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

I. Alexiev, I. Ivanov, I. Stoikov, D. Donchev, L. Grigorova, R. Dimitrova, A. Gancheva, N. Korsun, I. Trifonova, V. Dobrinov, T. Kantardjiev, I. Christova

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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.

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

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

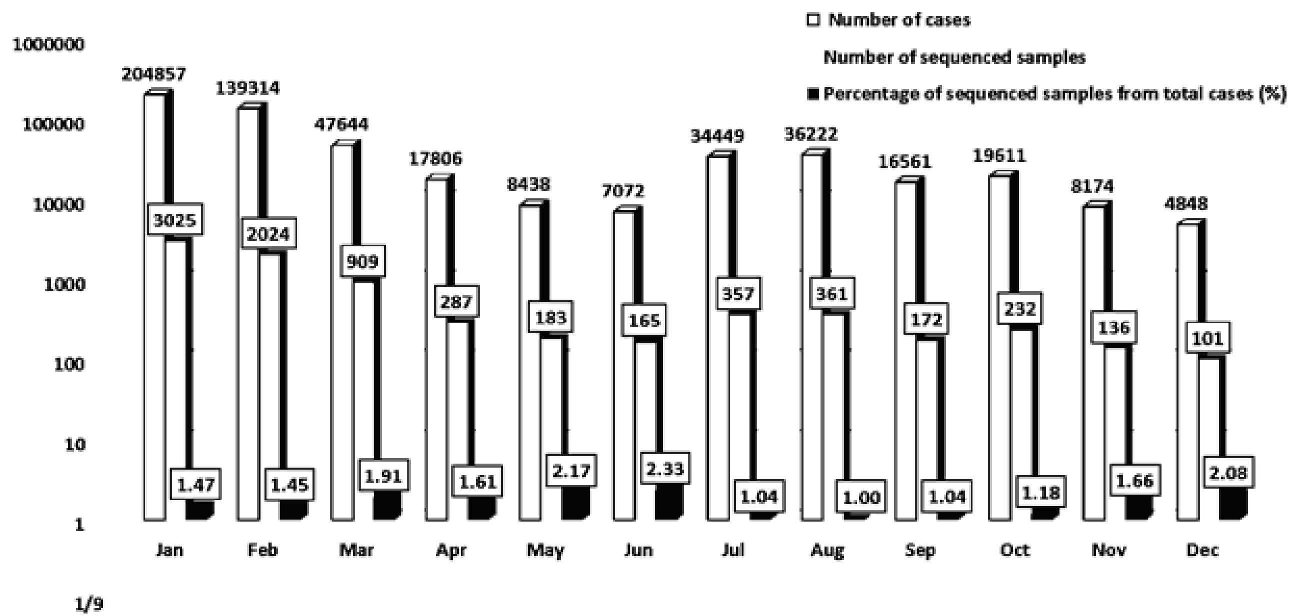
3.1. Population characteristics

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

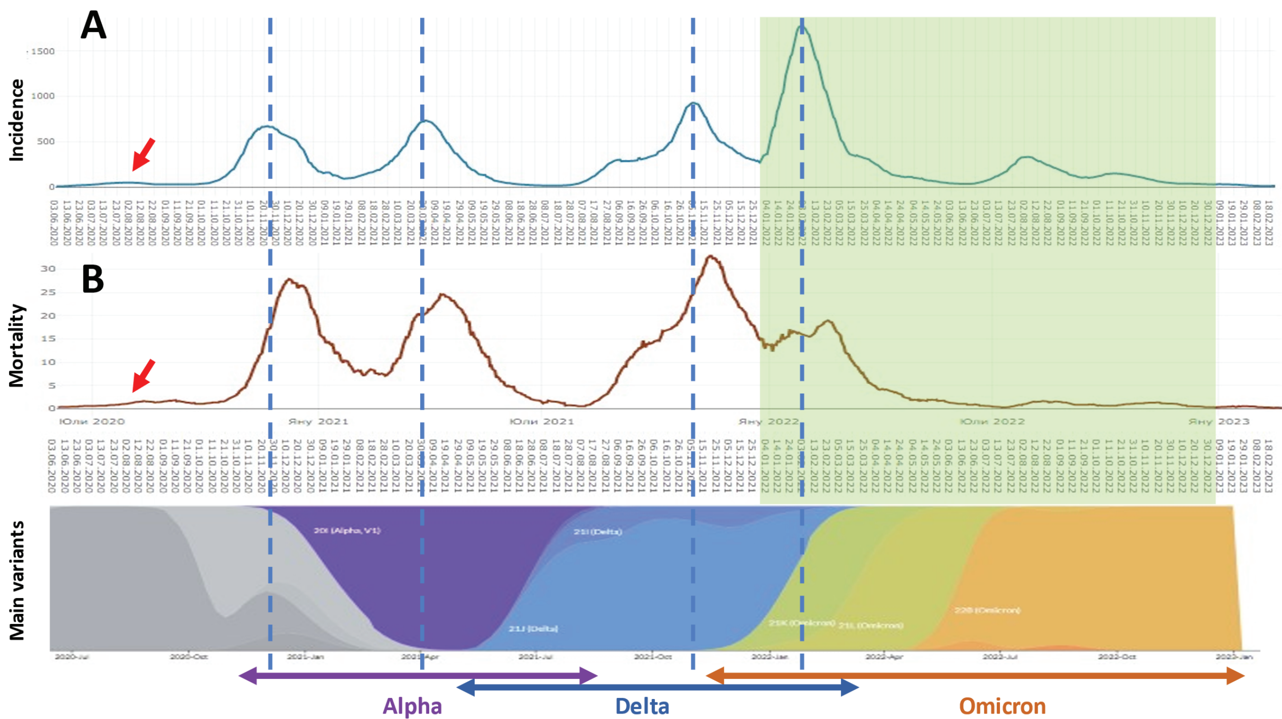


Figure 1. Incidence and mortality of COVID-19 in Bulgaria, 2020 – 2022. A. Incidence cases (Number per 100,000 population). B. Mortality rate (Number per 100,000 population). The green rectangle represents 2022, which is characterized by a period of the gradual disappearance of the Delta Variant and 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

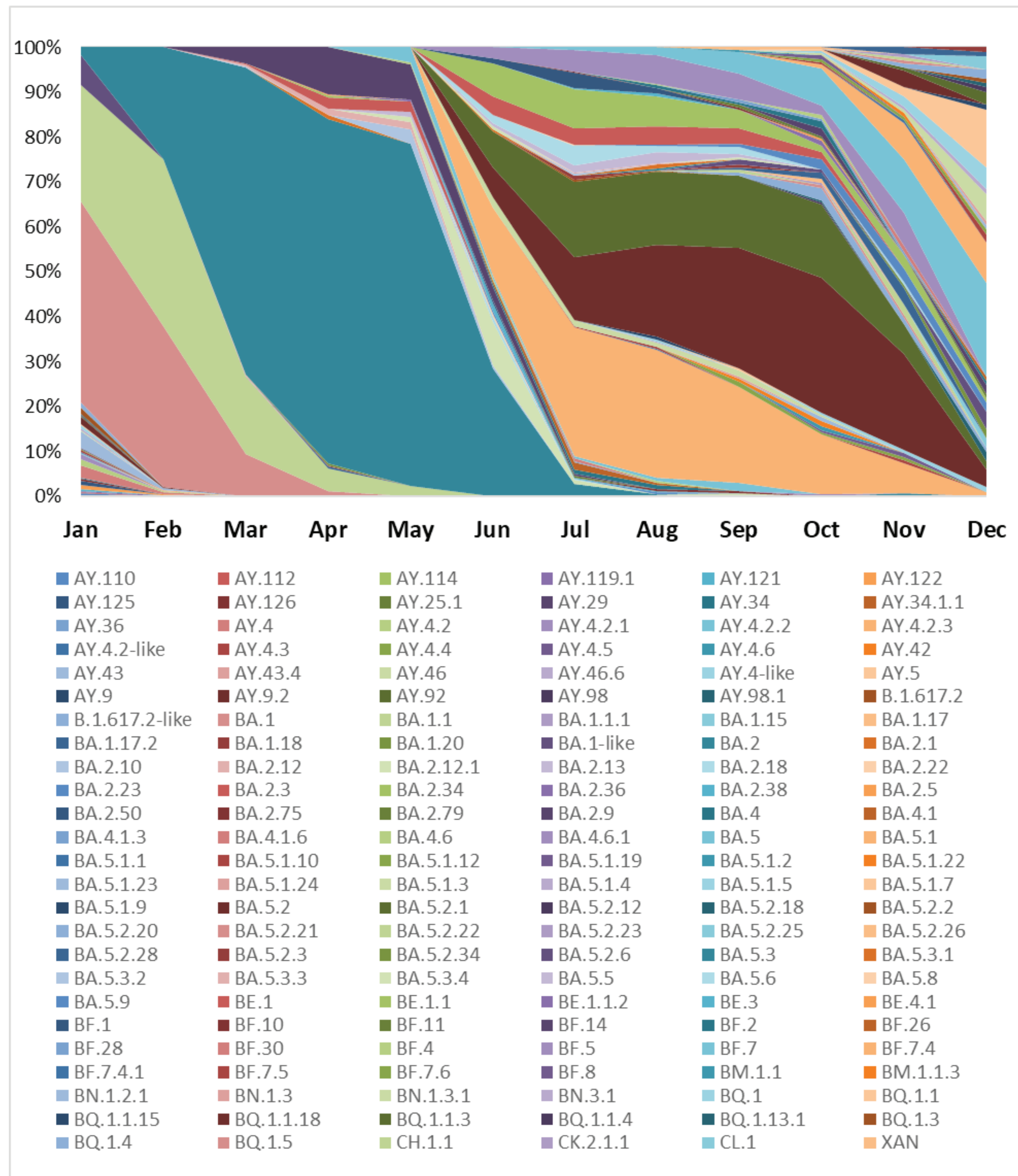


Figure 2. Dynamics in the prevalence of the SARS-CoV-2 sub-variants over time in 2022 in Bulgaria. In the beginning of the year, the Delta variant prevailed, further replaced by the much faster spreading Omicron. AYx/B.1.617.2 represent Delta; BA/BE/BF/BM/BN/BQ/CH/CK/CK represents Omicron; XAN/XBB represents 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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ASSESSMENT OF SARS-COV-2 SPECIFIC B-CELL IMMUNE MEMORY: EVIDENCE FOR PERSISTENCE UP TO 1 YEAR POST-INFECTION

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ABSTRACT

Background: SARS-CoV-2, the virus responsible for COVID-19 pandemic, has posed huge global health challenges. Understanding the immune response to SARS-CoV-2 infection, and in particular – the role of B cells in the generation of immune memory is crucial for assessing the durability of protective immunity.

Materials and Methods: In this longitudinal prospective study, individuals who had recovered from SARS-CoV-2 infection were included. Peripheral venous blood samples were collected at three time intervals post symptom onset (PSO): 1-3 mo, 4-8 mo, and 9-12 mo. The humoral immune response was evaluated by measuring anti-SARS-CoV-2 IgG, virus-neutralizing antibody activity, total S1-specific B-cells, and B cell subpopulations.

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Results: The levels of anti-SARS-CoV-2 specific IgG antibodies decreased from 390.3 to 204.5 BAU/ml in the first 6-8 months PSO but did not significantly decrease further until the 12th mo (126.6 BAU/ml). Virus-neutralizing antibodies (activity decreased by 20.4% between the 1st and 6-8th mos but remained relatively stable thereafter and could be detected up to 12 months PSO. In peripheral blood, the amount of S1-specific plasmablasts was highest one month after COVID-19 infection, and the level of memory B cells at 6 months. Those were detected even 12 months PSO, albeit in smaller quantities.

Conclusion: The study provides evidence for the persistence of SARS-CoV-2-specific B-cell immune memory up to 1year post-infection. The presence of virus-specific memory B cells and plasmablasts suggests potential for sustained protection against reinfection. Further research is needed to elucidate the role of B-cell immune memory in preventing infection and to understand the individual variations of immune response.

Key words: SARS-CoV-2, Immune memory, Humoral immunity, B-cell immunity

INTRODUCTION

SARS-CoV-2, the novel coronavirus responsible for COVID-19 pandemic, has posed a significant global health challenge (1). Understanding the nature of immune response induced by SARS-CoV-2 infection is crucial for describing the dynamics of protective immunity and development of public health strategies. Among the key components of immune response, B cells play a vital role in combating viral infections by producing specific antibodies and generating immune memory.

