



Genomic evolution of human respiratory syncytial virus during a decade (2013–2023): bridging the path to monoclonal antibody surveillance

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SUMMARY

Objectives: This study investigated the prevalence, genetic diversity, and evolution of human respiratory syncytial virus (HRSV) in Barcelona from 2013 to 2023.

Methods: Respiratory specimens from patients with RTI suspicion at Hospital Universitari Vall d'Hebron were collected from October 2013 to May 2023 for laboratory-confirmation of respiratory viruses. Next-

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generation sequencing was performed in randomly-selected samples with Illumina technology. Phylogenetic analyses of whole genome sequences were performed with BEAST v1.10.4. Signals of selection and evolutionary pressures were inferred by population dynamics and evolutionary analyses. Mutations in major surface proteins were genetic and structurally characterised, emphasizing those within antigenic epitopes.

Results: Analyzing 139,625 samples, 5.3% were HRSV-positive (3008 HRSV-A, 3882 HRSV-B, 56 HRSV-A and -B, and 495 untyped HRSV), with a higher prevalence observed in the paediatric population. Pandemic-related shifts in seasonal patterns returned to normal in 2022–2023. A total of 198 whole-genome sequences were obtained for HRSV-A (6.6% of the HRSV-A positive samples) belonging to GA2.3.5 lineage. For HRSV-B, 167 samples were sequenced (4.3% of the HRSV-B positive samples), belonging to GB5.0.2, GB5.0.4a and GB5.0.5a. HRSV-B exhibited a higher evolution rate. Post-SARS-CoV-2 pandemic, both subtypes showed increased evolutionary rates and decreased effective population size initially, followed by a sharp increase. Analyses indicated negative selective pressure on HRSV. Mutations in antigenic epitopes, including S276N and M274I in palivizumab-targeted site II, and I206M, Q209R, and S211N in nirsevimab-targeted site Ø, were identified.

Discussion: Particularly in the context of the large-scale use in 2023–2024 season of nirsevimab, continuous epidemiological and genomic surveillance is crucial.

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Introduction

The epidemics of human respiratory syncytial virus (HRSV) have a major public health impact, as it is the primary cause of lower respiratory tract infections (LRTI) in children and infants. These infections impose a substantial health burden on those under 1 year of age, requiring intensive care and respiratory support in some cases.^{1–3} Reinfections are common throughout life, usually associated with mild disease or asymptomatic infections, but they can also lead to severe respiratory illnesses in vulnerable adults, such as those with comorbidities and the elderly.⁴ HRSV circulation is observed primarily during the months of November to January, when it has been historically predominant in countries in the temperate northern hemisphere, like Spain.^{5,6}

HRSV, a member of the *Pneumoviridae* family along with human metapneumovirus (HMPV), is an enveloped virus with a linear, negative-sense, single-stranded RNA genome. This 15-kb-genome contains 10 genes encoding 11 proteins, which are the non-structural proteins 1 (NS-1) and 2 (NS-2), the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the small hydrophobic protein (SH), the attachment glycoprotein (G), the fusion protein (F), the M2 (which codes for M2-1 and M2-2), and the polymerase or large protein (L).⁷

The classification of HRSV into HRSV-A or -B subtypes, and its further subdivision into multiple genotypes, is based on the G protein-coding sequence, although a lack of standardization led to the proposal of a new system in 2020.⁸ Unlike other RNA viruses, there is no documented evidence of recombination events generating diverse offspring for HRSV in vivo.⁹ Instead, mutations in HRSV arise spontaneously due to the RNA-dependent RNA polymerase's lack of proofreading capabilities. This deficiency contributes to the frequent emergence of new mutations, obtaining a dynamic pool of genetically distinct viral variants enabling rapid adaptation to changing environments. Specifically, a notably higher occurrence rate of single nucleotide polymorphisms (SNPs) has been observed in the G gene of both HRSV-A and -B compared to other genes.⁹ This heightened SNP frequency underscores the specific genetic dynamics in the G gene, suggesting a potential role in the virus' adaptive strategies. This variability influences evolutionary dynamics, which may correlate with inter-seasonal transmission and the co-circulation dynamics of this virus.¹⁰

Given the significant morbidity and public health impact of HRSV infection, substantial efforts have been made to develop highly effective and cost-efficient HRSV immunoprophylaxis based on vaccination or neutralizing monoclonal antibodies (mAb). As reviewed by Mazur et al.,¹¹ more than 30 HRSV prevention candidates are

currently in clinical development. The F protein, with up to six antigenic sites described, has been selected as the primary target for vaccine and mAb development due to its immunogenic properties and low mutation rate.^{12,13} While palivizumab [EMA/H/C/000257] has been the only licensed mAb since 1999, recently a new mAb, nirsevimab [EMA/H/C/005304], has been approved for widespread use in young children. This targets antigenic site Ø in the pre-F conformation and will be administered to all healthy children under 6 months old and the high-risk paediatric population under 2 years old starting from October 2023 in many Spanish regions.¹⁴ Additionally, clesrovimab (MK-1654), an mAb against site IV in both pre-F and post-F conformations, is in phase 3 clinical trials and may become a future alternative. At the protein level, the antigenic epitopes for these mAbs are highly conserved based on currently available sequence data, and therefore, the acquisition of single mutations into the coding sequence is very restricted. Moreover, the limited use of palivizumab to a small paediatric population has not exerted significant selective pressure, resulting in the emergence of only a minimal number of monoclonal antibodies resistant mutants (MARMs).¹⁵ However, the emergence of MARMs for previous promising candidates such as suptavumab¹⁶ highlights the importance of global genomic surveillance of viral genomes, especially of these epitopes.

The aim of this study was to describe the genetic diversity, genotype circulation and evolution of HRSV detected in paediatric and adult populations attended at a tertiary university hospital in Barcelona from the 2013 to the 2023 seasons based on the whole genome sequence, focusing on the antigenic epitopes targeted by licensed and promising mAbs.

Materials and methods

Sample collection and respiratory viruses' laboratory confirmation

From October 2013 (week 40/2013) to May 2023 (week 20/2023), respiratory specimens from paediatric and adult patients with suspicion of RTI attended at the Hospital Universitari Vall d'Hebron (HUVH) were collected for the laboratory confirmation of respiratory viruses. Detection of respiratory viruses was performed as previously described.¹⁷ Briefly, during HRSV and Influenza epidemics, rapid tests were performed for a fast diagnosis (Alere BinaxNOW Influenza A&B/RSV, Alere, USA until 2015; Sofia RSV FIA, Quidel, USA until 2018; GenXpert Flu/RSV XC, Cepheid, USA since 2018). Those samples with a negative result for these tests or received out of the above-mentioned epidemics, were analysed by

immuno-fluorescence (D3 Ultra 8 DFA Respiratory Virus, Diagnostic HYBRIDS, USA) or mainly by real-time RT-PCR (Anyplex II RV16, Seegene, Korea, until 2015; Allplex Respiratory Panels 1-3, Seegene, Korea, since 2015). Institutional Review Board approval (PR(AG)78/2014) was obtained from the hospital's Clinical Research Ethics Committee.

