

# DYNAMIC OF SARS-COV-2 SPREAD IN BULGARIA, 2020-2022

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## ABSTRACT

The COVID-19 pandemic is associated with high morbidity and significant mortality worldwide. The objective of this study was to track the circulation pattern of SARS-CoV-2 in Bulgaria over three consecutive years (2020-2022) and to analyze the involvement of SARS-CoV-2 in cases of co-infections. A total of 98 247 clinical samples were tested for SARS-CoV-2 using a Real-Time RT-PCR method and 25.2% of them were positive. The positive rate for SARS-CoV-2 was greater among hospitalized patients compared to outpatients ( $p < 0.05$ ). Approximately 48.3% of all SARS-CoV-2-positive cases were male and 51.7% were female ( $p < 0.05$ ). SARS-CoV-2 positivity was highest in the group of oldest adults ( $\geq 65$  years) (average 40.6%), and lowest in the group of youngest children (0-5 years) (average 9.4%). Several peaks in the spread of SARS-CoV-2 infections were observed. Among the 1 463 SARS-CoV-2 positive clinical samples examined for the presence of other respiratory viruses, 109 (7.5%) cases of co-infections were found. The greatest variety of co-infections with SARS-CoV-2 and other respiratory viruses was detected during the Omicron wave. Surveillance of SARS-CoV-2 is important to continue in the future in order not to miss the emergence of new genetic variants with increased infectivity, virulence or immune escape.

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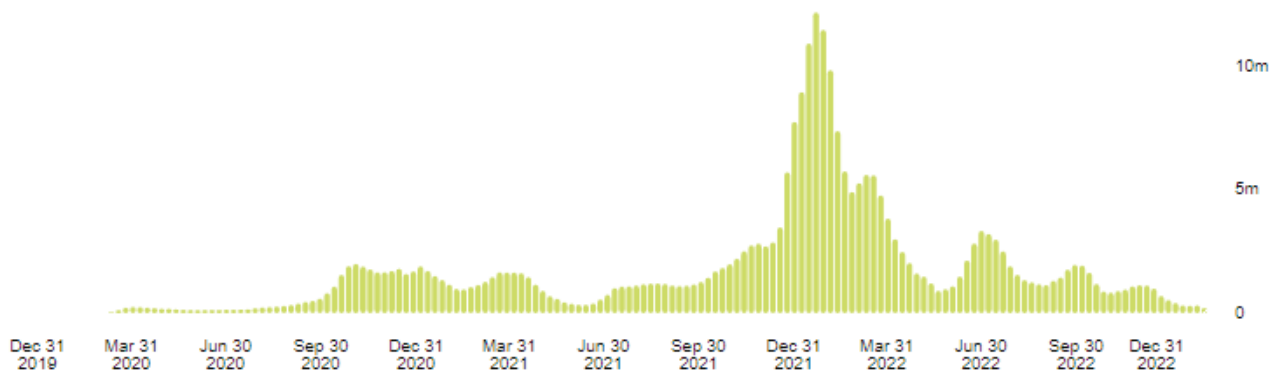
## INTRODUCTION

SARS-CoV-2, the etiological agent of COVID-19, emerged in Wuhan, China in late 2019 and quickly spread throughout the world, causing an unprecedented health and economic crisis (1). As of February 10, 2023, 755 385 709 confirmed cases of SARS-CoV-2 infection have been reported worldwide, with 6 833 388 deaths (2) (Figure 1). On March 11, 2020, the WHO officially declared the outbreak of COVID-19 as a pandemic. In the course of the pandemic, numerous genetic variants of the SARS-CoV-2 carrying different combinations of mutations appeared, including several variants of concern (VOC). The latter were characterized by increasing transmissibility, and/or change in the clinical presentation, and/or decrease in the effectiveness of available diagnostics, vaccines, and therapeutics (3).

