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Genetic diversity and its impact on disease severity in respiratory syncytial virus subtype-A and -B bronchiolitis before and after pandemic restrictions in Rome

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SUMMARY

Objectives: To scrutinize whether the high circulation of respiratory syncytial virus (RSV) observed in 2021–2022 and 2022–2023 was due to viral diversity, we characterized RSV-A and -B strains causing bronchiolitis in Rome, before and after the COVID-19 pandemic.
Methods: RSV-positive samples, prospectively collected from infants hospitalized for bronchiolitis from 2017–2018 to 2022–2023, were sequenced in the G gene; phylogenetic results and amino acid substitutions were analyzed. Subtype-specific data were compared among seasons.
Results: Predominance of RSV-A and -B alternated in the pre-pandemic seasons; RSV-A dominated in 2021–2022 whereas RSV-B was predominant in 2022–2023. RSV-A sequences were ON1 genotype but quite distant from the ancestor; two divergent clades included sequences from pre- and post-pandemic seasons. Nearly all RSV-B were BA10 genotype; a divergent clade included only strains from 2021–2022 to 2022–2023. RSV-A cases had lower need of O_2 therapy and of intensive care during 2021–2022 with respect to all other seasons. RSV-B infected infants were more frequently admitted to intensive care units and needed O_2 in 2022–2023.
Conclusions: The intense RSV peak in 2021–2022, driven by RSV-A phylogenetically related to pre-pandemic strains is attributable to the immune debt created by pandemic restrictions. The RSV-B genetic divergence

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observed in post-pandemic strains may have increased the RSV-B specific immune debt, being a possible contributor to bronchiolitis severity in 2022–2023.

Introduction

Respiratory syncytial virus (RSV) bronchiolitis is the worldwide cause of 1.4 million hospitalizations, and 45,700 deaths in infants less than 6 months, estimated in 2019.[1] RSV infection is common in the first years of life but only a fraction of infected children develops severe lower respiratory tract infection.[2]

RSV is a single-stranded, negative-sense RNA virus belonging to the *Pneumoviridae* family; the enveloped virion is composed of two main surface glycoproteins, G and F, involved into attachment and entry by membrane fusion in the host cells.[3] G is a mucin-like protein composed of two hypervariable regions presenting several N- and numerous O-glycosylation sites, separated by an un-glycosylated central conserved domain (CCD)[3]; the second highly variable region (HVR2) of the G gene has been conventionally used to differentiate the RSV genotypes within subtypes A and B.[4] RSV subtype A is thought to be more prevalent in the first infection in children and to cause a more severe disease than RSV-B[5–7]; nonetheless, other reports showed no significant clinical differences between subtypes.[8,9] Indeed, a different disease course may also reflect different RSV genotypes.[10–12]

Contemporary RSV-A and -B strains derive from ancestors that underwent an insertion in the HVR2 of the G gene: RSV-A ON1, detected in Ontario, Canada, in 2010[13] characterized by a 72-nucleotides (nt) insertion causing the duplication of 23 amino acids (aa) and RSV-B BA found between 1997 and 1999 in Buenos Aires, Argentina, characterized by a 20 aa duplication in a similar position.[14] The fitness advantage of ON1 and BA led to the disappearance of RSV strains without the insertion[15–19]; they further differentiated in what are defined genotypes, sub-genotypes or clades, by different classification methods.[4,20,21]

In the first period of circulation in Rome, RSV-A ON1 caused a milder clinical course with respect to the previously circulating RSV-A without the insertion.[22] In the following years, ON1 strains differentiated into at least three different clades and were associated to a higher number of pediatric hospitalizations and to bronchiolitis severity in the epidemic seasons 2016–2017 and in 2017–2018 in Rome.[22]

In this study we aimed to further characterize RSV molecular epidemiology, extending the study after the emergence of the coronavirus disease 2019 (COVID-19) pandemic. In the first pandemic period, the sanitary measures adopted to reduce SARS-CoV-2 transmission also prevented the spread of other respiratory viruses.[23] The successive decrease of restrictions has caused a surge of RSV cases, likely due to the waning population immunity caused by the nearly complete absence of RSV circulation in the preceding winter.[24] The sharp reduction of infections could have caused a genetic bottleneck resulting in reduced viral diversity,[23] and the local emergence of distinct RSV clades as seen in Australia.[25] Therefore, to understand whether the increased number of RSV cases seen in the post-pandemic period, was due to novel strains and whether they cause severe illness, we characterized the genetic variability of RSV-A and -B causing bronchiolitis cases in Rome over six epidemic seasons.

Patients and methods

Study group

Infants hospitalized for bronchiolitis in the Pediatric Emergency Department, or in the pediatric intensive care unit (PICU) of Sapienza University hospital in Rome, were prospectively enrolled, during the epidemic seasons (September-April) starting from 2017–2018 to 2022–2023, after the informed consent was obtained from infants' parents. Demographic and clinical data were obtained from medical files. In line with confidentiality requirements, the database was anonymized; the ethic committees of Policlinico Umberto I, Sapienza University Hospital, approved the study (Prot. 107/12). Infants with underlying chronic diseases (e.g., cystic fibrosis, congenital heart disease, immunodeficiency) or prematurity were excluded. Moreover, in the post pandemic period, all infants attending the hospital were routinely subjected to SARS-CoV-2 molecular tests; those resulted positive to SARS-CoV-2 were excluded. Bronchiolitis was defined as the first lower respiratory tract infection in infants < 12 months, characterized by a history of upper respiratory tract infection followed by the onset of cough, respiratory distress, and diffuse crackles on chest auscultation.[12]

RSV detection and sequencing

In the first day of hospitalization, nasopharyngeal washings or bronchoalveolar lavages were obtained from infants, delivered on ice within 1–2 h to the laboratory of Virology, and tested with reverse transcriptase polymerase chain reactions (RT-PCR) for 14 respiratory viruses (RSV, influenza virus A/B, coronaviruses OC43, 229E, NL-63, HUK1, adenovirus, rhinovirus, parainfluenza 1–3, metapneumovirus, and bocavirus) as described.[12]

Moreover, RSV-positive residual samples were sequenced in the second half of the G gene using subtype specific forward primers (position 481–498 of the RSV-A2 reference strain G gene), and the F1 reverse primer targeting both subtypes at the 5′ end of the fusion protein gene[12]; the amplicons of around 500 bp were subjected to Sanger sequencing.

Phylogenetic analysis

The sequence tract starts with nucleotide 514 of the G gene (aa position 172), in the middle of the CCD (aa 157–198), and includes the CX3C motif (aa 182–186) that mediates binding to the cell receptor CX3CR1,[26] the heparin binding site (aa 184–198), and the HRV2 up to the stop codon. RSV-A and -B study sequences were aligned with reference sequences using Bioedit v7.1.3; sequencing errors were removed and identical sequences from the same epidemic season were grouped. Phylogenetic trees were constructed with IQ-TREE v2.2.0, using the Maximum Likelihood method based on the Tamura-Nei model and a discrete Gamma distribution with 5 categories (+G), with bootstrap values of 1000, and edited with FigTree v1.4.4. A clade was defined as a group of at least 20 study sequences with a common ancestor having a bootstrap support of at least 90%. A group of at least 10 study sequences from the same epidemic season with a bootstrap value > 90%, was considered a monophyletic node. The average p-distance values (i.e. the proportion of nucleotide sites that differ between sequences) of study strains over the epidemic seasons were calculated by pairwise comparison. Study sequences were submitted to GenBank: the RSV-B accession numbers are OR048822-OR048928 and the RSV-A accession numbers are OR048881-OR048928.

