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COMPARATIVE ANALYSIS OF *MXA*, *OAS1*, *PKR* GENE EXPRESSION LEVELS IN LEUKOCYTES OF PATIENTS WITH INFLUENZA AND CORONAVIRUS INFECTION

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BACKGROUND: The innate immune response, particularly the interferon system, plays a crucial role in defending the host against viral pathogens. Interferon signaling induces the expression of specific antiviral proteins known as interferon-stimulated genes, which inhibit viral replication through various mechanisms.

AIM: This study aimed to develop a quantitative PCR system to assess the molecular regulation of human interferon-stimulated genes *MxA*, *OAS1*, and *PKR*, and to determine their expression in blood leukocytes in response to RNA-containing viruses.

MATERIALS AND METHODS: Leukocytes were isolated from patients with laboratory-confirmed influenza and COVID-19 infections 3–4 days after symptom onset. *Ex vivo* viral infection was induced using influenza viruses A/California/07/09pdm (H1N1pdm09), B/Malaysia/2506/04 (Vic), strain A2 respiratory syncytial virus, and SARS-CoV-2 HCoV-19/Russia/SPE-RII-3524V/2020.

RESULTS: A multiplex qPCR assay was developed for analyzing human *MxA*, *OAS1*, and *PKR* gene expression, with high amplification efficiency. The test system was used to study the molecular regulation of these genes in leukocytes in influenza and COVID-19 patients. The expression levels of *MxA*, *OAS1*, and *PKR* genes were significantly increased in blood leukocytes of hospitalized patients 3–4 days after symptom onset. Stimulation of leukocytes by influenza A, influenza B, and respiratory syncytial virus led to increased mRNA levels of these genes, while stimulation by SARS-CoV-2 did not result in changes in gene expression.

CONCLUSIONS: The multiplex test system can be used to characterize the expression of antiviral effector interferon-stimulated genes, aiding in the study of virus evasion from the innate immune response.

Keywords: respiratory viruses; multiplex PCR; influenza; SARS-CoV-2; leukocytes; immune response; interferon-stimulated genes, *MxA*, *OAS1*, *PKR*, antiviral effect.

СРАВНИТЕЛЬНОЕ ОПРЕДЕЛЕНИЕ УРОВНЯ ЭКСПРЕССИИ ГЕНОВ *MXA*, *OAS1* И *PKR* В ЛЕЙКОЦИТАХ ПАЦИЕНТОВ С ГРИППОЗНОЙ И КОРОНАВИРУСНОЙ ИНФЕКЦИЕЙ

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Обоснование. Первой линией защиты хозяина против инвазии вирусных патогенов служит врожденный иммунный ответ, наиважнейшее звено которого — система интерферонов. Передача сигналов интерфероном индуцирует в клетке-мишени экспрессию широкого спектра специфических противовирусных белков, так называемых интерферон-стимулируемых генов. Наиболее эффективные интерферон-стимулируемые гены напрямую ингибируют репликацию вирусов РНК-центричным образом посредством деградации вирусной РНК, нарушения ее транспорта, ингибирования вирусной трансляции и т. п.

Abbreviations

ISGs, interferon-stimulated genes; PCR, polymerase chain reaction; IVA, influenza virus A; IVB, influenza virus B; RSV, respiratory syncytial virus.

Цель — разработка количественной тест-системы на основе полимеразной цепной реакции для оценки молекулярной регуляции интерферон-стимулируемых генов *MxA*, *OAS1* и *PKR* человека, а также определение экспрессии этих генов в лейкоцитах крови в ответ на инфицирование РНК-содержащими вирусами.

Материалы и методы. Исследование проводили с использованием лейкоцитов, выделенных из крови пациентов с лабораторно подтвержденными заболеваниями грипп и COVID-19 на 3–4-й день после манифестации болезни. Для *ex vivo* индукции вирусного инфицирования использовали вирусы гриппа A/California/07/09pdm (H1N1pdm09), B/Malaysia/2506/04 (Викторианской генетической линии), штамм A2 респираторно-синцитиального вируса и вирус SARS-CoV-2 hCoV-19/Russia/SPE-RII-3524V/2020.

Результаты. Разработана мультиплексная тест-система на основе полимеразной цепной реакции для оценки экспрессии генов *MxA*, *OAS1* и *PKR*, эффективность амплификации которых составила 101,9, 92,5 и 101,5 % соответственно. Разработанная тест-система была предложена для исследования молекулярной регуляции *MxA*, *OAS1* и *PKR* в лейкоцитах при социально значимых заболеваниях, таких как грипп и COVID-19. Согласно полученным нами результатам у госпитализированных пациентов на 3–4-й день после появления симптомов заболевания уровни экспрессии генов *MxA*, *OAS1* и *PKR* были значимо повышены на системном уровне в лейкоцитах крови. Показано, что стимуляция лейкоцитов, выделенных от здоровых волонтеров, вирусами гриппа A, B и респираторно-синцитиальным вирусом приводила к значимому увеличению уровней мРНК генов *MxA*, *OAS1* и *PKR* через 24 ч после инфицирования. В то же время стимуляция лейкоцитов вирусом SARS-CoV-2 не приводила к изменению экспрессии этих генов.

Заключение. Разработанную тест-систему можно использовать для характеристики экспрессии противовирусных эффекторных интерферон-стимулируемых генов, что потенциально поможет в изучении эволюционно выбранных механизмов ускользания вирусов от врожденного иммунного ответа.

