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Development of a Test System to Detect the Omicron Variant of SARS-CoV-2 and the Frequency of Its Detection in Patients

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We developed a new test system to detect the omicron variant of SARS-CoV-2 using allele-specific reverse transcription PCR and estimated the frequency of its detection in patients living in the Novosibirsk Region. Clinical samples were divided into 3 groups: samples collected from December 1 to December 30, 2021 (group 1; n=66), from December 30, 2021 to January 10, 2022 (group 2; n=20), and from January 11 to January 22, 2022 (group 3; n=101). Based on the identification of 5 mutations specific to SARS-CoV-2 (B.1.1.529), two systems of oligonucleotide primers and probes were developed for detecting this coronavirus genotype in clinical samples. Limit of detection (LOD_{95}) was 4×10^3 genome equivalents per 1 ml of clinical sample for the first test system and 2×10^3 for the for the second test system. The omicron variant of SARS-CoV-2 was absent in group 1 of studied samples, but was detected in 20% (4/20) of group 2 samples and 88% of group 2 samples collected within less than 2 weeks of January 2022. Using developed test system, we showed that in less than 2 weeks the omicron variant has become dominant in patients, which confirms previously published data on its exceptional contagiousness.

Key Words: *SARS-CoV 2; coronavirus; mutations; omicron variant; allele-specific reverse transcription PCR (AS-RT-PCR)*

The SARS-CoV-2 virus, a representative of the *Betacoronavirus* genus, is the cause of the COVID-19 coronavirus pandemic that began in December 2019 and continues in January 2022. During the two years of the pandemic, SARS-CoV-2 coronavirus has gradually evolved and accumulated mutations in its genome. The transmissivity and virulence of new SARS-CoV-2 strains differ; the symptom complexes of infections caused by different strains do not coincide. In addition, the effectiveness of vaccines against SARS-CoV-2 also depends on a particular strain. All these circumstances necessitate careful epidemiological control over the emergence and spread of new SARS-CoV-2 strains.

The classification of SARS-CoV-2 strains proposed by WHO [13] is based on nucleotide substitutions in the SARS-CoV-2 genome. Newly discovered isolates can be assigned the statuses of interest or concern and a name based on the letter of the Greek alphabet. The variant of interest status is assigned in the pres-

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ence of mutations with a known or predicted effect on transmissibility, the severity of infection symptoms, avoidance of the immune response, effectiveness of therapy, and diagnosis. The variant of concern status is assigned, if there is an association with increased transmissibility or deterioration of the epidemiological situation, an increase in virulence or severity of symptoms, a decrease in the health system effectiveness or diagnostic tests, vaccines, and therapeutic agents.

The first worrisome variant was variant alpha (B.1.1.7), first discovered in the UK in September 2020. This variant had a combination of 23 mutations and was characterized by increased transmissivity [2] with a reproduction index R_0=~4-5, while for the initial variant SARS-CoV-2 R_0=2.4-2.6. Lower effectiveness of antibodies against the alpha variant RBD domain was also shown [8]. Within 6 months of the first reports from the UK, this variant was detected in all parts of the world and caused serious concern about the possible decrease in vaccine effectiveness and higher pathogenicity. However, these concerns were not subsequently confirmed [4,9].

The delta variant (B.1.617.2) was first found in India in October 2020 as a combination of 13 nucleotide substitutions leading to amino acid substitutions. Like the alpha variant, the delta variant has increased transmissivity with a reproduction index R_0=~5 [10]. In July 2021, the delta variant was recognized by WHO as the dominant or close to dominant strain; later, higher virulence of this variant was established [3]. In addition, vaccine effectiveness against the delta variant turned out to be lower by 10-13% than against the alpha variant [12].

