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Original Article

TARGETED IN VITRO- CONFIRMATION OF THE ANTIVIRAL ACTIVITY OF TENVIR (TENOFOVIR) DRUG AGAINST THE SARS-COV-2 VIRUS VARIANT B. IN KAZAKHSTAN AND IDENTIFYING NSP12 IN THE VIRAL GENOME

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ABSTRACT

COVID-19 became a true medication target worldwide due to its pandemic scale in 2020. Viral infections are generally hard to cope with, especially, if the viral load and spreading speed seriously surpass the immune reaction, both innate and adaptive. Thus, only relatively few drugs were used clinically to stop either in the early stages of viral reproduction like with the help of Tenvir (Tenofovir) or vaccination to gain collective immunity in a particular population. None of the interferon-based drugs showed clear medical effects in medical trials and during pandemic hospitalization protocols. However, purine-analogs like Tenvir and others showed sustainable survival and recovery rates among SARS-COV-2 virus-infected patients at moderate and severe stages of pneumonia caused by this virus. This article reveals the invitro testing of the Tenvir drug on virus strain, Variant B.1.1, Sampled from Kazakhstan and molecular-genetic characterization of NSP12 (non-structural protein 12) located in ORF1b region of the SARS-COV 2 genome. Tenvir or tenofovir was broadly used against HIV infection and this study shows a sufficient effect on viral spread due to RdRP-inhibiting properties in-vitro.

Keywords: COVID-19, NSP-12, RNA dependent RNA-Polymerase (RdRP), purine-analogs, RT-PCR, Gene-product

INTRODUCTION

Tenvir (Tenofovir or T705) is most promising antiviral drug against SARS-COV2 viral infection in post-soviet countries. COVID-19 (Corona virus disease), Wuhan, China, 2019 became pandemic scale headache for global health care system in early 2020 [1]. Virus Strain, ***Variant B.1.1,*** Sampled from Kazakhstan has about 30K bp genome [2-3], divided into three main groups from 5' UTR start, it has ORF1a and ORF1b which contain 16 non-structural proteins (NSPs) that are responsible for proteins synthesis, among them RNA dependent RNA-polymerase (RdRp) the non-structural protein 12 (nsp12) is responsible for expressing this gene product (non-catalysis featured) The second group is so called structural proteins -**S**-protein-Spike, **E**-protein -Envelope, **M**-protein-membrane and **N**-protein-nucleocapsid [4]. The third group is so called accessory proteins (ORF3ab, ORF6, ORF7ab, ORF8, ORF9bc and ORF10), Accessory proteins are shed between structural proteins and form isles or gaps between structural proteins closer to 3'UTR end. **[6].** All these three main parts of viral genome have functions, the structural proteins are responsible to build up four main structures that are needed to build the new virions to proceed the further infection through its reproduction i.e. viral replication. The accessory proteins provide protection of structural proteins from non-specific immune response like accessory proteins 8b and 8ab (ORF8ab) suppress the interferon signaling pathway by mediating ubiquitin-dependent rapid degradation of interferon regulatory factor 3 (IRF3) **[5].** And of course, the ORF1a and ORF1b the non-structural proteins are responsible both for replicating the viral genome and maintaining the intact viral replication machinery. It is also known that RNA dependent polymerases provide the viral genome with genetical diversity through the controlled positive mutations. The open reading frame **ORF1a** as well as **ORF1b** together combined occupy two thirds of entire viral genome and therefore these ORFs represent a great concern for scientist to study, mostly because this part of genome's task which is to ensure the viral replication processes. The NSP12 synthesizes the RdRp that ensures smooth viral replication on host ribosomes, moreover ORF1a and neighboring NSPS support NSP12 to sustain the viral replication. Even the spike protein expression is essential in early stages of viral expression [4].

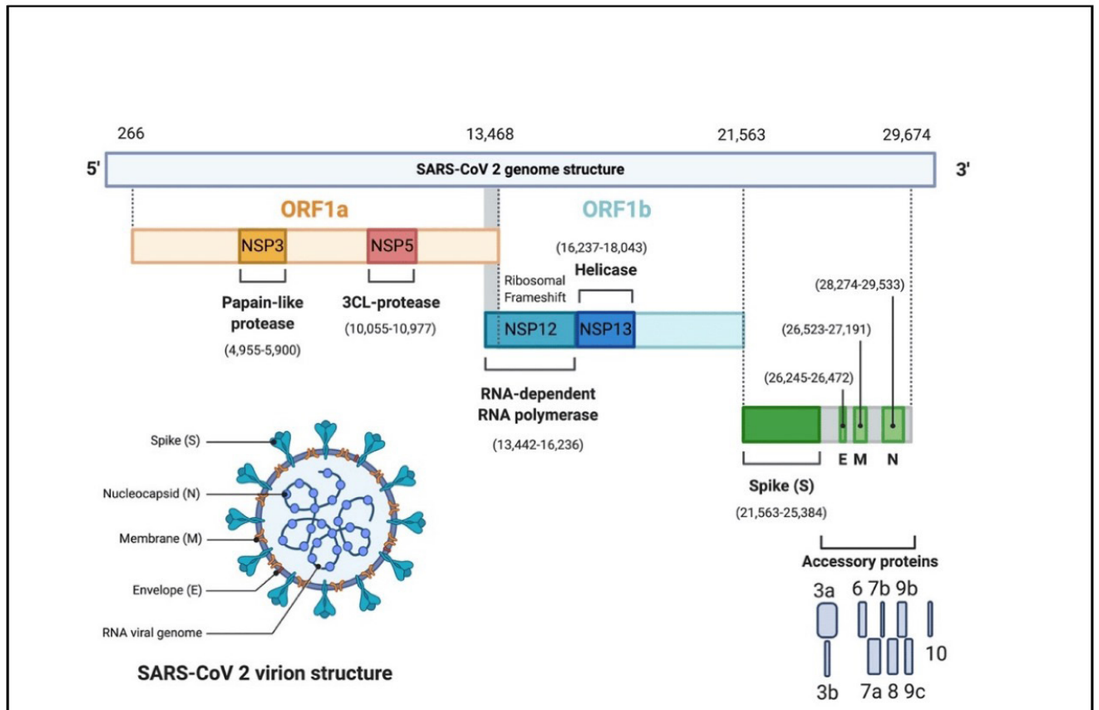
Figure 1 – the genome overview of SARS-COV-2, Wuhan- Hu-1 strain, December 2019, from 5'-UTR to 3'-UTR with four main antiviral sites: a) ORF1a (NSP3 and NSP5 for instance) – innate immune system induction – interferon activity-viral replication. b) ORF1b (NSP12 -RNA dependent RNA polymerase and NSP13 for instance) – nucleotide analogs in our case purine analogs- *Tenvir (Tenofovir)- RdRP inhibitor* –is used primarily against HIV-infection before COVID19 pandemics occurred c) structural proteins, especially, spike protein is main target for vaccination strategies worldwide to induce adaptive immunity-IgGs that neutralize viruses and virions effectively d) the accessory proteins (ORF8a and ORF8ab for instance) help to suppress the host interferon cellular activity during innate immunity encounter, these ORFs are located close and dense to structural proteins, spike protein recognition by naturally and synthetically derived monoclonal antibodies neutralize the accessory proteins activity too [6].