Whole genome sequencing

Samples were randomly selected throughout all the seasons studied, selecting at least one sample ($Ct < 30$) per week of each HRSV subtype (HRSV-A and B), from patients of both sexes and all age groups (0–2, 2–5, 5–15, 15–64 and ≥ 64 years old) when available. Amplification of HRSV genome was performed in four overlapping 4-kb-amplicons with the SuperScript IV One-Step RT-PCR (Invitrogen, USA) with the primers designed by Bose et al. and Schobel et al.,^{18,19} the PCR protocol was kindly provided by the WHO Collaborating Centre for Reference and Research on Influenza in Australia.

PCR products were purified with AMPure Beads (Beckman Coulter, USA), and quantified with NanoDrop One (ThermoScientific, USA). Amplicons were normalized and pooled. NGS library was performed with DNA Prep (Illumina, CA, USA), and indexed with IDT for Illumina DNA/RNA UD Indexes Set A-D (384 IDX) (Illumina, USA). Products were quality tested with 4200 TapeStation System (Agilent, USA). The final pool was loaded in a MiSeq Reagent Kit 600v3 cartridge (Illumina, CA, USA) and sequenced using a MiSeq platform (Illumina, CA, USA).

FASTQ files were parsed to Trimmomatic v0.39.²⁰ Sliding window trimming option was enabled, using a 10-base wide window and cutting when the average quality dropped below Q30. Leading and trailing bases below Q30 were removed. FASTQ files were processed by Bowtie2 v2.3.5.1²¹ for host sequences removal (human genome assembly GRCh38) and primer sequences were trimmed with BBDuk v38.91 (script bbdduk.sh; BBDuk - Bushnell B. - sourceforge.net/projects/bbmap). High-quality filtered reads obtained after pre-processing were *de novo* assembled by SPAdes v3.15.2,²² using `-naviral` option. Resulting scaffolds were classified by subtype through BLASTN v2.14.0+.²³ Scaffolds were mapped to its subtype's reference genome with Minimap2 v2.26-r1175.²⁴ Metadata from GenBank available sequences was downloaded from RSV page of NextStrain²⁵ for both subtypes. Sequences underwent filtering based on specific criteria: 100% coverage of the G and F genes, at least 99% coverage of the viral genome, complete sampling date information, and classification as the most prevalent genotypes within our region (GA2.3.5, formerly ON1, for HRSV-A, and GB5.0.1, formerly BA9, for HRSV-B). Following this filtering process, the samples obtained ranged from 2011-08-22 to 2023-12-23 and from 2002-01-08 to 2009-09-24, for HRSV-A and B, respectively. The oldest sequence identified was designated as the reference for HRSV-A (accession number MH447951). However, in the case of HRSV-B, the oldest sequence exhibited an insertion of 3 nucleotides (A3246AGAA) that was not found in any of the other analysed samples. Consequently, this sequence was discarded, and the reference for HRSV-B was selected as the oldest from the resulting dataset (accession number JX576761). Non-overlapping scaffolds were concatenated by N-string based on mapping positions. Overlapping scaffolds were joined based on sequence homology, the CIGAR and kmer coverage, defined by SPAdes. In cases where the overlapped sequences exhibited less than 100% identity, the sequence with greater coverage was designated as the consensus. Read coverage for each position was determined, masking positions with a coverage below 20 reads. Sequences with a percentage of N above 1% were excluded.

Genetic characterization of whole genome sequences

Whole genome sequences were aligned to a set of references (Supplementary Table 1) representative of all genotypes, for HRSV-A and HRSV-B separately, with MAFFT v7.475.²⁶ Phylogenetic analyses were carried out with BEAST v1.10.4²⁷ with a chain length of 100,000,000, sampling every 1000 steps, reaching ESS values over 200 for all parameters. Nucleotide substitution model was inferred through IQ-TREE v1.6.12 (ModelFinder integrated),^{28,29} and GTR was included as a prior in all BEAST analyses in both cases. Substitution rates were calculated with a shorter BEAST run with a chain length of 10,000,000 and included as a prior in the final analyses. The clock type was set to "Strict clock" and "Uncorrelated relaxed clock" with lognormal distribution for HRSV-A and -B, respectively. Tree prior was configured in both cases to "Coalescent: Bayesian SkyGrid" for effective population size estimation. Time at last transition point was set to 95 for HRSV-A, and 56 for HRSV-B, and the number of parameters was established at 380 and 224 for HRSV-A and -B, respectively. Convergence was achieved after combining several runs. Consensus trees were computed with TreeAnnotator v1.10.4 using a 10-states burnin. Finally, genotyping was based on these phylogenetic analyses using the new nomenclature proposed by Goya et al.⁸

Additionally, four more BEAST analyses were performed, comparing pre- and post-pandemic samples for both subtypes. TempEst was used to infer temporal signal of datasets, due to the reduction in time range and number of samples. The same parameters used earlier were employed, except for tree prior, which was changed to "Coalescent: Constant Size."

Amino acid substitutions of all 11 proteins were determined using a combination of custom Python and Bash scripts. High-quality, previously filtered reads were mapped to defined references by Minimap2 v2.26-r1175.²⁴ Variant calling was performed by LoFreq v2.1.5,³⁰ and mutations were subsequently filtered to retain only those with a minimum allele frequency of 5%.

Selection pressures were inferred for protein coding sequences (CDS) and individual codons, applying Tajima's D³¹ and dN/dS ratio, respectively. Tajima's D was calculated by the `tajima.test()` function of `pegas` (Population and Evolutionary Genetics Analysis System) package³² in R. Additionally, dN/dS ratio was computed through the Datamonkey Webserver using the single likelihood ancestor counting (SLAC) algorithm.³³ Positively and negatively selected sites for both F and G proteins were identified through Datamonkey Webserver³⁴ with 4 different algorithms: single likelihood ancestor counting (SLAC),³³ fixed effects likelihood (FEL),³³ mixed effects model of evolution (MEME)³⁵ and a fast, unconstrained Bayesian approximation for inferring selection (FUBAR).³⁶ The best-fit substitution model was also determined by Datamonkey Webserver. Each site was considered under diversifying or purifying selection only when two or more algorithms agreed with statistical significance ($p < 0.05$ or Bayes > 50).

Genetic characterization of major surface proteins

Amino acid changes for proteins F and G were analysed, specifically those located in F protein's antigenic sites II (residues 254–277), IV (residues 426–447) and Ø (residues 62–69/196–212), targeted by palivizumab, clesrovimab and nirsevimab, respectively.^{37,38} Maximum likelihood trees were generated using IQ-TREE v1.6.12 software,²⁸ using a bootstrap of 1000, for proteins F and G of each subtype. Mutations were annotated in phylogenetic trees for each protein using `treeSub` software.³⁹

Table 1

Demographic data of total patients and patients with laboratory-confirmed HRSV. Seasons (yyyy–yyyy) start at week 40 and last until week 20 of the following natural year, while inter-seasonal periods (yyyy) last from week 21 until week 39 of the same year.

