The Journal of Infectious Diseases

MAJOR ARTICLE

Genomic Surveillance and Antigenic Characterization of Respiratory Syncytial Virus (RSV) in Spain During the 2023-2024 Season of Nirsevimab Administration

María Iglesias-Caballero,[1] Vicente Mas,[1] Albert Campoy,[1] Cristina Calvo,[2,3] María Luz García,[3,4] Sonia Alcolea,[2,3,4] Laura Moreno-Parrado,[5] Jordi Reina,[6] Ana Navascués,[7] Andrés Antón,[3,8] Carlos Davina-Nunez,[9,10] Sonia Perez-Castro,[9,10,11] José Costa,[12] Ana Fernández-González,[13] José Antonio Lepe,[14] Cristina Gaona,[15] Zulema Pérez,[16] Diego García-Martínez de Artola,[17] Sonia Vázquez-Morón,[1,18] Sara Camarero,[1] Olga Cano,[1] Francisco Pozo,[1,18] and Inmaculada Casas[1,18]

[1]Reference and Research Laboratory for Respiratory Virus, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain; [2]Paediatric Infectious and Tropical Diseases Department, Universitary Hospital La Paz, Hospital La Paz Institute for Health Research (IdiPAZ Foundation), Madrid, Spain; [3]Consortium for Biomedical Research in Infectious Diseases (CIBERINFEC), Madrid, Spain; [4]Paediatric Department, Severo Ochoa University Hospital, Leganés, Biomedical Sciences Research Institute, Puerta de Hierro-Majadahonda University Hospital, Madrid, Spain; [5]Microbiology Department,Universitary Hospital Virgen de Arrixaca, Murcia, Spain; [6]Microbiology Department,Universitary Hospital Son Espases, Mallorca, Spain; [7]Clinical Microbiology Unit, Navarra Universitary Hospital, Pamplona, Navarra, Spain; [8]Respiratory Viruses Unit, Microbiology Department, Vall d'Hebron Institut de Recerca (VHIR), Vall d'Hebron Hospital Universitari, Vall d' Hebron Barcelona Hospital Campus, Universitat Autònoma de Barcelona, Barcelona, Spain; [9]Microbiology and Infectology Research Group, Galicia Sur Health Research Institute (IIS Galicia Sur), Vigo, Spain; [10]Microbiology and Infectology Department, University of Vigo, Vigo, Spain; [11]Microbiology Department, Complexo Hospitalario Universitario de Vigo, Vigo, Spain; [12]Microbiology Department, Santiago Universitary Hospital Complex, Santiago de Compostela, Spain; [13]Microbiology Department, A Coruña Universitary Hospital Complex, A Coruña, Spain; [14]Microbiology Department, Universitary Hospital Virgen del Rocío, Sevilla, Spain; [15]Microbiology Department,Badajoz Universitary Hospital Complex, Badajoz, Spain; [16]Microbiology Department, Central Universitary Hospital of Asturias, Oviedo, Spain; [17]Microbiology Unit, Nuestra Señora de Candelaria University Hospital, Instituto de Investigación Sanitaria de Canarias, Santa Cruz de Tenerife, Spain; and [18]Consortium for Biomedical Research in Epidemiology and Public Health (CIBERESP), Madrid, Spain

Respiratory Syncytial Virus (RSV) is a leading cause of respiratory infections in infants and older adults. In Spain, surveillance is supported by the SiVIRA system and the RELECOV genomic sequencing network. The 2023–2024 season marked the first nationwide administration of nirsevimab, a monoclonal antibody for preventing severe RSV in infants.

This study analyzes the genomic evolution of RSV during this period, focusing on potential escape mutations in the F protein. RSV-A showed high genetic diversity with eleven circulating lineages, while RSV-B was dominated by lineage B.D.E.1. Phylogenetic analysis revealed three distinct B.D.4.1.1 groups, one sharing mutations with B.D.E.1 in the nirsevimab binding site.

Despite these changes, neutralization assays confirmed that nirsevimab and other monoclonal antibodies remained effective. No significant antigenic drift compromising immunoprophylaxis was observed. These findings support the continued efficacy of nirsevimab and highlight the importance of genomic surveillance for tracking RSV evolution and informing future immunization strategies.

Keywords. RSV; nirsevimab; genomic and antigenic surveillance; RELECOV multicentre study.

Respiratory Syncytial Virus (RSV) is a leading cause of respiratory infections in children, particularly in those under 2 years of age, and adults over 60 years old [1, 2]. Given its impact, international health organizations, including ECDC and WHO [3, 4], recommend RSV surveillance within respiratory infection monitoring systems. Spain has implemented the Acute Respiratory Infection Surveillance System (SiVIRA) since 2020-2021, covering both primary (mild) and hospital care (severe) [5]. This system allows integrated monitoring of respiratory viruses like influenza, SARS-CoV-2, and RSV. The National Network of Spanish Laboratories for Genomic Sequencing (RELECOV), originally established for SARS-CoV-2 [6], was extended to include influenza and RSV [7].

In 2023, Spain approved nirsevimab to prevent severe RSV infections in infants under six months and other high-risk individuals [8]. The introduction of this mAb and upcoming vaccines [9] underscores the need for strong surveillance, including RSV genomic sequencing.

Data from a prospective study in Madrid confirm nirsevimab's effectiveness with ~92% coverage reducing RSV-related hospitalizations by 93.6% and ICU admissions by over 90% [10]. SiVIRA data showed 9364 to 9875 fewer RSV hospitalizations in children under one year in 2023-2024 season, a ~75% reduction compared with 2022–2023 after adjusting for RSV circulation [11]. A nationwide study comparing epidemic periods from 2018 to 2024 found a 79.3% reduction in RSV-related hospitalizations in infants under three months

Received 29 May 2025; accepted 08 October 2025; published online 16 October 2025

Correspondence: Prof. María Iglesias-Caballero, PhD, Reference and Research Laboratory for Respiratory Virus, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, 28220, Spain (miglesias@isciii.es), Prof. Inmaculada Casas, PhD (icasas@isciii.es).

https://doi.org/10.1093/infdis/jiaf530

after nirsevimab introduction [12]. Furthermore, nirsevimab significantly reduced RSV-related hospitalizations and severe lower respiratory tract infections (LRTI) needing respiratory support [12].

RSV is classified into two antigenic groups, RSV-A and RSV-B [13]. Traditionally, clades were defined by the highly variable G protein [14, 15]. Ramaekers *et al* [16] and Chen *et al* [17] emphasized classifying RSVs using whole genome sequences or at least considering the F protein. In 2024, Goya et al [18] proposed a standardized RSV nomenclature using letters to indicate subgroup and duplications, and numbers for monophyletic clusters with >10 sequences and >5 amino acid changes relative to the parental lineage.

