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SINGLE NUCLEOTIDE POLYMORPHISM TEST FOR RAPID DETECTION OF SARS-COV-2 LINEAGES IN THE REPUBLIC OF NORTH MACEDONIA

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ABSTRACT

Introduction The coronavirus pandemic represents one of the most significant medical crises in recent history; therefore, rapid virus detection has become a critical component of public health practice. As the virus has undergone continuous mutations, the emergence of new variants has resulted in altered transmission dynamics, changes in disease severity, and implications for diagnostic testing. Although genomic sequencing is the most effective method for mutation detection, it remains time-consuming and expensive process.

Aim To present a testing algorithm and evaluate the use of single nucleotide polymorphism (SNP) melting curve PCR for the detection of SARS-CoV-2 lineages, which may inform modifications in public health control and preventive measures, as well as potential adjustments to PCR-based diagnostic tests.

Material and methods RNA extracted from 140 SARS-CoV-2 positive samples received in the National Reference Laboratory for Virology, at the Institute of Public Health – Skopje as part of the COVID-19 surveillance system where Ct value ≤ 25 were subjected to SNP testing.

Results Analysis of 140 SARS-CoV-2–positive samples collected between January and September 2022 using SNP testing revealed a predominance of the BA.4/BA.5 Omicron sublineages, accounting for 55.7% of cases.

Conclusions Targeted SNP assays enable rapid and accurate detection of mutations associated with specific SARS-CoV-2 Omicron sublineages, facilitating early identification of emerging variants. These results may subsequently be subjected to further investigation, ultimately contributing to an improved public health response.

Keywords Coronaviruses, Omicron, SARS-CoV-2, Melting curve analysis, Single Nucleotide Polymorphism.

TESTUL DE POLIMORFISM CU UN SINGUR NUCLEOTID PENTRU DETECTAREA RAPIDĂ A TULPINILOR SARS-COV-2 ÎN REPUBLICA MACEDONIA DE NORD

Introducere Pandemia de coronavirus reprezintă una dintre cele mai mari crize medicale din ultima vreme, prin urmare, detectarea rapidă a virusului a devenit o normalitate. Pe măsură ce virusul a suferit mutații, apariția de noi variante a dus la modificări ale dinamicii de transmitere și ale severității bolii, precum și remanieri în utilizarea testelor de diagnostic. Cea mai eficientă modalitate de a detecta mutațiile este secvențierea, care este un proces de durată și costisitor.

Scop Prezentarea unui algoritm de testare și elaborare a utilizării PCR cu curbă de topire a polimorfismului cu un singur nucleotid (SNP) pentru detectarea tulpinilor SARS-CoV-2, ceea ce poate duce la modificarea măsurilor de control al sănătății publice și a măsurilor preventive, dar și la posibile modificări ale testelor PCR, utilizate pentru detectarea SARS-CoV-2.

Material și metode ARN-ul extras din 140 de probe pozitive la SARS-CoV-2 recepționate în Laboratorul Național de Referință pentru Virusologie, la Institutul de Sănătate Publică - Skopje, ca parte a sistemului de supraveghere a COVID-19, unde valoarea Ct ≤ 25 a fost supusă testării SNP.

Rezultate Analiza a 140 de probe pozitive la SARS-CoV-2 din ianuarie până în septembrie 2022, utilizând testarea SNP, a identificat o prezență dominantă a subtulpinilor BA.4/BA.5 Omicron, reprezentând 55,7% dintre cazuri.

Concluzii Testele SNP specifice permit detectarea rapidă și precisă a mutațiilor legate de o subtulpină specifică a SARS-CoV-2 Omicron, ajutând la identificarea timpurie a variantelor emergente. Rezultatele obținute pot fi supuse, ulterior, unor examinări suplimentare, ceea ce ar putea genera un coeficient mai mare a cazurilor depistate.

Cuvinte-cheie Coronavirusuri, Omicron, SARS-CoV-2, analiza curbei de topire, polimorfism cu un singur nucleotid.

