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Genetic characterization of respiratory syncytial virus highlights a new BA genotype and emergence of the ON1 genotype in Lyon, France, between 2010 and 2014☆

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ABSTRACT

Background: Respiratory syncytial virus (RSV) is a well-recognized cause of respiratory tract infections. Based on G gene variations, 11 RSV-A and 36 RSV-B genotypes have been described to date. The ON1 genotype was detected in Ontario in 2010 and subsequently reported in several countries.
Objectives: The objective of the present study was to investigate for the first time the RSV epidemiology and genotype diversity in France between 2010 and 2014.
Study design: All respiratory samples received from patients with influenza-like illness or respiratory tract infection were screened for RSV infection by RT-PCR. The results were stratified according to winter season. Among the RSV-positive cases, 117 samples were further investigated for phylogenetic analysis out of 150 randomly selected for sequencing.
Results: Among the 20,359 cases screened, 14% of the cases were RSV-positive. RSV-A was predominant during the four winter seasons. The first ON1 variant was detected during the 2010–2011 winter and reached 85% of all RSV-A-positive cases in 2013–2014. Most RSV-B was classified as BA9 and BA10 genotypes but a new genotype (BA-Ly) was described.
Conclusion: As reported in different countries, ON1 variants were firstly detected in 2011 and became the predominant RSV-A genotype in Lyon. Among RSV-B, BA9 was predominant but detected alongside BA10 or a transient genotype (BA-Ly).

1. Background

Human respiratory syncytial virus (RSV) is one of the main causes of severe respiratory tract infections (RTI) and death in children [1]. Almost all children before two years of age have been infected by RSV and multiple reinfections may occur throughout their lifetime [2]. This virus is also reported in adult patients, especially after 65 years old [3,4]. During an acute RSV infection, very few therapeutics are available: there are no specific antiviral treatment and ribavirin is the only antiviral approved for RSV treatment but hardly used due to important side effects. The monoclonal antibody palivizumab binds postfusion F protein and is the only option to prevent RSV disease in infants. Moreover, to date, there are no licensed vaccines available for RSV even if studies are currently investigating candidates. Characterization of the evolving RSV epidemiology is a major issue in the context of these preventive strategies development.

RSV have been splitted into two groups based on the antigenic variation of the G-protein, RSV-A and RSV-B [5]. This protein is a surface glycoprotein, involved in virus attachment to the host cells and in virus immunogenicity as it is the main antigen. The G-protein can sustain large genetic variations without functionality loss leading to the emergence of new variants [6]. This variability is mainly observed in the two hypervariable regions of the ectodomain [7] and the sequencing of the second hypervariable region, located at the G-protein C-terminal end, is widely used to subdivide RSV-A and RSV-B into genotypes [8]. So far, 11 RSV-A genotypes (GA1 to GA 7, SAA1, NA1, NA2,

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and ON1) [8–10] and at least 36 RSV-B genotypes (GB1 to GB13, SAB1 to SAB4, BA1 to BA14, BA-CCA and BA-CCB, THB, URU1, and URU2) have been described [8,10–15].

During the 2010–2011 winter season, a novel RSV-A genotype (ON1) with a 72-nucleotides-long duplication in the G-gene was detected in Ontario [9]. Since then, this genotype has been reported in Europe [16–19] and worldwide [20].

2. Objectives

Considering the upcoming RSV vaccine, characterization of the evolving RSV epidemiology has become a major concern. Data are still limited due to the absence of national and international surveillance systems. The objective of this retrospective study was to analyze RSV prevalence and genotype patterns among patients, both children and adults, with respiratory illness. Thus, we investigated the RSV genetic diversity by sequencing the second hypervariable region of the G gene on samples collected between 2010 and 2014 at the teaching hospital of Lyon, France.

