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Epidemiology and genetic variability of respiratory syncytial virus in Portugal, 2014–2018

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ABSTRACT

Introduction: Respiratory syncytial virus (RSV) is associated with substantial morbidity and mortality since it is a predominant viral agent causing respiratory tract infections in infants, young children and the elderly. Considering the availability of the RSV vaccines in the coming years, molecular understanding in RSV is necessary.

Objective: The objective of the present study was to describe RSV epidemiology and genotype variability in Portugal during the 2014/15–2017/18 period.

Material and methods: Epidemiological data and RSV-positive samples from patients with a respiratory infection were collected through the non-sentinel and sentinel influenza surveillance system (ISS). RSV detection, subtyping in A and B, and sequencing of the second hypervariable region (HVR2) of G gene were performed by molecular methods. Phylogenetic trees were generated using the Neighbor-Joining method and p-distance model on MEGA 7.0.

Results: RSV prevalence varied between the sentinel (2.5%, 97/3891) and the non-sentinel ISS (20.7%, 3138/16779), being higher ($P < 0.0001$) among children aged < 5 years. Bronchiolitis (62.9%, 183/291) and influenza-like illness (24.6%, 14/57) were associated ($P < 0.0001$) with RSV laboratory confirmation among children aged < 6 months and adults ≥ 65 years, respectively. The HVR2 was sequenced for 562 samples. RSV-A (46.4%, 261/562) and RSV-B (53.6%, 301/562) strains clustered mainly to ON1 (89.2%, 233/261) and BA9

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(92%, 277/301) genotypes, respectively, although NA1 and BA10 were also present until 2015/2016.

Conclusion: The sequence and phylogenetic analysis reflected the relatively high diversity of Portuguese RSV strains. BA9 and ON1 genotypes, which have been circulating in Portugal since 2010/2011 and 2011/2012 respectively, predominated during the whole study period.

1. Text

1.1. Background

Respiratory syncytial virus (RSV) A and RSV-B are antigenically different, often co-circulate although one of them usually predominates [1,2]. Genetic diversity increased with the spread of new genotypes among both of subtypes in last years. The second hypervariable region (HVR2), which carries the C-terminus of the G attachment glycoprotein, has a high degree of divergence and, thereby, it has been used as the main indicator for studies on RSV evolution [3,4]. To date, based on this region, 15 RSV-A and 29 RSV-B genotypes have been described [3,5–11].

RSV is associated with substantial morbidity and mortality since it is the major cause of lower respiratory tract infections (LRTI) during childhood resulting in hospitalization due to the severity of infection in many cases [12]. Moreover, it causes severe respiratory tract infection in adults, especially elderly, and immunosuppressed patients [13,14]. Symptomatic supportive care and Palivizumab are currently available options to RSV disease clinical management, which reduce the symptoms and severity, decreasing hospitalization rate but not mortality [15–18]. Fortunately, RSV vaccines are progressing in phase III clinical trials and could be available in the coming years [19], being G protein suggested as a plausible target [20]. Therefore, ongoing global surveillance and characterization of circulating RSV strains are required for evidence-based vaccination policies. In Portugal, RSV cases have been detected since 2010 through the national influenza surveillance system (ISS), during the influenza season, from week 37 (September) to week 24 (June) of the next year.

1.2. Objectives

This study aimed to describe the prevalence and genetic variability of RSV during the 2014/15–2017/18 period in Portugal, as well as to evaluate the association between subtype, age and clinical diagnose among patients with laboratory-confirmed RSV infection.

2. Study design

2.1. Portuguese influenza surveillance system (ISS)

The Portuguese ISS accounts with the sentinel and non-sentinel components. Sentinel ISS is made up of General Practitioners' (GP) Sentinel Network and GP from the EuroEva Portuguese component of the I-MOVE project [21], and the Emergency/Obstetric Departments Networks in hospitals. Non-sentinel ISS integrates the Portuguese Laboratory Network for the Diagnosis of Influenza Infection (PLNDII), which comprises 14 hospital-based laboratories and is coordinated by the National Institute of Health Doutor Ricardo Jorge (INSA). This network is integrated by general hospitals with pediatric and adults emergency rooms and medical wards, and one reference pediatric hospital in Lisbon. ISS weekly reports laboratory data of tested samples to the European Influenza Surveillance Network, which is coordinated by the European Centre for Disease Prevention and Control (ECDC) and WHO/Europe.

2.2. Study population and sample collection

Demographic, clinical and laboratory data from RSV-positive cases

were collected through the ISS. RSV-positive respiratory samples were collected from three populations: (i) all age influenza-like illness (ILI) [22] patients reported by the sentinel ISS during the 2014/15–2017/18 period; (ii) children aged < 5 years with respiratory infection diagnosed by the PLNDII during the 2015/16–2017/18 period; and (iii) adults aged ≥65 years with respiratory infection diagnosed by the PLNDII during the 2017/18 season. Samples included nasopharyngeal or oropharyngeal swabs, and nasopharyngeal aspirates and lavages. This study was approved by the Health Ethic Committee of INSA.