INTRODUCTION

In December 2019, cases with similar symptoms and clinical presentation caused by a new human pathogen were reported in Wuhan, China. The virus was named SARS-CoV-2 and the disease COVID-19. On March 11, 2020, the World Health Organization officially categorized the COVID-19 outbreak, a pandemic. The SARS-CoV-2 genome (30kb) has a single-stranded, positive-sense RNA. The main structural components of the virus are: glycoprotein S (Spike), membrane protein M, envelope protein E, and nucleocapsid protein N. In addition, the virus has six open reading frames. The RT-PCR test for detection of SARS-CoV-2 is recommended by WHO as first line test for detection of this virus. Rapid tests for antigen detection have also been developed (1, 2).

Since the appearance of SARS-CoV-2 in the human population, mutations had appeared in the virus's genome resulting in emergence of new variants (3). According to the infectious potential and virulence of the variants, the WHO classified them as Variants of Concern (VOCs), Variants of Interest (VOIs) and Variants Under Monitoring (VUMs). The VOCs are: Alfa (B.1.1.7), Beta (B.1.351), Gama (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529). Currently the Omicron variant is circulating, from which several sub lineages evolved (4-15).

The Omicron variant was first detected on November 9, 2021, in the South African province of Gauteng. The number of confirmed infections per variant and the rapid emergence of each variant indicated that Omicron is more infectious than Delta and Beta (7). To date, it is the VOC with the highest number of non-synonymous mutations, most of which in the Spike protein and they cause increased transmission, disease severity, and immune response evasion (7). As such, the Omicron variant has received a lot of attention in healthcare around the world (11-14). More than 60 mutations have been identified in the Omicron variant, 30 of which are located in the S protein, with 15 in the RBD region. Compared to the other VOCs, Spike protein mutations in Omicron are 3-4 times more numerous including 30 substitutions: A67V, T95I, Y145D, L212I, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, и L981F (13, 14, 16-18).

The Omicron variant initially gave rise to the BA.1 sublineage, followed by the more transmissible BA.2 sublineage, which rapidly became globally dominant. Comparison of the genetic profiles of the BA.1 and BA.2 sublineages revealed a total of 51 mutations across the genome, of which 32 are shared between the two sublineages (7, 13). Among the shared mutations, 21 occur in the spike (S) protein. Nineteen mutations define the unique genetic profiles of the respective sublineages, including 13 spike protein mutations specific to BA.1 (A67V, HV69del, T95I, VYY143del, N211del, L212I, 215EPEins, S371L, G446S, G496S, T547K, N856K, L981F) and seven spike protein mutations specific to BA.2 (T19I, LPPA24S, V213G, S371F, T376A, D405N, R408S) (7, 13, 16–20).

As the virus continued to evolve, the genetic sequences of the BA.4 and BA.5 sublineages were found to contain elements derived from both the BA.2 sublineage and the earlier Delta variant. At the Department of Virology, Institute of Public Health (IPH)–Skopje, a testing algorithm was developed using available commercial kits for the indirect detection of the emerging BA.4 and BA.5 sublineages. The assay targeted the L452R mutation, which is present in the Delta variant and in the BA.4 and BA.5 sublineages, but absent in BA.1 and BA.2. Consequently, in samples initially identified as BA.2, the detection of the L452R mutation suggested the presence of BA.4 or BA.5, prompting further sequencing for definitive genotyping (21).

To enhance epidemiological surveillance and ensure a rapid public health response, North Macedonia established the *National Program for Sequencing of SARS-CoV-2 and Other Highly Pathogenic Agents with Public Health Importance* in December 2021, with support from the World Health Organization. As part of this strategy, SNP testing was implemented as a pre-screening tool, particularly in view of the high cost of next-generation sequencing in low- and middle-income countries. SNP testing was used to differentiate between the BA.1 and BA.2 Omicron sublineages for diagnostic purposes and to select representative samples for sequencing in order to monitor viral evolution. This approach enables a strengthened public health response by facilitating timely implementation of appropriate control measures and optimization of diagnostic methods for the detection of emerging variants.

The aim of this study is to present a testing algorithm based on SNP melting curve PCR for the detection and differentiation of SARS-CoV-2 sublineages, with the potential to inform modifications of existing PCR-based diagnostic strategies. This method is particularly useful for targeted sampling during outbreak or cluster investigations. Routine screening of circulating variants and sublineages can thus be efficiently performed with high throughput, allowing for the selection of samples for additional next-generation sequencing while providing rapid and accurate results at a substantially lower cost.

