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SHORT COMMUNICATION

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Initial introduction and spread of the SARS-CoV-2 AY.4.2.1 Delta variant in Bulgaria, a genomic insight

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Abstract

The evolution of the emerging SARS-CoV-2 variants carrying mutations in the spike protein raises concerns about the possibility of accelerated transmission in the ever-evolving COVID-19 pandemic worldwide. AY.4.2, a sublineage of the Delta variant, was considered a variant under investigation (VUI) and also gained the nickname "Delta Plus," due to its extra mutations, Y145H and A222V. In this study, using genomic epidemiology, we provide the first insights into the introduction of AY.4.2 in Bulgaria and the AY.4.2.1 sublineage that found larger dissemination only in Bulgaria and the United Kingdom.

KEYWORDS
epidemiology, mutation/mutation rate, SARS coronavirus

1 | INTRODUCTION

The COVID-19 pandemic was caused by the emerging coronavirus SARS-CoV-2 in 2019, which led to an unprecedented pandemic related to the challenges facing national health systems, social systems, and the global economy.[1] Since the emergence of SARS-CoV-2, many variants of the virus have branched off, some of which are of particular importance due to their potential for increased transmissibility, virulence, or reduced vaccine effectiveness.[2,3] In late 2020, the emergence of variants that posed an increased risk to global public health prompted the characterization of specific variants of concern (VOCs), variants of interest (VOIs), and variant under investigation (VUI) so as to prioritize global monitoring and research, and ultimately to inform the ongoing response to the COVID-19 pandemic. WHO provides regular updates on currently circulating VOCs, and as of May 25, 2022, these are Delta (B.1.617.2) and Omicron (B.1.1.529). The Delta variant was first detected in India in late 2020, and in June 2021 already became the dominant strain globally and was designated as VOC.[4] The advancement of the Delta variant may be attributable to multiple factors, including increased host cell entry efficiency and improved evasion of neutralizing antibodies.[5] Moreover, several Delta sublineages that harbor

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additional mutations in the S protein have branched off from the parental B.1.617.2 lineage, including sublineages from AY.1 to AY.134 and their subdivisions.[6] The AY.4.2 sublineage, in particular, was detected in January 2021 and is distinguished by two additional genetic mutations, Y145H and A222V, in the spike protein. Since the beginning of the pandemic, both Y145H and A222V spike mutations have been identified in other SARS-CoV-2 lineages but neither was found in VOC variants suggesting that these mutations are not likely to cause significant increases in transmissibility and/or in the immune escape. In mid-October, the AY.4.2 Delta sublineage rapidly expanded in the United Kingdom, with an increase of about 15% faster per week and was defined as a "variant under investigation" (VUI).[7] Later, AY.4.2 was further divided into five sublineages (AY.4.2.1–AY.4.2.5) and all of them were present in circulation up to February 2022. Finally, with the gradual displacement of Delta by the more transmissible Omicron, the AY.4.2 subvariant of Delta was de-escalated from the register of the European Center for Disease Prevention and Control.[8]

In this study, we analyzed the introduction of the newly circulating SARS-CoV-2 AY.4.2 variant in Bulgaria, to provide the first preliminary insight regarding the circulation of this strain in the country.

2 | MATERIALS AND METHODS

Viral RNA was extracted from 400 µl of nasal swab suspensions obtained for routine COVID-19 diagnostic/genomic surveillance purposes using an automated ExiPrep 48 Dx (Bioneer) system (Bioneer) following the manufacturer's instructions. Real-time polymerase chain reaction (RT-qPCR) was performed using GeneFinder™ COVID-19 Plus RealAmp Kit (OSANG Healthcare Co., Ltd.), targeting the RdRp (RNA-dependent RNA Polymerase), E (envelope), and N (nucleocapsid) 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 v3 tailed amplicon method.[9] 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.[10] 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). The reads were trimmed, quality filtered, the primer sequences were removed, and full genomes were assembled in Geneious Prime 2021.1 (https://www.geneious.com). Lineage assignment was performed on the obtained consensus sequences using the Pangolin COVID-19 lineage classification software tool.[11] The identified AY.4.2 isolates were compared to a diverse pool of genome sequences (n = 13 434) sampled worldwide collected up to November 20, 2021. All sequences were aligned using the ViralMSA tool,[12,13] and IQ-TREE2[14] was used for phylogenetic analysis using the maximum likelihood approach. TreeTime[15] was used to transform this raw maximum likelihood (ML) tree topology into a dated tree using a constant mean rate of 8.0×10^{-4} nucleotide substitutions per site per year, after the exclusion of outlier sequences. The mutation pattern of the VUI was analyzed using the NextClade online tool.[16]

3 | RESULTS

The sequenced samples obtained in this study were collected from a total of 40 patients, 52.5% females and 47.5% males (Supporting Information: Table 1), with a mean age of 48 years (range: 1–86 years of age) originating from eight geographically distinct districts. Samples were collected between August 27 and October 12, 2021, and sent for sequencing and analysis in the National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria, following the regulations in the country. All patients from whom SARS-CoV-2 sequences were isolated were alive at the time of study data collection; information on patients' accompanying symptoms was not available.

