time RT-PCR test-systems include their high sensitivity and specificity. This is achieved through the use of species-specific primer sets and fluorescent probes, which, combined with specially selected reaction conditions, enable the detection of trace amounts of microbial RNA molecules in the analyzed sample.

Influenza A virus is one of the most widespread and variable viruses causing epidemics and pandemics. The viral genome consists of eight segments of negative-sense single-stranded RNA. Direct detection of viral RNA avoids errors associated with antigenic variability and cross-reactions in immunodiagnostic tests [16, 17]. When developing a test-system, it is important to carefully select primers and probes to ensure maximum specificity and sensitivity. The most commonly used primer targets are the NP and M genes, which are highly conserved among influenza A viruses [18].

Coronaviruses (*Coronaviridae*) are a group of viruses with the largest genomes among RNA viruses. Their genetic structure includes four main structural proteins: S, M, E, and N, which play a key role in disease pathogenesis and are the primary targets for diagnostics [19]. The S protein, responsible for virus binding to host cells, has received the most attention, making it a promising target for both diagnostics and therapeutic drug development [17].

Comparative analysis of the nucleotide sequences of viral genomes is conducted to design specific primers. Conserved regions of viral genomes become targets in the development of diagnostic molecular genetic tests [20]. The selection of specific primers and probes is a key step in the development of PCR-based diagnostic tests.

The aim of this study is to select primers and probes for real-time RT-PCR for the detection of influenza A and SARS-CoV-2 virus RNA.

Experimental

The SARS-CoV-2/KZ Almaty/04.2020 coronavirus strain and the A/Almaty/5/98(H1N1) influenza virus strain were used in this experiment.

Viral RNA was isolated under BSL-2 laboratory conditions using the innuPREP Virus DNA/RNA Kit according to the manufacturer's instructions. The quality and concentration of the obtained viral RNA were verified using a Nano Drop 2000 spectrophotometer.

Nucleotide sequences for selecting specific primers were searched in the NCBI GenBank international database (http://www.ncbi.nlm.nih.gov/GenBank).

Primers and probes were constructed using programs MEGA v.10, BLAST and Invitrogen Vector NTI for diagnostics of influenza A and SARS-CoV-2. The synthesis of oligonucleotide primers was carried out on an automatic synthesizer from K&A Laborgeraete, model DNA/RNA Synthesizer H-16 (made in Germany) using the phosphoramidite method according to the instructions supplied with the device.

Reverse transcription PCR and Real-Time Reverse transcription PCR

Reverse transcription PCR (RT-PCR) was performed using a ProFlex™ PCR System gradient thermal cycler, Applied Biosystems. Real-Time Reverse transcription PCR (Real-time RT-PCR) was performed on a Rotor-Gene Q thermal cycler, Qiagen.

RT-PCR amplification results were analyzed by electrophoresis at 400 mA in a 1.5 % agarose gel supplemented with SYBR Safe DNA gel stain, Invitrogen. The 100 bp DNA Ladder length marker, NEB, was used to estimate fragment length. Real-time RT-PCR results were detected and analyzed using Rotor-Gene Q software, version 1.8.187.5.

Results and Discussion

Numerous international studies are devoted to the development of highly specific and sensitive primers and probes for the detection of influenza A and SARS-CoV-2 viruses using real-time RT-PCR. Selecting a target gene is a key step in developing a diagnostic test, as it affects its analytical sensitivity and specificity.

For influenza A virus, the optimal target is the highly conserved matrix (M) gene, as confirmed by international studies, including the work of Spackman et al. (2002) and subsequent WHO protocols [21, 22]. For SARS-CoV-2, international recommendations indicate the RdRp gene as the most specific confirmatory target, and the E gene as a screening target, according to the protocol of Corman et al. (2020) [23].