MATERIALS AND METHODS

Materials

RNA was extracted from 140 samples received at the National Reference Laboratory for Virology, Institute of Public Health (IPH), Skopje, as part of the COVID-19 surveillance system. All samples tested positive for SARS-CoV-2 by real-time RT-PCR and had a cycle threshold (Ct) value ≤ 25. These samples were subjected to melting curve analysis.

Methods

RNA was extracted manually using the QIAamp Viral RNA (Qiagen) based on the manufacturer instructions. The principle is based on a combination of the selective binding capabilities of silica gel membranes and the centrifugation speed (22).

Melting Curve PCR

The principle of Melting Curve PCR is based on determining the melting point (Tm) of the DNA molecule. The Tm of DNA is the temperature at which half of the DNA molecules in the solution are denatured. It is monitored with fluorescent dyes intercalating with the DNA molecule and emitting a signal once bound to the still paired, double-stranded DNA. It depends on the number of G+C pairs in the target sequence, it's length and the ionic strength of the solution. The peak occurs at the temperature at which 50% of the DNA molecules are denatured. The analysis's dynamic approach enables rapid detection and genotyping. The result is used to confirm or exclude the presence of a specific single nucleotide polymorphism (SNP) characteristic for specific variants or sublineages (23, 24).

Reagents and Kits

Two commercial kits were used for SNP melting curve analysis:

1. VirSNiP SARS-CoV-2 Spike S371L/S373P Kit (Cat. No. 53-0827-96; TIB Molbiol) together with the corresponding Lyophilized 1-step RT-PCR Polymerase Mix (Cat. No. 90-9999-96; TIB Molbiol).
2. SARS-CoV-2 Delta Real-Time PCR Genotyping Kit (DNA Technology), which detects the L452R and T478K mutations. All procedures were performed according to the manufacturers' instructions.

The primers and probes in the VirSNiP kit target a specific region of the SARS-CoV-2 genome, generating distinct melting profiles depending on the mutation present. Detection of the S371L/S373P mutations indicates the BA.1 sublineage, whereas detection of the S371F/S373P mutations indicates the BA.2 sublineage (25). An additional assay targeting the L452R mutation, originally developed for Delta variant detection, was used to identify BA.4 or BA.5 sublineages (26).

The mastermix was prepared in a separate room under sterile conditions, in a PCR cabinet, according to the kit's protocol.

For the VirSNiP assay, the lyophilized 1-step RT-PCR Polymerase Mix was reconstituted with 990 µL buffer and gently mixed by pipetting. Primers and probes were rehydrated with 50 µL nuclease-free water, vortexed, and centrifuged before being added to the mastermix along with buffer, enzyme, and nuclease-free water. A volume of 15 µL mastermix was dispensed into each reaction tube, followed by the addition of 5 µL RNA sample (25).

For the purposes of this research, only the primers and probes for the L452R mutation were used from the SARS-CoV-2 DELTA REAL-TIME PCR Genotyping Kit. The buffer, enzyme and the PCR mix (primers and probes) were added to a 1,5µl tube and briefly vortexed. 15µl mastermix was placed into each tube, then 10µl from the RNA sample was added (26).

PCR amplification and melting curve analysis were performed using: PCR thermocycler (used: QuantStudio 5 Real-Time PCR System (manufacturer: Thermo Fisher Scientific – Waltham, Massachusetts, United States) and DTlite real-time PCR instrument (manufacturer: DNA Technology – Moscow, Russia).

Negative and positive controls were included in each PCR run to validate the reaction and monitor potential contamination. Two negative controls were used: one during nucleic acid extraction and one during RNA pipetting, along with a single positive control. Negative controls were required to show no fluorescence signal, whereas the positive control confirmed proper assay performance.

Results were interpreted based on the amplification curve and the cycle at which the fluorescence's exponential growth started. Where there is no signal the result is negative ("Undetermined"). This result should appear in the negative control. When analyzing the results, the focus is on the Amplification Plot and the Melting Curve Plot. Amplification should appear on the Amplification Plot in SARS-CoV-2 positive samples and a peak according to the detected mutation on the Melting Curve Plot. Negative controls should show no fluorescence in either plot. The detected Tm values were interpreted as follows: Tm = 53 °C indicated the BA.2 sublineage (S371F/S373P), Tm = 62 °C indicated the BA.1 sublineage (S371L/S373P), and Tm = 45 °C indicated a non-Omicron variant. Detection of two melting peaks in a single sample suggested either contamination or co-infection with multiple sublineages (25).