Diagram 1. Genome of the first Wuhan strain sequenced in March 2020

Source: https://doi.org/10.3390/pathogens9050331

Tenvir (Tenofovir)

Tenofovir belongs both for anti-HIV drugs and Antihepa-



titic drug according to the producer's manual. Tenofovir represents the reverse transcriptase inhibitors or nucleoside reverse transcriptase inhibitors (NRTIs) are structural analogues of nucleic acids, **adenosine monophosphate** which competitively inhibit the reverse transcription by causing the chain termination after they got involved into viral DNA. This viral DNA-incorporation causes so-called 'lethal mutagenesis'. Tenofovir is also used as antiviral drug against chronic hepatitis B as nucleotide analogue. Tenofovir inhibits the HBV (hepatitis B virus) polymerase by competing with natural substrate for in cooperation with growing viral DNA-strand causing as in HIV (human immune deficit virus) chain termination, subsequently stalls the reverse transcription and synthesis of viral DNA. Tenofovir is yet another nucleotide analogue that was initially designed to inhibit the HIV (human immunogenicity virus) reverse transcriptase by interfering the ATP-Polymerization in the growing nucleic acid chain [7,8]. Tenofovir was also assumed to be effective against COVID-19 as it showed the tendency to dock the RNA- dependent RNA-polymerase (RdRP) and silence its activity in replication as well as in transcription and translation of structural and accessory proteins making virions assembly almost impossible [9]. Tenofovir that is used in our study is for oral administration medicine in a form of disoproxil furmate (TDF) has many side effects if it is used in high dosage, such as renal toxicity, bone density degradation etc. [10].

In-vitro studies suggest that at concentrations under 100µM, tenofovir does not inhibit the viral replication in VeroE6 cells at the multiple infections in a so-called preventive way, when tenofovir was administered 1h prior to infection and up to 48h post infection. In the discussion of results, researchers came to idea that tenofovir in ATP-forms require the activation by host kinase and any cell type has probably the proper kinase activity to launch the tenofovir antiviral features and was suggested to try a study on human airway epithelial cells [11,12,13].

2.0 Materials and methods of research

2.1. Biomaterial/sample

The biological samples taken from sick patients during the COVID-19 pandemic thorough their consent in the favor of scientific research, from the Scientific and Practical Center for Sanitary and Epidemiological Expertise and Monitoring, Almaty. Strain: ***SARS-CoV-2/human/KAZ/B1.1/2021***

2.2. Viral RNA isolation

Isolation of viral RNA from virus-containing material was carried out using the *QIAamp Viral RNA Mini Kit, Qiagen,* according to the manufacturer's instructions.

2.3. Selection and synthesis of primers

The primers were designed with various computer programs, mainly Oligo 6 and Vector NTI Suite 10. The reaction composition and the temperature-time regimes were selected according to the annotation attached to the enzyme and the properties of the primers.

The designed primers were synthesized on the H-16 oligonucleotide synthesizer (manufactured in Germany) according to the instructions attached to the device. The elution of synthesized primers from the columns was carried out with a concentrated ammonia solution. The primers were then dried on a rotary evaporator and purified by alcohol precipitation.

2.4. Conducting PCR

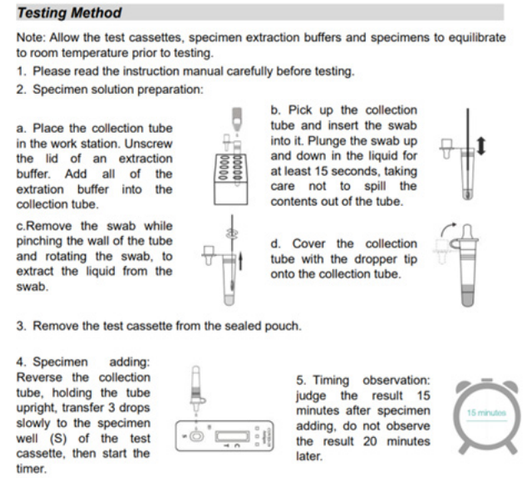


Diagram of usage 1. Express COVID19 Antigen count of GenSure-kit

A set of **superscribed III One Step RT-PCR with platinum Taq**, invitrogen, was used to perform the PCR. The reaction composition and the temperature-time regimes were selected according to the annotation attached to the enzyme and the properties of the primers. Techne performed the production of specific DNA sites using a GeneAmp PCR 9600 thermal cycler, Applied Biosystems and TC512. Further detection of amplification products was carried out in the device for horizontal electrophoresis «G-100», manufactured by Pharmacia. For electrophoresis, **a 1% solution of agarose in TA** was used. The results were visualized and recorded with the «Quantity One» program. «DNA Ladder 1 kb» from invitrogen was used as a comparative marker for molecular weights.