In this study, the M gene for influenza A virus and the RdRp gene for SARS-CoV-2 were selected as target genes. This choice is consistent with the results of studies by Vogels et al. (2020), indicating high diagnostic sensitivity of the RdRp target [24]. Nucleotide sequences of the analyzed genes were retrieved from the GenBank database, taking into account the geographic and temporal diversity of the isolates. The list of GenBank IDs used is provided in Table 1.

Table 1

GenBank-registered influenza A virus and coronavirus isolates used for multiple nucleotide alignment

Name of the virus	**Isolate identification numbers in the GenBank database**
Influenza A virus (M gene)	EF541447.1, AJ410572.1, DQ021776.2, HM144894.1, AB299810.2, MG280269.1, JX673922.1, GU051365.1, KU679934.1, KJ781226.1, MG976713.1, KY644319.1, KT932366.1, KP414902.1, KC881293.1, GU182162.1, EF593105.1, DQ064385.1, MN530740.1, MK237090.1, KY130988.1, GU186693.1, GQ257419.1, GQ257456.1, AB916670.1, EU124190.1, DQ320993.1, MN253808.1, MG366527.1, MH932132.1, MH134599.1, KJ907546.1, KY644227.1, KX215215.1, KU289771.1, KP336340.1, KP336329.1, KP417011.1, GU083624.1, HM144813.1, CY178327.1, CY178031.1, CY178015.1, CY177927.1, CY177895.1
SARS-CoV-2	MT192765.1, MT159721.1 MT159714.1, MT159713.1 MT159710.1, MT039873.1, MN996528.1, MN988668.1, MT192772.1, MT184912.1, MT159709.1, MT159707.1, MT118835.1, MT019533.1, MT159722.1, MT159718.1, MT121215.1, MT123290.1, MT066175.1, MN996530.1, MT066156.1, MT093631.2, LC529905.1, MT159720.1, MT027062.1, MN996529.1, MT123291.2, MT135041.1, MN996531.1, MT184908.1, LC528232.1, LR757996.1, MT039887.1, MT123293.2, LR757995.1, MN988713.1, MT163719.1, MT019530.1, MT192759.1, MT106054.1, MT072688.1, MT184911.1, LR757998.1, MT050493.1, MT012098.1, MN996527.1, MN938384.1, MT188340.1, MT188339.1, MT044258.1.
SARS-CoV	AY395003.1, AY394996.1, AY304488.1, AY304486.1, AY394985.1, EU371559.1, AY394994.1, JX163927.1, JX163923.1, JQ316196.1, AY559096.1, AY274119.3, AY394999.1, AY394987.1, AY282752.2, JX163925.1, JX163924.1, EU371560.1, AY395002.1, AY357076.1, GU553365.1, AY338175.1, FJ429166.1, AY461660.1, AY568539.1, HQ890541.1, AY772062.1, FJ882953.1, FJ882962.1
Bat coronavirus	MN996532.1, MG772933.1, MG772934.1, KF367457.1, MK211378.1, MK211375.1, KY417151.1, JX993988.1, KJ473813., KJ473811.1, MK211376.1, KY417146.1, KY417145.1, KY417152.1

Multiple sequence alignment was performed using MEGA v.10. This analysis allowed us to identify conserved regions of the genome used in the design of primers and fluorescent probes (Figs 1-2).

Figure 1. Results of a multiple alignment of the nucleotide sequences of the influenza A virus M gene, performed in MEGA v.10.

Color coding reflects nucleotide type:
adenine (A) — green, thymine (T) — red, guanine (G) — purple, cytosine (C) — blue