On January 2022, the last SARS-CoV-2 variant raising concern (omicron-variant or B.1.1.529) was first registered in November 2021 in South Africa; it carries 60 mutations compared to the original SARS-CoV-2 strain; $^2/_3$ of these mutations are located inside the S-protein and have not been detected previously in other SARS-CoV-2 lines [1]. It is stated that the omicron variant reproduction index is 2-3 times higher compared to the delta variant [6,11], which correlates with the rapid SARS-CoV-2 omicron variant spread. At the same time, viral load for the omicron variant was shown to not differ from that of the delta variant [7]. A large number of accumulated mutations in the omicron variant can also affect the effectiveness of diagnosis and require verification of methods used for its detection. The data on the effectiveness of vaccines and monoclonal antibodies are still sketchy, but preliminary results show that the effectiveness of these drugs decreases and boosting is required to increase individual and population immunity against the omicron variant [15]. However, the first studies showed that disease severity and the probability of hospitalization in omicron variant infection decreased by several times in comparison with the delta variant [14].

The purpose of this study was to develop a test system for detecting the omicron variant of SARS-CoV-2 in clinical samples and to study its occurrence in patients living in the Novosibirsk region. To this end, we developed an allele-specific reverse transcription PCR (AS-RT-PCR) for detection of the omicron variant in SARS-CoV-2-positive patients.

MATERIALS AND METHODS

Clinical samples. Clinical RNA samples (*n*=187) after depersonalization were obtained from patients of the Center of New Medical Technologies of the Institute of Chemical Biology and Fundamental Medicine and the laboratory of the SKR-Test Company from patients who applied to the laboratory privately due to signs of acute respiratory viral infections, having a mild form of the disease (temperature less than 38°C, cough, runny nose, and headache). Clinical samples were divided into 3 groups: samples collected from December 1 to December 30, 2021 (group 1; *n*=66), from December 30, 2021 to January 10, 2022 (group 2; *n*=20), and from January 11 to January 22, 2022 (group 3; *n*=101). The research was approved by the Local Ethical Committee of the Institute of Chemical Biology and Fundamental Medicine (July 21, 2019) and Academic Council of the Institute of Chemical Biology and Fundamental Medicine (Protocol No. 18, December 23, 2021).

Plasmids with fragments of the nucleotide sequence of SARS-CoV-2 omicron variant. DNA fragments of the S-protein gene (length 200 bp) corresponding to regions 22,857-23,056 (mutations (E484A, G496S, and Q498R) and 24,297-4496 (mutations N969K and L981F) of the SARS-CoV2 OL677199 virus genome were synthetically obtained by PCR from individual oligonucleotides using the Polymerase Cycling Assembly (PCA) method. The obtained DNA fragments were hydrolyzed by EcoRI and HindIII restriction endonucleases (SibEnzyme) and ligated with the pBluescript vector SK (+) hydrolyzed by the same endonucleases using 100 U of T4 DNA ligase (Biosan). Competent cells of *E. coli* XL1-Blue strain (Stratagene) were transformed with a ligase mixture. In plasmid clones selected according to the restriction analysis results, the insertion nucleotide sequence was determined by Sanger sequencing to confirm the structure. Recombinant plasmid DNA was isolated from 50 ml of nocturnal culture in LB medium using the Plasmid Midi Kit (QIAGEN) according to the manufacturer's instructions. The concentration of the obtained standard plasmid DNA was determined spectrophotometrically and fluorometrically using the Qubit BR kit (Invitrogen).

RNA synthesis ***in vitro.*** The plasmid DNA pBluescript-CoV2-OME384A and pBluescript-CoV2-OMN969K linearized by HindIII restriction endonuclease were used as a matrix for RNA synthesis. The synthesis was carried out in a 50 µl reaction volume containing 2 µg DNA matrix, 1 mM NTP, 100 U Phage T7 RNA polymerase (SibEnzyme), 1× reaction buffer (50 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine). The samples were incubated for 2 h at 37°C followed by treatment with 100 U DNase I (Worthington Biochemical) addition and incubation for 15 min at 37°C. The synthesized RNA fragments were purified by phenol—chloroform extraction and precipitated with isopropanol. The purified RNA fragments were dissolved in water treated with diethyl pyrocarbonate and stored at -80°C. The exact standard DNA (plasmids) and RNA (SARS-CoV-2 transcripts and RNA from clinical samples) concentrations were clarified using digital PCR on the QX200 Droplet Digital PCR System (Bio-Rad) platform according to the manufacturer's instructions.