2.5. Gene sequencing

Viral RNA and DNA are isolated using a set of QIAamp virus RNA and trizol reagents according to the manufacturer's instructions. The RNA is eluted with water, 2 times 40 µl each. Given the fact that the full genome of the SARS-COV-2 virus includes about 30 thousand nucleotides, a set of primers is used to amplify the full genome for sequencing. PCR is performed using a set of single-stage RT-PCR elevated III systems. Cleaning of PCR products is carried out using the AMPure kit according to the manufacturer's instructions. The quality of PCR products is checked by electrophoretic analysis. PCR sequencing products were obtained using the BigDye® Terminator v3.1 cyclic sequencing kit. The purification of the sequencing reaction was carried out with the Clean Seq Kit. The sequencing is carried out on 16 capillary sequencers of the genetic analyzer 3130xl (Applied Biosystems / Hitachi). Genomic assembly, genome annotation, comparative genomics and phylogenetic analysis were performed using the CLC Genomic Workbench 11.0.1 program. Genome-wide sequencing of ILT strains is performed using the new generation NGS sequencing method, the Ion GeneStudio™ sequencer. The S5 system is bundled with the Ion Chef SmartStart system.

2.7. Comparative and phylogenetic analysis of the nucleotide sequence of genes

The available complete genomes of the SARS-COV-2 virus downloaded from the GenBank are used for the complete analysis. Phylogenetic trees were created using the maximum similarity method on the CLC Genomics Server 12.0 using the «neighbor Joining» method and the Jukes-Cantor model with gamma distribution 1.0 and 100 replications to assign confidence levels to branches. The MEGA 7.0 application is also used for phylogenetic analysis.

2.8. Determination of cytotoxicity of Tenvir (Tenofovir) for cell culture

Tenvir (Tenofovir) was selected to study antiviral activity against the SARS-CoV-2 virus. Before determining the antiviral activity, a working dose was established that did not cause toxicity to cell culture.

2.9. CCK8 test for cell-viability in Tenofovir of concentration 50µg/ml-200 µg/ml

The CD8-Kit-8 (KK-8) enables sensitive colorimetric analyses to determine the viability of cells in the analysis of cell proliferation and cytotoxicity. The dojindo-tetrazole salt, **WST-8, soluble in water**, is restored in the cells by the activity of dehydrogenase, forming a yellow formazan dye, soluble in a medium for tissue culture. The amount of the dye formazan, which is formed as a result of the activity of dehydrogenases in cells, is directly proportional to the number of living cells. Three steps: Step 1: Add 10 Pl of Cell Counting Kit-8 to each well in a 96 well microplate. Step 2: Place in a CO2 incubator for 1-4 hours to react. Step 3: Measure the absorbance at 450 nm with a microplate reader.

2.10. COVID19 Antigen count *of GenSure-kit* from a specimen swab: TID50

Equipment

- oligonucleotide synthesizer Synthesizer H-16, K&Laborgeraete, Germany;

- thermal cycler GeneAmp PCR System 9600, Applied Biosystems;

- thermal cycler TC-512, Techne;

- thermal boards DryBlockHeater, Techne;

- shakers, vortexes Vortex Genie 2 Shaker, Cole-Parmer;

- automatic micropipettes, Eppendorf;

- apparatus for electrophoresis of nucleic acids G100,

Table 1. The TID50 (viral load) scheme count (viral load pattern)

Microorganism	Concentration	Crossreactivity (YES/NO)
Influenza A (H1N1, H3N2	1.0 x 10^5 TCID50/mL	NO
Avian Influenza (H5N1, H7N9)	1,7 x 105 TCID50/mL	NO
Influenza B (Victoria, Yamagata)	2,5 x 10^5 TCID50/mL	NO
Respiratory Syncytial Virus	3,8 x 10^5 TCID50/mL	NO
Rhinovirus	1,4 x 10^5 TCID50/mL	NO
Adenovirus	1,8 x 10^5 TCID50/mL	NO
Measales	1,0 x 10^6 TCID50/mL	NO
Human coronavirus (OC43,229E, NL63)	1,0 x 105 TCID50/mL	NO
Coronavirus, MERS	1,2 x 10^6 TCID50/mL	NO
Mycoplasma pneumoniae	1,0 x 10^6 CFU/m	NO

Pharmacia;

- gel documenting system “BioRad”, USA;

- microcentrifuge “MiniSpin”, Eppendorf;

- refrigerator – 20 °C;

- package of application programs for analysis of DNA sequences - DNASYS MAX 1.0, Sequencher, Vector NTI, BioEdit, GENEDOC, Staden package.

- **microplate reader OD (optical density)** Plate Verification instrument for Hipo MPP-96, BioSAN-for CCK-8

Materials, reagents, and solutions

- Recombinant Taq DNA Polymerase 5000 unit/mL, SIGMA.

- T4 DNA Ligase;

- ProtoScript® II First Strand cDNA Synthesis Kit;

- RNAZap decontamination solution;

- SuperScript IV One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, USA) (100 reaction);

- 310 and 31xx Running Buffer, 10X;

- BigDye™ Terminator v3.1 Cycle Sequencing Kit, 100 reactions;

- Reagent for safe staining of agarose gel SYBR Safe DNA gel stain;

- UltraPure™ nuclease-free distilled water;

- Microcentrifuge tubes, 1.5 ml, 500 pcs. pack; Eppendorf;

- Microtubes 0.2 ml with flat cap 1000 pcs/pack Tubes, 0.2 mL, flat cap 1000/pc Eppendorf;

- Microtubes 0.5 ml with flat cap 1000pcs/pack Tubes, 0.5 mL, flat cap 1000/pc Eppendorf.

The vero cells E6

Vero C1008 [Vero 76, clone E6, Vero E6], from African green monkey kidney, by Sigma Aldrich. Vero cells are derived from the kidney of an African green monkey. These are anchorage-dependent cells that have applications in molecular and cell biology research. Vero E6 cells enable achieving high titers of severe acute respiratory syndrome coronavirus (SARS-CoV virus) [18]. Split sub-confluent cultures (70-80%) 1:3 to 1:10, i.e., seeding at 1-3x10,000 cells/cm2 using 0.25% trypsin or trypsin/EDTA; 5% CO2; 37°C.

The culture medium

DMEM (D6546) + 2 mM L-Glutamine (G7513) + 10% FBS / FCS (F2442): DMEM with 2% bovine serum and 0,1% (100U/ml) antibiotics (Penicillin-Streptomycin) was prepared.