3. Study design

3.1. Patients and specimen collection

All respiratory samples (nasopharyngeal aspirates, nasal/throat/oral swabs, trachea-bronchial aspirates and bronchoalveolar lavages) received by the virology laboratory of the Hospices Civils de Lyon, France, from September 2010 to April 2014 were retrospectively included in this study. These samples were taken from patients with influenza-like illness (ILI) or RTI (upper or lower/mild or severe). All duplicates (samples collected on the same day) or follow-up samples (samples taken less than 28 days after the last positive sample) were excluded.

In France, RSV epidemic season is observed during winter, usually from late October to March, and therefore data were divided into four periods according to the sample date and centered on the epidemic season. Clinical data (age and clinical unit) were retrospectively extracted from the laboratory database.

3.2. RSV molecular detection

RNA was extracted using EasyMag® platform (bioMérieux, Marcy l'Etoile, France). Briefly, 200 µL of clinical samples were extracted in 50 µL of elution buffer. Screening for RSV was performed using standard diagnostic protocol (MWS RSV/hMPV r-gene® PCR kit, bioMérieux) on the ABI PRISM® 7500 system (Applied Biosystems, Foster city, CA, USA).

3.3. Gene sequencing

For genotyping, we selected RSV-positive samples from RSV peak weeks of each period (the week of the peak plus one week before and one after). Among samples with Ct values under 30, we randomly select 150 cases using excel software: 60 from 2010 to 2011, 30 from 2011 to 2012, 30 from 2012 to 2013, and 30 from 2013 to 2014. After usual diagnostic procedure, all samples were extracted then stored at −80 °C before genotyping. Genotyping targeted the second hypervariable region of G-gene and was performed by a one-step RT-PCR adapted from a previously reported protocol [21]. RT-PCR was performed using Superscript III reverse transcriptase and Pfx polymerase system (Invitrogen, Saint Aubin, France). After size verification (around 500 bp), the amplified DNA was sequenced using ABI 3730xl sequencer (Biofidal, Vaulx-en-Velin, France). The GenBank accession numbers for the sequences produced during this study are MF589001 to MF589082 for RSV-A and MF510885 to MF510919 for RSV-B.

3.4. Sequence analysis and phylogenetic analysis

For each sequence successfully obtained, related sequences were searched using the basic local alignment search tool (BLAST). The RSV-A and RSV-B sequences were aligned with representative sequences retrieved from GenBank using MUSCLE in MEGA7 software [22]. Phylogenetic trees based on partial G gene sequences were generated using the neighbour-joining method consolidated per 1000 replicates. The evolutionary distances were derived using the Tamura-Nei method [23].

Genotypes were assigned as described previously [10]. Sequences were considered as a genotype when they clustered together with a bootstrap value greater than 70 with p-distance to all other members in the same phylogenetic cluster below 0.07 [11–13]. The most closely related sequences to the new genotype found in GenBank using BLAST were included in the phylogenetic analysis.

3.5. Statistical analysis

Group comparisons were performed using Fisher's exact or Chi-2 test for categorical variables and by Student's *t*-test or analysis of variance (ANOVA) for continuous variables, as appropriate. Pearson's test was used to assess intergroup difference. The statistical analyses were performed on EpiInfo software (V 7.2.0.1; CDC, Atlanta, GA, USA) and/or Graphpad Prism software (V5.0) (GraphPad Software Inc, La Jolla, CA, USA). Because of the sample size, results were considered significant when p-values $< .01$.

3.6. Ethics statement

In compliance with French law at the time of sampling, information was given to each patient consulting at the Hospices Civils of Lyon about the collection and use of biological samples for regular disease management and further epidemiological studies. For the purpose of this study, patient confidentiality was strictly protected.

4. Results

4.1. Population studied

A total of 28,156 respiratory samples were received during the study period. Among these samples, 7797 were considered as duplicates or follow-up samples. Therefore 20,359 cases of potential RTI were included. These cases were classified according to sample date and patient age (Tables 1 and 2). There was an increase in the number of suspected RTI sampled each period until 2012–2013 period; a decrease was observed the year after. The patients mean age was lower during the 2010–2011 period as compared to each of the three following periods (between which there was no significant difference in patients' age). There was also no significant difference between periods studied in terms of intensive care unit (ICU) hospitalization for patients with a RSV infection (Table 1). Male were more sampled than woman (sex ratio = 1.3) but the sex ratio was not significantly different between periods.