One essential aspect of B cell immune memory is the duration of antibody production and the persistence of circulating protective antibodies following recovery from SARS-CoV-2 infection (2,3). Understanding the longevity of B cell immune memory is important for assessing the risk of reinfection and the effectiveness of acquired immunity against subsequent exposures to the virus (4). Several studies showed that after the acute phase of SARS-CoV-2 infection, there is an initial rapid elevation of antibody levels. However, the antibody levels tend to decrease over time reaching a plateau 8 months PSO, and gradually declining thereafter (5). This is a normal dynamic of the immune

response, and it does not necessarily indicate a loss of immune memory. Instead, it suggests a transition from short-lived plasma cells to long-lived memory B cells that can persist in the body for an extended period. (6)

The generation of virus-specific B-cell immune memory involves the formation of germinal centers within the secondary lymphoid organs, where B cells undergo clonal expansion, affinity maturation, and class-switching. These processes contribute to the production of high-affinity antibodies with enhanced neutralizing capacity. Moreover, memory B cells can persist in circulation or reside in specialized niches, poised to respond promptly and effectively upon re-exposure to virus (7). Several publications indicated that memory B cells generated in response to SARS-CoV-2 infection can persist for several months after recovery (9,10) and even up to one year post-infection (11,12,13).

In this study, we demonstrated that one year PSO S1-binding and neutralizing antibodies along with S1-specific B cells and plasma cells were detected in the circulation. This supports the hypothesis that SARS-CoV-2 infection induces durable humoral immunity.

MATERIALS AND METHODS

This longitudinal prospective study included individuals recovered from SARS-CoV-2 infection, confirmed with RT-PCR. The participants were aged between 18 and 75 years. Peripheral venous blood samples for isolation of peripheral blood mononuclear cells (PBMCs) and serum were taken and analyzed at three-time intervals: (1-3 mos, 4-8 mos, and 9-12 mos PSO). In 20% of the cases, the analysis was performed on different patients in the mentioned time periods. (Table 1).

The amount of anti-SARS-CoV-2 IgG binding antibodies was assessed by ELFA (Enzyme-linked fluorescent assay, VIDAS PC) and expressed as BAU/ml (Binding antibody units per mL). The presence of SARS-CoV-2-neutralizing antibodies was measured by the percentage of inhibition in a surrogate virus neutralization test (sVNT, GenScript kit).

Table 1. Characteristics of the included individuals included in the study

Testing period (mos PSO)	Male (n)	Female (n)	Age (years, X±SD)
1-3	20	46	44.5±22.5
4-8	7	41	45.5±21.5
9-12	14	19	49.5±22.5

Total S1-specific B cells were evaluated by B-ELISpot (Mabtech). B-ELISpot test was performed after a preliminary stimulation of $2x10^5$ PBMCs with IL-2 and R848 for 5 days. The number of SARS-CoV-2-specific IgG-secreting B cells was measured as spot-forming units (SFU) per million PBMCs (SFU/10^6 PBMC) using an automated system – BIOREADER 700.

Multiparameter flow cytometry of PBMCs was used to identify peripheral blood S1-specific memory B cells. Based on the expression of specific CD markers, we developed a 13-parameter panel for the analysis of B-cell subpopulations: CD45 (BUV496), CD24 (BV421), CD27 (BV480), CD19 (BV605), CD20 (BV786), CD38 (PE), IgD (BB515), IgG (PE-Cy7), IgM (APC), CD138 (PE-CF594), CD21 (BUV395), Streptavidin (APC-R700), Streptavidin (BV605). To exclude T cells, NK cells, and monocytes from the analysis we used CD3, CD14, CD16 and CD56 mAbs stained with the same fluorochrome (PerCPCy5.5).

Antigen-specific B cells were identified using tetramers of four biotinylated S1 proteins and streptavidin conjugated with two different fluorochromes (BV421 and BV711). They were added to the CD marker panel along with streptavidin labeled with a third fluorochrome (BUV395 – decoy streptavidin). Stained cells were collected by FACSAria III flow cytometer and analyzed with DIVA v.8 software. The cells which were simultaneously stained by both tetramers and not stained by decoy streptavidin, were considered antigen-specific. An algorithm was created for the analysis of B-cell subpopulations and the identification of SARS-CoV-2-specific memory B cells and plasmablasts.

Statistical analysis was performed by GraphPad Prizm 8.0.1 and IBM SPSS 28.0. Data were presented as mean values ± SD. P was considered statistically significant at $p<0.05$.

RESULTS

Levels of S1 binding and virus-neutralizing antibodies.

The results for anti-SARS-Cov-2 IgG binding antibodies are presented in Figure 1A. The mean levels of anti-

SARS-CoV-2 IgG decreased significantly during the first 6-8 mos PSO from 390.3 to 204.5 BAU/ml (p<0.001). Afterwards, no additional significant decrease was observed up to the 12th mo PSO [126.6 BAU/ml (p>0.05)]. The levels of virus-neutralizing antibodies decreased by 20.4% between 1st and 6-8 mos after recovery (from 79.9% to 63.6% inhibition, p<0.001), followed by insignificant changes up to one year, (59.8% inhibition, p>0.05). Thus, a virus-neutralizing activity could still be detected up to 12 mos after the last contact with the viral antigens (Figure 1B).

S1-specific memory B-cells evaluated by B-ELISpot

During the first period (1-3 mos PSO), an average of 143.5±59 SFCs (Spot-forming cells) per 10^6 PBMCs were detected. During the second period (4-8 mos PSO), the number of S1-specific B-cells significantly decreased (11.4±13.8, p<0.05), and at the end of the first year (9-12 mos PSO), an insignificant increase was registered (47.5.3±40.7, p>0.05). These results are presented in Figure 2.

Memory B-cell subpopulations evaluated by flow cytometry

The results of the peripheral blood B-cell subset analysis are presented in Table 2. The percentages of naive, transitional, and memory B cells did not show significant differences between the three studied periods. As might be expected, the percentages of plasmablasts and plasma cells were highest 1-3 mos PSO (6.3% and 6.9%, respectively). The share of these subpopulations decreased twice at the end of the first year (2.9%, and 3.2%, respectively, p<0.05 for both). The average share of NCSMB (non-class-switched memory B cells) increased between the first (1-3 mos) and second period (9-12 mos) from 39.2% to 59.4% (p<0.05). In parallel, the percentage of CSMB (class-switched memory B cells) decreased insignificantly during the same time, from 30.5% to 26.3% (p>0.05).

Antigen-specific B-cells and plasmablasts evaluated by flow cytometry.

The results about virus-specific B cells and plasmablasts are presented in Figure 3. During the first period (1–3 mos PSO) the percentage of S1-specific CSMB exceeded that of NCSMB (0.6% vs 0.2%, p<0.05). The amount of CSMB remained stable until 6 mo and decreased threefold afterwards (0.7% vs 0.2%, p<0.05). The level of NCSMB was low during the first period (1–3-mos PSO) (0.2%, followed by a 5-fold increase between 4-8 mos, (1.3%, p<0.05), and a subsequent decrease

Table 1. Percentage distribution of B cell subpopulations.

B cell subset (phenotype)	1-3 mos PSO	4-8 mos PSO	9-12 mos PSO
Naive B cells CD45$^+$/CD19$^+$/CD20$^+$/CD21$^+$/CD24$^+$/ IgD$^+$/IgM$^+$	6.9%	5.4%	5.9%
Transitional B cells CD45$^+$/CD19$^+$/CD20$^+$/CD21$^+$/CD24$^+$/CD38^{+++}/IgD$^+$	56.0%	65.0%	63.5%
Memory B cells CD45$^+$/CD19$^+$/CD20$^+$/CD21$^+$/ CD27$^+$/CD24$^+$	22.6%	19.6%	19.7%
NCSMB (% from memory B cells) CD45$^+$/CD19$^+$/CD20$^+$/CD21$^{+/}$ CD27$^+$/CD24$^+$/ IgD-/ IgM+/IgG-	39.2%	47.1%	59.4%
CSMB (% from memory B cells) CD45$^+$, CD19$^+$, CD20$^+$, CD21$^+$, CD27$^+$, CD24$^+$, IgD-, IgM$^{-/low}$, IgG$^+$	30.5%	26.3%	26.3%
Plasmablasts CD45$^+$/ CD19$^+$/ CD20$^{-/low}$/ CD27$^+$/ CD38$^{++/+++}$/ CD1$^{38+/-,}$ IgD$^{-/low}$/ IgM$^{-/low}$	6.3%	4.2%	2.9%
Plasma cells CD45$^+$, CD19$^+$, CD20$^{-/low}$, CD27$^+$, CD38^{++}, CD138^{++}, IgD$^{-/low}$, IgM$^{-/low}$.	6.9%	4.2%	3.2%

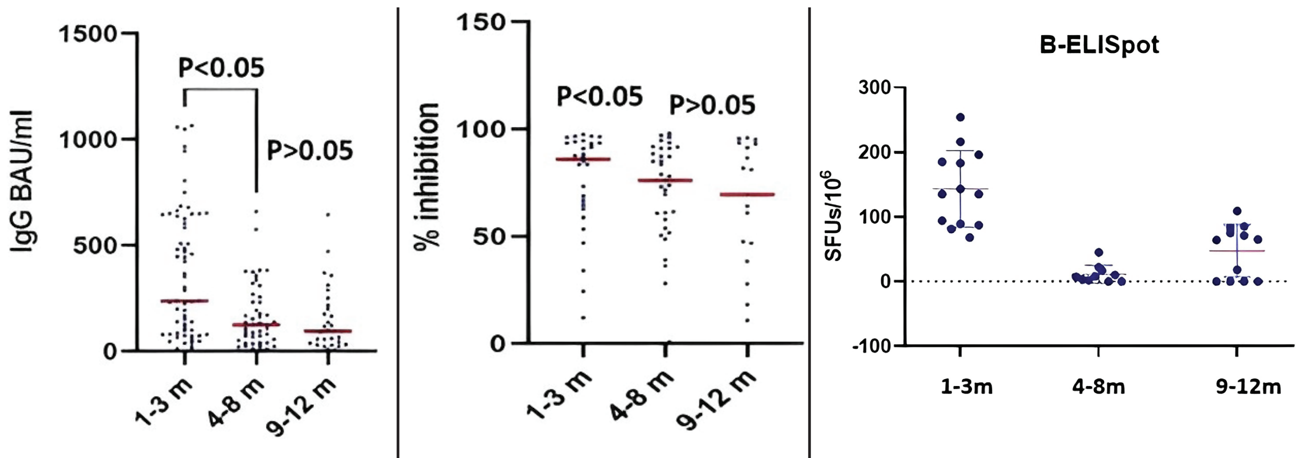

Figure 1. Dynamics of A) S1-specific anti-SARS-CoV-2 IgG and B) percentage of inhibition of virus-neutralizing antibodies.

Figure 2. Number of S1-SARS-CoV-2-specific plasma cells detected by B-ELISpot

between 9-12 mos to 0.3% (p<0.05). The share of S1-specific plasmablasts in peripheral blood was highest between 1 and 3 mos PSO (4%). During the second period (4-8 months PSO) plasmablasts decreased to 0.7% (p<0.05), followed by a new increase to 3.8% between 9 and 12 mos (p<0.05).

DISCUSSION

In this study, we investigated the duration of humoral immunity after recovery from COVID-19 according to the following parameters: S1-binding and neutralizing antibodies, S1-specific total memory B cells, as well as their subpopulations. We found that SARS-Cov-2 infection induced the formation of S1-binding and neutralizing antibodies. Their levels were highest during the first 3 months after recovery. Although their level decreased later, it was still detectable until the end of the first year PSO. This is consistent with the results of other authors and supports the notion that SARS-Cov-2 likely builds long-lasting immunity. To answer the question about the longevity of B cell immunity, we investigated the circulating total S1-specific memory B cells as well as their subpopulations. We found that the amount of total S1-specific memory B cells in the blood was highest 4-8 mos PSO, followed by a decrease. However, during the third period (9–12 mos PSO), a significant amount of S1-specific total memory B cells were found in circulation. These results are in line with other authors' published data (13-17). The presence of S1-B memory cells one year after infection supports the hypothesis of lasting B-cell immune memory.