RSV expresses two main surface glycoproteins: G and F. Notably, the F protein, targeted by nirsevimab and new vaccines, is key to understand how these measures influence viral evolution. Six major antigenic sites (Ø and I to V) have been described on RSV F protein´s prefusion conformation. Sites Ø, II, IV, and V are crucial for immunoprophylactic strategies. Site Ø, at the trimer's membrane-distal apex, is the main target of potent neutralizing antibodies like nirsevimab and RSM01.

As a complement to the clinical and epidemiological studies already conducted [10–12], this study presents RSV genomic and antigenic data from Spain´s 2023–2024 season, the first after nationwide nirsevimab immunoprophylaxis campaign. The study analyzes RSV evolution, potential F protein escape mutations affecting nirsevimab efficacy, and implications for future immunoprophylaxis strategies based on the mAb Clesrovimab [19] and RSM01 [20], both in clinical development.

METHODS

Source of the Sequences for Genomic Analysis

For the viral characterization study in this report, 1,164 complete RSV consensus genome sequences from Spain were used, covering the period between epidemiological weeks 40 of 2023 and 39 of 2024. The sequences were downloaded from the database GISAID [21], including 150 which were obtained at the Reference and Research Laboratory for Respiratory Viruses, National Center for Microbiology (CNM-ISCIII). The full list of GISAID accession numbers for the sequences analyzed in this study is available at: https://doi.org/10.5281/zenodo.17184379. Consensus sequences included had >80% genome coverage, good or moderate quality by Nextclade v3.15.1 [22], and complete coverage of both G and F proteins. CNM-ISCIII samples also met a minimum depth threshold of 10X. These sequences reflect viral diversity across Spain, covering 13 of its 17 regions (Supplementary Fig. 1)

Sequencing and Phylogenetic Analysis Methods

Sequences from CNM-ISCIII were obtained following a previously published protocol [23], which is available on the protocols.io platform (dx.doi.org/10.17504/protocols.io.kqdg3xbzqg25/v4). Sequences were analyzed for viral genome reconstruction using the viralrecon pipeline v2.6.0. (https://github.com/nf-core/viralrecon) [24] that comprised mapping and de novo assembly.

Sequences from Galicia, Asturias and Canary Islands were obtained following a previously published protocol [25].

The phylogenetic analysis of Spanish sequences included 554 RSV-A and 610 RSV-B whole genome sequences from Spain, aligned with reference sequences of Orthopneumovirus subtype A and subtype B (NCBI GenBank, NC_038235.1 and NC_001781.1, respectively). These references were also used for characterization of genomic changes in the genomes.

Lineages were assigned from consensus sequences using Nextclade v3.15.1 on March 7, 2025.

Multiple sequence alignment was performed using MAFFT v.7 [26], and phylogenetic trees were constructed using FastTreeMP v. 2.1.10, employing a GTR model with 1,000 bootstrap replicates [27]. A downloadable version of the full tree files is available at https://doi.org/10.5281/zenodo.17184379.

Cell Culture and Virus for Antigenic Analysis

The HEp-2 cell line (ATCC CCL-23) was maintained in DMEM supplemented with 2.5% of Fetal Bovine Serum, 2 mM L-glutamine, non-essential amino acids (Invitrogen, Carlsbad, CA, USA), penicillin (100 IU/mL), streptomycin (100 µg/mL), and 1 mM sodium pyruvate, and was kept at 37°C in a 5% CO_2 atmosphere. Circulating RSV-B strains hRSV/B/Spain/MD-ISCIII-0688/2024 (GISAID EpiRSV EPI_ISL_19345755) and hRSV/B/Spain/MD-ISCIII-2484/2023 (GISAID EpiRSV EPI_ISL_18927346) were isolated after three passages in HEp-2 cells from two RSV PCR-positive nasopharyngeal aspirate samples collected in Spain during the 2023–2024 season. Viral stocks of these viral isolates, as well as reference viruses RSV-A2 (ATCC VR-1540) and RSV-CH18537 (ATCC VR-1580), were propagated following three days of infection at 37°C. Viral titers were determined using a fluorescence foci-forming unit (FFFU) assay. The viral stock sequences after passaging were analyzed confirming the absence of selection adaptation mutations in G and F. The sequences are available in GISAID under the accession numbers EPI_ISL_20055308 and EPI_ISL_20055309.

Production and Purification of Mouse Antibodies

Mouse mAbs specific for RSV F and G glycoproteins (2F, 47F, 56F, 101F, 021/1G, 021/21G) [28] were generated from hybridoma supernatants. Recombinant human mAbs nirsevimab, palivizumab, clesrovimab, and ADI-15618 were produced by

transient transfection of Freestyle 293-F cells. Expression plasmids were based on pcDNA 3.4 TOPO (GeneArt, Thermo Fisher) and designed according to published sequences [19, 29, 30]. After 5 days, antibodies were purified using protein A affinity chromatography (Cytiva).

Production and Purification of Stabilized Prefusion F Proteins (F DSCav-1)

Recombinant F DSCav-1 proteins, corresponding to RSV-A2 (UniProtKB/Swiss-Prot: P03420.1) modified with the naturally occurring P102A, I379 V, and M447 V changes to increase expression efficiency, RSV-B 9320 (GeneBank: AY353550.1), and the MD-ISCIII-0688/24 (EPI_ISL_19345755) clinical isolate, were produced by transient transfection of Freestyle 293-F cells (Thermo Fisher Scientific). The expression plasmids pHα-F DSCav-1 (RSV-A2 and RSV-B 9320) were generously provided by Dr. Jason S. McLellan (University of Texas at Austin, USA), while the pcDNA 3.4-TOPO plasmid encoding F DSCav-1 of RSV-B isolate 240122 was purchased from GeneArt (Thermo Fisher Scientific). Five days post-transfection, supernatants were clarified and proteins purified using Ni2+ columns, followed by gel filtration.

RSV Microneutralization Assays

Microneutralization assays were performed in 96-well plates using 90% confluent HEp-2 cell monolayers. The cells were infected with reference RSV strains or circulating RSV B isolates at a multiplicity of infection (MOI) of 0.001, in the presence or absence of serial dilutions of RSV-neutralizing antibodies. After 36–48 hours of infection, the medium was removed, and the cells were washed with 0.05% Tween-20 in PBS, then fixed and permeabilized with methanol. RSV infectivity was assessed by staining with a cocktail of mouse monoclonal antibodies against the RSV F and G glycoproteins, followed by detection with an Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody. Fluorescent foci were visualized and quantified using a Thunder Imaging System (Leica Microsystems). Antibody IC50 values, expressed in ng/ml, were calculated as the antibody concentration required to reduce the number of fluorescent foci-forming units (FFFU) by 50% compared to the untreated virus control.