Real-time RT-PCR. RNA from clinical samples was isolated using a set of reagents for isolating RNA/DNA from the clinical material RealBest Sorbitus (Vector-Best) according to the manufacturer's instructions.

Real-time RT-PCR was performed on a CFX96 amplifier (Bio-Rad) in a volume of 20 µl containing 1× buffer for PCR (65 mM Tris-HCl, pH 8.9, 24 mM $(NH_4)_2SO_4$, 0.05% Tween-20, and 2.5 mM $MgCl_2$), 0.3 µM primers and 0.1 µM probes (Table 1), 1 U of hot-start Taq polymerase (Biosan), 100 U of reverse transcriptase M-MuLV (Biosan), and RNA matrix. Amplification was carried out according to the following program: reverse transcription at 50°C for 15 min; activation of Taq polymerase and denaturation of the DNA matrix at 95°C for 3 min; 50 cycles with the following stages: denaturation at 95°C for 10 sec, annealing and elongation at 60°C for 40 sec, fluorescence signal registration via FAM, HEX, and ROX channels.

Sequencing. To confirm the specificity of the analysis, reverse transcription and amplification of RNA samples positively and negatively identified by the omicron genotype status were performed using primers 371-U and SS-R, a fragment of 440 bp was sequenced by Sanger, the resulting nucleotide sequence was compared to omicron and delta SARS-CoV-2 variants' sequences. Sequencing was performed on an automatic sequencer ABI 3130XL GeneticAnalyzer (Applied Biosystems) using a set of BigDye 3.1 (Common Use Center "Genomics", Institute of Chemical Biology and Fundamental Medicine).

RESULTS

Selection and validation of primers for AS-RT-PCR. It was previously shown that the SARS-CoV-2 omicron variant contains 25 unique S-protein mutations (*A67V*, *ΔV143*, *ΔN211*, *L212I*, *ins214EPE*, *G339D*, *S371L*, *S373P*, *S375F*, *N440K*, *G446S*, *S477N*, *E484A*, *Q493R*, *G496S*, *Q498R*, *Y505H*, *T547K*, *N679K*, *N764K*, *D796Y*, *N856K*, *Q954H*, *N969K*, and *L981F*) [1]. Five of them (*E484A*, *G496S*, *Q498R*, *N969K*, and *L981F*) we selected as targets for primer design for AS-RT-PCR. The sequence OL677119 (GISAID EPI_ISL_6826713; NCBI database) deposited by Canadian researchers at the end of November 2021 was chosen as the basic nucleotide sequence of the SARS-CoV-2 variant omicron genome.

TABLE 1. Oligonucleotide Primers and Fluorescently Labeled Hydrolyzable Probes Used for RT-PCR

Gene	Sequences of oligonucleotide primers and probes
S-SARS-CoV2	371-U CTGTGTTGCTGATTATTCTGTCCT
	SS-R AGTTCAAAAGAAAGTACTACTACTCTGTA
S-SARS-CoV2	N969K-PH HEX-TGGTGCAATTTCAAGTGTTTTAAATG-BHQ1
	N969K-U CTTGTTAAACAACTTAGCTCCTAA
	N969K-R TCAACTTTGTCAAGACGTGAAAA
S-SARS-CoV2	E384-PH HEX-TGATCGTAAAGGAAAGTAACAATTAAAACC-BHQ1
	E384-U AACAAACCTTGTAATGGTGTTACC
	E384-R ACACCATAAGTGGGACGGAAAA
E-SARS-CoV2	E_Sarb_F ACAGGTACGTTAATAGTTAATAGCGT
	E_Sarb_R ATATTGCAGCAGTACGCACACA
	E_Sarb_P HEX-ACACTAGCCATCCTTACTGCGCTTCG-BHQ2
MS2 phage	MS2-5-F GTACGAGGAGAAAGCCGGTTTC
	MS2-5-R GTTCTGCGGCACTTCGATG
	MS2-5-P FAM-TCCCTCGACGCACGCTCCTGCT-BHQ1