RESULTS

The Laboratory for Virology conducted routine testing as part of its central role in national surveillance and outbreak response, including the characterization of pathogens such as SARS-CoV-2 and identification of circulating sublineages to monitor their regional distribution. The results presented include analyses performed using the VirSNiP SARS-CoV-2 Spike S371L/S373P assay for the detection of BA.1 and BA.2 sublineages, as well as the SARS-CoV-2 Delta Real-Time PCR Genotyping Kit for the detection of sublineages indicative of BA.4 and BA.5.

Omicron sublineages circulating in Macedonia in January-September 2022

A total of 140 SARS-CoV-2 positive samples were selected for detection of Omicron sublineages with Melting Curve analysis. Using two different kits BA.1 was detected in 16 samples (11.4%), BA.2 was detected in 46 samples (32.9%) and sublineages indicative of BA.4 and BA.5 in 78 (55.7%) samples (fig. 1, 2).

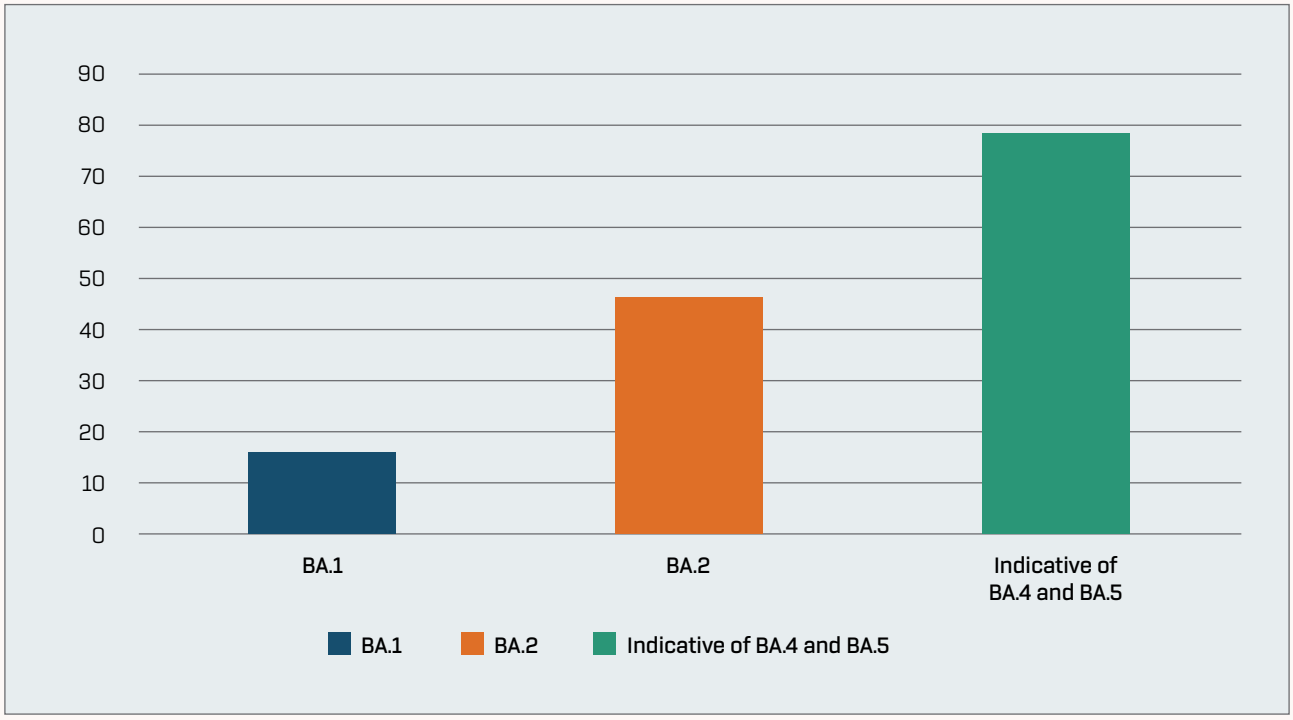

Figure 1. Number of Omicron sublineages between January-September 2022.