Season	Total samples received	Total patients	Total samples positive for at least 1 respiratory virus		HRSV laboratory-confirmed			
					Samples		Patients	
2013–2014	3588	2273	1056	29.4%	334	9.3%	320	14.1%
2014	796	510	81	10.2%	5	0.6%	5	1.0%
2014–2015	5190	3683	2050	39.5%	449	8.7%	409	11.1%
2015	1017	741	310	30.5%	4	0.4%	3	0.4%
2015–2016	6499	4802	3187	49.0%	608	9.4%	543	11.3%
2016	1046	786	435	41.6%	7	0.7%	5	0.6%
2016–2017	6381	4759	3391	53.1%	734	11.5%	691	14.5%
2017	1455	1041	587	40.3%	5	0.3%	4	0.4%
2017–2018	8033	6118	4642	57.8%	846	10.5%	781	12.8%
2018	1131	849	474	41.9%	4	0.4%	4	0.5%
2018–2019	7237	5383	4210	58.2%	692	9.6%	639	11.9%
2019	1259	813	465	36.9%	2	0.2%	2	0.2%
2019–2020	7061	5294	3478	49.3%	603	8.5%	569	10.7%
2020	2257	1696	428	19.0%	5	0.2%	5	0.3%
2020–2021	9547	6244	3491	36.6%	215	2.3%	210	3.4%
2021	6922	5440	2260	32.6%	661	9.5%	642	11.8%
2021–2022	32,656	23,335	6267	19.2%	751	2.3%	688	2.9%
2022	13,619	10,681	1608	11.8%	153	1.1%	151	1.4%
2022–2023	23,931	17,136	7288	30.5%	1363	5.7%	1256	7.3%
Overall	139,625	101,584	45,708	32.7%	7441	5.3%	6927	6.8%

Results

HRSV epidemiology

Over these 10 consecutive seasons, from 2013 to 2023, 139,625 samples from 101,584 patients were collected for respiratory viruses' detection, of which 45,708 (32.7%) were laboratory-confirmed for at least one respiratory virus. Overall, 7441 (5.3%) samples from 6927 (6.8%) patients were laboratory-confirmed for HRSV (Table 1).

Concerning the distribution of HRSV laboratory-confirmed cases between paediatric (< 18 years old) and adult (≥18 years old) cohorts, there was a higher prevalence in the paediatric population (9.30%) compared to the adult population (2.65%), as expected (Table 2).

However, this prevalence decreased upon the SARS-CoV-2 pandemic, despite an increase in the absolute number of patients attended, and coincided with a rise in the median age. For the adult population, there was an increase in the number of patients and prevalence of HRSV laboratory-confirmed samples since the 2015–2016 season. This trend reversed during the SARS-CoV-2

pandemic season (2020–2022) but increased again during the last season, reaching levels similar to those in pre-pandemic seasons.

HRSV showed a marked seasonality until the emergence of SARS-CoV-2 (Fig. 1). Prior to the 2020–2021 season, HRSV consistently circulated between weeks 45 and 5 of the following year, displaying a characteristic epidemic peak in weeks 49–01. However, this seasonality changed upon the SARS-CoV-2 pandemic, not being detected during the 2020–2021 season and presenting two successive epidemic peaks afterwards, which were separated by the fifth wave of SARS-CoV-2 lineage Delta. Due to the proximity of these two peaks, they have been considered as an only epidemic event in this study, merging seasons 2020–2021 and 2021–2022 in a single season (2020–2022). Over the last season, 2022–2023, HRSV recovered its pre-pandemic seasonality, and peaked on week 47. Despite the re-establishment of the seasonality, HRSV detection diminished but did not stop between epidemic peaks, and HRSV was detected during summer 2022.

Regarding HRSV typing, both genetic groups (HRSV-A and HRSV-B) co-circulated, showing a triennial shift in predominance, alternating B-B-B-A (Fig. 2).

Table 2

Characteristics of HRSV laboratory-confirmed patients. Distribution of age and sex for each cohort (paediatric and adult populations).

Season	Paediatric population					Adult population				
	n (%)	Median age (percentil 25; percentil 75)	M	F	M:F	n (%)	Median age (percentil 25; percentil 75)	M	F	M:F
2013–2014	306 (14.23%)	0.65 (0.18; 1.53)	178	128	1.39:1	28 (1.95%)	61.32 (54.02; 65.43)	12	16	0.75:1
2014	4 (0.91%)	1.25 (0.68; 1.87)	3	1	3:1	1 (0.28%)	61.08 (61.08; 61.08)	1	0	-
2014–2015	385 (14.06%)	0.53 (0.17; 1.82)	233	152	1.53:1	64 (2.61%)	61.22 (51.81; 67.57)	42	22	1.91:1
2015	2 (0.26%)	0.04 (0.04; 0.05)	0	2	-	2 (0.81%)	67.62 (61.97; 73.26)	2	0	-
2015–2016	474 (14.87%)	0.82 (0.19; 2.1)	281	193	1.46:1	134 (4.05%)	66.23 (51.99; 75.8)	77	57	1.35:1
2016	6 (0.78%)	5.24 (0.44; 9.62)	4	2	2:1	1 (0.36%)	70.23 (70.23; 70.23)	1	0	-
2016–2017	494 (17.62%)	0.87 (0.3; 2.55)	264	230	1.15:1	240 (6.71%)	72.96 (57.29; 84.07)	109	131	0.83:1
2017	4 (0.50%)	0.36 (0.2; 0.96)	1	3	0.33:1	1 (0.15%)	40.65 (40.65; 40.65)	0	1	0:1
2017–2018	505 (15.80%)	0.83 (0.23; 2.41)	262	243	1.08:1	341 (7.05%)	74.23 (61.39; 84.13)	173	168	1.03:1
2018	3 (0.50%)	0.17 (0.15; 7.62)	3	0	-	1 (0.19%)	72.19 (72.19; 72.19)	0	1	-
2018–2019	461 (13.91%)	0.81 (0.23; 2.2)	245	216	1.13:1	231 (5.89%)	71.02 (58.4; 81.19)	110	121	0.91:1
2019	2 (0.33%)	0.58 (0.35; 0.82)	1	1	1:1	-	-	-	-	-
2019–2020	416 (14.59%)	0.90 (0.25; 2.41)	221	195	1.13:1	187 (4.44%)	68.9 (59.01; 82.67)	90	97	0.93:1
2020	5 (0.28%)	4.59 (1.34; 5.07)	2	3	0.67:1	-	-	-	-	-
2020–2022	1413 (6.36%)	1.84 (0.86; 3.22)	732	681	1.07:1	214 (0.80%)	67.36 (56.24; 80.58)	98	116	0.84:1
2022	114 (0.0475)	1.28 (0.49; 2.4)	69	45	1.53:1	39 (0.35%)	66.22 (45.69; 84.75)	14	25	0.56:1
2022–2023	633 (11.30%)	1.28 (0.36; 3.57)	317	315	1.01:1	730 (3.98%)	74.18 (55.88; 85.19)	284	446	0.64:1
Total	5227 (9.30%)	1.13 (0.32; 10.53)	2816	2410	1.17:1	2214 (2.65%)	71.28 (57.15; 83.12)	1013	1201	0.84:1