Surface Plasmon Resonance (SPR) Analysis

SPR experiments were performed using a Biacore T200 system in a single-cycle kinetic format. Anti-His tag antibodies (Cytiva) were immobilized to 10,000 response units (RU). Approximately 200 RU of purified F DSCav-1 proteins were captured onto the sensor surface. Purified antibodies were injected over the immobilized F DSCav-1 proteins at seven different concentrations, at a flow rate of 50 µL/min. Association and dissociation phases were set to 180 s and 300–900 s, respectively, depending on the antibody. The sensor chip was regenerated using 30 mM HCl, and the binding kinetics were analyzed using a 1:1 Langmuir binding model. The equilibrium apparent dissociation constant (KD_{app}) was calculated as the ratio of the association (ka_{app}) and dissociation (kd_{app}) rate constants.

RESULTS

Lineage Distribution in Subgroups A and B

Significant lineage variability was observed for each RSV subtype based on Spanish sequences deposited in GISAID database. In RSV-A, a total of eleven distinct lineages were identified: A.D.1, A.D.1.4, A.D.1.5, A.D.1.7, A.D.1.8, A.D.3, A.D.3.1, A.D.4, A.D.5.1, A.D.5.2, and A.D.5.3, with A.D.1.5 (30%), A.D.3 (21%), and A.D.1 (15%) being the most prevalent throughout the 2023-2024 season (Figure 1*A*). Regarding the temporal distribution of these lineages (Figure 1*B*), an increased variability was noted toward the end of 2023, with several lineages co-circulating. Some lineages, such as A.D.5.3, A.D.4, and A.D.1.4, were no longer present at the start of 2024, while others, including A.D.5.2 and A.D.1.8, began to emerge early in the year. The three dominant lineages were presented throughout the whole season, with a notable increase in the prevalence of A.D.1 and A.D.3 towards the end. For RSV-B, two primary lineages were identified: B.D.4.1.1 and B.D.E.1, with B.D.E.1 predominating, accounting for 88% of the sequences (Figure 1*C*). In terms of temporal distribution, B.D.4.1.1, the parental lineage of B.D.E.1, showed a slight increase in prevalence in early 2024 (Figure 1*D*). According to geographic representation, most RSV-A and -B lineages were present across all regions performing sequencing, except for A.D.1.7 and A.D.4, which showed more limited representation both temporally and geographically.

Phylogeny and Genomic Context

To contextualize the Spanish RSV sequences, phylogenetic trees of the different RSV subgroups were constructed using complete sequences collected between epidemiological week 40 of 2023 and week 39 of 2024, as outlined in the Materials and Methods section.

RSV-A exhibited greater lineage diversity than RSV-B, previously described in the text, divided into two major groups: one cluster consisting of lineages A.D.1 and A.D.3, and the other containing lineages A.D.4 and A.D.5 along with their subgroups (Figure 2). Within lineage A.D.5.1, two distinct subgroups were identified despite sharing the same taxonomic classification. Across the entire genome, group A.D.5.1 (1) differed from group A.D.5.1 (2) by only three substitutions in the G protein (S100I, S275N, and a serine at position 284 in the 72-nt duplication, which occurs in the second hypervariable region of the G gene. This duplication, first reported in 2011, is now dominant among RSV-A strains [15]. Additionally, two substitutions were observed in the F protein (M115 V, V127A). These two groups do not show sufficient differences

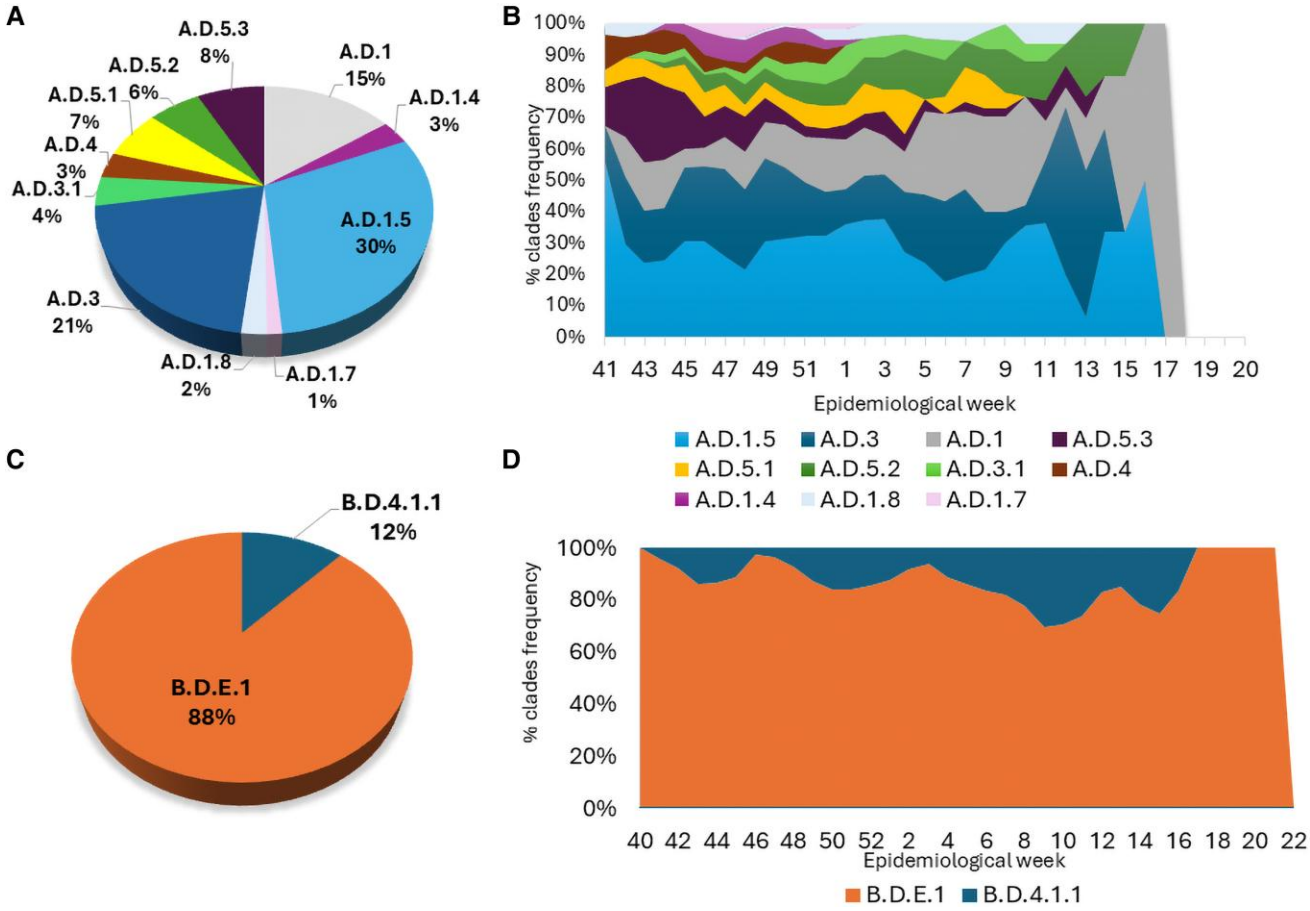


Figure 1. Percentage of different lineages described for RSV-A (*A*) and RSV-B (*C*) sequences published in GISAID database from the 2023-2024 season. Temporal distribution of RSV-A (*B*) and RSV-B (*D*) lineages throughout the epidemiological weeks of the 2023-2024 season.

to be considered new lineages but allowed us to monitoring future changes that could define them.

