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Adaptation of the MTT assay for detection of neutralizing antibodies against the SARS-CoV-2 virus

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Abstract

Introduction. The ability of SARS-CoV-2 antibodies to neutralize the virus is the primary indicator of their specific activity. The test for virus neutralizing antibodies (NAbs) is much needed in different biomedical studies.

The aim of the study is to find optimum conditions for microscopic and spectrophotometric detection of SARS-CoV-2 NAbs by inhibition of cytopathic effect (CPE) in cell cultures.

Materials and methods. Blood sera collected from COVID-19 convalescent patients and healthy individuals (n = 96) were tested using the ELISA method. The SARS-CoV-2 coronavirus, Dubrovka strain (GenBank accession no. MW514307.1) was grown in culture medium of Vero cell line CCL-81 (ATCC). Real-time RT-PCR, ELISA, and Sanger sequencing were used for identification of the virus. The results of the neutralization test (NT) were assessed through the microscopic examination for CPE and by the methyl thiazolyl tetrazolium (MTT) assay.

Results. SARS-CoV-2 was isolated from a COVID-19 patient and adapted to grow in cell culture. At a low dose of infection (MOI = 0.00001), the virus caused a pronounced CPE with the cell viability less than 3%, thus making it possible to assess NT results by CPE inhibition. The NT and ELISA-based comparative study of sera showed positive correlation between virus NAb titers and antibodies titers to S-protein RBD (Spearman's r = 0.714; p < 0.001). The results of NAbs microscopic and spectrophotometric detection (the MTT assay) also demonstrated positive correlation (Spearman's r = 0.963; p < 0.05).

Conclusion. The SARS-CoV-2 virus adapted to Vero cell culture served to develop a NAb titer assessment system, which allows to detect the result both by microscopic examination and spectrophotometrically in the MTT-test. The MTT assay provides automated reading of NT results, optimizes the statistical analysis of the obtained data, and minimizes subjectivity in assessment of results. Being a vital dye, MTT detect only viable cells, thus contributing to the reliability of the obtained results compared to other dyes.

Keywords: *neutralization test, SARS-CoV-2 coronavirus, MTT assay, virus neutralizing antibodies*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the I. Mechnikov Research Institute of Vaccines and Sera.

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Научная статья
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Адаптация МТТ-теста для определения нейтрализующих антител к вирусу SARS-CoV-2

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Аннотация

Введение. Основным показателем специфической активности антител к вирусу SARS-CoV-2 является их способность нейтрализовать вирус. Тест на вируснейтрализующие антитела (ВНА) широко востребован в различных направлениях биомедицинских исследований.

Целью работы являлся подбор оптимальных условий для определения ВНА к вирусу SARS-CoV-2 по ингибированию цитопатогенного действия (ЦПД) в культуре клеток с возможностью как микроскопического, так и спектрофотометрического учёта результата.

Материалы и методы. Сыворотку крови реконвалесцентов COVID-19 и здоровых лиц (n = 96) изучали методом ИФА. Коронавирус SARS-CoV-2, штамм Dubrovka (номер GenBank: MW514307.1) выращивали в культуре клеток Vero CCL81 (ATCC). Идентификацию вируса проводили методами ОТ-ПЦР-РВ, ИФА и секвенирования по Сэнгеру. Результаты реакции нейтрализации (РН) учитывали по ЦПД микроскопически и в метилтетразолиевом (МТТ) тесте.

Результаты. От больного COVID-19 изолирован коронавирус SARS-CoV-2 и адаптирован к выращиванию в культуре клеток. При заражении низкой дозой (MOI = 0,00001) вирус вызывал выраженное ЦПД с выживаемостью клеток менее 3%, что позволяло учитывать результаты РН по ингибированию ЦПД. Сравнительный анализ сывороток в РН и методом ИФА показал достоверную корреляцию между титрами ВНА и титрами антител к RBD-домену S-белка (Спирмен r = 0,714; p < 0,001). Результаты определения ВНА с микроскопической и спектрофотометрической детекцией (тест МТТ) также достоверно коррелировали (Спирмен r = 0,963; p < 0,05).

Заключение. На основе адаптированного к культуре клеток Vero вируса SARS-CoV-2 разработана система оценки титра ВНА, позволяющая учитывать результат как с помощью микроскопического исследования, так и спектрофотометрически в МТТ-тесте. Применение теста МТТ позволяет автоматизировать учёт результатов РН, проводить статистическую обработку получаемых данных, снижает субъективизм при оценке результата. Являясь витальным красителем, МТТ выявляет только живые клетки, что повышает надёжность получаемых результатов по сравнению с другими красителями.

Ключевые слова: *реакция нейтрализации, коронавирус SARS-CoV-2, МТТ-тест, вируснейтрализующие антитела*

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Introduction

A novel human coronavirus, SARS-CoV-2, first identified in China in December 2019, caused a pandemic, which claimed over 2 million lives worldwide in 2020. Studies of regularities of the immune response to novel coronavirus infection are essential for development of effective preventive options and methods of immunotherapy. The scope of present-day laboratory diagnostics has been expanded by multiple immunochemical (primarily, immunoenzyme) test systems designed for detection and quantification of SARS-CoV-2 specific IgG and IgM antibodies [1, 2]. However, these tests cannot be used for assessment of functional activity of specific antibodies, for example, for assessment of their ability to neutralize a virus. Virus neutralizing antibodies (NAbs) generally target the receptor binding domain (RBD) of the SARS-CoV-2 surface (S) glycoprotein and block the ability of the virus to bind to its receptor ACE2 on the target cell surface [3, 4] (**Fig. 1**).