Appearance of Omicron sublineages according to collection date

All samples were collected between 10 January and 23 September 2022. The BA.1 sublineage circulated primarily from 10 January to 5 February 2022. The BA.2 sublineage subsequently emerged and increased in prevalence between 27 June and 30 June 2022, followed by a marked decline during the period from 3 August to 11 August 2022. From 23 August to 23 September 2022, a pronounced increase in cases indicative of the BA.4 and BA.5 sublineages was observed, while BA.2 circulation further decreased and BA.1 was no longer detected in the samples analyzed.

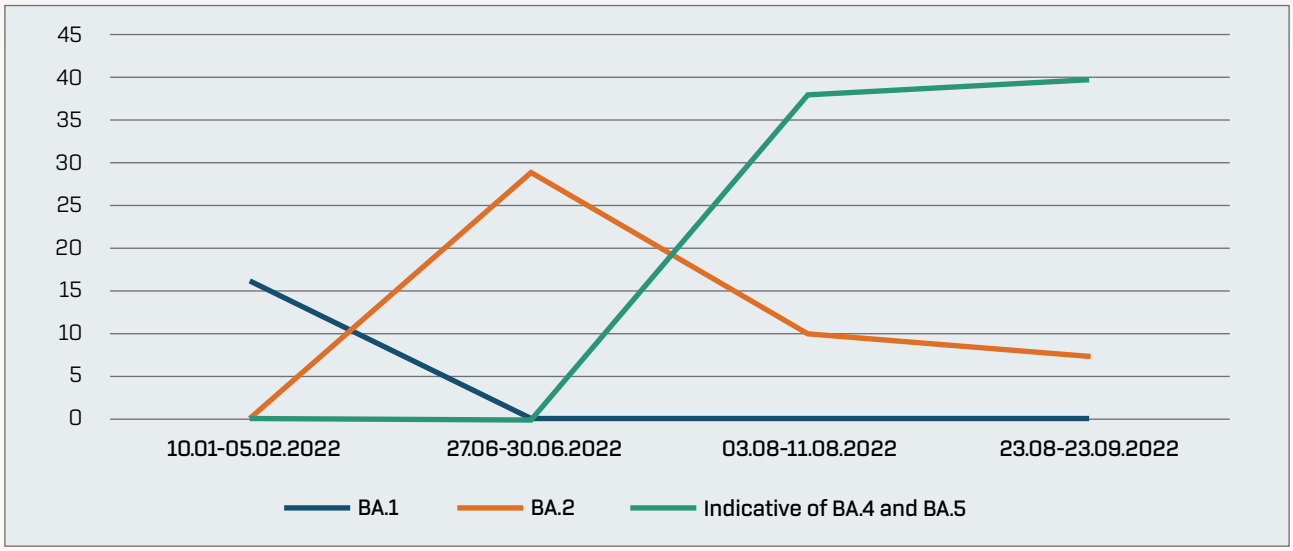

Figure 2. Emergence of Omicron sublineages, their increase and decrease according to their collection date.

City distribution

Figure 3 shows the distribution and number of patients in which the Omicron sublineages were detected.

