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# Genomic and Molecular Characterisation of Respiratory Syncytial Virus in the National SARI Surveillance System Across Three Consecutive Seasons in North Macedonia (2022-2025)

Teodora Karevska[1], Golubinka Boshevska[1, 2], Elizabeta Jancheska[1], Maja Vukovikj[1], Senada Karishik[1], Gala Matevska[1], Gordana Nikolovska[1], Aneta Peshnachka[1], Dragan Kochinski[1], Kristina Stavridis[1], Enkela Polozhani[1], Milica Simova[1], Marija Andonovska[1], Gorica Popova[2, 3], Icko Gjorgoski[4]

[1]Institute for Public Health of the Republic of North Macedonia, Skopje, [2]Faculty of Medical Sciences, Goce Delchev University, Shtip, [3]PHI University Clinic for Respiratory Diseases in Children "Kozle", Skopje, [4]Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss.Cyril and Methodius University, Skopje

**Correspondence:** *teodorabuzarova@yahoo.com*

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**Abstract**

**Objective.** To describe the epidemiological, molecular, and genomic characteristics of respiratory syncytial virus (RSV) detected through the national severe acute respiratory infection (SARI) surveillance system in North Macedonia across three consecutive seasons (2022/2023-2024/2025). **Materials and Methods.** This study analysed SARI surveillance data from seven hospital-based sentinel sites. RSV detection and subgroup determination were performed using reverse transcription quantitative polymerase chain reaction (RT-qPCR), following season-specific testing strategies, which should be considered when interpreting seasonal differences in RSV positivity. Consensus genomes were generated, and phylogenetic analyses were performed using contemporaneous European reference sequences. Amino acid substitutions in the G and F proteins were assessed descriptively. **Results.** Among 1,899 laboratory-tested SARI cases, 53.3% were tested for RSV, of which 24.8% were positive. RSV-A predominated overall, although seasonal shifts in subgroup dominance were observed. RSV infections were concentrated among young children, particularly those aged 0-2 years. Whole-genome sequencing of 85 RSV-positive samples revealed a dynamic seasonal turnover of RSV-A lineages, whereas RSV-B circulation remained largely confined to a single dominant lineage across seasons. Analysis of antigenically important genes showed greater variability in the G gene compared with the more conserved F gene in both subgroups. **Conclusion.** The integration of epidemiological, molecular, and whole-genome data within routine SARI surveillance provides a comprehensive overview of RSV circulation and evolution in North Macedonia. These findings contribute genomic data from an under-represented region and provide a foundation for future evaluation of RSV vaccination and monoclonal antibody effectiveness by detecting viral genetic changes, should such measures be implemented in North Macedonia.

**Key Words:** Respiratory Syncytial Virus ▪ SARI ▪ Genomic Surveillance ▪ Whole-Genome Sequencing ▪ Molecular Epidemiology.

## Introduction

Respiratory syncytial virus (RSV) represents a major public health threat worldwide due to its ability to cause seasonal epidemics and severe respiratory disease, particularly among infants, young children, older adults, and immunocompromised individuals. RSV is responsible for more than 3.6 million hospitalisations and approximately 100,000 deaths annually among children under five years of age worldwide (1).

RSV is an enveloped, negative-sense, single-stranded RNA virus belonging to the *Pneumoviridae* family and *Orthopneumovirus* genus. The viral genome encodes multiple structural and non-structural proteins; however, the fusion (F) and attachment (G) glycoproteins are particularly important due to their central roles in viral entry and immune recognition (2). Both proteins are the main targets of neutralising antibodies and are therefore critical for the development and evaluation of RSV

preventive strategies, with the F glycoprotein representing a key target of current immunoprophylactic approaches. The F protein mediates fusion between the viral and host cell membranes and is less variable at the sequence level than the G protein. In contrast, the G protein is characterised by extensive genetic diversity and plays a key role in antigenic variation and immune evasion.

Whole-genome sequencing provides critical insights into RSV transmission dynamics, genetic diversity, and the seasonal replacement of circulating strains. In particular, G gene analysis remains central to RSV molecular surveillance, as genotype and lineage assignments are primarily based on sequence variability within this region. Based on antigenic and genetic differences in the attachment (G) glycoprotein, RSV is classified into two major subgroups: RSV-A and RSV-B. Both subgroups co-circulate globally and are further divided into multiple genotypes and lineages; however, one subgroup often predominates during a given season, with shifts in the dominant subgroup frequently observed between consecutive seasons (3). Previous large-scale analyses of RSV genetic diversity have shown that among the numerous genotypes described, RSV-A NA1 (including ON1 viruses) and RSV-B BA genotypes have been the most frequently detected globally in recent decades, coinciding with a reduction in overall genotype diversity since the early 2000s (4).

Lessons learned from previous influenza pandemics and, more recently, from the coronavirus disease 2019 (COVID-19) pandemic, underscore the critical importance of integrated respiratory virus surveillance systems for timely detection and effective public health responses. In North Macedonia, these experiences served as a major impetus for establishing the national SARI surveillance system, which began operating in an initial form during the 2014/2015 season and has progressively expanded and strengthened over time. As a result of this gradual development, the national SARI surveillance system has evolved into a comprehensive framework for monitoring severe respiratory infections, supporting enhanced characterisation of circulating respiratory viruses, and providing a foundation for molecular and genomic surveillance activities.

Publicly available sequence repositories provide an important overview of the geographic distribution of RSV genomic data and reveal substantial heterogeneity in data availability across Europe. In this context, Southeast Europe and the Western Balkans remain less well represented compared with other European regions, and RSV genomic data generated through the national SARI surveillance system in North Macedonia contribute valuable information to improve regional representation and understanding of RSV circulation.

In parallel with advances in RSV surveillance, important progress has also been made in RSV prevention. In the European context, currently authorised immunisation products include the long-acting monoclonal antibody Beyfortus (*nirsevimab*) for infant protection and the monoclonal antibody Synagis (*palivizumab*) for selected children at high risk of severe RSV disease. Vaccination strategies include Abrysvo, authorised for maternal immunisation and older adults, as well as Arexvy and mRESVIA, which are also authorised for older adults. In addition, Enflonsia (*clesrovimab*), another long-acting monoclonal antibody for infant protection, had previously received a positive opinion from the European Medicines Agency Committee for Medicinal Products for Human Use and was subsequently authorised by the European Commission in April 2026 (5). Across Europe, the implementation of these products varies according to national epidemiological and economic considerations; however, access remains limited in many settings, highlighting the ongoing need for RSV surveillance (6).

This study aimed to describe the epidemiological, molecular, and genomic characteristics of respiratory syncytial virus detected through the national SARI surveillance system in North Macedonia across three consecutive seasons (2022/2023, 2023/2024, and 2024/2025).

## Materials and Methods

### *Study Design, SARI Surveillance System, and Case Definition*

This study analysed data generated through the national SARI surveillance system in North Macedonia across three consecutive RSV seasons (2022/2023, 2023/2024, and 2024/2025). Within the SARI surveillance framework, seven hospital-based sentinel sites nationwide submit nasopharyngeal swabs collected in universal transport medium to the Virology Laboratory at the Institute of Public Health of North Macedonia, which serves as the national reference laboratory and performs the laboratory component of SARI surveillance.

The SARI surveillance system in North Macedonia was established in its initial form during the 2014/2015 season and was initially implemented primarily for the monitoring of influenza. During the COVID-19 pandemic, the system was expanded to include severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and since 2022, RSV testing has been incorporated into the SARI surveillance framework. Over time, this system has evolved beyond basic molecular detection. Currently, it integrates influenza virus typing and subtyping, RSV subgroup determination, and whole-genome sequencing of a subset of positive samples across respiratory pathogens covered by the surveillance system, thereby supporting the genomic surveillance of these pathogens.

Laboratory testing results generated through SARI surveillance are routinely communicated to the Department for Epidemiology of Infectious Diseases of the Institute of Public Health for surveillance data analysis. SARI cases were defined in accordance with national surveillance protocols and international guidelines, aligned with the World Health Organisation (WHO) surveillance case definition of SARI (7).

### *RSV Testing Algorithm*

All specimens were initially tested using a multiplex RT-qPCR assay for SARS-CoV-2, Influenza A, and Influenza B. RSV RT-qPCR testing was performed and applied according to season-specific testing strategies, as summarised in Figure 1. During the 2022/2023 season, RSV testing was performed based on clinical indication. In the 2023/2024 season, RSV testing was performed on all SARI samples that tested negative for both SARS-CoV-2 and influenza viruses. In the 2024/2025 season, RSV testing was prioritised for patients with SARI who belonged to high-risk age groups, specifically children under four years of age and adults aged 60 years and older.

