SARS-COV-2 GENOMIC SURVEILLANCE IN BULGARIA INDICATES DIVERSE DYNAMICS DRIVEN BY MULTIPLE INTRODUCTIONS OF DIFFERENT VIRAL VARIANTS IN 2022

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ABSTRACT

Background. Evolution of the emerging SARS-CoV-2 variants raises concerns about the possibility of accelerated transmission, disease severity, diagnostic challenges, and reduced vaccine effectiveness in the ever-evolving COVID-19 pandemic worldwide. Objectives for this study were to build a comprehensive national system for monitoring and genomic surveillance of SARS-CoV-2 and to identify the introduced virus variants in the country.

Methods. We analyzed SARS-CoV-2 infections in 7948 representative clinical samples collected in medical institutions in different geographical regions of the country in 2022. Whole-genome next-generation sequencing of SARS-CoV-2 was performed on samples from randomly selected SARS-CoV-2-positive individuals by using a modified ARTIC v3-tailed amplicon method. A bioinformatic and phylogenetic analyses of the obtained sequences was carried out.

Results. Significant dynamics was observed in the spread of viral variants in 2022, which is characterized by the introduction and spread of multiple SARS-CoV-2 variants. The phylogenomic analysis identified a high genetic heterogeneiety composed of a total of 152 different viral clades divided into 3 main supergroups: 114 (75.0%) of which were Omicron sub-variants, 35 (23.0%) Delta sub-variants, and 3 (2.0%) recombinant forms.

Conclusion. Viral variants and their sub-clades with different potentials to impact disease severity were identified and the information was immediately published for use by decision-makers and the scientific community. The global pandemic of COVID-19 has shown the importance of molecular biological surveillance, which is an indispensable element of the modern approach in the fight against infectious diseases.

Keywords: SARS-CoV-2, COVID-19, sequencing, Viral variants

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1. INTRODUCTION

The newly emerging coronavirus SARS-CoV-2 in 2019, led to an unprecedented pandemic challenging the global healthcare systems, social systems, and economy (1). Viruses including SARS-CoV-2 mutate and as a result of accumulated mutations over time new variants emerge. Many new variants and sublineages have branched off from the original virus Wuhan-Hu-1, some variants disappear, while others successfully continue to spread and may replace previous ones. Certain variants are of particular importance due to their potential for increased transmissibility, virulence, or reduced vaccine effectiveness (2,3). The circulation of different viral lineages is a dynamic process with uneven distribution in different geographical regions and favors the dominance of a particular local clade in certain places and time frames.

Some of the variants posed an exceptional risk to public health and their global monitoring was given priority. WHO and ECDC defined those lineages as specific variants of concern (VOCs), variants of interest (VOIs), and variants under investigation (VUI). VOCs include B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta), and B.1.1.529 (Omicron), some of

which have been de-escalated in time due to their diminishing prevalence globally.

WHO provides regular updates on currently circulating VOCs and as of 2022 the most widespread variants in the world were Delta (B.1.617.2) and Omicron (B.1.1.529) (4). The latter split into numerous sub-variants and currently (March 2023) continues to be the dominant one in the world as well as in Bulgaria. In addition to the different classifications of variants, and the observation of the impact of the different variants on public health, the Centers for Disease Control and Prevention (CDC) proposed to reclassify variants based on their attributes and prevalence in the United States as follows: Variants being monitored (VBM), Variant of interest (VOI), Variant of Concern (VOC), Variant of high consequence (VOHC) (5).

While Delta was far more deadly and dominating globally during 2021 (4.44 million cases) it was rapidly replaced by the more adapted Omicron variant (7.11 million cases) (6). Omicron was first reported by the Network for Genomics Surveillance in South Africa on 24 November 2021 (7). It was first detected in Botswana and has spread to become the predominant variant in circulation around the world as well as in Bulgaria (8). The emergence of the original Omicron variant (B.1.1.529) was followed by several major successor sub-variants, which were designated as: BA.1, BA.2, BA.3, BA.4, and BA.5 and these sub-variants further split into over 200 sub-variants worldwide (9). Since October 2022, two subvariants of BA.5 called BQ.1 and BQ.1.1 have emerged.