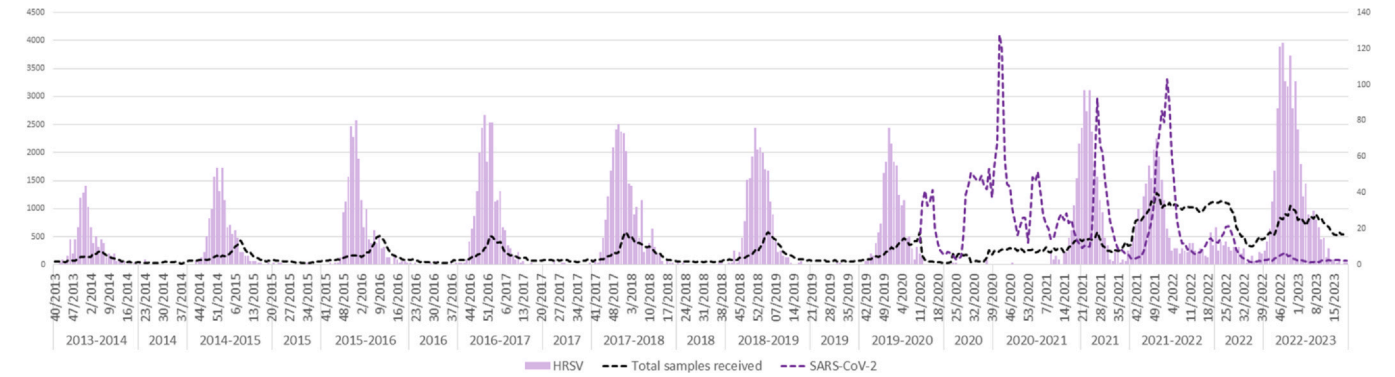


Fig. 1. Seasonality of HRSV. The X-axis represents the timeline of the study, where epidemiological seasons were considered to last from week 40 to week 20 of the following year. The left Y-axis represents the number of total samples received (black dotted line) and the number of SARS-CoV-2 laboratory-confirmed samples (purple dotted line); the right Y-axis represents the number of HRSV laboratory-confirmed samples (pink bars).

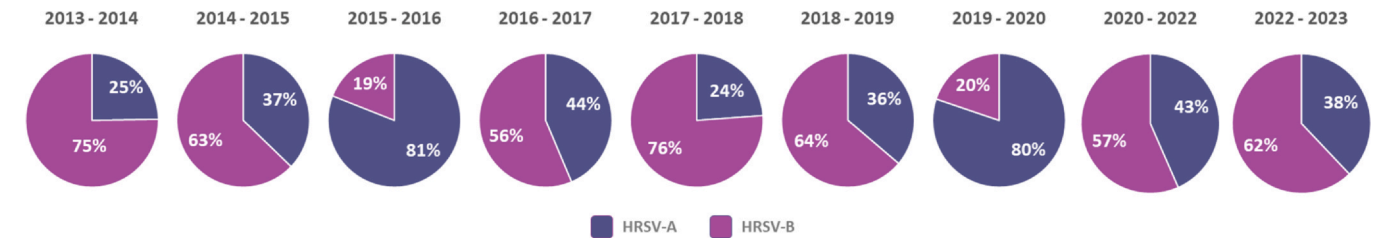


Fig. 2. Circulation of HRSV-A and -B throughout 2013–2023 seasons. Seasons 2020–2021 and 2021–2022 are comprised in the represented season 2020–2022, as both peaks were considered to belong to the same epidemic.

Table 3
Distribution of samples selected for whole genome sequencing per season and subtype.

	HRSV-A	HRSV-B
2013–2014	6	8
2014–2015	13	11
2015–2016	21	9
2016–2017	26	22
2017–2018	10	26
2018–2019	21	13
2019–2020	40	7
2020–2022	37	43
2022–2023	24	28
Total	198	167

Genetic characterization of viral strains

Over the entire study period, between October 2013 and October 2023, a total of 365 samples (198 for HRSV-A and 167 for HRSV-B) were successfully sequenced, ranging from 6 to 43 samples per season and genotype (Table 3). The efficiency of this protocol with samples with a Ct < 30 is 81.0% for HRSV-A and 83.2% for HRSV-B. Whole genome sequences are available at GISAID repository (identifiers EPI_ISL_18682082 to EPI_ISL_18682446).

Phylogenetic analyses of the whole genome were ultimately conducted using the GTR model. Phylogenetic trees of HRSV-A and -B (Fig. 3) unveiled that HRSV-B exhibited a higher evolution rate than HRSV-A throughout the study period. All HRSV-A samples were attributed to the GA2.3.5 lineage, while HRSV-B samples were distributed across three different genetic groups. The oldest sequences belonged to GB5.0.2 and GB5.0.4a, and samples since 2015 were categorized under GB5.0.5a. Furthermore, a noticeable divergence trend was observed for HRSV-B, where samples from the same seasons tended to group together, whereas no such differentiation was observed for HRSV-A. However, samples circulating since the emergence of SARS-CoV-2 group differed from pre-pandemic samples.

The evolutionary rate for HRSV-A was 5.1989×10^{-4} nucleotide substitutions/site/year, with a 95% highest probability density (HPD) interval of $[4.4295 \times 10^{-4}, 5.8102 \times 10^{-4}]$. In contrast, HRSV-B displayed a higher rate at 7.5365×10^{-4} nucleotide substitutions/site/year, with a 95% HPD interval of $[7.1262 \times 10^{-4}, 7.9537 \times 10^{-4}]$. The time to most recent common ancestor (tMRCA) for the tree root was inferred in 1933 (95% HPD [1924,1948]) for HRSV-A, and 1968 (95% HPD [1967,1970]) for HRSV-B.

Regarding the Bayesian SkyGrid reconstruction, HRSV-A exhibited a fluctuating effective population size (N_e) of approximately 1000 since the 1950s, whereas HRSV-B N_e varied within the range of 10–100 since the 1980s (Fig. 3). Phylogenetic trees corroborated this distinction, with HRSV-A pre-pandemic sequences maintaining variability across seasons and grouping in the same nodes. HRSV-B nodes showed stronger season-to-season linkage, indicating replacement from one season to another and subsequent variability reduction over consecutive years. Both subtypes experienced a notable N_e decrease in 2020, in the SARS-CoV-2 pandemic's onset, leading to a substantial reduction in genetic variability. At the resurgence of HRSV after the emergence of SARS-CoV-2, both subtypes rapidly increased N_e , suggesting recovery in the genetic diversity pool.

Coefficients of correlation for pre- and post-pandemic datasets with TempEst were 0.8183 and 0.9903 for HRSV-A, and 0.9944 and 0.9216 for HRSV-B, showing a high temporal signal. Evolutionary rates estimated by BEAST were 6.543×10^{-4} and 8.441×10^{-4} nucleotide substitutions/site/year for pre- and post-pandemic HRSV-A, and 7.907×10^{-4} and 1.051×10^{-3} for pre- and post-pandemic HRSV-B (Fig. 4), showing an increase in evolutionary rates after SARS-CoV-2 pandemic in both subtypes.