RSV subgroup determination was performed using RT-qPCR either during initial RSV detection using assays capable of subgroup differentiation or by subsequent testing with an available RT-qPCR assay allowing RSV subgroup identification. Whole-genome sequencing was performed on a selected subset of RSV-positive samples. Priority was given to samples with cycle threshold (Ct) values ≤30, although not all RSV-positive samples meeting this criterion were sequenced. Among the eligible samples, the selection further aimed to include specimens collected at different time points within each RSV season. Because different RT-qPCR assays were used during the study period, the Ct threshold for sequencing selection was applied across heterogeneous assays, and cross-kit Ct equivalence was not formally validated.

To provide context on the representativeness of the sequencing subset, sequenced and non-sequenced RSV-positive samples were compared based on Ct value distribution, age group, collection period, and RSV subgroup (Supplementary Table S1).

### *Laboratory Methods*

Viral RNA extraction was performed using either the QIAamp Viral RNA Mini Kit (Qiagen; REF:52906) or the MagCore Viral Nucleic Acid Extraction Kit High Sensitivity (RBC Bioscience; REF: MVN400-05) (8, 9).

RSV detection was performed by RT-qPCR using commercially available assays, with different kits used during the study period depending on availability, including the RevoDx RSV qPCR

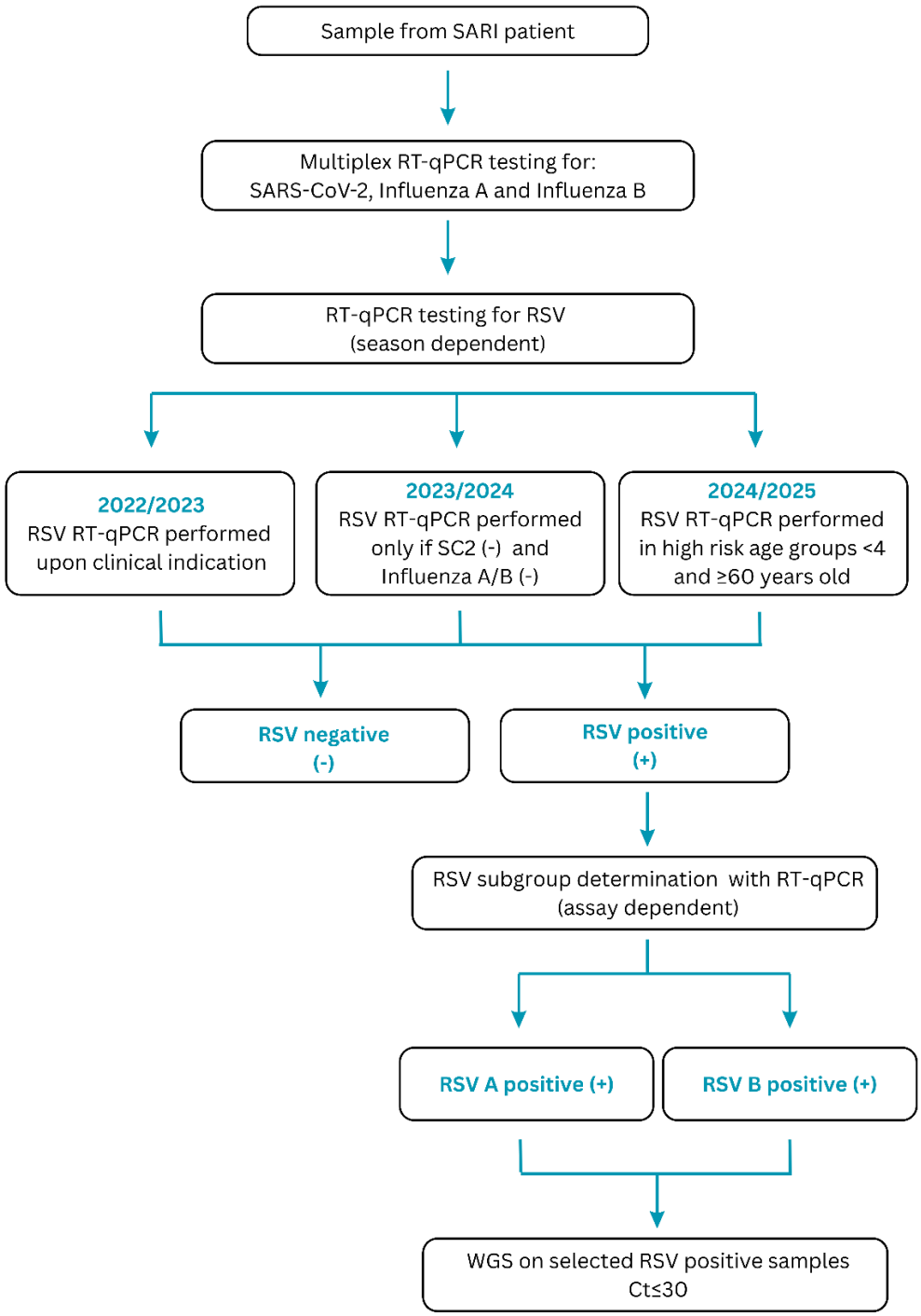



Figure 1. Overview of the RSV testing algorithm applied within the national SARI surveillance system in North Macedonia across three consecutive seasons (2022/2023 – 2024/2025). All respiratory specimens were initially tested for SARS-CoV-2 and influenza A/B using multiplex RT-qPCR. RSV testing criteria differed by season, and RSV-positive samples were subsequently subjected to subgroup determination and whole-genome sequencing.

Kit (Idil Biotech; REF: IP202231-100) (10), FTD Respiratory Pathogens 33 (Fast Track Diagnostics, Luxembourg; REF: FTD-2P.3-64-RUO) (11), RealStar RSV RT-PCR Kit 3.0 (Altona; REF: 193013) (12), and the Respiratory Syncytial Virus Multiplex Real-time RT-PCR Kit (Centers for Disease Control and Prevention - CDC; REF: GR-1365); the Altona and CDC assays additionally enabled RSV subgroup determination. Real-time polymerase chain reaction (PCR) was performed using QuantStudio5 (Applied Biosystems) and CFX96 (Bio-Rad) PCR thermocyclers.

Whole-genome sequencing was performed using a hybridisation-capture-based enrichment approach. Sequencing libraries were prepared using the Illumina DNA Prep with Enrichment kit in combination with the Respiratory Virus Oligos Panel v2 (Illumina) and sequenced on an Illumina MiniSeq platform (13). Library quantification was performed using a Qubit4 Fluorimeter with the Qubit 1X dsDNA High Sensitivity Assay Kit (Invitrogen; Q33231). All assays and kits were used according to the manufacturers' instructions.

### *Bioinformatic Analysis*

Raw sequencing data (FASTQ files) generated from RSV-positive samples were processed for quality control, reference-based genome assembly, and the generation of consensus sequences. During the initial phase of the study, raw read processing and consensus generation were performed using UGENE (v45.0), specifically the *Raw DNA-seq data processing* and *Extract consensus from assemblies* tools (14). Subsequently, a more automated and standardised bioinformatic workflow was implemented, and raw sequencing data were processed using the INSaFLU platform for quality control, reference-based genome assembly, and consensus sequence generation (15). The bioinformatic approach used for each sequence included in the study is indicated in Supplementary Table S2. Reference-based genome assembly was performed using hRSV/A/England/397/2017 (EPI_ISL_412866) and hRSV/B/Australia/VIC-RCH056/2019 (EPI_ISL_1653999) as the reference sequences for RSV-A and RSV-B, respectively.

For phylogenetic analyses, contemporaneous European reference sequences were retrieved from the Global Initiative on Sharing All Influenza Data (GISAID) EpiRSV database to provide epidemiological context. Sequences collected between 29 November 2021 and 7 April 2025 were selected from European countries with publicly available data, with selection guided by the aim of including the same or closely related lineages as those identified among the Macedonian sequences, based on Nextclade lineage assignment. Only sequences with an overall good quality control (QC) status in Nextclade were included. In total, 21 reference sequences were included, comprising 12 RSV-A and 9 RSV-B sequences. The reference sequences were downloaded from GISAID in November 2025, and their accession IDs and corresponding information are provided in Supplementary Table S3.

Multiple sequence alignment of RSV consensus genomes was performed using MAFFT v7.526 (16). Prior to downstream analyses, consensus genome quality was assessed using Nextclade v3.18.1 (17), which evaluates sequence quality based on flagged issues, including missing data (Ns), excess private mutations, mixed sites, frameshifts, and stop codons. No separate study-specific numeric QC threshold was applied; genomes were retained for downstream analyses if assigned an overall QC status of good by Nextclade. Nextclade was also used to assign RSV genetic clades and lineages. Phylogenetic trees were inferred using FastTree v2.1.10 under a generalised time-reversible (GTR) nucleotide substitution model with CAT rate heterogeneity approximation (18). Branch support was assessed using SH-like local support values, as implemented in FastTree. Nodes with SH-like local support values ≥0.80 were considered well supported. Phylogenetic trees were visualised and annotated using the Interactive Tree of Life (iTOL) platform (19).