4.2. RSV prevalence over four consecutive winters

During the study, RSV-positive samples ranged from 12 (2012–2013) to 18% (2010–2011). There was a significant decrease of RSV-positive samples between 2010 and 2011 period and the three other periods but also between the 2011–2012 (14%) and the 2012–2013 (12%) period (Table 1). In Lyon, the epidemic peak was reached for each season during December except for the 2010–2011 period (peak in January) (Fig. 1). RSV-positive samples were mostly found in patients ≤6 months of age and the prevalence of RSV infection decreased significantly according to age until 10 years of age

Table 1
Patient characteristics and RSV infection according to sample date.

Sampling period	Total				RSV +				RSV −			
	Patients, n	Sex ratio (M/F)	Mean age, years ± SD	Patient in ICU (%)	Patients, n (%)	Sex ratio (M/F)	Mean age, year ± SD	Patient in ICU (%)	Patients, n (%)	Sex ratio (M/F)	Mean age, year ± SD	Patient in ICU (%)
2010–2011	4016	1.2	14.6 ± 23.6	536 (13%)	721 (18%)	1.0	4.1 ± 13.9	61 (8%)	3295 (82%)	1.2	16.9 ± 24.6	472 (14%)
2011–2012	4807	1.3	28.2 ± 31.7*	848 (18%)	696 (14%)*	1.2	7.5 ± 20.4*	78 (11%)	4111 (86%)	1.3	31.7 ± 31.9*	760 (18%)
2012–2013	6087	1.3	34.6 ± 32.7*	1047 (17%)	760 (12%)*♯	1.4*	9.6 ± 22.9*	109 (14%)	5327 (88%)	1.3	38.1 ± 32.3*	929 (17%)
2013–2014	5449	1.3	38.1 ± 33.2*	900 (17%)	695 (13%)*	1.1	10.6 ± 24.9*	81 (12%)	4754 (87%)	1.3	42.2 ± 32.3*	814 (17%)
Total	20,359	1.3	30.1 ± 32.1	3331 (16%)	2872 (14%)	1.2	8.0 ± 21.0	329 (11%)	17,487 (86%)	1.3	33.7 ± 32.2	2975 (17%)

Number of patients, sex ratio, mean age in years and number of patients hospitalized in per period for all patients studied, RSV positive patients and RSV negative patients from September 2010 to April 2014 in Lyon, France. *: p-value < .01 for the appropriate test against results from the 2010–2011 period. ♯: p-value < .01 for the appropriate test against the results from the 2011–2012 period. Only significant results were reported. The percentage presented here, were calculated for each period. F: female. ICU: intensive care units. M: male. RSV: respiratory syncytial virus. SD: standard deviation.

Table 2
Patient characteristics and RSV infections according to age group and sample date.

Sampling period	≤6 months old			> 6 months and ≤2 years old			> 2 and ≤10 years old		
	Total, n (%)	RSV + (%)	RSV + in ICU (%)	Total, n (%)	RSV + (%)	RSV + in ICU (%)	Total, n (%)	RSV + (%)	RSV + in ICU (%)
2010–2011	1212 (31%)	419 (35%)	34 (8%)	731 (18%)	168 (23%)	9 (5%)	848 (21%)	82 (10%)	8 (10%)
2011–2012	1201 (25%)	433 (36%)	37 (9%)	559 (12%)	117 (21%)	14 (12%)	679 (14%)	63 (9%)	5 (8%)
2012–2013	1196 (20%)	460 (38%)	63 (14%)	526 (8%)	104 (20%)	10 (10%)	782 (13%)	81 (10%)	9 (11%)
2013–2014	1023 (19%)	424 (41%)	48 (11%)	437 (8%)	108 (25%)	8 (7%)	582 (11%)	59 (10%)	6 (10%)
Total	4632 (23%)	1736 (37%)*	182 (10%)	2253 (11%)	497 (22%)♯	41 (8%)	2891 (14%)	285 (10%)⌉	28 (10%)