Ключевые слова: респираторные вирусы; мультиплексная полимеразная цепная реакция; грипп; SARS-CoV-2; лейкоциты; иммунный ответ; интерферон-стимулируемые гены; гены *MxA*, *OAS1*, *PKR*; противовирусное действие.

Background

Innate immune response is the host's first line of defense against viral pathogen invasion, the most important link of which is the interferon system. Interferon signaling induces the expression of a wide range of specific antiviral proteins, called interferon-stimulated genes (ISGs), in the target cell.

The most effective ISGs directly inhibit viral replication in an RNA-centric manner by degrading viral RNA, disrupting its transport, inhibiting viral translation, etc. [1].

Various recent publications have provided new insights on the diversity and complexity of the mechanisms by which different ISGs inhibit viruses with RNA genome [2].

The present study was conducted to design and create a multiplex polymerase chain reaction (PCR) to assess the expression of ISGs such as double-stranded RNA-dependent protein kinase R (PKR), 2′-5′-oligoadenylate synthetase (OAS1), and myxovirus resistance protein (MxA) in human cells.

MxA expression is controlled by type I and III interferons. MxA exhibits a direct antiviral effect against various viruses [3], binding directly to the viral ribonucleoprotein and blocking the nuclear import of viral RNAs. PKR and OAS1 (in addition to RIG-1 and MDA5) are sensors of foreign double-stranded RNA (dsRNA). Binding to dsRNA activates OAS1, which causes 2′-5′-oligoadenylate synthesis, which then activates RNase L involved in the direct cleavage of cytoplasmic RNAs [4]. Similarly, interaction with dsRNA (or other polyanions) leads to PKR dimerization and activation. Activated PKR suppresses translation initiation through eukaryotic initiation factor 2 (EIF2AK2) phosphorylation [5] and acts as a signal transducer for pro-inflammatory gene expression [6].

Some viruses can manipulate the expression of antiviral ISGs, suppressing the cellular innate immune response. For example, suppression of *PKR*, *OAS1*, and *Mx2* expression by the porcine epidemic diarrhea virus (family *Coronaviridae*) in the early stages of reproduction causes an intense inflammatory response [7]. The presence of some polymorphisms in the *PKR, OAS1, and MxA* genes is associated with the progression and course of HIV infection [8] and hepatitis C [9].

Hence, the identification and characterization of direct antiviral effector ISGs can reveal evolutionarily selected pathogen defense mechanisms that can be imitated or manipulated to generate novel treatment methods.

This study aimed to develop a quantitative PCR system to assess the molecular regulation of ISGs of human *MxA*, *OAS1*, and *PKR* and determine the expression of these genes in blood leukocytes in response to RNA virus infection.

Materials and methods

The study involved 14 healthy donors, 14 patients with influenza A/H3N2 (epidemic season 2018/2019), and 14 patients with pneumonia caused by the SARS-CoV-2 virus who were treated at the S.P. Botkin Clinical Infectious Diseases Hospital (St. Petersburg, Russia) in April–May 2020. The patients had various symptoms as the most obvious clinical manifestations, namely, fever, intoxication (weakness, headache, muscle pain), and/or catarrhal-respiratory syndrome (nasal stuffiness, rhi-

norrhea, sore throat, cough, chest pain). Patients were included in the A/H3N2 and SARS-CoV-2 groups based on positive reverse transcription PCR diagnostic results for the corresponding pathogens in nasopharyngeal smears using certified Amplisense kits.

Blood for leukocyte isolation was collected from patients on days 3–4 after the onset of the first clinical symptoms into vacuum tubes with sodium heparin. Then, 8 ml of blood diluted with DPBS to a volume of 12 ml was added to a new tube, avoiding mixing, and 9 ml of Lymphosep reagent (BioWest, #L05600-500, USA) was added and centrifuged at 400 g for 20 min. The opaque interphase with leukocytes was selected and washed twice in a solution of 2% fetal serum FBS (Gibco, USA) prepared in RPMI-1640 nutrient medium (BioloT, Russia).

This study used viral strains from the collection of viruses and cell cultures of A.A. Smorodintsev Research Institute of Influenza, Russian Ministry of Health. For infection, we used strains of influenza virus A/California/07/09pdm (H1N1pdm09), influenza virus B/Malaysia/2506/04 (Victorian line), respiratory syncytial virus (RSV) strain A2, and coronavirus hCoV-19/Russia/SPE-RII-3524V/2020 (GISAID ID EPI_ISL_415710). For stimulation, influenza viruses were accumulated in 10–11-day developing chicken embryos and RSV and SARS-CoV-2 in Hep2 and Vero cell cultures, respectively. SARS-CoV-2 was handled in a BSL-3 biosafety laboratory. Virus titers were determined using permissive systems used for accumulation. Leukocytes isolated from healthy donors were stimulated with viruses in doses of 0.5 MOI (SARS-CoV-2), 1 MOI for influenza A and B viruses (IVA and IVB), and 1.5 MOI (RSV), added in a volume of 100 µl of viral suspension for $1.4 \cdot 10^6$ leukocytes in 100 µl of RPMI-1640 medium. After incubation for 1 h (virus contact was performed in a serum-free medium) at 37°C and 5% CO_2, medium with FBS was added to the cells to a final concentration of 10%. Analysis of the expression patterns of ISGs was performed after 24 h.

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen) according to manufacturer instructions. The quality and RNA concentration obtained were tested using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The degree of purity of the isolated RNA was determined using the A260/A280 value (norm $\geqslant$ 1.9).