### *Ethical Statement*

Informed consent was not required, as the study was conducted within the framework of the national SARI surveillance system, and all data and specimens were analysed anonymously in accordance with national surveillance protocols.

### *Statistical Analysis*

Descriptive statistical analyses were performed to summarise the epidemiological and laboratory data. Categorical variables are presented as counts and percentages. No inferential statistical tests were applied, and no predefined level of statistical significance (P-value) was specified, as the study was descriptive. Statistical analyses were performed using Microsoft Excel 2019 (Microsoft Corp.).

### *Data Availability*

All 85 RSV consensus genome sequences analysed in this study are publicly available in the GISAID EpiRSV database. Their GISAID accession IDs and corresponding sample information are provided in Supplementary Table S2.

## Results

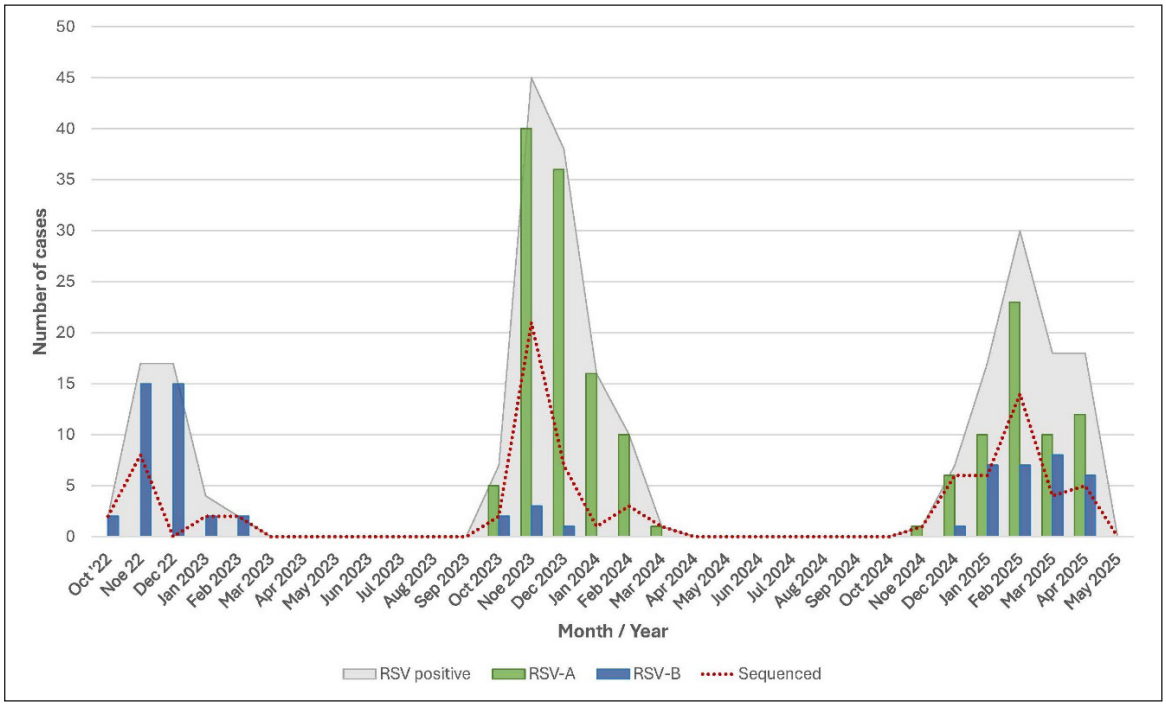
### *Epidemiological Characteristics of RSV Within the SARI Surveillance System*

During three consecutive RSV seasons (2022/2023, 2023/2024, and 2024/2025), a total of 1,899 SARI cases were laboratory-tested within the national SARI surveillance system in North Macedonia, of which 53.3% (N=1012) were tested for RSV. Overall, RSV was detected in 24.8% (N=251) of the tested SARI samples. In the 2023/2024 and 2024/2025 seasons, SARI surveillance was conducted year-round; to ensure uniformity across seasons, all seasonal comparisons in the analysis refer to weeks 40-20.

The 2022/2023 season was the first season in which RSV testing was incorporated into the national SARI surveillance system. RSV-positive cases were identified in all three analysed surveillance seasons, with variations observed in both testing coverage and RSV positivity rates. As RSV testing strategies differed by season, the epidemiological patterns described below, particularly testing coverage and RSV positivity rates, should be interpreted in that context. During the 2022/2023 season, RSV testing was performed for 16.2% (N=94) of all reported SARI cases, of which 44.7% (N=42) tested positive for RSV. In contrast, during the 2023/2024 season, which was characterised by the highest testing coverage, 75.8% (N=589) of SARI cases were tested for RSV, with 20.0% (N=118) of the tested samples yielding positive results. In the 2024/2025 season, 60.9% (*N*=329) of SARI cases were tested for RSV, of which 27.7% (N=91) were RSV-positive. Across all three seasons, the majority of RSV-positive SARI cases were classified as RSV-A, accounting for 67.3% (N=169), whereas RSV-B accounted for 27.9% (*N*=70). RSV subgroup was not determined in 4.4% (N=11) of cases, and one case was identified as a co-infection with both RSV-A and RSV-B.

When analysed by season, distinct patterns of RSV subgroup dominance were observed (Figure 2). During the 2022/2023 season, RSV-B was the predominant subgroup, detected in 85.7% (N=36) of cases. The remaining six RSV-positive cases in 2022/2023 had an undetermined subgroup status, and no RSV-A cases were identified in that season. In contrast, during the 2023/2024 and 2024/2025 seasons, co-circulation of both RSV subgroups was observed, with a clear predominance of RSV-A. In the 2023/2024 season, RSV-A accounted for 90.7% (N=107) of the RSV-positive cases. During the 2024/2025 season, a more balanced distribution between the two RSV subgroups was observed; although RSV-A remained predominant, accounting for 68.1% (N=62) of cases compared to 31.9% (N=29) for RSV-B.

As shown in Figure 2, RSV activity followed a typical seasonal pattern, with the highest activity observed during the winter months. In the 2022/2023 and 2023/2024 seasons, peak RSV activity occurred in November and December, whereas during the 2024/2025 season, a delayed peak was observed in February.




Monthly distribution of laboratory-confirmed RSV-positive SARI cases detected through the national SARI surveillance system in North Macedonia from October 2022 to May 2025. Total RSV-positive cases are shown in grey, with RSV-A and RSV-B cases indicated in green and blue, respectively; in 2022/2023, RSV-positive cases not shown as RSV-B had an undetermined subgroup status. The red dotted line represents the number of RSV-positive samples selected for whole genome sequencing. Interseasonal periods are shown for completeness; however, seasonal comparisons were restricted to weeks 40-20, as described in the Methods. RSV testing differed by season (see Figure 1); therefore, seasonal positivity patterns are not directly comparable.

Figure 2. Seasonal distribution of RSV-positive SARI cases and sequenced samples across three consecutive seasons.

Among the RSV-positive SARI cases, 51.0% (N=128) occurred in males and 49.0% (N=123) in females. RSV-positive cases were predominantly observed among young children, with the highest burden in the youngest age groups. Children aged 0-2 years accounted for 90.0% (N=226) of all RSV-positive cases, followed by those aged 3-4 (4.8%; N=12) and 5-14 years (3.6%; N=9). RSV infections were rare among adults, with one case (0.4%) identified in the 30-64 years age group and three cases (1.2%) among individuals aged 65-79 years, while no RSV-positive cases were detected in the 15-29 or ≥80 years age groups.

Since RSV detections originated from SARI surveillance, cough and fever (≥38°C) were consistently reported among all RSV-positive cases. Beyond these core SARI manifestations, rhinitis/coryza and dyspnoea were commonly observed. Gastrointestinal symptoms (diarrhoea and vomiting/nausea) were less frequent, whereas systemic symptoms such as headache, myalgia, and arthralgia occurred in a smaller proportion of cases. The detailed demographic and epidemiological characteristics of the RSV-positive SARI cases are summarised in Table 1.

### *Whole-Genome Sequencing*

Whole-genome sequencing was successfully performed on 85 RSV-positive samples, representing a subset of cases selected across all three surveillance seasons. Sample selection aimed to ensure temporal coverage by including specimens collected during different months within each season.