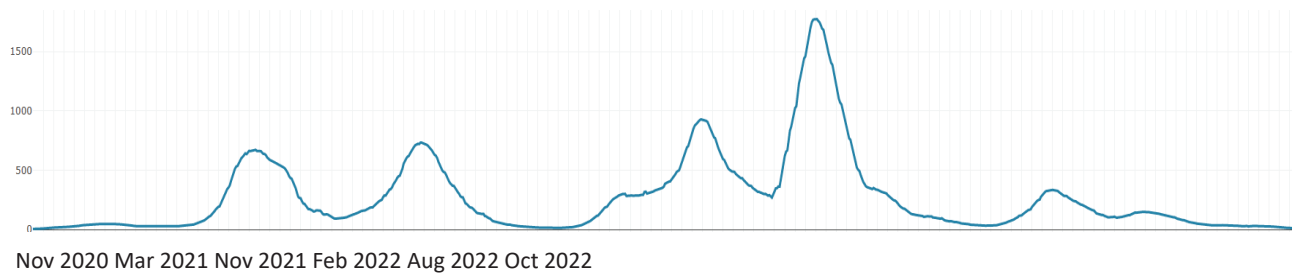
Bulgaria was severely affected by the COVID-19 pandemic, which resulted in more than 1.2 million confirmed cases including over 38 000 deaths. There were 6 peaks in the incidence of COVID-19 in the country (Figure 2). The highest peak was registered in February 2022 as a result of the large number of people infected with the Omicron variant of SARS-CoV-2.

In the National Reference Laboratory (NRL) "Influenza and ARD", SARS-CoV-2 testing began in the end of January 2020 using Real Time (RT-PCR), and the primers and probes developed by Corman VM at Charite, Berlin, as recommended by WHO (5). Initially, clinical samples from persons arriving from countries affected by the epidemic were examined. Later on, hospitalized patients treated for pneumonia in the hospitals of the country were tested. On 08.03.2020, the country's first two cases of SARS-CoV-2 infection were detected in the laboratory.

The objective of the present study was to track the circulation pattern of SARS-CoV-2 in Bulgaria over three consecutive years (2020-2023) based on the results of research conducted in the NRL "Influenza and ARD". The involvement of SARS-CoV-2 as co-infection with other respiratory viruses was also analyzed.



**Figure 1.** Number (in millions) of confirmed COVID-19 cases in the WHO European Region, December 2019 – December 2022 (2)



**Figure 2.** Incidence of COVID-19 in Bulgaria, 2020 - 2022 (Number of cases per 100 000 population) (4)

**MATERIALS AND METHODS**

From February 2020 to December 2022, patients with symptoms of acute respiratory illness (ARI) or their asymptomatic contacts from different regions of the country were examined. Nasopharyngeal samples were collected using polyester collection swabs placed in containers containing 2 mL of virus transport medium. The samples were transported under refrigeration to the NRL “Influenza and ARD”, where they were processed immediately.

Viral nucleic acids were extracted using automated systems: ExiPrep 16DX (BioNeer, Korea), SaMag 12 System (Sacace Biotechnologies, Italy.), and EXM3000 (Zybio Inc., China) according to the manufacturer’s instructions. SARS-CoV-2 and other respiratory viruses were identified by RT-PCR assays using the following PCR detectors: QuantStudio™ 3 real-time PCR system, 96 wells (ThermoFisher Scientific); CFX96 Touch PCR Real-Time Detection System (Bio-Rad) and Gentier 96E/R real-time PCR system. In the course of pandemic, several commercial PCR kits were used for the detection of SARS-CoV-2 (Table 1). Since the beginning of 2022, clinical samples have been tested with a Multiplex real-time RT-PCR kit (FluSC2) for simultaneous detection of influenza A/B and SARS-CoV-2 viruses.