The bioinformatics tools NetNGlyc and NetOGlyc (http://www.services.healthtech.dtu.dk) were used to predict N- and O-linked glycosylation sites in aa sequences.

Statistical analysis

For categorical variables, either Pearson's chi-square or Fisher's exact test were used to test the statistical difference in proportion among independent groups. For age values in months, ANOVA was run to compare the mean values among epidemic seasons.

Results

RSV detection rates and subtype distribution in pre- and post-pandemic seasons

Infants suffering from bronchiolitis were prospectively tested by molecular assays from September to April during three pre-pandemic (2017–2018, 2018–2019, 2019–2020) and three post COVID-19 pandemic seasons (2020–2021, 2021–2022, and 2022–2023) for respiratory virus detection. No RSV-positive case was detected among the few bronchiolitis cases hospitalized during September 2020 to April 2021 and this period was excluded from further analysis. After obtaining informed consent from parents, 343 RSV positive infants fulfilling inclusion criteria were enrolled in this study (Table 1); bronchiolitis clinical data are analyzed in a separate study from our group.

In 263/343 RSV-positive samples (76.7%), the subtype could be determined by Sanger sequencing targeting the second-half of the G gene, resulting in 150 RSV-A (57.0%) and 113 RSV-B (43.0%). RSV subtype distribution significantly differed among seasons ($p < 0.001$): RSV-A dominated in three epidemic seasons: 2017–2018 (42/61 cases = 68.8%), 2019–2020 (25/28 cases = 89.3%) and 2021–2022 (58/78 cases = 74.4%) whereas RSV-B was predominant in 2018/19 (23/30 cases = 76.7%) and 2022–2023 (48/66 cases = 72.2%) (Table 1). Monthly distribution of RSV-A and -B cases over the study period is shown in Fig. 1.

Sequence analysis

Out of 263 respiratory samples identified as RSV-A or -B strains carrying the insertions of 72 and 60 nucleotides in the HRV2, respectively (Table 1), 21 sequences were not further analyzed because of poor chromatograms and/or of mixed signals. A total of 242 sequences were aligned and identical sequences of the same epidemic season were grouped. To observe differences in the rate of genetic differentiation over the study period, the average p-distance among RM sequences within each epidemic season, between each epidemic season and the preceding one, and between each epidemic season and the first season of the study, 2017–2018, was calculated (Table 2). These analyses showed no differences in genetic divergence among RSV-A sequences before and after the pandemic period whereas for RSV-B strains, the p-distance between 2021–2022 and 2019–2020 epidemics was about twice that of the 2019–2020 and 2018–2019 seasons (Table 2), suggesting higher genetic divergency in the RSV-B strains in the post pandemic period.

Phylogenetic analysis was performed on datasets of 60 unique RSV-A and of 59 unique RSV-B sequences from Rome (RM), together with reference RSV genotypes and with the strains from different countries that showed the higher similarity with RM representative sequences from each clade. Phylogenetic reconstruction of RM sequences and representative strains of RSV-A and RSV-B is shown in Fig. 2; as this study is based on sequences of the second-half of the G gene and extends our previous findings,[12,22] the traditional classification[4] has been followed to describe RM strains. As expected, RSV-A and RSV-B CCD tract (aa 157–198) was well conserved, with a few exceptions, whilst numerous nucleotide substitutions, mainly non-synonymous, were found throughout the HRV2, including both copies of the duplicated tract, that were not identical in most cases (Fig. 3). The aa substitutions found more than twice in one epidemic season and in more than two seasons are shown in supplementary Table 1.

All RM RSV-A sequences were ON1 genotype but evolutionary distant from the reference Ontario strain, now named ON1–1.1[17]; overall, RSV-A sequences from the post-pandemic period grouped together with sequences from pre-pandemic seasons. Most RM sequences were included in two divergent and well-supported clades, here named A1 and A2 (Fig. 2A); besides, sequences from a single season grouped in divergent monophyletic nodes, having high bootstrap support (circled in Fig. 2A).

Table 1
Demographic characteristics of RSV-positive cases and subtype distribution by epidemic season.

RSV-positive cases	2017–2018 N = 75	2018–2019 N = 40	2019–2020 N = 39	2021–2022 N = 93	2022–2023 N = 96	p-value
Female/Male	36/39	11/29	16/23	44/49	42/54	0.247
Mean age in months (standard deviation)	3.06 ± 2.07	2.99 ± 2.37	1.99 ± 1.42	3.21 ± 2.31	2.84 ± 1.99	0.045*
RSV-A-positive (%)[a]	42/61 (68.8%)	7/30 (23.3%)	25/28 (89.3%)	58/78 (74.4%)	18/66 (27.3%)	< 0.001
RSV-B-positive (%)[a]	19/61 (31.1%)	23/30 (76.7%)	3/28 (10.7%)	20/78 (25.6%)	48/66 (72.2%)	< 0.001

* Bonferroni post hoc test was significant (p = 0.026) only between 2019–2020 and 2021–2022.
[a] Denominator indicates the number of RSV-positive samples in which the subtype could be determined.

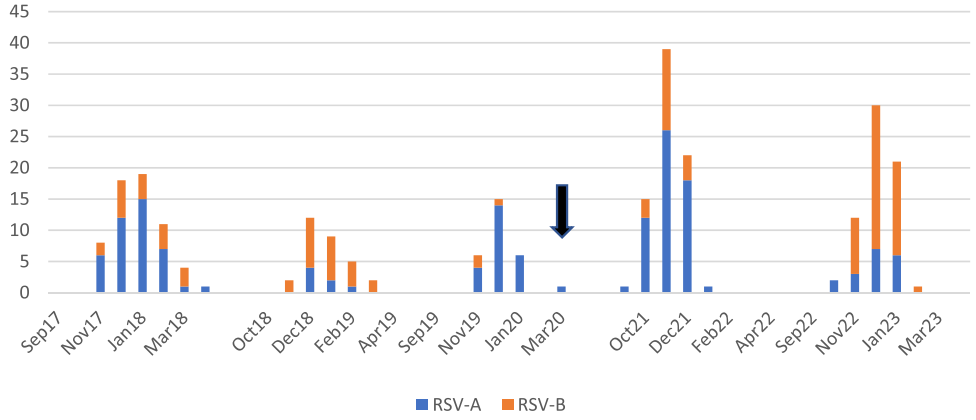

Fig. 1. RSV-A and -B cases monthly distribution. On the X-axis, the calendar month of the five epidemic seasons (September-April) included in the study is reported; the season 2020–2021 is not represented. On the Y-axis, the number of RSV positive children is reported; RSV-A cases are represented in blue and those positive for RSV–B in orange. The black arrow indicates when the lockdown in Italy was declared (March 09, 2020).

Table 2
Between and within group average distance estimation. The average p-distance (p-dist) among nucleotide sequences of the four epidemic seasons was calculated by pairwise comparison using Tamura-Nei model, Gamma Distributed including transitions and transversions, in MEGA 11.

