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# Respiratory Syncytial Virus in Greece, 2016–2018

Katerina Tsergouli[a] Styliani Pappa[a] Katerina Haidopoulou[b] Maria Gogou[b] Andreas Giannopoulos[b] Anna Papa[a]

[a]Department of Microbiology, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece; [b]Second Pediatric Department, AHEPA Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece

**Keywords**
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**Abstract**
Human respiratory syncytial virus (RSV) is the leading cause of acute bronchiolitis in infants and young children. Children under the age of 2 years, hospitalized for bronchiolitis in the pediatric clinic of a tertiary hospital in northern Greece, were tested for RSV infection during two RSV seasons (2016–2017 and 2017–2018). RSV was detected in 37 of 71 (52.1%) patients, most of them younger than 6 months. Both RSV subtypes were detected – RSV-A (54.1%) and RSV-B (45.9%) – with predominance of RSV-A during the 2016–2017 and RSV-B during the 2017–2018 season. RSV-A and RSV-B sequences clustered within the ON1 and BA genotypes, respectively. Compared to the prototype strains, several amino acid substitutions were observed in the duplication region of the G gene. The study provides a first insight into the molecular epidemiology of RSV in Greece. 

## Introduction

Human respiratory syncytial virus (RSV) (genus Orthopneumovirus, family Pneumoviridae) is the leading cause of acute bronchiolitis and pneumonia in infants and young children. It is estimated that the majority of children are infected by the age of 2 years [1, 2]. RSV epidemics have a seasonal pattern, with cases in the northern hemisphere being observed between November and March [3]. Based on variations in the sequence of the attachment G protein gene, RSV strains are classified into two subtypes, RSV-A and RSV-B, while based on the high variable second region of the G protein gene, they are classified into at least 13 RSV-A (GA1–7, SAA1, NA1–4, and ON1) [4] and 36 RSV-B genotypes (GB1–13, SAB1–4, BA1–14, URU1–2, THB, BA-CCA and BA-CCB) [5]. Since the available knowledge on genetic diversity of RSV strains in Greece is limited, the aim of the present study was to identify the subtypes of RSV strains which caused infections in children hospitalized during 2016–2018 in a pediatric clinic in Greece.

## Materials and Methods

The study included 71 children (38 male, 53.5%) with acute bronchiolitis hospitalized during two RSV seasons, 2016–2017 ($n$ = 30) and 2017–2018 ($n$ = 41), in the pediatric clinic of a tertiary hospital in northern Greece. The mean age of the patients was 4 months (range 0.57–24 months). Patients were classified into two age groups: 0–6 months ($n$ = 48) and >6 months ($n$ = 23). From each patient a throat swab was collected 1–9 days (median 4 days) after onset of symptoms. Viral RNA was extracted using the QIAamp viral RNA mini kit (QIAGEN, Lübeck, Germany). A nested RT-PCR which amplifies a highly conserved region of the

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Prof. Dr. Anna Papa, MD, PhD
Department of Microbiology, Medical School
Aristotle University of Thessaloniki
GR–54124 Thessaloniki (Greece)
annap@auth.gr

**Table 1.** Number and mean age of patients tested for RSV infection during two RSV seasons in Greece and RSV subtypes per season

| Season | Patients, *n* | Mean age, months (IQR) | RSV-positive, *n* (%) | RSV-A, *n* (%) | RSV-B, *n* (%) |
|---|---|---|---|---|---|
| 2016–2017 | 30 | 4.5 (6.2) | 18 (60.0) | 13/18 (72.2) | 5/18 (27.8) |
| 2017–2018 | 41 | 4.0 (7.5) | 19 (46.3) | 7/19 (36.8) | 12/19 (63.2) |
| Total | 71 | 4.0 (6.0) | 37 (52.1) | 20/37 (54.1) | 17/37 (45.9) |

RSV, respiratory syncytial virus.

RSV fusion protein gene was applied; the same PCR was also used for the identification of the subtypes since the PCR products for RSV-A and RSV-B differ in size (363 and 661 bp, respectively) [6]. To genotype the RSV-positive samples, a one-step RT-PCR was applied which amplifies a fragment of the second hypervariable region of the G protein gene (527 and 512 bp for subtypes A and B, respectively) [7]. The obtained sequences were aligned using CLUSTAL W and phylogenetic trees were constructed using MEGA7 after applying the best-fit model method [8].