The passive transfer of RBD-specific monoclonal antibodies to animals sensitive to SARS-CoV-2 (mice,

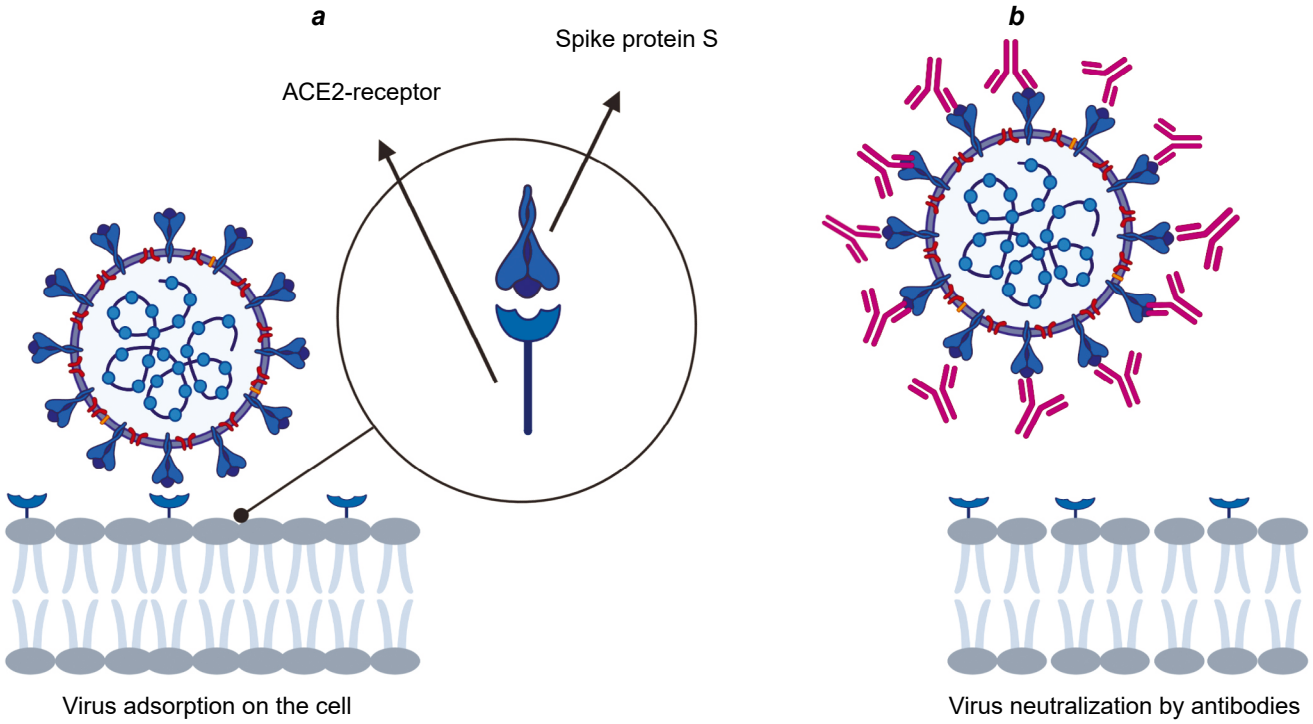

Fig. 1. Schematic representation of SARS-CoV-2 neutralization by antibodies.
a — virion adsorption on the SARS-CoV-2 target cell by binding the S glycoprotein and the ACE receptor;
b — NAbs inhibit the virus adsorption on the target cell.
The representation is created with BioRender's web-based software.

hamsters, and rhesus monkeys) demonstrated their protective activity [3–5]. There is positive clinical experience of using convalescent plasma for treatment of COVID-19 patients [6, 7]. The presence of monoclonal NAbs in convalescent plasma products is indicative of their protective activity. The presence of NAbs in blood sera from vaccinated individuals is the primary indicator of the specific activity in most of the COVID-19 preventive vaccines [6]. Therefore, tests for NAbs are much needed in biomedical studies.

Traditional methods used in assessment of activity of SARS-CoV-2 neutralizing antibodies are based on the ability of NAbs to inhibit plaque formation or a virus-induced cytopathic effect (CPE) in a sensitive cell culture [8]. As tests involve the virulent strain of the virus, they are performed in laboratories, which comply with the biosafety level 3. NAbs can also be detected by using alternative techniques, which do not involve viruses. These are surrogate test systems based on solid-phase ELISA with recombinant RBD peptides and ACE receptor [9, 10], using pseudoviral particles derived from recombinant viruses and different reporter systems [11, 12]. Some alternative NAb detection techniques can give much quicker results that techniques aimed at identification of viral CPE. Nevertheless, despite the above limitations, the traditional titration methods remain the gold standard for detection of NAbs, as they most accurately simulate the natural virus neutralization by antibodies. Note that, contrary to the established opinion, SARS-CoV-2 neutralizing antibodies target not only RBD epitopes, but also epitopes in other S protein domains [5, 13, 14]. Therefore, only traditional culture-based methods of NAb assessment can detect the entire range of circulating SARS-CoV-2 neutralizing antibodies, while solid-phase ELISA-based surrogate test systems detect only antibodies targeting RBD epitopes.

The aim of the study was to find optimum conditions for detection of SARS-CoV-2 NAbs by CPE inhibition in the cell culture, including visual and instrumental detection.

The following objectives were set to achieve the aim:

- to isolate the SARS-CoV-2 coronavirus from the clinical sample from a COVID-19 patient in sensitive cell culture;
- to use passages in the cell culture to obtain a laboratory strain of SARS-CoV-2, which would cause the death of cells at low doses of infection;
- to develop and test the method of assessment of the SARS-CoV-2 NAb titer, including detection of the result in the MTT colorimetric assay.

