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Unravelling respiratory syncytial virus outbreaks in Buenos Aires, Argentina: Molecular basis of the spatio-temporal transmission

Gabriel Lihue Rojo[a,c], Stephanie Goya[a], Mariana Orellana[b,c], Andrea Sancilio[d], Alberto Rodriguez Perez[e], César Montali[f], Carolina García[g], Lilian Sosa[e], Alejandra Musto[b,d], Daniela Alvarez[b], Alejandro Castello[b,c], Mariana Viegas[a,*]

[a] Hospital de Niños "Dr. Ricardo Gutiérrez", Ciudad Autónoma de Buenos Aires, Argentina
[b] Universidad Nacional Arturo Jauretche, Florencio Varela, Buenos Aires, Argentina
[c] Universidad Nacional de Quilmes, Bernal, Buenos Aires, Argentina
[d] Hospital Interzonal General de Agudos "Evita", Lanús, Buenos Aires, Argentina
[e] Hospital Interzonal "Dr. Alberto Eurnekian", Ezeiza, Buenos Aires, Argentina
[f] Hospital El Cruce "Dr. Néstor Carlos Kirchner", Florencio Varela, Buenos Aires, Argentina
[g] Hospital Zonal General de Agudos "Evita Pueblo", Berazategui, Buenos Aires, Argentina

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ABSTRACT

Respiratory syncytial virus (RSV) is the main viral cause of hospitalization due to acute lower respiratory tract infections in infants worldwide. Several vaccines against RSV are under research and development, which are about to be approved. We evaluated transmission patterns in different settings to determine age-specific vaccination targets from a viral perspective. We sequenced the G glycoprotein's ectodomain of a constant clinical sampling between two epidemic outbreaks in a limited geographical region and performed phylogeographic analyses. We described a spatio-temporal transmission between local strains, which were originated in the center of the analyzed area and then spread to others. Interestingly, that central area reported the highest population density of the region and also showed overcrowding. This information should be considered by public health systems to evaluate vaccination at all ages in those areas to decrease viral transmission and in lower density populations only susceptible children should be vaccinated.

1. Introduction

Respiratory syncytial virus (RSV) is known as the main viral cause of acute lower respiratory tract infections (ALRTI) in infants and children worldwide (Nair et al., 2010; Holberg et al., 1991). Viral transmission is established from one individual to another due to direct and indirect contact with nasal and oral secretions. Reinfections may occur throughout life (Glezen et al., 1986), being asymptomatic in adults but causing severe disease in the elderly population (Walsh and Falsey, 2012). In countries with temperate climates, as Argentina, RSV outbreaks occur mainly in the coldest months peaking annually from April to September, with a peak in June (Viegas et al., 2004).

RSV is a negative-sense, single stranded RNA virus from the *Pneumoviridae* family (Afonso et al., 2016), with a genome of approximately 15,200 nt in length (Collins and Karron, 2013). RSV genome presents 10 genes that codify for 11 proteins, three of which are located on the viral surface: small hydrophobic protein (SH), fusion glycoprotein (F) and the attachment glycoprotein (G). There is a single serotype of RSV, but there are two different genetic groups that consist of the antigenic groups A and B (Anderson et al., 1985; Cristina et al., 1990) which differ mostly genetic and antigenically in the sequence of the G glycoprotein (Kim et al., 2007). The G gene presents two hypervariable regions in its external domain, or ectodomain, which are the target to carry out molecular epidemiology and evolutionary studies of this virus. By those studies, it was recently described an unusual genetic event, a duplication of a segment located on the second hypervariable region of the G gene's ectodomain in strains of both antigenic groups. The first event was a 60 nt duplication described on B antigenic group, found between 1997 and 1999 in Buenos Aires, Argentina, named as BA genotype (Trento et al., 2003, 2006). The second one was a 72 nt duplication on A antigenic group from the GA2 genotype that circulated for the first time during 2010–2011 winter season in Ontario, Canada, named as ON1 lineage (Eshaghi et al., 2012; Duvvuri et al., 2015). Since their discovery, those strains have evolved

* Correspondence to: Laboratorio de Virología, Hospital de Niños "Dr. Ricardo Gutiérrez", Gallo 1330, 1425 Ciudad Autónoma de Buenos Aires, Argentina.
E-mail address: viegasmariana@conicet.gov.ar (M. Viegas).

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acquiring different mutations in both duplicated segments, leading to the diversification of RSV. However, ON1 and BA have particularly replaced almost all genotypes and lineages from both antigenic groups globally (Trento et al., 2010). Some authors proposed the idea that the duplication brought them an evolutionary advantage over strains lacking it, giving them structural and antigenic changes, which alter immunogenicity and pathogenicity (Hotard et al., 2015). In Argentina, the molecular epidemiological studies of RSV have been limited to Buenos Aires City, where the ON1 lineage and BA lineages have been circulating during the last few years (Trento et al., 2006; Viegas et al., 2016).