2.3. RSV molecular detection

Viral nucleic acid extraction was performed using the automated acid extraction platform EasyMAG. Molecular detection of RSV and identification of the subtypes A and B were performed using real-time PCR adapted from a previous protocol described by Gunson et al. [23].

2.4. Gene sequencing

For genotyping, sequencing of RSV-positive ISS cases was prospectively performed since the 2015/16 season and retrospectively carried out before that time. In addition, the PLNDII voluntarily selected the first two RSV positive samples per week from children aged < 5 years (2015/16–2017/18) and adults aged ≥65 years (2017/18). Conventional PCR and sequencing analysis of the HVR2 was performed by a one-step RT-PCR adapted from previously published protocols. For the first-round PCR, RSV3 (GGCAATGATAATCTCAAC) [24] and F164 (GTT ATG ACA CTG GTA TAC CAA CC) [25] were used, whereas for the second round PCR and sequencing, OGCH496+ (GAT GATTACCATTTTGAAGTGTTC) [25] and F1 (CAACTCCATTGTTATT TGCC) [26] were selected. The size of the product was around 500 pb.

2.5. Sequence and phylogenetic analysis

Potentially N-glycosylation sites (Asn-X-Ser/Thr) were identified by NetNGlyc 1.0. server (<http://www.cbs.dtu.dk/services/NetNGlyc/>). Alignments were performed with the MUSCLE method and phylogenetic trees were generated using the Neighbor-Joining method and p-distance model (bootstrap re-sampling of 1000 replicates) on MEGA 7.0. In addition to worldwide reference strains (RSV-A and RSV-B) that were retrieved from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>), 20 RSV-A strains from a previous study in Portugal (2010/11–2013/14) [27] were used for the tree construction together with the sequences generated in this study. Sequences with 100% homology in the nucleotide sequence were represented by one strain and submitted to GenBank database with accession numbers MN122441–MN122562 (RSV-A) and MN122563–MN122694 (RSV-B).

2.6. Statistical analysis

Demographic and epidemiological characteristics were described using proportions, and group comparisons were assessed through Chi-square test or Fisher's exact test. Tests with p-value < 0.05 were considered statistically significant. The statistical analysis was performed using STATA 12.

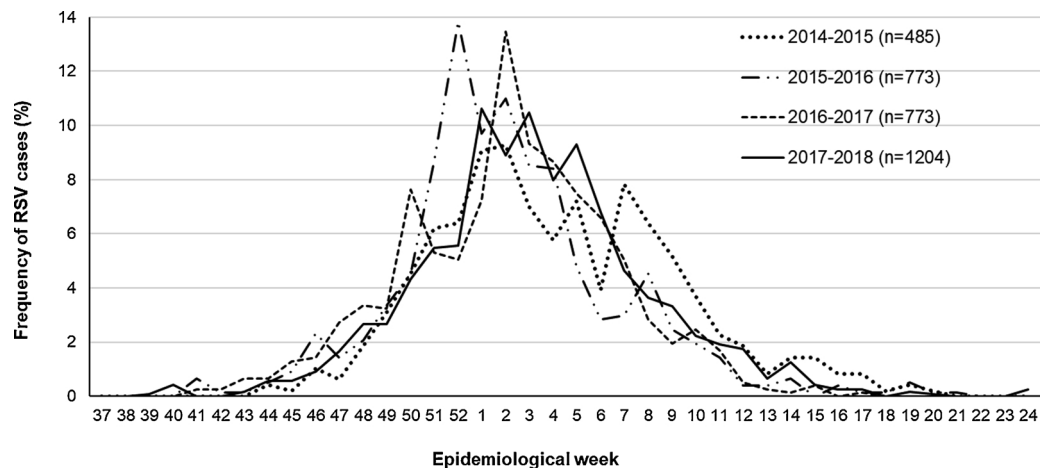


Fig. 1. Weekly distribution of detected RSV cases between 2014 and 2018 during the influenza season through the Portuguese influenza surveillance system. RSV, Respiratory syncytial virus. (2-column fitting image).

Table 1
Prevalence of respiratory syncytial virus according to the component of the influenza surveillance system and sample date.

Component of the ISS	Total	Season			
		2014-15	2015-16	2016-17	2017-18
Sentinel					
Studied patients	3891	900	1093	921	977
RSV-positive cases (%)	97 (2.5)	25 (2.8)	35 (3.2)	18 (2)	19 (1.9)
Non-sentinel (PLNDII)					
Studied patients	16779	2342	3452	2773	8212
RSV-positive cases (%)	3138 (20.7)	460 (19.6)	738 (21.4)	755 (27.2)	1185 (14.4)

ISS, Influenza Surveillance System; PLNDII, Portuguese Laboratory Network for Diagnosis Infection; RSV, Respiratory syncytial virus.