The detection of non-influenza respiratory viruses: respiratory-syncytial virus (RSV), human metapneumovirus (hMPV), parainfluenza viruses (PIV) types 1/2/3, rhinoviruses (RV), adenoviruses (AdV), bocaviruses (BoV), and human coronaviruses (hCoV) was performed using Multiplex real-time PCR assays with primers and probes, previously described (6,7). Four PCR mixtures were developed, including the SuperScript III Platinum® One-Step qRT-PCR system (Invitrogen, USA) and combinations of primers and TaqMan probes labeled with different fluorescent dyes:

- Mix 1: AdV + RSV + PIV1
- Mix 2: BoV + RV + PIV2
- Mix 3: hMPV + PIV3
- Mix 4: hCoV: 229E + OC43 + NL63 + HKU-1

**Table 1.** Diagnostic kits used for the detection of SARS-CoV-2

Diagnostic kits	SARS-CoV-2 genes identified
CerTest (Spain)	ORF1ab, N
GeneFinder (Korea)	RdRp, E, N
BGI (China)	N
Sansure Biotech (China)	ORF1ab, N
Sacace (Italy)	ORF1ab, E, N
TaqPath ThermoFisher (UK)	ORF1ab, S, N
FluSC2 (USA)	N

**Table 2.** Results of the SARS-CoV-2 detection during the 3 years of the COVID-19 pandemic

	2020	2021	2022	Total
Number of tested patients	76 987	18 468	2 792	98 247
Number of positive patients	16 268	7 833	615	24 716
% positive	21.1%	42.4%	22%	25.2%

The result of the real-time RT-PCR assays was considered positive at  $Ct \leq 38$ .

The Whole Genome Next Generation Sequencing (INGS) method was used to analyze the SARS-CoV-2 genome and to determine its genetic variants. The ARTIC protocol v3 with tail amplicon and lumina MiSeq v2 reagents with 500 luminal cycles was used. Phylogenetic analysis was performed using a Pangolin COVID-19 Lineage Assigner Tool v3.1.7 to define variant classification and reference sequence from GISAID (EPI\_ISL\_402124).

Differences in the detection rates of the studied viruses were analyzed using chi-square or Fisher's exact tests for categorical variables. *P* values less than 0.05 were considered significant.

## RESULTS

### Patients' characteristics

A total of 98 247 clinical samples were tested for SARS-CoV-2: 76 987 in 2020, 18 468 – in 2021, and 2 792 – in 2022. Of these, 24 061 (31.3%), 1 486 (8%), and 966 (34.6%), respectively, were samples of patients attending outpatient healthcare centers, and the remaining were samples of hospitalized patients. There was a consistent decline in the number of clinical samples examined in 2022 since at that time over 130 laboratories in the country already offered PCR diagnostics of SARS-CoV-2. The age of the study subjects ranged from 1 day to 102 years (mean  $46.7 \pm 25.2$ ). The distribution of patients by age was as follows: 0-5 (1 979, 2%), 6-16 (2 412, 2.5%), 17-64 (70 736, 72%), and  $\geq 65$  years (23 120, 23.5%). Approximately 44.4% of the study subjects were male and 55.6% were female.

### SARS-CoV-2 detection

In Table 2, the results of the identification of SARS-CoV-2 during the 3 years of the pandemic are shown. The highest percentage of positive results for SARS-CoV-2 was found in 2021 – 42.5% ( $p < 0.05$ ). The positive rates in 2020 and 2022 were similar –

21.1% and 22%. SARS-CoV-2 was detected in 3009 (12.5%), 357 (24%), and 132 (13.7%) outpatients, tested in 2020, 2021, and 2022, respectively, and in 13 259 (25.1%), 7 476 (44%), and 483 (26.5%) inpatients, respectively. Approximately 48.3% of the SARS-CoV-2-positive cases were male and 51.7% were female ( $p < 0.05$ ). The male/female ratio of confirmed cases was 0.93:1.

### Monthly distribution of SARS-CoV-2 infections

During the 3-year period, five peaks in the share of SARS-CoV-2-positive cases stood out (Figure 3). In March-September, 2020 there was a gradual and smooth increase in the number of infections, followed by a sharp spike in the incidence. The first peak occurred in November 2020 with 46.6% positivity. In the beginning of 2021, a slight decline in the positivity rate was observed, but the incidence of SARS-CoV-2 remained relatively high. A second incidence peak, the highest for all studied periods (59.4%), was reached in March-April 2021, followed by a significant decline in the incidence of SARS-CoV-2 infections during the summer months. In the fall of 2021, the number of people infected with the pandemic coronavirus increased again and reached a third peak with 47.3% positivity in October 2021. In January 2022, a 43.3% surge in the percentage of infected people was outlined. This fourth peak was followed by a gradual decline in the percentage of SARS-CoV-2 infections. The fifth smallest peak of SARS-CoV-2-positivity (28.6%) was observed in October 2022.

