Heliyon 8 (2023) e12650

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

The SARS-CoV-2 Delta (B.1.617.2) variant with spike N501Y mutation in the shadow of Omicron emergence

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HIGHLIGHTS

- The study describes the presence of rare Delta GK/478K.V1 (B.1.617.2; AY.4.3) isolates.
- Isolates differ from the most related clade GK AY.4.3 by the presence of Spike N501Y and L54F changes.
- Spike_N501Y + Delta variant seems not to have large-scale consequences to general population.

ARTICLE INFO

Keywords:
SARS-CoV-2
N501Y
Delta variant
Omicron variant
COVID-19
Pandemic
Spike protein

ABSTRACT

Circulation of the Omicron variant with the reemergence of the N501Y mutation along with many others in the spike protein has once again stirred the academic community. Interestingly, tracing the genetic diversity of SARS-CoV-2 shed light on a less frequent N501Y + Delta variant which has been in the global circulation for some time before the Omicron appearance. This paper aims to present the molecular characteristics of the SARS-CoV-2 Spike_N501Y + Delta variant detected in Bosnia and Herzegovina. The study was conducted during November and December 2021. All patients were tested using real-time RT-PCR for detection of SARS-CoV-2. A representative number of SARS-CoV-2 positive samples was pre-screened using VirSNiP SARS-CoV-2 Spike N501Y kit. The characterization of the viruses was carried out with Illumina RNA Prep with enrichment and the Respiratory Virus Oligo Panel kit. Among the analyzed sequences, we found two isolates of the Delta variant that differ from their most related clade- GK AY.4.3 in additional mutations N501Y and L54F. In this study, we described the presence of a rare form of Delta variant with Spike_N501Y mutation in the shadow of the Omicron emergence. Despite the set of mutations in the Spike protein, this form of Delta variant does not indicate the large-scale consequences for the general population. Further functional studies of this form could provide more information about its antigenicity and infectivity.

1. Introduction

According to the World Health Organization (WHO) (WHO, 2021a) and the latest Centers for Disease Control and Prevention (CDC, 2021) classification and definition of the SARS-CoV-2 variants, Delta (Pango lineages B.1.617.2 and AY; Nextstrain 21A/S:478K) and Omicron (Pango B.1.1.529 and BA lineages; Nextstrain 21K) are classified as variants of concern (VOC) (CDC, 2021; Hadfield et al., 2018; Rambaut et al., 2020; WHO, 2021a).

The Delta variant was first identified in India in October 2020 with characteristic set of spike protein substitutions T19R, T95I, G142D, E156-, F157-, R158G, L452R, T478K, D614G, P681R, D950N, and V70F, A222V, W258L, K417N changes detected in some but not all sequences. It has been shown that Delta lineages have increased transmissibility compared to previous variants, which suggested the possibility of more hospitalized cases and deaths (Allen et al., 2022). To date, most Delta lineages (except AY.1 and AY.2) can be treated with monoclonal

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https://doi.org/10.1016/j.heliyon.2022.e12650
Received 5 April 2022; Received in revised form 5 October 2022; Accepted 20 December 2022

antibodies under the Emergency Use Authorization (EUA) (Deng et al., 2021a; EUA, 2022).

As of December 21, 2021, the CDC designated the three previous VOC variants (Alpha- Pango lineages B.1.1.7 and Q lineages; Beta-B.1.351 and descendent lineages; Gamma- Pango lineages P.1 and descendent lineages) as Variants Being Monitored (VBM) (CDC, 2021). On December 22, 2021 Joint European Centre for Disease Prevention and Control (ECDC)/WHO Euro Virus Characterization Working Group included Omicron as a VOC for Europe region (WHO, 2021b), beside Beta, Gamma and Delta variants, and reported de-escalation of Alpha (previous VOC) variant.

The specific feature of Alpha, Beta, Gamma and Omicron SARS-CoV-2 variants has been their common mutation N501Y in the receptor-binding domain (RBD) of the spike glycoprotein. This change played a key role in increased binding affinity of viral RBD to human angiotensin-converting enzyme 2 (ACE2) (Williams and Zhan, 2021) and immune evasion (Li et al., 2021; Uzoeto et al., 2022).

The first detection of the Omicron variant in South Africa on November 24, 2021, has once again stirred the academic community with the emergence of the N501Y along with many other mutations in the spike region of this variant (A67V, del69–70, T95I, del142-144, Y145D, del211, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F) (CDC, 2021).

According to the existing instructions of real-time reverse transcription polymerase chain reaction (rtRT-PCR) tests for monitoring the circulation of variants (e.g. TiBMolBiol VirSNiP assays), no targeting was found for this mutation as potentially present in the Delta variant, nor in available literature to date. With the emergence of Omicron variant and the lack of adequate low-budget pre-screening SARS-CoV-2 test, we targeted the N501Y mutation by rtRT-PCR/Melt-curve analysis and obtained positive results. However, the downstream analysis of these samples showed the presence of Delta variant. Tracing the genetic diversity of SARS-CoV-2 in GISAID, also showed that the N501Y + Delta variant itself has been in the global circulation for some time before the Omicron variant appeared.

This paper aims to present the molecular characteristics and epidemiology of the SARS-CoV-2 Spike_N501Y + Delta variant detected at the Clinical Center of the University of Sarajevo, Bosnia and Herzegovina.

2. Material and methods

2.1. Sample collection

The study was conducted during November and December 2021. In that period, a total of 43738 samples of nasopharyngeal swabs were collected and tested for the presence of SARS-CoV-2 infection. The nasopharyngeal swab samples were collected in a Citoswab Collection and Transport Kit (nal von Minden, GmbH, Moers, Germany) containing 3 ml of the virus transport medium (VTM) from patients referred to the Clinical Center of the University of Sarajevo, Unit for Clinical Microbiology, Bosnia and Herzegovina, for SARS-CoV-2 testing. Samples in VTM were stored at 4 °C and tested for the presence of SARS-CoV-2 within 2 h of admission. Then, they were aliquoted and stored at -70 °C until further analysis.