Materials and methods

Clinical samples. The serum samples from convalescent COVID-19 patients and healthy individuals (seronegative by ELISA) as well as the oropharyngeal swab from a patient with confirmed COVID-19 diag-

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nosis were obtained from the Clinical and Diagnostic Center of the Mechnikov Research Institute for Vaccines and Sera. The tests on the clinical material were performed in compliance with the international ethical standards and with the informed consent of the participants.

Virus and cell culture. We used the Dubrovka laboratory strain of the SARS-CoV-2 coronavirus (GenBank: MW514307.1). The virus was grown on African green monkey kidney epithelial cells, Vero CCL81 (ATCC) (hereinafter referred to as Vero cells). The cells were cultured at 37ºC in the DMEM medium with Earle's salts (PanEco, Russia) and 5% fetal bovine serum (Gibco), 300 μg/ml L-glutamine (PanEco), 40 μg/ml gentamicin (PanEco) in 5% CO_2.

Detection of the viral RNA. The viral RNA was extracted from the clinical and cell culture samples by using MAGNO-sorb (InterLabService, Russia) and RealBest extraction 100 (Vector-Best, Russia) reagent kits. The SARS-CoV-2 virus was identified through the real-time reverse transcription (RT) PCR using RealBest RNA SARS-CoV-2 kit (Vector-Best). The virus accumulation and viral RNA replication in the cell culture were monitored through the quantitative real-time RT-PCR by using the set of primers and probes for the N-gene of SARS-CoV-2 [15].

To perform the real-time RT-PCR, we used the reagent kit — 2.5x reaction mixture for real-time PCR with Taq-polymerase and MMLV reverse transcriptase (Syntol). The 25 μl reaction mix contained 10 pmol of each primer and 5 pmol of the probe, Taq DNA polymerase, and 30 units of reverse transcriptase. The temperature and time requirements: 45°C — 10 min (1 cycle); 95°C — 5 min (1 cycle); 95°C — 5 sec, 55°C — 45 sec (45 cycles). The test was performed in a DTprime thermal cycler (DNA-Technology, Russia). All primers and probes were synthesized at Syntol.

Sequencing of the S-gene of SARS-CoV-2. To sequence the S-gene of SARS-CoV-2 through RT-PCR, we amplified S-gene fragments by using the in-house designed set of primers (**Table 1**). The RT was performed in the 100 μl reaction mix containing the RT buffer (Syntol), 20 pmol of CSr0 primer, 24 μl of viral RNA, 50 units of MMLV RT (Syntol); programm — 10 min at 42°C, 2 min at 95°C.

The PCR test was performed in 50 μl reaction mix containing LongAmp™ Taq 2X Master Mix (NEB), 5 μl of cDNA, 10 pmol of both F and R primers; temperature/time requirements: 94°C — 2 min (1 cycle), 94°C — 60 sec, 55°C — 40 sec, 65°C — 100 sec (40 cycles), 65°C — 10 min (1 cycle); storage at 4°C. The resulting amplicons were identified by their mobility through electrophoresis on 1% agarose gel; purification was performed with the Cleanup Mini reagent kit (Evrogen, Russia).

The concentration of PCR products was measured spectrophotometrically; the further Sanger sequencing was performed at Syntol. The full-length S-gene was assembled with the VectorNTI 11.0.0 software (Invitrogen Corp.).

Propagation of the SARS-CoV-2 coronavirus. Three days prior to the infection, the Vero cells were seeded in culture flasks with vented caps (Corning), with available growth area of 75 cm^2, in a 1:5 dilution. On day 3, after reaching a 100% monolayer, the culture liquid was removed from the flask and the viral material was added at MOI = 0.01–0.0001 $TCID_{50}$ per cell. The virus adsorption was performed in a CO_2 incubator for 60 min; then the maintenance medium (DMEM, L-glutamine — 300 μg/ml, gentamicin — 40 μg/ml, 1% fetal bovine serum) was added, and the incubation continued at 37°C in 5% CO_2 until CPE. Upon occurrence of CPE, the virus-containing cell-culture liquid was collected, clarified by centrifugation, aliquoted, and stored at –80°C.

Virus titration by the CPE endpoint. The SARS-CoV-2 titer was detected by the CPE endpoint in the Vero cell culture. Vero cells were seeded in 96-well plates in a 1 : 5 dilution. Three days later, the growth medium was removed from the wells; 10-fold serial dilutions of the virus in the maintenance medium were added and incubated for 5 days in a CO_2-incubator at 37°C. The titration results were assessed visually through microscopic examination of the cell monolayer for the

Table 1. Primers for amplification and sequencing of the SARS-CoV-2 S gene

Primer		Sequence
F	CSf0	AGGGGTACTGCTGTTATGTCTT
	CSf1	TTCTTCTTCAGGTTGGACAGC
	CSf2	ACATGCACCAGCAACTGTTT
	CSf3	GGGCTGAACATGTCAACAAC
	CSf4	AACAAATTTACAAAACACCACCAA
	CSf5	GAACCAAAAATTGATTGCCA
	CSf6	CTTCCCTCAGTCAGCACCTC
	CSf7	CTCAATGAGGTTGCCAAGAA
R	CSr0	GCTTGTATCGGTATCGTTGCAG
	CSr1	TTGTGGTAATAAACACCCAAAAA
	CSr2	TTTCCAGTTTGCCCTGGAG
	CSr3	CCTGTGCCTGTTAAACCATTG
	CSr4	CGCCGAGGAGAATTAGTCTG
	CSr5	TCTTGCTTGGTTTTGATGGA
	CSr6	GACAAATGGCAGGAGCAGTT
	CSr7	CCATGGCCATTTTATATACTGCT

presence of specific CPE on day 5 post-infection (cell rounding and detachment from the monolayer). The virus titer was estimated by using the following method [16] and expressed as lg $TCID_{50}$/ml.