Regarding the selective pressure, Tajima's D values suggest negative selective pressure affecting all 11 proteins in both subtypes (Table 4). For HRSV-A, proteins SH, N and G showed non-significant p-values, while for HRSV-B only M2–2. Also, synonymous and non-synonymous mutations were analysed across protein coding consensus sequences (Supplementary Fig. 1). HRSV-B exhibited a higher

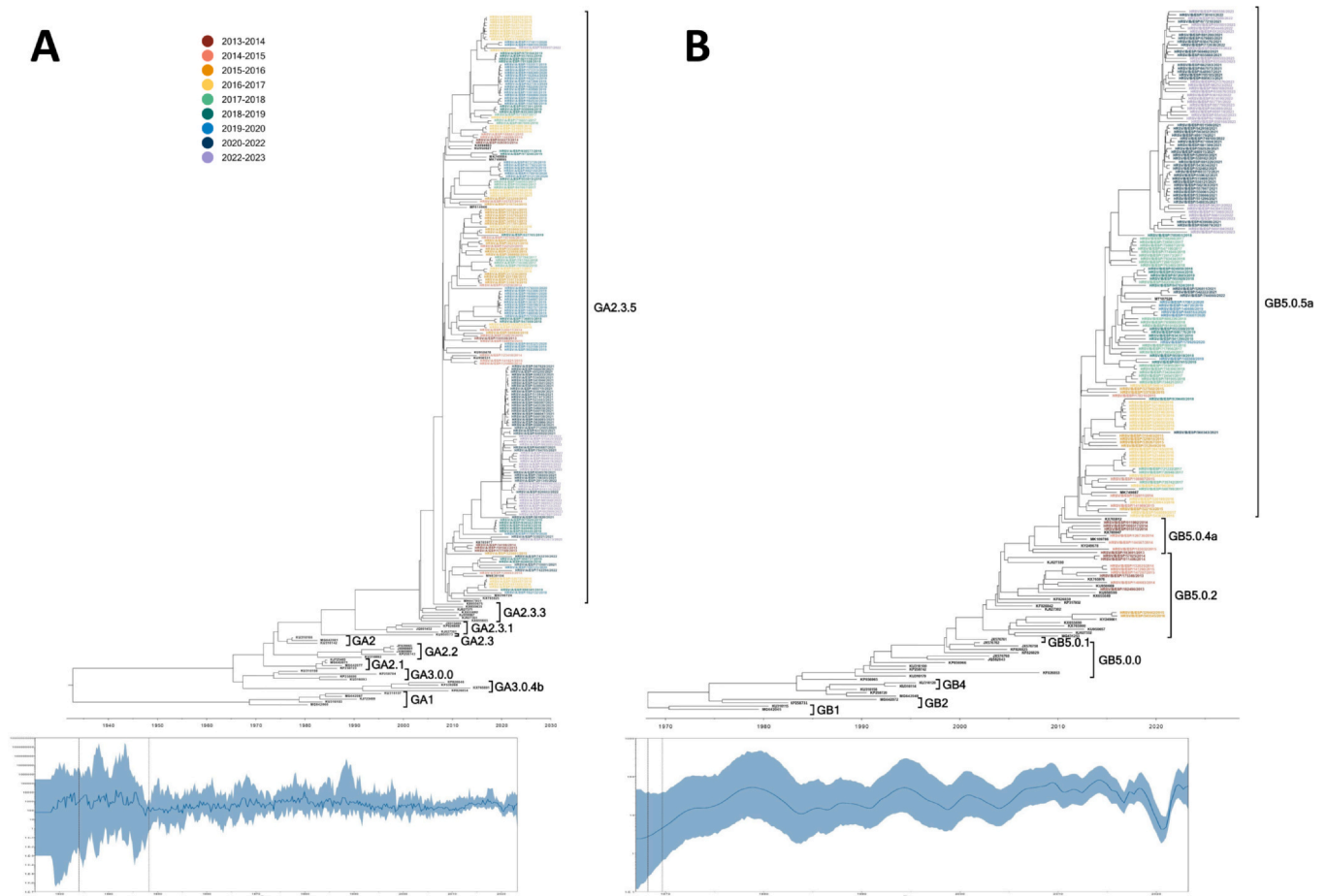


Fig. 3. Phylogenetic trees of whole-genome sequences of (A) HRSV-A and (B) HRSV-B. Both phylogenetic trees were inferred by using the maximum likelihood method, based on the General Time Reversible model, and performed with BEAST v1.10.4. Sequences generated in this study are marked with coloured solid circles, while reference sequences downloaded from GenBank are not marked. The effective population size estimation is represented with skygrid graphics scaled under the respective evolutive trees.

mutation count, both synonymous and non-synonymous, correlating with its elevated mutation rate. Both subtypes exhibited concentrated non-synonymous mutations in G, M2-2, F, and L proteins. In G proteins, there were more residues under diversifying selection in HRSV-A than in HRSV-B (6 vs 3, respectively), and more residues

under purifying selection in HRSV-B than in HRSV-A (22 vs 15) (Supplementary Tables 2 and 3). On the other hand, only one site was under diversifying selection in the F protein of both HRSV-A and -B (Supplementary Tables 4 and 5). In HRSV-A, this site was located at the residue 122, within the pep27 region, while in HRSV-B, it was

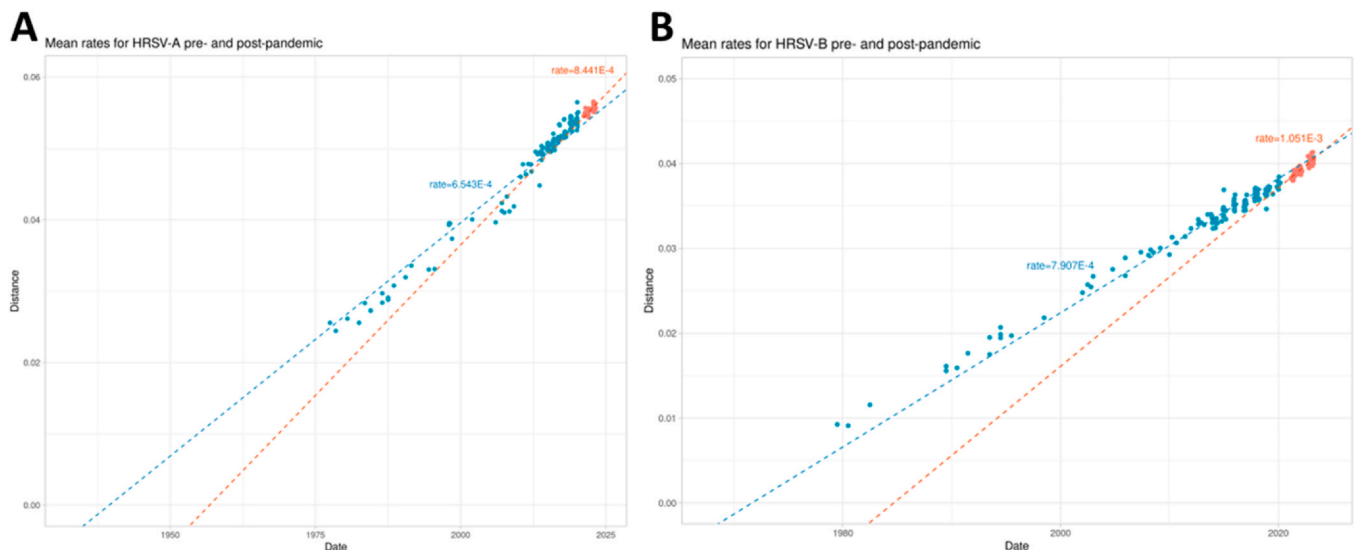


Fig. 4. Evolutionary rates of HRSV-A (A) and HRSV-B (B). Turquoise dots represent those sequences belonging to the pre-pandemic era, while orange dots represent those belonging to the post-pandemic era.

Table 4

Tajima's values for HRSV-A and HRSV-B proteins. Proteins with a statistically significant p-value are written in bold, while those with a p-value over 0.05 are written in italics.