The results presented in Figure 1 showed a nonlinear reduction of S1-specific total memory B cells, measured by B-ELISpot. Therefore, we investigated the B cell subpopulations in more details by multiparameter flow cytometry. The six B-cell subpopulations showed different dynamics during the studied PSO periods. The percentage of CSMB cells followed the dynamics of serum antibodies, maintaining the level until 6 mos and decreasing until

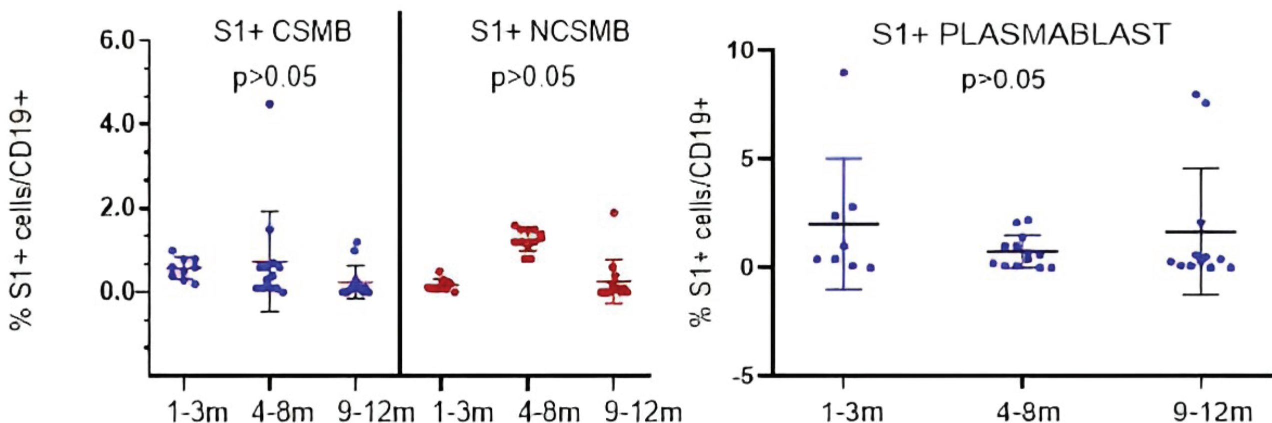

Figure 3. Analysis of flow cytometric data for specific memory B cells and plasmablasts. (1)

the 12th mo. The amount of NCSMB cells increased between the first and second period (4-6 mos PSO) of the study, followed by a decrease. The dynamics of S1-specific plasmablasts coincided with that of the total S1 memory cells detected by B-ELISpot. The presence of S1-specific plasmablasts in the circulation after recovery from COVID-19 was also described by other authors (18). In this study, at 9-12 mos PSO we detected also S1-CSMB and S1-NCSMB cells in the peripheral blood. This finding gives us the reason to assume that following SARS-CoV-2 infection, B-cell immune memory dynamically develops for at least 12 mos, which is the basis for establishment of long-lasting immunity. Further research is needed to establish the longevity of this immune memory and its protective effect.

CONCLUSION

Our results show that using S1 tetramers and multiparameter flow cytometry, virus-specific B-cell immune memory can be assessed in detail. S1-specific plasmablasts and memory B cells were detected by B-ELISpot and S1 tetramers 12- mos PSO albeit in small amounts. This finding supports the hypothesis that long-lasting B-cell immune memory is possible after COVID-19, although its protective role remains to be clarified.

LIMITATIONS

A major limitation of the study is the decreased number of tested individuals during the subsequent periods of testing, due to reinfection or vaccination of the original donors. Another limitation is the fact that in 20% of the cases, the analysis in the studied time periods was performed on different patients.

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HEALTHCARE WORKERS IN BULGARIA – ARE THEY PROTECTED FROM VACCINE-PREVENTABLE INFECTIONS?

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ABSTRACT

Background: Healthcare workers (HCWs) are at increased risk of exposure to many viral infections, including vaccine preventable diseases (VPDs) such as measles, mumps and rubella (MMR) as compared to non-HCWs. Immunity of HCWs against these viruses is mandatory in a healthcare setting due to possible exposure from patients or colleagues.

Aim: To provide an assessment of anti-measles, mumps and rubella IgG seropositivity among Bulgarian HCWs employed in hospitals and regional health inspectorates (RHI), as an indicator of protective immunity against MMR in this risk group.

Materials and Methods: In the current study, 181 HCWs from Infectious Units in regional hospitals in the country, and HCWs from the RHI, involved in the monitoring and surveillance of MMR cases in Bulgaria were screened. Serum specimens from all participants were tested by a commercial indirect enzyme-linked immunosorbent assay (Anti-Measles, Anti-Mumps, Anti-Rubella IgG EIA-Euroimmun®, Germany) for presence of IgG antibodies against measles, mumps and rubella, as an indicator of protective immunity.

Results: The study included 181 HCWs, 25 male and 156 female, aged 22 to 66 years. The average protective seroprevalence for measles, mumps and rubella was 82.9%, 76.2% and 92.3% percent, respectively. The highest share of negative results were obtained for mumps-specific IgG – 23.2% (42/181), followed by measles 16.6% (60/181) and rubella-specific IgG 7.7% (19/181). Regarding the age distribution, the highest number of HCWs non-immune to measles and mumps was found among the 31- 40-year olds, and against mumps – among the 41-50-year-olds.

Conclusion: HCWs are at greater risk of contracting infections than the general population because of contact with sick patients or infectious material. Infected healthcare workers can spread nosocomial diseases to vulnerable patients with more severe illness, leading to complications and even death. Therefore, the vaccination status of HCWs must be strictly monitored.

Key words: HCWs, measles, mumps, rubella, IgG immunity

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INTRODUCTION

HCWs are exposed to much more viral infections, including VPDs such as measles, mumps and rubella as compared to non-HCWs. Immunity of HCWs against these viruses is mandatory in a healthcare setting due to possible exposure from patients or colleagues [1-3].

The high contagion index (>90% for measles and rubella, and >50% for mumps), the high frequency of severe, debilitating complications and the significant mortality determine the great healthcare and socio-economic importance of these infections. Approximately 30% of reported measles cases have one or more complications, with disabling effects most common in children under five years of age. The public health importance of rubella infection is determined by the teratogenic effect of rubella virus during pregnancy. Rubella is associated with a high rate of miscarriages, stillbirths or congenital rubella syndrome, manifested by blindness, deafness, heart defects and other severe organ damages in the

newborn. The mumps virus, in turn, is one of the main causes of viral meningitis and meningoencephalitis in about 10 to 30% of infected and non-immune persons. Epidemic outbreaks of MMR are recorded mainly among unvaccinated and non-immune individuals, and nosocomial transmission has also been reported, making it extremely important to maintain optimal immunity among HCWs involved in the care and monitoring of such patients [4]. Beacuse of their professional duties, HCWs are more likely to acquire and transmit vaccine-preventable diseases such as influenza, measles, rubella and whooping cough [5].

In the pre-vaccination era, MMR were endemic in Europe with regular outbreaks occurring each 2–5 years, so that most people would be infected during childhood. In the pre-vaccination era, MMR were endemic in all ages of the world and in all age groups that had immunity to the viruses was acquired through exposure to the disease in infancy or adolescence. In 1998, the Regional Committee of the World Health Organization (WHO) for Europe defined nine vaccine-preventable diseases as the main targets of healthcare policy, including measles elimination, and reducing the incidence of Congenital Rubella Syndrome (CRS) [6]. Since 2004, Bulgaria has been included in the WHO Program for the Elimination of Measles and Rubella (including Congenital Rubella) in the European Region and conducts active seroepidemiological surveillance of all reported cases [7, 8]. In parallel, case-based mumps surveillance is being introduced in the country.

The present study was carried out in 2022, and **aims** to provide an assessment of anti-measles, mumps and rubella IgG seropositivity among HCWs in Bulgaria, who are employed in hospitals and RHI, as an indicator of protective immunity against MMR in this risk group.

MATERIALS AND METHODS

Study design

The study was focused on medical staff from seven country regions (Sofia capital, Burgas, Blagoevgrad, Dobrich, Pazardzhik, Veliko Tarnovo, and Sofia region) working at the Infectious Units of regional hospitals, and HCWs from the RHI, involved in the monitoring and surveillance of MMR cases. The samples were taken during the period of measles outbreaks in Bulgaria (2017 – 2020). After the tests, the HCWs were informed about their MMR IgG titers.

MATERIALS

Serum samples collected from 181 HCWs were tested for presence of IgG antibodies specific for measles, mumps and rubella viruses, as an indicatorof protective immunity. The laboratory assays were carried out at the National Reference Laboratory "Measles, Mumps, Rubella", Department of Virology, National Center for Infectious and Parasitic Diseases (NCIPD), Sofia.

METHODS

Serological analysis

All serum specimens were tested for the presence of anti-Measles, anti-Mumps and anti-Rubella IgG with a commercial indirect enzyme-linked immunosorbent assay (Anti-Measles, Anti-Mumps, Anti-Rubella IgG EIA-Euroimmun®, Germany). The extinction of eachtested sample was divided by the extinction of the calibrator and the results were interpreted qualitatively as positive, negative or equivocal. in accordance with the manufacturer’s instructions (a test was considered positive for MMR if the calculated ratio was above 1.1). Quantitative analysis was also performed and the level of protective antibodies was calculated in international units per milliliter (IU/ml) by plotting a standard curve. The assay specificity and sensitivity was more than 95%, respectively according to the manufacturer.

Statistical Analysis

We calculated overall and group-specific percent seropositivity. In order to compare seropositivity among the different groups under investigation, we used the Fisher’s exact test and the results were considered as significant if the p-value was ≤0.05.

RESULTS

Characteristic of subjects

The study included 181 participants HCWs, 25 male and 156 female aged 22 to 66 years The demographic characteristics of the study population are given in Table 1.

Table 1. Demographic characteristics of the study population

Characteristics		N	(%)
HCWs tested		181	(100)
Gender	Male	25	(13.8)
	Female	156	(86.2)
Occupation	Employed in regional hospitals	137	(75.7)
	RHI employees	44	(24.3)
		Median (range):	SD*
SD*:		46.5 (22 – 66)	13.6

**SD – Standard deviation*

Overall, protective seroprevalence for measles, mumps and rubella was 82.9% (150/181), 76.2% (138/181) and 92.3% (167/181) percent, respectively. The mumps seronegative HCWs were the highest share 23.2% (42/181), as compared to measles 16.6% (60/181) and rubella- seronegative ones7.7% (19/181) (Table 2).

Regarding the age groups, the lowest number protected against measles and mumps was found among the 31-40-year-olds (15/26, 57.7% and 16/3, 61.5%), and against mumps – among the 41-50-year-olds (35/47, 74.5%). On the other hand, calculated protective immunity against rubella was lowest in the 20-30-year-olds (23/27, 85.2%) and enhanced with increasing age to 32/33, 97% in those aged above 60 (Figure 1).

The analysis with Fisher's exact test identified statistically significant differences between age-specific positivity and overall positivity for two age groups, regarding measles and mumps : 31- 40 and > 60 (Figure 1). The positivity among the 31-40 year-olds was particularly low (57.7% as compared to the overall 82.9% for measles and 61.5% as compared to the overall 76.2% for mumps). This difference was statistically significant regarding measles ($p<0.001$). Additionally, the positivity among those >60 years of age (93.9% for measles and 84.8% for mumps) was higher than the overall positivity which was calculated for these VPDs ($p=0.1223$ for measles and $p=0.2601$ for mumps).

DISCUSSION

HCWs are at high risk of contracting serious and sometimes fatal diseases, including VPDs. According to the Center for Disease Control and Prevention (CDC) recommendations if a HCW was born in 1957 or later and has not received MMR vaccine or does not have a positive serological result for the presence of protective MMR IgG antibodies, should receive one or two doses of MMR (1 dose immediately and a 2nd dose at least 28 days later) [9]. The present study involved 181 HCWs whose MMR immune status was determined. The highest IgG seropositivity was calculated against rubella (92.3%), followed by measles (82.9%) and mumps (76.2%). Similar studies from other regions revealed that in Australia 91.5% of HCWs were seropositive to measles, 88.7% to mumps, 91.1% to rubella [10]. In Italy and Turkey, 98.2% and 98.6% were seropositive to measles, 85.9% and 92.2% to mumps, 97.6% and 98.3% to rubella, respectively [11, 12]. In Saudi Arabia, seropositivity rates were shown to be 87% to measles and 90% to rubella [13]. Our MMR seroprevalence results were also similar

Table 1. Seroprevalence for MMR among 181 participants.