To remove genomic DNA, which may be contaminated with total RNA preparations after isolation with TRIzol, DNase treatment was performed. All incubation steps were performed using the RQ1 RNase-Free DNase kit (Promega, USA) according to manufacturer instructions. Moreover, 1 µg of total RNA was used in the reaction.

For the reverse transcription reaction, 1 µg of RNA was used (immediately after DNase treatment). Complementary DNA (cDNA) synthesis required a reaction mixture with RNA-dependent Moloney murine leukemia virus DNA polymerase (M-MLV reverse transcriptase). Additionally, 0.5 µg of oligo $(dT)_{16}$ primers and water were added to the RNA to a final volume of 10 µl. The resulting mixture was incubated for 5 min at 70°C to anneal the primers and then transferred to ice for 2–3 min. Further, 15 µL of the mixture according to the protocol for reverse transcription (M-MLV, 1 µL; dNTP, 1.5 µL; 7.5 µL water) was added to 10 µL of sample. The prepared mixture was added to each RNA sample and incubated for 1 h at 42°C; inactivation occurred for 5 min at 65°C. Reagents from Biolabmix (Novosibirsk) were used for reverse transcription.

Real-time PCR was performed using a ready-made BioMaster HS qPCR kit (2×) (Biolabmix), into which 1–2 µl of cDNA was added. Primers and oligonucleotide probes were synthesized using DNA Synthesis (Moscow). The reaction was performed in a 25 µl preparation containing 6.25–12.5 pmol of forward and reverse primers and TaqMan probe. A two-stage temperature profile was used for PCR, namely, primary denaturation at 95°C for 5 min, followed by 40 two-stage cycles with denaturation at 95°C for 10 s and primer annealing and chain elongation at 61°C for 30 s. Amplification was performed using a CFX96 Touch thermal cycler (Bio-Rad) and detection by fluorescence growth; the presence of nonspecific products was assessed by electrophoretic separation of products in an agarose gel.

Gene amplification efficiency was calculated from the slope of the standard curve. For each of the three amplicons *MxA*, *OAS1*, and *PKR*, a series of tenfold dilutions were prepared, and PCR was performed using one set of specific primers and probes (monoplex format) or a mixture thereof (multiplex format). For the obtained linear functions ($y = \alpha \cdot x + b$), reflecting the dependence of the PCR cycle on the sample dilution logarithm, the slope angles α were determined. Then, the efficiency was calculated using the equation $E(\%) = (E - 1) \cdot 100\%$. By varying the concentrations of primers and oligonucleotide probes in PCR, the efficiency of amplification of genes of interest in a multiplex format was maximally equalized.

In performing multiplex PCR, the amplification of *MxA, OAS1,* and *PKR* genes was performed simultaneously in one tube. Simultaneously, all selected pairs of primers and oligonucleotide probes that detect specifically the declared genes were added to the PCR sample containing DNA-dependent DNA polymerase and the buffer attached to it. The final concentrations of primers and probes contained in the sample during multiplex PCR were hMxA_F

250 nM, hMxA_R 250 nM, hMxA_O 100 nM, hOAS1_F 500 nM, hOAS1_R 500 nM, hOAS1_O 200 nM, hPKR_F 500 nM, hPKR_R 500 nM, and hPKR_O 200 nM.

Relative gene expression was calculated using the ΔΔ*Ct* method, with *GAPDH* as a normalization gene. The relative gene expression level was obtained using the inductive equation $R = 2^{-[\Delta\Delta Ct]}$. All calculations were performed using Microsoft Office Excel software. The statistical significance of differences was assessed using the GraphPadPrism 6 computer program.

Results

Initially, primers and oligonucleotide probes were designed, which detect specifically messenger RNA (mRNA) of the human *MxA*, *OAS1*, and *PKR* genes (EIF2AK2 subunits). The original sequences (Table) were selected for the protein-coding region of the genes in that the primers were separated by an intron region, their melting temperatures were similar, and the length of the amplicons formed during the PCR process was not >300 bp. The glyceraldehyde-3-phosphate dehydrogenase gene *GAPDH* was proposed as an endogenous control used for normalization, and a PCR system used for its determination was previously developed by the authors [10].

The selected primers detected all mRNA transcriptional variants of the studied genes presented in the NCBI database (3 *MxA*, 4 *OAS1*, and 3 *PKR*).

To implement the multiplex PCR format in the test system being developed, TaqMan probes containing various fluorescent tags (FAM, ROX, and CY5) at the 5′-terminus. Using different fluorophores, the amplification efficiency of specific products was further investigated using a probe tailored to the human *MxA* gene. For this purpose, five oligonucleotide probes that specifically detect the human *MxA* gene were ordered, identical in nucleotide sequence and differing only in fluorescent tags and quenchers contained at the 5′- and 3′-terminals, respectively. According to the growth curves (results are presented in the Appendix, Fig. 1), fluorophores had little effect on the accumulation rate of a specific product. In the reactions considered, the specific product was detected at the PCR threshold cycle 20–21, which corresponds to a method error of ± cycle.

As a template for optimizing the conditions of multiplex PCR, we used cDNA samples obtained by reverse transcription of total RNA preparations isolated from A549 cells infected with IVA. The optimization criteria were the accumulation rate of amplification products according to the fluorescence growth curve (Appendix, Fig. 2) and absence of nonspecific amplicons when analyzing PCR products by electrophoretic separation in an agarose gel.