Among the sequenced samples, 57 were classified as RSV-A and 28 as RSV-B. The cycle threshold (Ct) values of the sequenced samples ranged from 16 to 30.7. All 85 consensus genomes obtained in this study had an overall good QC status in Nextclade and were therefore included in the

Table 1. Characteristics of RSV-Positive Cases Detected Through the National SARI Surveillance System in North Macedonia, 2022/2023 – 2024/2025

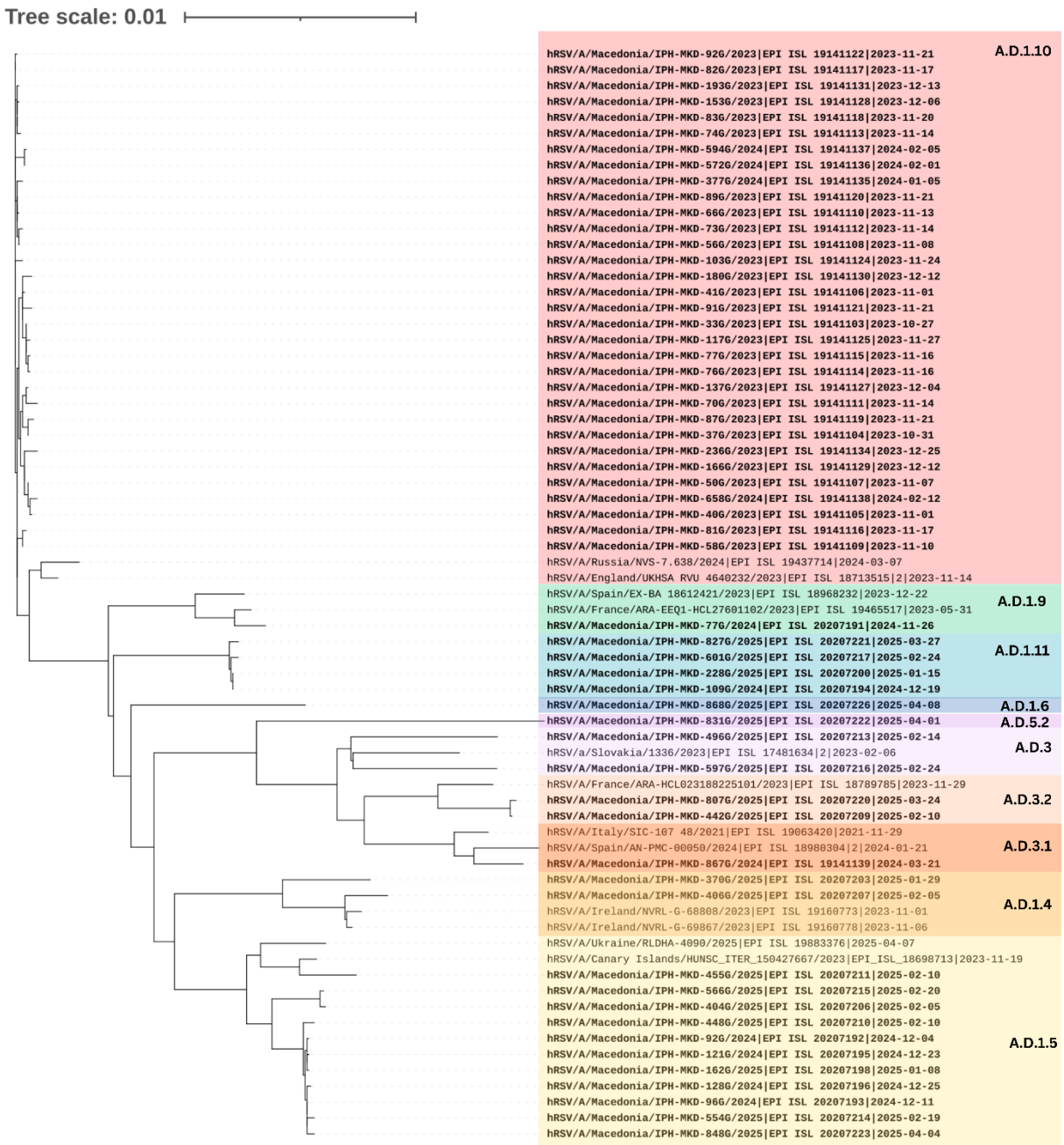
| Season | 2022/2023 | 2023/2024 | 2024/2025 | Total |
|---|---|---|---|---|
| Total tested SARI cases (N) | 582 | 777 | 540 | 1899 |
| SARI cases tested for RSV (N; %) | 94 (16.2) | 589 (75.8) | 329 (60.9) | 1012 (53.3) |
| RSV-positive cases (N; %) | 42 (44.7) | 118 (20.0) | 91 (27.7) | 251 (24.8) |
| RSV-positive cases sequenced (N; %) | 14 (33.3) | 35 (29.7) | 36 (39.6) | 85 (33.9) |
| RSV Subgroup (N; %) | | | | |
| RSV A | 0 (0.0) | 107 (90.7) | 62 (68.1) | 169 (67.3) |
| RSV B | 36 (85.7) | 5 (4.2) | 29 (31.9) | 70 (27.9) |
| RSV A/B coinfection | 0 (0.0%) | 1 (0.8%) | 0 (0.0) | 1 (0.4) |
| Undetermined subgroup | 6 (14.3) | 5 (4.2) | 0 (0.0) | 11 (4.4%) |
| Sex (N; %) | | | | |
| Male | 27 (64.3) | 63 (53.4) | 38 (41.8) | 128 (51.0) |
| Female | 15 (35.7) | 55 (46.6) | 53 (58.2) | 123 (49.0) |
| Age group (N; %) | | | | |
| 0-2 | 34 (81.0) | 104 (88.1) | 88 (96.7) | 226 (90.0) |
| 3-4 | 5 (11.9) | 7 (5.9) | 0 (0.0) | 12 (4.8) |
| 5-14 | 3 (7.1) | 6 (5.1) | 0 (0.0) | 9 (3.6) |
| 15-29 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| 30-64 | 0 (0.0) | 1 (0.8) | 0 (0.0) | 1 (0.4) |
| 65-79 | 0 (0.0) | 0 (0.0) | 3 (3.3) | 3 (1.2) |
| 80 + | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Symptoms (N; %) | | | | |
| Cough + fever ≥38°C | 42 (100.0) | 118 (100.0) | 91 (100.0) | 251 (100.0) |
| Rhinitis/Coryza | 36 (85.7) | 98 (83.1) | 70 (76.9) | 204 (81.3) |
| Dyspnoea | 37 (88.1%) | 105 (89.0) | 76 (83.5) | 218 (86.9) |
| Diarrhoea | 2 (4.8) | 15 (12.7) | 8 (8.8) | 25 (10.0) |
| Vomiting/Nausea | 11 (26.2) | 25 (21.2) | 13 (14.3) | 49 (19.5) |
| Headache | 4 (9.5) | 7 (5.9) | 5 (5.5) | 16 (6.4) |
| Myalgia | 7 (16.7) | 9 (7.6) | 4 (4.4) | 20 (8.0) |
| Arthralgia | 4 (9.5) | 5 (4.2) | 5 (5.5) | 14 (5.6) |

downstream analyses. Accordingly, the same set of 85 genomes was used for both phylogenetic and substitution analyses.