Tested VPDs	Positive n (%)	Equivocal n (%)	Negative n (%)
Measles	150 (82.9)	1 (0.6)	30 (16.6)
Mumps	138 (76.2)	1 (0.6)	42 (23.2)
Rubella	167 (92.3)	0 (0)	19 (7.7)

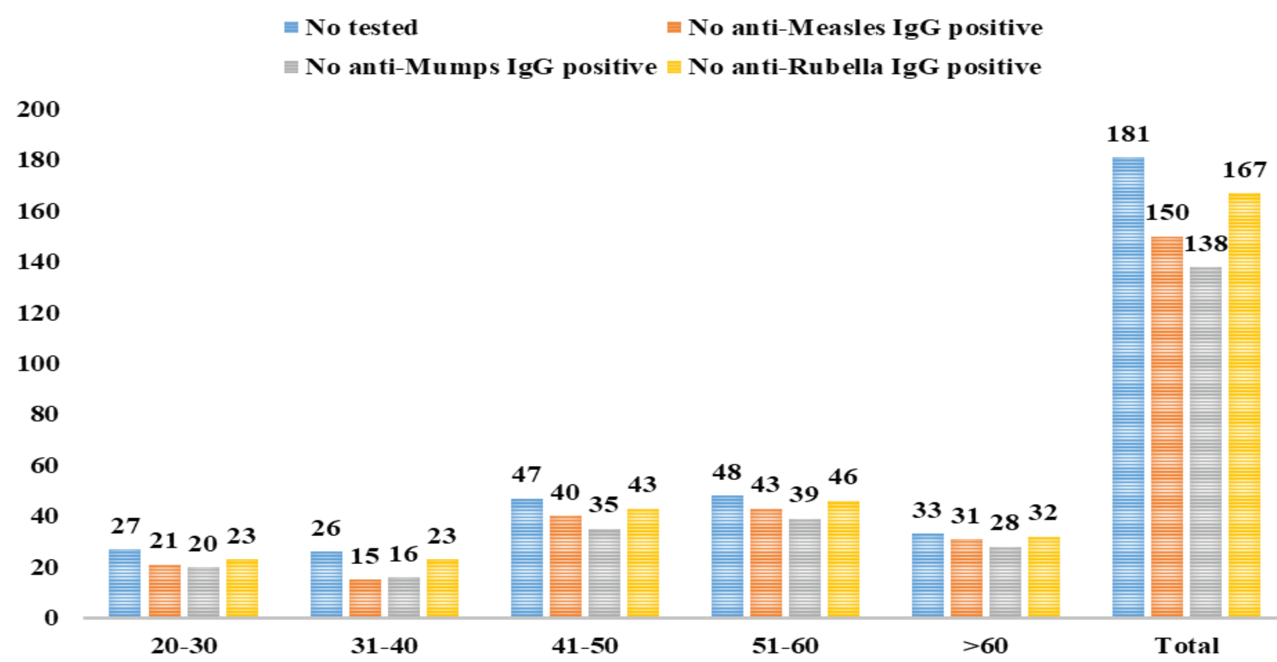

Figure 1. Distribution of the tested HCWs by age groups and the presence of anti-Measles, Mumps and Rubella IgG marker (n=181)

to data obtained with studies performed in HCWs in Japan which showed the highest IgG immunity against rubella [14-16]. There are some differences between regional seropositivity rates which are perhaps attributable to differences in the design of early childhood immunization programmes of each country. The main part of the Bulgarian HCWs included in the study were in the age groups above 20 years (154/181, 85.1%), in which immunizations against mumps and rubella have been selective and the combined MMR vaccine has not been used. For this reason, their MMR immunity could be due to a viral infection. On the other hand, the high percentage of seronegativity against mumps (23.2%, 42/181) can be explained by the lower contagious index of the virus (~ 50%) and its lower spread over the years in the country.

The monitoring of f HCWs immunity to VPDs is important to define potential risk groups for the spread of nosocomial infections, such as those recently described in Bulgaria in relation to measles outbreak [17].

A limitation of the present study is the relatively small number of participants included. However, the studied HCWs were staff of Infectious Units in regional hospitals in the country, and RHI, involved in the monitoring and surveillance of MMR cases, who have primary contact with patients suspected of measles, mumps and rubella infection.

CONCLUSION

HCWs are at a greater risk of contracting infections than the general public because they have contact with sick patients or infectious material. Infected healthcare workers can spread nosocomial diseases to vulnerable patients with more severe illness, complications and even death. Therefore, the HCWs vaccination status must be strictly monitored to limit the spread of nosocomial infections in hospital settings.

Competing Interest

The authors do not have any competing interest.

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SARS-COV-2 SEROSURVEY AMONG BULGARIAN HEALTHCARE WORKERS BEFORE AND AFTER VACCINATION

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ABSTRACT

Background: In the response to the current COVID-19 pandemic caused by the novel SARS-CoV-2, one of the groups at higher risk were healthcare workers (HCWs), especially those who worked on the frontline. The presence of SARS-CoV-2 specific IgG antibodies (seropositivity) in certain populations provides better understanding of virus circulation and transmission. Our aim was to study the seroprevalence rates of anti-SARS-CoV-2 antibodies among a group of healthcare workers before and after vaccination/ COVID-19 infection.

Material and Methods: We determined the presence of SARS-CoV-2 specific IgG and IgA antibodies against S-antigen of by ELISA method. In this study, we enrolled 74 healthcare workers and three months later, 48 of the participants were followed up. At the baseline, none of the participants was vaccinated or had suffered COVID-19.

Results: SARS-Cov-2 specific IgG antibodies were found in 32.4% of the participants. Higher prevalence of class IgA antibodies – 44.6% was detected. All samples that were IgG seropositive were also positive or borderline for IgA antibodies. Overall, virus-specific antibodies were not detected in 40.6% of HCWs in the group. During the follow-up (after vaccination and/or COVID-19 infection) high rates of both IgG and IgA seroprevalence were established. SARS-C0V-2 specific IgG antibodies were detected in 95.8% of the participants. Statistically significant difference was found in the levels of IgG and IgA antibodies both before and after vaccination, p<0.0001.

Conclusions: Based on detection of anti-SARS-CoV-2 IgG antibodies, seroprevalence of 32.4% was established in an unvaccinated group of HCWs. Our survey demonstrated that asymptomatic COVID-19 infection may induce weaker humoral immune response, with production of IgA but not of IgG antibodies.

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INTRODUCTION

The Coronavirus Disease 2019 (COVID-19) pandemic caused by the novel SARS-CoV-2 exerted immense pressure on the health and public systems around the world over the past three years (2020-2022). In response to the pandemic, one of the groups at highest risk were healthcare workers (HCWs), especially those who worked on the frontline in COVID-19 units. Some authors considered that in countries with lower vaccination coverage as Bulgaria, the risk was even higher (1). According to the official statistics, as of June 2023, 26 519 Bulgarian health workers were infected with SARS-CoV-2 (2). This number is probably underestimated.

Seroepidemiological studies might be a helpful tool to give insight into asymptomatic infections, as well as those not registered in official statistics. The presence of SARS-CoV-2 specific IgG antibodies (seropositivity) in certain populations provides a better understanding of the viral circulation and transmission. Also, large-scale population studies could predict the future development of the pandemic (3).

Since the beginning of COVID-19 pandemic, many serological studies have been carried out, both in specific groups and in the general population. The reported seroprevalence rates vary widely. A study from Poland reported 25.2% seroprevalence of anti-SARS-CoV-2 antibodies among HCWs prior vaccination availability (4). Socan at all. observed a seroprevalence rate of 20.4% among hospital staff during the second COVID-19 wave in Slovenia (5).

After the first COVID-19 wave in France, SARS-CoV-2 IgG seroprevalence rate among personnel in hospitals was 1.1 % (6). In the UK, 28.0% seropositivity was found after the first COVID-19 wave (7). A prospective cohort study from Saudi Arabia, conducted in June 2020, reported a seroprevalence of 10.8% (8). Seroprevalence of 45.3 % – twice as high in comparison with the local community, was established among HCWs from Western Switzerland after the second wave of the infection (10). Many factors, such as the timepoint, vaccination coverage, demographic, socio-economic characteristics, etc., should be taken into account when comparing data for the seroprevalence in different areas (3).

Studies have shown that higher IgG seroprevalence rates were found among medical personnel with frequent exposure to COVID-19 patients. It was estimated that the exposure together with the use of personal protective equipment are important and specific risk factors (10-12).

Our aim was to study the presence of anti-SARS-CoV-2 antibodies among healthcare workers from the Military Medical Academy, Sofia, which is one of the biggest hospitals treating COVID-19 patients in Bulgaria.

MATERIALS AND METHODS

Sample collection and testing was conducted twice – in November 2021 and in March 2022. At the start of the study we enrolled 74 healthcare workers from one hospital (Military Medical Academy, Sofia, Bulgaria). Three months later, 48 of the participants were followed up. At the time of the first sampling, none of the participants was vaccinated nor had evidence or history of previous SARS-CoV-2 infection. We determined presence rates of IgG and IgA antibodies against the S1 domain of the Spike (S) protein of SARS-CoV-2 by ELISA kits (EUROIMMUN, Germany). The samples were processed in accordance with the manufacturer`s instructions. The results are presented semi-quantitatively as the ratio of the extinction of the tested sample over the extinction of the calibrator (S/CO). The results were interpreted as follows: positive if S/CO ratio ≥1.1, borderline if S/CO ratio ≥0.8 to <1.1 and negative if S/CO ratio <0.8.

Statistical analysis was performed with SPSS software using the Kruskal-Wallis independent samples test. A p value < 0.05 was considered statistically significant. Graphs were made with GraphPad Prism 9 software. The study was approved by the Institutional Review Board of NCIPD (approval number 4/17.02.2021).

RESULTS

The baseline study in November 2021 involved 74 HCWs, of whom 48 males, and 26 females, with an average age of 41.74 ±12.02 years. Positive values of SARS-CoV-2 specific IgG antibodies were found in 32.4% (24/74) of participants, and in 4.1% (3/74) borderline levels were detected. Interestingly, the prevalence of IgA antibodies was higher than the prevalence of IgG antibodies. In 44.6% (33/74) of the participants, IgA antibodies were detected and in additional 13.5% (10/74) borderline values of IgA antibodies were found **(Figure 1).** The number of HCWs with positive and borderline results for IgA antibodies (43/74) was higher than the number of HCWs with positive and borderline results for IgG antibodies (27/74) and this difference was statistically significant (p<0.05).

We analysed the relationship between the presence of IgG and IgA antibodies. All samples that were seropositive for IgG antibodies were also positive or borderline for IgA antibodies (32.4%). Only one sample with borderline levels of IgG was negative for IgA antibodies. Almost 23.0% of the tested serum

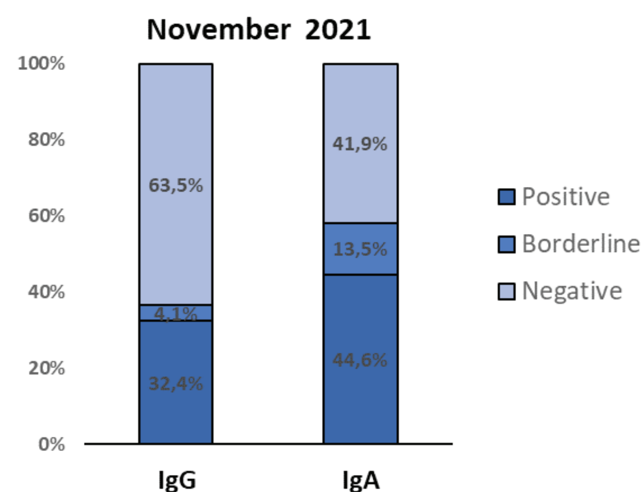

Figure 1. Seroprevalence rates of IgG and IgA SARS-CoV-2 antibodies in a group of unvaccinated healthcare workers. The results are presented as percentage (%) of the positive, borderline and negative values.

Table 1. SARS-CoV-2 IgA antibodies in healthcare workers with negative, borderline and positive IgG antibodies with 95.0% confidence interval.