Table 2
Demographic characteristics of respiratory syncytial virus positive and negative cases, which were detected through the influenza surveillance system in Portugal, 2014-2018.

Demographic characteristics*	RSV + (n = 3235)		RSV- (n = 17,435)	
	n	%	n	%
Age group**				
0-4 y (n = 6743)	2343	72.5	4400	25.3
5-14 y (n = 1378)	91	2.8	1287	7.4
15-44 y (n = 3550)	185	5.7	3365	19.4
45-64 y (n = 3830)	232	7.2	3598	20.7
≥ 65 y (n = 5099)	379	11.7	4720	27.2
Total (n = 20,600)	3230		17370	
Sex				
Female (n = 10,088)	1608	49.7	8942	51.3
Male (n = 10,568)	1626	50.3	8480	48.7
Total (n = 20,656)	3234		17422	

y, years.

* Cases with missing age or sex included in the study. Age was missing for five RSV-positive and 65 RSV-negative cases; sex was missing for one RSV-positive and 13 RSV-negative cases.

** Observed frequencies where higher than those expected among the 0-4 years age group (P < 0.0001).

Table 3
Demographic and clinical characteristics from subtyped RSV samples through the sentinel influenza surveillance system (n = 56).

Characteristics	Total (n = 56)		RSV subtype		p-value		
	n	%	RSVA (n = 34)	RSVB (n = 22)			
Age group (years)							
1-4	7	12.3	5	14.7	2	9.1	0.037 ^a
5-14	1	1.8	1	2.9	0	0	
15-44	6	10.5	0	0	6	27.3	
45-64	26	45.6	17	50	9	40.9	
≥ 65	14	24.6	9	26.5	5	22.7	
Unknown	2	3.5	2	5.9	0	0	
Sex							
Male	20	35.1	17	50	3	13.6	0.012 ^b
Female	35	61.4	16	47.1	19	86.4	
Unknown	1	1.8	1	2.9	0	0	
Clinical characteristics							
Cough	52	91.2	32	94.1	20	90.9	0.448
Malaise	50	87.7	33	97.1	17	77.3	0.061
Sore throat	44	77.2	27	79.4	17	77.3	0.064
Sudden onset of symptoms	43	75.4	25	73.5	18	81.8	0.644
Myalgia	41	71.9	25	73.5	16	72.7	0.460
Fever or feverishness	36	63.2	23	67.6	13	59.1	0.691
Headache	34	59.6	20	58.8	14	63.6	0.358
Shortness of breath	24	42.1	15	44.1	9	40.9	0.565

RSV, Respiratory syncytial virus.

^a Observed frequencies of RSV-B in 15-44 years old age group higher than expected.

^b Observed frequencies of RSV-B in females and RSV-A in males higher than expected.

3. Results

3.1. RSV detection

RSV was identified in 3235 samples through the ISS from week 37 of 2014 to week 24 of 2018 (Fig. 1). A total of 407 (12.6%) RSV cases were co-detected with other respiratory viruses, mainly Picornavirus (29.5%, 120/407), Coronavirus (11.5%, 47/407), and Adenovirus (10.1%, 41/407). The majority of RSV cases (97%, 3138/3235) were

Table 4
Demographic and clinical characteristics from subtyped RSV samples through the non-sentinel influenza surveillance system (n = 486).

Characteristics	Total (n = 486)		RSV subtype				p-value
			RSVA (n = 207)		RSVB (n = 279)		
	n	%	n	%	n	%	
Age group							0.018 ^a
Children (< 5 y)	429	88.3	191	92.3	238	85.3	
0-6 m	291	59.9	137	66.2	154	55.2	
7-12 m	63	13.0	30	14.5	33	11.8	
13 m - 4 y	75	15.4	24	11.6	51	18.3	
≥ 65	57	11.7	16	7.7	41	14.7	
Sex							0.721
Male	249	51.2	108	52.2	141	50.5	
Female	237	48.8	99	47.8	138	49.5	
Diagnostic							0.150
Bronchiolitis	242	49.8	114	55.1	128	45.9	
Other	93	19.1	40	19.3	53	19	
Unknown	57	11.7	20	9.7	37	13.3	
Respiratory distress syndrome	46	9.5	20	9.7	26	9.3	
Pneumonia	27	5.6	7	3.4	20	7.2	
ILI	21	4.3	6	2.9	15	5.4	
Hospitalization							0.069
Yes	336	69.1	134	64.7	202	72.4	
No	42	8.6	16	7.7	26	9.3	
Unknown	108	22.2	57	27.5	51	18.3	

RSV, Respiratory syncytial virus; y, years; m, months; ILI, Influenza-like illness.