Epidemic season	p-dist within group	Epidemic seasons	p-dist between groups	Epidemic seasons	p-dist between groups
RSV-A					
2017–2018	0.0388				
2018–2019	0.0301	2018–2019 vs 2017–2018	0.0396		
2019–2020	0.0441	2019–2020 vs 2018–2019	0.0487	2019–2020 vs 2017–2018	0.0497
2021–2022	0.0311	2021–2022 vs 2019–2020	0.0482	2021–2022 vs 2017–2018	0.0500
2022–2023	0.0474	2022–2023 vs 2021–2022	0.0476	2022–2023 vs 2017–2018	0.0562
RSV-B					
2017–2018	0.0250				
2018–2019	0.0161	2018–2019 vs 2017–2018	0.0252		
2019–2020	0.0197	2019–2020 vs 2018–2019	0.0166	2019–2020 vs 2017–2018	0.0264
2021–2022	0.0262	2021–2022 vs 2019–2020	0.0324	2021–2022 vs 2017–2018	0.0432
2022–2023	0.0247	2022–2023 vs 2021–2022	0.0251	2022–2023 vs 2017–2018	0.0469

A monophyletic node (circled in black in Fig. 2A), included eleven 2017–2018 sequences derived from ON1–1.1 and characterized by six signature changes (T200P, P215L, N255D, S275N, V279I, E295V)[22]; no other sequence clustered with ON1–1.1 in the subsequent seasons. The sub-genotype ON1–1.3, first identified in Rome in 2016–2017 and characterized by the changes I243S and E262K,[22] was represented by only twelve sequences from the pre-pandemic period. Differently, 82 strains derived from the ON1–1.2 sub-genotype, characterized by the substitutions L274P and L298P and present in Italy since December 2012.[16,17,27] The substitution L274P varied in frequency along the study (Fig. 3A and supplementary Table 1); in the post-pandemic period, L274P was found in all 2021–2022 strains but only in 37.5% of those from 2022 to 2023 (Fig. 3 and supplementary Table 1). In fact, L274P is a site under strong positive selection, showing a "flip-flop" pattern, i.e. the reversion to a previous state over time[28]; the other "flip-flop" sites K233E, D237N, N260S, L286P and P290L were not changed in this study. ON1–1.2 further differentiated during the pre- and post-pandemic seasons forming the divergent clade A1 (Fig. 2A), characterized by P206Q, K209R, L248I, V303A, found in similar sequences from different countries (Fig. 3A); within A1, ten RM 2021–2022 sequences formed a monophyletic node characterized by N191G and T238I (circled in green in Fig. 2A). The other divergent and well-supported clade, A2 (Fig. 2A), was characterized by aa substitutions N178G, H258Q, and H266L and included sequences from 2019 to 2020 and post-pandemic strains (Fig. 3A and supplementary Table 1); the same set of aa changes has been described in a sub-lineage circulating in China during 2017–2019.[29] Interestingly, the change N178G is located in the CCD, four aa before the conserved CX3C motif, and could influence its function and antigenicity.[30] Furthermore, ten RM 2022–2023 sequences formed a monophyletic node characterized by T210A and S277P (circled in red in Fig. 2A).

All RM RSV-B strains were derived from the BA10 genotype, with the exception of one sequence that was close to BA9 (Fig. 2B). In the RSV-B, CCD only singleton substitutions were found (Fig. 3B). Most RM strains (55/64) were characterized by the anticipated stop codon at position 313 (Fig. 3B and supplementary Table 1), typical of the BA10 genotype, that reduces the G length to 312 amino acids, the shortest of three alternative glycoprotein forms.[18] Together with Q313stop, the substitutions P219S, I227T, P235L, L237S, T255A, P257S, T270I, L286P were described as evolutionary reversible[28]; in these "flip-flop" sites, we detected the change P219L in nearly all RM strains and I270T in most post-pandemic strains (Fig. 3B and supplementary Table 1). Derived from BA10, a divergent monophyletic node was formed by eleven sequences from 2017 to 2018 node (circled in black in Fig. 2B), characterized by the aa substitutions T228S, L252F and E292K (Fig. 3B and supplementary Table 1). All other pre-pandemic sequences grouped together and with five 2021–2022 and eight 2022–2023 sequences (Fig. 2B). Interestingly, a divergent and well-supported clade (B1, Fig. 2B) was formed by RM sequences found in the post-pandemic period only, characterized by the aa substitutions P216S, P223L, K258N (Fig. 3B and supplementary Table 1); the latter is located in a positively selected site,[28] and introduced a new predicted N-Glyc site (see below). Moreover, a monophyletic node (circled in red in Fig. 2A) included fourteen 2022–2023 sequences characterized by the aa change K209N (Fig. 3B and supplementary Table 1), conferring a further predicted N-glyc site (see below). Notably, K209N is not present among RSV-B sequences in NextStrain [https://nextstrain.org/rsv/b/genome?c=gt-G_209, accessed on 30/06/2023]. The evolutionary divergence of RSV-B post-pandemic strains is confirmed by the between group p-distance, that is higher between 2019–2020 and 2021–2022, with respect to the other values (Table 2).

Analysis of predicted glycosylation sites in RSV-A and -B study sequences

All RSV-A RM strains were predicted to have two NetNGlyc such as the ON1–1.1 genotype, with the exception of a sequence found in December 2022 with the substitution K232N leading to the acquisition of one NetNGlyc site. Predicted NetOGlyc differed among RM RSV-A strains between and within epidemic seasons (Supplementary Table 2). Categorizing OGlyc patterns in OGlyc-low (30–39 sites) and OGlyc-high (40–50 sites), in 2017–2018 all strains were OGlyc-high and in 2018–2019 all were OGlyc-low. In the subsequent seasons, 2019–2020 and 2021–2022, both OGlyc patterns were represented, whereas in 2022–2023 nearly all RM strains were OGlyc-high (Supplementary Table 2).

The analysis of RSV-B predicted glycosylation sites revealed interesting differences with RSV-A. The predicted number of NetNGlyc sites varied among epidemic seasons: strains circulating in the pre-pandemic seasons had two NetNGlyc sites whereas sequences from 2021 to 2022 and most from 2022 to 2023 acquired one NetNGlyc site due to the K258N substitution. In addition, the monophyletic node of 14 divergent 2022–2023 RSV-B sequences acquired a fourth NetNGlyc site, due to the substitution K209N. With respect to RSV-A, RSV-B G gene had more predicted NetOGlyc sites (N = 45–50, Supplementary Table 2) but no modification was observed during the study period.

Subtype-specific clinical data from bronchiolitis cases in the pre- and post-pandemic seasons

Having found some genetic diversity in RSV-B strains that caused hospitalization for bronchiolitis in Rome in the post pandemic seasons, we sought to analyze subtype-specific demographic and clinical data (Table 3), after ensuring that cases with and without identified RSV subtype did not significantly differed in sex, age, PICU admission, and O_2 therapy (data not shown).