There is only one prophylaxis method against RSV, named Palivizumab. It consists of a humanized monoclonal antibody against the F glycoprotein, and can be used to prevent RSV severe disease in extremely premature infants or those with congenital heart disease or chronic obstructive pulmonary disease, but its high cost and the need of several administrations makes it difficult to use. There are non-approved vaccines to protect against RSV, but currently several vaccine candidates are under research and development, and some of them are next to be released (Anderson et al., 2013; Higgins et al., 2016). Different age groups are targeted, as young children, adults and older adults. It has been proposed the vaccination of pregnant women to decrease transmissibility and to protect newborns by placental antibody transfer or the vaccination of the elderly population which also develops severe disease, but recent studies showed that vaccination of children less than 5 years of age is the most effective strategy to avert RSV in children and elderly (Yamin et al., 2016). Children are responsible for transmission because they present the highest infectious viral loads, which were found to increase disease severity as it was reported in children and adult volunteers (DeVincenzo et al., 2010; Wathuo et al., 2016), and also have greater frequency and duration of contacts between children of their own age group, enabling the infection with RSV within susceptible individuals (Hall et al., 1976).

Previously to a vaccine implementation, the epidemiological and molecular characteristics of the circulating viruses should be considered to know if the vaccine will be effective in a studied population. In addition, it is important to consider the transmission patterns of a studied virus in order to identify the most vulnerable population. Phylogeographic tools are useful for estimating these patterns, and at the same time to predict the location of the most recent common ancestor that gave origin to that group of viruses.

It has been shown that areas characterized by critical social determinants such as high overcrowding, stunting, and environmental factors such as in-house smokers, air humidity, temperature, air pollution might increase the RSV infection severity as well as favor the viral transmission (Viegas et al., 2004; Okiro et al., 2008; Caballero et al., 2015; Yamin et al., 2016). In recent years, there have been many studies attempting to describe the chains of transmission of RSV from the individuals' perspective, describing contacts between the susceptible population and their cohabitants, contacts in schools, etc (Read et al., 2012; Munywoki et al., 2015). The aim of this work was to describe the dynamic of RSV transmission from the viral perspective by performing phylogeographic analyses taking advantage of having a constant clinical sampling between two epidemic outbreaks and in a limited geographical region.

2. Materials and methods

2.1. Study area

For this study, the pediatric units from four public hospitals participated. Those hospitals are part of a localized region of Buenos Aires Province known as the VI Sanitary Region, which is located in the south of Buenos Aires City, Argentina. Those hospitals were: Hospital Interzonal General de Agudos “Evita” (Lanús district), Hospital Interzonal “Dr. Alberto Eurnekian” (Ezeiza district), Hospital Zonal General de Agudos “Evita Pueblo” (Berazategui district) and Hospital El Cruce “Dr. Néstor Carlos Kirchner” (Florencio Varela district), denoted with a white H in Fig. 1. The last hospital mentioned admits patients with severe disease from other hospitals of the region that are part of the VI Sanitary Region health service network: Hospital Zonal General “Dr. Arturo Oñativia” (Rafael Calzada – Almirante Brown district); Hospital General de Agudos “Evita Pueblo” (Berazategui district); Hospital Zonal de Agudos “Mi Pueblo” (Florencio Varela district); Hospital Zonal General de Agudos “Dr. Isidoro Iriarte” (Quilmes district); Hospital Subzonal Especializado Materno Infantil “Dr. Oller” (San Francisco Solano, Quilmes district); Hospital Zonal General de Agudos “Lucio Meléndez” (Adrogué district) (denoted with a green H in Fig. 1).

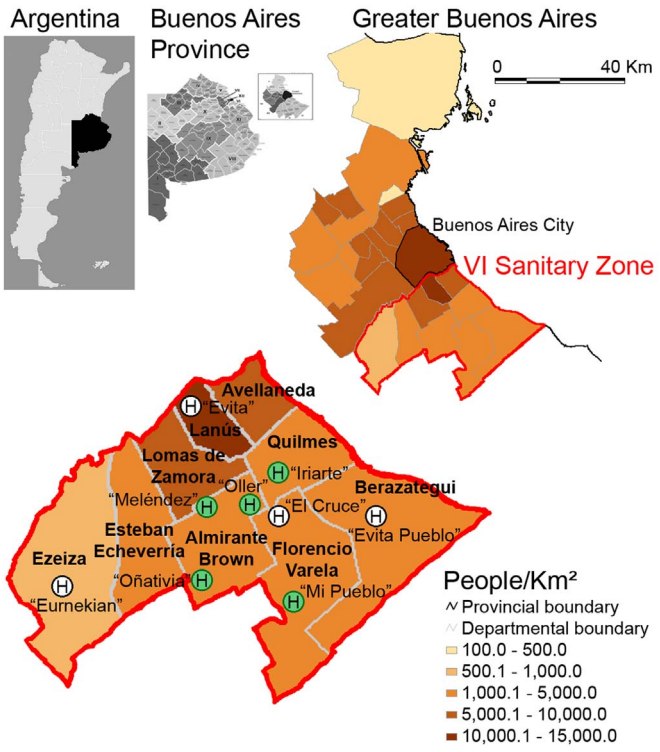

Fig. 1. Map of the studied area. The VI Sanitary Zone is delimited with red color and the districts names within are in bold. Population density of the Greater Buenos Aires from 2010 census is shown (http://www.sig.indec.gov.ar/censo2010/). The H surrounded by circles indicates the localization of hospitals: in white are shown the participating hospitals and in green are those that derived patients to the Hospital “El Cruce” (All hospitals names are between quotation marks).