Variants with a high epidemic potential require more specific measures proportional to the risk for the public health system. Being the country with the lowest vaccination coverage (30.1%) in EU, Bulgaria still shows a COVID-19 case fatality rate 4.4-fold higher than the EU average (2.95% versus 0.66%) (10). Therefore, particular vigilance is needed incorporating early recognition and response protocols in likely scenarios of local emergence of variants with elevated epidemic potential.

The Bulgarian SARS-CoV-2 sequencing group at the National Center of Infectious and Parasitic Diseases (NCIPD) with its commitment to the Ministry of Health and the European Center for Disease Control (ECDC), aims to identify and monitor introduced and spread viral variants and mutations by conducting whole genome sequencing (WGS) and reporting this essential information to the health authorities in the country as well as to the international scientific community (e.g GISAID).

2. MATERIALS AND METHODS

2.1. Study Design and Patient Samples

We analyzed SARS-CoV-2 infections in clinical samples confirmed locally in medical institutions in different geographical regions of the country. Following the national regulations, samples were sent to the NCIPD, where PCR-confirmation tests and sequencing analysis were conducted. Epidemiological, demographic, and clinical data about the patients were obtained from the National electronic system for COVID-19 following the national regulations. Patient samples were linked to epidemiological data by using anonymous numerical codes following ethical standards and medical standards (11).

2.2. Real-time PCR and Sequencing Analyses

Viral RNA was extracted from 400 µl of nasal swabs using an ExiPrep 16DX (BioNeer, Korea), SaMag 12 System (Sacace Biotechnologies, Italy.), or EXM3000 (Zybio Inc., China) according to the manufacturer's instructions. Reverse-transcription Real-time polymerase chain reaction (RT-qPCR) was performed using QuantStudio™ real-time PCR system (ThermoFisher Scientific), CFX96 Touch PCR Real-Time Detection System (Bio-Rad), or Gentier 96E/R real-time PCR system, targeting at least one of the following genes: RdRp (RNA-dependent RNA Polymerase), E (envelope), N (nucleocapsid), ORF1ab (open reading frames, ORF1a and ORF1b) of the SARS-CoV-2 genes.

Whole-genome next-generation sequencing of SARS-CoV-2 was performed on samples from randomly selected SARS-CoV-2-positive individuals by using a modified ARTIC-tailed amplicon method (12). Briefly, after the RT step, 3 µl of cDNA was used in four multiplex PCRs (20 µl each). The ARTIC v3-tailed primer concentrations were normalized according

to the protocol developed by Benjamin Farr et al. to improve the evenness of genome coverage (13). The indexed libraries were purified by HighPrep™ PCR Clean-up (MagBio Genomics Inc.), quantified, normalized, and pooled to 4 nM for sequencing on Illumina MiSeq with v2 reagent kit and 500 cycles (Illumina). In addition to the sequencing carried out at the NCIPD, samples were sequenced by a collaboration funded by the European Commission at Eurofins, Germany (14). The reads were trimmed, and quality filtered, the primer sequences were removed, and full genomes were assembled in Geneious Prime 2021.1 (https://www.geneious.com). The current version of the Pangolin COVID-19 Lineage Assigner Tool was used to define the variant classification (15).