The virus preparation and safety procedures

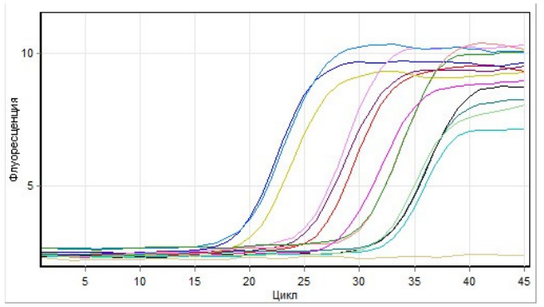
An isolate of the ***SARS-CoV-2/human/KAZ/B1.1/2021 strain***, was passaged on various occasions in Vero E6 cells to establish a high titer stock that was used in all our experiments. Since SARS-CoV-2 is classified as a high-risk pathogen in Kazakhstan, all procedures performed with the virus, including infecting cell lines and their further supervision in BSL-2 (Biosafety level) class laboratory.

3.0. Results

3.1.1. RT-PCR test results

Figure 1a. the initial data is the fluorescent signal Cycling A. Red and **Figure1b.** Quantitative data for Cycling A. Red. According to the peaks report the following samples were taken for further proceedings with subsequent CT (cycle threshold) value: untreated- samples #1-20, -sample #2-21, -sample#3-23, -sample #4 - 24, -sample #5-25, Other samples above 25cycles and lover 20cycles were excluded. Over CT25 not informative, lower CT20 too high viral load. maximum of 40 thermal cycles. The lower the CT value, the higher the amount of viral genetic material in the sample (as an approximate indicator of viral load). An increase in the CT value by 3 points approximately corresponds to a 10-fold decrease in the amount of viral genetic material.

There is no difference between Ct and Cq values. All these values are the same, but have different names. Ct stands for a threshold value cycle and Cq for a quantification cycle. In order to standardize the nomenclature of PCR analysis, the MIQE manual (minimal information on the publication of quantitative real-time PCR experiments) recommends using the more general term «quantitative evaluation cycle» (Cq). Real-time PCR usually determines the absolute number of the target sequence or compares the relative amounts of the target sequence in the samples. Although fluorescent dyes and real-time PCR probes must be sequential, there is significant background fluorescence in most real-time PCR experiments. Therefore, it is extremely important to bypass this background signal in order to get meaningful information about your goal. This problem is solved by two values in the PCR in real time, namely the threshold line and the Cq value. A threshold line



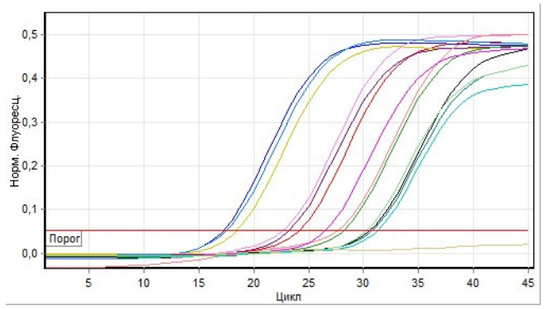


Table 2. Quantitative report, information about the test as well as Quantitative analysis parameters. The samples taken from sick patients during the COVID-19 pandemic, from the Scientific and Practical Center for Sanitary and Epidemiological Expertise and Monitoring, Almaty. During identification in Real-Time PCR, all samples showed a positive result, where the peak range was from 17 to 30 cycles.

Name of test	Test 2021-03-29 (1)
Begin of test	29.03.2021 10:24:30
End of test	29.03.2021 12:10:50
Operator	Nurlan
Notices	None
Test are completed on program version	Rotor-Gene 1.8.17.5
Test signature	Test signature: appopriate
Signal level Green	5,
Signal level Orange	5,
Signal level Red	5,
Thresehold	0,05139
Exclude the cycles up to	1,000
Standard curve imported	*Hem*
Graph standard (1)	N/A
Graph standard (2)	N/A
Begin the cycle normalization from (**Cq**)	**11**
Slope adjustment	*Hem*
Background Threshold (NTC)	0%
The Threshold for The Effectiveness of The Reaction	Off
Normalization method	Dynamic. Background normalization

is a point or detection stage at which the intensity of the fluorescence in a reaction reaches a value greater than the background level. Before performing the PCR, the software will set a threshold value in your cycler. It is literally a line in your graph that represents a level greater than the background fluorescence, which also crosses your reaction curve somewhere at the beginning of your exponential phase. The Cq value is the cycle number of the PCR at which the reaction curve of your sample exceeds this threshold line. This value indicates how many cycles it took to detect a real signal in your samples. If the PCR is performed in real time, a reaction curve and thus a large number of Cq values are generated for each sample. Your cycler’s software calculates and displays the Cq value for each of your samples in a graph.

The Cq values are inversely proportional to the amount of nucleic acid contained in your sample and correlate with the number of target copies in your sample. Lower Cq values (usually less than 28 cycles) indicate a large number of target sequences. Higher Cq values (more than 38 cycles) mean a lower amount of your target nucleic acid. However, high Cq values may also indicate some problems with the target or PCR setting.

3.1.1. Determination of morphological characteristics of viral strains of the SARS- COV-2 virus.

To determine and compare morphological characteristics, viral preparations related to various variants of the SARS-COV-2 virus were taken: Wuhan, British, Delta and Omicron. As a result, it was found that virions are spherical, with a size of 115-125 nanometers in the presence of spikes (surface glycoproteins) with a length of about 10 nanometers. Electron microscopy of the virus is presented below.

3.1.2 Study of biological properties of isolated isolates and their subsequent deposition

Isolates were isolated from samples supplied by the Scientific and Practical Center for Sanitary and Epidemiological Expertise and Monitoring. After studying the biological properties, these isolates were deposited in collections of microorganisms under the following names: SARS-CoV-2 / human / KAZ / B1.1/2021 and SARS-CoV-2 / human / KAZ / Britain / 2021.

Sequencing of significant viral genes. Comparative and phylogenetic analysis of the nucleotide sequence of viral genes.

The main indicators of PCR (sensitivity, specificity, product yield and the possibility of further manipulations with the final product) in most cases are determined by how well the specific structures of the primers are selected and developed [45]. Currently, there are several information databases (Ref Seq, GenBank) and many programs (Primer3, Fast PCR, etc.).) for the design of primers depending on the purpose.