## Results

RSV was detected in 37/71 (52.1%) patients. All RSV-positive patients except one were younger than 1 year. The prevalence of RSV infection was higher in children younger than 6 months (27/48, 56.3%). The mean hospitalization time of the RSV patients was 6.7 days. Their dyspnea score ranged from 1 to 10 (mean 4.54). Compared to non-RSV cases, no significant differences were seen regarding sex, prematurity, hospitalization days, and days of oxygen and intravenous fluids supplementation. All children recovered uneventfully, and none required admission to intensive care.

The RSV prevalence did not differ significantly between the two RSV seasons (60% during the 2016–2017 season and 46.3% during the 2017–2018 season, $p = 0.401$) (Table 1). Almost half of the cases (18/37) occurred in January.

Twenty cases (20/37, 54.1%) were caused by RSV-A and 17 (17/37, 45.9%) by RSV-B. Patients with RSV-A infection were significantly younger than those with RSV-B infection (2.5 months [IQR 3.7] and 6.0 months [IQR 7.0], respectively, $p = 0.030$). All infants younger than 1 month were infected with RSV-A. No significant difference was seen in the median hospitalization time of children infected with RSV-A and RSV-B (6 and 7 days, respectively, $p = 0.876$) nor in the mean dyspnea score (4.75 and 4.29, respectively, $p = 0.112$). Similarly, no significant differences were observed in sex, prematurity, or days of need for oxygen and intravenous fluids supplementation.

Although both RSV subtypes were co-circulating during the two seasons, a significant difference was observed between seasons, as RSV-A predominated during 2016–2017 (13/18, 72.2%) and RSV-B predominated during 2017–2018 (12/19, 63.2%) ($p = 0.031$).

Genotyping was successful on 23 samples (13 RSV-A and 10 RSV-B). All RSV-A sequences clustered within the ON1 genotype (Fig. 1a), and all RSV-B sequences clustered within the BA genotype (Fig. 1b). The genetic distances among RSV-A and RSV-B sequences between the two seasons were 3.2 and 2% at the nucleotide level and 6.2 and 3.8% at the amino acid level, respectively, suggesting that most nucleotide changes were non-silent. Sequences were submitted to the GenBank database and assigned the accession numbers MN704880 to MN704902.

The RSV-A ON1 genotype is characterized by a 72-nucleotide-long duplication in the C-terminal region of the G gene (positions 261–283) which encodes a 23-amino acid duplication with seven new O-glycosylation sites [9]. Based on differences in this region, the Greek isolates were divided into three subgroups: one included two isolates from 2018 (GR21 and GR23), with sequences identical to the prototype ON1 strain (GenBank accession number JN257693); the second included one isolate from 2018 (GR18) which had three characteristic substitutions (L274P, L298P, and Y304H), which are also present in other ON1 sequences available in GenBank such as KJ710402, MF589080, and KM042388 [10]; the third included the isolates from 2017 which had the three above-mentioned substitutions together with two additional ones (E262K and L289P), a pattern which is also present in other sequences such as MK749886 (Fig. 2a).