### *Phylogenetic Analysis*

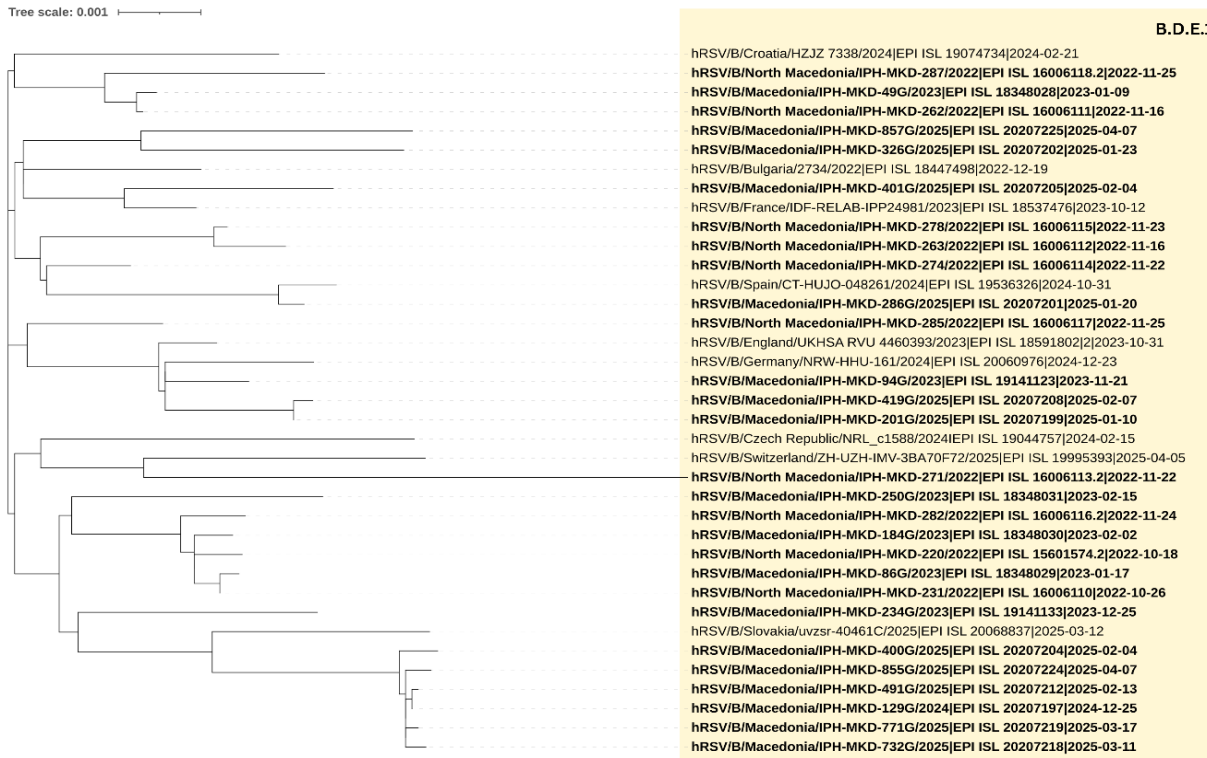
Phylogenetic analysis was performed on 85 RSV whole-genome sequences to describe the genetic relationships of Macedonian RSV strains across the three surveillance seasons, in the context of contemporaneous European reference sequences. As shown in Figure 3, Macedonian RSV-A isolates clustered within the ON1 genotype and demonstrated seasonal differences in clade distribution. No RSV-A cases were identified in the 2022/2023 season; therefore, the RSV-A phylogenetic analysis included only sequences from the 2023/2024 and 2024/2025 seasons. In 2023/2024, almost all RSV-A sequences were assigned to clade A.D.1.10 (32/33; 97.0%), and a single sequence was assigned to A.D.3.1. (1/33; 3.0%). In 2024/2025, a broader clade diversity was observed, with the

predominance of A.D.1.5 (11/24; 45.8%), followed by A.D.1.11 (4/24; 16.7%). Additional clades were detected at lower frequencies, including A.D.1.4 (2/24; 8.3%) and A.D.3.2. (2/24; 8.3%), while A.D.1.9, A.D.3, A.D.3.11, A.D.5.2, and A.D.1.6 were each represented by a single sequence (1/24; 4.2%). Overall, RSV-A showed higher genetic diversity, as reflected by a wider range of detected clades, whereas RSV-B displayed comparatively lower diversity.




Phylogenetic tree of RSV-A whole-genome sequences generated in this study and 12 selected contemporaneous European reference strains. Macedonian isolates cluster within multiple RSV-A genetic lineages across the three seasons, demonstrating lineage turnover between seasons. Distinct genetic lineages are indicated by different colours and labelled alongside the tree. Macedonian isolates are shown in bold. SH-like local support values were computed using FastTree but are not shown on the tree for clarity; values ≥0.80 were considered well supported.

Figure 3. Phylogenetic analysis of RSV-A genomes.




Phylogenetic tree of RSV-B whole-genome sequences generated in this study and 9 selected contemporaneous European reference strains. Macedonian isolates cluster predominantly within a single dominant RSV-B genetic lineage (B.D.E.1) across all three seasons, indicating more stable circulation compared with RSV-A. Genetic lineages are indicated by colour coding and labelled next to the tree. Macedonian isolates are shown in bold. SH-like local support values were computed using FastTree but are not shown on the tree for clarity; values ≥0.80 were considered well supported.

Figure 4. Phylogenetic analysis of RSV-B genomes.

### *Amino Acid Substitutions in G and F Proteins*

Amino acid substitutions in the antigenically important G and F proteins were assessed in RSV-A (N=57) and RSV-B (N=28) whole-genome sequences. Substitutions were summarised according to their frequency across genomes (*n*/*N, %*) and are presented in Table 2. Singleton substitutions were excluded from the main summary to maintain a focus on recurrent substitutions. The bolded numbers in Table 2 indicate the total number of amino acid substitutions identified for each gene and RSV subgroup.

### *G protein*

Across RSV-A genomes (N=57), multiple amino acid substitutions were identified in the G protein, occurring at varying frequencies (Table 2). One substitution, H90Y, was present in all RSV-A genomes (57/57; 100%). Several substitutions were detected at high frequency (≥80%), including I134K, K262E, and I265L (each 56/57; 98.25%), P71L and D284G (each 55/57; 96.49%), Y304H (54/57; 94.74%); S243I (53/57; 92.98%), L142S and T320A (each 51/57; 89.47%), and L101F (46/57; 80.70%). Substitutions detected at intermediate frequency (31-79%) included G224E (44/57; 77.19%), V225A (39/57; 68.42%), A116V, P143L, P234L, and Y273H (each 32/57; 56.14%), L247P, H266Q, and Y297H (each 31/57; 54.39%), and G272D (28/57; 49.12%). Additional substitutions detected at lower frequencies included G224V and T319I (each 12/57; 21.05%), L101S (11/57; 19.30%), V131A (8/57; 14.04%), P120T, N135S, L314P (each 7/57; 12.28%), T113I, V131D, N178G,

Table 2. Frequency Distribution of Amino Acid Substitutions in G and F Genes of RSV-A and RSV-B Genomes

| Subgroup / gene | High frequency (80-100%) | Intermediate frequency (31-79%) | Low frequency (0-30%) |
|---|---|---|---|
| RSV-A / G (N=57) | 11; H90Y (100%), I134K K262E, and I265L (98.25% each), P71L and D284G (96.49% each), Y304H (94.74%), S243I (92.98%), L142S and T320A (89.47% each), and L101F (80.70%) | 10; G224E (77.19%), V225A (68.42%), A116V, P143L, P234L, and Y273H (56.14% each), L247P, H266Q, and Y297H (54.39% each), and G272D (49.12%) | 91; highest within this bin: G224V and T319I (21.05% each), L101S (19.30%), V131A (14.04%), P120T N135S, and L314P (12.28% each), T113I, N178G, P276L, V131D, H258Q, and H266L (8.77% each), I83V and E295V (7.02% each), P88S and E295K (5.26% each), and T4N, A57T, I75T, N103T, A122V, I133V, S144I, P217S, P230L, P230T, T245I, G254R, S270P, Y280H, S294P, P298L, P298S, and L310P (3.51% each) |
| RSV-A / F (N=57) | 0 | 1; S377N (59.65%) | 16; V127I (19.30%), L375I (15.79%), T12I (8.77%), A518V and L119H (7.02% each), S276N (5.26%), L3S (3.51%) |
| RSV-B / G (N=28) | 11; A74V and I252T (100% each), S100G, T131A, and I137T (96.43% each), P214S, P221L, and I268T (92.86% each), K256N and S275P (89.29% each), and Y285H (85.71%) | 0 | 49; highest within this bin: S265P (21.43%), E303K (14.29%), S267P (10.71%), and T78P, S138P, T253I, and L284F (7.14% each) |
| RSV-B / F (N=28) | 3; S190N, S211N and S389P (100% each) | 1; R42K (46.43%) | 13; N105S (21.43%), I292V and C550F (7.14% each) |

Note: Only substitutions detected in at least two genomes are shown; singleton substitutions were excluded.

H258Q, H266L, and P276L (each 5/57; 8.77%), I83V, E295V (each 4/57; 7.02%), P88S and E295K (each 3/57; 5.26%), and T4N, A57T, I75T, N103T, A122V, I133V, S144I, P217S, P230L, P230T, T245I, G254R, S270P, Y280H, S294P, P298L, P298S, and L310P (each 2/57; 3.51%).

In RSV-B genomes (N=28), two G protein substitutions, A74V and I252T, were present in all sequences (28/28; 100%). Additional high-frequency substitutions included S100G, T131A, and I137T (each 27/28; 96.43%), P214S, P221I, and I268T (each 26/28; 92.86%), K256N and S275P (each 25/28; 89.29%), and Y285H (24/28; 85.71%). Substitutions detected at lower frequency included S265P (6/28; 21.43%), E303K (4/28; 14.29%), S267P (3/28; 10.71%), and T78P, S138P, T253I, and L284F (each 2/28; 7.14%).

### *F protein*

In RSV-A genomes (N=57), no F protein substitution was detected in ≥80% of sequences. One substitution, S377N, occurred at an intermediate frequency (34/57; 59.65%). Additional substitutions detected at lower frequencies included V127I (11/57; 19.30%), L375I (9/57; 15.79%), T12I (5/57; 8.77%), A518V, and L119H (each 4/57; 7.02%), S276N (3/57; 5.26%), and L3S (2/57; 3.51%). Notably, S276N (3/57; 5.26%) maps within the F protein antigenic site II region targeted by palivizumab.