3. RESULTS

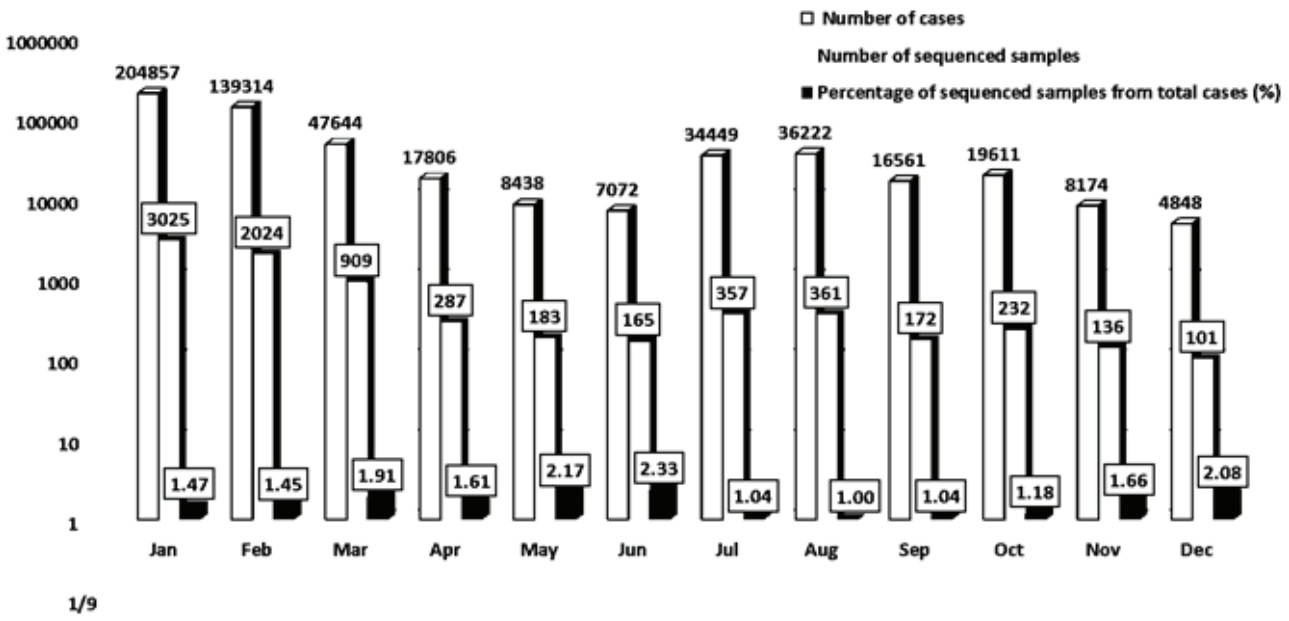
3.1. Population characteristics

A total of 7948 samples (1.46%) of 544,996 patients diagnosed with SARS-CoV-2 in Bulgaria during 2022 were included in this study. Of them, 45.3% were men and 54.7% were women. The clinical specimens (nasopharyngeal swabs) were collected

Table 1. Population structure in Bulgaria and the corresponding number of SARS-CoV-2 sequenced samples from the respective region.

Region	Population		Samples	
	(n)	(%)	(n)	(%)
Sofia (capital)	1307439	19,1	1919	24,1
Plovdiv	662907	9,7	949	11,9
Varna	468614	6,9	730	9,2
Burgas	408704	6,0	829	10,4
Stara Zagora	307140	4,5	501	6,3
Blagoevgrad	298251	4,4	118	1,5
Pazardzhik	247360	3,6	281	3,5
Sofia	233607	3,4	148	1,9
Pleven	228300	3,3	358	4,5
Veliko Tarnovo	225674	3,3	155	2,0
Haskovo	220269	3,2	51	0,6
Ruse	209084	3,1	717	9,0
Sliven	180058	2,6	43	0,5
Shumen	169423	2,5	117	1,5
Dobrich	167314	2,4	34	0,4
Kyustendil	161024	2,4	97	1,2
Vratsa	153700	2,2	32	0,4
Montana	122179	1,8	117	1,5
Lovech	119780	1,8	30	0,4
Pernik	118023	1,7	296	3,7
Yambol	114361	1,7	32	0,4
Kurdzhali	113440	1,7	8	0,1
Targovishte	108117	1,6	127	1,6
Razgrad	107764	1,6	11	0,1
Silistra	104869	1,5	27	0,3
Gabrovo	103404	1,5	134	1,7
Smolyan	99318	1,5	68	0,9
Vidin	78814	1,2	19	0,2
Total	**6838937**	**100,0**	**7948**	**100,0**

Table 2. Number of sequenced samples for SARS-CoV-2 and their percentage compared to the number of COVID-19 cases in Bulgaria in the corresponding month of 2022.



in 181 medical facilities and laboratories in all 28 administrative regions of the country.

According to the data of the National Statistical Institute, the population of the country as of December 31, 2021, was a total of 6,838,937 distributed in 28 administrative regions, the most populated of which was Sofia (capital), Table 1 (16). The largest number of clinical samples were isolated in the region of Sofia (capital) with almost a quarter 24.1% of all samples in the study, followed by Plovdiv, Varna and Burgas with 11.9%, 10.4% and 6.3% respectively. A total of 55.7% of the sequenced samples belonged to these four main urban areas, which in turn constituted 41.6% of the country's population. The remaining 24 regions collectively accounted for 44.3% of the clinical samples, while they constituted 58.4% of the population, Table 1.