^a Observed frequencies of RSV-A among children < 5 years and RSV-B among adults ≥ 65 years higher than expected.

detected through the PLNDII (Table 1). Furthermore, during the four studied seasons, RSV prevalence ranged from 1.9% to 3.2% (2.5%, 97/3891) in the sentinel ISS and from 14.4% to 27.2% in the PLNDII (20.7%, 3138/16,779).

3.2. Epidemiological characteristics

Demographic characteristics for RSV-positive and negative cases were described (Table 2). RSV-positive frequency (72.5%, 2343/3230) was significantly ($P < 0.0001$) higher among children aged < 5 years than the one expected if there was not association between age and RSV infection. The sequence of the HVR2 was determined for 542 RSV-positive samples during the 2014/15–2017/18 period. Demographic and clinical characteristics for those 542 patients, including 56 through the sentinel ISS (Table 3) and 486 through the PLNDII (Table 4), were

Table 5
Distribution of respiratory syncytial virus subtype and genotype according to the sample date (n = 562).

Sampling period	Number of samples	RSV-A viruses n (%)	RSV-A (n = 261)		RSV-B viruses n (%)	RSV-B (n = 301)	
			NA1	ON1		BA10	BA9
			n (%)	n (%)		n (%)	n (%)
2010-14 ^a	20	20 (100)	4 (20)	16 (80)	0	0	0
2014-15 ^b	21	3 (14.3)	0	3 (100)	18 (85.7)	7 (38.9)	11 (61.1)
2015-16	163	99 (60.7)	24 (24.2)	75 (75.8)	64 (39.3)	17 (26.6)	47 (73.4)
2016-17	169	73 (43.2)	0	73 (100)	96 (56.8)	0	96 (100)
2017-18 ^c	189	66 (34.9)	0	66 (100)	123 (65.1)	0	123 (100)
Total	562	261 (46.4)	28 (10.7)	233 (89.3)	301 (53.6)	24 (8)	277 (92)

RSV, Respiratory syncytial virus.

^a 20 RSV from 2010-14 were included in the phylogenetic analysis. 2010-11: 3 ON1; 2011-12: 1 NA1 and 1 ON1; 2012-13: 7 ON1; 2013-14: 8 ON1.

^b All samples were collected through the sentinel influenza surveillance system (ISS).

^c All samples were collected through the non-sentinel ISS.

described and the association with the RSV subtype was assessed. On RSV cases detected through the ISS, observed RSV-B subtype frequency (86.4%, 19/22) was significantly higher in females ($P = 0.012$) and in 15–44 years age group ($P = 0.037$). Samples from RSV cases that were detected through the PLNDII, belonged mostly to children (88.3%, 429/486) and 59.9% (291/486) were aged < 6 months. They presented a RSV-A frequency (66.2%, 137/207) significantly ($P = 0.015$) higher than the one expected. Three hundred thirty-six (69.1%) cases were hospitalized patients. Bronchiolitis in children aged < 6 months (62.9%, 183/291) and ILI in adults aged ≥ 65 years (24.6%, 14/57) were found to be significantly ($P < 0.0001$) associated with RSV laboratory confirmation. Moreover, observed frequencies of RSV-positive ILI (8.1%, 6/74) and pneumonia (10.8%, 8/74) among children from 13 months to 4 years old were also significantly ($P = 0.002$) higher than the ones expected. Therefore, these diagnostics were associated with laboratory-confirmed RSV infection among those age groups.

3.3. Subgroups and genotype

In addition to the 542 samples from the studied period, 20 RSV-A strains from the 2010/14 period were included in the phylogenetic analysis (Table 5). RSV-B strains before 2014/15 could not be included due to the poor quality of the sequences. Both of subtypes co-circulated during the study period (2014/15–2017/18), being 46.4% (261/562) of samples subtyped as RSV-A strains and 53.6% (301/562) subtyped as RSV-B strains. Most of RSV-A strains belonged to ON1 genotype (89.3%, 233/261). The remaining ones (10.7%, 28/261) belonged to NA1 genotype, which was undetected since 2015/16 (Fig. 2). All RSV-B strains clustered in two Buenos Aires genotypes, BA9 (92%, 277/301) and BA10 (8%, 24/301), the latter being only present until the 2015/16 season (Fig. 3).

3.4. Amino acid sequence analysis

The HVR2 of all NA1 strains was aligned with the reference NA1 strain (AB470478) whereas ON1 strains were compared with the reference ON1 strain (JN257693) (Supplementary Fig. 1). Eight NA1 and 114 ON1 strains showed at least one different nucleotide, being the highest number of amino acid substitutions found in the ON1 genotype. I243S, E262 K, L274 P, L298 P, and Y304H were the most common substitutions among ON1 strains. In addition, they contained E232 G/R/K and T253 K/R substitutions in comparison to NA1 strains and, in fact, the last substitution led to loss of one potential N-glycosylation site. Another site loss was due to N273Y/H/S substitution in all strains with the exception of one NA1 strain (2010/11). However, in addition to two conserved N-glycosylation sites (N237 and N318), two novel sites due to I243 N and S299 N substitutions were observed in one and two ON1 strains, respectively.