For RSV-B, noteworthy patterns were identified that are beyond simple lineage classifications. In Spain, lineage B.D.4.1.1 was represented by three distinct groups, each with unique mutations in the G and F proteins (Figure 3*A*). To highlight the key differences between these groups, a heatmap shows the genetic changes in the F protein relative to the RSV-B reference sequence (NCBI GenBank NC_001781.1) (Figure 3*B*).

The B.D.4.1.1 (1) group contains the lineage-defining mutations and serves as the parental lineage for both other B.D.4.1.1 groups and the predominant lineage B.D.E.1. The B.D.4.1.1 (2) group exhibits several unique mutations in the G and F proteins, including R8H, R98 M, and V99A in G, as well as a premature stop codon occurring three amino acids earlier than in the B.D.4.1.1 (1) group. This mutation pattern is also present in the B.D.4.1.1 (3) group and in lineage B.D.E.1. In the F protein, the characteristic mutations include K65Q, located within antigenic site Ø, and T529 V (Figure 3*B*).

The B.D.4.1.1 (3) group shows additional changes in the G protein, such as R98 K, P150S, and T245I, along with mutations shared with lineage B.D.E.1, including R32 K, S100G, P216S, and T233L. This group is particularly relevant for its incorporation of F protein mutations that are also found in lineage B.D.E.1, such as S190N, Q209R, S211N and S389P (Figure 3*B*). These mutations, which began to emerge globally in B.D.4.1.1 in 2023, were already present in lineage B.D.E.1 in previous seasons. The widespread occurrence of these mutations in RSV-B may suggest evolutionary selection, potentially contributing to the global persistence of lineage B.D.E.1.

F Protein Analysis and Key Mutations

Comparing the Spanish sequences to previously described reference sequences revealed a common pattern across both subgroups being a high number of unique mutations or mutations occurring at very low frequencies, mostly reported by a single submitter. In RSV-A, a total of 90 mutations were identified, 51 of which had a frequency below 0.5%. In RSV-B, 70 mutations were detected, with 50 of these showing a frequency under 0.5%.

Regarding site Ø, the membrane-distal apex of the F protein targeted by nirsevimab and ADI-15618 antibodies, only two

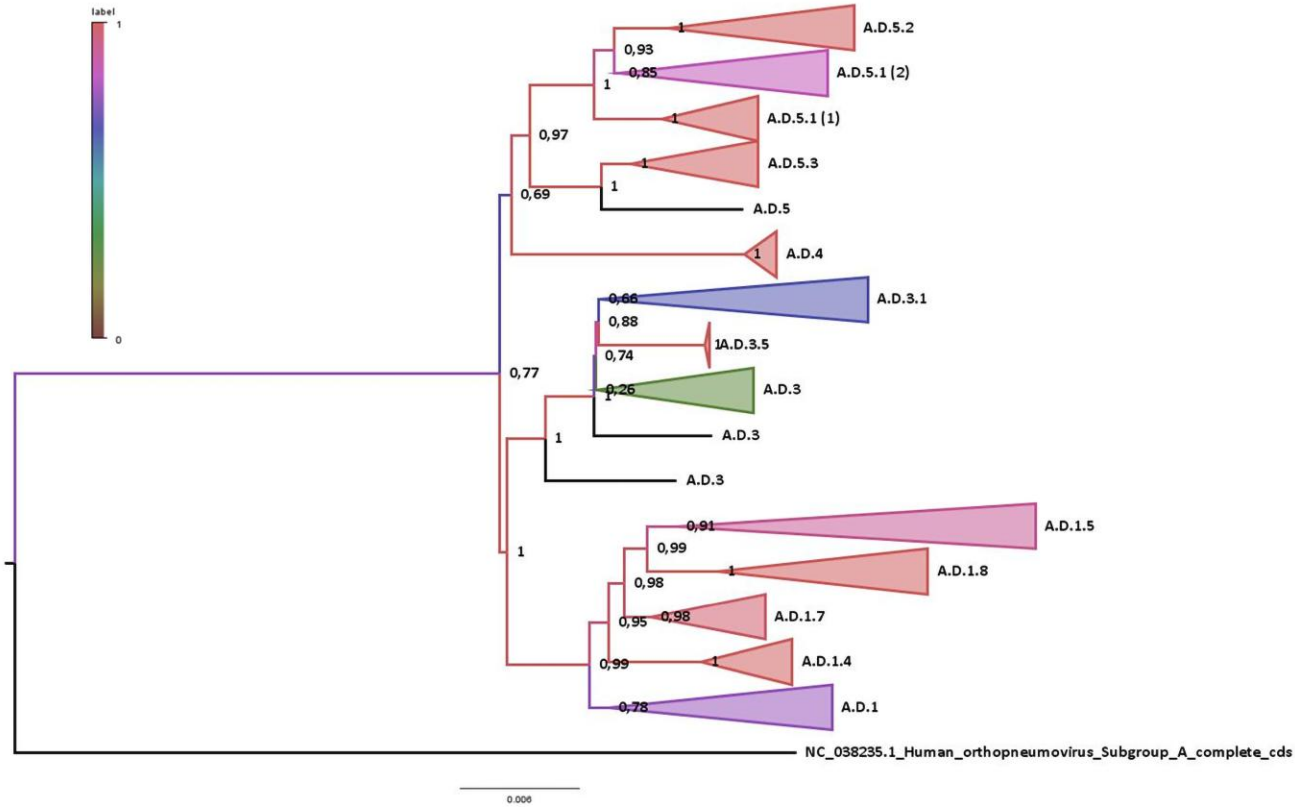


Figure 2. Phylogenetic tree constructed based on the analysis of the complete RSV-A genome sequences available in GISAID database for Spain during the 2023-2024 season (n = 554). The tree was generated using a GTR model with a bootstrap analysis of 1,000 replicates. The different clades are collapsed and labeled according to their respective annotations, with color coding used to indicate bootstrap support values, which reflect the robustness of the phylogenetic relationships. The specific sequences used from each lineage are: A.D.1 (87), A.D.1.4 (17), A.D.1.5 (162), A.D.1.7 (7), A.D.1.8 (11), A.D.3 (114), A.D.3.1 (20), A.D.4 (18), A.D.5.1 (39), A.D.5.2 (35) and A.D.5.3 (44).

mutations were identified in RSV-A, N63S (3% frequency) and K65R (3.6% frequency). In RSV-B, four amino acid changes (K65Q, I206 M, Q209R, and S211N) were found at site Ø (Figure 3*B* and Figure 4). The I206 M substitution was found in the parental B.D.4.1.1 group 1 and 3 but absent in group 2, while K65Q was restricted to B.D.4.1.1 group 2. A triple mutation I206M:Q209R:S211N, formed by the acquisition of Q209R and S211N onto the preexisting I206 M variant, was present in both the B.D.E.1 lineage and B.D.4.1.1 group 3. This triple mutation represents the most prevalent polymorphism at site Ø in circulating RSV-B viruses in Spain (Figure 3*B*).