For multiplex PCR, temperature profile with primary denaturation at 95°C for 5 min was used, followed by 40 two-stage cycles of denaturation at 95°C for 10 s and primer annealing and chain elongation at 61°C for 30 s.

To accurately perform relative quantitative analysis of the expression of ISGs in a multiplex format, the amplification efficiencies of the corresponding cDNAs should be identical or as close as possible to each other. Therefore, under selected optimal conditions, the efficiencies of PCR performed in monoplex and multiplex formats were calculated (Fig. 1) using the slope of a curve obtained by PCR with a series of sequential dilutions of the prepared amplicons.

The calculated amplification efficiencies of the *MxA*, *OAS1, and PKR* genes during multiplex PCR were 101.9%, 92.5%, and 101.5% (Fig. 1*a*), respec-

Table / Таблица

Selected primers and TaqMan probes for *MxA*, *OAS1*, and *PKR* gene expression analysis
Подобранные праймеры и TaqMan-зонды для определения экспрессии генов *MxA*, *OAS1* и *PKR*

Gene	mRNA	Primer name	Selected primers (5′-3′)	PCR product length, bp.
MxA	NM_001144925.2 NM_002462.5 NM_001178046.3	qH-MxA_F	GAGACAATCGTGAAACAGCAAATCA	105
		qH-MxA_R	TATCGAAACATCTGTGAAAGCAAGC	
		qH-MxA_O	FAM-CACTGGAAGAGCCGGCTGTGGATATG-BHQ2	
OAS1	NM_016816.4 NM_002534.3 NM_001032409.3 NM_001320151.2	qH-OAS1_F	CCAAGGTGGTAAAGGGTGGCT	200
		qH-OAS1_R	CTGGACCTCAAACTTCACGGAAA	
		qH-OAS1_O	ROX-AGGCCGATCTGACGCTGACCTGGTTGT-BHQ3	
PKR	NM_002759.3 NM_001135652.2 NM_001135651.3	qH-PKR_F	GAAAGCGAACAAGGAGTAAGG	175
		qH-PKR_R	CCATCCCGTAGGTCTGTGAAA	
		qH-PKR_O	Cy5-AGCCCCAAAGCGTAGAGGTCCACTTCC-BHQ1	

tively. These efficiencies were obtained by optimizing primer and probe concentrations (presented in the Materials and Methods section). According to the results of electrophoretic separation of multiplex and monoplex PCR products in an agarose gel, no unwanted nonspecific products were formed during the reaction (Fig. 1*b*).

Using the developed test system, the expression levels of the *MxA*, *OAS1*, and *PKR* genes were assessed in leukocytes isolated from the blood of patients diagnosed with influenza and COVID-19 and in leukocytes obtained from healthy donors. Laboratory confirmation of diagnoses was previously performed by identifying the relevant etiological agents (their genetic material) in nasopharyngeal smears using RT-PCR. Leukocytes were obtained from the peripheral blood of hospitalized patients on days 3–4 after disease onset.

According to the results presented in Fig. 2, the mRNA expression of the *MxA*, *OAS1*, and *PKR* genes in white blood cells in infected people significantly increased on days 3–4 after the disease manifestation compared with that in healthy volunteers. Notably, the expression of the analyzed genes in samples obtained from COVID-19 patients was more dispersed. Thus, *MxA* and *PKR* expression levels of