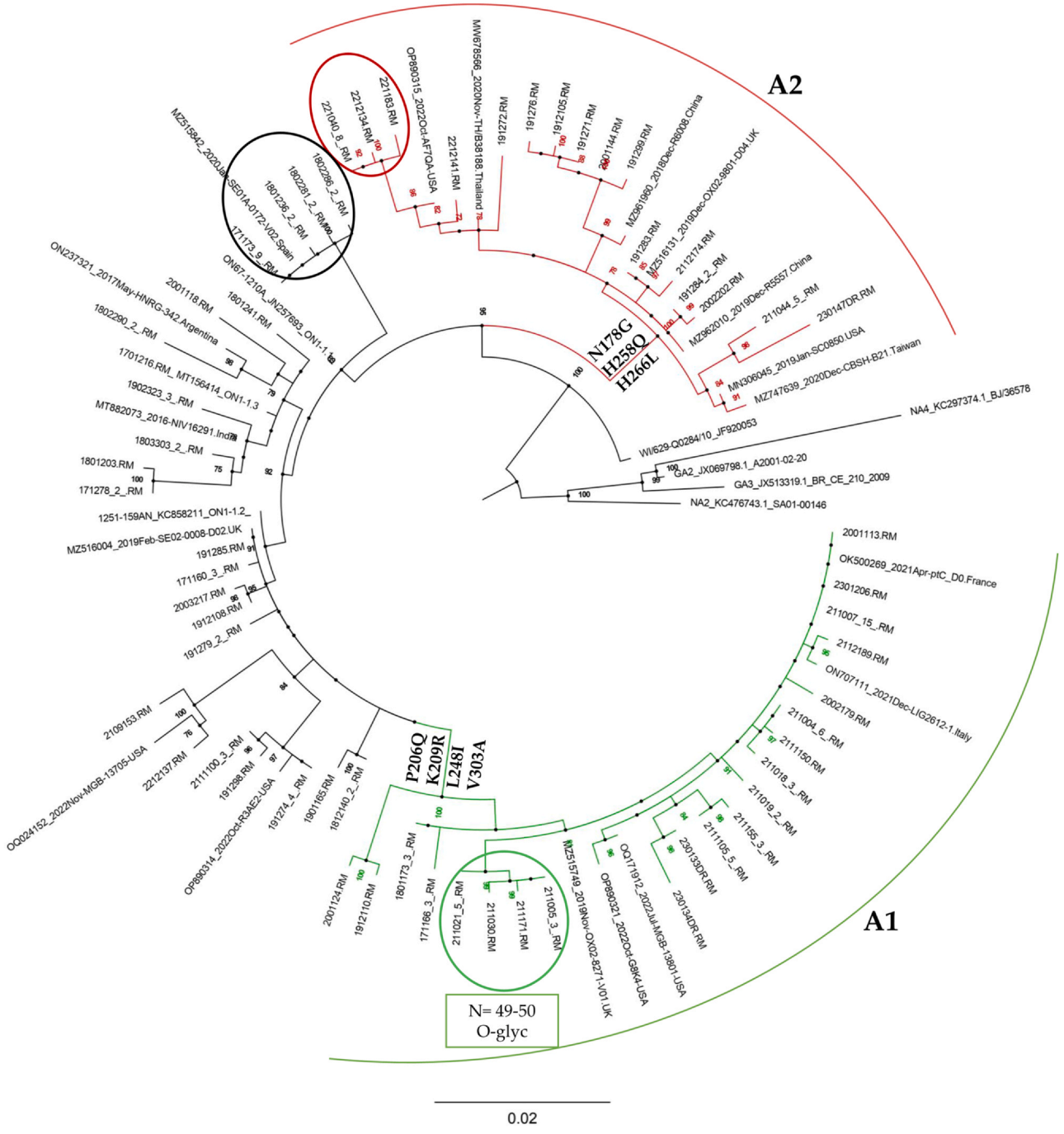

Fig. 2. Phylogenetic analysis of the 2nd half of the G gene of the RSV-A (upper) and RSV-B strains (lower) circulating in Rome (September 2017–April 2023). Labels at the branch node show bootstrap support > 70%. Scale bar indicates nucleotide substitutions per site. RM sequences are identified by year (2 digits), month (2 digits), sample number (2 or 3 digits). If more than one identical strain was found, their total number is indicated in parenthesis following the strain id. The phylogenetic tree is drawn to scale, and below the tree, scale bar shows the number of substitutions per site. The divergent clades are evidenced and named; the monophyletic nodes are circled. The distinctive amino acid changes are reported at the base of clades A1, A2, and B1. The number of predicted O-glyc (for RSV-A) and N-glyc (for RSV-B) sites in monophyletic nodes are boxed. Name (GenBank accession number) of RSV-A reference strains are: A2001–02–20 (JX069798.1) for GA2, BJ/36578 (KC297374.1) for NA4, BR_CE_210_2009 (JX513319.1) for GA3, SA01–00146 (KC476743.1) for NA2, WI/629-Q0284/10 (JF920053) for NA1, GA2ON67–1210A (JN257693) for ON1–1.1; 1302–319AN (KC858211) for ON1–1.2; and 1701216.RM (MT156414) for ON1–1.3. Name (GenBank accession number) of RSV-B reference strains are: GB4 (KU316159.1); GB1 (AF065250.1), SAB1 (AY660684.1), GB3 (JX908835.1), BA1 (MF185752.1), BA2 (AY751123.1), BA3 (DQ227370.1), BCH-Y2016 (KY924878.1) for BA9, SC2225 (KY684758) for BA10, BA12 (KF246586).

Subtype-specific demographic data of 150 RSV-A and 113 RSV-B positive patients did not significantly differ among epidemic seasons (Table 3). Bronchiolitis cases caused by RSV-A appeared to be milder (lower PICU admission and need of O_2 therapy) in the 2021–2022 season with respect to all others (Table 3). Contrastingly, more RSV-B infected infants were admitted to PICU, needed O_2 therapy and were subjected to mechanical ventilation in the 2022–2023 season (Table 3).

Discussion

During the COVID-19 pandemic, we have learnt that SARS-CoV-2 variants may have different infectivity, replicative potential and clinical characteristics; similar studies are needed to evaluate RSV strains. To our knowledge, this study is the first describing RSV-A and -B variability and analyzing subtype-specific data of infants