Among RSV-B genomes (N=28), three F protein substitutions – S190N, S211N, and S389P – were present in all sequences (28/28; 100%). One additional substitution, R42K, was detected at an intermediate frequency (13/28; 46,43%). Lower-frequency substitutions included N105S (6/28; 21.43%) and I292V and C550F (each 2/28; 7.14%). Notably, S211N maps within the prefusion F antigenic site Ø region targeted by nirsevimab, whereas S190N is located at or immediately adjacent to this region.

## Discussion

In this study, we describe the epidemiological, molecular, and genomic characteristics of RSV detected through the national SARI surveillance system in North Macedonia across three consecutive seasons. The findings highlight both the substantial

contribution of RSV to severe respiratory illness among hospitalised SARI cases and the dynamic seasonal turnover of RSV subgroups and genetic lineages, while illustrating the added value of integrating genomic approaches into an evolving national surveillance framework.

The epidemiological patterns observed across the three RSV seasons confirm that RSV accounts for a substantial proportion of severe acute respiratory illness among laboratory-tested SARI cases in North Macedonia, while also illustrating how evolving surveillance implementation can shape the observed season-to-season patterns. RSV testing within SARI surveillance has evolved through continuous adaptations designed to optimise case detection among hospitalised SARI patients, and these changes likely influenced both the number of patients tested and the observed RSV positivity. In the first season (2022/2023), RSV testing was newly introduced into the surveillance framework, and as in many settings during an early implementation phase, testing was comparatively limited and more selective. Such selectivity – driven by clinical suspicion – can enrich the tested subset for "typical" RSV presentations and may contribute to higher positivity. This interpretation is consistent with the pattern observed in this study, in which the season with the lowest testing coverage showed the highest RSV positivity, emphasising the potential role of clinician-based selection in identifying patients with a higher probability of RSV infection. In 2023/2024, RSV testing covered a larger share of SARI cases, and the observed RSV positivity was lower. Therefore, season-to-season differences in positivity likely reflect differences in testing practices rather than true changes in RSV circulation in the community. Importantly, although SARI surveillance was conducted year-round in 2023/2024 and 2024/2025, restricting seasonal comparisons to weeks 40-20 strengthens comparability and aligns the analysis with the expected RSV seasonality in temperate climates.

Beyond testing coverage, the seasonal subgroup dynamics observed in North Macedonia align with broader regional patterns and reinforce the value of molecular and subgroup-level surveillance. The predominance of RSV-B in 2022/2023, followed by co-circulation with a clear RSV-A predominance in the subsequent two seasons, reflects a well-documented phenomenon of alternating dominance, commonly attributed to multiple mechanisms, including population-level immunity that accumulates after intense circulation of one subgroup, thereby creating space for the other subgroup in the following season. In addition, ongoing viral evolution and the emergence and spread of fitter lineages can influence the subgroup prevalence and lineage turnover. The concordance between the subgroup pattern observed in North Macedonia and contemporaneous European circulation patterns – as suggested by publicly available sequences on GISAID – supports the interpretation that local RSV epidemiology is embedded within broader regional circulation, likely shaped by shared climatic drivers and population mobility. The timing of peak RSV activity differed between seasons, occurring in November-December in 2022/2023 and 2023/2024 but shifting to February in 2024/2025. This seasonal shift highlights that the timing of RSV activity can vary from year to year and that peak timing should be compared cautiously across seasons.

The age distribution of RSV-positive SARI cases further supports established epidemiological knowledge: severe RSV disease and hospitalisation burden are concentrated in the youngest children, especially those aged 0-2 years. This is biologically plausible, given immunological immaturity, limited prior exposure, and the higher likelihood of severe lower respiratory tract disease in infants and young children, making them more likely to meet SARI criteria and require hospital admission. At the same time, the low number of adult RSV detections should not be interpreted as the absence of adult infection, but rather as a reflection of the SARI-based design of the surveillance platform: adults more frequently experience milder RSV illness that does not necessitate hospitalisation and therefore is less likely to be captured by SARI surveillance. Nonetheless, adults remain epidemiologically relevant as potential reservoirs and transmitters, particularly those in

close contact with children (e.g. parents, caregivers, and grandparents). Finally, the symptom profile must be interpreted in a surveillance-specific manner: cough and fever (≥38°C) were uniformly present because they are integral to the SARI case definition and therefore reflect the inclusion criteria rather than an independent clinical observation. The distribution of additional manifestations – such as rhinitis/coryza and dyspnoea, and less frequent gastrointestinal and systemic symptoms – adds descriptive granularity and complements the demographic and subgroup findings.

The under-representation of RSV genomes from several European countries – particularly in south-eastern Europe – limits the resolution with which regional RSV evolution and cross-border spread can be interpreted from public databases alone. In this context, our contribution of season-resolved whole-genome data from North Macedonia provides timely molecular evidence that local RSV circulation mirrors broader European patterns, while adding the needed geographic coverage to an otherwise sparse area. However, the relatively small number of European reference sequences included for phylogenetic contextualisation may have limited the resolution with which potential cross-border introduction events could be reconstructed. The phylogenies indicate that RSV circulation during 2022/2023 – 2024/2025 is better explained by repeated introductions and turnover of variants than by long-term persistence of a single lineage across seasons, with a notably more dynamic pattern for RSV-A; multiple ON1 sublineages were observed across consecutive seasons, and the dominant genetic groups differed between the 2023/2024 and 2024/2025 seasons. In contrast, RSV-B genomes were largely confined to the BA9 genotype and clustered predominantly within a single lineage (B.D.E.1) across all three seasons.

A methodological limitation of this study relates to the evolving laboratory and analytical approaches applied across the three surveillance seasons. In studies spanning multiple seasons, such refinements are often introduced as surveillance systems develop and analytical capacities expand. In the present study, different RT-qPCR assays were used during the study period, and the Ct values obtained with these assays were used as an operational criterion for prioritising samples for sequencing. However, the Ct threshold for sequencing selection was applied across heterogeneous assays, and the cross-kit Ct equivalence was not formally validated. In addition, two different bioinformatic tools were used for consensus sequence generation, and as they differ in their analytical workflows, a direct comparison of their output characteristics was not feasible. Therefore, potential minor inter-seasonal differences related to assay heterogeneity and bioinformatic processing should be considered when interpreting season-to-season comparisons, phylogenetic relationships, and reported frequencies of amino acid substitutions. The bioinformatic approach used for each Macedonian RSV sequence included in the study is provided in Supplementary Table S2.

An additional consideration when interpreting seasonal differences in lineage composition is that the SARI testing framework changed across the study period, which may have influenced the profile of the sampled and sequenced cases between seasons. In the present dataset, sequenced cases from all three seasons were predominantly concentrated in children aged 0-2 years, who accounted for 90.6% of all sequenced samples, with only a small number of sequences obtained from older age groups (Supplementary Table S1). This distribution does not allow for a meaningful assessment of lineage distribution across age categories. Likewise, because the surveillance platform included only hospitalised cases, the study design does not allow reliable evaluation of whether particular lineages were associated with differences in clinical severity or symptom profiles, as all included patients already met the criteria for severe acute respiratory infection requiring hospital care. This cautious interpretation is further supported by the literature, where data on lineage-specific clinical differences remain limited, and even studies assessing associations between the broader RSV subgroups and disease severity have yielded inconsistent findings, with some suggesting greater severity for RSV-A, others reporting less

favourable outcomes for RSV-B, and others showing no significant subgroup-related difference (20-23). Therefore, although the observed lineage turnover likely reflects true viral circulation dynamics, the contribution of sampling-related differences cannot be excluded, and the findings should be interpreted with appropriate caution. Importantly, both globally successful genetic backgrounds represented in our dataset – RSV-B BA9 genotype and RSV-A ON1 – are defined by duplications in the G gene: a 60-nucleotide duplication in RSV-B first reported in 1999, and a 72-nucleotide duplication in RSV-A reported in 2010. Since their independent emergence, the BA (RSV-B) and ON1 (RSV-A) genotypes have replaced previously circulating RSV variants, suggesting that G-gene duplication confers a selective advantage (24). Because the G protein is exposed to immune pressure, higher variability in this gene is expected. In our dataset, this is reflected by the broader spectrum of G substitutions in RSV-A, whereas RSV-B showed several high-frequency substitutions together with additional low-frequency variants. In comparison, the F gene exhibited fewer substitutions overall in both subgroups, with most changes occurring at low frequencies, consistent with the more conserved nature of the F protein.

An additional limitation of this study is the selection of samples for whole-genome sequencing. Prioritisation of samples with lower Ct values was used as a practical approach to increase the likelihood of successful sequencing while making reasonable use of the available resources. However, this strategy enriched the sequenced subset for samples with higher viral RNA concentrations and may, therefore, have limited its representativeness relative to the broader RSV-positive population. Consequently, bias in lineage representation cannot be excluded if certain viral lineages were more frequently present in samples with lower RNA concentrations and were therefore less likely to be included in the sequencing dataset. A descriptive comparison of sequenced and non-sequenced RSV-positive samples is provided in Supplementary Table S1.