HRSV subtype	Protein	D	Normalized p-value
HRSV-A	NS1	-2.103901	0.03538706
	NS2	-2.086542	0.03692959
	<i>N</i>	<i>-1.916436</i>	<i>0.05530968</i>
	P	-2.335867	0.01949818
	M	-2.117348	0.03423037
	<i>SH</i>	<i>-1.860875</i>	<i>0.06276186</i>
	<i>G</i>	<i>-1.840889</i>	<i>0.06563777</i>
	F	-2.42811	0.01517773
	M2-1	-2.248454	0.02454727
	M2-2	-2.160878	0.03070477
	L	-2.162455	0.03058309
HRSV-B	NS1	-2.403953	0.01621885
	NS2	-2.496599	0.01253906
	N	-2.500574	0.01239922
	P	-2.359214	0.01831371
	M	-2.147428	0.03175923
	SH	-2.268595	0.02329297
	G	-2.129397	0.03322141
	F	-2.168629	0.03011088
	M2-1	-2.341687	0.0191968
	<i>M2-2</i>	<i>-1.904437</i>	<i>0.05685326</i>
	L	-2.452322	0.01419375

located in residue 4, within the signal peptide. No sites within known antigenic epitopes were under diversifying selection. Besides, there were 40 and 41 sites under purifying selection in HRSV-A and HRSV-B, respectively, of which 9 and 8 were located within known antigenic epitopes, respectively.

Genetic characterization of the major surface proteins

A more in-depth analysis was made for major surface proteins, for these are the most immunogenic ones. Phylogenetic analyses of F and G proteins of both HRSV-A and -B (Fig. 5) revealed a similar pattern within each subtype, though with a higher divergence in the G protein, as expected. Clusters were better defined in the analyses of the G protein due to its higher evolutionary rate,^{13,40–43} compared to F.^{44–46} At single mutation level, a mutation (A65V) was found in cleavage site in sequence HRSV-A/ESP/105727/2014.

Several mutations were fixed over the multiple seasons, some of them into known antigenic epitopes. In relation to antigenic epitope II, two changes exhibited low frequencies throughout the seasons, none of them surpassing 25% frequency: the S276N substitution, present in both subtypes, and the M274I substitution, exclusive to HRSV-B (Fig. 6). The antigenic epitope Ø demonstrated a higher degree of variability in HRSV-B, with seven distinct mutations described along the study period, while HRSV-A displayed a greater conservation degree with only two changes. The dynamics of these mutations followed a pattern of sporadic occurrence at very low frequencies in both subtypes, except for three mutations specific to HRSV-B at residue positions 206, 209 and 211. The substitutions I206M and Q209R emerged during the 2017–2018 season, with a frequency of occurrence reaching 70% in both cases (18 out of 26 sequences) and increasing up to 96% (27/28) in the 2022–2023 season. It is noteworthy that both mutations emerged simultaneously at a phylogenetic node in the tree depicted in Fig. 5. In addition, the S211N mutation emerged during the 2020–2022 season with a frequency of 91% (39 out of 43 samples), increasing to 100% (28/28) one season later. In contrast, no mutations were identified in epitope IV.

Discussion

HRSV outbreaks significantly affect the community and health-care system. The absence of robust surveillance hampers resource management; therefore, understanding circulation patterns and their genetic features is crucial. Prevalence and seasonality of HRSV in our hospital mirrored that of northern hemisphere countries.^{5,6} However, the SARS-CoV-2 emergence altered prevalence and seasonality, as previously described,^{47–51} probably linked to pandemic control measures (strict lockdown during March–May 2020 and non-pharmaceutical interventions). HRSV circulation resumed post-lockdown, coinciding with HMPV and after non-enveloped viruses' resurgence.⁵² Phylogenetic analysis based on WGS showed a unique epidemic with a double-peaked pattern, possibly disrupted by the Delta SARS-CoV-2 wave. Moreover, this viral interference would explain that this pattern was observed in Delta wave coinciding countries, while in countries with other timing for this wave presented only one peak of HRSV.^{47,49,52,53}

The median age of paediatric and adult patients was similar to other regions until the emergence of SARS-CoV-2.⁴⁸ Thereupon, paediatric median age drastically increased up to 1 year in 2020–2022. This shift is widely discussed and attributed to an increase in immunologically vulnerable children due to limited exposure to HRSV during the early SARS-CoV-2 pandemic stages.^{48,51,54–56} Similar trends have been observed for other respiratory viruses, such as HMPV.⁵²

HRSV-A and -B co-circulation displayed cyclic shifts, where HRSV-A predominated every 3 seasons, as observed in other studies.^{17,57,58} This has been suggested to be associated both to a short-living acquired immune response, which enables the reinfection of a previously circulating HRSV type, and to a higher genetic diversity in HRSV-B, as measured in this study and congruent with previous works,^{9,59} favouring the predominance of HRSV-B for 3 consecutive years.

Whole genome sequences analyses revealed that all HRSV-A samples belonged to GA2.3.5 lineage, which is the equivalent to the old ON1 genotype, and all HRSV-B samples belonged to several lineages within GB5 genotype, equivalent to the old BA genotypes (GB5.0.5a and GB5.0.4a to the old BA9 genotype, while GB5.0.2 to the old BA10 genotype). Though these new genotypes seem to correspond to similar clusters of former genotypes, the new classification is more uniform and robust, as it is based on high values of bootstrap and high posterior probabilities values,⁸ as opposed to the previous classification, which used a variable criterion based on different phylogenetic analyses methodologies and a scanty region of G protein (HRV-2, of approximately 300 bp). This study provides further evidence that the new classification is applicable in phylogeny based on whole genome sequences, which together with the fact that having the characterisation of the entire HRSV genome can strengthen surveillance for mutations associated with clinical prognosis or reduced susceptibility to treatment, reinforces the importance of conducting this type of virological surveillance.

As previously stated, HRSV-B had a higher evolutionary rate than HRSV-A.^{9,59} Interestingly, despite the period of no detection of this virus during the beginning of the SARS-CoV-2 pandemic, it seems to have continued circulating, accumulating mutations, and differentiating in phylogenetic trees from previous seasons. This circulation could have occurred asymptotically and thus, without detection, or could be in a low rate shed by immunocompromised patients who act as a reservoir, as suggested by other authors.⁵⁷ The tMRCA estimated in this study for the tree root went back to mid-1930s for HRSV-A and late 1960s for HRSV-B, which was comparable to the results in previous studies,^{9,59} being all estimations within a