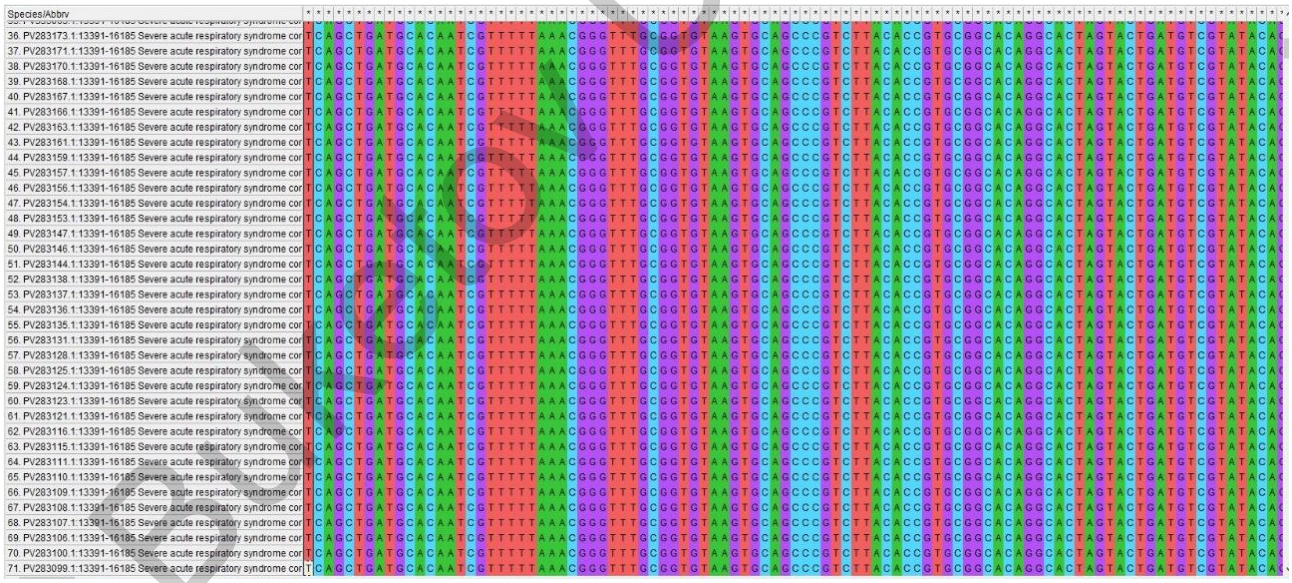
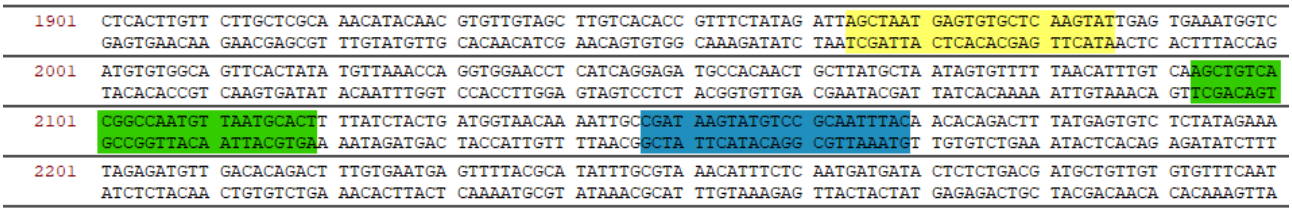


Figure 2. Results of a multiple alignment of the nucleotide sequences of the SARS-CoV-2 virus genomes, performed in MEGA v.10.

Color coding reflects nucleotide type:
adenine (A) — green, thymine (T) — red, guanine (G) — purple, cytosine (C) — blue

For each target, a primer pair and probe with the best specificity based on BLAST analysis were selected. For influenza A, primers InfM2 F/InfM2 R (153 bp amplicon) were used, and for SARS-CoV-2 RdRp-1 F/RdRp-2 R (205 bp amplicon) were used. Conserved regions selected for primer and probe attachment are shown in Figures 3-4. The parameters of the synthesized oligonucleotides are presented in Table 2.