The HVR2 of all RSV B strains was analyzed in comparison to the

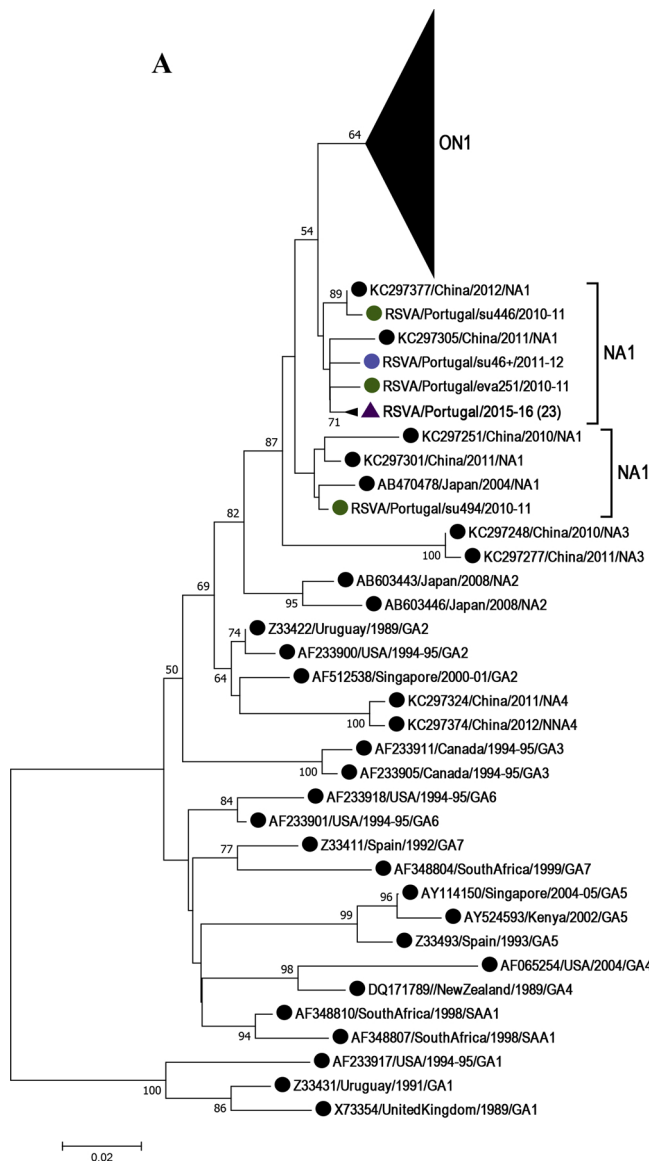


Fig. 2. Phylogenetic tree of RSV-A strains based on the C-terminal second hypervariable region (HVR2) of the G gene. (A) Complete phylogenetic tree, (B) ON1 strains. The phylogenetic tree was constructed with the Neighbor-Joining method with 1000 replicates for the bootstrap using MEGA 7.0. Only bootstrap values greater than 50 were represented at the branch nodes. The evolutionary distances were computed using the p-distance method and the scale bar represented the number of nucleotide substitutions per site. Reference strains (n = 50) were represented by a black circle. The accession number from GenBank, the country, the year and the genotype from reference strains are shown in the phylogenetic trees. Reference GA1 (AF233917, Z33431, X73354) strains served as outgroup strains. Sequences from this study with 100% nucleotide homology were represented with only one sequence. Different colors distinguished different periods (green, 2010-11; light blue, 2011-12; red, 2012-13; grey, 2013-14; turquoise, 2014-15; purple, 2015-16; orange, 2016-17; strong blue, 2017-18). A circle represented one sequence and a triangle represented more than one sequence from the same period with a bootstrap value greater than 50 (the number of sequences included is indicated between parentheses). Representative RSV-A sequences from this study were deposited in GenBank under accession numbers MN122441-MN122562. (In color online only, 2-column fitting image). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