Sampling period	> 10 and ≤20 years old			> 20 and ≤65 years old			> 65 years old		
	Total, n (%)	RSV + (%)	RSV + in ICU (%)	Total, n (%)	RSV + (%)	RSV + in ICU (%)	Total, n (%)	RSV + (%)	RSV + in ICU (%)
2010–2011	325 (8%)	15 (5%)	2 (13%)	613 (15%)	19 (3%)	3 (16%)	286 (7%)	18 (6%)	5 (28%)
2011–2012	299 (6%)	11 (4%)	1 (9%)	1173 (24%)	32 (3%)	9 (28%)	896 (19%)	40 (4%)	12 (30%)
2012–2013	376 (6%)	16 (4%)	2 (13%)	1708 (28%)	47 (3%)	9 (19%)	1498 (25%)	52 (3%)	16 (31%)
2013–2014	248 (5%)	5 (2%)	1 (20%)	1606 (29%)	40 (2%)	7 (18%)	1552 (28%)	59 (4%)	11 (19%)
Total	1248 (6%)	47 (4%)	6 (13%)	5100 (25%)	138 (3%)	28 (20%)	4232 (21%)	169 (4%)	44 (26%)

Detection rates of RSV infections in all patients and in patients hospitalized in ICU per year and age category from September 2010 to April 2014 in Lyon, France. Percentage in total column represents the proportion of sample done for each age category during one period. Percentage in RSV+ column represents the proportion of positive RSV diagnoses for each age category during one period. Percentage in RSV+ in ICU column represents the proportion of patients hospitalized in ICU which are also RSV+ for each age category during one period. Three cases did not have birth date information and were excluded. *: p-value < .01 for the appropriate test between "≤6 months" category and the other age categories. ♯: p-value < .01 for the appropriate test between " > 6 months and ≤2 years" category and the other age categories, ⌉: p-value < .01 for the appropriate test between " > 2 and ≤10 years" category and the other age categories. Only significant results are represented. RSV: respiratory syncytial virus. ICU: intensive care units.