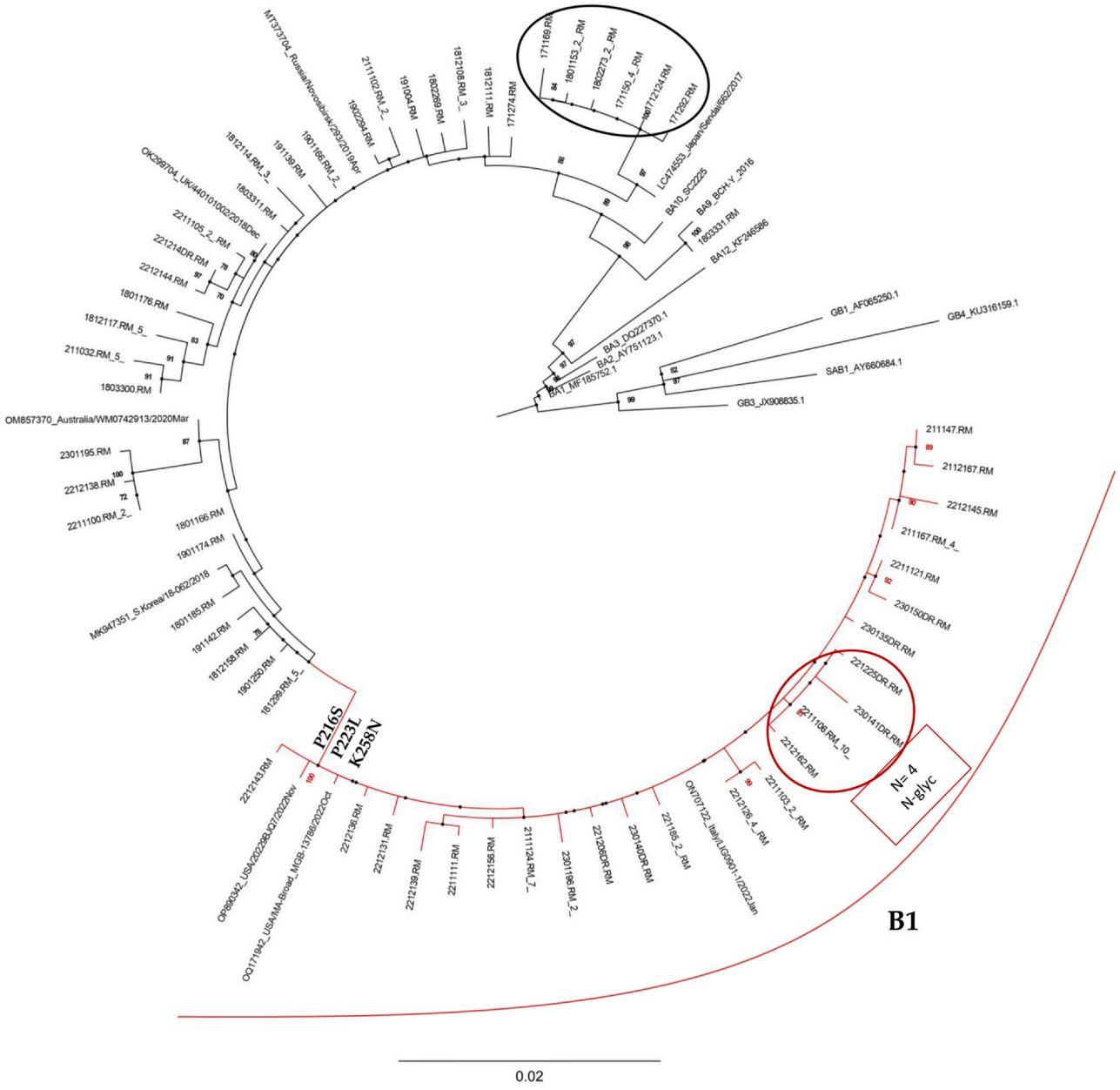

Fig. 2. (*continued*)

hospitalized with bronchiolitis over several respiratory virus seasons before and after the COVID-19 pandemic up to 2022–2023.

Excluding 2020–2021 in which no RSV hospitalization was recorded, in the post-pandemic seasons, there were a high number of RSV-associated hospitalizations for bronchiolitis in otherwise healthy children, being more than double than that in 2018–2019 and 2019–2020 and higher also than that recorded in 2017–2018, an epidemic characterized by high RSV clinical severity in Rome.[22] The surge of RSV-associated hospitalizations in early autumn 2021, reported in many countries worldwide,[23,25,31–35] was explained by a decrease in population immunity due to the interruption of RSV circulation following pandemic restrictions.[24] Besides a decline in the general population in 2020 and 2021,[24] RSV antibody levels were reduced in infants and in women of childbearing age,[36] as well as in human milk,[37] supporting the notion that antibody immunity against RSV is short-lived and that the interruption of RSV circulation created an immune debt.[34] Accordingly, higher rates of a first RSV infection and lowered protection from severe disease due to reduced maternal antibodies are likely explanations for the autumn 2021 surge of RSV hospitalizations that was observed in several Italian centers, including Rome.[31] This epidemic was RSV-A dominated; by sequencing most RSV strains infecting infants hospitalized for bronchiolitis in Rome, we did not identify the presence of a particular RSV-A variant surviving the pandemic restrictions but several strains evolving from those previously circulating in Rome. The extinction of RSV-A sub-genotypes and of divergent clusters was observed, not exclusively after the interruption in viral circulation in 2020–2021, but over the entire study period. Indeed, introduction from abroad, local evolution, and extinction of strains are frequent in RSV-A and -B evolutionary history.[19,27,28,38] In addition to amino acid substitutions in conserved and in more variable sites, we also found RSV-A variability in predicted O-Glyc sites; in recent strains, O-Glyc sites would sum up at 48–50, far more than those predicted in the ancestor genotype ON1 (N = 34,[13]). Indeed, modifications in G glycosylation patterns contribute to RSV antigenic escape and may also impact pathogenicity, as G glycosylation contributes to cell receptor

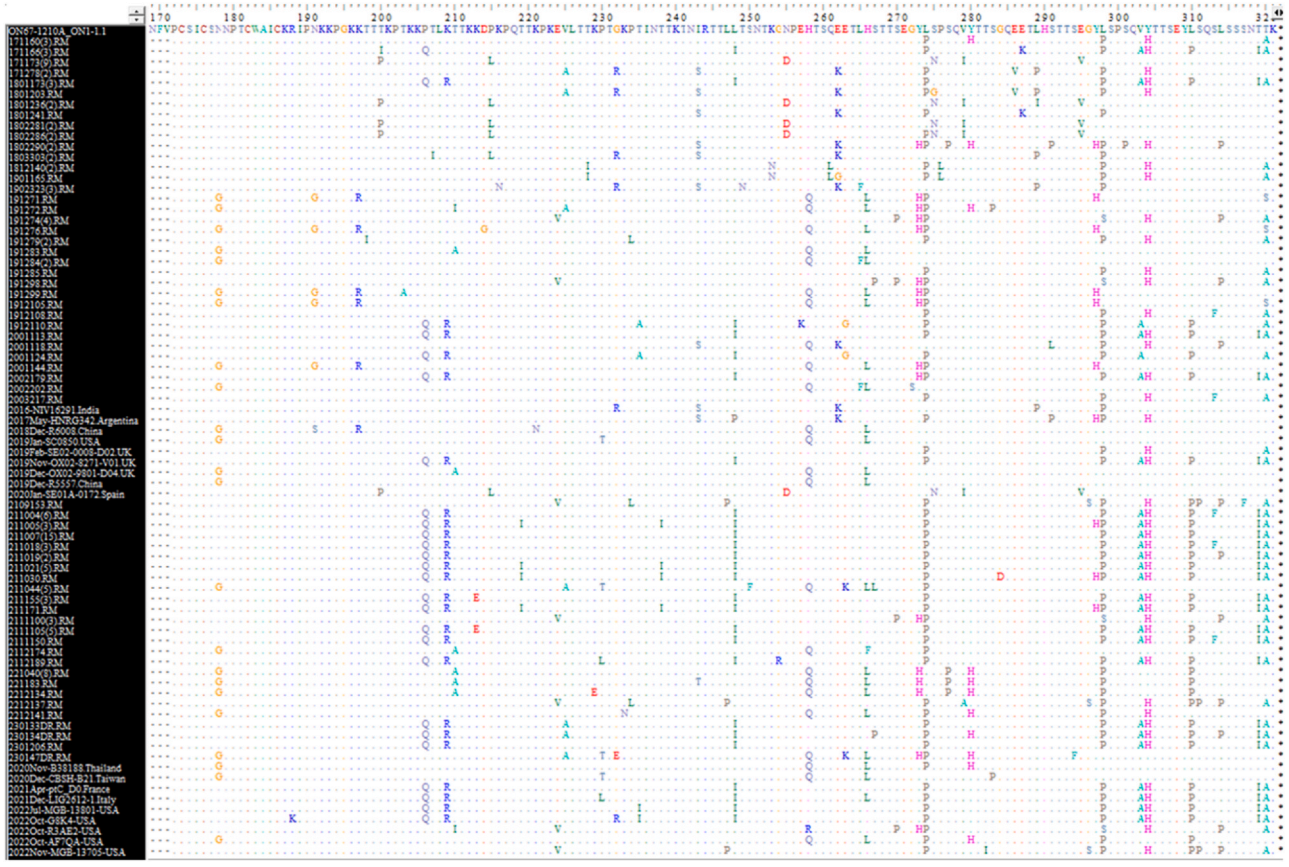

Fig. 3. Alignments of the second half (from aa position 172 to the stop codon) of the G protein sequences of RSV study strains. Alignments of 60 unique RSV-A and of 59 unique RSV-B sequences from Rome (RM), together with strains from different countries having the highest similarity with RM sequences, are illustrated. Substitutions are shown relative to ON67–1210A (JN257693) used as the reference strain for RSV-A ON1–1.1 and to SC2225 (KY684758) used as the reference strain for RSV-B BA10. Dots indicate aa identical to ON1–1.1 or to BA10; the amino acids are colored according to Bioedit v7.1.3 color codes for ease in visual identification of aa polymorphisms.