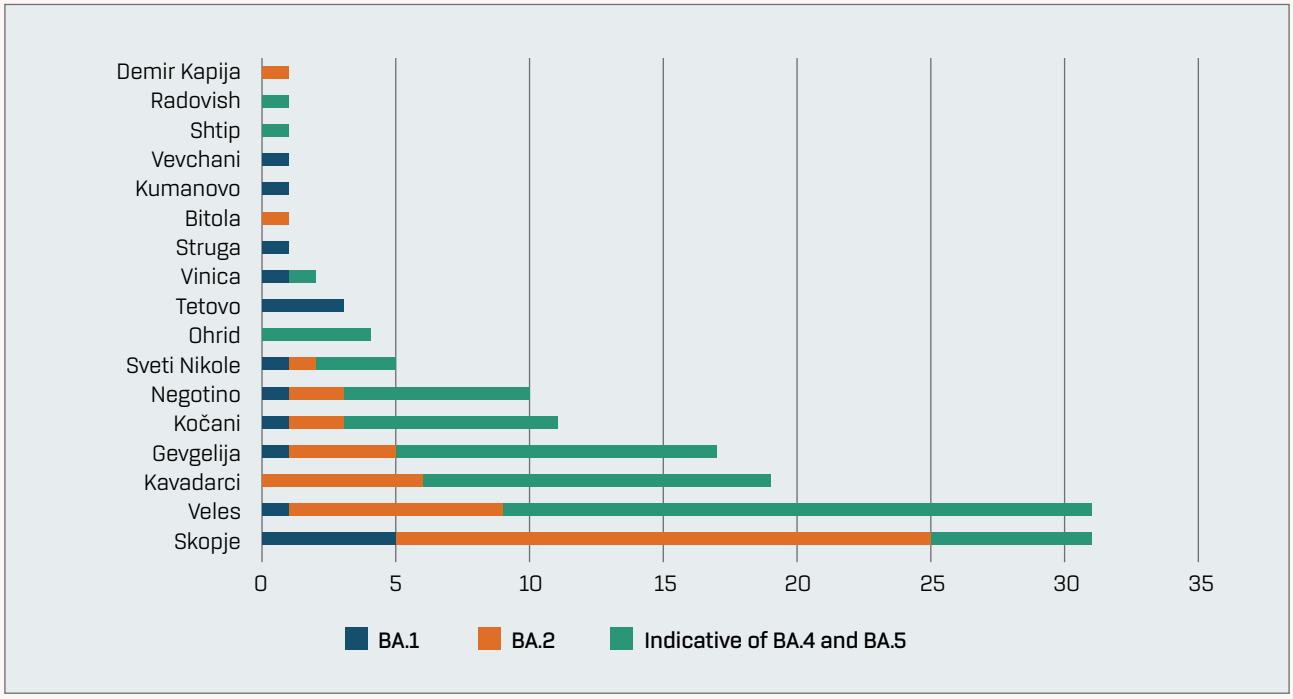

Figure 3. Distribution of Omicron sublineages across cities in Macedonia.

Distribution by sex and age

From all analyzed patients, 66 were male and 74 were female (fig. 4).

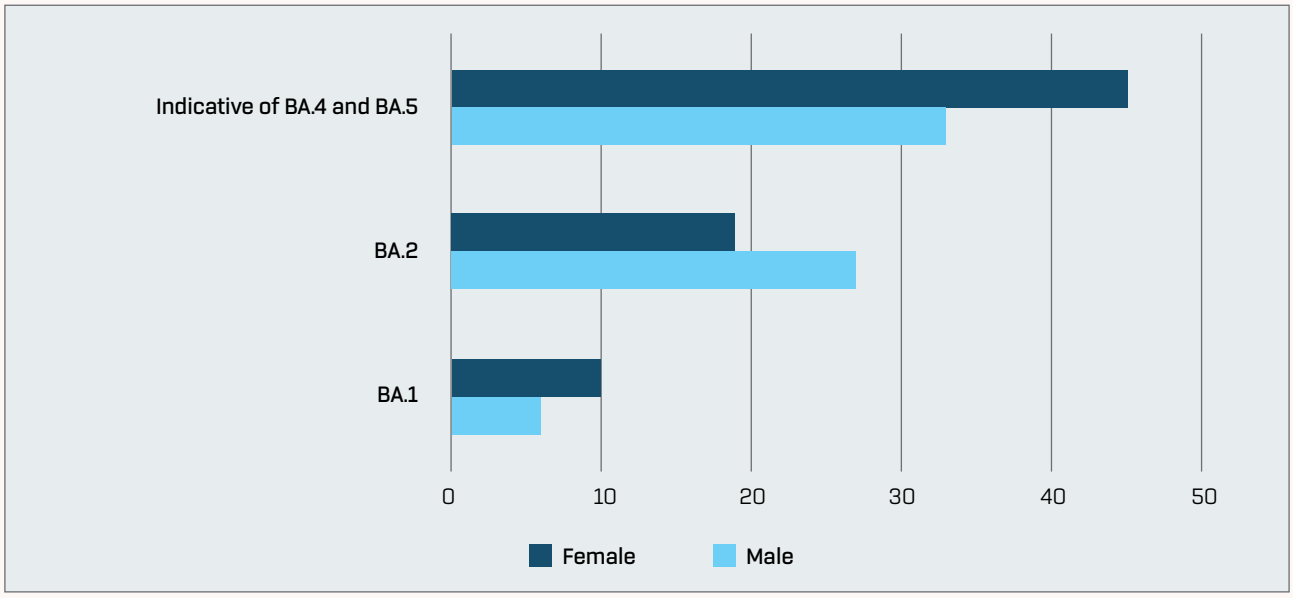

Figure 4. Distribution of Omicron cases and their sublineages, in men and women separately.