3.2. Sequencing and analysis

Following ECDC guidelines for in-depth surveillance of the introduction and spread of viral variants in the country, national measures were taken to sequence and analyze a sufficiently large representative sample set from patients with SARS-CoV-2 in Bulgaria (17). Samples were obtained from patients with various disease course, age and sex in 181 hospitals and clinical laboratories in different regions, in order to produce a representative population sample. A total of 7948 samples were successfully sequenced and analyzed, representing 1.5% of all COVID-19 cases during the study period (January-December 2022), Table 2.

3.3. Dynamics of the pandemic waves against the background of the introduction of different SARS-CoV-2 variants in Bulgaria.

The spread of the newly emerged SARS-CoV-2 and the large waves of the pandemic in Bulgaria followed those across other European countries. The first peak as a result of an epidemic outbreak in the summer of 2020 was of limited size due to the strict anti-epidemic measures that were applied in the country. The next four waves with corresponding high peaks of cases and mortality were caused by several major viral variants, including Alpha, Delta, and Omicron, Figure 1. As a rule, each subsequent peak was increasingly high in the number of cases, as well as in the death rate, except for the last wave caused by the rapidly spreading Omicron. After the introduction and dissemination of Omicron, there was a sharp increase in the number of infections, yet for the first time since the beginning of the pandemic, the death rate decreased, Figure 1.

3.4. Dynamics of the Omicron sub-variants in 2022.

After the emergence of Omicron in South Africa on 24 November 2021, under evolutionary pressure in