binding.[39] RSV-A post-pandemic strains circulating in Rome caused an anticipated and intense peak of hospitalization for bronchiolitis but were not associated to increased clinical severity in individual patients, with respect to the other seasons included in this study. Concordantly, clinical severity of the RSV cases in summer/autumn 2021 was comparable to that observed in previous epidemics in UK[33] and in Denmark for children younger than one year.[35] The latter study reported higher hospital admissions with atypical complications for RSV disease among older children,[35] a group that was not included in this study. In Croatia, during the RSV epidemic in summer/autumn 2021, children in their second year of life were more likely to have a severe RSV disease, followed by infants under six months, whereas those older than six months and younger than one year experienced a clinical course similar to that in pre-pandemic seasons.[40]

Extending from late October to February with a peak in December, the 2022–2023 RSV season in Rome resembled the typical RSV circulation that commonly starts in November, peaks in late December or in January and ends in March ([12,16] and this study); differently from the previous one, the 2022–2023 season was characterized by RSV-B predominance. The 2022–2023 RSV epidemic was intense and RSV-B dominated also in Denmark[41] whereas the RSV surge in Autumn 2022 was dominated by RSV-A cases in the Washington State[42] and the Boston Area.[43] Both studies showed that RSV clades circulating in 2021 and in 2022 in US were pre-existing, not monophyletic and were unlikely to be more transmissible or more pathogenic, but no clinical data were reported; the surge of cases was attributed to non-viral factors, mainly the pre-existing immunity lower than in the pre-pandemic period. Notably, a recent paper comparing RSV pediatric hospitalizations between 2018 and 2023 in Colorado, found, in accordance with our data, a greater rate of PICU admission in the 2022–2023 season with respect to the previous one, despite the age of the 2022–2023 cases was the highest.[44] The authors suggest that more severe disease was attributable to waning immunity but raised also the possibility of higher virulence of 2022–2023 RSV strains (but they were not characterized) and/or that immune dysregulation from prior SARS-CoV-2 infection was associated with increased susceptibility to subsequent RSV infections.[44] In our patients, the occurrence of a previous SARS-CoV-2 infection was not known but it is not likely, because of their very young age.

In accordance with the immune debt hypothesis, it is likely that RSV-B caused a high number of RSV-associated hospitalization in the 2022–2023 season in Rome, because the immune debt was paid only for RSV-A that circulated abundantly during autumn 2021. Indeed, subtype alternance every two or three years is a common occurrence in RSV evolution at the population level.[4,5,10] Our previous studies documented that in the same hospital in Rome, in ten pre-pandemic seasons, bronchiolitis cases were mostly caused by RSV-A[12,16] whereas RSV-B caused around 55% of the RSV-bronchiolitis in 2014–2015[12] and was predominant only in 2018–2019 (this study). Similar to RSV-A, the interruption of RSV-B circulation and the consequent decrease of pre-existing immunity would have originated the 2022–2023 intense epidemic. However, this consideration accounts for the high number of RSV-B hospitalizations in 2022–2023 but cannot adequately explain the clinical severity observed in the RSV-B bronchiolitis that was relatively higher than in our historical series of cases. Conversely, it is not clear why the

Fig. 3. (*continued*)

Table 3
Demographic and clinical data of 150 RSV-A and 113 RSV-B positive patients hospitalized with bronchiolitis during the epidemic seasons from 2017 to 2018–2022–2023.

RSV-A	**2017–2018 N = 42**	**2018/19 N = 7**	**2019–2020 N = 25**	**2021–2022 N = 58**	**2022–2023 N = 18**	**p-value**
Female/Male	19/23	3/4	8/17	31/27	11/7	0.329
Mean age in months (SD)	2.96 ± 1.73	4.26 ± 3.65	1.99 ± 1.39	3.21 ± 2.40	2.81 ± 1.68	0.065
PICU[a]	10/42 (23.8%)	0/7 (0.0%)	9/25 (36.0%)	9/58 (15.5%)	7/18 (38.9%)	0.064
O_2 therapy	27/42 (64.3%)	5/7 (71.4%)	17/24 (70.8%)	23/58 (39.6%)	13/18 (72.2%)	0.011
O_2 administration[b]						
No O_2	*15*	2	7	*35*	5	0.045[c]
LFNC	*10*	3	12	*13*	1	
HFNC	*13*	2	5	*7*	8	
MV	*4*	0	0	*3*	4	
RSV-B	**2017–2018 N = 19**	**2018–2019 N = 23**	**2019–2020 N = 3**	**2021–2022 N = 20**	**2022–2023 N = 48**	**p-value**
Female/Male	11/8	6/17	3/0	7/13	18/30	
Mean age in months (SD)	3.24 ± 2.13	2.93 ± 2.18	2.38 ± 1.92	3.27 ± 2.15	3.08 ± 2.31	0.958
PICU[a]	2/19 (10.5%)	3/23 (13.0%)	0/3 (0.0%)	3/20 (15.0%)	18/48 (37.5%)	0.036
O_2 therapy	5/19 (26.3%)	11/23 (47.8%)	1/3 (33.3%)	8/20 (40.0%)	38/48 (79.2%)	< 0.001
O_2 administration[b]						
No O_2	14	*12*	2	14	*10*	0.019[c]
LFNC	3	*3*	0	4	*5*	
HFNC	1	*7*	1	4	*20*	
MV	1	*1*	0	0	*13*	

[a] Children with admission to pediatric intensive care unit (PICU);
[b] O_2 administration methods were: low flow nasal cannula (LFNC), high flow nasal cannula (HFNC), mechanical ventilation (MV).
[c] the Freeman-Halton extension of Fisher's exact test was used to compute the distribution of O_2 administration methods in a 2 × 4 contingency table, comparing 2017–2018 and 2021–2022 (in italics) for RSV-A and 2018–2019 and 2022–2023 (in italics) for RSV-B.

immune debt toward RSV-A apparently did not result in a more severe disease in 2021. Considering that RSV-A circulated abundantly in Rome in the months immediately preceding the pandemic, it is possible that the transfer of RSV-A-primed lymphocytes in breast-feeding mothers,[36] conferred some protection to infants despite the decrease in milk antibodies,[37] because there were no relevant genetic changes in 2021 RSV-A strains (this study). Contrastingly, the RSV-B specific immune debt would have been higher because of the longer time elapsed since the last RSV-B dominated season (2018–2019) and the higher divergence of RM RSV-B post-pandemic strains contributing to immune escape; in addition, a particular genetic configuration might have enhanced RSV-B pathogenicity during the 2022–2023 epidemic. Strains similar to RM sequences clade B1, were found in the US studies[42,43] but their prevalence was low and clinical data were not reported; the study from Colorado reporting RSV hospitalizations in 2022–2023 did not present subtype-specific data.[44] Comparable clinical data have not yet published by other groups and in general there is a paucity of

studies comparing specific RSV-B genotypes/clades; a recent Chinese study have documented the same severity associated to two BA9 sub-lineages circulating during 2017–2019.[29] Undoubtedly, whole-genome sequencing (WGS) of post-pandemic RSV strains would have contributed more information on genetic diversity and the relationships with disease severity, but this study has been approved by the ethical committee for research on residual diagnostic respiratory samples, insufficient for WGS. Another possible limitation is the single center nature of this study; however, with this study design, we were able to compare bronchiolitis cases of the pre- and -post pandemic period in the same hospital setting, using the same inclusion criteria, in an age-homogeneous population, with no pre-existing risk factors.