The expanding use of RSV preventive interventions in Europe further strengthens the relevance of integrated RSV surveillance. In the European context, long-acting monoclonal antibodies for infant protection and maternal RSV vaccination are already part of the evolving prevention landscape, while vaccines for older adults have also been authorised. To our knowledge, such preventive interventions were not part of the routine national implementation in North Macedonia at the time of writing. However, if introduced in the future, the established RSV genomic surveillance framework within the national SARI system could provide an important baseline for monitoring possible changes in the RSV burden, age distribution, seasonal dynamics, and circulation of viral variants over time. In our dataset, two RSV-B F substitutions present in all RSV-B genomes, S190N and S211N, mapped at or near the prefusion F antigenic site Ø targeted by nirsevimab, whereas one RSV-A substitution, S276N, was detected within antigenic site II of the F protein, the region targeted by palivizumab (25, 26). Importantly, available data indicate that S211N retains susceptibility to nirsevimab, which is supported both by phenotypic analyses showing preserved neutralisation potency and the OUTSMART-RSV surveillance program, in which S211N variants showed full susceptibility to nirsevimab (IC50 fold change = 1.24 versus reference) (27, 28). In contrast, although S190N is located in close proximity to this antigenically relevant region, its functional significance remains insufficiently characterised. Similarly, although S276N lies within the palivizumab-targeted site II region, the available data do not support an association with reduced palivizumab activity (29). More broadly, the detection of a substitution within a monoclonal antibody binding site should be regarded as noteworthy from a surveillance perspective, while avoiding default assumptions regarding altered binding or functional significance in the absence of phenotypic evidence. In this context, the value of genomic surveillance lies in enabling the timely detection and monitoring of changes with potential relevance,

should preventive interventions be introduced in North Macedonia in the future.

Together, these findings reinforce the importance of sustained RSV genomic surveillance in undersampled European settings, as the inclusion of genomes from North Macedonia improves regional representativeness and strengthens the interpretation of lineage turnover, viral introductions, and subgroup-specific evolutionary patterns across seasons.

## Conclusion

This study provides an integrated epidemiological, molecular, and genomic overview of RSV circulation within the national SARI surveillance system in North Macedonia over three consecutive seasons. The findings confirm that RSV is a substantial contributor to severe respiratory disease among hospitalised SARI cases, particularly in young children, and demonstrate marked season-to-season variations in subgroup dominance and lineage composition. Phylogenetic analyses indicate dynamic turnover of RSV-A lineages and more stable circulation of RSV-B within a single dominant lineage, consistent with broader European patterns. Together, these results highlight the value of sustained RSV surveillance that combines epidemiological data with whole-genome sequencing, particularly in undersampled regions. The established surveillance framework provides a robust baseline for future monitoring of RSV evolution and circulation, should pharmacological preventive measures be implemented.

**What Is Already Known on This Topic:**

*Respiratory syncytial virus is a leading cause of severe lower respiratory tract infections and hospitalisation in infants and young children worldwide. RSV circulates in two major subgroups, RSV-A and RSV-B, which co-circulate globally and often show alternating dominance between seasons. Molecular surveillance has demonstrated that contemporary RSV circulation is dominated by several globally successful genotypes, most notably ON1 (RSV-A) and BA9 (RSV-B), both of which are characterised by duplications in the G gene. Whole-genome sequencing has been increasingly applied to understand RSV transmission dynamics, lineage turnover, and genetic diversity. However, RSV genomic data remain unevenly distributed across Europe, with limited representation from south-eastern European countries, constraining the comprehensive regional interpretation of RSV evolution and spread.*

**What This Study Adds:**

*This study provided an integrated analysis of RSV epidemiology, molecular characteristics, and whole-genome data across three consecutive seasons within the national SARI surveillance system in North Macedonia. It documents the dynamic seasonal turnover of RSV-A lineages alongside the more stable circulation of RSV-B within a dominant lineage and contributes whole-genome data from an under-represented region of Europe. By embedding genomic analysis within routine SARI surveillance, this study provides new insights into the circulation of RSV subgroups and lineages associated with severe disease in North Macedonia.*

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**Authors' Contributions:** Conception and design: TK, GB and IGj; Laboratory analyses: TK, MV, SK, GM, GN and AP; Bioinformatic analysis: TK; Acquisition, analysis and interpretation of data: TK, GB, EJ, MV, SK, GM, DK, KS, EP, GP and IGj; Drafting the article: TK; Revising it critically for important intellectual content: all authors; Approved final version of the manuscript: all authors.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

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## Supplementary Material

Table S1. Comparison of Sequenced and Non-sequenced RSV-Positive Samples by Ct Value Distribution, Age Group, Collection Month, and RSV Subgroup

| Variable | Category | Sequenced RSV-positive samples (N=85) | Non-sequenced RSV-positive samples (N=166) |
|---|---|---|---|
| Ct value | <20 | 14 | 4 |
| | 20-29.9 | 69 | 105 |
| | ≥30 | 2 | 57 |
| Age group | 0-2 | 77 | 149 |
| | 3-4 | 4 | 8 |
| | 5-14 | 3 | 6 |
| | 15-29 | 0 | 0 |
| | 30-64 | 0 | 1 |
| | 65-79 | 1 | 2 |
| | 80+ | 0 | 0 |
| Month of collection | October 2022 | 2 | 0 |
| | November 2022 | 8 | 9 |
| | December 2022 | 0 | 17 |
| | January 2023 | 2 | 2 |
| | February 2023 | 2 | 0 |
| | March 2023 | 0 | 0 |
| | April 2023 | 0 | 0 |
| | October 2023 | 2 | 5 |
| | November 2023 | 21 | 25 |
| | December 2023 | 7 | 31 |
| | January 2024 | 1 | 15 |
| | February 2024 | 3 | 7 |
| | March 2024 | 1 | 0 |
| | April 2024 | 0 | 0 |
| | October 2024 | 0 | 0 |
| | November 2024 | 1 | 0 |
| | December 2024 | 6 | 1 |
| | January 2025 | 6 | 11 |
| | February 2025 | 14 | 16 |
| | March 2025 | 4 | 14 |
| | April 2025 | 5 | 13 |
| RSV subgroup | RSV-A | 57 | 112 |
| | RSV-B | 28 | 42 |
| | RSV-A/B coinfection | 0 | 1 |
| | Not determined | 0 | 11 |

Note: The months of collection are presented within the analytical surveillance period used for seasonal comparisons, spanning week 40 of one year to week 20 of the following year.

Table S2. GISAID Accession IDs and Corresponding Sample Information for Macedonian RSV Consensus Genome Sequences Obtained Through National SARI Surveillance During the 2022-2025 Seasons

| Virus name | Accession ID | Collection date | Pipeline |
|---|---|---|---|
| hRSV/A/Macedonia/IPH-MKD-137G/2023 | EPI_ISL_19141127 | 2023-12-04 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-153G/2023 | EPI_ISL_19141128 | 2023-12-06 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-117G/2023 | EPI_ISL_19141125 | 2023-11-27 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-103G/2023 | EPI_ISL_19141124 | 2023-11-24 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-91G/2023 | EPI_ISL_19141121 | 2023-11-21 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-92G/2023 | EPI_ISL_19141122 | 2023-11-21 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-89G/2023 | EPI_ISL_19141120 | 2023-11-21 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-81G/2023 | EPI_ISL_19141116 | 2023-11-17 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-82G/2023 | EPI_ISL_19141117 | 2023-11-17 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-76G/2023 | EPI_ISL_19141114 | 2023-11-16 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-77G/2023 | EPI_ISL_19141115 | 2023-11-16 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-73G/2023 | EPI_ISL_19141112 | 2023-11-14 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-74G/2023 | EPI_ISL_19141113 | 2023-11-14 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-83G/2023 | EPI_ISL_19141118 | 2023-11-20 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-87G/2023 | EPI_ISL_19141119 | 2023-11-21 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-658G/2024 | EPI_ISL_19141138 | 2024-02-12 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-867G/2024 | EPI_ISL_19141139 | 2024-03-21 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-572G/2024 | EPI_ISL_19141136 | 2024-02-01 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-594G/2024 | EPI_ISL_19141137 | 2024-02-05 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-236G/2023 | EPI_ISL_19141134 | 2023-12-25 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-377G/2024 | EPI_ISL_19141135 | 2024-01-05 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-180G/2023 | EPI_ISL_19141130 | 2023-12-12 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-193G/2023 | EPI_ISL_19141131 | 2023-12-13 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-166G/2023 | EPI_ISL_19141129 | 2023-12-12 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-40G/2023 | EPI_ISL_19141105 | 2023-11-01 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-41G/2023 | EPI_ISL_19141106 | 2023-11-01 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-33G/2023 | EPI_ISL_19141103 | 2023-10-27 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-37G/2023 | EPI_ISL_19141104 | 2023-10-31 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-66G/2023 | EPI_ISL_19141110 | 2023-11-13 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-70G/2023 | EPI_ISL_19141111 | 2023-11-14 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-58G/2023 | EPI_ISL_19141109 | 2023-11-10 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-50G/2023 | EPI_ISL_19141107 | 2023-11-07 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-56G/2023 | EPI_ISL_19141108 | 2023-11-08 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-77G/2024 | EPI_ISL_20207191 | 2024-11-26 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-92G/2024 | EPI_ISL_20207192 | 2024-12-04 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-96G/2024 | EPI_ISL_20207193 | 2024-12-11 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-109G/2024 | EPI_ISL_20207194 | 2024-12-19 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-121G/2024 | EPI_ISL_20207195 | 2024-12-23 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-128G/2024 | EPI_ISL_20207196 | 2024-12-25 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-162G/2025 | EPI_ISL_20207198 | 2025-01-08 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-228G/2025 | EPI_ISL_20207200 | 2025-01-15 | INSaFLU |