2.2. Screening SARS-CoV-2 real-time RT-PCR test

All patients were screened by real-time RT-PCR (rtRT-PCR) test for the qualitative detection of SARS-CoV-2 specific genes (Artus SARS-CoV-2 Prep&Amp UM Kit; Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. The kit constitutes a ready-to-use system with a simple sample preparation step followed by detection of the SARS-CoV-2 ribonucleic acid (RNA) N1 and N2 regions detected with the same fluorescence channel, without differentiation. The Primers and Probes mix contains the oligonucleotides required for the RNAse P amplification needed to monitor the presence of human cells in biological samples. To reveal a possible RT-PCR inhibition in the reaction, an internal RNA control (IC) is included in the kit.

Briefly, 50 µl of the samples in VTM were inactivated at 70 °C for 10 min in a thermocycler and cooled down at 4 °C for at least 2 min. The 8 µl of inactivated samples were mixed with 2 µl of UM Prep Buffer for extraction of viral RNA. The isolated RNAs were immediately used for rtRT-PCR. Fifteen µl of the Reaction Mix containing 7.25 µl of Amp Primers (1x), 1.5 µl of IC (10 cp/µl), and 6.25 µl (1x) of UM Amp Buffer completed with ROX Reference Dye, were added to the 10 µl of sample RNA, No Template Control (NTC, nuclease-free water-contamination check), No Extraction Control (NEC, 8 µl of nuclease-free water and 2 µl of UM Prep Buffer-to verify the absence of rtRT-PCR inhibitors in the preparation buffer) and SARS-CoV-2 specific Positive Control. The rtRT-PCR program was set up as follows: reverse transcription (50 °C, 10 min), PCR initial heat activation (95 °C, 2 min), and 2-step cycling (40 cycles of the steps: 95 °C, 5 s, and 58 °C, 30 s-data collection step). The test was performed as a multiplex reaction measuring the fluorescence through three channels in each well/sample simultaneously for specific viral targets (N1 and N2 regions-green channel), RNAse P (yellow channel), and IC (red channel), using standard two replicate testing.

According to the manufacturer's instructions, data were analyzed by absolute quantification using the standard curve method. The fluorescence threshold was set as follows: FAM- 0.13, VIC/HEX- 0.05, and CY5- 0.025, while the baseline was set on Automatic for each dye. The Ct values $\leq$39 for virus-specific targets (FAM dye) are considered to represent the positive SARS-CoV-2 result with or without the presence of internal RNase P (VIC dye) or IC signal (Cy5 dye). The rtRT-PCR was performed on CFX96 C1000 (Bio-Rad Laboratories, Hercules, CA, USA), ABI7500 and QuantStudio5 (Applied Biosystems, Waltham, MA, USA), and Cobas z480 (Roche Molecular Systems, Inc., Pleasanton, CA, USA) instruments.

2.3. VirSNiP N501Y mutation screening

A representative number of SARS-CoV-2 positive samples, in terms of age, gender, and disease severity of cases, with Ct < 27, was selected for a 130 bp PCR long fragment amplification with an absolute quantification and melting curve analysis, using a virus single nucleotide polymorphism (VirSNiP) SARS-CoV-2 Spike N501Y kit (TIB Molbiol, Berlin, Germany) following the manufacturer's instructions. The sample size for testing was calculated to estimate 10% of confirmed SARS-CoV-2 cases per week, using random numbers generated by computer program (Excel).

One-step rtRT-PCR was performed for multiplex detection of viral RNA targets using a 501Y- specific probe. Reactions were set up using LightCycler Multiplex RNA Virus Master containing a unique reverse transcriptase enzyme solution and the aptamer-mediated hot start AptaTaq Fast DNA Polymerase (Roche Molecular Systems, Inc., Pleasanton, CA, USA). Fifteen µl of the Reaction Mix containing 10.4 µl of Water (PCR-grade), 0.5 µl Parameter-Specific Reagents (PSR), 4.0 µl of Roche Mastermix, and 0.1 µl of RT Enzyme were added to the 5 µl of sample RNA, No Template Control (NTC, nuclease-free water-contamination check), and SARS-CoV-2 specific Positive Control, respectively. The kit manufacturer did not provide detailed information about the PSR (primer and probe sequences). Run set-up for absolute quantification was done according to the protocol for cobas z 480 Analyzer (Roche Molecular Systems, Inc., Pleasanton, CA, USA), UDF (User Defined Workflow) module as follows: reverse transcription (55 °C, 5 min), denaturation (95 °C, 5 min), 2-step cycling (45 cycles of the steps: 95 °C, 5 s, and 60 °C, 15 s-data collection step), and cooling (40 °C, 30 s). Ramp rates (°C/s) were adjusted for a 96-well plate according to the manufacturer's instructions. Melting analysis was programmed as a second run consisting of a cycle of three steps (95 °C, 30 s; 40 °C, 2 min; 75 °C, 0 seconds/Acquisition Mode-Continuous), and cooling at 40 °C, 30 s (Acquisition Mode- 2.0). The results of amplification and melting analysis (Tm calling) were read in the 530 nm channel (cobas z 480 Analyzer-open channel: 465–510 nm).

The amplification (absolute quantification mode) of isolates containing the 501N variant has not been visible using Roche polymerase. The melting curve of the amplified product carrying the N501Y mutation was observed at around 60 °C, while Spike 501N amplicons had a melting curve with a peak at around 54 °C.

Samples-candidates for genomic sequencing were selected after VirSNiP testing according to the mutation profile of interest, including N501Y positive results.