In-cell ELISA. The Vero cells grown to the 100% monolayer in the 96-well plate were infected with the virus at MOI = 0.3. In 24 hours the cells were fixed for 15 minutes with the 8% paraformaldehyde solution prepared in phosphate-buffered saline (PBS; pH 7.2), then washed twice with PBS; the plate was stored at 4°C prior to ELISA. Before the ELISA test, 150 µl 0.02 M PBS containing 1% Triton X-100 were added to the wells for 30 minutes; then the blocking buffer (0.02 M PBS containing 0.09% sodium caseinate) was added for 1 hour. After the blocking buffer was removed, 100 µl of sera in a 1 : 100 dilution in the 0.02 M PBS containing 0.2% bovine serum albumin and 0.05% Tween 20 were added to the wells for 1 hour at 37°C. After the washing, we added 100 µl of horseradish peroxidase conjugated monoclonal mouse anti-human IgG antibodies. The incubation and washing steps were repeated to be followed by addition of 100 µg of 33 mM citrate buffer with pH 4.0 containing 0.01% hydrogen peroxide and 0.5 mM 3.3', 5.5'-tetramethylbenzidine. After 15 minutes, the test was stopped by adding 50 µl of 2N sulfuric acid; the optical density (OD) was measured at the test wavelength of 450 nm and the reference wavelength of 680 nm.

Detection of virus antibodies with ELISA. IgG antibodies against the RBD of SARS-CoV-2 were detected by using the SARS-CoV-2-ELISA-IgG reagent kit (Medipaltekh, LLC).

The titer of SARS-CoV-2 antibodies was set as the last dilution where the OD value was higher than the cutoff threshold in each study.

MTT assay. The viability of virus-infected Vero cells was assessed by using the methylthiazolyl tetrazolium (MTT) bromide vital dye. On day 5 post-infection, 20 µl of the MTT solution, 5 mg/ml (PanEco) was added to the cell-containing wells of the 96-well plate to be further incubated at 37°C in 5% CO_2 for 2 hours. Then, the cell culture liquid was removed, and 100 µl of dimethyl sulfoxide (Sigma-Aldrich) was added to each well. By using the plate spectrophotometer, the OD of each well was measured at 530 nm, taking into account the baseline values at 620 nm. The viability of cells was calculated by the formula:

$$\text{Viability} = (OD_{530}\ \text{test sample}/OD_{530}\ \text{cell control}) \times 100\%,$$

where OD_{530} test sample is the mean value of OD_{530} in the wells with infected cells; OD_{530} cell control – the mean value of OD_{530} in the wells with uninfected cell culture.

Neutralization test. Measuring the titer of SARS-CoV-2 NAbs was performed as described in the study [17] with some modifications. The serum samples were aliquoted by 100 µl and stored at –20°C. Prior to the neutralization test (NT), the sera was defrosted and heated at 56°C for 30 min; two-fold serial dilutions with maintenance medium were prepared. The serum dilutions were mixed with the equal amount of SARS-CoV-2 viral material in the titer of 2×10^3 $TCID_{50}$/ml and incubated at 37°C in 5% CO_2 for 1 hour. The medium was removed from the 96-well plate with the 3-day monolayer of Vero cells; the wells were filled with the mixture of virus and serum in 4 repeats by 100 µl (the virus dose — 100 $TCID_{50}$ per well) in accordance with the scheme (**Table 2**) and incubated for 5 days at 37°C in 5% CO_2.

In addition to the studied samples, NT included the following controls: cell control (CC — uninfected cell culture), virus control (VC — cells infected with working dilution of the virus), serum control (SC — serum in a 1:20 dilution), dose control (DC — five-fold dilutions of the virus).

The NT results were assessed visually by microscopic examination of cells or spectrophotometrically by using the colorimetric MTT assay. To protect the personnel, prior to the optical density measurement, the uncovered plate was exposed to UV light from different sides for 5 minutes in a biosafety cabinet. For visual assessment, the serum neutralizing titer was expressed as the reciprocal value of the last dilution of the serum, which had no signs of CPE presence in 2 or more wells. In the MTT assay, the neutralizing titer was measured spectrophotometrically based on the last dilution where the mean value of $OD_{530–620}$ (hereinafter OD_{530}) was equal to or higher than the threshold value (TV) calculated by the formula:

$$TV = (OD_{530}\ CC – OD_{530}\ VC)/2,$$

where OD_{530} CC – mean value of OD_{530} in control wells with uninfected cell culture; OD_{530} VC – mean value of OD_{530} in control wells containing the sample dilution of the virus.

The control values were assessed as follows: CC — the cell monolayer in the control wells had to be preserved completely. VC — total degeneration of the cell monolayer resulting from the viral CPE. The VC OD_{530} values must not exceed 0.2, while the OD_{530} CC/OD_{530} VC ratio must be equal at least to 8.

Statistical analysis of the data. The statistical significance of the difference among the antibody titers measured by different methods was assessed by using Spearman's rank correlation coefficient. The difference at $p < 0.05$ and $p < 0.001$ was considered significant. The qualitative assessment was performed by using the Chaddock scale; the results were processed with the help of Microsoft Excel and GraphPadPrism programs.