In conclusion, this study demonstrated that the first RSV post-pandemic surge in Rome, concentrated in October-December 2021, was mainly due to RSV-A strains very similar to those previously circulating. The increased number of total RSV cases, with no enhanced virulence, was probably due to the immune debt brought about by pandemic restrictions. The still high number of RSV bronchiolitis observed in the 2022–2023, were mostly caused by divergent RSV-B strains; a waned RSV-B population immunity and genetic divergence are both possible contributors to clinical severity. These results strengthen the concept that extensive local data on RSV molecular epidemiology are needed to observe patterns of genomic diversity associated with transmissibility and virulence or with resistance to therapeutics. The integration of regional data into the national surveillance system could provide a timely alert for variants that may represent health concerns, causing out of season and/or severe outbreaks. In addition, since vaccines and innovative treatments for RSV are about to enter the market, RSV virological surveillance may contribute to define strategies to prevent severe disease.

Ethical approval

This study was conducted in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration. The institutional review board and the Ethics Committee of Rome University Hospital approved this study (Prot. 107/12); informed consent was obtained from infants' parents. Clinical data were extracted from patients' healthcare records and, in line with confidentiality requirements, the database was anonymized.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: GUIDO ANTONELLI reports financial support was provided by EU funding within the NextGeneration EU-MUR PNRR. ANNA TERESA PALAMARA reports financial support was provided by Italian Ministry of Health.

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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.2023.07.008.

References

1. Li Y, Wang X, Blau DM, Caballero MT, Feikin DR, Gill CJ, et al. *Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in children younger than 5 years in 2019: a systematic analysis. Lancet* 2022;**399**:2047–64. https://doi.org/10.1016/S0140-6736(22)00478-0
2. Toivonen L, Karppinen S, Schuez-Havupalo L, Teros-Jaakkola T, Mertsola J, Waris M, et al. *Respiratory syncytial virus infections in children 0-24 months of age in the community. J Infect* 2020;**80**:69–75. https://doi.org/10.1016/j.jinf.2019.09.002
3. McLellan JS, Ray WC, Peeples ME. *Structure and function of respiratory syncytial virus surface glycoproteins. Curr Top Microbiol Immunol* 2013;**372**:83–104. https://doi.org/10.1007/978-3-642-38919-1_4
4. Peret TC, Hall CB, Schnabel KC, Golub JA, Anderson LJ. *Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. J Gen Virol* 1998;**79**:2221–9. https://doi.org/10.1099/0022-1317-79-9-2221
5. Hall CB, Walsh EE, Schnabel KC, Long CE, McConnochie KM, Hildreth SW, et al. *Occurrence of groups A and B of respiratory syncytial virus over 15 years: associated epidemiologic and clinical characteristics in hospitalized and ambulatory children. J Infect Dis* 1990;**162**:1283–90.
6. Gilca R, De Serres G, Tremblay M, Vachon ML, Leblanc E, Bergeron MG, et al. *Distribution and clinical impact of human respiratory syncytial virus genotypes in hospitalized children over 2 winter seasons. J Infect Dis* 2006;**193**:54–8. https://doi.org/10.1086/498526
7. Jafri HS, Wu X, Makari D, Henrickson KJ. *Distribution of respiratory syncytial virus subtypes A and B among infants presenting to the emergency department with lower respiratory tract infection or apnea. Pediatr Infect Dis J* 2013;**32**:335–40. https://doi.org/10.1097/INF.0b013e318282603a
8. Devincenzo JP. *Natural infection of infants with respiratory syncytial virus subgroups A and B: a study of frequency, disease severity, and viral load. Pediatr Res* 2004;**56**:914–7. https://doi.org/10.1203/01.PDR.0000145255.86117.6A
9. Martinello RA, Chen MD, Weibel C, Kahn JS. *Correlation between respiratory syncytial virus genotype and severity of illness. J Infect Dis* 2002;**186**:839–42. https://doi.org/10.1086/342414
10. Saravanos GL, Ramos I, Britton PN, Wood NJ. *Respiratory syncytial virus subtype circulation and associated disease severity at an Australian paediatric referral hospital, 2014-2018. J Paediatr Child Health* 2021;**57**:1190–5. https://doi.org/10.1111/jpc.15419
11. Rodriguez-Fernandez R, Tapia LI, Yang CF, Torres JP, Chavez-Bueno S, Garcia C, et al. *Respiratory syncytial virus genotypes, host immune profiles, and disease severity in young children hospitalized with bronchiolitis. J Infect Dis* 2017;**217**:24–34. https://doi.org/10.1093/infdis/jix543
12. Midulla F, Nenna R, Scagnolari C, Petrarca L, Frassanito A, Viscido A, et al. *How respiratory syncytial virus genotypes influence the clinical course in infants hospitalized for bronchiolitis. J Infect Dis* 2019;**219**:526–34. https://doi.org/10.1093/infdis/jiy496
13. Eshaghi A, Duvvuri VR, Lai R, Nadarajah JT, Li A, Patel SN, 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. https://doi.org/10.1371/journal.pone.0032807
14. Trento A, Galiano M, Videla C, Carballal G, García-Barreno B, Melero JA, 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. https://doi.org/10.1099/vir.0.19357-0
15. Prifert C, Streng A, Krempl CD, Liese J, Weissbrich B. *Novel respiratory syncytial virus A genotype, Germany, 2011-2012. Emerg Infect Dis* 2013;**19**:1029–30. https://doi.org/10.3201/eid1906.121582
16. Pierangeli A, Trotta D, Scagnolari C, Ferreri ML, Nicolai A, Midulla F, et al. *Rapid spread of the novel respiratory syncytial virus A ON1 genotype, central Italy, 2011 to 2013. Eur Surveill* 2014;**19**:20843. https://doi.org/10.2807/1560-7917.es2014.19.26.20843
17. Duvvuri VR, Granados A, Rosenfeld P, Bahl J, Eshaghi A, Gubbay JB. *Genetic diversity and evolutionary insights of respiratory syncytial virus A ON1 genotype: global and local transmission dynamics. Sci Rep* 2015;**5**:14268. https://doi.org/10.1038/srep14268
18. Trento A, Casas I, Calderón A, Garcia-Garcia ML, Calvo C, Perez-Breña P, et al. *Ten years of global evolution of the human respiratory syncytial virus BA genotype with a 60-nucleotide duplication in the G protein gene. J Virol* 2010;**84**:7500–12. https://doi.org/10.1128/JVI.00345-10
19. Otieno JR, Kamau EM, Oketch JW, Ngoi JM, Gichuki AM, Binter Š, et al. *Whole genome analysis of local Kenyan and global sequences unravels the epidemiological and molecular evolutionary dynamics of RSV genotype ON1 strains. Virus Evol* 2018;**4**:vey027. https://doi.org/10.1093/ve/vey027
20. Goya S, Galiano M, Nauwelaers I, Trento A, Openshaw PJ, Mistchenko AS, et al. *Toward unified molecular surveillance of RSV: a proposal for genotype definition. Influenza Other Respir Virus* 2020;**14**:274–85. https://doi.org/10.1111/irv.12715
21. Chen J, Qiu X, Avadhanula V, Shepard SS, Kim DK, Hixson J, 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. https://doi.org/10.1111/irv.12936
22. Midulla F, Di Mattia G, Nenna R, Scagnolari C, Viscido A, Oliveto G, et al. *Novel variants of respiratory syncytial virus A ON1 associated with increased clinical severity of bronchiolitis. J Infect Dis* 2020;**222**:102–10. https://doi.org/10.1093/infdis/jiaa059
23. Chow EJ, Uyeki TM, Chu HY. *The effects of the COVID-19 pandemic on community respiratory virus activity. Nat Rev Microbiol* 2022:1–16. https://doi.org/10.1038/s41579-022-00807-9
24. den Hartog G, van Kasteren PB, Schepp RM, Teirlinck AC, van der Klis FRM, van Binnendijk RS. *Decline of RSV-specific antibodies during the COVID-19 pandemic. Lancet Infect Dis* 2023;**23**:23–5. https://doi.org/10.1016/S1473-3099(22)00763-0