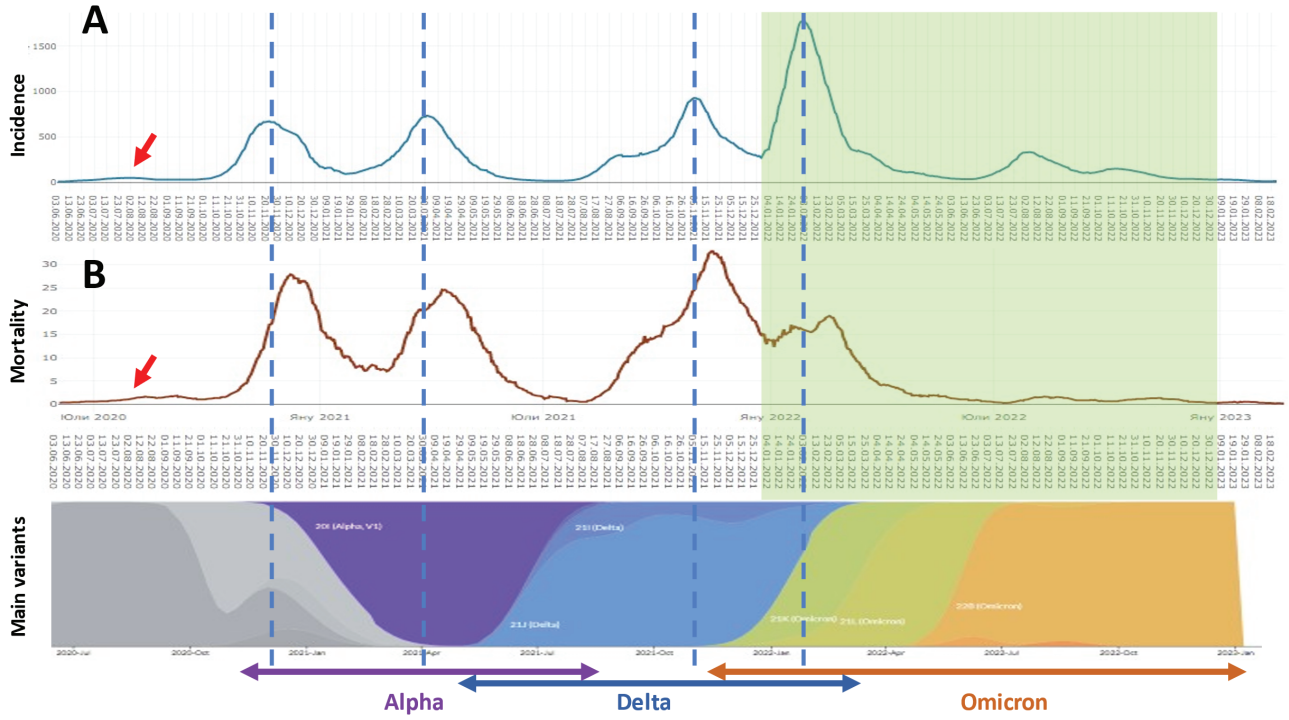


Figure 1. Incidence and mortality of COVID-19 in Bulgaria, 2020 – 2022. A. Incidence cases (Number per 100,000 population). B. Mortality rate (Number per 100,000 population). The green rectangle represents 2022, which is characterized by a period of the gradual disappearance of the Delta Variant and its displacement by the Omicron. The red arrows indicate the first very limited peak of covid-19 in Bulgaria. The figure is adapted from the NCIPD website and Nextstrain. Graphical analysis of data from the National Information System for COVID-19, Bulgaria and Nextstrain (18,19).

2022, Omicron subvariants diverged into a swarm of numerous clades, some of which had a greater chance of spreading worldwide. This led to the introduction and spread of multiple of these variants worldwide as well as in Bulgaria, Figure 2. Our sequencing and phylogenetic analysis conducted on patient samples in 2022 identified an incredibly wide variety of a total of 152 different viral variants that could be classified into three main supergroups:

A) 114 (75.0%) Omicron sub-variants, indicated in the legend of Figure 2 as derivatives of BA/BE/BF/BM/BN/BQ/CH/CK/CK;

B) 35 (23.0%) Delta sub-variants, indicated as derivatives of AY/ B.1.617.2; and

C) 3 (2.0%) recombinant forms indicated as XAN/XBB, Figure 2.

Amid the fading Delta morbidity which disappeared by the end of February 2022, Omicron peaked in March 2022 and branched off into 114 different successor clades. The most prevalent Omicron sub-variants (January – December 2022) were as follows: January – February BA.1 and BA.1.1, March – June BA.2, July BA.5.1, August –October BA.5.2, November BF.x, and in December BF.x and BQ.1.x.

The dissemination of the different clades in time is shown in Figure 2 in different colors representing different sub-variants. The most abundant infections with a particular variant are indicated by the largest proportion of a particular color on the diagram which is also indicated in the figure legend. The initial introduction and spread of the respective viral clades start with a thin line in the upper left corner that expands down and to the right and then thins again until it disappears. In this way, the growth of the specific viral population during its spread over time (indicated at the bottom horizontal part of the figure) and eventual disappearance and replacement by another viral lineage is presented.

4. DISCUSSION

The global COVID-19 pandemic required urgent measures to analyze the molecular characteristics