### Age distribution of SARS-CoV-2 positive cases

The results from SARS-CoV-2 testing among the total number of examined patients for four age groups are shown in Figure 4 for each of the studied years. The positivity rate for SARS-CoV-2 infection was highest in the group of the oldest adults ( $\geq 65$  years), (average 40.6%), and lowest - in the group of the youngest children (0-5 years), (average 9.4%). During the

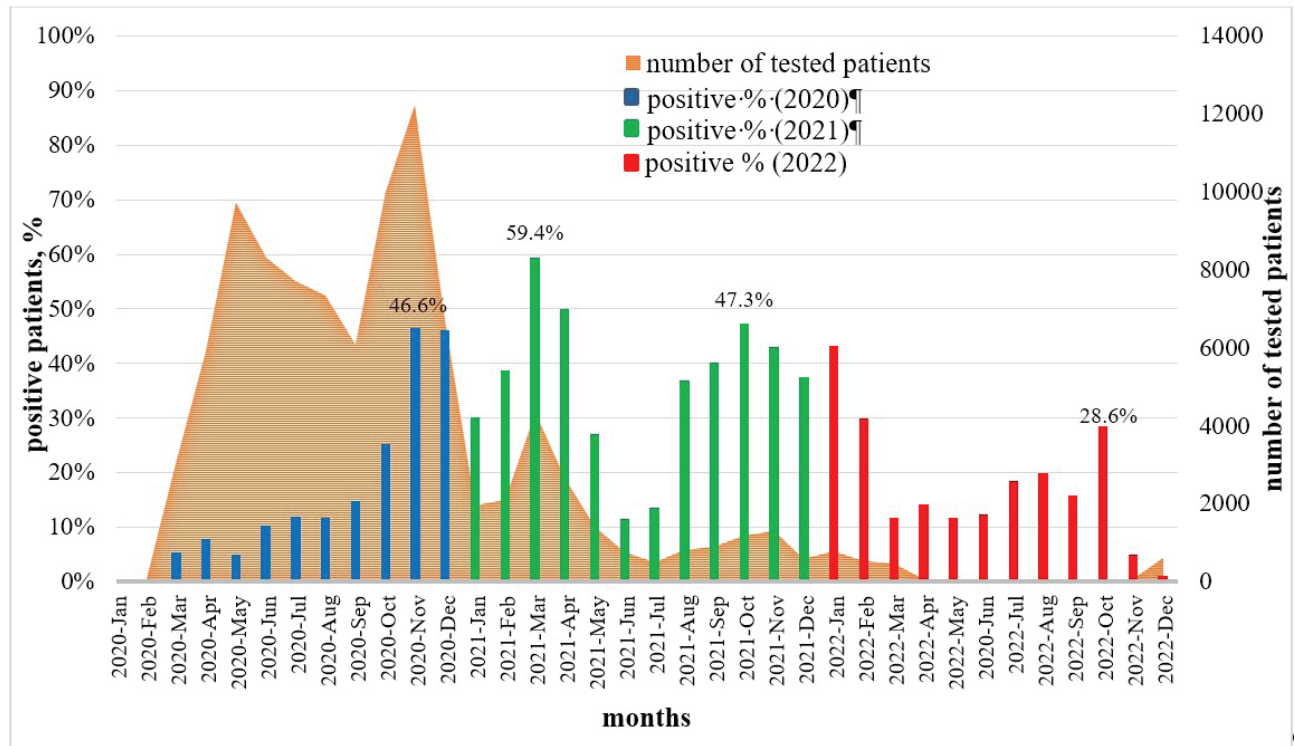


Figure 3. Monthly distribution of confirmed SARS-CoV-2 cases in Bulgaria, 2020-2022