2.4. Whole genome sequencing of SARS-CoV-2 and data analysis

In order to perform a detailed analysis of the SARS-CoV-2 variants by whole genome sequencing (WGS) of the virus, the Respiratory Virus Oligo Panel with Illumina RNA Prep with Enrichment (hybrid-capture methods) were used. The workflow integrated sample isolation (QIAamp Viral RNA mini kit, Qiagen GmbH, Hilden, Germany), library preparation, sequencing, and data analysis.

After sequencing on the MiniSeq system (Illumina, San Diego, CA, US) and obtaining FASTQ files, data analysis was carried out using the DRAGEN COVID Lineage pipeline v3.5.6 (Illumina, San Diego, CA, US) and the Galaxy platform (Galaxy, 2022). The genomic architecture of the isolates was constructed at the Stanford University Coronavirus Antiviral & Resistance Database (Tzou et al., 2020). Monitoring of nucleotide and amino acid variations and 3D structural mapping was carried out with the CoVsurver tool, performing sequence alignments and annotations, highlighting phenotypically or epidemiologically interesting candidate amino acid changes and constructing 3D structural mapping (GISAID, 2021). Whole genome sequences of SARS-CoV-2 isolates from Bosnia and Herzegovina were deposited in the GISAID database. The CLUSTAL multiple sequence alignment by MUSCLE 3.8 (EMBL-EBI Hinxton) was performed prior to the phylogenetic analysis using the PhyML 3.1/3.0 aLRT (doc + aLRT) online tool (Anisimova and Gascuel, 2006; Dereeper et al., 2008, 2010; Guindon and Gascuel, 2003).

2.5. Ethical statement

The research has complied with all relevant national regulations, institutional policies, and in accordance with the tenets of the Helsinki Declaration. This research has been approved by the Ethics Committee of the Clinical Center of the University of Sarajevo, No. 51-45-1-49529/21, dated 22nd October 2021. Informed consent of all participants for this research was obtained.

3. Results

A total of 43738 nasopharyngeal swab samples were tested during November–December 2021, of which 8656 (19.79%) were positive for SARS-CoV-2. 10% of representative SARS-CoV-2 confirmed cases per week (865 in total) were further characterized for the presence of the Spike_N501Y mutation. While pre-screening the positive SARS-CoV-2 samples with the VirSNiP Spike_N501Y rtRT-PCR assay (on December 3, 2021) to detect a possible presence of the emerged Omicron variant, we found two N501Y + samples (2/865; 0.23%). The samples were taken from an elderly couple living in the same household, aged 85 and 78 years, located in the municipality of Ilidža (Sarajevo Canton, Bosnia and Herzegovina). Both patients showed symptoms of moderate COVID-19, including fever (up to 39 °C), fatigue, cough, difficulty breathing, and developed bilateral pneumonia. The female has been suffering from essential (primary) hypertension and cardiomyopathy since 2015, while the male did not have any underlying condition. None of the patients required oxygen support or hospitalization. Both patients responded favorably to the recommended therapy, and recovery occurred around the 15th day of treatment. The patients were not vaccinated and were without recent traveling history.

After WGS, we confirmed the presence of a VOC Delta GK/478K.V1 (B.1.617.2 + AY.x) first detected in India (Pango lineage AY.4.3; Prob = 1.0; pangolin: 3.1.17; pangoLEARN: 2022-01-20) carrying the Spike_N501Y mutation in both samples (GISAID IDs: EPI_ISL_7547062 and EPI_ISL_7547063). The genome architecture of these SARS-CoV-2 isolates was identical and is shown in Figure 1.

The overall amino acid identity of the SARS-CoV-2 Spike_N501Y + Delta variant isolates and the Wuhan reference sequence (hCoV-19/Wuhan/WIV04/2019|EPI_ISL_402124) was 98.90%, with 14 amino acid changes (Table 1). The lowest percentage of identity was found in the genes NS7b (97.7%), NS7a, N (99.0%), and Spike protein (99.1%). The obtained sequences differ from the phylogenetically closest clade of Delta variant (example hCoV-19/England/ALDP-188D2DD/2021, EPI_ISL_2925424|2021-07-01, Clade GK AY.4.3 (Pango v.3.1.19 2022-01-20), Delta (AY.4-like) (Scorpio)) in two substitutions positioned in Spike protein, L54F and N501Y. The lack of these two mutations increases the total identity of the given amino acid sequence with the Wuhan reference sequence from 98.90% to 99.06%.

According the spike glycoprotein sequence homology analyzed by Stanford University Coronavirus Antiviral & Resistance Database, the best and the same match (Delta, 0.10%) was observed with the sequences from Japan (2021; EPI_ISL_4349091 and EPI_ISL_4414869), United Kingdom (2021; EPI_ISL_2513528 and EPI_ISL_2568065), United States (2021; EPI_ISL_4295481 and EPI_ISL_4360704), Ireland (2021; EPI_ISL_3298531), Italy (2021; EPI_ISL_3233067 and EPI_ISL_4339200), and Canada (2021; EPI_ISL_4416231). However, all these whole genome sequences do not contain Spike_ N501Y substitution.

Phylogenetic analysis of two SARS-CoV-2 Spike_N501Y Delta isolates from Bosnia and Herzegovina with the most related clade sequence (England, EPI_ISL_2925424), one of the best matching spike glycoprotein sequence according to Stanford University Coronavirus Antiviral & Resistance Database (Japan, EPI_ISL_4349091), as well as Delta VOC first detected in India (EPI_ISL_9232324), Wuhan reference sequence (EPI_ISL_402124) and Omicron VOC first detected in Hong Kong (EPI_ISL_6590782.2) is represented in Figure 2.