Antibodies result	Number of participants	%, (95.0% CI)
IgG(neg.) IgA(neg.)	30	40.5% (95.0% CI: 29.4, 51.8)
IgG(neg.) IgA(pos.)	8	10.8% (95.0% CI: 3.7, 17.9)
IgG (neg.) IgA(bord.)	9	12.2% (95.0% CI: 4.7, 19.6)
IgG (bord.) IgA (neg.)	1	1.4% (95.0% CI:-1.3, 4.0)
IgG(bord.) IgA(pos.)	2	2.7% (95.0% CI: –1.0, 6.4)
IgG(pos.) IgA(bord.)	1	1.4% (95.0% CI:-1.3, 4.0)
IgG(pos.) IgA(pos.)	23	31.0% (95.0% CI: 20.5, 41.6)
Total:	**74**	**100%**

neg. = negative; bord. = borderline; pos. = positive; CI = Confidence interval

samples were positive or borderline only for IgA antibodies. The presence of specific antibodies at this time point may be considered indicative of previous exposure to the pathogen and/or asymptomatic infection. Overall, 40.5% of HCWs in the group were seronegative for both IgG and IgA antibodies **(Table 1).**

Three months after the initial testing, we performed a follow up of 48 HCWs from the original group. During this time, all participants had been vaccinated and/or recovered from COVID-19. As expected, high seroprevalence rates were established. Class IgG antibodies were detected in 95.8% (46/48) and only two participants were seronegative – 4.2% (2/48). With one exception, all participants were found to have class IgA antibodies, or 97.9% (47/48) of them. Of the participants, 91.7% (44/48) were positive and 6.3% (3/48) – borderline (Figure 2). Both seronegative participants were vaccinated; one of them had neither IgG nor IgA antibodies, while the other had borderline IgA values.

We compared the results from November 2021 with those from March 2022 in order to evaluate

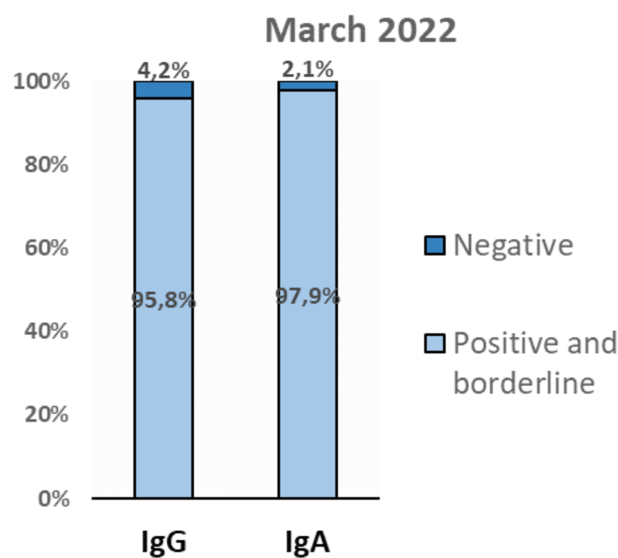

Figure 2. Seroprevalence rates of IgG and IgA SARS-CoV-2 antibodies during the follow-up. The results are presented as percentage (%) of the positive, borderline and negative values.

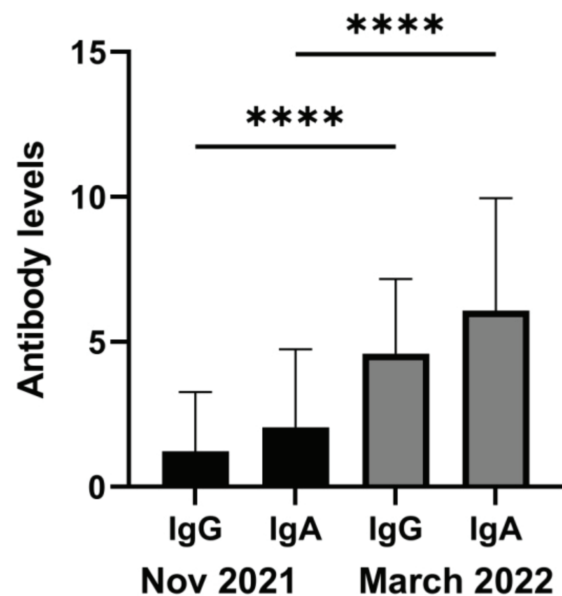

Figure 3. A comparison of mean S/CO ratio of IgG and IgA antibody levels in November 2021 and March 2022. The statistical differences were determined by the Kruskal–Wallis test (****, $p<0.0001$)

the alteration in mean antibody levels. We found statistically significant differences between the levels of both IgG and IgA antibodies detected during the two studies, $p<0.0001$ **(Figure 3)**

In this study no correlation between the seropositivity and the participants' age or sex was observed.

DISCUSSION

Based on the detection of anti-SARS-CoV-2 IgG antibodies, we observed a seroprevalence of 32.4% in an unvaccinated group of Bulgarian HCWs. During the follow up, after vaccination and/or COVID-19, we observed an increase of the seroprevalence rate to 95.8%. The relatively high baseline IgG antibody rates should be considered in the context of the COVID-19 pandemic situation in the country at the time of the study. At the same time (autumn, 2021), we had the most active circulation of Delta SARS-Co-2 variant, which led to the highest COVID-19 wave with a peak incidence rate of 928.3 $_{(100000)}$ (13).

Production of IgA antibodies without IgG antibodies was detected in about 23% of the HCWs. One possible explanation for the higher seroprevalence of IgA antibodies in the baseline study might be that asymptomatic infection induces a weaker immune response without IgG engagement. Cordova et all. report lower levels of IgG seroprevalence in asymptomatic HCWs with confirmed COVID-19 in comparison with the symptomatic ones (14). Madureira et all. reported that among previously asymptomatic HCWs 12.9% were positive for neutralizing and IgG antibodies against SARS-CoV-2 (9). Others found similar seropositivity rates in both asymptomatic and symptomatic COVID-19 patients, but established significantly higher levels of IgG in the symptomatic group in comparison to those without symptoms (15).

According to Brehm et al., individuals who had received three doses of vaccine or had a previous infection plus two doses of vaccine elicited the strongest humoral immune response to SARS-CoV-2 (16). During the follow up, we found significant differences between the mean levels of both IgG and IgA before and after vaccination and/or COVID-19 infection. Our results also indicated that almost all participants had acquired virus-specific IgG antibodies. Only two HCWs were IgG/IgA seronegative after COVID-19 infection/vaccination. This finding could be explained by a short-lived antibody immune response or other reasons such as immunodeficiency or incomplete vaccination course (17, 18). One of the main limitations of the seroprevalence studies is that they do not take into account the T-cell response, which has a leading role in anti-SARS-CoV-2 immunity (19, 20).

Numerous studies on COVID-19 indicated a wide range of seroprevalence rates, with time of conduction and study design mainly accounting for the differences in the results. A study from Belgium reported seroprevalence of 15.1% before vaccination (December 2020) and an increase to 84.2% after the first vaccination among primary healthcare providers, underlining the importance of vaccination for occupational health and protection of medical personnel (21). Recently, a study from Serbia, similar to ours, reported 93.0% overall prevalence of anti-SARS-CoV-2 antibodies among HCWs before the emergence of the Omicron variant (22).

In general, the seropositivity levels have increased since the first and second waves of COVID-19 and after the mass vaccination. This is expected because, as the pandemic progresses, the proportion of individuals with either previous COVID-19 infection and/or at least one vaccine dose is growing. On the other hand, the levels of circulating IgG and IgA antibodies normally decline over time. The duration of acquired immunity after illness and vaccination is still being discussed. With the emergence and spread of new variants of SARS-CoV-2, reinfections continue to occur and affect the seroprevalence in the general population.

One limitation of this study is the relatively small group of participants. Further investigations among larger groups, and following the dynamics of seropositivity rates are necessary.

Conclusions

Our survey demonstrates that asymptomatic COVID-19 infection may induce a weaker humoral immune response, with a predominant production of class IgA antibodies and a weaker IgG antibody response. The study also emphasizes the importance of vaccination for acquiring strong protective immunity against SARS-CoV-2.

ACKNOWLEDGMENTS

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EVALUATION OF INTERACTIONS OF SARS-COV-2 STRUCTURAL PROTEINS WITH SPECIFIC ANTIBODIES BY SPR ASSAY

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ABSTRACT

Background: The World Health Organization admitted that the vaccination against Covid 19 limited the deaths, but not the spread of the disease. This requires a method allowing a specific, rapid and accurate diagnosis of the disease. We report a SPR assay that meets the requirements and can be applied no only for SARS Cov-2 diagnosis but as a tool for early diagnosis of otherinfections. (2) Methods: Surface plasmon resonance (SPR) method was used to identify the binding of S/N protein to monoclonal antibodies. N-protein monoclonal antibody (NP mAb), S-protein monoclonal antibody (SP mAb), and receptor bind domain (RBD) antibody were used as recognition molecules. Ligands were deposited by the matrix-assisted laser evaporation (MAPLE) method, which guarantees maximum interaction specificity. (3) Results: We registered S/N protein binding to the corresponding mAbs and S protein to RBD antibody with high sensitivity: the interactions were observed at protein concentration about 130 femtomoles (fM). A very good specificity was observed: the measured S protein binding activity to NP mAb was below the limit of detection (LOD). The same was noticed for N protein binding to SP mAb. (4) Conclusions: The presented SPR assay possesses high sensitivity and selectivity and provides quantitative analysis. This makes it applicable for following the evolution of acute SARS-CoV-2 infection, especially at the early stages of viral replication which can be clinically useful.

Keywords: SARS-CoV-2, spike (S-) protein, nucleocapsid (N-) protein, anti-SARS-CoV-1/2 antibodies, Surface Plasmon Resonance (SPR) assay.

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INTRODUCTION

Despite the significant increase in the numbers of people vaccinated for coronavirus 2019 (COVID-19) disease, additional waves of the pandemic of COVID-19 were registered worldwide. This required a rapid, cost- effective, quantitative, on-site assay that could explore coronavirus 2 (SARS-CoV-2) severe acute respiratory syndrome. This is of particular importance for hospitals and any place where humans spread the virus (1, 2). COVID-19 diagnostic tests that are commercially available can be classified into three groups. The tests in the first group are based on molecular methods involving real-time polymerase chain reaction (RT-PCR), acknowledged as the gold standard for diagnosing COVID-19 (3, 4). However, they require a long turnaround time, well-equipped laboratory facilities, as well as qualified and trained personnel. Furthermore, this group of assays is not suitable for point-of-care testing (5, 6). treatment efficacy monitoring, or identification of a past infection. Serology tests form the second group. They have been established to detect antibodies against the SARS CoV-2 virus in the infected patients (7, 8). Antigen detection methods belong to the third group. They are designed to detect specific SARS-CoV-2 structural proteins (nucleocapsid protein (NP) and spike proteins (SP)). Antigen tests provide a fast and on-side diagnosis but have insufficient sensitivity, as compared to RT-PCR (9, 10).

Hence, there is a high demand for alternative techniques that are able to provide a diagnosis

with higher reliability and accuracy than the ones used to date. Biosensors based on Surface Plasmon Resonance (SPR) have already proved their feasibility as an accurate and sensitive diagnostic method. In (11) they were shown to be efficient for real time detection of an antigen-antibody interaction. In (12) S- and N-proteins were used on SPR transducer as recognition molecules for detecting SARS-CoV-2 antibodies.

The integration of two methods – generation of local SPR by nanoparticles and SPR excited on a planar chip – provides highly sensitive detection (13). This technique was used to achieve ultrasensitive SARS-CoV-2 N-protein detection (14).

SARS detection by an SPR biosensor was reported in (15) where corona viral surface antigen (SCVme) was immobilized on an SPR transducer. The lower limit of detection has been evaluated at 200 ng/mL for anti-SCVme antibodies within 10 min. Comprehensive reviews of SPR-based sensors for SARS-CoV-2 show recent achievements and limitations (16, 17).

Herein, we report SPR sensing of SARS-CoV-2 N/S-proteins at about 130 fM levels using monoclonal antibodies (mAb) and receptor binding domain (RBD) antibody as ligands immobilized directly (without built-in matrix) on the gold surface of an SPR transducer., Using a variety of SARS-CoV-2 specific antibodies as ligands, we evaluated the possibilities of the CPR assay to study the binding affinity of structural proteins.

MATERIALS AND METHODS

Reagents and materials

All the chemicals and reagents used were of analytical grade. We used the following SARS-CoV-2 specific structural proteins for evaluation of the bimolecular interaction:

1. SARS-CoV-2 Spike S1 subunit protein fused to a C-terminal poly-histidine (6x Histidine) tag with a tri-amino acid linker (Molecular weight (Mw) ~ 123 kDa) were purchased from InvivoGen Company USA. Stock solutions for the experiments were prepared at initial concentration 100 µg/ml in endotoxin and nuclease-free water (DEPC-treated water, ThermoFisher Scientific, USA). Aliquots were prepared and stored at –20°C until use. Working concentrations were propagated in DEPC-treated water in the concentration range 13 fM – 13 pM.