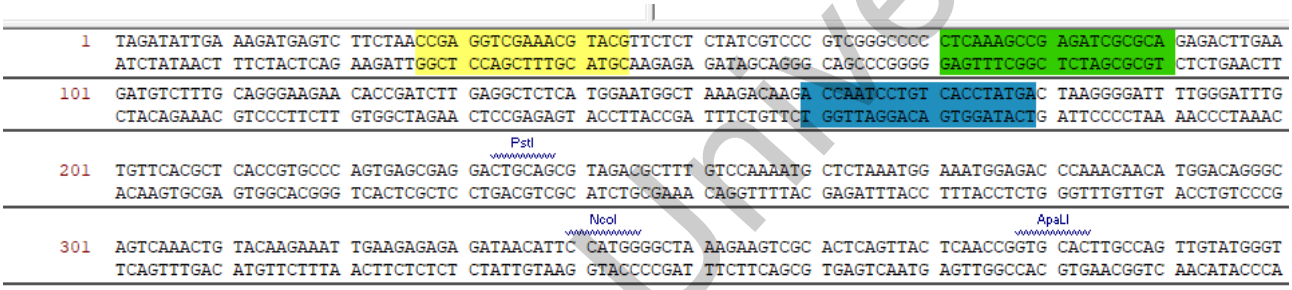


Forward primer (RdRp-1 F)
Probe (RdRp-2 Probe)
Reverse primer (RdRp-2 R)

Figure 3. Selection of sites for primers and a probe for the RdRp gene of the SARS-CoV-2

Figure 3 shows a fragment of the nucleotide sequence of the SARS-CoV-2 RdRp gene used to select oligonucleotide primers and a fluorescent probe for the detection of SARS-CoV-2 RNA by real-time RT-PCR.

The highlighted region was selected based on conservation analysis across different SARS-CoV-2 strains (GISAID and GenBank data), taking into account minimal variability and the absence of mutations at the primer and probe binding sites. The amplicon length is 205 base pairs, which meets the requirements for real-time RT-PCR. The probe is labeled with the ROX fluorophore and the BHQ-2 quencher.



Forward primer (InfM2 F)
Probe (InfM2 Probe)
Reverse primer (InfM2 R)

Figure 4. Selection of sites for primers and a probe for the M gene of the influenza A virus

Figure 4 shows a fragment of the nucleotide sequence of the M gene of the influenza A virus, one of the most conserved fragments of the genome, selected as the target for amplification.

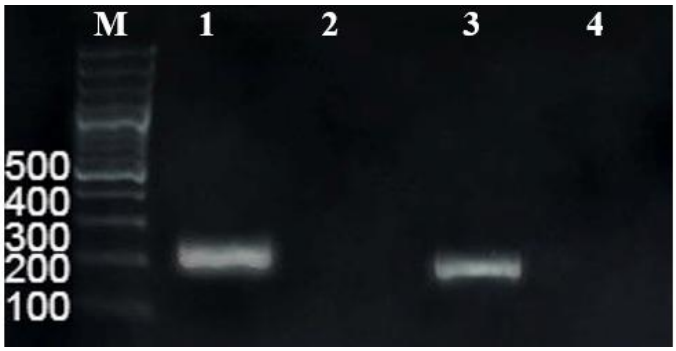
The designed primers and probes were tested for specificity using the BLAST program (https://blast.ncbi.nlm.nih.gov). Based on these studies, primers were designed; their parameters are listed in Table 2. FAM, ROX, and quenchers BHQ-1 and BHQ-2 were used as fluorescent dyes.

Table 2

Parameters of designed primers and probes

Name of primers and probe	Sequence 5' — 3'	Tm	GC, %	Size of product
InfM2 F	ACCGAGGTCGAAACGTAYGT	58	55	153 bp
InfM2 Probe	FAM- CTCAAAGCCGAGATMGCGCAG-BHQ-1	65	62	
InfM2 R	TCAGAGGTGACARGATTGGTC	59	52	
RdRp-1 F	AGCTAATGAGTGTGCTCAAGTAT	59	40	205 bp
RdRp-2 Probe	ROX-AGCTGTCACGGCCAATGTTAATGCACT-BHQ-2	68	48	
RdRp-2 R	GTAAATTGCGGACATACTTATCG	59	40	