Interaction of the SARS-CoV-2 Spike glycoprotein (PDB- Protein Data Bank code: 6ACJ, grey ribbon) in complex with the host cell receptor ACE2 (green ribbon) is visualized in Figure 3. A. List of variations shown in the structure of isolated SARS-CoV-2 Spike_N501Y + sequences in Bosnia and Herzegovina (nearest residue if in loop/termini region) is shown as colored balls: pink balls-T19R(20), blue balls- P26S, L54F, T95I, G142D, E156G; turquoise balls- F157del, R158del; ocher balls-L452R, T478K, N501Y, D614G, P681R(674); blue balls- D950N. The closest mutations in RBD, L452R, T478K and N501Y, are given in an enlarged view of the interaction. The host cell interaction corresponding to position Spike_N501Y and human ACE2 chain have been shown in Figure 3. B. In fact, the figure shows the Asparagine to Tyrosine change corresponding to position 501 marked with red balls on viral chains A, B and C, respectively (yellow backbone), while the purple backbone depicts the host chain of Protein Data Bank (PDB) entry- 7ct5 (Spike glycoprotein of SARS-CoV-2 in complex with ACE2 of *Homo sapiens*). The mutation is within 6 Å from host chain (D, E and F, respectively).

By retrieving SARS-CoV-2 Spike_N501Y + Delta WGSs from the GISAID database, we observed the presence of such sequences in other parts of the world. As of January 24, 2022, 1717 WGSs of the Spike_N501Y + Delta variant were deposited in GISAID, representing 0.02% of the total entries of 7411310 SARS-CoV-2 WGSs, and 0.04% of 4109686 Delta WGSs, respectively. From Bosnia and Herzegovina, only two WGSs of the Spike_N501Y + Delta variant were deposited in GISAID (described in this paper), representing 0.15% of the total entries of 1324 SARS-CoV-2 WGSs, and 0.18% of 1140 Delta WGSs, respectively. At the time of the manuscript revision, its prevalence was comparable to earlier results (worldwide 0.02% and 0.05%, respectively; B&H 0.15% and 0.18%; GISAID accessed September 17, 2022).

4. Discussion

In addition to the emerged Omicron variant of SARS-CoV-2 and associated lineages, the WHO and the CDC still consider Delta as a VOC

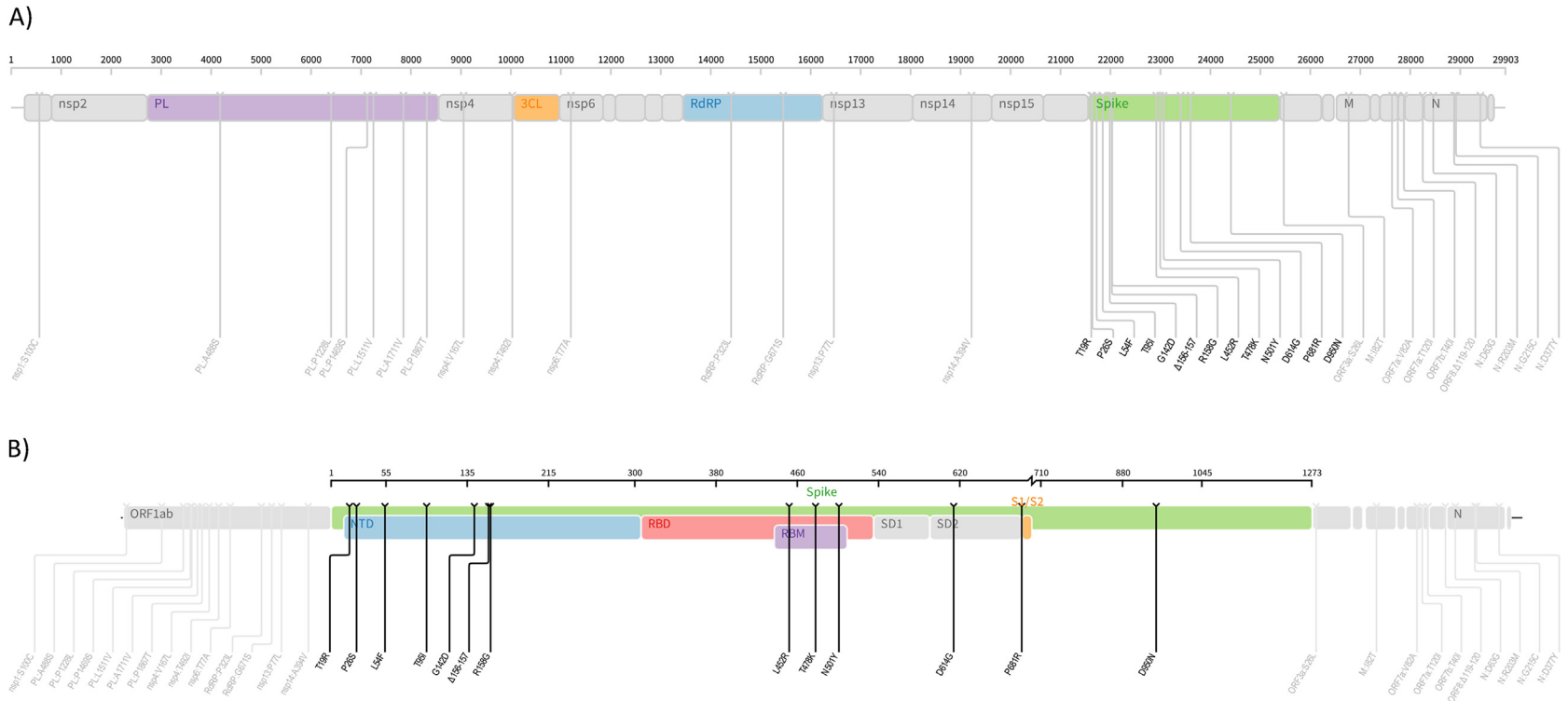

Figure 1. Amino acid mutations of SARS-CoV-2 Spike_N501Y + Delta variant isolates from Bosnia and Herzegovina plotted on a genome map of SARS-CoV-2 (A) with a focus on Spike glycoprotein (B).Figure represents the detailed genome architecture of two identical SARS-CoV-2 isolates (GISAID IDs: EPI_ISL_7547062 and EPI_ISL_7547063) from Bosnia and Herzegovina.