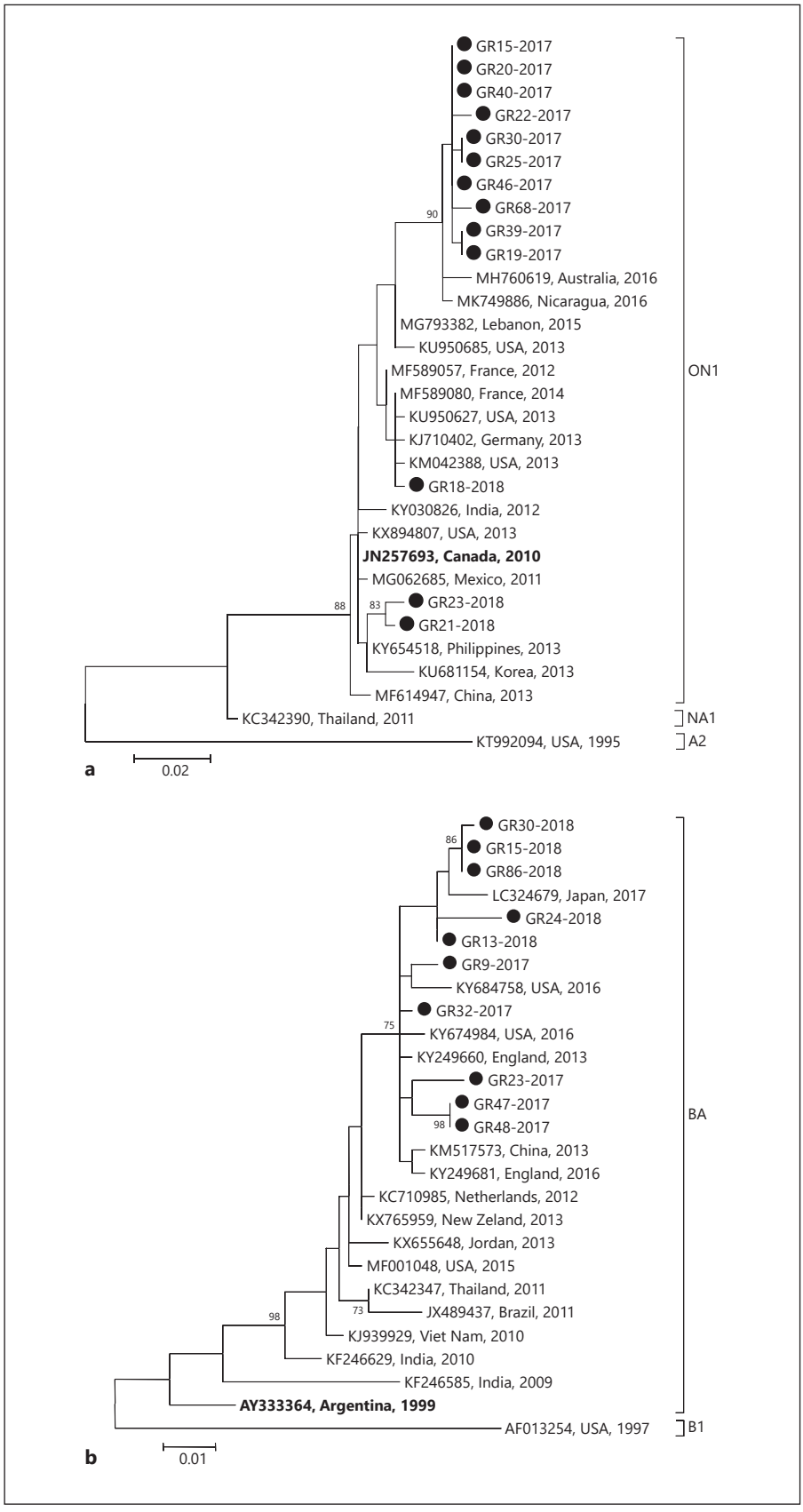


**Fig. 1.** Maximum likelihood phylogenetic trees based on 420- and 414-bp fragments of the second hypervariable region of the G gene of RSV-A (**a**) and RSV-B (**b**), respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Sequences of the present study are marked. Prototype strains are shown in bold. RSV, respiratory syncytial virus.