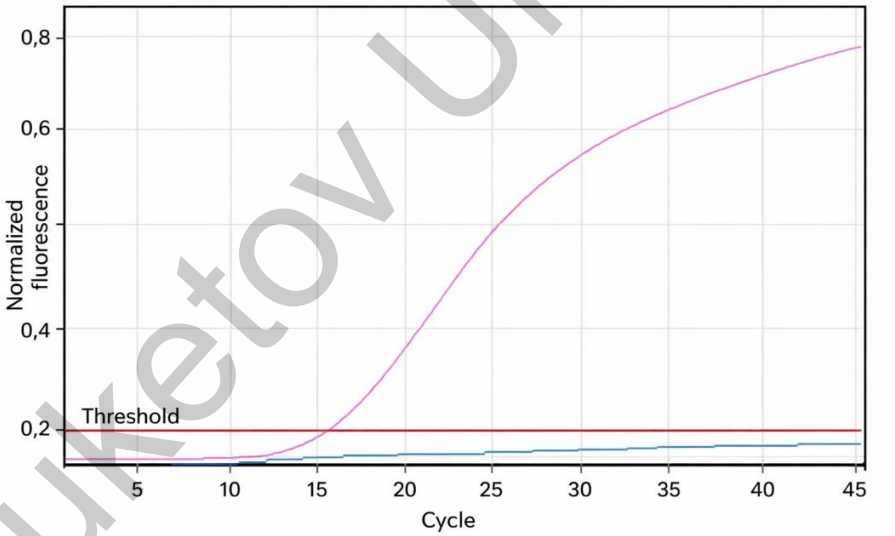
Based on the results of research, the performance of the primers and probes we developed for detecting influenza A virus and SARS-CoV-2 coronavirus RNA was tested using conventional PCR. The results are presented in Figure 5.



M — 100 bp DNA Ladder, NEB, 1 — positive control — plasmid DNA with an inserted fragment of the RdRp gene of the SARS-CoV-2 coronavirus, 2 — negative control, 3 — positive control–plasmid DNA with an inserted fragment of the M gene of the influenza A virus, 4 — negative control

Figure 5. Electropherogram of amplification products using primers RdRp-1 F and RdRp-2 R, InfM2 F and InfM2 R

The efficiency and specificity of the primers were further assessed using real-time PCR. Positive controls for influenza A and SARS-CoV-2 demonstrated early crossing of the fluorescence threshold (Ct = 15,75 and Ct = 18,70, respectively), while negative controls showed no signal (Figs 6-7, Tables 3-4). This confirms high specificity and the absence of nonspecific amplification.



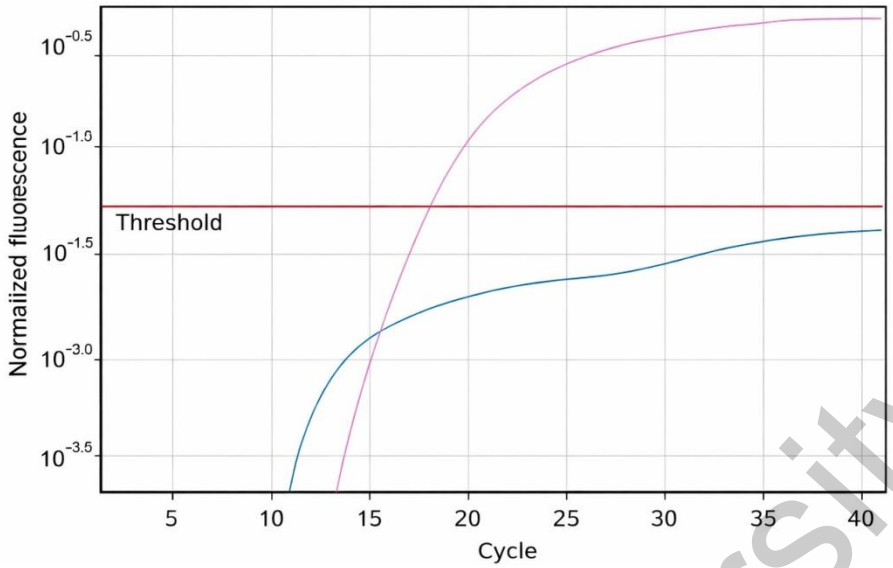
No. 1 — positive control — plasmid DNA with an inserted fragment of the M gene of the influenza a virus;
No.2 –NC — negative control– deionized water