CTGTGTTGCTGATTATTCTGTCCTATATAATCTCGCACCATTTTTCACTTTTAAGTGTTATG
GAGTGTCTCCTACTAAATTAAATGATCTCTGCTTTACTAATGTCTATGCAGATTCATTTGT
AATTAGAGGTGATGAAGTCAGACAAATCGCTCCAGGGCAAACTGGAAATATTGCTGATT
ATAATTATAAATTACCAGATGATTTTACAGGCTGCGTTATAGCTTGGAATTCTAACAAGCT
TGATTCTAAGGTTAGTGGTAATTATAATTACCTGTATAGATTGTTTAGGAAGTCTAATCTC
AAACCTTTTGAGAGAGATATTTCAACTGAAATCTATCAGGCCGGTAACAAACCTTGTAA
TGGTGTTGCAGGTTTTAATTGTTACTTTCCTTTACGATCATATAGTTTCCGACCCACTTAT
GGTGTTGGTCACCAACCATACAGAGTAGTAGTACTTTCTTTTGAACT

Fig. 1. Nucleotide sequence of the S-gene fragment (440 bp) of the SARS-CoV-2 omicron variant isolated from a patient living in Novosibirsk. Codons encoding omicron-specific nucleotide substitutions, which correspond to the following amino acid substitutions highlighted with grey (*S371L*, *S373P*, *S375F*, *N440K*, *G446S*, *S477N*, *E484A*, *Q493R*, *G496S*, *Q498R*, and *Y505H*).

To improve the discrimination of products with "mutant" (omicron variant) and "normal" (other SARS-CoV-2 variants) RNA, we tested the effectiveness of introducing additional unpaired nucleotide bases at the 3' end of the primer (in -2 and -3 positions) for further duplex destabilization to reduce the elongation effectiveness. To do this, plasmid DNA containing omicron-specific mutations and a "wild" type sequence were used at the first stage. Based on the results of this test, optimal oligonucleotide primers were selected that did not allow wild-type DNA amplification and had maximum efficiency (lower Ct) for the omicron variant sequence. The optimal primer set was further validated in AS-RT-PCR using artificial RNA transcripts obtained from a plasmid DNA matrix with omicron-variant sequence fragments and the original SARS-CoV-2 variant.

Omicron-variant detection in clinical samples. After the development of the PCR system, we conducted primary screening of RNA from clinical samples positive for SARS-CoV-2 RNA detected by coronavirus E-gene fragment amplification. For several samples, fluorescence growth was observed using primers E384-U/R (set 1, mutations *E484A*, *G496S*, and *Q498R*) and N969K-U/R (set 2, mutations *N969K* and *L981F*). The RNA samples we obtained were analyzed by sequencing the S-gene fragment (440 bp), which includes a set of mutations specific to the omicron variant. The nucleotide sequence analysis (the same in 4 clinical samples, Fig. 1) showed 100% homology with the SARS-CoV-2 omicron variant, now common in the United States (reference sequence OM327305.1) and Pakistan (reference sequence OM327526.1). Analysis of previously identified RNA sequencing samples of the delta variant of SARS-CoV-2 showed high specificity of primer sets 1 and 2. Thus, primer sets 1 and 2 yielded increments of the fluorescent signal even during examination of clinical samples with high viral load of the delta variant of coronavirus (Ct 12-16 with primers and a probe to the coronavirus E-protein gene via the ROX fluorescence channel). Due to lower PCR efficiency, Ct values for specific omicron variant sequence amplification were 4-9 cycles higher compared to Ct with primers and a probe to the coronavirus E-protein gene. The difference in Ct values was 6-9 cycles for set 1 and 4-7 cycles for set 2. Therefore, we increased the total number of amplification cycles to 50. To determine the limit of detection (LOD_{95}), a clinical sample identified as positive for the SARS-CoV-2 omicron variant was used. The concentration of the clinical sample was preliminarily determined using digital droplet PCR, after which serial dilutions of this RNA sample were analyzed in 20 repeats. LOD_{95} was 4×10^3 genome equivalents/ml for primer set 1 and 2×10^3 genome equivalents/ml for set 2.