Considering antigenic sites II and IV, targeted by palivizumab and clesrovimab, respectively, no mutations with significant frequency were detected either in Spain (Figure 4).

Antigenic Analysis of the F Protein

The predominant polymorphism (I206M:Q209R:S211N) at site Ø which has been previously detected in earlier seasons, and it has become the most prevalent variant among circulating RSV-B strains during the 2023–2024 season. Given its widespread presence, its potential antigenic impact is particularly relevant with respect to antibody effectiveness. A panel of four neutralizing antibodies was selected for the analysis: nirsevimab (site Ø), palivizumab (site II), clesrovimab (site IV) and ADI-15618 (site Ø), the parental antibody of RSM01.

Microneutralization assays demonstrated that circulating RSV-B isolates carrying the three changes, hRSV/B/Spain/MD-ISCIII-0688/2024 (GISAID EpiRSV EPI_ISL_19345755) lineage B.D.4.1.1 group 3 and hRSV/B/Spain/MD-ISCIII-2484/2023 (GISAID EpiRSV EPI_ISL_18927346) lineage B.D.E.1, remain susceptible to neutralization by antibodies targeting site Ø. Nirsevimab maintained full neutralization activity against both RSV-B strains, while ADI-15618 showed a modest reduction in efficiency against strain hRSV/B/Spain/MD-ISCIII-2484/2023, exhibiting a 17.1-fold decrease in neutralization. A comparable reduction was observed for the reference RSV-B strain CH18537 (5.3-fold), in line with previous reports working with RSM01 antibody (Table 1) [20].

RSV-B strains were efficiently neutralized by clesrovimab (targeting site IV) and palivizumab (targeting site II), showing little to no reduction in efficacy compared to the A2 virus. As expected, palivizumab demonstrated lower neutralization potency compared to site Ø-targeting antibodies and clesrovimab (Table 1) [20].

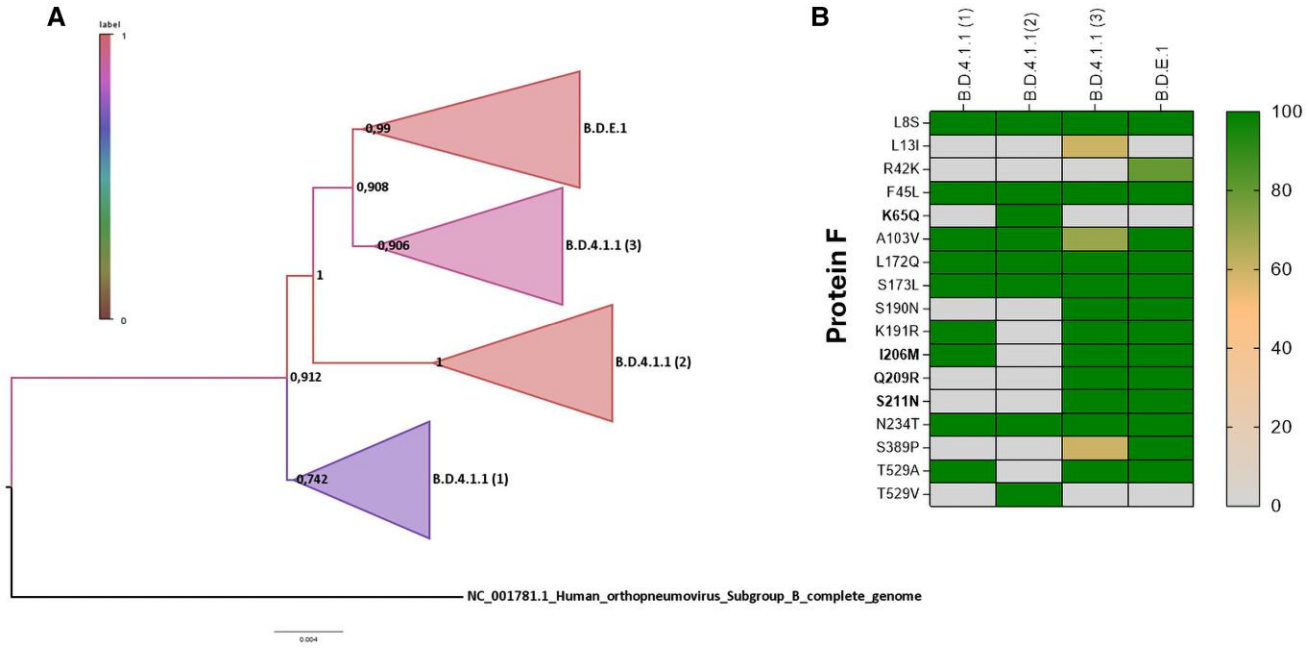


Figure 3. (*A*) Phylogenetic tree constructed based on the analysis of the complete RSV-A genome sequences available in GISAID database for Spain during the 2023-2024 season (n = 610). The tree was generated using a GTR model with a bootstrap analysis of 1,000 replicates. The different clades are collapsed and labeled according to their respective annotations, with colour coding used to indicate the bootstrap support values, which reflect the robustness of the phylogenetic relationships. Triangle size is not indicative of the number of sequences. The specific sequences used from each lineage are: 13 for lineage B.D.4.1.1 (1), 6 for lineage B.D.4.1.1 (2), 71 for B.D.4.1.1 (3), and 464 for B.D.E.1. (*B*) Heatmap of mutations detected in the F protein of lineages B.D.4.1.1 and B.D.E.1.

The binding affinity of site Ø of both nirsevimab and ADI-15618 exhibited strong affinity for the prefusion-stabilized F protein ectodomain derived from B.D.4.1.1 group 3. The dissociation constants (KD) for nirsevimab and ADI-15618 binding to F DSCav-1 240122 were in the picomolar (pM) range, comparable to those for F DSCav-1 proteins derived from reference RSV strains (A2 and B 9320) (Table 2). In contrast, palivizumab displayed a significantly weaker binding affinity, with KD values in the nanomolar (nM) range for all the three proteins assessed.