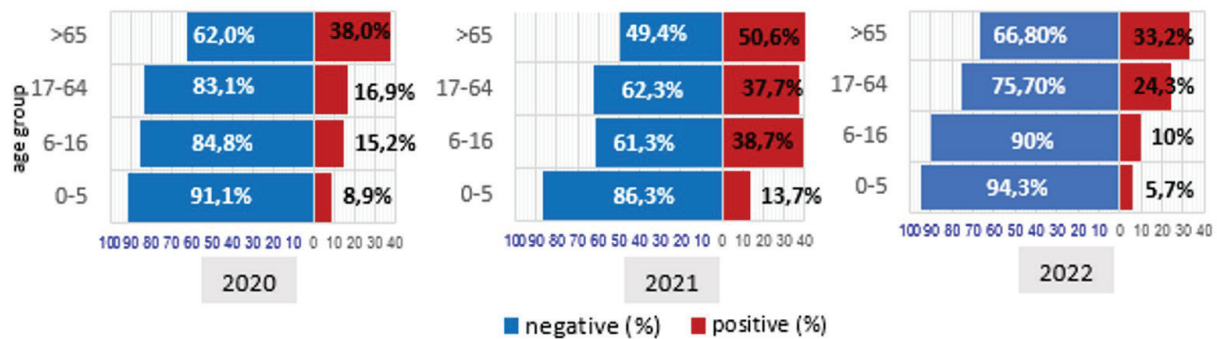


Figure 4. Age distribution of patients/contacts with confirmed SARS-CoV-2 infection in Bulgaria, 2020-2022

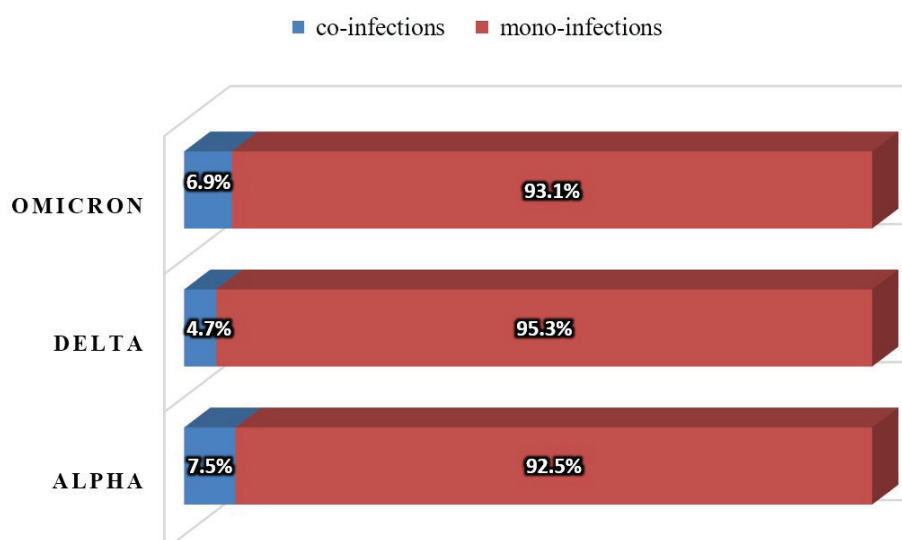
study period, the share of SARS-CoV-2 positive cases increased consistently with age. For all age groups, the highest incidence of SARS-CoV-2 positive cases was registered in 2021.