Figure 6. Results of real-time PCR analysis of primers and probe for detection of influenza A virus RNA

Table 3

Quantitative RT-PCR data in the Green channel

No.	Name	Type	CT	Avg. Ct
1	Influenza A	Positive control	15,75	15,75
2	NC	Negative control		



No. 1 — positive control — plasmid DNA with an inserted fragment of the RdRp gene of the SARS-CoV-2 coronavirus; No.2 — NC — negative control — deionized water

Figure 7. Results of real-time PCR analysis of primers and probe for detection of SARS-CoV-2 coronavirus RNA

Table 4

Quantitative RT-PCR data in the Orange channel

No.	Name	Type	CT	Avg. Ct
1	SARS-CoV-2	Positive control	18,70	18,70
2	NC	Negative control		

The RT-PCR results in Tables 2 and 3 show that the amplification curve for the influenza A virus positive control crossed the established fluorescence threshold as early as cycle 15,75 (Ct=15,75) during RT-PCR, and for the SARS-CoV-2 virus positive control, it crossed the established fluorescence threshold as early as cycle 18,70 (Ct=18,70), whereas no amplification occurred in the negative controls (no Ct) (Figs 6-7, Tables 3-4). The early appearance of a signal (low Ct) indicates a high initial target concentration and efficient target doubling in each PCR cycle. The absence of a signal in the negative control means that the reaction lacked the target nucleic acid and no non-specific amplification was observed; this confirms the high specificity of the primers and probes used. Thus, the experimental data demonstrate that the presence of positive controls of influenza A and SARS-CoV-2 viruses leads to an increase in fluorescence.

The presented data demonstrate the suitability of the selected primers and probes for further use in diagnostic PCR test-systems. High reaction efficiency (early Ct in the positive control) and the complete absence of amplification in the negative control indicate a reliable combination of efficacy and specificity of the selected oligonucleotides [25]. Thus, the developed primers and probes ensure selective and efficient detection of influenza A and SARS-CoV-2 viruses and can be recommended for integration into diagnostic test-systems using real-time PCR.

Conclusion

During the study, optimal primer pairs and fluorescent probes were selected for the detection of influenza A and SARS-CoV-2 viruses using real-time RT-PCR. Highly conserved regions were chosen as targets: the M gene of influenza A virus and the RdRp gene of SARS-CoV-2 virus, ensuring high sensitivity, specificity, and resistance to viral genome variability.

The developed oligonucleotide set provides the basis for the creation of reliable domestically produced diagnostic test-systems adapted to the epidemiological conditions of the Republic of Kazakhstan. Use of the-

se test-systems will improve the availability and efficiency of molecular diagnostics for acute respiratory infections, as well as strengthen epidemiological surveillance during the seasonal peak in cases.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. **M.D. Almezhanova, A.M. Melisbek, M.Zh. Shirinbekov —** investigation, **N.S. Kozhabergenov, A.T. Junushov —** discussion of research results, **O.V. Chervyakova, K.T. Sultankulova —** conceptualization and management of work.