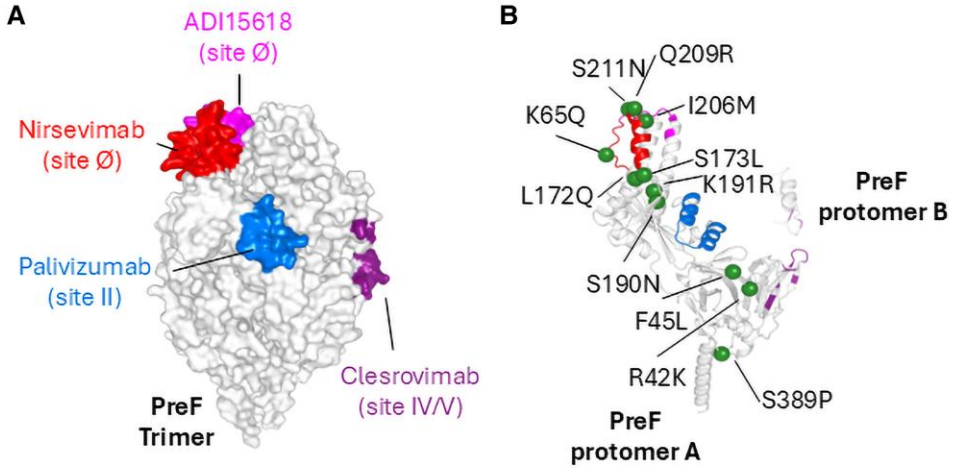
Overall, these findings indicate that the I206M:Q209R:S211N polymorphism has limited impact on RSV-B antigenicity, as neutralizing antibodies targeting site Ø remain highly effective against circulating RSV-B strains. Moreover, nirsevimab consistently exhibited greater neutralizing potency compared to palivizumab, reinforcing its superior capacity to neutralize RSV.

DISCUSSION

The study provides an analysis of the genetic and antigenic landscape of RSV in Spain during the 2023-2024 season, showing lineage diversity and ongoing viral evolution. For RSV-B, lineage B.D.E.1 emerged as the predominant, whereas RSV-A exhibited considerable variability, circulating across eleven different lineages. This contrast, also observed in studies from other countries [31, 32], suggests divergent evolutionary strategies between the subtypes, RSV-A sustained co-circulation with a broad lineage diversity, while RSV-B underwent greater homogenization. Notably, this homogenization coincided with the widespread emergence of the I206M:Q209R:S211N polymorphism at site Ø. According to data from the *Emerging Variants* section on the GISAID platform [21], these polymorphisms rose from undetectable levels at the beginning of 2023 to near ubiquity in RSV-B sequences by the end of the study. Previous research on temporal dynamics suggested that new lineages of both RSV-A and RSV-B periodically emerge and become the dominant circulating strains [33].

The lineage-specific analysis presented in this study is particularly valuable for characterizing the circulating viruses across Spain. Unlike previous studies that primarily focused on RSV subtypes in restricted geographic regions [34, 35], or faced limitations due to insufficient sequence diversity [25], this study provides a broader overview of the viral ecosystem. This achievement was made possible by the technological advancements in genomic surveillance developed through the RELECOV sequencing network established during the COVID-19 pandemic [36].

Our genomic and phylogenetic analyses identified key mutations in surface proteins, particularly the F protein, which is critical for immunoprophylaxis and vaccine efficacy [1]. The observation that more than half of the detected mutations in



C

Antibody	Antigenic Site	Footprint on F protein	References
Nirsevimab	Ø	F2: 62-69 F1:196-212	Zhu *et al* [29]
ADI15618	Ø	F2: 68,70-72 F1: 201-203,205,206, 209-216, 219	Bonavia *et al*. [20]
Palivizumab	II	F1: 255-276	Mas *et al*. [38]
Clesrovimab	IV	F1 protomer A: 426-429, 432, 440-441, 443, 445-447 F1 protomer B: 160, 182	Tang *et al*. [19]

Figure 4. Location of amino acid changes in RSV-B viruses mapped onto the 3D structure of the prefusion F protein and their association with neutralizing antibody binding sites. (*A*) Structure of the RSV prefusion F protein with antibody binding sites highlighted in color on one protomer. (*B*) Ribbon representation of the F protein showing antibody binding sites in color and the positions of amino acid changes detected in RSV-B viruses indicated as green alpha-carbon spheres. (*C*) List of amino acid residues that define each antibody binding site examined in this study.

both RSV subgroups were minor underscores the importance of rigorous sequence validation, particularly in regions crucial for mAb targeting.

Phylogenetic analyses revealed the evolutionary trajectory of RSV-B, highlighting the transition from the parental lineage B.D.4.1.1 to the predominant B.D.E.1. Additionally, as was previously mentioned, we identified the emergence of the I206M:Q209R:S211N polymorphism at site Ø, accompanied by the S190N and S389P mutations within the parental lineage, likely driven by adaptive pressures.

Despite viral evolution, 2023–2024 RSV-B strains remained susceptible to neutralizing antibodies, suggesting the I206M:Q209R:S211N polymorphism at site Ø has limited impact on current monoclonal immunoprophylaxis. This is consistent with previous studies using recombinant RSV variants, which showed that this triple mutation, identified in the MELODY clinical study, did not confer resistance to nirsevimab [37]. Moreover, the combination I206 M/Q209R, identified both in the phase 2b and MELODY studies, was even associated with enhanced susceptibility to nirsevimab [37]. These observations are further supported by seven clinical isolates from a recent real-world study reported during the preparation of this work, all of which carried the same triple mutation (I206M:Q209R:S211N) and showed no reduced susceptibility to nirsevimab [32]. Altogether, these findings confirm our results and reinforce the effectiveness of the recently approved nirsevimab-based immunoprophylaxis in preventing severe RSV infections in newborns, despite ongoing viral genetic variability.

Furthermore, our findings also support the clinical development of RSM01 [20], derived from the ADI-15618 antibody, as a cost-effective alternative, alongside Clesrovimab [19], as potential future immunoprophylaxis options against currently circulating RSV-B strains. Although a partial reduction in ADI-15618 effectiveness was observed in vitro against one RSV-B virus, no resistance-associated substitutions compromising its activity, or that of its derivative RSM01, have been identified to date. Further studies are needed to assess its significance.

Table 1. Susceptibility of Circulating RSV-B and Reference Viruses to Neutralizing Antibodies

		Nirsevimab		ADI-15618		Clesrovimab		Palivizumab	
Lineage	Virus	IC50 (ng/ml)	Fold Change to A2 Virus	IC50 (ng/ml)	Fold Change to A2 Virus	IC50 (ng/ml)	Fold Change to A2 Virus	IC50 (ng/ml)	Fold Change to A2 Virus
A	A2	1.4	—	0.9	—	1.1	—	187.1	—
B	CH18537	34	30.9	4.8	5.3	3.4	3.1	176.2	1.1
B.D.4.1.1 group3	MD-ISCIII-0688/24[a]	1.3	0.9	2.6	3.0	4.7	4.3	354.4	1.9
B.D.E.1	MD-ISCIII-2484/23*	2.7	1.9	15.4	17.1	3.7	3.4	453.9	2.4

Both circulating B viruses harbor the three site Ø mutations (I206 M, Q209R, S211N) along with all other lineage-defining substitutions.

[a]Additionally carries the minority substitutions L13I and A103 V, whereas * lacks the minority change R42 K. Fold changes for each antibody were calculated by dividing the IC50 values obtained for clinical isolates or CH18537 by the corresponding IC50 value for the A2 virus. Data represent the means of two or three independent experiments.