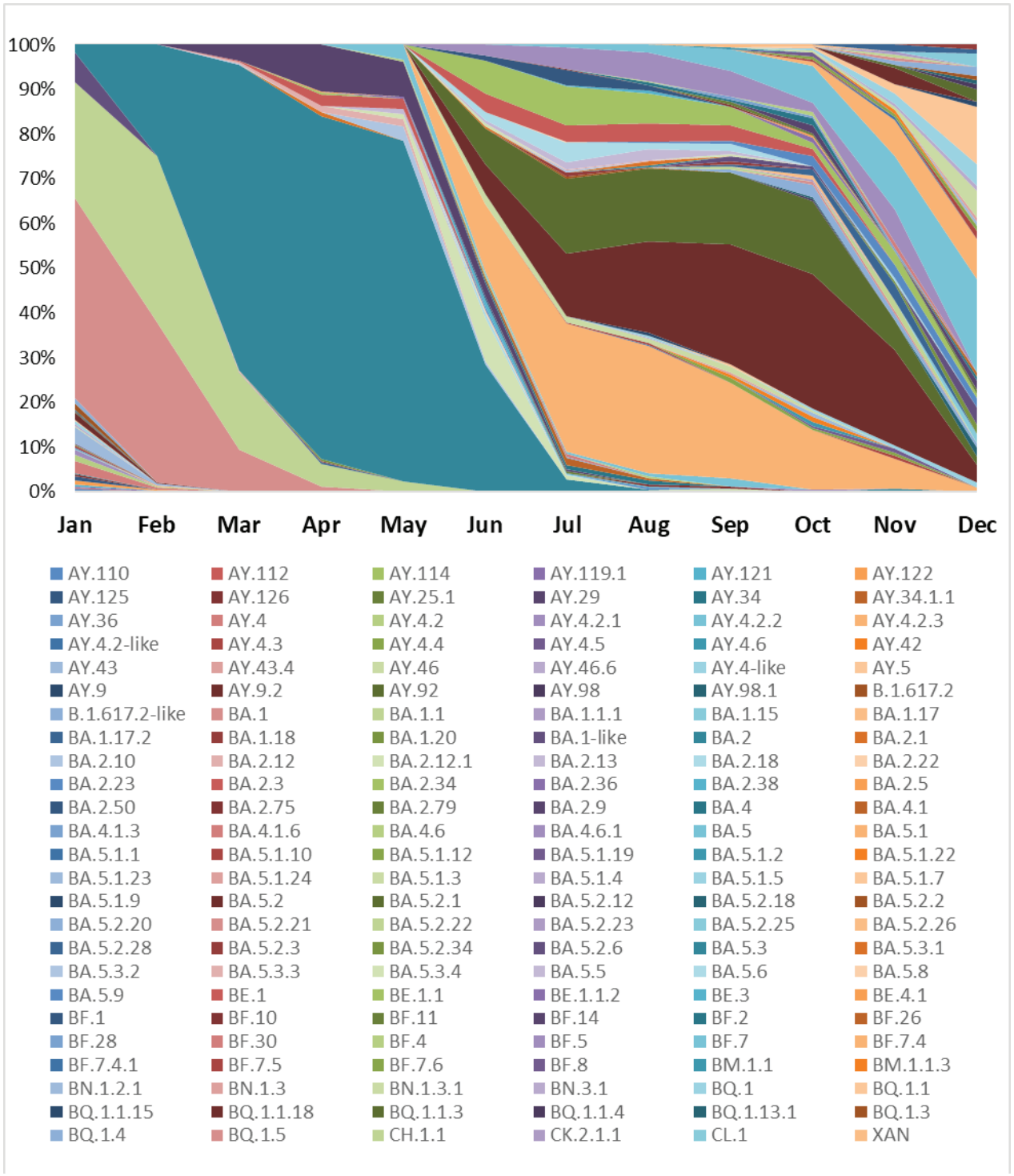


Figure 2. Dynamics in the prevalence of the SARS-CoV-2 sub-variants over time in 2022 in Bulgaria. In the beginning of the year, the Delta variant prevailed, further replaced by the much faster spreading Omicron. AYx/B.1.617.2 represent Delta; BA/BE/BF/BM/BN/BQ/CH/CK/CK represents Omicron; XAN/XBB represents recombinant variants (20).

of the newly emerged virus SARS-CoV-2. Genomic surveillance of SARS-CoV-2 is essential to detect and monitor the branching of virus variants which can result in increased transmissibility, disease severity, reduced vaccine effectiveness and diagnostic challenges. Timely and sufficiently detailed information on circulating variants among the populations is essential for public health decisions concerning reduction of general transmission and assessment of the effect of vaccination programs (21). Global genomic surveillance systems were integrated into national community-based and hospital-based COVID-19 surveillance systems, with a well-defined sampling and sequencing strategy to ensure

representativeness and reliability of findings (22,23). ECDC recommended EU/EEA Member States to implement into practice genomic SARS-CoV-2 surveillance, including recommendations for the number of samples that need to be sequenced to achieve surveillance objectives.

After the emergence of the global COVID-19 pandemic, the National Center of Infectious and Parasitic Diseases, in cooperation with the Ministry of Health in Bulgaria and with the help of the European Commission, urgently built a comprehensive national system for monitoring and genomic surveillance of SARS-CoV-2.

In this study, we analyzed SARS-CoV-2 and its variants introduced and disseminated in Bulgaria in 2022 by using sequencing and phylogenetic analysis methods. For this purpose, we analyzed 7948 representative clinical samples from patients diagnosed with COVID-19 in different geographical regions of the country. To meet the condition of a representative national sample required by ECDC, the samples in our study were randomly collected in all 28 administrative regions of the country to. Men and women were almost equally represented in the study, respectively 45.3% of the samples were isolated from men and 54.7% from women, which also corresponds to the fact that Covid-19 affects both sexes equally (24). The largest number of samples was collected in the four largest regions, including Sofia (capital), Plovdiv, Varna, and Burgas, where more than 40% of the population was concentrated, and also where the biggest health facilities and hospitals were located, Table 1. From the smaller towns and villages inhabited by 58.4% of the population, 44.3% of the clinical samples were collected. However, many of the patients from the smaller settlements received medical care in the larger medical centers of the country, assuming that the entire population of the country in the various urbanized areas was relatively evenly covered.