Table 1. List of amino acid changes of hCoV-19/Bosnia and Herzegovina/KCUS154814/2021 (EPI_ISL_7547063), Delta variant (Clade GK) in comparison to hCoV-19/Wuhan/WIV04/2019 reference sequence.

Best reference hit	% id	% coverage	Number of aa changes	List of aa changes
NSP1	99.4%	100%	1	S100C
NSP2	100%	100%	0	no aa changes
NSP3	99.7%	100%	6	A488S$^{\#o}$, P1228L, P1469S, L1511V, A1711V, P1867T
NSP4	99.6%	100%	2	V167L, T492I$^{\#o}$
NSP5	100%	100%	0	no aa changes
NSP6	99.7%	100%	1	T77A
NSP7	100%	100%	0	no aa changes
NSP8	100%	100%	0	no aa changes
NSP9	100%	100%	0	no aa changes
NSP10	100%	100%	0	no aa changes
NSP11	100%	100%	0	no aa changes
NSP12	99.8%	99.0%	2	P323L$^{\#o}$, G671S
NSP13	99.8%	100%	1	P77L
NSP14	99.8%	100%	1	A394V
NSP15	100%	100%	0	no aa changes
NSP16	100%	100%	0	no aa changes
Spike	99.1% *(99.2%)	99.8%	14 *(12)	T19R, P26S, *L54F, T95I, G142D, E156G, F157del, R158del$^{\#a}$, L452R$^{\$\#a}$, T478K$^{\$\#rao}$, *N501Y$^{\$\#rao}$, D614G$^{\$\#lo}$, P681R$^{\$}$, D950N$^{\#o}$
NS3	99.6%	100%	1	S26L
E	100%	100%	0	no aa changes
M	99.5%	100%	1	I82T
NS6	100%	100%	0	no aa changes
NS7a	98.3%	100%	2	V82A$^{\$}$, T120I
NS7b	97.7%	100%	1	T40I
NS8	100%	97.5%	0	no aa changes
N	99.0%	100%	4	D63G$^{\$,\#o}$, R203M, G215C, D377Y

Abbreviations: id, identity; aa, amino acid.
The data shown in the table were analyzed by GISAID database tool.
$^{\$}$ corresponding position of the amino acid change has been described in literature.
$^{\#}$ followed by the character(s) in superscript on the amino acid change means that the corresponding structural position of the amino acid change is found to be within 6 Å from host cell surface receptor binding (^{r}), host cell protein/RNA interaction (^{h}), antibody (^{a}), ligand (^{l}) or viral oligomerization interfaces (^{o}).
* The numbers given in brackets represent the difference in identity (%), coverage (%) and number of amino acid substitutions (N501Y, L54F) of the most related clade (example) hCoV-19/England/ALDP-188D2DD/2021, EPI_ISL_2925424|2021-07-01, Clade GK AY.4.3 (Pango v.3.1.19 2022-01-20), Delta (AY.4-like) (Scorpio) to isolates identified by the study, in comparison to the reference Wuhan sequence. The lack of these two mutations increases the total identity of the given amino acid sequence with the Wuhan reference sequence from 98.90% to 99.06%.

(WHO, 2021a). According to the regional distribution of variants (GISAID, on 2022-02-01), the Omicron variant is dominating (now) in six continents (Africa, Asia, Europe, Oceania, North America and South America). However, the recent prevalence of the Delta G/478K.V1 variant (B.1.617.2 + AY.x) ranges from 12.7% (Europe) to 32.3% (Africa), making it significant when considering SARS-CoV-2 circulation globally.

In this study, we described a rare form of the Delta variant with the Spike_N501Y mutation in Bosnia and Herzegovina in two patients who developed a moderate disease, not differing from the original COVID-19 or that caused by other Delta forms. The detection occurred at a time when other VOCs containing this substitution were no longer present (primarily Alpha). However, globally, cases of Omicron have already been reported.

A detailed insight into the genome composition and crystallography of the described isolates (GISAID ID: EPI_ISL_7547062 and EPI_ISL_7547063) shown the difference between these isolates and their most closely related clade - GK AY.4.3 (Pango v.3.1.19 2022-01-20), Delta (similar to AY.4), which are additional N501Y and L54F substitutions.

The N501Y is an ACE2-binding site RBM (receptor binding motif) mutation already present in the earlier Alpha, Beta, Gamma, and recently designated Omicron VOC. According to a number of studies, this change increases affinity for human ACE2 receptor (Cheng et al., 2021; Starr et al., 2020; Zhu et al., 2021) and virus replication in human upper respiratory route cells (Liu et al., 2021; Wibmer et al., 2021). N501Y influences the binding and neutralization of several EMA (European Medical Agency) approved monoclonal antibodies (mAbs) among which Xevudy (sotrovimab), Evusheld (tixagevimab/cilgavimab), and Regkirona (regdanvimab; Regkirona, INN- regdanvimab, 2021). The emergence of the N501Y substitution in different backgrounds and the combination of mutations occurring in a specific variant has led to dissimilar responses to mAbs. The pseudotyped virus-like particle (VLP) neutralization data for sotrovimab indicate a 16-fold reduction in activity relative to wild-type against the B.1.1.529/BA.2 spike variant (FDA, 2022). On the other hand, the pseudotyped VLP neutralization data for Evusheld has shown that the presence of the N501Y substitution in the Alpha background leads to a 5.2-fold reduction in susceptibility, while its co-emergence in the B.1.1.529/BA.1 variant leads to a 183-fold reduction (Evusheld, 2022). Regarding Regkirona, evidence has shown that the combination of substitutions K417N/E484K/N501Y occurring in the Beta, Gamma and Omicron variants has been detrimental for the efficacy of this mAb, leading to loss of activity against the variants (Regkirona, 2021).