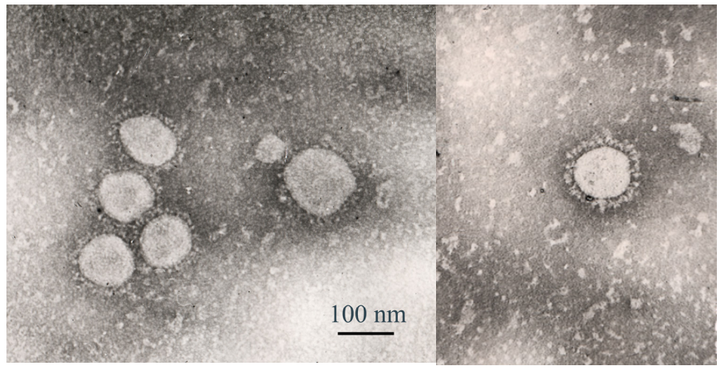


Figure 2. Electron microscopy of the SARS-COV-2 virus. Uv. 120,000, on the microscope Jeol Jem 100 XC, (provided by Kozhabergenov N.S.)

The search and development of nucleotide sequence primers was carried out manually on the NCBI website using the GenBank database. The nucleotide sequence of specific primers was selected based on the reference strain MN908947.3. The specificity of the primers was verified with the NCBI Primer BLAST Service. The primers were selected so that each pair of primers overlapped, and their sequence was conservative among all variants of the SARS-CoV-2 virus. As a result, 65 pairs of sequencing primers were selected to develop the complete genome of SARS-CoV-2 virus variants with an overlap of about 100 nucleotide pairs (bp). The estimated amplitude length ranges from 604 to 772 bp.

Table 3. Sequencing primer parameters, the main gen product of SARS-*CoV-2/human/KAZ/B1.1/2021 on* ORF1ab here is NSP12 that is responsible for RNA dependent RNA polymerase with **2697**bp long as same as Wuhan-Hu-1 strain has **2696 bp [Wuhan-Hu-1 -GenBank MN908947.3]**.

#	Primer orientation	Sequence (5'->3')	Start	Stop	T C^0(primer heat temp)	GC%	Product size(bp)
PP 28	Forward	TGGAACCACCTTGTAGGTTT	12891	12910	56.57	45.00	652
	Reverse	AGCCCTGTATACGACATCAG	13542	13523	56.52	50.00	
PP 29	Forward	ACCCTGTGGGTTTTACACTT	13341	13360	56.86	45.00	706
	Reverse	AACAATACCAGCATTTCGCA	14046	14027	56.32	40.00	
PP 30	Forward	TACGCCAACTTAGGTGAACG	13963	13982	57.93	50.00	639
	Reverse	TAGATTACCAGAAGCAGCGT	14601	14582	56.36	45.00	
PP 31	Forward	CCACTTCAGAGAGCTAGGTG	14478	14497	57.04	55.00	713
	Reverse	CTCTAGTGGCGGCTATTGAT	15190	15171	56.88	50.00	
PP 32	Forward	CCAAGTCATCGTCAACAACC	14913	14932	57.03	50.00	644
	Reverse	CATTAACATTGGCCGTGACA	15556	15537	56.71	45.00	
PP 33	Forward	GTGTTGTAGCTTGTCACACC	15372	15391	56.96	50.00	659
PP 34	Forward	ATGTTGGACTGAGACTGACC	15834	15853	56.86	50.00	669
PP35	Forward	TCCGTATGTTTGCAATGCTC	16374	16393	56.80	45.00	712

Table 4**.** RNA dependent RNA polymerase nucleotide positioning of SARS-*CoV-2/human/KAZ/B1.1/2021*on NSP12, almost the same nucleotide position as Wuhan-Hu-1 strain has: 13442 – 16236 **[Wuhan-Hu-1 -GenBank MN908947.3]**.

Gene: ORF1ab	Gene product/region	Nucleotide position
5'UTR 241		106
Non-structural protein NSP 12		14120
Non-structural protein NSP 12		14408
Non-structural protein NSP 12		14676
Non-structural protein NSP 12		15017
Non-structural protein NSP12		15279
Non-structural protein NSP12		16176

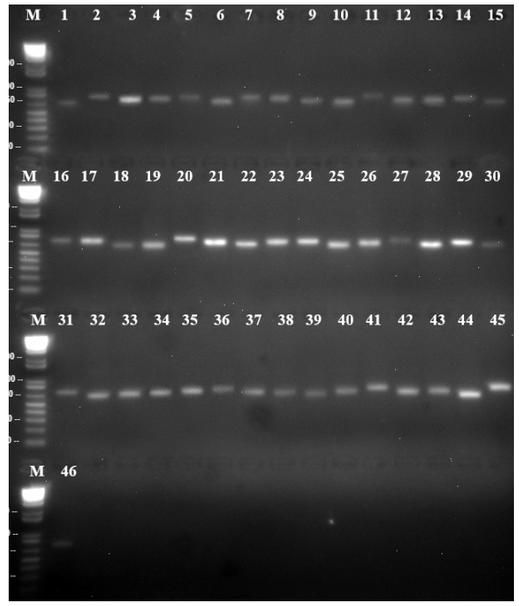


Figure 3. Electropherogram of results of RT-PCR genes of **ORF1ab** variant B. Virus SARS-CoV-2 As it can be seen from Figure 1, the developed primers make it possible to generate specific PCR products. Electrophoretic analysis yielded products with molecular weights between **604 and 772 bp**. from the ORF1ab gene. The length of the amplicon absolutely corresponds to the length of the synthesized primers.

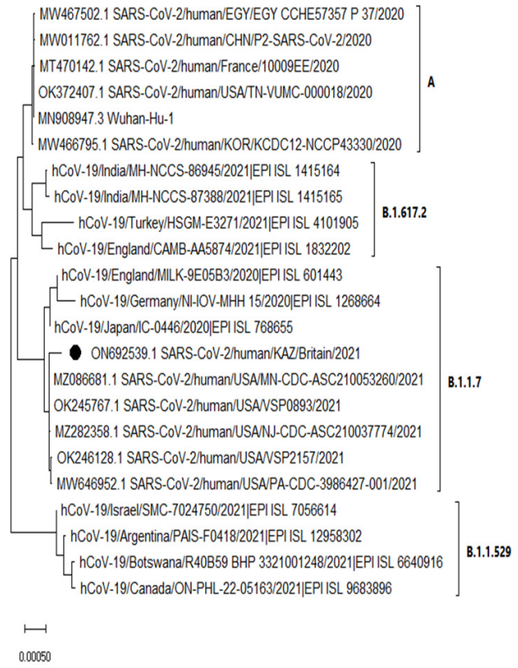


Figure 4. Phylogenetic analysis of the SARS-CoV-2 **strain/human/KAZ/Britain/2021** [2].