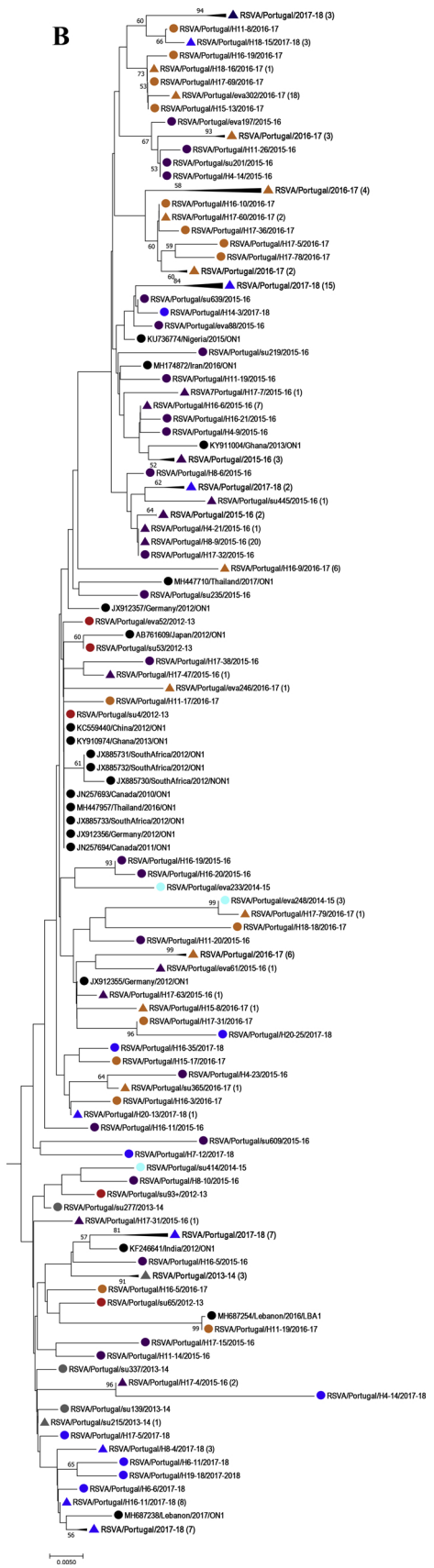
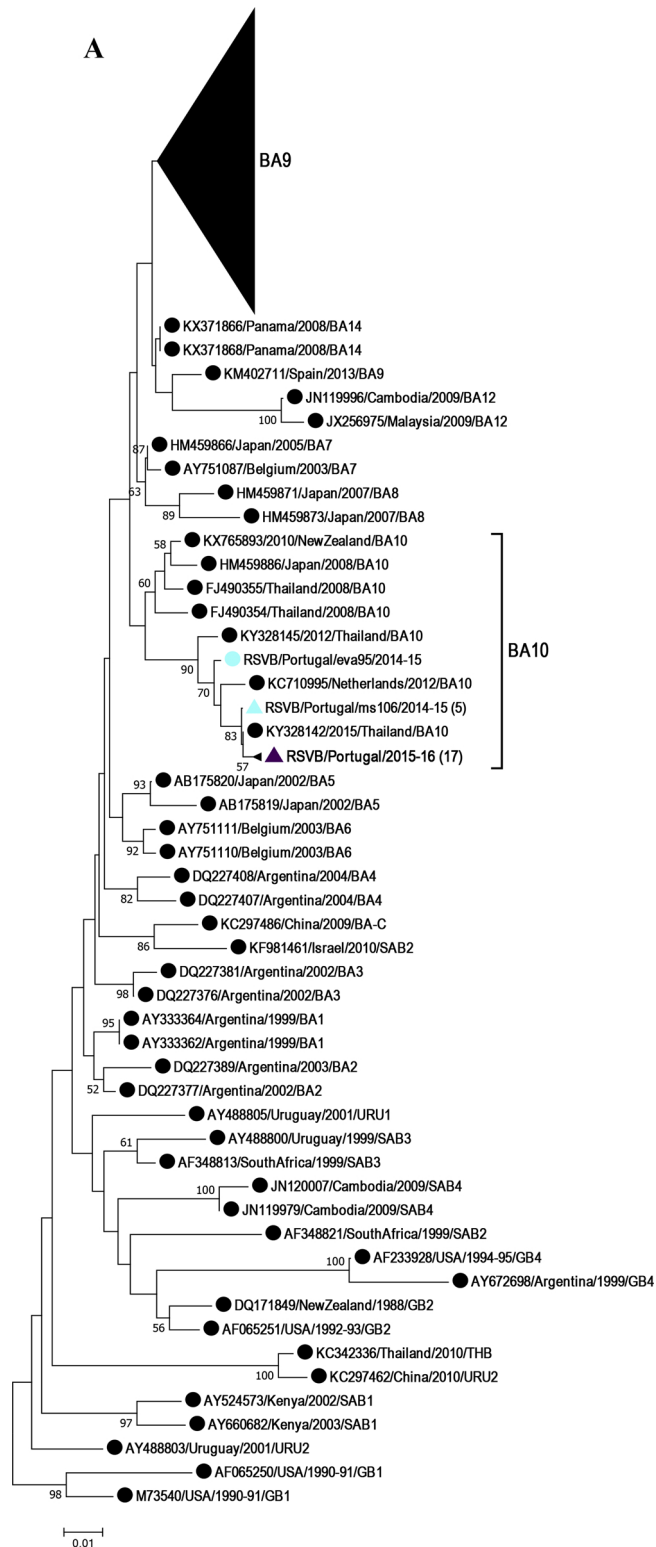