25. Eden JS, Sikazwe C, Xie R, Deng YM, Sullivan SG, Michie A, et al. *Off-season RSV epidemics in Australia after easing of COVID-19 restrictions. Nat Commun* 2022;**13**:2884. https://doi.org/10.1038/s41467-022-30485-3
26. Johnson SM, McNally BA, Ioannidis I, Flano E, Teng MN, Oomens AG, et al. *Respiratory syncytial virus uses CX3CR1 as a receptor on primary human airway epithelial cultures. PLoS Pathog* 2015;**11**(12):e1005318. https://doi.org/10.1371/journal.ppat.1005318
27. Hirano E, Kobayashi M, Tsukagoshi H, Yoshida LM, Kuroda M, Noda M, et al. *Molecular evolution of human respiratory syncytial virus attachment glycoprotein (G) gene of new genotype ON1 and ancestor NA1. Infect Genet Evol* 2014;**28**:183–91. https://doi.org/10.1016/j.meegid.2014.09.030
28. Botosso VF, Zanotto PM, Ueda M, Arruda E, Gilio AE, Vieira SE, et al. *Positive selection results in frequent reversible amino acid replacements in the G protein gene of human respiratory syncytial virus. PLoS Pathog* 2009;**5**(1):e1000254. https://doi.org/10.1371/journal.ppat.1000254
29. Luo Q, Li M, Li A, Gong C, Dong M, Huang Q, et al. *Genetic diversity and epidemiological features of respiratory syncytial virus, Beijing, 2015-2019: a multicenter and all-age groups study. J Infect* 2022;**85**:75–85. https://doi.org/10.1016/j.jinf.2022.04.046
30. Fedechkin SO, George NL, Wolff JT, Kauvar LM, DuBois RM. *Structures of respiratory syncytial virus G antigen bound to broadly neutralizing antibodies. Sci Immunol* 2018;**3**(21):eaar3534. https://doi.org/10.1126/sciimmunol.aar3534
31. Nenna R, Matera L, Licari A, Manti S, Di Bella G, Pierangeli A, et al. *An Italian multicenter study on the epidemiology of respiratory syncytial virus during SARS-CoV-2 pandemic in hospitalized children. Front Pediatr* 2022;**10**:930281. https://doi.org/10.3389/fped.2022.930281
32. Tenenbaum T, Doenhardt M, Diffloth N, Berner R, Armann JP. *High burden of RSV hospitalizations in Germany 2021-2022. Infection* 2022;**50**:1587–90. https://doi.org/10.1007/s15010-022-01889-6
33. Lumley SF, Richens N, Lees E, Cregan J, Kalimeris E, Oakley S, et al. *Changes in paediatric respiratory infections at a UK teaching hospital 2016-2021; impact of the SARS-CoV-2 pandemic. J Infect* 2022;**84**:40–7. https://doi.org/10.1016/j.jinf.2021.10.022
34. Bardsley M, Morbey RA, Hughes HE, Beck CR, Watson CH, Zhao H, et al. *Epidemiology of respiratory syncytial virus in children younger than 5 years in England during the COVID-19 pandemic, measured by laboratory, clinical, and syndromic surveillance: a retrospective observational study. Lancet Infect Dis* 2023;**23**:56–66. https://doi.org/10.1016/S1473-3099(22)00525-4
35. Nygaard U, Hartling UB, Nielsen J, Vestergaard LS, Dungu KHS, Nielsen JSA, et al. *Hospital admissions and need for mechanical ventilation in children with respiratory syncytial virus before and during the COVID-19 pandemic: a Danish nationwide cohort study. Lancet Child Adolesc Health* 2023;**7**:171–9. https://doi.org/10.1016/S2352-4642(22)00371-6
36. Reicherz F, Xu RY, Abu-Raya B, Majdoubi A, Michalski C, Golding L, et al. *Waning immunity against respiratory syncytial virus during the coronavirus disease 2019 pandemic. J Infect Dis* 2022;**226**:2064–8. https://doi.org/10.1093/infdis/jiac192
37. Grobben M, Juncker HG, van der Straten K, Lavell AHA, Schinkel M, Buis DTP, et al. *Decreased passive immunity to respiratory viruses through human milk during the COVID-19 pandemic. Microbiol Spectr* 2022;**10**(4):e0040522. https://doi.org/10.1128/spectrum.00405-22
38. Kamau E, Otieno JR, Lewa CS, Mwema A, Murunga N, Nokes DJ, et al. *Evolution of respiratory syncytial virus genotype BA in Kilifi, Kenya, 15 years on. Sci Rep* 2020;**10**:21176. https://doi.org/10.1038/s41598-020-78234-0
39. Bergeron HC, Tripp RA. *RSV replication, transmission, and disease are influenced by the RSV G protein. Viruses* 2022;**14**:2396. https://doi.org/10.3390/v14112396
40. Mrcela D, Markic J, Zhao C, Viskovic DV, Milic P, Copac R, et al. *Changes following the Onset of the COVID-19 pandemic in the burden of hospitalization for respiratory syncytial virus acute lower respiratory infection in children under two years: a retrospective study from Croatia. Viruses* 2022;**14**:2746. https://doi.org/10.3390/v1412274
41. Munkstrup C, Lomholt FK, Emborg HD, Møller KL, Krog JS, Trebbien R, et al. *Early and intense epidemic of respiratory syncytial virus (RSV) in Denmark, august to december 2022. Eur Surveill* 2023;**28**(1). https://doi.org/10.2807/1560-7917.ES.2023.28.1.2200937
42. Goya S, Sereewit J, Pfalmer D, Nguyen TV, Bakhash SAKM, Sobolik EB, et al. *Genomic characterization of respiratory syncytial virus during 2022-23 outbreak, Washington, USA. Emerg Infect Dis* 2023;**29**:865–8. https://doi.org/10.3201/eid2904.221834
43. Adams G, Moreno GK, Petros BA, Uddin R, Levine Z, Kotzen B, et al. *Viral lineages in the 2022 RSV surge in the United States. N Engl J Med* 2023;**388**:1335–7. https://doi.org/10.1056/NEJMc2216153
44. Rao S, Armistead I, Messacar K, Alden NB, Schmoll E, Austin E, et al. *Shifting epidemiology and severity of respiratory syncytial virus in children during the COVID-19 pandemic. JAMA Pediatr* 2023;**177**:730–2. https://doi.org/10.1001/jamapediatrics.2023.1088