3.2.1. Phylogenetic analysis of the SARS-CoV-2 strain/ human/KAZ/Britain/2021

3.2.2. Phylogenetic analysis of the SARS-CoV-2 strain/ human/KAZ/B 1.1/2021

3.2.3. Uploading a genome-wide nucleotide sequence to the GenBank database

The virus data obtained were collected using the program Sequencer V.5.4. The isolated nucleotide sequence of the ge-

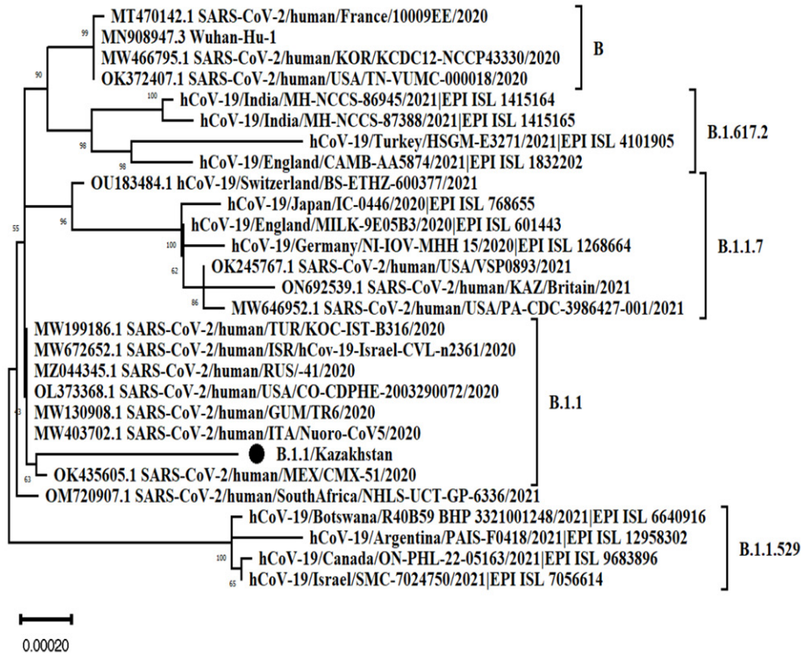


Figure 5. Phylogenetic analysis of the SARS-CoV-2 **strain/human/KAZ/B 1.1/2021 [2].**

nome of the virus strain was uploaded to the NCBI database under registration number ON692539.1 dated June 07, 2022, and OP684305.1 dated October 20, 2022. The obtained sequences were analyzed using the Pangolin COVID-19 database (https://pangolin.cog-uk.io), as a result of which it was established that they belong to lines B.1.1.7 and 1.1 (Graph 3 and Graph 4, respectively)

The analysis of the nucleotide sequence of the whole genome showed that the strains isolated in the Almaty region are 100% similar to SARS CoV-2/human/KAZ/Britain/2021 and SARS-CoV-2/human/KAZ/B1.1/2021 and have 99.80% and 99.82% of the total similarity. identity with the reference strain SARS-CoV-2, the Wuhan-Hu-1 isolate belonging to the B-line (see table4, as well as graph 3 and graph 4)

3.3.1. Determination of cytotoxicity of drugs for cell culture

The cytotoxicity results showed that Tenvir at a dosage of **50 µg** is non-toxic to cell culture and will be used to study the

Figure 6. Identification of the SARS-CoV-2 **strain variant/human/KAZ/Britain/2021** according to the Pangolin COVID-19 database

Figure 7. Determination of the SARS-CoV-2 **strain variant/human/KAZ/B 1.1/2021** according to the Pangolin COVID-19 database

Table 4. Analysis of the nucleotide sequence of the entire genome of SARS-CoV-2 viruses isolated in the Almaty region in the Republic of Kazakhstan

№	Variant	Strain	Country	Date	Genome identity%	GenBank
1	B	*SARS-CoV-2 isolate Wuhan-Hu-1*	PRC	18.03.2020	Ref.	MN908947.3
2	B 1.1.7	*SARS-CoV-2/human/KAZ/Britain/2021*	Kazakhstan	07.06.2022	99.80%	ON692539.1
3	B 1.1	*SARS-CoV-2/human/KAZ/B1.1/2021*	Kazakhstan	20.10.2022	99.82%	OP684305.1