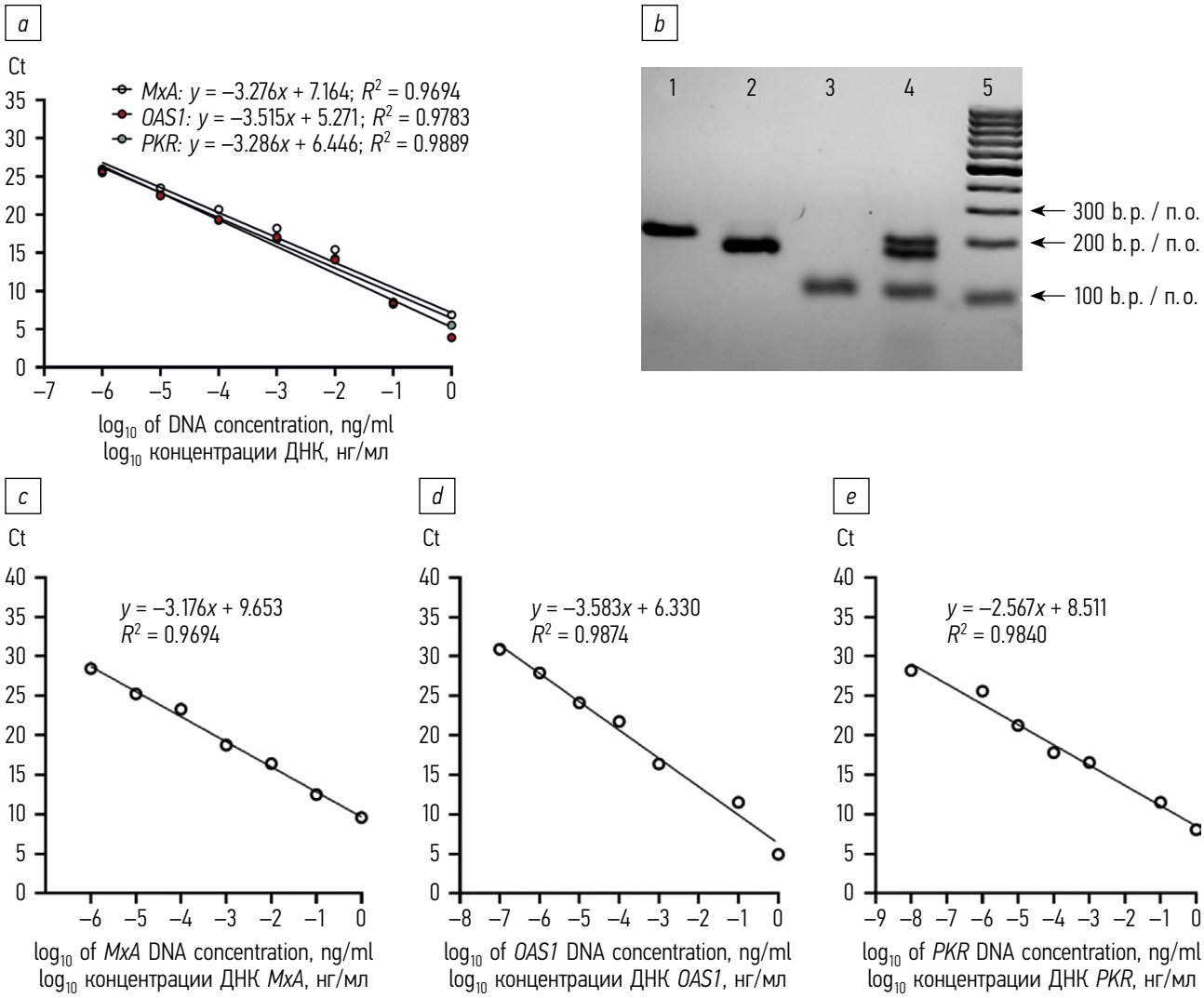

Fig. 1. Determination of PCR efficiencies in monoplex and multiplex approaches: *a* — multiplex amplification of *MxA*, *OAS1*, and *PKR* genes (simultaneous detection in one tube); *b* — separation of PCR products using agarose gel electrophoresis: 1 — *OAS1*, 2 — *PKR*, 3 — *MxA*, 4 — simultaneous amplification of three genes in multiplex PCR, 5 — DNA Ladder, 100 bp (Fermentas); *c* — standard curve for *MxA* gene in monoplex approach; *d* — standard curve for the *OAS1* gene in monoplex approach; *d* — standard curve for the *PKR* gene in monoplex approach

Рис. 1. Расчет эффективностей ПЦР в моноплексном и мультиплексном форматах: *a* — мультиплексная амплификация генов *MxA*, *OAS1* и *PKR* (одновременная детекция в одной пробирке); *b* — разделение продуктов ПЦР в агарозном геле: 1 — *OAS1*, 2 — *PKR*, 3 — *MxA*, 4 — одновременная амплификация трех генов в мультиплексной ПЦР, 5 — маркер длин, 100 bp (Fermentas); *c* — стандартная калибровочная кривая, полученная для гена *MxA* в моноплексном формате ПЦР; *d* — стандартная калибровочная кривая, полученная для гена *OAS1* в моноплексном формате ПЦР; *e* — стандартная калибровочная кривая, полученная для гена *PKR* в моноплексном формате ПЦР

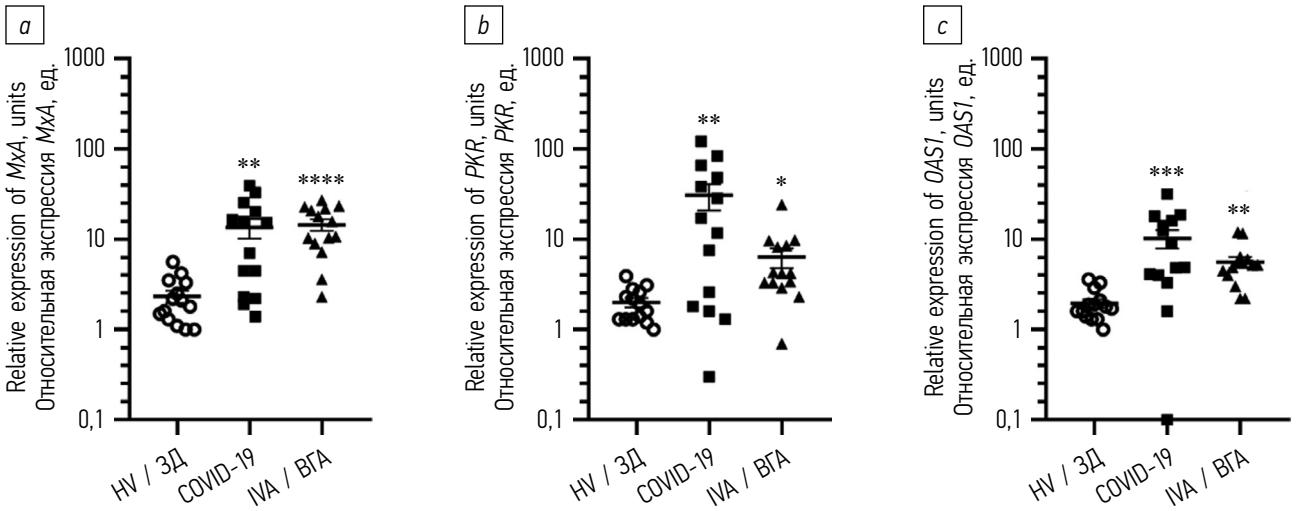

Fig. 2. Relative expression of *MxA*, *OAS1*, and *PKR* genes in leukocytes of patients with influenza infection A (IVA), coronavirus disease (COVID-19), and in healthy volunteers (HV). Statistical significance was determined for groups of infected people compared with a group of healthy volunteers by Kruskal–Wallis test (with pairwise Dunnett's multiple comparisons test): $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$