Safety requirements. All the works with the SARS-CoV-2 virus were carried out in compliance with the safety requirements applicable to work with pathogenic biological agents of Group 3. The employees working with the virus were briefed on the safety re-

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Table 2. Arrangement of samples in the 96-well plate during NT

Serum dilution	Serum-1				Serum-2				Controls			
	1	2	3	4	5	6	7	8	9	10	11	12
1 : 20									DC			
1 : 40												
1 : 80												
1 : 160												
1 : 320									CS-1		CS-2	
1 : 640									CC		VC	
1 : 1280												
1 : 2560												

quirements and had valid certificates confirming their training in bacteriology, virology, and biosafety; the certificates were issued by the Russian Research Anti-Plague Institute "Microbe" of the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor).

Results

The SARS-CoV-2 virus for NT was obtained by isolation of the virus from the clinical sample in Vero cell culture. An oropharyngeal swab from a 61-year-old female patient was used for this purpose. The real-time RT-PCR detected a high amount of SARS-CoV-2 RNA (8.82 lg copies/ml) in the swab. Later on, the patient developed clinical symptoms of COVID-19: cough, shortness of breath, fever, loss of taste and smell. The computed tomography of the thoracic organs showed typical consolidation of the pulmonary tissue with the 50% affected area from both sides. The patient was diagnosed with COVID-19, multilobar community-acquired viral pneumonia; the virus was identified (U07.1, ICD 10).

The clinical material was used to infect the Vero cell culture, which was further incubated in a CO_2-incubator for 5 days until appearance of CPE signs displayed as cell rounding; then, the next passage was made. To identify the virus, the material obtained at different passage levels was examined for presence of SARS-CoV-2 RNA by using the real-time RT-PCR and the primers for N-gene [15]. The SARS-CoV-2 RNA in high amounts (9.0; 9.7; 9.2, and 9.9 lg copies/ml, respectively) was detected at the 2nd, 7th, 14th and 21st passages in the cell culture liquid. The in-cell ELISA test showed that the native viral antigen obtained in the infected Vero cells reacted with the COVID-19 convalescent sera and did not react with the sera from healthy individuals.

The taxonomic affilation of the isolate to the severe acute respiratory syndrome-related coronavirus and SARS-CoV-2 coronavirus (clade GH) was identified through sequencing of the S-gene (GenBank: MW161041.1) and complete genome (GenBank: MW514307.1), including the subsequent phylogenetic analysis (**Fig. 2**). The isolated virus strain was named Dubrovka. The sequence of the Dubrovka strain S-gene demonstrated 99.2% similarity with the Wuhan-Hu-1 strain, which caused the epidemic outbreak in Wuhan (China) in December 2019. The Dubrovka strain differs from the Wuhan-Hu-1 strain by a 27 nucleotide deletion in the S-gene (encodes 9 amino acids from 68 to76 a.a. – YMSLGPMVL in the S-protein), thus entailing a relatively high level of differences (0.8%) between these strains. Note that the above deletion was detected in the genome of the Dubrovka strain after the 1st passage in the Vero cell culture and it continued to be observed at the 40th passage level. In GenBank, there are only 2 SARS-CoV-2 strains detected in Belgium and Taiwan in February and March 2020 (GenBank: MW368439.1, MT479224.1) and having the similar deletion.

During the passaging of the virus in the Vero cell culture, the virus titer was increasing (from 4.3 lg $TCID_{50}$/ml at the 2nd passage to 9.0 lg $TCID_{50}$/ml at the 30th passage), and CPE was becoming more and more pronounced. The visual assessment showed that the percentage of dead cells increased on day 5 post-infection at higher passage levels (**Fig. 3**).

The MTT assay shows that if during the infection of the 2nd-passage, the viability of Vero cells was 92%, it reached the minimum level (2–4%) by the 14th passage (**Fig. 4**).

Thus, at low doses of infection, the SARS-CoV-2 strain adapted to the Vero cell culture caused a pronounced CPE, which could be easily detected by microscopic examination of the monolayer. The developed indicator system served as a basis for a neutralization test (NT). The relationship between the value of NAb titer and the cell infection dose was studied. For this purpose, the COVID-19 convalescent serum

with a high titer of antibodies against the SARS-CoV-2 virus was examined through NT at 4 infection doses: 200, 100, 50, and 25 $TCID_{50}$ per well. As a result, the NAb titer values were distributed inversely in relation to the infection dose and amounted to 160, 320, 640, and 1280, respectively. In our further work, we used the dose of 100 $TCID_{50}$ per well for NT, relying on the scientific publications addressing the studies using this dose [16, 18, 19].

Then, we performed NT to examine the blood sera previously described by the amounts of antibodies against SARS-CoV-2. The examination included 46 samples containing IgG antibodies against the virus in dilutions (the titer) ranging from 1:200 to >1:3200 (**Fig. 5**, *a*). To assess the specificity, we also examined 20 sera not containing antibodies against SARS-CoV-2. The NAb titers positively correlated (Spearman's r = 0,714; $p < 0.001$) with the titers obtained in ELISA. All the sera identified as negative by ELISA also demonstrated total absence of virus neutralization. More than half of sera with titers ranging from 1:200 to 1:400 demonstrated neutralizing activity; only one serum with the 1:800 titer did not have NAbs, while all the sera with titers from 1 : 1600 to > 1 : 3200 had neutralizing activity (Fig. 5, *b*).

The study was further focused on spectrophotometric detection of NT results in the MTT assay. During NT with the subsequent MTT staining, the visual examination of the plates showed that the wells with serum dilutions containing the protective NAb titer assumed purple color, which was completely consistent with the microscopic assessment of the cell status. The increasing dilution of the sera led to disappearance of staining reactions in the wells, indicating the cell death at Nab concentrations insufficient for virus neutralization. In the MTT assay, the ratio between OD_{530} in the wells with uninfected cells (CC) and OD_{530} in the infected cells (VC) on average was 28 ± 16, thus implying the reliability of the virus-mediated CPE assessment. **Fig. 6** shows an example of detection of NT results with the MTT assay to measure the NAb titer in serum 1 (1:40), sera 2 and 3 (1:80).