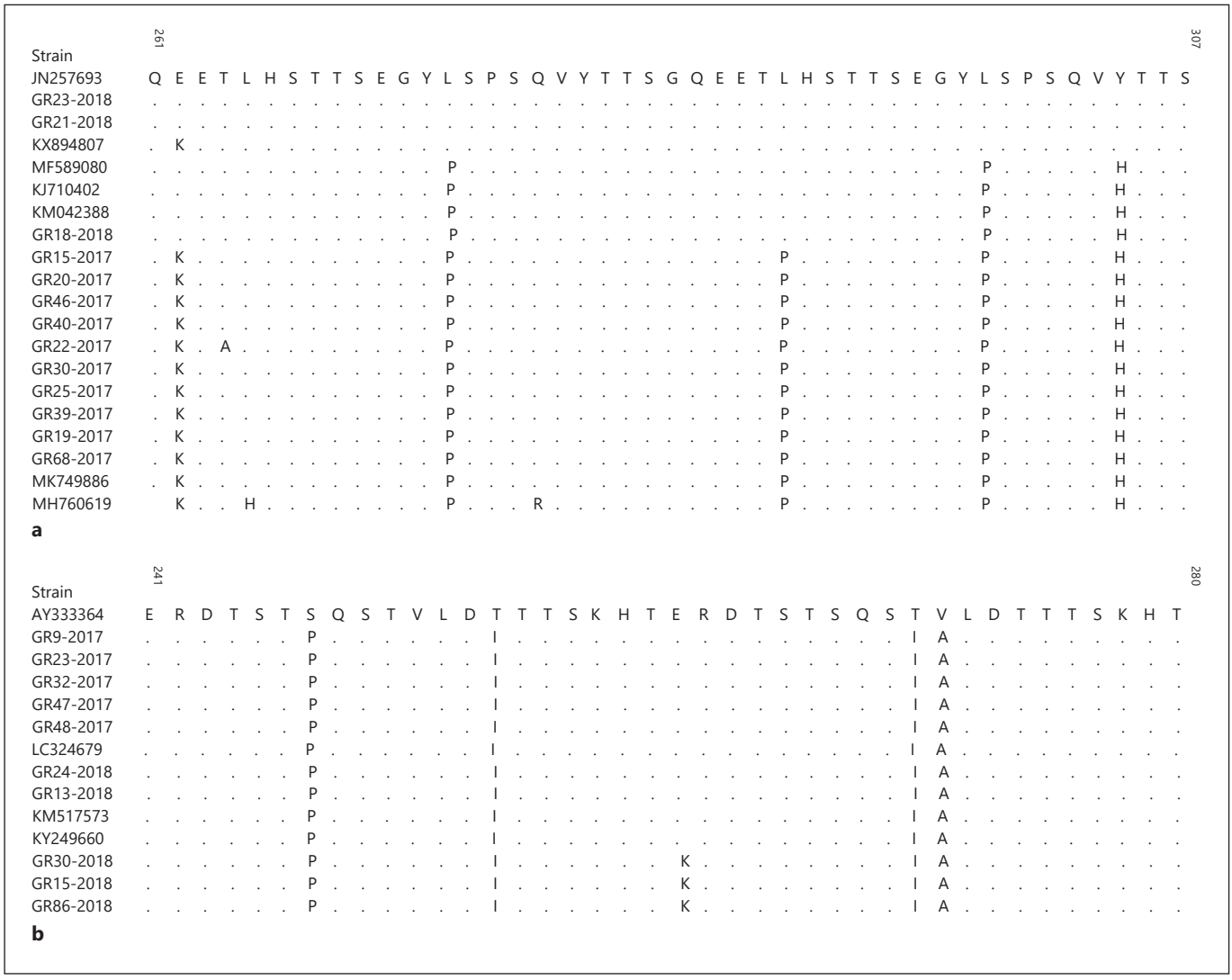


**Fig. 2.** Alignment of deduced amino acid sequences of the G protein. **a** RSV-A, relative to the sequence of the prototype ON1 strain (JN257693), amino acid positions 261–307. **b** RSV-B, relative to the sequence of the prototype BA strain (AY333364), amino acid positions 241–280. Identical residues are indicated by dots. RSV, respiratory syncytial virus.

The RSV-B BA genotype is characterized by a 60-nucleotide-long duplication in the C-terminal region of the G gene [11]. Compared with the prototype BA strain (GenBank accession number AY333364), the Greek strains presented four substitutions (S247P, T254I, T270I, and V271A), which are present also in other BA strains, such as KY249660, KM517573, and LC324679. The first three of these substitutions (S247P, T254I, and T270I) result in changes from polar and hydrophilic to hydrophobic amino acids, which may affect the protein structure and fold. One additional amino acid substitution, E261K, was seen in three Greek RSV strains of 2018 (Fig. 2b).