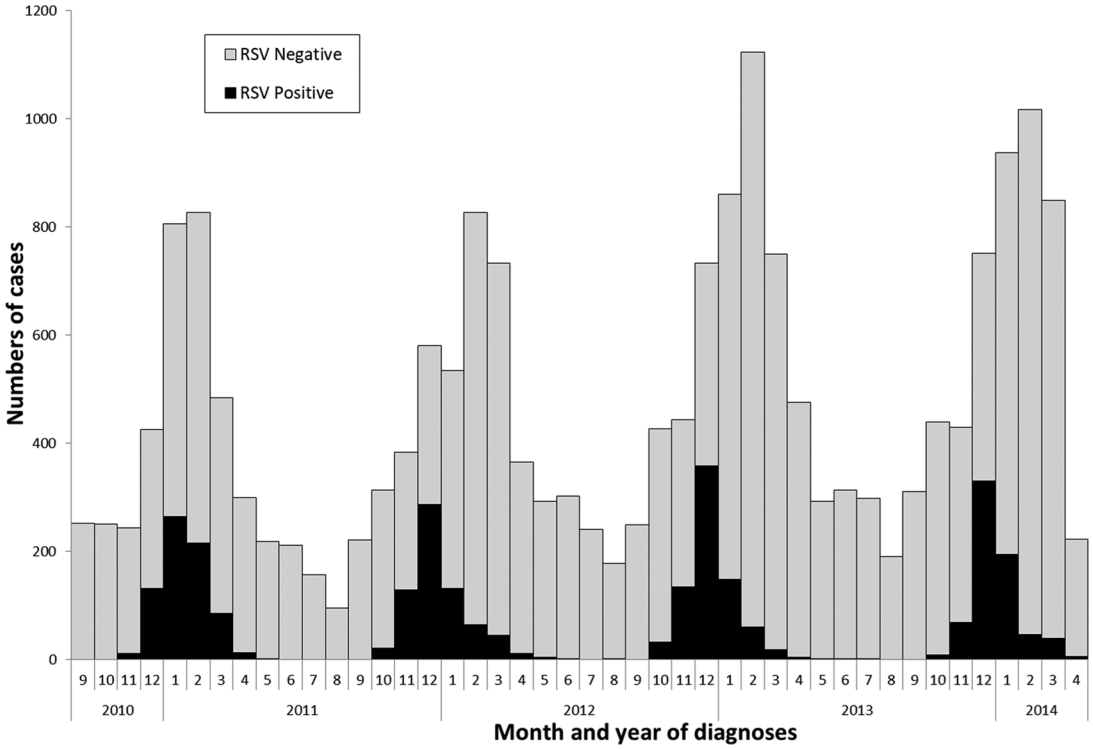

Fig. 1. Monthly distribution of RSV diagnoses in Lyon.
Distribution of all the samples tested between September 2010 and April 2014 in Lyon, France. RSV positive samples were represented in black and RSV negative sample in grey. RSV: respiratory syncytial virus.

($p < .001$) after which there was no significant difference. For each age category, the prevalence of RSV infection was not significantly different between the four periods considered (Table 2). Among the RSV-positive cases, the ICU hospitalizations were significantly lower for patients under 10 years of age compared to adults over 20 years of age ($p < .001$) (data not shown). However there were no significant differences between the four periods for ICU hospitalizations, regardless of the age category. Regarding the sex repartition, male were significantly more infected by RSV than woman ($p < .001$) only during the 2012–2013 period and the sex ratio during this period was significantly higher than what was noted during the other periods ($p = .003$).

4.3. Phylogenetic analysis and RSV epidemiology

Among all the RSV-positive cases randomly selected for sequencing (n = 150), 33 (22%) samples could not be sequenced due to insufficient viral load or RNA degradation during conservation. Therefore the sequence of the second hypervariable region of the G-gene could be determined for 117 RSV-positive samples: 36 from 2010 to 2011, 26 from 2011 to 2012, 27 from 2012 to 2013, and 28 from 2013 to 2014. Among the RSV sequenced, 82 (70%) were RSV-A, and 35 (30%) were RSV-B. Over the total study period, RSV-A was predominant but in 2012–2013 there was almost the same proportion of RSV-A and RSV-B detected (Fig. 2).

All RSV-A strains clustered with strains previously assigned either to the new ON1 genotype or to the NA1 genotype (Fig. 3A). The RSV-A strains found in Lyon clustered mostly with the NA1 genotype in the 2010–2011 period (n = 25, 95%) as well as in the 2011–2012 period (n = 20, 90%); later they clustered mostly with the new ON1 genotype (n = 10, 65% in 2012–2013, and n = 16, 85% in 2013–2014; Fig. 2). The second hypervariable region in the G-gene of all NA1 genotypes was aligned with the reference NA1 strain (AB470478; Supplementary data 1A) and the same region of all ON1 genotypes was aligned with the reference ON1 strain isolated first in Canada (JN257393; Supplementary data 1B). Amino Acid (AA) positions are based on the ON1 numbering for all RSV-A strains. The majority of the most recent ON1 strains isolated herein seemed to have three specific substitutions (L274P, L298P and Y304H) found also in other countries [24].

RSV-B strains clustered separately into at least three distinct genotypes: 26 BA9 (74%), 5 BA10 (14%), and 2 BA-Ly (6%). Two strains did not cluster with any other strain but they contained the characteristic 60-nucleotides duplication and were therefore named BA-like strains, (Fig. 3B). For each period, the BA9 genotype was the most frequent and ranged from 50% (2011–2012) to 100% (2010–2011). The BA10 genotype was found only during the two more recent periods when it represented 15% and 33% of the RSV-B strains sequenced.