A total of 30 sera were examined in NT by using spectrophotometric detection with the MTT assay. SARS-CoV-2 NAbs in different titers were detected in 16 COVID-19 convalescent sera, while 14 sera seronegative in ELISA did not contain any NAbs. The data obtained through visual microscopic examination and MTT assay (**Table 3**) demonstrated positive correlation; Spearman's rank correlation coefficient (r) was

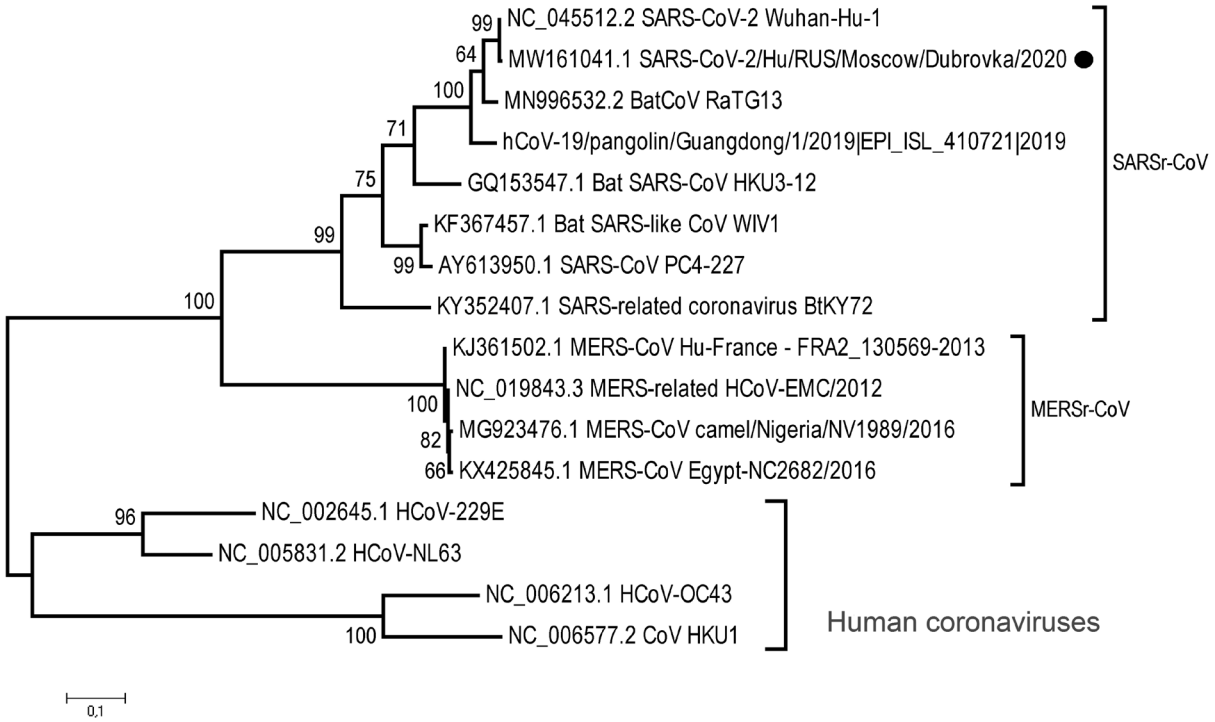

Fig. 2. The phylogenetic tree built on the comparison of nucleotide sequences of the S-gene (3794) of the SARS-CoV-2 Dubrovka strain, coronaviruses pathogenic for humans and phylogenetically related coronavirus strains of animals.

The description of strains includes the GenBank or GISAID strain identifier and the name of a strain. The tree was built with the maximum likelihood method and Tamura-Nei three-parameter evolution model by using the MEGA 5 program. The numbers above the tree nodes show the proportion (%) of 1,000 alternative trees supporting this group. The Dubrovka strain is marked by the ● sign.
SARSr-CoV — representatives of the species Severe acute respiratory syndrome-related coronavirus;
MERSr-CoV — representatives of the species Middle East respiratory syndrome-related coronavirus.