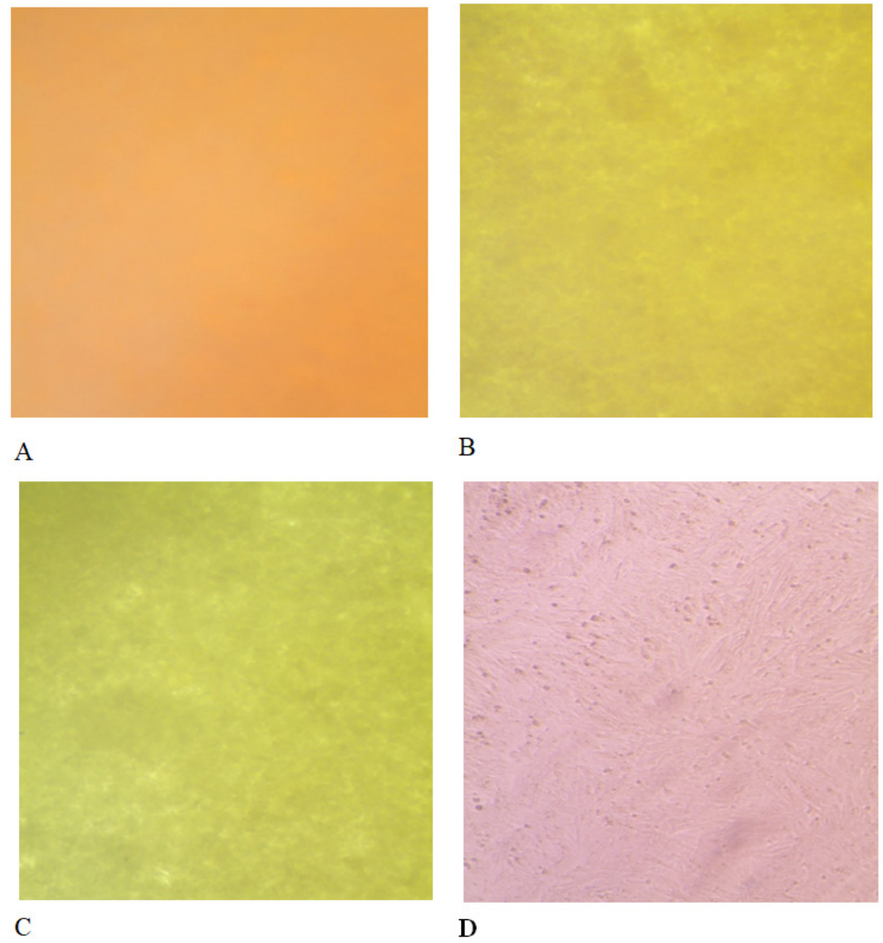


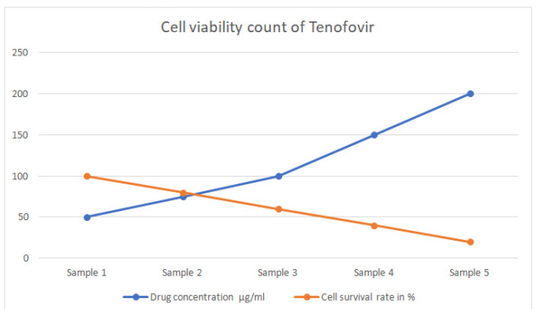
Figure 8. Determination of Tenvir cytotoxicity level in Vero cell culture

A – 200 µg, B – 150 gµ, C – 100 µg, D – 50 µg.

Tenvir also shows high toxicity rates (cell layers are clearly torn even at minimum concertation) because only relatively few concentrations are recommended to achieve therapeutical effect.

inhibition of antiviral activity. Also, with an increase in the dosage of the Tenvir drug, alkalization of the medium and detachment of cells from the surface are observed.

3.3.2. CCK8 test for cell-viability in Tenofovir in concentration of 50µg/ml



Graph 1. VeroE6 cells survival rates in percentage in a presence of 5 drug concentrations of Ribavirin, Favipiravir and Tenofovir starting from 50µg/ml to 200 µg/ml. The highest survival rates belong to sample 1.

3.3.3 Antiviral activity

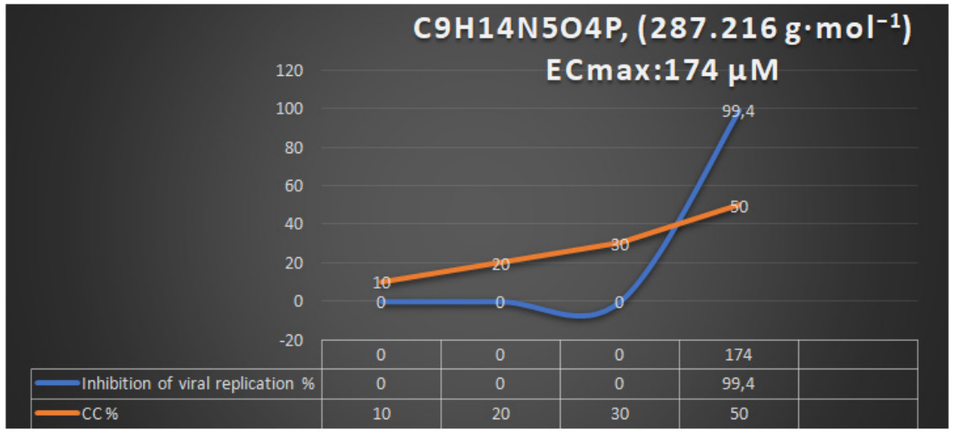
COVID19 Antigen count *of GenSure-kit* from a specimen swab: TID50 is 10 or MOI:2 Significant viral load 24H Incubation with Antivirals. The study of the antiviral activity of the drugs was carried out in a culture of Vero cells infected with the **SARS-CoV-2 virus, variant B**, based on the coefficient of inhibition of the cytopathic activity of the virus and virus reproduction. After 24 hours of incubation after infection of cells (at a dose of 10 CPD50) with a, when drugs were added in a concentration range of **50 µg/ml**, the cytopathic effect of the virus was detected to varying degrees. The results of viral suppression are presented in Table 5.

Discussion

The Tenvir – antiviral drug is a purine analog that was well established since 2003 MERS-epidemics, furthermore they have been recommended themselves as effective pharmaceutical therapy against chronic diseases caused by long term viral infections like HIV, turning soon into AIDS, or Hepatitis B/C lifelong viral load control. Cellular toxicity depends on

Table 5. Results of assessing the antiviral activity of drugs against the SARS-CoV-2 virus, variant B, as a result of a study of the antiviral activity of the drugs Tenvir 99.31% Tenvir showed the degree of inhibition and is one of the most effective drugs for the treatment of viral infection

Drug	Concentration mkg\ml	Virus accumulation lg, PFU/ml	Suppression of reproduction virus, lg	Inhibition coefficient, Percentage %
Tenvir	50	5,03±0,15	2,17	**99,31**



Graph 2. The intact VeroE6 cells seeded in 96-well plates were inoculated with SARS-CoV-2 titer during a **48**-hour infection phase, then **174µM** Tenvir (Tenofovir) was added. The **174µM** concentration and below of Tenofovir -cytotoxic safe concentration, therefore, the tested inhibitor concentration did not affect cell viability (CC-levels) and viral stock dilution 10^{-7}. The EC max is almost 100% inhibition, the cell viability does decrease with higher drug concentration with no higher antiviral effect.

many factors, not only increasing drug concentration for instance, but also how long a drug can suppress the viral replication rates with significant side effects factor. Thumb rule – the lower daily concentration is prescribed the less damage it brings during pharma dynamics both in-vivo and in-vitro. The importance of detecting ORF1ab products represent a great interest to fight current viral infections and develop future antiviral strategies using purine-analogs medication to increase the positive income among infected patients.