**Co-infections involving SARS-CoV-2**

During the period 2020-2022, 1 463 SARS-CoV-2 positive clinical samples were examined for the presence of other respiratory viruses. Among them, 107 (7.3%) cases of double infections were detected. Two patients were co-infected with SARS-CoV-2 and two other respiratory viruses (triple infection). A total of 999 samples tested for SARS-CoV-2 co-infections underwent sequence analysis to determine the genetic lineage of pandemic coronavirus. Co-infections were proved in 59

samples. The number (%) of detected co-infections was analyzed depending on the genetic variant of the SARS-CoV-2 involved in the co-infection. Mixed infections were confirmed in 7.5% (7/94) of patients infected with the Alpha variant; 4.7% (22/441) of those infected with the Delta variant and 6.9% (30/433) of those infected with the Omicron variant (Figure 5). The greatest variety of co-infections with SARS-CoV-2 and other respiratory viruses was found during the Omicron wave. Co-pathogens in the mixed infections were RSV (1.6%), RV (0.5%), AdV (0.7%), PIV2 (0.2%), BoV (0.9%), PIV3 (1.8%), HMPV (0.2%), NL36 (0.7%), and OC43 (0.2%). In the cases with Alpha variant, only three co-pathogens were identified: AdV (3.2%), HMPV (4.2%), and BoV (1.2%).





**Figure 5.** Distribution of confirmed co-infections according to the SARS-CoV-2 variant

## DISCUSSION

In the present study, a number of virological and epidemiological aspects of SARS-CoV-2 infection in Bulgaria during the period 2020-2022 were analyzed. The highest percentage of positive results for SARS-CoV-2 was found in 2021, which can be explained by the consecutive widespread in the country of two genetic variants, characterized by increased infectivity: Alpha (during the winter and spring) and Delta (during the fall). The highly contagious Omicron variant caused high morbidity during the winter and spring of 2022, but thereafter the incidence dropped sharply as the majority of the population had already encountered the virus (8). During the 3 years of the study, the SARS-CoV-2 positive rate was greater among hospitalized patients as compared to outpatients ( $p < 0.05$ ). SARS-CoV-2 positivity was the highest among the oldest adults (65+ years) and lowest among the youngest children (0-5 years). Our results showed that the rate for SARS-CoV-2 positivity increased consistently with age, which is in line with the findings of other researchers (9,10). In our study, the share of SARS-CoV-2-positive female individuals was significantly higher as compared to male individuals, indicating a possibly greater susceptibility of females to this infection. In a study analyzing data from 20 European countries, USA and Canada, male patients also accounted for less than half of the confirmed cases (11). The monthly distribution of SARS-CoV-2 cases showed

that this pathogen circulates throughout the year with increased frequency during the cold months (October-April). This autumn-winter-spring seasonality is also characteristic of endemic low pathogenic coronaviruses (229E, OC43, NL63, and HKU1) (12). The spread of SARS-CoV-2 outlined several waves of increased morbidity, each associated with the appearance and wide spread of some new genetic variant to which the population immunity was not sufficiently strong (13).

Various public health and social distancing measures (e.g., wearing masks, working from home, school closure, limited social gatherings, travel restrictions, quarantines, patient isolation, etc.) implemented to mitigate the spread of SARS-CoV-2 reduced the prevalence of other seasonal respiratory viruses. Our large-scale testing for co-infections between the SARS-CoV-2 and other respiratory viruses established a small number of such mixed infections, which can be explained by the low circulation of seasonal respiratory viruses during periods of intensified anti-epidemic measures. Our previous studies from the pre-pandemic years demonstrated a significantly higher rate of coinfections with seasonal respiratory viruses : 15.9% (14) and 14.3% (15) . A number of authors in other countries have also reported a low incidence of co-infections with the participation of the SARS-CoV-2 as a result of non-pharmaceutical protective measures introduced to limit the pandemic (16,17).

## CONCLUSION

Our study presents a versatile picture of COVID-19 pandemic in Bulgaria during the first three years of this extremely serious hardship for the health system and society as a whole. According to the lessons learned, the intensity of viral spread was determined by the transmissibility of the pathogen, its ability to evade pre-existing immunity, the anti-epidemic measures applied, and the level of herd immunity. In the future, surveillance of the SARS-CoV-2 should continue, integrating with that of influenza and other respiratory viruses (18). Continuous monitoring of this pathogen is necessary in order not to miss the emergence of new genetic variants with altered antigenicity, increased virulence, or reduced susceptibility to antiviral drugs. Such research is important for public health decision-making and policy formation.

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