Рис. 2. Относительная экспрессия генов *MxA*, *OAS1* и *PKR* в лейкоцитах пациентов с инфекцией вирусом гриппа А (ВГА), новой коронавирусной инфекцией (COVID-19) и у здоровых добровольцев (ЗД). Достоверность различий экспрессии у групп инфицированных людей по сравнению с группой здоровых добровольцев определяли с использованием непараметрического критерия Краскела – Уоллиса с поправкой Даннетта для множественного сравнения: $*p < 0,05$; $**p < 0,01$; $***p < 0,001$; $****p < 0,0001$

approximately 4–5 of 14 patients were comparable to those of controls.

Additionally, we investigated the mRNA levels of the *MxA*, *OAS1*, and *PKR* genes upon leukocyte stimulation with RNA viruses (Fig. 3). Remarkably, 24 h after infection, the expression levels of the studied genes when stimulated with the SARS-CoV-2 virus did not differ from the levels in control unstimulated cells. Simultaneously, *in vitro* cell stimulation with IVA, IVB, and RSV resulted in a significant increase in *MxA* and *OAS1* expression and an increase in *PKR* (in the case of RSV, despite the insignificant differences, a tendency to increased expression was also registered).

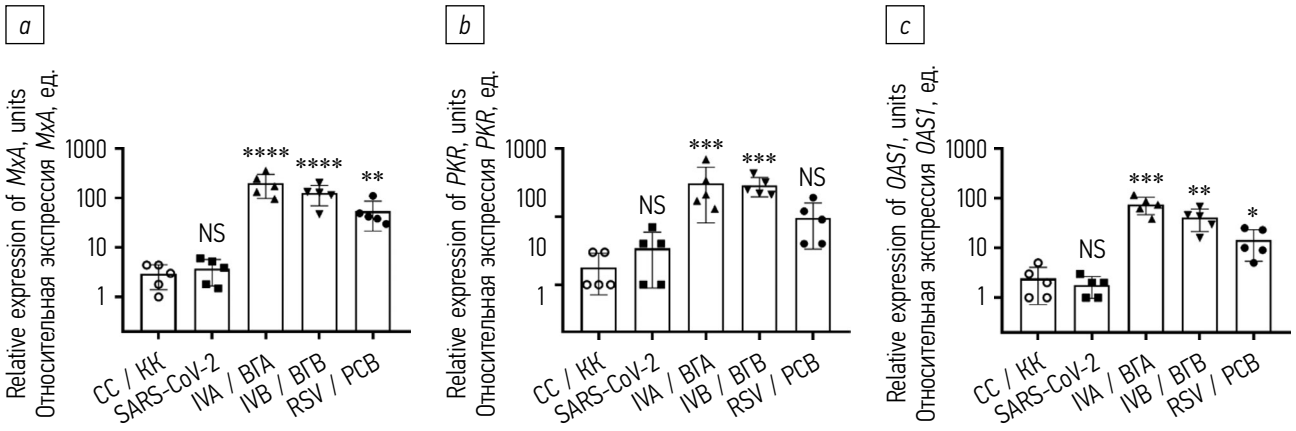

Fig. 3. Patterns expression of *MxA* (*a*), *OAS1* (*b*) and *PKR* (*c*) genes in leukocytes (from healthy volunteers) in response to *in vitro* stimulation of leukocytes by influenza viruses A (IVA), B (IVB), SARS-CoV-2, and respiratory syncytial virus (RSV) compared to uninfected cells (CC). Statistical significance was determined using single-factor analysis of variance (ANOVA) for paired samples with Holm–Sidak correction for groups stimulated by viruses relative to control cells group: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; NS is non-significant, the differences are not reliable

Рис. 3. Изменение экспрессии генов *MxA* (*a*), *OAS1* (*b*) и *PKR* (*c*) в лейкоцитах, полученных от здоровых добровольцев, в ответ на *in vitro* стимуляцию лейкоцитов вирусами гриппа А (ВГА), В (ВГВ), коронавирусом SARS-CoV-2 и респираторно-синцитиальным вирусом (РСВ) по сравнению с неинфицированными клетками (КК). Достоверность различий в группах, стимулированных вирусами, относительно группы контрольных клеток определяли с использованием однофакторного дисперсионного анализа (ANOVA) для парных образцов с коррекцией Холма – Шидака: $*p < 0,05$; $**p < 0,01$; $***p < 0,001$;$****p < 0,0001$; NS — non-significant, различия недостоверны

Discussion

Combined determination of the expression level of the *MxA*, *OAS1*, and *PKR* genes enables determination of the activation level of the body's innate immune system and assessment of the productivity of the interferon-mediated antiviral response. This assessment becomes relevant in analyzing the pathogenesis of acute respiratory viral infections that are capable of exploiting the immune response. The most sensitive and specific method for determining the level of gene expression is real-time PCR. In the present study, a multiplex test system was proposed, developed, and validated, which allows the expression of three ISGs with a direct antiviral effect to be measured simultaneously in one sample.