Two viruses found during the 2011–2012 period were genetically distinct and gather in a group named BA-Ly which was separated from the BA9 genotype using the criteria previously described for dividing genotypes [10–13]. This new genotype was detected during only one period in two unrelated patients hospitalized in two different medical centers. These two strains clustered together with a bootstrap value of 99 (Fig. 3B). The p-distance between these strains were null and the average p-distance between the new BA-Ly and the BA9 was only 0.06 but the BA-Ly also had specific genetic markers. The second hypervariable region in the G-gene of RSV-B was aligned with the reference RSV-B strain (M17213; Supplementary data 2). AA positions are based on the BA-Ly numbering for all RSV-B strain. The BA-Ly strains (2011-50-429B and 2011-51-305B) were characterized by the insertion of three AA (PKR) after K234, and three specific substitutions (K241E, P250L and S300F; Supplementary data 2).

5. Discussion

As reported in different countries, ON1 variants were first detected in 2011 and became the predominant RSV-A genotype in Lyon (France). Among RSV-B, BA9 was predominant but detected alongside BA10 or a transient genotype (BA-Ly). To the best of our knowledge, this study is the first to explore RSV genotype diversity in France and the results presented herein complete data already available in other European

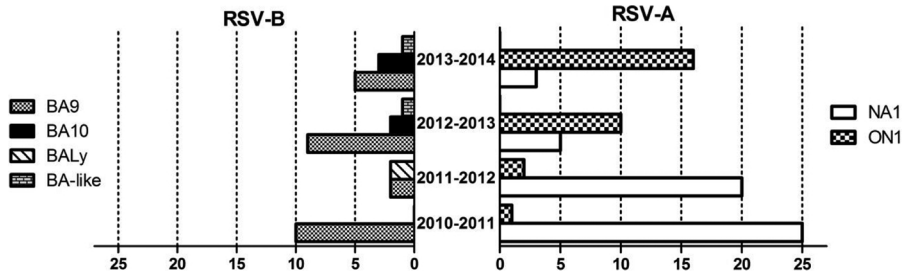

Fig. 2. Distribution of RSV genotype per period.
Distribution of proposed RSV genotypes (NA1 and ON1 for RSV-A/BA-9, BA-10, BA-Ly and BA-like for RSV-B) over the four consecutive periods studied in Lyon, France. The abscissa represents the absolute number of cases sequenced. RSV: respiratory syncytial virus.

countries (Italy, Belgium, Germany, Croatia) [16–19] and worldwide [20].

Between 2010 and 2014, among the 20,359 cases screened, 14% were RSV-positive in Lyon. This proportion of RSV positive samples decreased between 2010 and 2014, which may be related to the increased number of respiratory samples taken, especially among older patients. This increase could be associated to the progressive implementation of more efficient and broader molecular testing for respiratory virus detection, but also to a post-2009 influenza pandemic "effect" as described in Belgium [17].

In our study, RSV infected mainly children but was also detected among adults and the elderly, but at a lower rate than that is observed in other countries. In the USA, this rate differed widely (3%–17%) according to clinical setting and year studied [3] which suggests that a multicenter national study is needed to conclude on RSV prevalence in adults in France.

Among RSV-A, the ON1 genotype was first detected in a sample taken in February 2011 and the switch from NA1 to ON1 genotype was observed in the following winter season (2012–2013). The ON1 genotype with a 72-nucleotides duplication was first described in 2011 in Ontario [9] and then rapidly reported in different countries and all over the world [18,20,25]. The first ON1 RSV detected in Lyon was found only two months after the first cases described in Ontario. The ON1 genotype epidemiology seems to be the same in most European countries with only sporadic detections during the winter of 2010–2011 or 2011–2012, and detection as the predominant genotype during the 2012–2013 winter in Italy [16,25], Germany [18], Cyprus [26], and in the present study in France. The rapid spread of ON1 has to be related to relevant fitness of this new variant that has not yet been elucidated.