Table 2. The binding Kinetics of Neutralizing Antibodies to Capture Prefusion F DSCav-1 Proteins

F DSCav-1	Antibody	KD_{app}	Ka_{app} ($M^{-1}s^{-1}$)	Kd_{app} (s^{-1})
A2	Nirsevimab	< 11pM	8.96×10^5	$< 1.00 \times 10^{-5}$
	ADI-15618	< 25pM	4.65×10^5	$< 1.00 \times 10^{-5}$
	Palivizumab	4.55nM	1.66×10^5	7.53×10^{-4}
B 9320	Nirsevimab	< 28pM	3.56×10^5	$< 1.00 \times 10^{-5}$
	ADI-15618	180pM	2.51×10^5	4.58×10^{-5}
	Palivizumab	3.97nM	3.75×10^5	1.49×10^{-3}
MD-ISCIII-0688/24[a]	Nirsevimab	120pM	9.90×10^5	1.22×10^{-4}
	ADI-15618	840pM	4.29×10^5	3.59×10^{-4}
	Palivizumab	3.30nM	3.54×10^5	1.17×10^{-3}

[a]F DSCav-1 protein containing the three site Ø mutations (I206 M, Q209R, S211N), all other B.D.4.1 group 3 defining substitutions, and the minority substitutions L13I and A103 V.

Although our study was limited by assessing only two RSV-B isolates phenotypically and by not including any RSV-A strains, the identification of the K65Q mutation in the B.D.4.1.1 group 2 lineage, detected in a small number of sequences and previously linked to partial reductions in nirsevimab neutralization [30], suggests that viral adaptation may have occurred within the study population in Spain. This observation is consistent with data from clinical trials and real-world studies, where such variants have appeared predominantly in RSV-B and in only a small fraction of cases following nirsevimab use [32, 37]. It would be of interest to determine whether this trend continues in future nirsevimab immunization campaigns.

The absence of frequent mutations at site Ø in RSV-A, along with evidence that the most common RSV-B mutations did not compromise the activity of site Ø-targeting antibodies, indicates that even when occasional escape mutations arise, they do not become dominant. As a result, RSV has not undergone significant antigenic changes to evade monoclonal antibody recognition, despite the extensive administration of nirsevimab to newborns, representing about 1% of the total population.

Moreover, the global emergence of similar mutations and parallel genomic evolution in other geographic regions [31] further supports this conclusion. These findings suggest that mutation selection was likely influenced by factors beyond immunoprophylaxis-driven pressure, such as the structural and functional constraints of the F protein or immune responses from seasonal natural infections.

Based on these findings, it is important to highlight the success of the 2023-2024 nirsevimab immunization campaign in Spain, which effectively reduced hospitalizations without driving the selection of predominant antibody escape mutations. This outcome further validates the current administration schedule for the targeted population.

Finally, this study highlights the critical role of robust genomic surveillance systems, such as Spanish RELECOV network and the integration in the surveillance system SiVIRA for monitoring RSV evolution. These systems provide an essential framework for assessing the impact of emerging RSV variants, particularly in the context of immunoprophylaxis programs and vaccine implementation. Comprehensive monitoring is crucial for ensuring the continued efficacy of preventive measures and adapting strategies in response to the evolving viral landscape.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank Noelia Reyes, Silvia Moreno, Vanessa Montero, Mar Molinero and Mónica Vázquez from the Reference and Research Laboratory for Respiratory Virus, National Centre for Microbiology for their exceptional technical support. We thank the Genomics and Bioinformatics units at the Instituto de Salud Carlos III who collaborated in obtaining genomic sequences. We would also like to thank the RELECOV network members for the experiences shared in the sequencing of respiratory virus genomes. We gratefully

acknowledge all data contributors, authors and their originating laboratories responsible for obtaining the specimens, and their submitting laboratories for generating the genetic sequence and metadata and sharing them via the GISAID initiative and GenBank database (www.ncbi.nlm.nih.gov/genbank/), from which we retrieved the sequences used in this research.

Data availability statement. All the sequences are available in GISAID.

Meeting where the information has previously been presented. 13th INTERNATIONAL RSV SYMPOSIUM (RSV2025). 15th March of 2025. Iguazu (Brazil).

Financial support. This research was partially funded by EU4H-2022-Grant Agreement-101113109, by UNESPA "COVIDSEQ-UNESPA" (MPY 226/22), by Instituto de Salud Carlos III through the Projects PI21CIII/00019, PI21/00377 and PI22/00054, and by Cabildo Insular de Tenerife ["Apuestas científicas del ITER para colaborar en la lucha contra la COVID-19"]. Also, partial funding was received from CLARITY-Horizon Europe Program Grant Agreement 101137201.