During the pandemic, a large evolutionary diversity of SARS-CoV-2 was observed in a short period of time, for which there are still no clear explanations. However, it can be assumed that the ability of a virus to adapt and replicate in a given host plays an important role during its evolution. It is quantitatively determined by its effective reproductive number (Rt), which represents the number of new cases of infection transmitted from an infected person (Gostic et al., 2020). The higher the Rt value, the more likely the virus is to transmit to a larger number of hosts. The virus can best express this ability by circulating among the vulnerable population, when it becomes more contagious. According to data from the literature, the first variants of concern (Alpha, Delta) evolved so that each was about 50% more contagious than the previous one, which ensured its dominance over the previous one (Kraemer et al., 2021). Indeed, the period of cocirculation of variants may be a favorable time for the evolution of transitional forms with combined genetic changes of the virus. In contrast, circulating among the immune population will contribute little to virus transmission, as it is prevented by host immunity to infection. However, with the increase in the population that is immune to the circulating form, it is assumed that SARS-CoV-2 is likely to evolve rapidly antigenically so that it will be capable of reinfection, due to selective pressure (Markov et al., 2022). In Bosnia and Herzegovina, satisfactory immunization coverage has not been achieved to date. In fact, it is minimal (25.8% of those fully vaccinated against SARS-CoV-2 infection) (Coronavirus (COVID-19) Vaccinations, 2022). Furthermore, in Bosnia and Herzegovina, a country with a population of 3.281 million, from 3 January 2020 to 18 March 2022, 373,799 confirmed cases of COVID-19 were reported to the WHO (WHO, 2022). These statistics suggest that there is a large group of at-risk individuals who are susceptible to SARS-CoV-2 infection, suggesting the possibility of high transmission and replication of the virus, and thus the development of new mutant forms.

Genome recombination plays an essential role in the evolution of emerging and re-emerging viral agents. This mechanism results in recombinant forms of the two virus strains infecting the same cell. Different

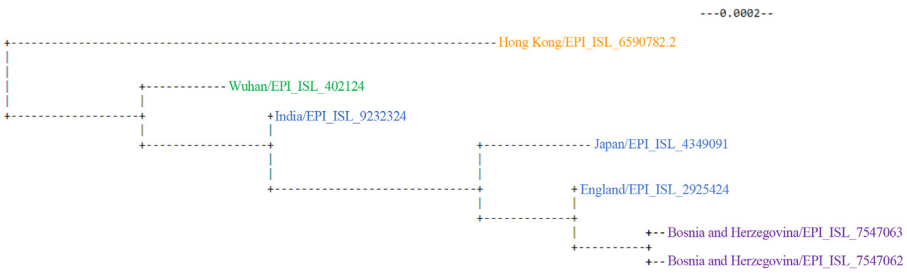

Figure 2. Phylogenetic analysis of two SARS-CoV-2 Spike_N501Y Delta isolates from Bosnia and Herzegovina. The CLUSTAL multiple sequence alignment by MUSCLE (3.8) and the phylogenetic analysis using the PhyML 3.1/3.0 aLRT (doc + aLRT) online tool was performed (Parameters: Substitution model: GTR; Gamma shape parameter: 99.141; Number of categories: 4; Proportion of invariant: 0.982). Whole genome sequences were obtained from GISAID database and the original names of the analyzed sequences are listed below: >hCoV-19/Bosnia and Herzegovina/KCUS154813/2021|EPI_ISL_7547062|2021-12-02 (in violet) >hCoV-19/Bosnia and Herzegovina/KCUS154814/2021|EPI_ISL_7547063|2021-12-02 (in violet) >hCoV-19/England/ALDP-188D2DD/2021|EPI_ISL_2925424|2021-07-01 (The most related clade to Bosnia and Herzegovina isolates according to GISAID; in blue) >hCoV-19/Japan/IC-1974/2021|EPI_ISL_4349091|2021-09-06 (One of the best matching Spike gene sequence to Bosnia and Herzegovina isolates according to Stanford University Coronavirus Antiviral & Resistance Database; in blue) >hCoV-19/India/TN-CLRI-CIC0568/2020|EPI_ISL_9232324|2020-09-05 (Delta VOC, first detected in India; in blue) >hCoV-19/Wuhan/WIV04/2019|EPI_ISL_402124|2019-12-30 (Wuhan reference sequence; in green) >hCoV-19/Hong Kong/VM21044713-1/2021|EPI_ISL_6590782.2|2021-11-13 (Omicron VOC, first detected in Hong Kong; in orange). The scale bar on the upper right corner indicates the number of substitutions per site.