Regarding RSV-B genetic diversity, the BA9 genotype was always the predominant genotype and was detected alongside other minority genotypes such as BA10 The first BA genotype was detected in Argentina [15], and since 1999, has become predominant among circulating RSV-B in most countries and has evolved rapidly to at least 16 new BA genotypes (BA1 to BA14, CCA, and CCB) [13,15]. In Europe, the BA10 genotype is frequently detected, but the BA9 genotype is always the predominant genotype as reported in Spain [14], Italy [16], Germany [18] and Croatia [19], as in the present study in France. The worldwide spread of BA genotypes reported for more than 15 years has to be related to an improved fitness, which could itself be related to the duplicated region characteristic of BA genotypes that is involved in virus attachment to target cells [27].

In contrast, the new BA-Ly genotype that was detected herein during the 2011–2012 period appears to be a transient evolution with no detection during the two following winter seasons. This genotype was characterized by an insertion and three substitutions. The three AA (PKR) insertions and the P250L substitution have never been described before. However, the other two specific substitutions have been reported: the K241E in the BA12 genotype [28], and the S300F in the BA9 and BA14 genotypes [13]. The transient detection of BA genotypes has recently been described for the BA14 genotype which was only detected between July and November 2008 from two different regions in Panama [13]. The disappearance of these transient genotypes has to be explored by studies focusing on their antigenicity and fitness which might be linked to the characteristic modifications (substitution/insertion) observed for each genotype.

To better understand the transient genotypes and the broad diversity of the BA genotypes, it is interesting to note that all new genotypes are defined using the common criteria as detailed in Materials and methods section [11–13]. However, these criteria have been adapted from what is used for other viruses and may not be perfectly suitable for RSV. Indeed, a large number of newly reported RSV genotypes could not be associated with a clinical impact, or a large dissemination as observed for ON1. It could be interesting to further correlate genetic changes with antigenic switch in order to be truly able to predict the risk of broad diffusion when a new variant is detected. RSV molecular studies should also be improved using, for instance, both F-gene and G-gene because of their involvement during immune escape. The F-gene codes for the fusion (F) protein which is a highly conserved surface glycoprotein existing within two conformations: pre-fusion and post-fusion. The pre-fusion conformation contribute to the high titer of neutralizing antibodies in human sera and is used in vaccine candidate [29].

To conclude, studies on RSV epidemiology have to be pursued and RSV surveillance might need to be copied from influenza surveillance system, especially as a new vaccine candidate is currently being investigated in a phase III study [30].

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Conflict of interest

AG, MBB, JSC, EF, and JV have no conflict of interest to declare.
MP declares potential conflicts of interests with Theradiag.
FM declares potential conflicts of interests with bioMerieux.
BL is a member of the scientific board of the Global Influenza Initiative (GII) and of the Global Hospital Influenza Surveillance Network, and received support for travel from GSK, Sanofi-Pasteur and Roche.