Fig. 2. (continued)

first reported BA strain (AY333364), which belongs to BA1 genotype (Supplementary Fig. 2). A total of 132 RSV-B group strains, including six BA10 and 126 BA9 strains with 98 different amino acidic combinations, were observed. Substitutions K218 T, L223 P, S247 P, T270I, V271A, I281 T and H287Y were identified in most RSV-B viruses. All BA10 strains showed the same amino acid sequence and had the substitution E292 G, which was exclusive of this genotype. Three conserved

Fig. 3. Phylogenetic tree of RSV-B strains based on the C-terminal second hypervariable region (HVR2) of the G gene. (A) Complete phylogenetic tree, (B) BA9 strains. The phylogenetic tree was constructed with the Neighbor-joining method with 1000 replicates for the bootstrap using MEGA 7.0. Only bootstrap values greater than 50 were represented at the branch nodes. The evolutionary distances were computed using the p-distance method and the scale bar represented the number of nucleotide substitutions per site. Reference strains (n = 76) were represented by a black circle. The accession number from GenBank, the country, the year and the genotype from reference strains are shown in the phylogenetic trees. Reference GB1 (AF065250, M73540) strains served as outgroup strains. Sequences from this study with 100% nucleotide homology were represented with only one sequence. Different colors distinguished different periods (turquoise, 2014-15; purple, 2015-16; orange, 2016-17; strong blue, 2017-18). A circle represented one sequence and a triangle represented more than one sequence from the same period with a bootstrap value greater than 50 (the number of sequences included is indicated between parentheses). Representative RSV-B sequences from this study were deposited in GenBank under accession numbers MN122563-MN122694. (In color online only, 2-column fitting image). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



sites were identified at amino acid 230, 296 and 310, although few BA9 strains lost N296 and N310 due to different substitutions. However, two additional sites (amino acid substitutions T227 N and H279 N) were identified in specific RSV-B strains.

4. Discussion

Circulating Portuguese RSV strains, through sentinel and non-sentinel ISS between 2014 and 2018, were characterized in epidemiologic and genetic terms in order to provide a better understanding of the molecular epidemiology of RSV infection in the country.

RSV detection rate in the sentinel ISS (2.5%) was similar to frequencies reported in outpatients from Brazil [28], Korea [29] and China [30] although was lower than in other European countries [15,31]. In the PLNDII, RSV prevalence was higher than the one found in the sentinel ISS since this network is composed by hospital-based laboratories, collect data from inpatients and sentinel ISS use the European Union (EU) ILI case definition [22], which is not accurate for RSV detection in Portugal [32]. The remarkable number of tested samples (8212) during the 2017/18 season could be explained with the consciousness-raising of relevance of RSV disease among physicians. Therefore, from our point of view, the prevalence of this season (14.4%) might be the best representative in the country, being similar to findings from Senegal [33], Thailand [34], Spain [35] and different regions in India [36,37]. Nonetheless, a true comparison of RSV prevalence in different geographic areas is difficult to perform taking into account the differences in the study design and, even in our study, prevalence was wide-ranging according to the season. Bronchiolitis and pneumonia were significantly associated with RSV-positive cases among children aged < 5 years, which was consistent with previous studies that found RSV as a major cause of acute LRTI in this age group [12,38–41].

RSV-A and RSV-B subtypes co-circulated between 2010 and 2018 in Portugal [27,42]. RSV-A subtype was the most prevalent in the 2015/16 season, whereas RSV-B subtype dominated in the next two consecutive seasons (2016/17 and 2017/2018). Periodic shifts (1–3 years) in RSV subtype have been reported all over the world [40]. RSV-A subtype was significantly higher in children younger than 5 years and especially those aged below 6 months, which may be concerning since RSV-A infection has been associated with more severe bronchiolitis and higher disease severity scores [43,44]. However, discrepancies have been described [45], and indeed, specific substitutions may indicate the most virulent strains during a specific period of time better than the subtype [46]. RSV-B subtype was significantly higher in 15–44 years old and female patients. However, this fact could be explained due to the low number characterized cases through the sentinel ISS.