All tested samples contained sufficient viral genetic material (≥2 ng/µl) for library preparation. For positive samples, PCR cycle threshold (C_t) values were, on average, 22.1 (range: 16.5–28.9). The length of the generated sequences ranged from 29 720 to 29 841 bp. To accurately establish evolutionary relationships among the generated sequences and other isolates of SARS-CoV-2, we subjected a combined data set to phylogenetic inference.

Our time-stamped phylogeny revealed that the sequences obtained in this study are scattered throughout the tree and highlight that at least five independent introduction events have occurred through time. Furthermore, Bulgarian AY.4.2 clustered together with viral strains isolated mainly in European countries, suggesting that those regions have likely acted as probable steppingstone spots in the dissemination of the virus (Figure 1A), which might have been influenced by the increased human mobility. The mutational pattern of the newly generated Bulgarian strains (Figure 1B) defined using NextClade highlighted the characterized AY.4.2 substitutions. One less common substitution, S^{V36F}, emerged among 85% of Bulgarian strains, albeit present only in the 1.8% of the global AY.4.2 genomes.[17] Besides S^{V36F}, another mutation N^{S202I} was copresent in 33/40 (83.5%) of the sequences defining them as AY.4.2.1.[6] In addition, S^{R158G} carried by all Bulgarian AY.4.2 strains has been rarely found (<1%) in global AY.4.2 as well as in other AY sublineages and seem to be associated with B.4.8 strains with prevalence as low as 19% (Figure 1B).[18]

4 | DISCUSSION

This report provides the first insights into the introduction and circulation of the AY.4.2 SARS-CoV-2 variant in Bulgaria, highlighting the importance of consistent genomic monitoring of emerging virus variants for enhanced awareness and effective public health measures for infection control and prevention.

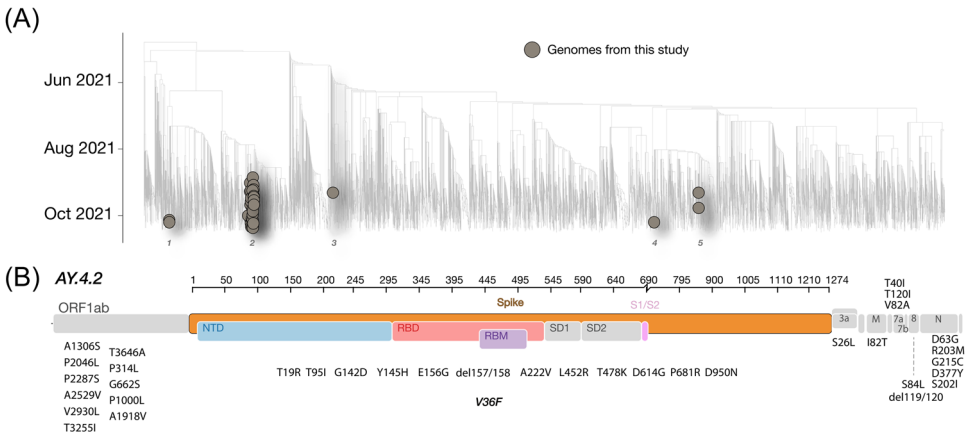

FIGURE 1 Genomic epidemiology of the SARS-CoV-2 AY.4.2 variant in Bulgaria. (A) Time-stamped phylogenetic tree including the $n = 40$ AY.4.2 isolates obtained in this study plus $n = 13\,434$ representative SARS-CoV-2 genomes collected up to November 20, 2021. The genomes generated in this study have been highlighted in the tree (gray circles). (B) Genome map of the AY.4.2 sublineage, where the spike region is shown in detail and in color, and the rest of the genome is shown in gray. NTD, N-terminal domain; ORF, open reading frame; RBD, receptor binding domain; RBM, receptor binding motif; SD1, subdomain 1; SD2, subdomain 2. AY.4.2 characterizing mutations highlighted included the additional ones identified within the Bulgarian strains (V36F and R158G in bold).