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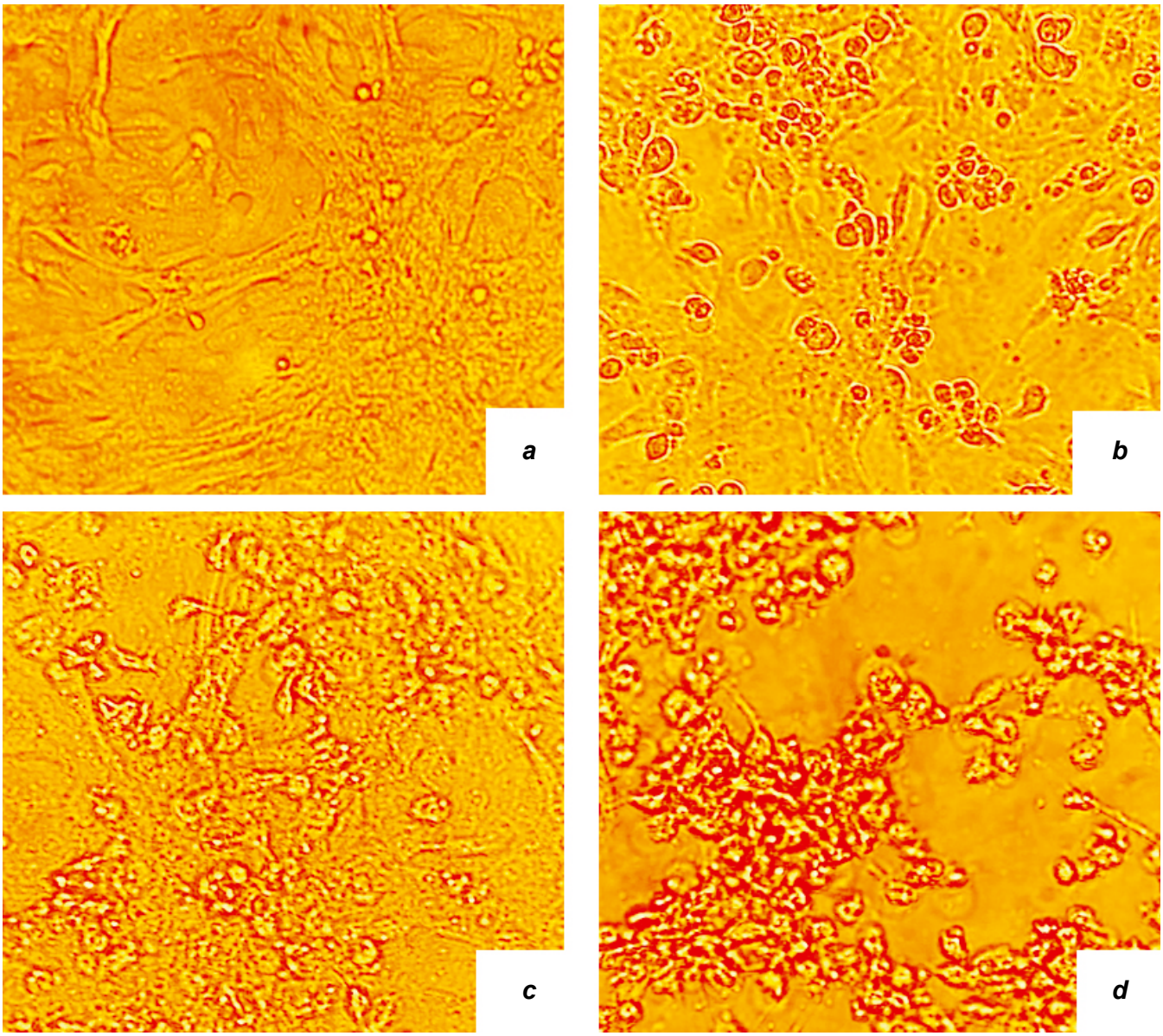

Fig. 3. CPE in Vero cells on day 5 post-infection with the virus of different passage levels.
a — uninfected cells; *b* — 2nd passage; *c* — 7th passage; *d* — 21st passage.

0.963 ($p < 0.05$), thus being consistent with a high correlation between the parameters by the Chaddock scale.

Discussion

The assessment of SARS-CoV-2 neutralizing antibodies is of critical importance for understanding of the potential protective immune response. This study offers methodological approaches to measurement of SARS-CoV-2 NAb titers through NT based on microscopic or spectrophotometric assessment of viability of Vero cells. Both approaches to assessment of NT results can be used when the pronounced CPE of the virus is present in the cell culture.

The SARS-CoV-2 virus obtained at early passages (from 2 to 7), when used for infection of the cell

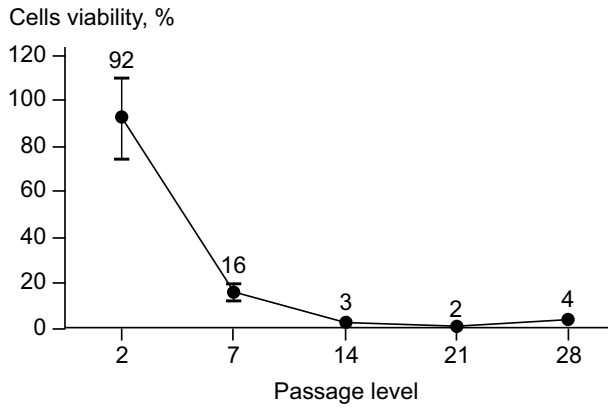

Fig. 4. Viability of Vero cells on day 5 post-infection depending on a passage level of the virus, MOI = 0.0001.