The majority of positive patients belong to the 30-64 age group (fig. 5). The exact ages of the patients range from 1 to 88 and the median age is 61,5.

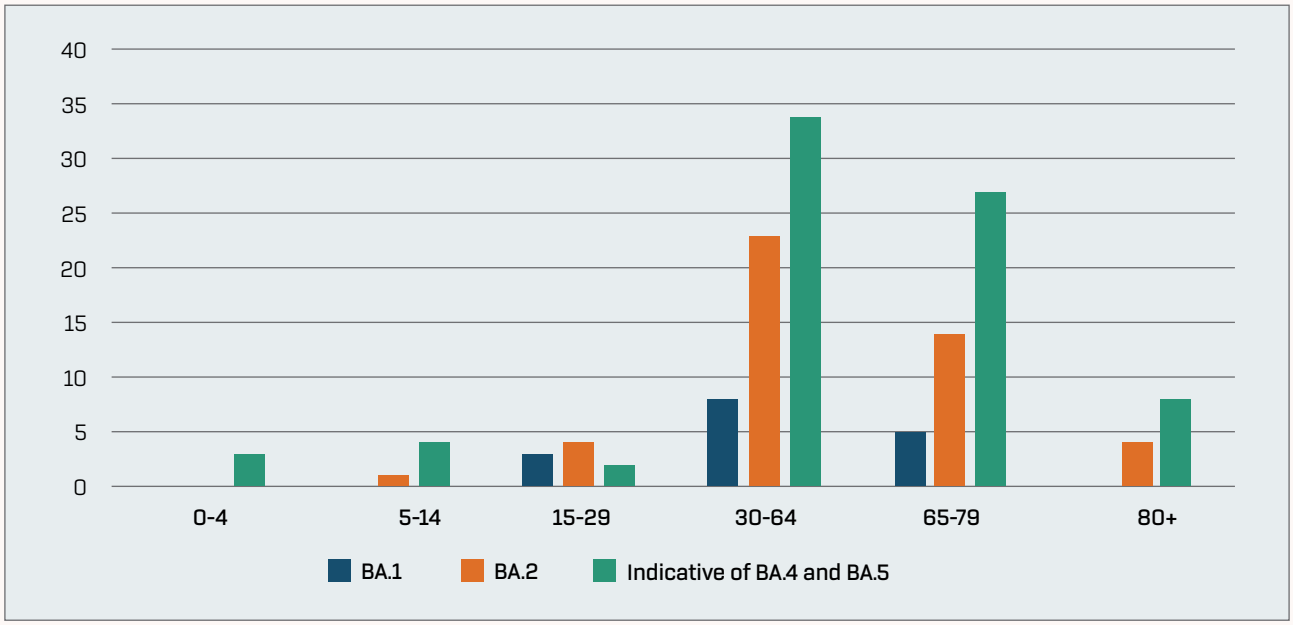

Figure 5. Distribution of Omicron patients and their sublineages across all age groups.

DISCUSSION

Real time surveillance of the prevalence and spread of circulating SARS-CoV-2 variants in the country enables early detection of new VOCs and VOIs with possible emergence of new SARS-CoV-2 variants and sublineages as a result of the appearance of new mutations in the genome.

The performance of laboratory diagnostic assays, particularly PCR-based methods, may be affected by the emergence of new variants. Therefore, rapid detection of genetic changes is critical to allow timely adaptation of diagnostic kits and to ensure continued accuracy of testing. For routine screening and differentiation of the BA.1 and BA.2 sublineages, melting curve analysis demonstrated satisfactory performance, particularly due to its short turnaround time of approximately 53 minutes. These assays complement sequencing-based surveillance by enabling monitoring of variants circulating at frequencies below 5%, thereby increasing the overall number of samples under surveillance (27). Furthermore, melting curve analysis facilitates the rapid design of new primer sets targeting mutations specific to newly emerging variants. In this study, only samples with Ct values ≤ 25 were included, as lower Ct values indicate higher viral RNA concentrations and are associated with improved assay reliability and result quality (26).