The developed test system was recommended for examining the molecular regulation of *MxA*, *OAS1*, and *PKR* in leukocytes in cases of socially significant infections such as influenza and COVID-19. According to our results, in hospitalized patients on days 3–4 after the onset of symptoms of the disease, the expression levels of the *MxA*, *OAS1*, and *PKR* genes significantly increased at the systemic level in blood leukocytes. The induction of these genes is due to the JAK/STAT intracellular signal transduction system activated by the type I and III interferon systems, which form the first line of defense against viral infections in mammals [11, 12]. Thus, numerous clinical studies have confirmed that MxA protein expression in peripheral blood is a sensitive and specific marker of viral infections [13]. However, we were interested in the fact that in COVID-19 patients, the expression values of the *PKR* and *MxA* genes were distributed relatively widely. In approximately 4–6 patients with COVID-19 (about a third of those examined), the measured mRNA levels of these genes were comparable to levels in uninfected volunteers. Virus-mediated suppression of the early interferon response at the infection site and unbalanced activation of immune signaling networks are known to regulate the excessive inflammatory immune response in severe COVID-19 [14, 15]. All the study patients were hospitalized in a state of moderate severity; no lethal outcomes were registered; however, they received appropriate therapy, which may have influenced the dispersion of our results.

The next stage of our work was to analyze the virus-induced expression of the *MxA*, *OAS1*, and *PKR* genes in leukocytes in response to respiratory virus stimulation. The leukocytes used in this study were isolated and obtained from healthy volunteers before 2018, i.e., the samples used were naive to the new coronavirus infection that appeared in 2019 (not influenza). Our results indicated that 24 h after infection, SARS-CoV-2 did not induce *MxA*, *OAS1*, and *PKR* expression in leukocytes, whereas IVA, IVB, and RSV naturally caused an increase in the mRNA levels of these genes. Our initial assumption was that the SARS-CoV-2 virus we used was not capable of infecting leukocytes. We proved virus infectivity using a back titration method (data not presented) on a permissive cell culture.

Kazmierski et al. [16] reported the inability of productive infection of SARS-CoV and SARS-CoV-2 in human leukocytes due to the absence of the ACE2 receptor on the surface of the latter, which coronaviruses use for invasion [17–19]. However, direct stimulation of monocytes by SARS-CoV-2 is accompanied by a strong induction of ISGs, despite the absence of detectable productive infection [16]. Moreover, the literature shows that SARS-CoV-2 infection in Calu-3 cells is accompanied by dynamic activation of the transcription of cytokines IL6, CXCL8, CXCL10, TNF-α, and IL1B and interferon-induced viral restriction factors, such as OAS1 and Mx1 [20]. In the first 24 hours, the expression values of OAS1 and Mx1 mRNA did not differ from the control values and reached maximum values 56–60 hours after infection.

SARS-CoV-2, when directly infecting leukocytes, potentially induces an aberrant interferon response in cells, which is reflected in reduced expression of key antiviral ISGs, such as *MxA*, *OAS1*, and *PKR*, in the early stages of viral infection. It is possible that the expression of these genes increases during the later stages of infection. This delayed antiviral response may provide a window for viral replication, prompting the SARS-CoV-2 manipulation strategy to target the innate immune response. Unfortunately, in our study, it was not possible to evaluate expression at later stages because the experimental design was a comparison of different viruses (IVA, IVB, RSV, and SARS-CoV-2), and the virus-mediated cytopathic effect during infection with influenza and RSV viruses at late terms results in inadequate measurement of gene expression in cells.

Thus, the developed multiplex system for determining the expression of the *MxA*, *OAS1*, and *PKR* genes, which have antiviral activity, may be crucial for determining the initiation of the immune system in response to viral infection, which enables the assessment of immune regulatory signaling pathway involvement in the cell antiviral state.

Additional information

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Conflict of interest. The authors declare no conflict of interest. All results and conclusions presented in the publication were personally produced by the authors of the article.

Ethics approval. Research involving leukocytes received approval from the local ethical commission of the Smorodintsev Research Institute of Influenza, Russian Ministry of Health, St. Petersburg, Russia (sessions No. 108 dated 03.09.2018 and No. 164 dated 12.02.2021).

Authors' contribution. All authors made a significant contribution to the development of the concept, research, and preparation of the article, read and approved the final version before publication. The largest contributions are distributed as follows: *S.A. Klotchenko*, *E.A. Romanovskaya-Romanko*, *M.A. Plotnikova* — idea of the work, planning the experiment, writing and editing the manuscript; *V.A. Oleynik*, *M.A. Egorova*, *V.S. Monakhova*, *E.V. Venev* — participation in the study, data collection; *E.A. Romanovskaya-Romanko*, *M.A. Plotnikova* — data analysis and interpretation.

Дополнительная информация

Источник финансирования. Исследование выполнено при финансовой поддержке Российского научного фонда, проект № 23-25-00433: «Изучение противовирусного действия мРНК, кодирующей MxA белок человека» (М.А. Плотникова), https://rscf.ru/project/23-25-00433/

Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов. Все результаты и выводы, представленные в публикации, выполнены лично авторами статьи.

Соблюдение этических норм. Исследования с использованием лейкоцитов получили одобрение локальной этической комиссии ФГБУ «НИИ гриппа им. А.А. Смородинцева» Минздрава России (заседания № 108 от 03.09.2018 и № 164 от 12.02.2021).