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Нақты уақыттағы КТ-ПТР әдісі арқылы А тұмауы мен SARS-CoV-2 вирустарының РНҚ-ын анықтауға арналған праймерлер мен зондтарды таңдау

Клиникалық вирусология зертханаларында кең таралған және жаңа вирустарды сынау және диагностикалау үшін үнемді, нақты және жылдам талдау қажет. КТ-ПТР талдауын қолдана отырып, А типті тұмауы вирусын және SARS-CoV-2 коронавирусын анықтау үшін олигонуклеотидтер таңдалған және құрастырылған. А тұмауы вирусы мен SARS-CoV-2 диагностикасы үшін отандық сынақ-жүйелерін құру маңызды міндет және ауруларды ерте диагностикалау қажеттілігіне байланысты. Зерттеудің мақсаты нақты уақыттағы КТ-ПТР (НУ КТ-ПТР) көмегімен А тұмауы және SARS-CoV-2 диагностикасына арналған праймерлер мен зондтарды таңдау. Мақалада А тұмауы және SARS-CoV-2 вирустарының РНҚ сәйкестендіруге арналған НУ КТ-ПТР үшін праймерлер мен зондтарды жобалау және синтездеу нәтижелері ұсынылған. Арнайы праймерлер мен зондтарды таңдау бойынша зерттеулерімізде А тұмауы вирусын анықтау үшін мақсатты ген ретінде М гені және SARS-CoV-2 вирусы үшін RdRp гені таңдалды. А тұмауы үшін InfM2 F және InfM2 R праймерлері мен InfM2 зонды, ал SARS-CoV-2 үшін RdRp-1 F және RdRp-2 R праймерлері мен RdRp-2 зонды жұпты олигонуклеотидтері таңдалып синтезделді. Таңдалған жұптық олигонуклеотидтер НУ КТ-ПТР көмегімен А тұмауы вирусын және SARS-CoV-2 анықтау кезінде 20 пмоль жұмыс концентрациясында жоғары тиімділігін көрсетті. Праймерлерді таңдау Primer Blast және Vector NTI компьютерлік бағдарламаларын қолдану арқылы жүзеге асырылды. Құрастырылған праймерлер мен зондтар отандық мультиплексті НУ КТ-ПТР сынақ-жүйесін құру кезінде қолданылады.

Кілт сөздер: А тұмауы, коронавирус, КТ-ПТР, диагностика, сынақ-жүйе

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Подбор праймеров и зондов для выявления РНК вирусов гриппа А и SARS-CoV-2 методом ОТ-ПЦР в реальном времени

Для тестирования и диагностики обычных и новых вирусов в клинических вирусологических лабораториях требуется экономичный, точный и быстрый анализ. Нами подобраны и сконструированы олигонуклеотиды для выявления вируса гриппа типа А и коронавируса SARS-CoV-2 с применением ОТ-ПЦР-анализа. Создание отечественных тест-систем для диагностики вируса гриппа А и SARS-CoV-2

является острой задачей и обусловлено необходимостью ранней диагностики заболеваний. Целью данного исследования является подбор праймеров и зондов для диагностики гриппа А и SARS-CoV-2 методом ОТ-ПЦР в реальном времени (ОТ-ПЦР РВ). В работе представлены результаты конструирования праймеров и зондов для постановки ОТ-ПЦР РВ для идентификации РНК вирусов гриппа А и SARS-CoV-2. В наших исследованиях по подбору специфических праймеров и зондов в качестве гена-мишени для обнаружения вируса гриппа А был выбран М-ген, вируса SARS-CoV-2 — RdRp. Были подобраны и синтезированы пары олигонуклеотидных праймеров для гриппа А: InfM2 F и InfM2 R, а также зонд InfM2 Probe, а для SARS-CoV-2 — RdRp-1 F и RdRp-2 R, зонд RdRp-2 Probe, которые при постановке ОТ-ПЦР РВ с рабочей концентрацией 20 пмоль показали высокую эффективность при выявлении вируса гриппа А и SARS-CoV-2. Подбор праймеров проведен с использованием компьютерных программ PrimerBlast и VectorNTI. Конструированные праймеры и зонды в дальнейшем будут использованы при создании отечественной мультиплексной ОТ-ПЦР РВ тест-системы.

Ключевые слова: грипп А, коронавирус, ОТ-ПЦР, диагностика, тест-система

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