## Discussion and Conclusion

RSV is a common respiratory pathogen worldwide affecting mainly infants and young children with bronchiolitis. In the current study, RSV was detected in 52.1% of children hospitalized with bronchiolitis. Previous studies in Greece reported RSV detection in 59% of infants hospitalized with bronchiolitis [12] and in 73% of children aged 0–4 years with respiratory tract infection [13]. The detection rate was lower (10.9%) when older children with community-acquired pneumonia were tested [14], and it was even lower (8.3%) when children under 2 years


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were tested, but they had infection of the upper respiratory tract [15]. RSV has also been detected in mixed viral infections, mainly together with influenza virus [13].

Both RSV subtypes were detected in the two study seasons, with significant predominance of RSV-A during 2016–2017 (72.2%) and RSV-B during 2017–2018 (63.2%). In general, the subtype predominance varies per year. In one study in Greece RSV-B accounted for 52% of cases [12], while in another study 10 of 11 RSV samples were positive for RSV-A [14].

Patients with RSV-A infection were significantly younger than those with RSV-B infection, and all infants younger than 1 month were infected with RSV-A. While there are studies in children showing predominance of RSV-A in younger patients [16, 17], other studies show predominance of RSV-B [12]. These contradictory findings can be explained by differences in study design, patient inclusion criteria, environmental factors, or differences in the pathogenicity of the various genotypes.

A possible association between RSV subtypes and disease severity has been investigated; the interest was renewed since the identification of the ON1 genotype, but it was concluded that it is not associated with disease severity [10, 18]. This is in accordance with the results of the current study, since none of the RSV ON1-infected patients required mechanical ventilation or admission to intensive care, and no relation was observed between subtype and disease severity.

Genotyping of the strains showed that one single genotype per subtype was detected, and RSV-A ON1 predominated during 2016–2017, and RSV-B BA predominated in the next season. Similarly, in Germany, a predominance of RSV-A (53.2%) was seen during 2016–2017, with ON1 being the only genotype detected [19]. However, the predominance of RSV subtypes in other European countries during the same winter seasons varied. In Italy, RSV-B predominated in 2016–2017 and RSV-A predominated in 2017–2018 [20], whereas in Catalonia, Spain, RSV-B was more prevalent than RSV-A during both seasons, with strains mainly classified into the BA9 and ON1 genotypes, respectively [21]. In Bulgaria, RSV-B predominated during both seasons (2016–1018) [22].

The ON1 genotype was first identified in 2010 in Ontario, Canada [9]. Since then it has been detected in several countries, and currently it is considered the predominant circulating RSV-A genotype. It has been suggested that the rapid spread of ON1 is related to the relevant fitness of this variant [5]. The BA genotype was first detected in 1999 in Buenos Aires, Argentina [11]. Since then, BA has become the predominant RSV-B genotype in most countries, including European ones. In the present study, BA was the only RSV-B genotype detected. In a study conducted in Cyprus, the BA genotype predominated during the winter season of 2011–2012, and ON1 was first detected in the next season (2012–2013) [23]. Since there are no available data on the molecular epidemiology of RSV in Greece, comparisons with previous seasons cannot be made.

In conclusion, the current study provides a first insight into the RSV genotypes in Greece, which present several genetic differences from the prototype strains. The study can be used as a basis for further comparative studies. Countrywide studies covering multiple seasons are needed to better understand the epidemiology of RSV.

## Statement of Ethics

The study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Samples were obtained for diagnostic purposes and data were extracted from the database and were anonymized and statistically analyzed in this study. Therefore, informed consent and ethics approval were not required.

## Disclosure Statement

The authors have no conflicts of interest to declare.


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## Author Contributions

A. Papa conceived the study, analyzed the results, and wrote the final version of the article. K. Haidopoulou organized the study and contributed to the writing of the article. S. Pappa performed the molecular tests and the phylogenetic analysis. K. Tsergouli performed the statistical analysis and wrote the first draft of the article. M. Gogou reviewed the inclusion and exclusion criteria and collected the patient data. A. Giannopoulos contributed to the writing of the article. All authors approved the current version of the article.

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