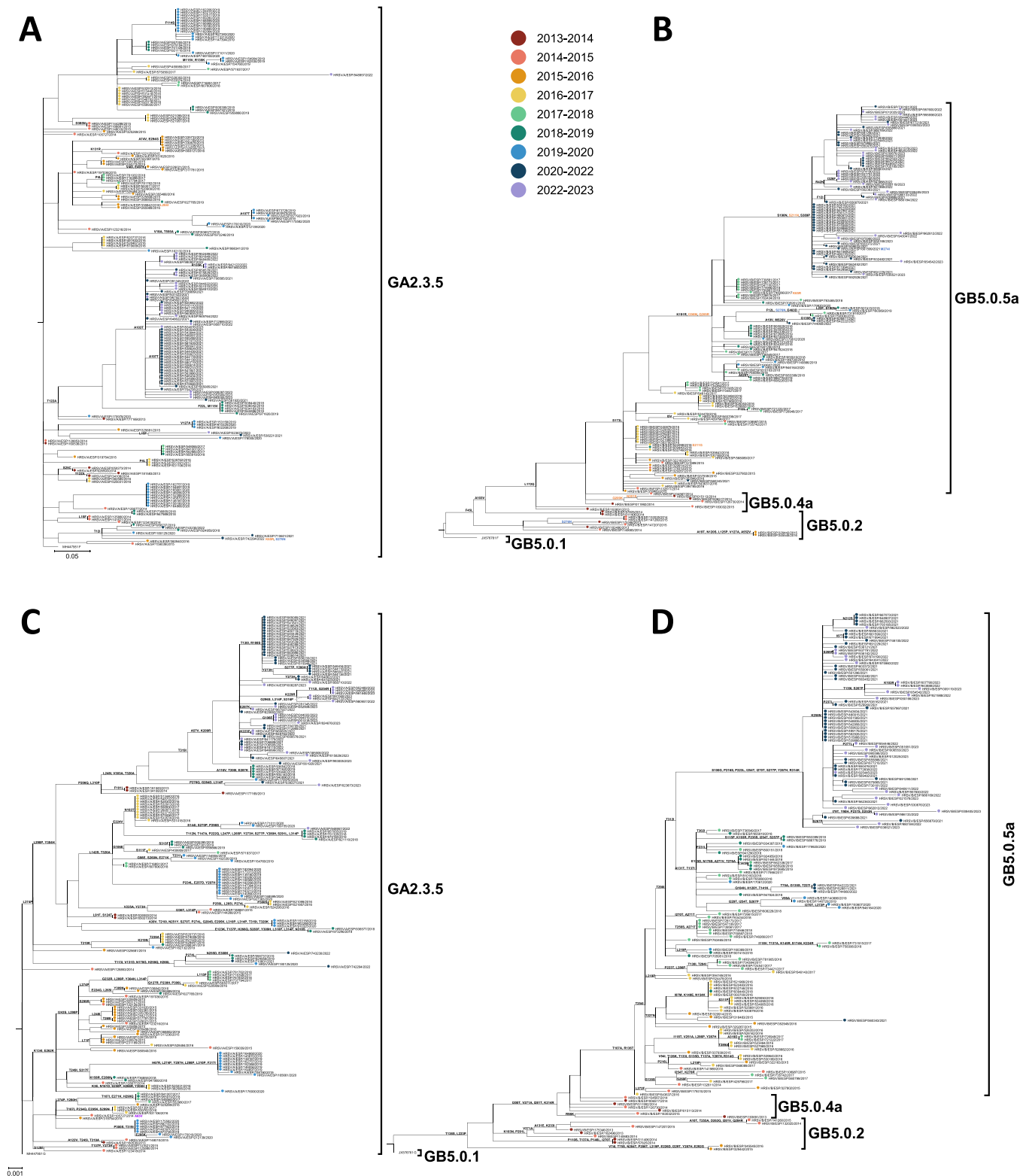


Fig. 5. Phylogenetic trees of F (A and B) and G (C and D) genes of HRSV-A (A and C) and HRSV-B (B and D). Both phylogenetic trees were inferred by using the maximum likelihood method, based on the General Time Reversible model. Sequences of our hospital are marked with coloured solid circles, while reference sequences downloaded from GenBank are not marked. A and B trees are scaled under the same pattern (0.05), and C and D trees are scaled under 0.001. Mutations defining the different genetic clades are annotated on the tree nodes.

range of 10 years. These findings suggest a relatively recent origin of circulating strains.

Effective population size estimation suggested a notable reduction in variability during the early stages of the SARS-CoV-2 pandemic, followed by a sudden expansion thereafter. Pandemic-related

restrictions resulted in a larger pool of individuals susceptible to infection due to reduced virus contact. The infection of a greater number of patients in a shorter time facilitated an elevated replication rate, consequently manifesting an increase in variability and evolution rate, as determined in this study. This could be

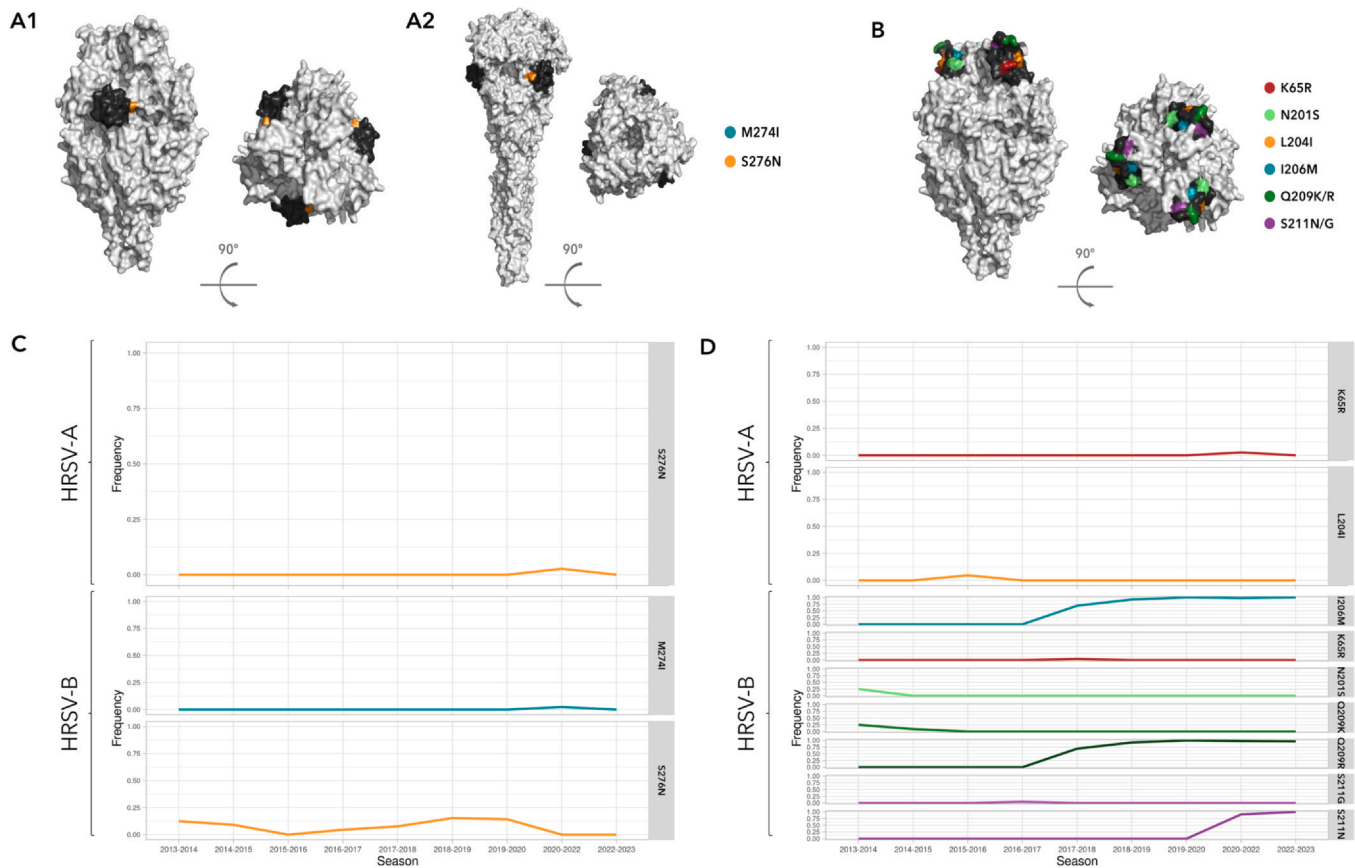


Fig. 6. Mutations identified for palivizumab epitope (A) in prefusion (A1) and postfusion (A2) protein and for nirsevimab epitope (B). Epitopes and mutations are colored in the 3D structure of the prefusion (PDB: 4MMT) and postfusion (PDB: 3RRR) protein. Epitopes are coloured in black, and mutations as shown in legend. Dynamics of the mutations found in palivizumab epitope for HRSV-A and B (C) and nirsevimab epitope for HRSV-A and B (D) are also shown. Mutation M274I is internalized (not shown in A1 and A2).

associated with the increase in the median age of the paediatric population, as mentioned above.