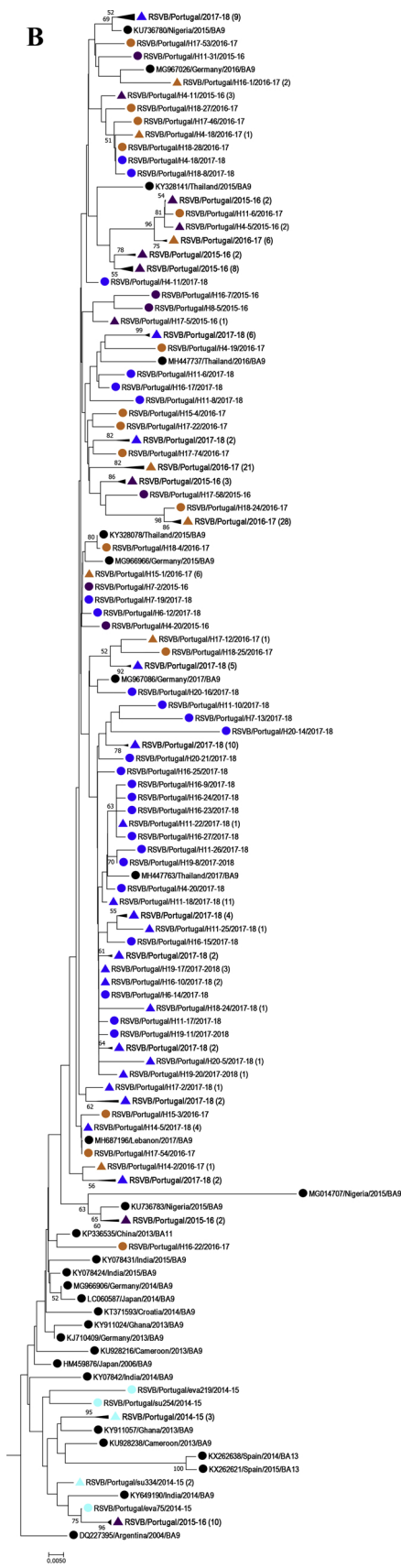


Fig. 3. (continued)

RSV-A strains (between 2010 and 2018) clustered in NA1 and ON1 genotypes, which was consistent with several studies reporting the almost exclusivity of these genotypes after 2005 [4,47]. NA1 genotype was detected from 2010 to 2012, and during the 2015/16 season. However, the fact that this genotype was not found between these two periods might be due to the sequencing bias. In Portugal, ON1 genotype was detected during the 2011/12 season for the first time [27], and since then, it has predominated in the country as reported worldwide [48,49]. Regarding RSV-B genetic diversity, all belonged to BA genotype, which has already been detected in at least 23 countries [50]. BA9 genotype has predominated in Portugal since 2010 [27,42] as in other countries [10,45,49,51], being detected alongside with BA10 genotype until 2015/16, when BA9 remained the only genotype detected onwards. Since 2001, new genotypes have been added over time by different authors following the proposed criteria by Venter et al. [5]. However, the addition of new sequences from different locations or more recent epidemics led to re-classify previous sequences into an existing genotype or even consolidate a new one [9,26,52,53]. In the present study, we considered that all RSV-A non-NA1 strains belonged to ON1 genotype since mostly of the identified clusters showed a bootstrap value < 70% and had the 72-nc insertion characteristic from this variant. Therefore, a standardized criteria may play a key role to assign an adequate genotype and assure the comparability between different studies regardless the addition of new sequences.

The alignment of the predicted amino acid sequences of strains from BA9, NA1 and ON1 genotypes confirmed the relatively high genetic variability associated with the HVR2. Strains from both of subtypes showed common changes, conserved N-glycosylation sites and substitutions leading to loss or gain of these sites that had been previously reported [11,40,41,45,46,49,50,54–59]. Within RSV-A strains, the number of substitutions among ON1 strains was higher compared with its NA1 counterparts. BA10 strains showed 100% homology in the HVR2 to reference strains from Thailand (KY328142, KY328145) and had the exclusive substitution E292 G, which has been observed in other studies [58,59]. Continual surveillance of amino acid changes as well as novel N-linked glycosylation sites should be performed since alterations in the antigenic characteristics of the viral surface glycoproteins may enable immune evasion leading to an evolutionary advantage [45,58]. In fact, the spread of BA genotype has been explained by the 60-nc duplication in the HVR2, which was associated with an increase of virus attachment and fitness [60].

The main limitation of this study was being retrospectively performed before the 2015/16 season. Consequently, there was a low number of successfully sequenced RSV samples before that time and through the sentinel ISS due to insufficient viral load or RNA degradation during conservation. However, the study had the advantage of including a large sample size from different regions during four consecutive seasons in Portugal, and thereby, it provides useful information on RSV epidemiology and genotype variability in the country from a recent period.

In conclusion, this study was the first to explore RSV epidemiology and genotype variability in Portugal from 2014 to 2018. It constitutes a contribution to RSV knowledge on evolution and strain circulation that is necessary for RSV vaccine development and future monitoring of vaccine effectiveness. Continuous surveillance of specific genomic changes and a standardized criteria for an optimized genotype assignment would be relevant as regards the vaccine design and RSV surveillance including virus characterization.

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CRedit authorship contribution statement

Emma Sáez-López: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Validation. **Paula Cristóvão:** Resources, Formal analysis, Writing - review & editing. **Inês Costa:** Resources, Formal analysis, Writing - review & editing. **Pedro Pechirra:** Resources, Writing - review & editing. **Patrícia Conde:** Resources, Writing - review & editing. **Raquel Guiomar:** Conceptualization, Project administration, Supervision, Writing - review & editing, Validation.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jcv.2019.104200>.

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