| Virus name | Accession ID | Collection date | Pipeline |
|---|---|---|---|
| hRSV/A/Macedonia/IPH-MKD-370G/2025 | EPI_ISL_20207203 | 2025-01-29 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-404G/2025 | EPI_ISL_20207206 | 2025-02-05 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-406G/2025 | EPI_ISL_20207207 | 2025-02-05 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-442G/2025 | EPI_ISL_20207209 | 2025-02-10 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-448G/2025 | EPI_ISL_20207210 | 2025-02-10 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-455G/2025 | EPI_ISL_20207211 | 2025-02-10 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-496G/2025 | EPI_ISL_20207213 | 2025-02-14 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-554G/2025 | EPI_ISL_20207214 | 2025-02-19 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-566G/2025 | EPI_ISL_20207215 | 2025-02-20 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-597G/2025 | EPI_ISL_20207216 | 2025-02-24 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-601G/2025 | EPI_ISL_20207217 | 2025-02-24 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-807G/2025 | EPI_ISL_20207220 | 2025-03-24 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-827G/2025 | EPI_ISL_20207221 | 2025-03-27 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-831G/2025 | EPI_ISL_20207222 | 2025-04-01 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-848G/2025 | EPI_ISL_20207223 | 2025-04-04 | INSaFLU |
| hRSV/A/Macedonia/IPH-MKD-868G/2025 | EPI_ISL_20207226 | 2025-04-08 | INSaFLU |
| hRSV/B/North Macedonia/IPH-MKD-220/2022 | EPI_ISL_15601574.2 | 2022-10-18 | INSaFLU |
| hRSV/B/North Macedonia/IPH-MKD-231/2022 | EPI_ISL_16006110 | 2022-10-26 | Ugene v45.0 |
| hRSV/B/North Macedonia/IPH-MKD-263/2022 | EPI_ISL_16006112 | 2022-11-16 | Ugene v45.0 |
| hRSV/B/North Macedonia/IPH-MKD-262/2022 | EPI_ISL_16006111 | 2022-11-16 | Ugene v45.0 |
| hRSV/B/North Macedonia/IPH-MKD-274/2022 | EPI_ISL_16006114 | 2022-11-22 | Ugene v45.0 |
| hRSV/B/North Macedonia/IPH-MKD-271/2022 | EPI_ISL_16006113.2 | 2022-11-22 | INSaFLU |
| hRSV/B/North Macedonia/IPH-MKD-282/2022 | EPI_ISL_16006116.2 | 2022-11-24 | INSaFLU |
| hRSV/B/North Macedonia/IPH-MKD-278/2022 | EPI_ISL_16006115 | 2022-11-23 | Ugene v45.0 |
| hRSV/B/North Macedonia/IPH-MKD-287/2022 | EPI_ISL_16006118.2 | 2022-11-25 | INSaFLU |
| hRSV/B/North Macedonia/IPH-MKD-285/2022 | EPI_ISL_16006117 | 2022-11-25 | Ugene v45.0 |
| hRSV/B/Macedonia/IPH-MKD-250G/2023 | EPI_ISL_18348031 | 2023-02-15 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-184G/2023 | EPI_ISL_18348030 | 2023-02-02 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-86G/2023 | EPI_ISL_18348029 | 2023-01-17 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-49G/2023 | EPI_ISL_18348028 | 2023-01-09 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-94G/2023 | EPI_ISL_19141123 | 2023-11-21 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-234G/2023 | EPI_ISL_19141133 | 2023-12-25 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-129G/2024 | EPI_ISL_20207197 | 2024-12-25 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-201G/2025 | EPI_ISL_20207199 | 2025-01-10 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-286G/2025 | EPI_ISL_20207201 | 2025-01-20 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-326G/2025 | EPI_ISL_20207202 | 2025-01-23 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-400G/2025 | EPI_ISL_20207204 | 2025-02-04 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-401G/2025 | EPI_ISL_20207205 | 2025-02-04 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-419G/2025 | EPI_ISL_20207208 | 2025-02-07 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-491G/2025 | EPI_ISL_20207212 | 2025-02-13 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-732G/2025 | EPI_ISL_20207218 | 2025-03-11 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-771G/2025 | EPI_ISL_20207219 | 2025-03-17 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-855G/2025 | EPI_ISL_20207224 | 2025-04-07 | INSaFLU |
| hRSV/B/Macedonia/IPH-MKD-857G/2025 | EPI_ISL_20207225 | 2025-04-07 | INSaFLU |

Table S3. GISAID Accession IDs and Corresponding Information for European RSV Reference Sequences Used in Phylogenetic Analyses

| Virus name | Accession ID | Collection date | Country |
|---|---|---|---|
| hRSV/A/Russia/NVS-7.638/2024 | EPI_ISL_19437714 | 2024-03-07 | Russian Federation |
| hRSV/A/England/UKHSA_RVU_4640232/2023 | EPI_ISL_18713515.2 | 2023-11-14 | United Kingdom |
| hRSV/A/Spain/EX-BA_18612421/2023 | EPI_ISL_18968232 | 2023-12-22 | Spain |
| hRSV/A/France/ARA-EEQ1-HCL27601102/2023 | EPI_ISL_19465517 | 2023-05-31 | France |
| hRSV/A/Slovakia/1336/2023 | EPI_ISL_17481634.2 | 2023-02-06 | Slovakia |
| hRSV/A/France/ARA-HCL023188225101/2023 | EPI_ISL_18789785 | 2023-11-29 | France |
| hRSV/A/Italy/SIC-107_48/2021 | EPI_ISL_19063420 | 2021-11-29 | Italy |
| hRSV/A/Spain/AN-PMC-00050/2024 | EPI_ISL_18980304 | 2024-01-21 | Spain |
| hRSV/A/Ireland/NVRL-G-68808/2023 | EPI_ISL_19160773 | 2023-11-01 | Ireland |
| hRSV/A/Ireland/NVRL-G-69867/2023 | EPI_ISL_19160778 | 2023-11-06 | Ireland |
| hRSV/A/Ukraine/RLDHA-4090/2025 | EPI_ISL_19883376 | 2025-04-07 | Ukraine |
| hRSV/A/Canary Islands/HUNSC_ITER_150427667/2023 | EPI_ISL_18698713 | 2023-11-19 | Spain |
| hRSV/B/Croatia/HZJZ_7338/2024 | EPI_ISL_19074734 | 2024-02-24 | Croatia |
| hRSV/B/Bulgaria/2734/2022 | EPI_ISL_18447498 | 2022-12-19 | Bulgaria |
| hRSV/B/France/IDF-RELAB-IPP24981/2023 | EPI_ISL_18537476 | 2023-10-12 | France |
| hRSV/B/Spain/CT-HUJO-048261/2024 | EPI_ISL_19536326 | 2024-10-31 | Spain |
| hRSV/B/England/UKHSA_RVU_4460393/2023 | EPI_ISL_18591802 | 2023-10-31 | England |
| hRSV/B/Germany/NRW-HHU-161/2024 | EPI_ISL_20060976 | 2024-12-23 | Germany |
| hRSV/B/Czech Republic/NRL_c1588/2024 | EPI_ISL_19044757 | 2024-02-15 | Czech Republic |
| hRSV/B/Switzerland/ZH-UZH-IMV-3BA70F72/2025 | EPI_ISL_19995393 | 2025-04-05 | Switzerland |
| hRSV/B/Slovakia/uvzsr-40461/2025 | EPI_ISL_20068837 | 2025-03-12 | Slovakia |