combinations of mutations of recombinant forms may have the potential for unique transmissibility, immune escape, and disease severity. Several studies have shown that recombinant SARS-CoV-2 genomes have been generated during the pandemic (Haddad et al., 2021; He et al., 2022). However, a high disequilibrium among polymorphic positions has been observed without affecting clonal inheritance (Wang et al., 2020). Furthermore, co-infections appear to occur rarely, with possible recombination at a later stage of infection when viral transmissibility is lower (Rockett et al., 2022; Combes et al., 2022). These data suggest that the prevalence of recombinant forms of SARS-CoV-2 may be consequently low (Nie et al., 2020; Wang et al., 2020; VanInsberghe et al., 2021). On the other hand, current tracking of SARS-CoV-2 mutations has provided evidence of the contribution of both inter- and intra-variant recombination of SARS-CoV-2 genomes developing emerged recombinants such as XD (Delta AY.4/Omicron BA.1) XE (Omicron BA.1/BA.2), XF (Delta/Omicron BA.1), XL (Omicron BA.1/BA.2), XG (Omicron BA.1/BA.2), XN (Omicron BA.1/BA.2), XQ (Omicron BA.1/BA.2), XR (Omicron BA.1/BA.2), and XAG (Omicron BA.1/BA.2) (Wang et al., 2022; Chakraborty et al., 2022; Silva et al., 2022). If we observe the target N501Y substitution of SARS-CoV-2 isolated from the patients described in our study and the possibility of co-infection with the 501N variant, both forms of the virus genome would be differentiated already with VirSNiP N501Y/melting curve analysis. However, we detected only one curve as a signal for the presence of a mutated form of SARS-CoV-2 (N501Y+). We also investigated the potential immunocompromise (oncological history) and immunodeficiency of our patients, as well as the longer duration of infection as a favorable situation for the development of the new viral forms within the host, but we did not record such circumstances. A plausible explanation could be that the mutations occurred spontaneously, as natural selection can cause rare but favorable mutations. Since the patients have not been vaccinated, a potential previous infection may have generated protective immunity, and the emergence of the N501Y + variant has been favored to avoid antibody neutralization. Another possible explanation could be that the substitution occurred earlier in persons with whom the described patients were in contact but remained unidentified.

The second mutation, L54F, found in our isolates, represents a kind of neutral mutation (according to a free energy change), but when combined with D614G, the resulting mutant becomes stable (Laha et al., 2020).

In addition to the N501Y change, the described Delta variant shares with Omicron other mutations in the Spike region, such as T95I, T478K, and D614G.

The T95I is an N-terminal domain (NTD) mutation present in many Delta VOC sublineages and in the Omicron VOC.

Before this study, the important RBD change T478K was described as a variant specific mutation which is coupled with L452R in the Delta VOC, and in the combination with multiple other RBD mutations (different from L452R) in the Omicron VOC. It was shown that the effects of T478K on mAbs and immune plasma retain the binding capacity to each of the EUA-approved mAbs (Starr et al., 2021a, 2021b).

Beside Delta variant, L452R is present also in Epsilon and Kappa VBM variants (Deng et al., 2021b). It was written that L452R is likely to affect the reduction of susceptibility to some RBM class II mAbs including bamlanivimab but not to the other FDA EUA-approved mAbs (Li et al., 2020; FDA, 2021). It has also been associated with low-level reductions in susceptibility to about one-third of convalescent and vaccine plasma samples (Ferreira et al., 2021). A deep mutational scanning analysis showed that Spike L452R escapes COV2-2096 antibody neutralization with an escape fraction of 0.3313, where any mutation conferring an escape fraction of an equal or more than 0.1 is considered high (Greaney et al., 2021).

D614G mutant became more prevalent since the late February 2020. This change increases the infectivity SARS-CoV-2 (Li et al., 2020), and may also be responsible for increasing the number of spike proteins per virion (Zhang et al., 2020) and higher rate of S1/S2 cleavage (Gobeil et al., 2021).

P681R positioned in the furin cleavage site, enhances the basicity of the poly-basic stretch, that might contribute in increased rate of membrane fusion, internalization and consequently better transmissibility (Cherian et al., 2021). Beside Delta VOC, this change is present in the Kappa VBM and in A.23.1 while the P681H form shares Alpha VBM, Omicron VOC, Theta and many other global lineages of SARS-CoV-2.

The Spike_N501Y + SARS-CoV-2/ACE2 complex structure highlighted its basic phenotypical characteristics and visual proximity of the L452R, N501Y and T478K mutations in the RBM region of RBD (CoV-surver, 11) presented in Figure 3. A, B. However, further investigation into the functional effects of this composition is needed to be done.

As of January 24, 2022, a relatively small number of Spike_N501Y + Delta variant forms have been deposited in the GISAID database (0.02% of the total entries). The Spike_N501Y + Delta variant was firstly detected in Romania (Europe; EPI_ISL_2100241), at the end of March 2021. This mutated form of the Delta variant has gradually spread to six of the seven world's continents (Europe, North America, South America, Asia, Africa, and Oceania) and has been recorded in 50 countries. It was most commonly recorded in Türkiye in 28.31% (486/1717), Israel 18.58% (319/1717), Germany 10.54% (181/1717), USA 8.56% (147/1717), England 7.98% (137/1717) and rarely in other countries until then (GISAID). It is interesting to observe the period of circulation of this form of the Delta variant, which dates back to the time when VOCs