After the designation of the Delta variant as VOC in May 2021 and its subsequent subdivision into AY.1–AY.95 sublineages in August 2021, as of mid-October 2021, AY.4.2 became a cause for concern because of its higher growth rate relative to other Delta lineages and sublineages in the population in the United Kingdom.[19] At that time AY.4.2 was also present in a number of other European countries with most cases detected in Germany, Denmark, Poland, and France. The time-stamped phylogenetic analysis presented in this study revealed that AY.4.2 and AY.4.2.1 were introduced in Bulgaria as early as August–September 2021 by at least five independent introduction events. The phylogenetic variant clustering suggests that those independent introductions occurred from European countries, which might have been influenced by the increased human mobility throughout the continent in the late summer of 2021. That presumption highlights the necessity of identification and tracking human mobility patterns in addition to genomic surveillance to timely implement infection prevention and control measures.

The AY.4.2 lineage carries the mutations of Delta and AY.4 related to increased risk of disease severity, risk of vaccine escape, and higher transmissibility.[20–22] In addition, AY.4.2 has two mutations in the N-terminal domain (NTD) of the spike protein (Y145H and A222V), one of which (Y145H) is located in the antigenic supersite, suggesting functional importance as well as possible immune evasion. The unusual mutations of the spike protein identified within the Bulgarian samples (V36F and R158G) were found uncommon within the global AY.4.2 lineage suggesting a rapid spread in the local community upon introduction. To date, the scientific evidence indicates that the AY.4.2 has increased transmissibility while its impact on immunity and severity remain similar to those of Delta variant.[19,23,24]

Interestingly almost 85% of the studied Bulgarian sequences had the AY.4.2.1 genotype. The data from outbreak.info show that the total AY.4.2.1 strains sequenced in the United Kingdom ($n = 17\,618$) and Bulgaria ($n = 213$) account for 95.8% of the global AY.4.2.1 (17 831/18 602). Moreover, in both countries, the peak cumulative prevalence has been reached simultaneously in early December 2021 but remained relatively low (up to 7%). Poland was another country affected by the AY.4.2.1, reporting a total of 142 sequences; however, the average cumulative prevalence was lower (0.5%) compared to Bulgaria (2%) and the United Kingdom (1%). According to outbreak.info, AY.4.2.1 appeared in over 40 countries around the world and remained relatively limited, with the most significant ratio in Bulgaria.

Although AY.4.2 variant of SARS-CoV-2 has been de-escalated based on the fact that it is no longer detected in circulation, European centre for disease prevention and control recommends Delta sublineages monitoring be continued within Delta VOC.[8] Variants with enhanced disease transmission and the ability to evade antibodies represent a threat to pandemic control and mitigation efforts. Furthermore, places with lower immunization coverage may upsurge emerging variants and initiate new epidemics.

In conclusion, consistent genomic surveillance of currently circulating variants remains of high importance for early detection and monitoring of emerging SARS-CoV-2 variants. Therefore, surveillance of SARS-CoV-2 evolution over time and in different geographical areas remains a priority to adapt our defenses against the pandemic.

AUTHOR CONTRIBUTIONS

Ivailo Alexiev and Massimo Ciccozzi conceived and designed the study; Marta Giovanetti, Eleonora Cella, Ivan Ivanov, and Ivailo Alexiev analyzed the data; Ivailo Alexiev, Marta Giovanetti, Eleonora Cella, Ivva Philipova, Ivan Ivanov, and Massimo Ciccozzi prepared the draft manuscript; Ivan Stoikov, Deyan Donchev, Lubomira Grigorova, Anna Gancheva, Reneta Dimitrova, Nelly Korsun, Ivelina Trifonova, Veselin Dobrinov, Iliana Grigorova, Todor Kantardjiev, and

Iva Christova performed molecular screening and produced SARS-CoV-2 genomic data. All authors reviewed and contributed to the draft, and approved the final version, for submission.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

SARS-CoV-2 sequence used for the study have been deposited in GISAID under accession numbers: EPI_ISL_4731435, EPI_ISL_5739135, EPI_ISL_5739272, EPI_ISL_5739283, EPI_ISL_5845625, EPI_ISL_5845717, EPI_ISL_5854826, EPI_ISL_5855123, EPI_ISL_5855126, EPI_ISL_5855137, EPI_ISL_5855145, EPI_ISL_5855169, EPI_ISL_5855177, EPI_ISL_5855180, EPI_ISL_5855190 and from EPI_ISL_6101740 to EPI_ISL_6101764.

ETHICS STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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