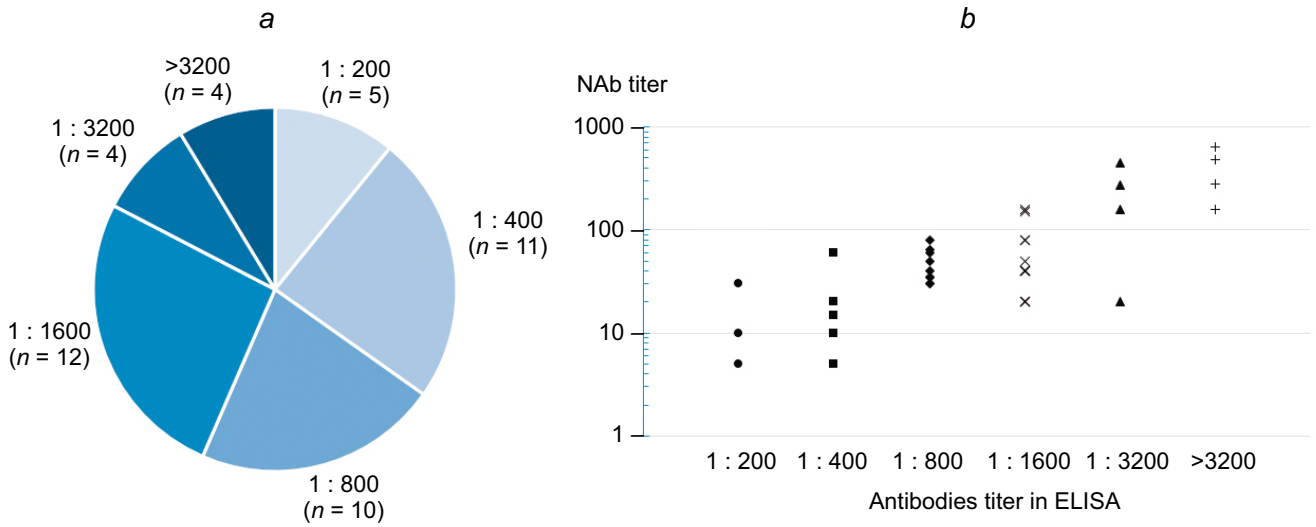

Fig. 5. Distribution of sera with detected IgG antibodies against SARS-CoV-2 by titers (the limiting dilution of the serum; *a*) and correlative relationship between the Nab titers and total antibodies against SARS-CoV-2 in the COVID-19 convalescent sera (n = 46; *b*).

Spearman's r = 0.714; p < 0.001.

culture, caused cell rounding and partial death at high doses of infection, while at low doses, CPE was not pronounced, making it difficult to assess it visually. The extensive adaptation of the virus to the Vero cell culture resulted in pronounced CPE, thus not only helping minimize the subjectivity of microscopic assessment, but also making it possible to assess NT results with the colorimetric MTT assay, by visual or spectrophotometric measurement of stained wells of the plate.

The instrumental measurement provides the numeric format of the results, thus making it possible to use software for statistical analysis of the obtained data and to minimize the employees' eye strain. The main advantage of the MTT assay is its affordability: The test results can be read by using the plate spectrophotometer, thus making it possible to do without expensive equipment and reagents required for other methods of assessment of cell viability [20–22].

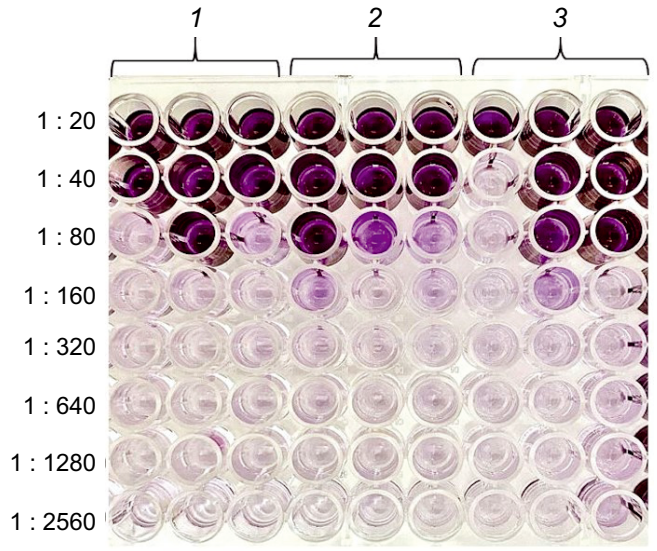

Fig. 6. MTT assay for NT assessment.

Numbers *1*, *2*, and *3* at the top mean the serum reference number; the values of serum dilutions are shown on the left.

In the colorimetric MTT assay, the yellow solution of methylthiazolyl tetrazolium bromide is reduced in viable cells containing NAD(P)H-dependent oxidoreductase enzymes to insoluble purple formazan crystals, while dead or dying cells do not show this effect [21, 22]. Therefore, the MTT assay has an advantage over the cell viability assessment with the crystal violet staining that is applied both to living and dead cells [23].

The study demonstrated that the viral infection dose could be reduced 4 times for NT — from 100 to 25 $TCID_{50}$ per well, thus increasing NT sensitivity 4 times. Note that the Dubrovka strain that is well-adapted to the cell culture provides the pronounced CPE at lower doses of infection (less than 25 $TCID_{50}$ per well), thus increasing NT sensitivity.

Low dilutions of the studied sera (1:20 and 1:40) can demonstrate cytotoxicity and, therefore, affect the accuracy of measurement of the NAb titer. An important requirement associated with reliable NT results, especially when using the MTT assay, is fasting before the blood test to minimize the risk of toxic effect produced by serum components and mandatory application of serum controls (the serum is added to the cells in a 1:20 dilution without the virus).

Conclusion

The SARS-CoV-2 virus adapted to Vero cell culture was used to develop a NAb titer measuring system employing the microscope and spectrophotome-

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Table 3. Comparison of NT results from microscopic examination and MTT assay (COVID-19 convalescent sera)

Serum number	NAb titer	
	microscopic accounting	MTT-test
1	640	640
2	640	640
3	20	40
4	20	40
5	20	20
6	20	20
7	40	40
8	20	40
9	20	0
10	80	40
11	40	80
12	80	80
13	320	320
14	1280	1280
15	160	160
16	80	80

ter-based MTT assay. The colorimetric assay is essential for statistical analysis of the obtained data, minimizing subjectivity in assessment of results and the eye strain associated with microscopic measurement. Thus, the MTT assay offers an efficient and high-throughput method of automated reading of the NT results. Being a vital stain, MTT targets only viable cells, thus increasing the reliability of the obtained results compared to other colorimetric techniques. The offered methodological approaches have a great potential for evaluation of antiviral (natural and vaccine) immunity, for assessment of specific activity of COVID-19 convalescent plasma used for urgent disease prevention and treatment, and for application in trials of vaccines and diagnostic tests as the gold standard for NAb measurement.

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