Appendix / Приложение

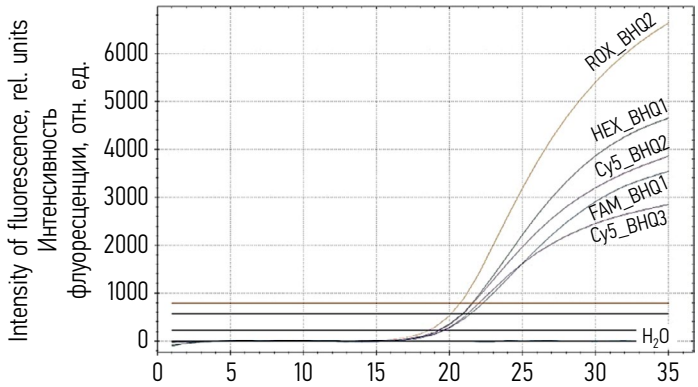

Fig. 1. *MxA* gene amplification using different fluorophores and quenchers

Рис. 1. Амплификация гена *MxA* при использовании разных флуорофоров и гасителей

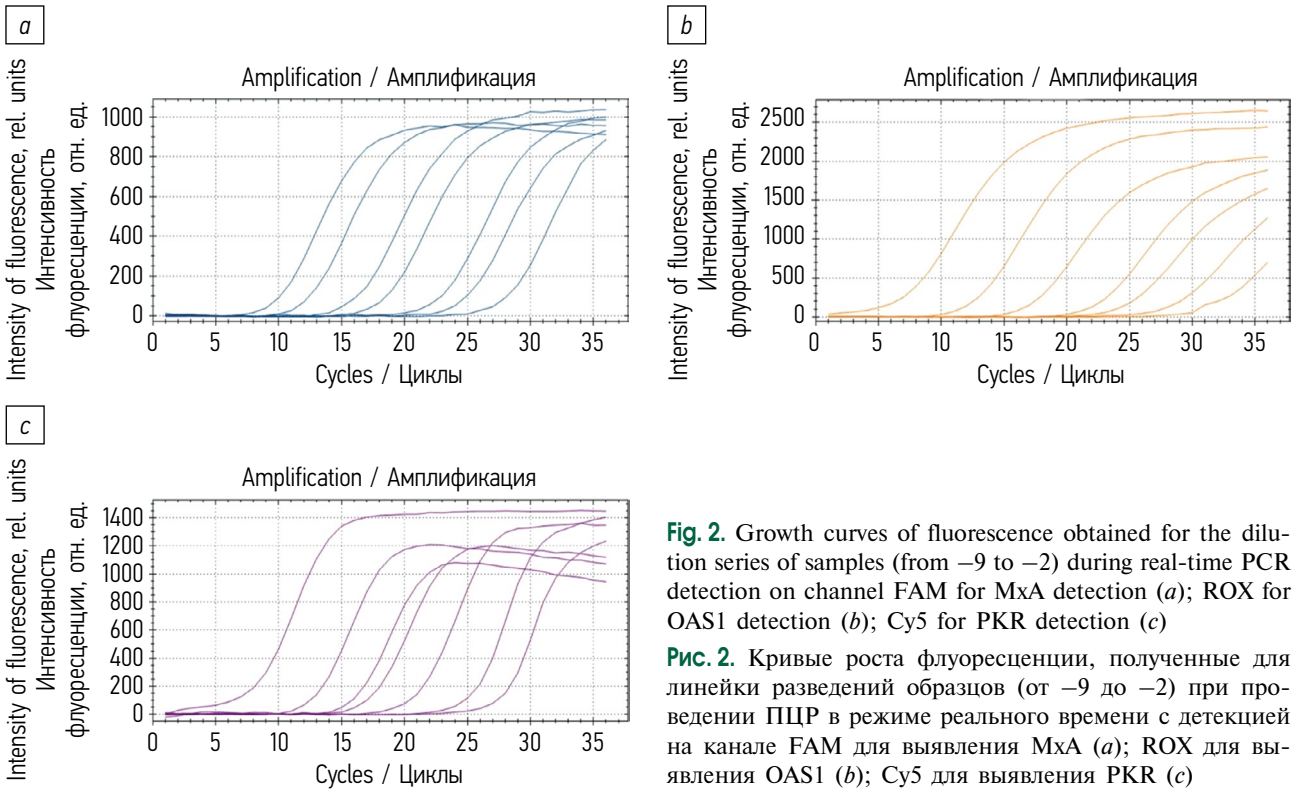

Fig. 2. Growth curves of fluorescence obtained for the dilution series of samples (from –9 to –2) during real-time PCR detection on channel FAM for MxA detection (*a*); ROX for OAS1 detection (*b*); Cy5 for PKR detection (*c*)

Рис. 2. Кривые роста флуоресценции, полученные для линейки разведений образцов (от –9 до –2) при проведении ПЦР в режиме реального времени с детекцией на канале FAM для выявления MxA (*a*); ROX для выявления OAS1 (*b*); Cy5 для выявления PKR (*c*)

Вклад авторов. Все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией. Наибольший вклад распределен следующим образом: *С.А. Клотченко*, *Е.А. Романовская-Романько*, *М.А. Плотникова* — идея работы, планирование эксперимента, написание и редактирование рукописи; *В.А. Олейник*, *М.А. Егорова*, *В.С. Монахова*, *Е.В. Венёв* — участие в исследовании, сбор данных; *Е.А. Романовская-Романько*, *М.А. Плотникова* — анализ и интерпретация данных.

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