2. SARS-CoV-2 nucleocapsid protein fused to a human IgG1 Fc tag with a TEV (Tobacco Etch virus) sequence linker (Mw ~ 79 kDa) were purchased from InvivoGen Company, USA. Stock solutions were prepared at initial concentration 100 µg/ml in DEPC-treated water. Aliquots were stored at –20°C until use. Working concentrations of the stock solution were propagated in DEPC-treated water in the concentration range 0.0025 – 2.5 µg/ml.

3. Anti-SARS-CoV-1/2 NP antibody, clone 1C7C7 ZooMAb® mouse monoclonal (mAb) (Sigma-Aldrich, USA) (Mw ~ 46 kDa) was prepared at a working concentration 2.5 µg/ml in DEPC-treated water and then stored at –20°C until use.

4. Anti-SARS-CoV-2 SP antibody (SP mAb) cleavage site (Lot No 9091), raised against a peptide corresponding to 12 amino acids near the center of SARS CoV-2 Spike glycoprotein; purchased from Sigma-Aldrich, USA.

5. SARS-CoV-2 (COVID-19) Spike glycoprotein RBD Antibody (Lot No 9087) raised against a peptide corresponding to 19 amino acids near the carboxyterminus of SARS-CoV-2 Spike glycoprotein RBD SPR chips were incubated for 20 minutes in N and S protein solutions of different concentrations at room temperature, then washed with deionized water (< 2µS/cm), after which the liquid phase was removed by centrifugation.

SPR method

The fundamental principles of SPR method rely on the propagation of plasmon wave along the interface of a thin, metal layer (commonly gold) and a dielectric. SPR biosensing takes advantage of the local refractive index changes of the transducer surface when monitoring molecular interactions between the target analyte and the immobilized biological receptor.

In contrast to the prism-coupling method, widely used in SPR biosensors, we use grating-based SPR. Fig. 1A illustrates what kind of a transducer is used in our study – this is a gilded diffraction grating. The gratings were supplied by DEMAX Ltd, Sofia, Bulgaria; for the purposes of the experiment we covered them with about 110 nm gold film coating obtained by vacuum evaporation. SPR conditions were fulfilled for P- polarized light beam that illuminated the

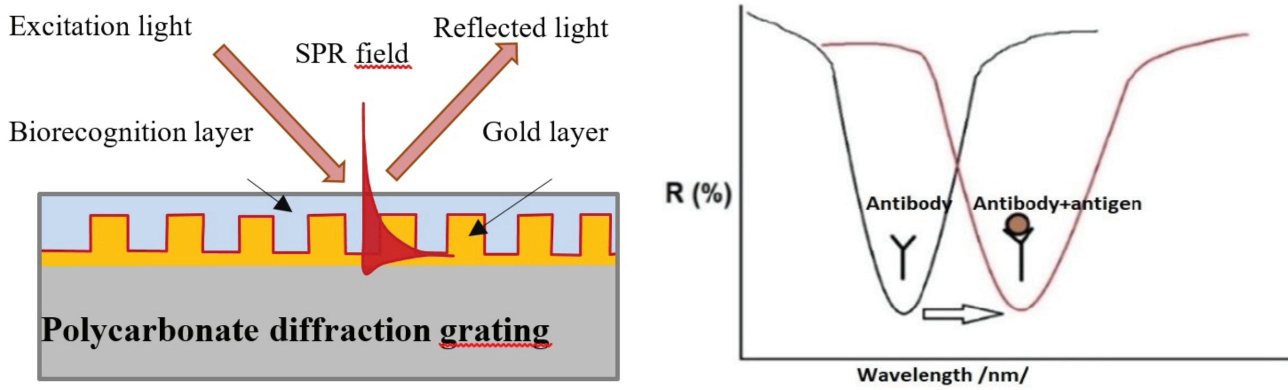

Figure 1. SPR principle: A/ SPR biochip: gilded diffraction grating with immobilized antibody.
B/ resonance wavelength shift occurs when proteins bind to antibody.

grating at an incidence angle of about 35 degrees. Typically, the resonance was excited in the range 690-710 nm for a bare grating having 80 nm high grooves at a distance of 1.55 µm from one another. More details about our SPR system can be found in (18). We elaborated three type SPR biochip that represent the grating with immobilized NP mAb, SP mAb or RBD antibody of certain thickness, as shown in Fig. 1A. The wavelength, at which the plasmon wave is excited, shifts significantly when structural proteins interact with the ligand, as illustrated in Fig. 1B. This wavelength shift corresponds to the number of interacting molecules, therefore SPR assay provides a quantitative assessment of interaction.

RESULTS AND DISCUSSION

Antibody immobilization

The main disadvantage of SPR biosensors is their low specificity. Even one hundred percent specificity of the ligand does not guarantee high specificity of the biosensor. This is due to the built-in matrix required for ligand immobilization. Protein immobilization is a delicate procedure – proteins tend to unfold and denature upon contact with metals and most other artificial substrates. However, these statements are valid for conventional methods of immobilization. We use another approach for ligand immobilization.

The matrix-assisted pulsed laser evaporation (MAPLE) method has been successfully applied for deposition of proteins. MAPLE immobilized proteins without using built-in matrix and preserving their bioactivity. Then, specificity of reactions depends only on specificity of the ligand. In a previous publication (19) we showed that this technique provides deposition of intact molecules, as well as high accuracy and sensitivity of detection (20).

For antibodies deposition we used frozen targets consisting of 19.2 pM/ml antibodies dissolved in

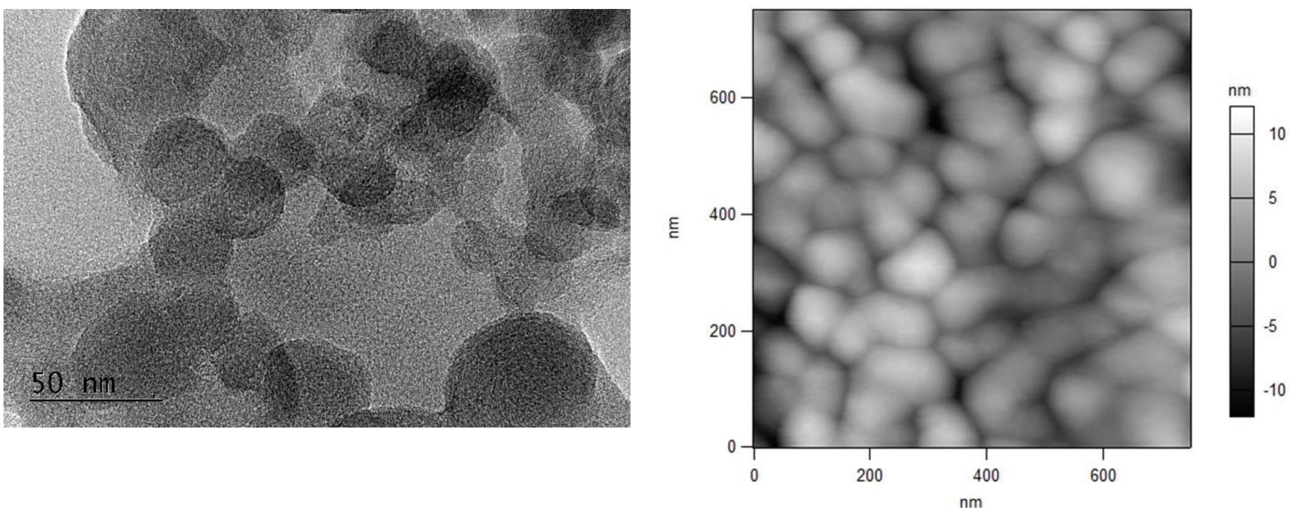

Figure 2. Mab Layer A/ TEM images of mAb layer. B/ AFM image of the same layer.

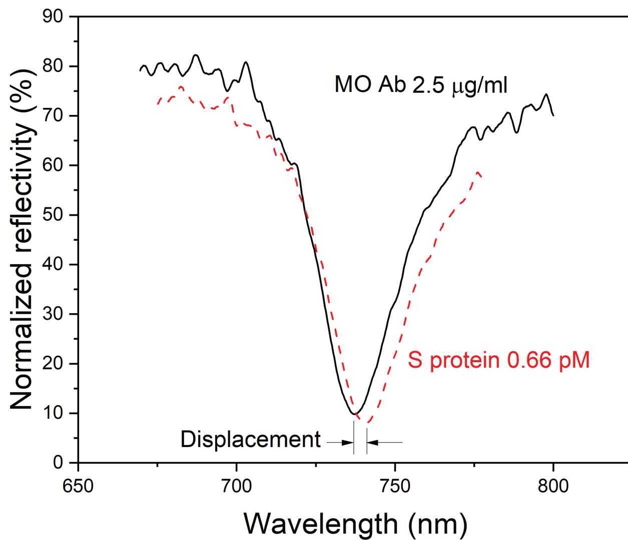

Figure 3. SPR detection of antigen–antibody interactions.

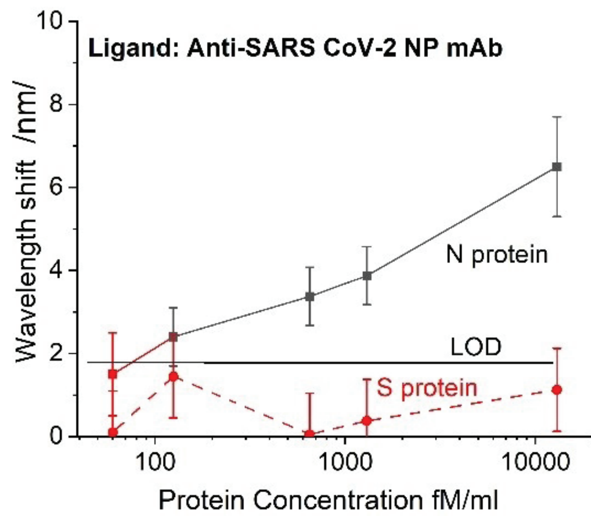

Figure 4. SPR assay with immobilized specific NP mAb: detection of S/N-proteins with different concentrations.

DEPC water. This concentration was established after many experiments as MAPLE technology requirements against detection sensitivity tradeoff. Details regarding the MAPLE technique and the parameters of the immobilization procedure can be found in (19).

Characterization of the mAb layer

The layer of anti-SARS-CoV-2 antibodies was deposited with a thickness of about 110 nm – a well controllable parameter of the MAPLE technique. At this thickness SPR ensures a maximum sensitivity of detection, since the field of plasmon wave entirely penetrates the deposited layer. Specificity is guaranteed, since the deposited layer consists only of antibody molecules. The only but significant problem is whether the deposited molecules are bioactive. To check this, we studied the deposited film by well adopted techniques for nanolayer characterization as Transmission Electron Microscope (TEM) (21) and Atom Force Microscope (AFM) (22). Fig. 2A shows TEM images of the MAPLE-deposited sensing layer covering the metal surface. The layer is uniform, dense and antibodies molecules are identified, which is confirmed by the AFM – Fig. 2B shows that the deposited molecules are well shaped. This is a convincing evidence that intact direct immobilization was performed and bioactivity of deposited layer has to be expected.

SPR-based assay

SPR is a very effective label-free technique for registering the real-time interaction of two binding molecules. It can provide useful information on the interaction's specificity and binding affinity. Today, it is used in many other life science areas.

Theoretically, a SPR sensing structure having three different ligand-analyte modes has been proposed in (23): (i) the monoclonal antibodies (mAb) as ligand and the SARS-CoV-2 virus spike RBD as analyte, (ii) the virus spike RBD as ligand and the virus anti-spike immunoglobulins (IgM, IgG) as analyte and (iii) the specific RNA probe as ligand and the virus single-stranded RNA as analyte.

In our study we realized experimentally three ligand-analyte strategies: (i) NP mAb as ligand and NP as analyte, (ii) SP mAb as ligand and SP as analyte, (iii) RBD antibody as ligand and SP as analyte.

Various modifications of the SPR platforms targeting amplification of the signal have bee applied for SARS-Cov-2 detection. A SPR assay with a graphene layer was proposed in (24) for the detection of SARS-Cov-2 N proteins. The reported LOD has been evaluated to 1.02 pM.