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Mazur NI, Terstappen J, Baral R, et al. Respiratory syncytial virus prevention within reach: the vaccine and monoclonal antibody landscape. Lancet Infect Dis **2023**; 23:e2–e21.
2. Nair H, Nokes DJ, Gessner BD, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet **2010**; 375:1545–55.
3. Operational considerations for respiratory virus surveillance in Europe [Internet]. [cited 2024 Sep 19]. Available at: https://www.ecdc.europa.eu/en/publications-data/operational-considerations-respiratory-virus-surveillance-europe.
4. Maintaining surveillance of influenza and monitoring SARS-CoV-2 – adapting Global Influenza surveillance and Response System (GISRS) and sentinel systems during the COVID-19 pandemic [Internet]. [cited 2024 Sep 19]. Available at: https://www.who.int/publications/i/item/maintaining-surveillance-of-influenza-and-monitoring-sars-cov-2-adapting-global-influenza-surveillance-and-response-system-(gisrs)-and-sentinel-systems-during-the-covid-19-pandemic.
5. Vigilancia centinela de Infeccin Respiratoria Aguda en Atencin Primaria IRAs y en Hospitales IRAG Gripe COVID-19 y VRS temporada 2022-2023. Espaa. Instituto de Salud Carlos III; 2023. Available at: https://www.isciii.es/documents/20119/537151/InformeAnualSiVIRATemporada2022-2023.pdf/dffa4c86-a31e-afd6-afc7-5096c6179588.
6. HERA-Incubator Program, ECDC 2021 Grant/2021/PHF/23776 (2021-2022) and EU4Health European Program (2023-2026) a genomic based surveillance of SARS-CoV-2. Available at: https://relecov.isciii.es/es/.
7. Grupo de Trabajo utilización de nirsevimab frente a infección por virus respiratorio sincitial de la Ponencia de Programa y Registro de Vacunaciones. Recomendaciones de utilización de nirsevimab frente a virus respiratorio sincitial para la temporada 2023-2024 Comisión de Salud Pública del Consejo Interterritorial del Sistema Nacional de Salud. Ministerio de Sanidad, julio 2023. Available at: https://www.sanidad.gob.es/areas/promocionPrevencion/vacunaciones/comoTrabajamos/docs/Nirsevimab_2023.pdf.
8. Francisco L, Cruz-Canete M, Perez C, et al. Nirsevimab for the prevention of respiratory syncytial virus disease in children. Statement of the Spanish Society of Paediatric Infectious Disease (SEIP). An Pediatr (Engl Ed) **2023**; 99:257–63.
9. Venkatesan P. First RSV vaccine approvals. Lancet Microbe **2023**; 4:e577.
10. Barbas Del Buey JF, Inigo Martinez J, Gutierrez Rodriguez MA, et al. The effectiveness of nirsevimab in reducing the burden of disease due to respiratory syncytial virus (RSV) infection over time in the Madrid region (Spain): a prospective population-based cohort study. Front Public Health **2024**; 12:1441786.
11. Mazagatos C, Mendioroz J, Rumayor MB, et al. Estimated impact of nirsevimab on the incidence of respiratory syncytial virus infections requiring hospital admission in children< 1 year, weeks 40, 2023, to 8, 2024, Spain. Influenza Other Respir Viruses **2024**; 18:e13294.
12. Jimeno Ruiz S, Pelaez A, Labourt A, et al. Evaluating the effectiveness of nirsevimab in reducing pediatric RSV hospitalizations in Spain. Vaccines (Basel) **2024**; 12:1160.
13. Salimi V, Viegas M, Trento A, et al. Proposal for human respiratory syncytial virus Nomenclature below the Species level. Emerg Infect Dis **2021**; 27:1–9.
14. Trento A, Galiano M, Videla C, et al. Major changes in the G protein of human respiratory syncytial virus isolates introduced by a duplication of 60 nucleotides. J Gen Virol **2003**; 84:3115–20.
15. Eshaghi A, Duvvuri VR, Lai R, et al. Genetic variability of human respiratory syncytial virus A strains circulating in Ontario: a novel genotype with a 72 nucleotide G gene duplication. PLoS One **2012**; 7:e32807.
16. Ramaekers K, Rector A, Cuypers L, Lemey P, Keyaerts E, Van Ranst M. Towards a unified classification for human respiratory syncytial virus genotypes. Virus Evol **2020**; 6: veaa052.
17. Chen J, Qiu X, Avadhanula V, et al. Novel and extendable genotyping system for human respiratory syncytial virus

based on whole-genome sequence analysis. Influenza Other Respir Viruses **2022**; 16:492–500.
18. Goya S, Ruis C, Neher RA, et al. Standardized phylogenetic classification of human respiratory syncytial virus below the subgroup level. Emerg Infect Dis **2024**; 30:1631–41.
19. Tang A, Chen Z, Cox KS, et al. A potent broadly neutralizing human RSV antibody targets conserved site IV of the fusion glycoprotein. Nat Commun **2019**; 10:4153.
20. Bonavia A, Levi M, Rouha H, et al. Rsm01, a novel respiratory syncytial virus monoclonal antibody: preclinical characterization and results of a first-in-human, randomised clinical trial. BMC Infect Dis **2024**; 24:1378.
21. Shu Y, McCauley J. Gisaid: global initiative on sharing all influenza data - from vision to reality. Euro Surveill **2017**; 22:30494.
22. Aksamentov I, Roemer C, Hodcroft EB, Neher RA. Nextclade: clade assignment, mutation calling and quality control for viral genomes. J Open Source Softw **2021**; 6: 3773.
23. Iglesias-Caballero M, Camarero-Serrano S, Varona S, et al. Genomic characterisation of respiratory syncytial virus: a novel system for whole genome sequencing and full-length G and F gene sequences. Euro Surveill **2023**; 28:2300637.
24. Ewels PA, Peltzer A, Fillinger S, et al. The nf-core framework for community-curated bioinformatics pipelines. Nat Biotechnol **2020**; 38:276–8.
25. Davina-Nunez C, Perez-Castro S, Cabrera-Alvargonzalez JJ, Montano-Barrientos J, Godoy-Diz M, Regueiro B. The modification of the Illumina((R)) CovidSeq workflow for RSV genomic surveillance: the genetic variability of RSV during the 2022-2023 season in northwest Spain. Int J Mol Sci **2023**; 24:16055.
26. Katoh K, Misawa K, Kuma K, Miyata T. Mafft: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res **2002**; 30: 3059–66.
27. Price MN, Dehal PS, Arkin AP. Fasttree 2–approximately maximum-likelihood trees for large alignments. PLoS One **2010**; 5:e9490.
28. Magro M, Andreu D, Gomez-Puertas P, Melero JA, Palomo C. Neutralization of human respiratory syncytial virus infectivity by antibodies and low-molecular-weight compounds targeted against the fusion glycoprotein. J Virol **2010**; 84:7970–82.
29. Gilman MS, Castellanos CA, Chen M, et al. Rapid profiling of RSV antibody repertoires from the memory B cells of naturally infected adult donors. Sci Immunol **2016**; 1: eaaj1879.
30. Zhu Q, McLellan JS, Kallewaard NL, et al. A highly potent extended half-life antibody as a potential RSV vaccine surrogate for all infants. Sci Transl Med **2017**; 9:eaaj1928.
31. Yunker M, Fall A, Norton JM, et al. Genomic evolution and surveillance of respiratory syncytial virus during the 2023-2024 season. Viruses **2024**; 16:1122.
32. Fourati S, Reslan A, Bourret J, et al. Genotypic and phenotypic characterisation of respiratory syncytial virus after nirsevimab breakthrough infections: a large, multicentre, observational, real-world study. Lancet Infect Dis **2025**; 25:301–11.
33. Yu JM, Fu YH, Peng XL, Zheng YP, He JS. Genetic diversity and molecular evolution of human respiratory syncytial virus A and B. Sci Rep **2021**; 11:12941.
34. Calderon A, Pozo F, Calvo C, et al. Genetic variability of respiratory syncytial virus A in hospitalized children in the last five consecutive winter seasons in central Spain. J Med Virol **2017**; 89:767–74.
35. Vila J, Lera E, Peremiquel-Trillas P, et al. Increased RSV-A bronchiolitis severity in RSV-infected children admitted to a reference center in Catalonia (Spain) between 2014 and 2018. J Pediatric Infect Dis Soc **2023**; 12:180–3.
36. Vazquez-Moron S, Iglesias-Caballero M, Lepe JA, et al. Enhancing SARS-CoV-2 surveillance through regular genomic sequencing in Spain: the RELECOV network. Int J Mol Sci **2023**; 24:8573.
37. Ahani B, Tuffy KM, Aksyuk AA, et al. Molecular and phenotypic characteristics of RSV infections in infants during two nirsevimab randomized clinical trials. Nat Commun **2023**; 14:4347.
38. Mas V, Nair H, Campbell H, Melero JA, Williams TC. Antigenic and sequence variability of the human respiratory syncytial virus F glycoprotein compared to related viruses in a comprehensive dataset. Vaccine **2018**; 36:6660–73.