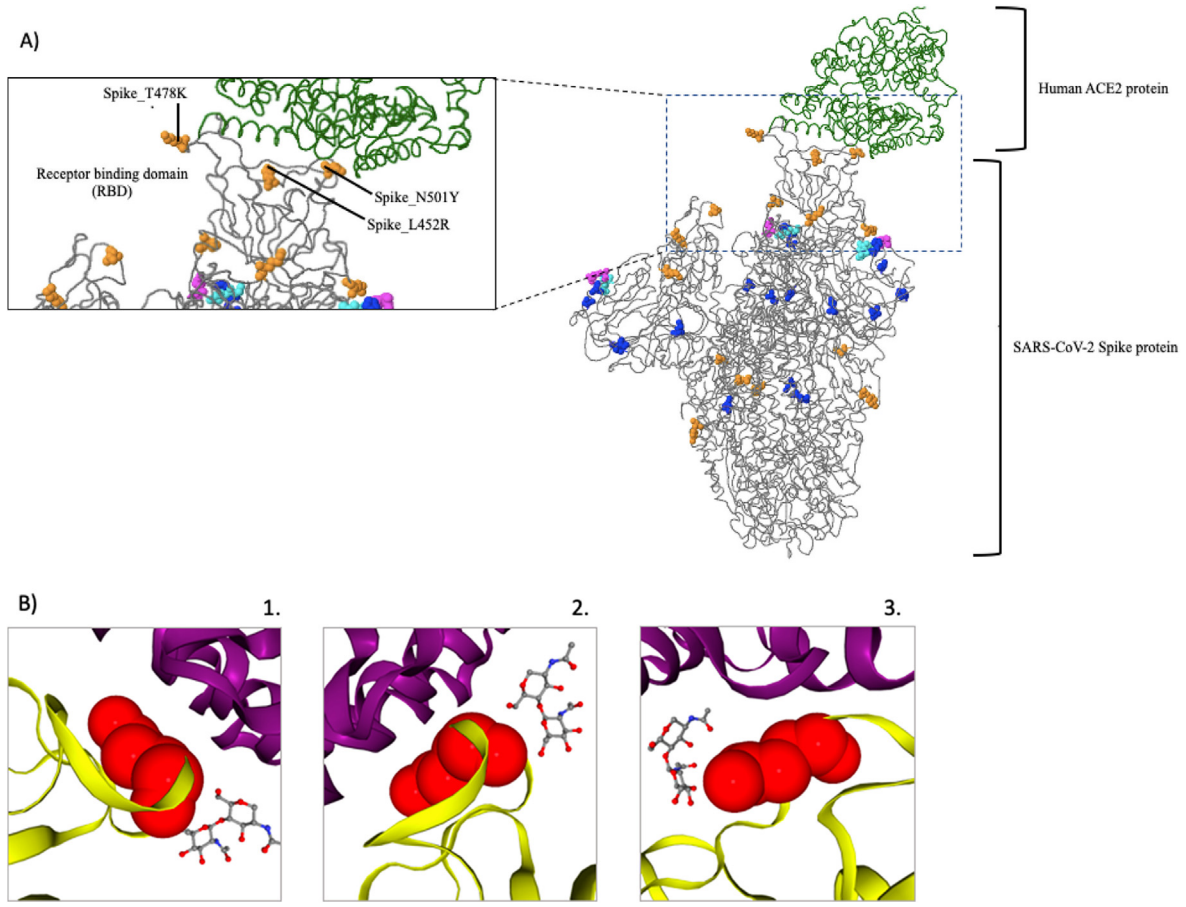

Figure 3. Structural visualization of the SARS-CoV-2 Spike glycoprotein in complex with host cell receptor angiotensin-converting enzyme-2 (ACE2) with amino acid changes. A) SARS-CoV-2 Spike glycoprotein (PDB- Protein Data Bank code: 6acj, EM-electron microscopy- 4.2 Å, grey ribbon) in complex with host cell receptor ACE2 (green ribbon). List of variations displayed in structure (nearest residue if in loop/termini region) shown as colored balls: pink balls-T19R(20), blue balls- P26S, L54F, T95I, G142D, E156G; turquoise balls- F157del, R158del; ocher balls- L452R, T478K, N501Y, D614G, P681R(674); blue balls- D950N. B) Host cell interaction corresponding to position Spike_N501Y and human ACE2 chain. The mutation position (red balls) corresponds to position 501 on viral chain A, B and C, respectively (yellow backbone) of protein entry PDB-7ct5 (Spike glycoprotein of SARS-CoV-2 in complex with ACE2 of *Homo sapiens*). The mutation is within 6 Å from host chain (D, E and F, respectively) (purple backbone); 1. viral chain A/host chain D; 2. viral chain B/host chain E and 3. viral chain C/host chain E.

containing the N501Y mutation (Alpha mainly, Beta, Gamma) predominated in the world. However, it remains unclear what mechanisms really influenced its development, which opens the space for further research. Considering the low prevalence and limited data on the clinical manifestations of COVID-19 caused by the N501Y + Delta variant and the current dominance of the Omicron variant, it seems that it is not necessary to develop new diagnostic tools, a vaccine, or a therapy against this form of SARS-CoV-2. Further functional studies of this form of SARS-CoV-2 could provide more information about its antigenicity and infectivity.

A limitation of this study is the small number of sequences presented. In fact, the only two sequences of this rare form detected in Bosnia and Herzegovina were analyzed.

5. Conclusions

Molecular epidemiological insight showed that the Spike_N501Y + Delta variant spread sporadically worldwide with a relatively low prevalence compared to other forms of this variant (0.04%). Despite the set of mutations in the Spike protein, this form of Delta variant does not indicate the large-scale consequences for the general population. More detailed studies are needed for the functional characterization of this rare form of SARS-CoV-2.

Declarations

Author contribution statement

Irma Salimović-Bešić: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Amela Dedeić-Ljubović: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Edina Zahirović: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Medina Hasanović: Performed the experiments; Wrote the paper.

Merima Šehić: Performed the experiments; Wrote the paper.

Maja Vukovikj: Analyzed and interpreted the data; Wrote the paper.

Golubinka Boshevska: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sandra Vegar-Zubović: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Enra Mehmedika-Suljić: Conceived and designed the experiments; Wrote the paper.

Sebija Izetbegović: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by the Federal Ministry of Education and Science of Bosnia and Herzegovina (05-35-1809-1/21) for financing and realization of the scientific research project "Monitoring the genomic and evolutionary diversity of SARS-CoV-2 in B&H (FB&H)".

Data availability statement

Data associated with this study has been deposited at GISAID under the accession number EPI_ISL_7547062, EPI_ISL_7547063.

Declaration of interest's statement

The authors declare no competing interests.

Additional information

No additional information is available for this paper.

Acknowledgements

Authors thank the WHO Regional Office for Europe and the WHO Country Office in Bosnia and Herzegovina for their support with equipment, reagents and staff training for sequencing and data analysis. We gratefully acknowledge the Authors from Originating and Submitting laboratories of sequence data on which the analysis is based on.

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