CONCLUSION

Tenvir is now most effective anti-viral orally administrated drug at concentration range of 50 µg/ml. The viral infection, particularly, the SARS-COVID-2 has many ways to invade the host cell, first due to high variability of mutation of spike protein (viral structural protein) that could be coupled by systematic vaccination – B-cells production – immunoglobulins (active immunity). The second strategy is to use purine-analogs that seriously inhibits the viral replication, thereby, stopping the viral load development. Purine-analogs also causes so called 'lethal mutagenesis' leading to viral extinction via already well known and established drugs that were already used against other diseases based on virus ontogenesis. The purine analogs could be used not only as main treatment strategy but also as option or in combination with others, furthermore, dexamethasone increases the survival rates in severe cases of COVID-19 infection, especially, in pneumonia complication [14]. This study is still important because it shows that traditional antiviral drugs is effective in vitro against new and local SARS-COV-2 variants and shows high conservatism of NSP12 that is responsible for RdRP expression in viral genome and therefore beneficial medication target. Unlike spike protein, that tends to mutate relatedly frequent into the new strains of concern appear where vaccination nowadays brings less effectiveness. In addition, even before pandemics of COVID-19, the corona viruses perfectly deal with interferon proteins during innate, non-specific immune response. It is worth to mention that purine analogs like adenine and guanine analogs are most capable to cause lethal mutagenesis rather that pyrimidine analogs [15-17]. The classical pyrimidine analogs are tested nowadays mostly against cancer development [18].

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ҚАЗАҚСТАНДАҒЫ SARS-COV-2 ВИРУСЫНЫҢ B НҰСҚАСЫНА ҚАРСЫ TENVIR (ТЕНОФОВИР) ПРЕПАРАТЫНЫҢ ВИРУСҚА ҚАРСЫ БЕЛСЕНДІЛІГІН IN VITRO- РАСТАУ ЖӘНЕ ВИРУСТЫҚ ГЕНОМДАҒЫ NSP12 АНЫҚТАУ

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2020 жылы пандемия ауқымына байланысты COVID-19 бүкіл әлем бойынша дәрі-дәрмектің шынайы нысанасына айналды. Вирустық инфекциялармен күресу әдетте қиын, әсіресе вирустық жүктеме мен таралу жылдамдығы иммундық реакциядан айтарлықтай асып кетсе, туа біткен және бейімделген. Осылайша, вирустық көбеюдің ерте кезеңдерінде, мысалы, Тенвирдің (Тенофовир) көмегімен немесе белгілі бір популяцияда ұжымдық иммунитетті алу үшін вакцинацияны тоқтату үшін клиникалық түрде салыстырмалы түрде аз ғана дәрілер қолданылды. Интерферон негізіндегі препараттардың ешқайсысы медициналық сынақтарда және пандемиялық ауруханаға жатқызу хаттамаларында айқын медициналық әсерлер көрсеткен жоқ. Дегенмен, Тенвир және басқалар сияқты пурин аналогтары SARS-COV-2 вирусын жұқтырған пациенттер арасында осы вирус туындаған пневмонияның орташа және ауыр кезеңдерінде тұрақты өмір сүру және қалпына келтіру көрсеткіштерін көрсетті. Бұл мақалада Қазақстаннан алынған B.1.1 нұсқасының вирус штаммына Tenvir препаратының invitro сынағы және SARS-COV 2 геномының ORF1b аймағында орналасқан NSP12 (құрылымдық емес ақуыз 12) молекулалық-генетикалық сипаттамасы ашылады. Тенвир немесе тенофовир АИВ-инфекциясына қарсы кеңінен қолданылды және бұл зерттеу in-vitro RdRP- тежеу қасиеттеріне байланысты вирустың таралуына жеткілікті әсерін көрсетеді.

Түйінді сөздер: COVID-19, NSP-12, РНҚ-ға тәуелді РНҚ-полимераза (RdRP), пурин-аналогтар, RT-ПТР, Ген-өнім.

УДК: 615.371

НАПРАВЛЕННОЕ IN VITRO ПОДТВЕРЖДЕНИЕ ПРОТИВОВИРУСНОЙ АКТИВНОСТИ ПРЕПАРАТА ТЕНВИР (ТЕНОФОВИР) В ОТНОШЕНИИ ВАРИАНТА Б ВИРУСА SARS-COV-2 В КАЗАХСТАНЕ И ВЫЯВЛЕНИЕ NSP12 В ВИРУСНОМ ГЕНОМЕ

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АБСТРАКТ

COVID-19 стал настоящей мишенью для лекарств во всем мире из-за масштабов пандемии в 2020 году. С вирусными инфекциями, как правило, трудно справиться, особенно если вирусная нагрузка и скорость распространения серьезно превосходят иммунную реакцию, как врожденную, так и адаптивную. Таким образом, лишь относительно небольшое количество препаратов применялось клинически для остановки либо на ранних стадиях репродукции вируса, например, с помощью Тенвира (Тенофовира), либо вакцинации для получения коллективного иммунитета в конкретной популяции. Ни один из препаратов на основе интерферона не продемонстрировал явного медицинского эффекта в медицинских исследованиях и во время протоколов госпитализации в связи с пандемией. Однако аналоги пурина, такие как Тенвир и другие, показали устойчивые показатели выживаемости и выздоровления среди пациентов, инфицированных вирусом SARS-COV-2, на средней и тяжелой стадиях пневмонии, вызванной этим вирусом. В данной статье представлены результаты испытаний in vitro препарата Тенвир на штамме вируса Вариант Б.1.1, отобранного из Казахстана, а также молекулярно-генетическая характеристика NSP12 (неструктурного белка 12), расположенного в регионе ORF1b генома SARS-COV 2. Тенвир или тенофовир широко использовались против ВИЧ-инфекции, и это исследование показывает достаточный эффект на распространение вируса благодаря свойствам ингибирования RdRP in vitro.

Ключевые слова: COVID-19, NSP-12, РНК-зависимая РНК-полимераза (RdRP), аналоги пурина, ОТ-ПЦР, ген-продукт.