During the period from January to August 2022, a clear replacement of the BA.1 sublineage by BA.2 was observed, followed by the emergence and increasing circulation of BA.4 and BA.5 sublineages (28). Consequently, BA.1 accounted for the smallest proportion of detected cases, while the majority were indicative of BA.4 and BA.5. Although these sublineages were initially classified as VOCs, the European Centre for Disease Prevention and Control (ECDC) de-escalated BA.2, BA.4, and BA.5 from VOC status on 3 March 2023, as they were no longer circulating at that time (29).

The majority of analyzed positive cases were from Skopje, as a capital city with largest number of SARS-CoV-2 cases, and from Veles. However, in order to get a representative picture of the Omicron positive cases in the country, it is recommended to select a specific number of samples for SNP assay based on the number of detected SARS-CoV-2 cases and the proportional distribution of circulating variants across regions.

No association was observed between SARS-CoV-2 infection and patient age or sex. The highest number of cases occurred in individuals aged 30–64 years, while the lowest number was observed among children aged 0–4 years. A limitation of this study is that only a small proportion of the total SARS-CoV-2–positive cases during the study period was included. However, existing literature indicates that SARS-CoV-2 infection and Omicron sublineages are distributed across all age groups and are not significantly influenced by patient age or sex (7).

Eventually, the existing tests may stop detecting the positive cases due to acquired mutations or may not be able to differentiate the sublineages. Therefore, it is recommended that diagnostic kits target multiple genomic regions to ensure sustained performance. In periods of high variant prevalence, melting curve analysis provides an efficient method for continuous variant monitoring, significantly reducing reliance on sequencing, which requires longer processing times, specialized bioinformatics expertise, higher reagent costs, and extensive instrument calibration and maintenance.

According to the *National Program for Sequencing of SARS-CoV-2 and Other Highly Pathogenic Agents with Public Health Importance*, a minimum of 10% of randomly selected positive pre-screening samples are planned to undergo sequencing to estimate the prevalence of circulating variants. Conse-

quently, mutation detection assays such as melting curve analysis are widely employed in resource-limited settings to monitor how genomic mutations influence viral transmission and clinical presentation. This information is essential for vaccine development and supports the modeling of public health prevention and control strategies aimed at limiting the spread of highly pathogenic variants.

LIMITATIONS

A limitation of this study is the potential for false-positive results, which may arise from non-specific binding of probes or primers, as well as from sample contamination. Conversely, false-negative results may occur due to improper sample storage conditions, including prolonged exposure to light, inappropriate temperatures, or repeated freeze–thaw cycles.

An additional limitation relates to the selected time intervals, which were determined by the availability of the diagnostic kits used in this study. These constraints resulted from global delays and increased demand for testing during the COVID-19 pandemic, particularly following the emergence of the Omicron variant. During these periods, the laboratory team employed alternative testing assays and surveillance strategies that are not included in the present analysis.

CONCLUSIONS

1. Molecular techniques, due to their high specificity, sensitivity, and flexibility, have a significant and broad applicability in the detection and monitoring of viral infections.
2. Melting Curve analysis, based on the detection of a specific SNP, enables rapid and accurate identification of mutations, allowing precise discrimination of Omicron sublineages.
3. This approach is particularly valuable for the selection of samples for sequencing, especially when changes in the clinical or epidemiological patterns of viral infections are observed.
4. The method facilitates early detection of emerging viral variants and supports the timely implementation of appropriate public health measures.
5. The results obtained can be further subjected to additional analyses of public health relevance, contributing to the optimization of diagnostic strategies and strengthening epidemiological surveillance systems.

CONFLICT OF INTEREST

None declared.

ETHICAL APPROVAL

Ethical considerations are addressed within the *National Program for Sequencing of SARS-CoV-2 and Other Highly Pathogenic Agents with Public Health Importance*. The program stipulates that all positive samples are anonymized through coding, ensuring that no direct link exists between patient identity and SNP or sequencing results. Metadata are linked to SNP and sequencing data solely for epidemiological analysis and the implementation of public health measures, without any possibility of identifying individual patients.

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