Analysis of SARS-CoV-2 omicron-variant occurrence in patients. The SARS-CoV-2 omicron variant was absent in group 1 samples, but was found in 20% (4/20) samples of group 2 and in 88% samples of group 3 (Fig. 2). Thus, in less than 2 weeks of January

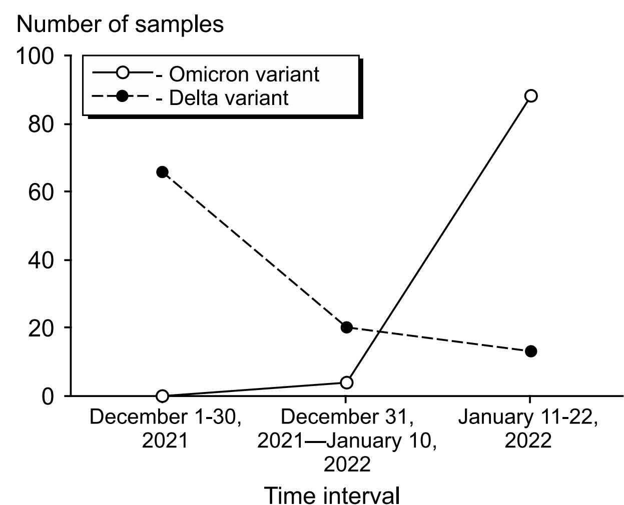

Fig. 2. The prevalence dynamics of SARS-CoV-2 omicron and delta variants in the Novosibirsk region during December 2021—January 2022.

2022, this variant became dominant in the sample of patients in the Novosibirsk region.

The data obtained are consistent with the pattern of SARS-CoV-2 omicron variant distribution observed in South Africa. Thus, among the SARS-CoV-2 samples sequenced in October 2021 in South Africa, 617 (84.2%) out of 733 were delta variants and only 1 (0.1%) was an omicron variant. In November 2021, 148 (13.7%) of 1082 SARS-CoV-2 samples were delta variant and 881 (81.4%) were omicron variant [14]. The high spread rate of the SARS-CoV-2 omicron variant and its delta variant displacement in South Africa was reproduced in the restricted sample on the territory of the Novosibirsk region. A possible reason for rapid spread of the omicron variant in the Novosibirsk region could be the long weekends in early 2022, which contributed to the active movement and contacts in the population.

However, the data obtained differ from the picture of the omicron variant distribution in France, where 62.4% of all diagnosed COVID-19 cases by December 30 were caused by the omicron variant. During the period from November 28 (the first case of the SARS-CoV-2 omicron variant registration) to December 31, 2021, 1119 (26.7%) cases of infection with the omicron variant were recorded in Marseille, 3075 (73.3%) cases of infection with the delta variant were detected during the same period [5]. There was a rapid increase in cases of infection with the omicron variant since mid-December and a simultaneous drop in the frequency of infection with the delta variant; at the end of December, the occurrence of the omicron variant exceeded the occurrence of the delta variant by about 2 times. A possible reason for the divergence could be the difference in the omicron variant subvariants found in Europe and the Novosibirsk Region. This assumption requires further verification based on the data of the SARS-CoV-2 omicron variant spread on the territory of other countries, primarily Pakistan and the United States, in which the omicron variant coincides with what we found.

We have developed a simple and easy-to-use test system for monitoring the SARS-CoV-2 omicron variant, based on the identification of 5 mutations specific to this genotype. Using it, it is shown that in less than 2 weeks the omicron variant has become the dominant variant in the Novosibirsk region, which confirms previously published data on its exceptional contagiousness. A further collecting of statistics and whole-genome sequencing data of SARS-CoV-2 omicron isolates identified with the developed simple PCR analysis will contribute to a more complete understanding of this variant biological properties.

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The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

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