In (25) was reported LOD of 0.22 pM in protein detection by photothermal enhanced plasmonic biosensor. The LOD achieved in (14) was 85 fM in N protein detection by nanoparticle-enhanced SPR. A record sensitivity was reported in (26) – 12 fg/ml in the detection of S protein by SPR excited in a

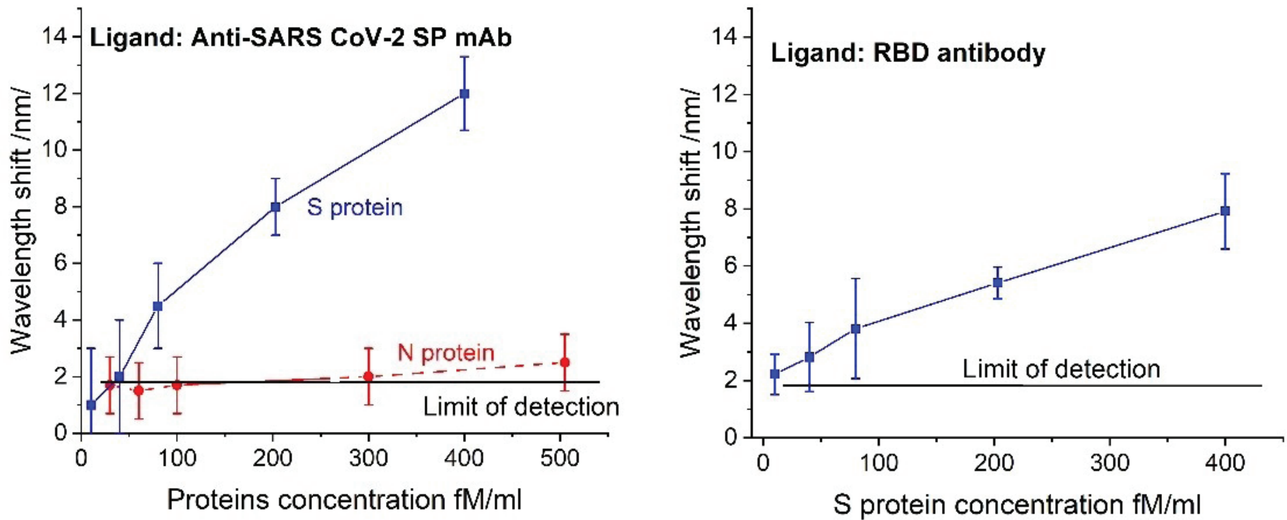

Figure 5. SPR wavelength shift as a function of proteins concentrations for chips functionalized with: A/ SARS CoV-2 SP mAb. B/ RBD antibody.

multilayer structure including graphene.

The SPR-based assay presented here was designed to study the binding affinity between structural SARS-CoV-2 S-and N-proteins and a specific anti-SARS-CoV-2 mAbs and RBD antibody.

Three MAPLE deposition procedures were performed to functionalize SPR gratings. A total of 75 SPR biochips were examined in order to establish the measurement accuracy, out of which 30 were NP mAb-functionalized, 30 were SP mAb-functionalized, and 15 were functionalized with RBD antibody.

The biochips functionalized with NP mAb were incubated as follows: 15 – with S protein of different concentrations, 15 – with N protein of different concentrations. The biochips functionalized with SP mAb were incubated as follows: 15 – with S protein of different concentrations, 15 – with N protein of different concentrations. I.e. when performing the above-mentioned ligand-analyte strategies (i) and (ii), 3 measurements were provided for each concentration of the N/S proteins in order to assess the accuracy of the measurements. The biochips functionalized with RBD antibody were incubated with S proteins: 3 measurements were provided for each concentration in order to assess the accuracy of the measurements.

After the gilded diffraction gratings were functionalized, the plasmon resonances were measured at six diffrent points on the biochip surface to evaluate the quality of the ligand layer. The spectral position of the resonances at each point was taken as a reference against which the shift due to the antibody – protein interaction was registered.

23. After incubation, the plasmon resonances were measured at the same 6 points on the biochip surface and the resonance wavelength shifts were estimated as differences from the reference resonances. Then the corresponding resonance shift average values and the absolute measurement errors were determined. Therefore, each of the experimental points in the graphs, as well as the errors, are the result of 18 measurements.

Fig. 3 shows an experimentally observed SPR resonance shift for a biochip treated with 0.66. pM/ml SARS-CoV-2 N protein, compared with the resonance of a biochip immobilized with a NP specific anti-SARS-CoV-2 mAb, accepted as reference resonance. The wavelength shift of the plasmon resonance results from the viral N-protein binding to mAb

We evaluated the dependence of the wavelength shift of the incubated chips on the viral structural S- and N-protein concentrations. For this purpose, various concentrations of structural SARS-CoV-2 S- and N-proteins in the concentration range 60 femtomoles/ml (fM/ml) – 13 picomoles/ml (pM/ml) were prepared.

Fig. 4 presents the wavelength shift plotted as a function of the S- and N-protein concentrations for SPR biochip having a ligand specified NP mAb. We observed pronounced mAb – N protein interaction

for concentration above 126 fM. For N-protein concentration of 126 fM the measured spectral displacement was 2.5 nm, which is above the limit of detection (LOD) accounting for measurement error (in this case–0.5 nm). LOD was evaluated by considering the accuracy of the spectrometer as well as the accuracy of the goniometer for setting-up the angle of light incidence. The probability of reliably measuring concentrations lower than 126 fM is small because the SPR displacement is compatible to the LOD and the measurement error increases.

The mAb – S proteins interactions generated an SPR response in the range of measurement accuracy, as illustrated in Fig.4. First of all, this was due to the specificity of the used anti-SARS-CoV-1/2 NP clone 1C7C7 ZooMAb® mouse monoclonal antibody. However, this result also shows the applicability of our method of detection.

Having laser deposited the specific SP mAb and RBD antibodies upon the grating surfaces we provided a similar measurement procedure for structural proteins detection. The results of SPR measurements are summarized in Figure 5.

The S protein detection by specified SP mAb (Fig. 5A) is better expressed than the detection of N protein by mAb (Fig.4), however the LOD is the same – 126 fM. N- proteins binding mAb generated a signal slightly above the detection limit, but within the measurement error zone, as shown in Fig. 5A which is partly due to the direct immobilization of the mAb, but also to its specificity.

S-protein binding the RBD antibody (Fig. 5B) is not so effective as S-protein/mAb binding (Fig. 5A). This fact indicates that RBD antibody affinity is lower that the affinity of SP mAb and hardly can be used for S-protein detection.

CONCLUSIONS

The SPR-assays presented here are able to evaluate a wide range of biomolecular interactions. Its high specificity, partly due to the specificity of immobilized antibodies and to the immobilization method, makes it applicable in a diversity of conditions, especially when studying SARS CoV-2.

The proposed SPR assay could be optimized for any new antibody (monoclonal or polyclonal). Most importantly, this type of assay design could assist the detection of a variety of viruses.

It is worth mentioning the high sensitivity of about 130 fM achieved in detecting structural proteins. As reported in our recent research (27) the comparison of SPR assay with clinically used ones shows that the SPR method ensures sensitivity and accuracy similar to those of the rapid antigen tests. Therefore, SPR assay is able to detect acute SARS-CoV-2 infection, especially at the early stages of viral replication and can be clinically useful.

DISCLOSURE OF CONFLICT OF INTEREST

All authors declare no conflict of interest.

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A CLINICAL CASE OF PULMONARY NOCARDIOSIS IN AN IMMUNOCOMPROMISED PATIENT

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ABSTRACT

Nocardia microorganisms are saprophytes, either non-pathogenic or pathogenic, causing nocardiosis. The clinically significant disease occurs in immunocompromised people, most often as pneumonia with cough, dyspnea, and fever. Antibiotic therapy, which is longer in time, is necessary. The main treatment is with sulfonamides, but the sensitivity of these bacteria varies. Therefore, the antibiotic susceptibility of the respective strain is important to apply combined therapy if needed. The risk of death without treatment is high, especially if the infection disseminates and the brain is involved. Antibacterial prophylaxis is therefore recommended in patients at high risk of nocardiosis. Our clinical case concerns an immunocompromised patient with isolated *Nocardia* from bronchoalveolar lavage (BAL).

Keywords: Nocardia, Aspergillus, MALDI TOF

INTRODUCTION

The species Nocardia are widespread saprophytes that can cause infections inhuman. These infections can be localized or disseminated, and are more common in immunocompromised patients (transplant on solid organs, HIV) (1, 2). Different Nocardia species are of medical importance. The most often isolated and responsible for most human infections are representatives of the former *Nocardia asteroides* complex. It includes *N. abscessus, N. brevicatena, N. farcinica, N. nova complex (N. africana, N. nova, N. veterana* and others (3). Nocardiosis is difficult to diagnose – clinically, radiologically, and histologically. *Nocardia nova* was isolated and first described by Tsukamura in 1982. Nocardia- microorganisms are gram-positive filamentous rods with branches. In culture, they require aerobic conditions, but growth on blood agar may require more than 48 hours. Literature on the role of this microorganism in lung infections is scarce (14). Clinical manifestations can vary from a cutaneous form, a pulmonary form to disseminated nocardiosis with the development of brain lesions and a mortality rate of over 85% (4). Nocardial endocarditis is suspected when there is no lung or central nervous system (CNS) involvement (5). In brain abscesses, CSF is positive in up to 20% of these cases (6). Pulmonary nocardiosis can most often appear with pneumonia, sometimes associated with cavitation (7). These infections can lead to pleural effusion, empyema, pericarditis, mediastinitis and, less often, to the development of local abscesses on the neck and chest wall.

Most often, the samples that are examined are sputum, broncho-alveolar lavage (BAL), exudate, or cerebrospinal fluid. In addition to cultural examination, the microscopic assessment is important. By Gram staining, branched delicate threads are visible. Histological examination by hematoxylin and eosin staining or Gomori staining is also very useful (8). These species are often acid-tolerant, unlike actinomycetes, which are generally not acid-tolerant.

Chest radiography in pulmonary nocardiosis usually shows consolidation, nodular lesions, cavitation, or abscesses. Computed tomography (CT) of the lungs can show the presence of an abscess earlier than plain radiography (12).

Regarding the therapy of nocardiosis, representatives of the following antimicrobial classes can be used: sulfonamides, aminoglycosides, beta-lactams, quinolones, macrolides and tetracyclines (18). The main drug of choice is trimethoprim-sulfamethoxazole (TMP-SMX), especially preferred in pulmonary nocardiosis. When TMP-SMX is not tolerated, the patient can be treated with the macrolide clarithromycin, although macrolides are less commonly used, possibly due to insufficient

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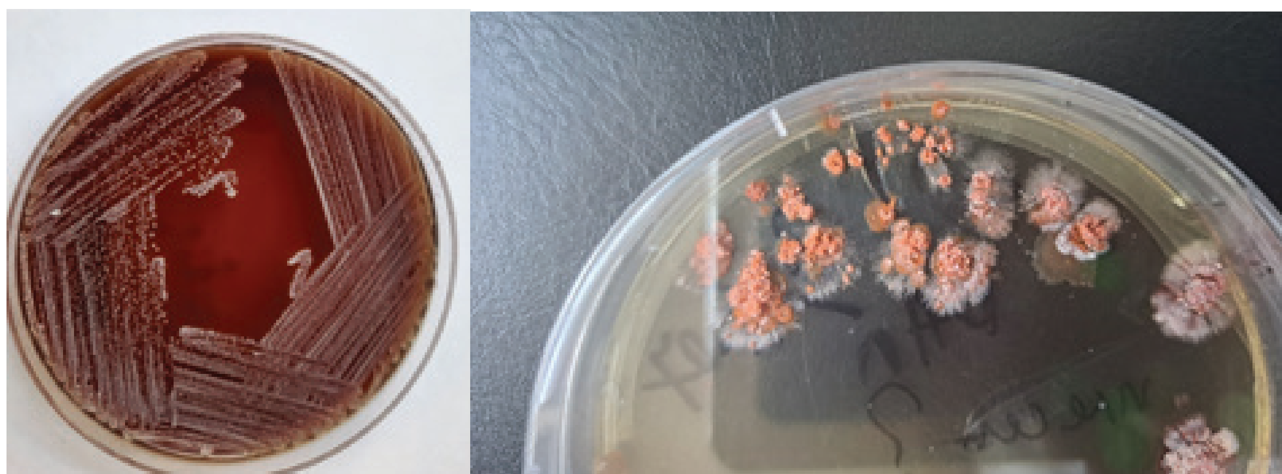

Figure 1. Macroscopic characterization of *Nocardia nova*

studies (9). The isolates of *Nocardia nova* are always sensitive to erythromycin (10).

Pathogenic Nocardia produces beta-lactamases and only 44% of *N.nova* isolates are sensitive to ampicillin (11). Most strains are resistant to cefuroxime, cefotaxime and ceftriaxone, although all isolates of *N.nova* are resistant to cefixime (12). Most *N.nova* isolates are resistant to ciprofloxacin and other quinolones (13). Combination therapies are also available to treat nocardiosis.