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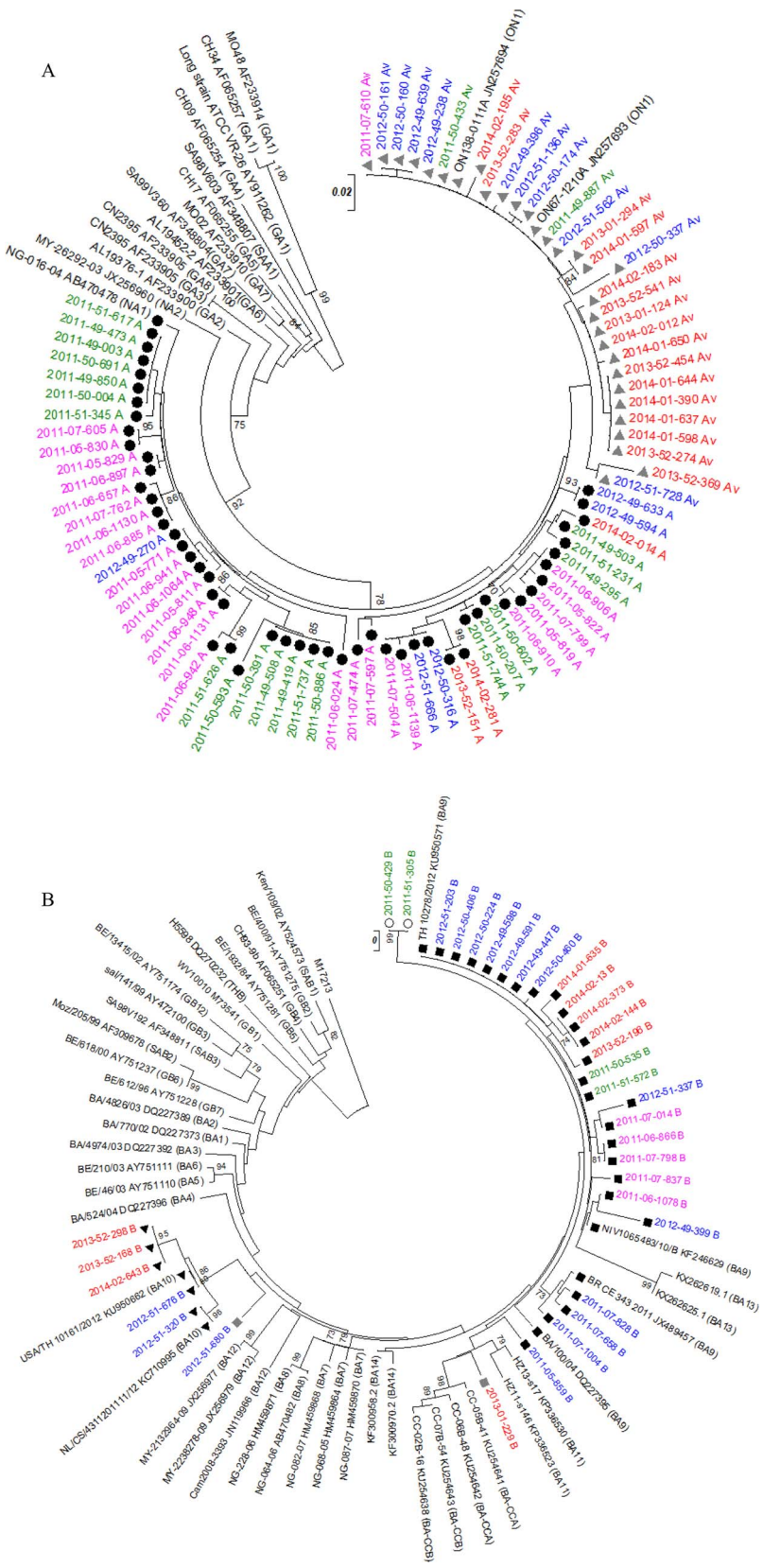

Fig. 3. Phylogenetic tree of RSV-A and RSV-B strains.
Phylogenetic trees for RSV-A (A) and RSV-B (B) strains were constructed with the neighbour-joining method and 1000 replicates for the bootstrap test using MEGA7 software. RSV strains names were written in purple for the 2010–2011 period, in green for the 2011–2012 period, in blue for the 2012–2013 period and in red for the 2013–2014 period. RSV strains written in black were reference strains from all over the world and published in NCBI. The reference strains genotype was written on the right of the strain name by brackets. For RSV-A, the NA1 genotype assignment was shown by a black circle and the ON1 genotype assignment was shown by a grey triangle. For RSV-B, the BA-Ly genotype assignment was shown by a white circle, the BA-9 genotype assignment was shown by a black square, the BA-10 genotype assignment was shown by a black triangle and the BA-like genotype assignment was shown by a grey square. Only bootstraps values greater than 70, considered as significant were represented at the branch nodes. The scale bar represents the number of nucleotide substitutions per site. RSV: respiratory syncytial virus.

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.2018.02.004.

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