The spread of the newly emerged SARS-CoV-2 was not gradual, but intensified at certain periods with a peak followed by a decline in the spread, thus causing waves of new infections in the population The first peak as a result of an epidemic outbreak in the summer of 2020 was of limited size due to the strict anti-epidemic measures that were applied in Bulgaria, (Figure 1). In this way, extremely valuable time was gained for the preparation of the healthcare system with its two main activities, laboratory diagnostic and treatment. The next four waves with corresponding high peaks of cases and mortality were caused by several major viral variants, including Alpha, Delta, and Omicron, Figure 1. As a rule, the peak of infections, (Figure 1, A.) preceded the peak of death rate by several weeks, (Figure 1, B). We observed that each subsequent wave was increasingly higher in the number of cases, as well as in the death rate, except for the last wave caused by the rapidly spreading Omicron, where the death rate dropped. After the introduction and dissemination of Omicron, there was a significant increase in the number of infections, yet for the first time since the beginning of the pandemic, the death rate decreased, Figure 1 (25).

The year 2022 was dominated by Omicron worldwide and in Bulgaria. The origin of Omicron is unclear and raises many questions because its genome has accumulated an unexpectedly high number of mutations, an indication that this clade has remained long time hidden from the scientific community. It is possible that Omicron did not evolve from any other variant, but instead diverged on a distinct track, perhaps in the mid-2020. Several hypotheses were proposed, including the long persistence of the virus in an immunocompromised patient; possible recombination with another coronavirus (known as HCoV-229E), or transmission of the virus from human to mouse and vice versa to humans (26-28). Whatever the reason for its appearance, Omicron caused a large number of infections, and on 26 Nov 2021 WHO classified it as a VOC. In 2022 Omicron subvariants diverged very quickly into a swarm of more than150 clades, some of which had a greater chance of spreading, (Figure 2.) Sequencing and phylogenetic analysis in our study identified that multiple of these variants were introduced in Bulgaria, Figure 2. A total of 152 different sub-variants of SARS-CoV-2 in 3 main supergroups were identified, including Omicron, Delta as well as recombinant forms indicated as XAN/XBB, Figure 2.

The dynamics of the different viral branches over time are depicted in Figure 2. Our analysis showed the gradual disappearance of Delta by the end of February 2022, while Omicron spread and peaked in March 2022 by branching into multiple clades. Some of the most common Omicron sub-variants in the population were: BA.1, BA.1.1, BA.2, BA.5.1, BA.5.2, BF.x, and BQ sub-variants were identified, Figure 2. The distribution of sub-variants was not uniform, some of the clades were responsible for a larger proportion of infections and, can thus be defined as viral lineages of greater public health importance, Figure 2. We also identified several recombinant viral lineages in Bulgaria, which demonstrates the need for continuous and focused SARS-CoV-2 genomic surveillance (29).

Our study has some limitations that may have affected the results. Not all patients diagnosed with COVID-19 were included in the sequencing, and some individuals were infected but did not seek medical care or were asymptomatic. Therefore, it is possible that not all virus clades introduced into the country have been identified and this may impact the rest of the findings of the study.

5. CONCLUSIONS

The rapid identification of the newly emerged SARS-CoV-2 by using modern molecular biological methods allowed the health community to urgently identify the virus, assess its impact on public health, take adequate measures to limit the spread of the virus in the population, and develop successful vaccine prophylaxis. The NCIPD carried out genomic surveillance of the introduced viruses, which helped to track the emerging variants, which was indispensable for taking timely, targeted, and adequate public health actions. Viral variants and their sub-clades with different potentials to impact disease severity were identified and the information was immediately published for use by decision-makers and the scientific community. The global pandemic of COVID-19 has shown the importance of molecular biological surveillance, which is an indispensable element of the modern approach in the fight against infectious diseases.

Institutional Review Board Statement: This study was approved by the Ethical Committee at the National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria (NCIPD IRB 00006384).

Author Contributions: IA and II conceived and designed the study; IA wrote the first draft of the manuscript; II, IS, DD, LG, and AG performed sequencing analysis; IA, II, IS, DD, and LG analyzed the data; RD, NK, IT, VD, TK, and IC reviewed the draft and all authors contributed to the final version, which was approved by all authors for submission.

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