Here, we describe a clinical case with lung disease in which *Nocardia nova* was isolated from broncho-alveolar lavage (BAL).

MATERIAL AND METHODS

The patient was a 34-year-old woman diagnosed with bronchial asthma, bronchiectasis, and Churg-Strauss syndrome. She was on regular corticosteroid therapy. According to the epicrisis, she reported episodic epistaxis associated with atrophy of the nasal mucosa and a previous operation to remove the middle conch on the left. She was allergic to pollen and house dust. In addition, the patient had established iron-deficiency anemia, thyroid hypofunction, otitis media with hearing loss affecting both ears, as well as chronic glomerulonephritis.

Churg-Strauss syndrome is an inflammation of the blood vessels leading to restricted blood flow to tissues and organs, and possible permanent damage. It is also called "eosinophilic granulomatosis with polyangiitis (EGPA)". The most common symptom in adulthood is asthma. In our patient, the disease debuted with proteinuria, otitis, and granulomas in the lung.

The woman was hospitalized in a satisfactory general condition, adequate and oriented, with the aim of diagnostic and therapeutic clarification. Chest – normosthenic, with bilateral vesicular breathing, no wheezing, sinus rhythm on electrocardiogram (ECG). The paraclinical examinations demonstrated an erythrocyte sedimentation rate (ESR) of 51.0 mm/h, and C- reactive protein (CRP) of 46.0 mg/l, proteinuria was also detected.

A chest radiograph visualized inhomogeneous shadowing in the left lung base, consistent with an inflammatory infiltrate. Dilated bronchial lumens with thickened walls were seen in the parenchyma of the right lung.

Computed tomography (CT) showed bilateral maxillary sinusitis, frontal sinusitis, as well as bilateral mastoiditis. Chest CT was without evidence of pleural effusions. The lungs showed multiple nodular areas of varying density, most of them with scars of excavation. Peripherally dilated bronchial lumens with thickened walls and bronchiectasis were also seen.

The histologic lung examination visualised pulmonary parenchyma with fibrotic interstitium, perivasal fibrosis around thin-walled vessels, and reactive pneumocytes. Histology confirmed granulomatous vasculitis. The morphological picture was defined as non-specific for Churg-Strauss syndrome, possibly with pronounced post-therapeutic changes.

RESULTS AND DISCUSSION

During a fibro-bronchoscopy (FBS), broncho-alveolar lavage (BAL) was taken and sent for microbiological examination to the National Reference Laboratory

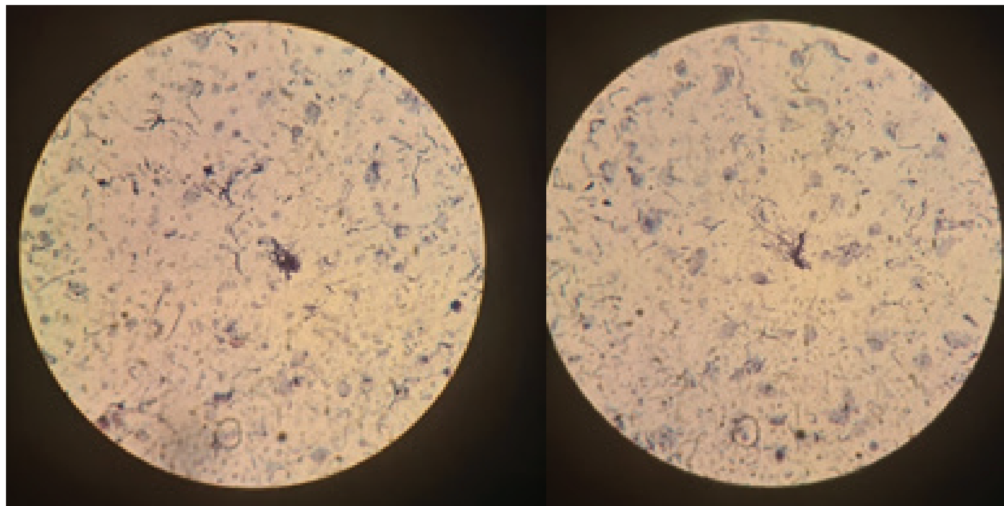

Figure 2. Microscopic characterization of *Nocardia nova*

"Mycoses" at the National Center of Infectious and Parasitic Diseases, Sofia.

ON microscopic examination by Gram staining, numerous leukocytes, lymphocytes, lymphoplasmic cells and epithelia were visualized. The cultural study on Blood Agar and on Sabouraud medium agar isolated no fungi. Instead, a microorganism with a whitish aerial and substrate mycelium, slightly buried in the agar (Fig. 1) was isolated. The microorganism was further identified by MALDI-TOF technology (Biotyper Bruker) as *Nocardia nova*.

The microscopic evaluation of the pure *N. nova* culture after Gram staining showed delicate gram-positive branched threads (Fig.2). Pathogenic *N. nova* act as facultative intracellular microorganisms in macrophages, where they inhibit the fusion of lysosomes with phagosomes (15).

The antimicrobial activity was evaluated by means of an antibiogram. The results are presented in Table 1.

The isolated *N. nova strain* was resistant to quinolones and aminoglycosides, to tetracyclines, and clindamycin. The bacteria also showed resistance to ampicillin and susceptibility to Amoxicillin/Clavulanic acid, probably because this was a beta-lactamase producing strain. Macrolide sensitivity for *N. nova* was expected.

According he European Committee on Antimicrobial Susceptibility (EUCAST 2022), there are data for interpretation only for gram-positive anaerobes, including Actinomyces, without specifying for representatives of Nocardia. Such criteria for interpretation are available at the Institute for Clinical and Laboratory Standards (CLSI) (12).

Given the demonstrated sensitivity of *Nocardia nova* to biseptol, (TMP/SMX), *p.o* was assigned as therapy in the next 6 months, as well as inhaled corticosteroid therapy.

After a control examination, FBS was performed and

Table 1. Results of the antibiotic susceptibility of *Nocardia nova*

Nocadia nova			
Ampicillin	R	Erythromycin	S
Amoxacillin/ Clav.acid	S	Clindamycin	R
Rifampicin	R	Tetracycline	R
Imipenem	S	**TMP/SMX** *	S
Ceftriaxone	S	Linezolid	S
Cefixime	R	Ciprofloxacin	R
Cefepime	S	Levofloxacin	R
Cefotaxime	S	Gentamycin	R

* *TMP/SMX – Trimethoprime-Sulfametoxazole*
S-susceptible R- resistent

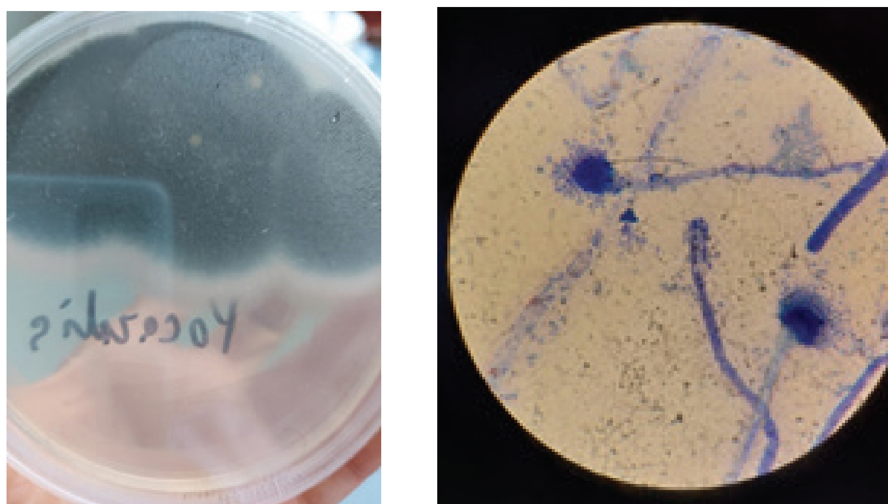

Figure 3. Macroscopic and microscopic characterization of *Aspergillus fumigatus*

BAL was again sent for microbiological examination in our laboratory. Microscopic evaluation after Gram staining visualized a pavement of leukocytes, lymphocytes, lymphoplasmacytic cells and epithelia. The cultural examination on blood agar at 37 °C, detected no bacterial growth while on Sabouraud agar for fungi, incubated at 30 °C, a significant amount of pure fungus-mold culture was isolated. The mold was identified by MALDI-TOF (Biotyper Bruker), as well as by microscopic and macroscopic analysis as *Aspergillus fumigatus*.

Aspergillus fumigatus is a fungus that has an aerial and substrate mycelium, conidiophores with a vesicle, and numerous spores (Fig. 3).

Antifungal susceptibility of *Aspergillus fumigatus* (antimycogram) was tested against the following antimycotics Itraconazole, Voriconazole, Nystatin, Isavuconazole, Anidulafungin, Amphotericin B and Posaconazole. Fluconazole resistance was congenital. The results are presented in Table 2.

The E-test method was used to determine the minimum inhibitory concentration (MIC) (EUCAST 2022). (Fig. 4)

After prescribed Itraconazole therapy, *Aspergillus fumigatus* was cleared in a post-therapy follow-up study.

The recommendation was the inclusion of antifungal agents in long-term antibiotic therapy, as well as in the case of inhaled corticosteroids as a proven risk factor for the occurrence of medically significant fungi.

CONCLUSION

No specific measures to prevent nocardiosis exist. However, it was shown that the concomitant administration of TMP/SMX prophylactically while receiving high-dose immunosuppressants after heart transplantation could reduce the risk of nocardial infections (16).

Recent studies, clinical observations, and taxonomic developments suggest that therapeutic decisions in nocardiosis regarding the most appropriate drug and duration of therapy are not straightforward. An individual approach may be required depending on the infection and the specific Nocardia species. Therefore, antimicrobial susceptibility tests are necessary (17;18).

Table 2. *Aspergillus fumigatus* antimycogram results

Aspergillus fumigatus			
Fluconazole	R – congenital	Isavuconazole	S
Itraconazole	S	Anidulafungin	S
Voriconazole	S	AmphotericinB	S
Nystatin	S	Posaconazole	S

S-susceptible R- resistent

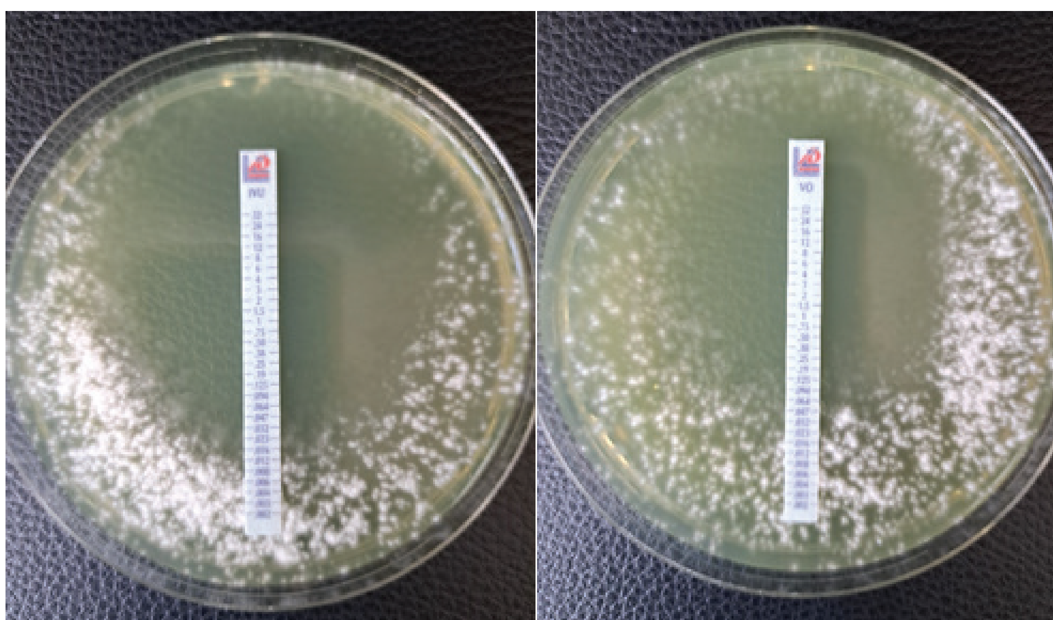

Figure 4. Antimycotic susceptibility of *Aspergillus fumigatus*

The application of appropriate identification methods, including the development of molecular-biological methods for diagnosis, increases the probability, respectively the number of Nocardia isolates (14).

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