Overall, Tajima's D results suggested viral evolution under a non-random process for all proteins despite the non-significant p-values, as it showed a clear trend to negative values, indicating an acting negative selective pressure. The dN/dS ratio per protein showed that G and M2-2 proteins exhibited a high degree of variability, as well as two regions in L protein, consistent with prior analyses.^{13,40} Given M2-2 function in transitioning from transcription to viral replication within the viral infectious cycle,⁶⁰ a higher level of constraint was expected. However, high sequence variability of this protein has been previously reported,^{13,40} suggesting a possible role in other infective processes as highlighted in previous studies.⁶¹ Further studies are needed to identify the origin of this variability despite its important role in efficient packaging and viral production. Regarding major surface proteins G and F, and congruent with evolutionary rates, greater variability was observed in the G protein across both HRSV subtypes compared to the F protein. This observation aligns with the presence of a higher number of sites subject to diversifying selection in the G protein relative to the F protein, specifically 3 vs 1 and 6 vs 1 for HRSV-B and -A, respectively. Such findings support the selection of F protein as the main target for mAb. Despite the non-detection of sites under diversifying selection within the known epitopes, mutation monitoring is crucial in a scenario where new selective pressures are imposed to this protein.

Given the high genetic diversity that HRSV exhibits, its impact on mAbs susceptibility has been one of the aims of this study. Since the approval of palivizumab, mAbs have emerged as a promising prophylaxis strategy for HRSV infection, especially in high-risk populations such as preterm infants and individuals with

immunosuppression and other underlying diseases. Monitoring emerging mutations in mAb targeted epitopes, although they are known to be conserved, is crucial to ensure their efficacy and to track the emergence of MARMs.^{9,59} It is well-known that G protein is the protein with the highest evolutionary rate,^{13,40} being a good candidate for genotyping but not a good choice as a target for therapies and immunoprophylaxis. Despite the lower rate of evolution of F protein compared to other proteins, this study has proven that amino acidic mutations can emerge and become fixed without a selective pressure caused by prophylaxis, being more common in HRSV-B than A due to a higher evolution rate. Among acquired mutations, some also arise in mAb epitopes and accumulate. In relation to epitope II, and despite 20 years of continuous use of palivizumab, no mutations associated with a reduced neutralising capacity of this mAb have been observed. This situation could be due to two main factors: first, the use in a small population size has resulted in an insufficient selective pressure for the selection of mutations; and second, the probable alteration of the structural and functional characteristics of the fusion protein caused by mutations might restrict their selection and thus, their fixation over time. On the other hand, the detection of mutations I206M, Q209R and S211N in epitope Ø, targeted by nirsevimab, suggest a lower level of restriction at the structural and functional level for epitope Ø, since the emergence of mutations at this site has been observed even without a selective pressure such as the administration of a monoclonal antibody. However, none of these mutations have been characterised to confer a susceptibility reduction to this mAb [EMA/H/C/005304].⁶² In fact, I206M:Q209R showed an increased susceptibility to nirsevimab neutralization (0.2-fold), and S211N was not linked to a reduction in susceptibility alone or with co-occurring

substitutions.⁶³ However, changes for residues not described in this study at positions 65 (K65Q/T), 201 and 206 have been previously associated with reduced susceptibility to nirsevimab neutralization [EMA/H/C/005304].⁶² Furthermore, mutations identified in this study have been described to confer resistance when accompanied by others (mutation constellation), such as I64T+K68E+I206M+Q209R (> 447-fold)⁶³ or K68N+N201S (> 13,000-fold) for subtype HRSV-B [EMA/H/C/005304]. This underlines the importance of close genomic surveillance of circulating strains in order to have an early detection of the emergence of viruses resistant to this mAb. Several resistant mutations have been identified in vitro and in clinical trials, although they have not been found in circulating viruses. Special attention must be paid to mutation constellations in which at least one of the residues with side chains forming hydrogen bonds or salt bridges with nirsevimab (65, 200, 202, 208, 209) is mutated.⁶² Regarding epitope IV, no mutations were detected, showing a higher level of conservation.

Together with genomic surveillance, phenotypic studies are also important to be performed. Previous studies have demonstrated the effectiveness of neutralization assays in identifying MARMs in clinical samples, particularly in the context of monoclonal antibodies such as palivizumab and motavizumab.^{64–66} Neutralization assays provide a robust platform for evaluating the susceptibility of HRSV strains to monoclonal antibody therapy, including those treated with nirsevimab. The combination of these assays facilitates the identification of viral escape mechanisms and provide invaluable insights into the efficacy of therapeutic interventions. As the field of monoclonal antibody therapy continues to advance, the importance of neutralization assays in vitro cannot be overstated in ensuring the effectiveness of these therapeutic interventions in combating viral pathogens.

This study presents some limitations which are important to consider. First and most importantly, the SARS-CoV-2 pandemic significantly reshaped the hospital's priorities. The entire healthcare staff directed all efforts towards halting the pandemic, with the diagnosis of any respiratory virus other than SARS-CoV-2 being a rare exception. This might be the reason why HRSV was not detected during all that period, as the mutation rate of the virus during that time suggests that it continued circulating. Other limitation in this study is that the inclusion criteria only accepted patients attended at this hospital, so results in this study might not reflect the genetic diversity on the community or in other Catalan regions. The ideal surveillance method should include samples from mild and severe cases from both primary attention centres and hospitals, as well as from asymptomatic population, and from different regions. This has been partially achieved since the implementation of the Catalan Information System for the Surveillance of Infections (SIVIC, <https://sivic.salut.gencat.cat/>) on the 2022–2023 season.

In conclusion, the findings in this study highlight the importance of global monitoring of emerging mutations to prevent MARMs spreading, with particular attention in upcoming months to hospitalized patients with HRSV infection post-nirsevimab administration. HRSV has exhibited fluctuations around a peak of fitness, and its ecology will be disrupted by the introduction of immunoprophylactics, thereby posing a challenge to its adaptive landscape during the 2023–2024 epidemic in some Spanish regions. After significant efforts in developing this effective immunoprophylaxis, it is crucial to actively monitor MARMs emergence, ideally through whole-genome analyses. This includes assessing combinations of mAbs targeting various epitopes for minimizing the risk of MARMs. Additionally, tracking virus evolution across populations can guide the development of new prophylaxis options in a near future.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2024.106153.

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