2.2. Ethics statement

A written informed consent to participate in this study was obtained from parents or legal guardians of all patients, and the Medical Ethics and Research Committees from the four participating hospitals approved the study protocol. Additionally, a survey form was also obtained from each patient, referring to their location and socio-sanitary information such as age, sex, exact location, inhabitants in a household, housing characteristics, etc. To preserve the identity of the patients, every sample was codified before being analyzed, according to the Declaration of Helsinki and the *Habeas Data* law on protection of personal data (Law no. 25326, Argentina). The sample codification contained an acronym corresponding to the hospital where each sample was collected ("B" for the Berazategui district hospital, "C" for the El Cruce hospital, "E" for the Ezeiza district hospital and "L" for the Lanús district hospital) followed by an internal number.

2.3. Samples collection and handling

Clinical samples were nasopharyngeal aspirates (NPA) collected from pediatric patients lower than 14 years of age who had been hospitalized due to ALRTI. The sampling period was from June 2014 to July of 2015 and it included the final portion of the epidemiological peak of 2014 and the beginning of the next one in 2015 to study the relation/connection between circulating strains from both years. Since there was a large number of patients per day attending each hospital only the first three samples from each day were considered and collected. Immunofluorescence assay (IFA) was used for the detection of respiratory viruses present in samples such as RSV, influenza virus A and B, parainfluenza 1–3, metapneumovirus and adenovirus (Light Diagnostics, Chemicon Int. Inc., USA) (Gardner and McQuilin, 1968). RSV positive samples were cooled and transported to the Virology Laboratory of the Ricardo Gutierrez Children's Hospital in Buenos Aires city, where the molecular and bioinformatic analyses were performed.

2.4. Laboratory methods

Viral RNA was extracted from NPA by QIAamp® Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions (as well as all kits mentioned in this study), and stored at −70 °C. The G gene's ectodomain sequence was retrotranscribed and amplified by using the OneStep RT-PCR Kit (QIAGEN, GmbH, Hilden, Germany). The primer pairs were published before, but were optimized to ensure the recognition of both antigenic groups. They were RSVBG10f-TAG: 5′-CACGACGTTGTAAAACGACCGCAATGATAATCTCAACCTC-3′(4825-4844)/RSVBF1r-TAG: 5′-CAGGAAACAGCTATGACCCAACTCCATKGTTATTTGCC-3′(5649-5668), with K=G or T. The universal M13 phage tag are shown underlined for later sequencing steps and the number following the primers refers to its position in the genome of the reference strain Long/56 (GenBank accession number: AY911262). In some cases, an alternative forward primer was used, which was RSVAG267f-TAG: 5′-CACGACGTTGTAAAACGACGATGCAACAAGCCAGATCAAG-3′(4918-4938). The retrotranscription and amplification cycles consisted of: 50 °C for 30 min; 95 °C for 15 min; 94 °C for 0.5 min, 59 °C for 0.5 min, 72 °C for 1 min for 35 cycles and 72 °C for 10 min. Amplified fragments were electrophoresed in a 1.5% w/v agarose gel with 1 μg/mL ethidium bromide and purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA, USA). The purified PCR products were labelled with M13 universal primers by using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and electrophoresed in an ABI3500 Genetic Analyzer (AppliedBiosystems). Each sample's raw sequence was used to determine its antigenic group by using the online Basic Local Alignment Search Tool (BLAST). Then, consensus sequences were obtained with the software SeqScape v2.7 (Applied Biosystems, Foster City, CA, USA). The sequence nomenclature indicates the viral agent (RSV), the country of isolation (Argentina, ARG), the internal laboratory coded number for clinical samples (explained in Ethics Statement) and the date of sample collection. All RSV sequences obtained in this study were submitted to GenBank (accession numbers KY634251 to KY634418).

2.5. Phylogenetic analyses

Sequences were aligned with G glycoprotein's ectodomain sequences from different reported genotypes and lineages downloaded from GenBank by using the software MUSCLE (Edgar, 2004). The most suitable nucleotide substitution model for both alignments was selected using jModelTest 2.1.3 (Darriba et al., 2012) by using the Akaike Information Criteria. Phylogenetic inferences were obtained by Neighbor-Joining (Mega v.6.06) (Tamura et al., 2013) and Maximum Likelihood (Mega v.6.06), for both inferences the branch support was evaluated by bootstrapping with 1000 pseudo replica. In addition phylogenetic inference by Bayesian criteria (MrBayes) was performed (Ronquist and Huelsenbeck, 2003). The convergence of Monte Carlo Markov Chains (MCMC) was evaluated with split frequencies ≤0.01 and an effective sample size (ESS) > 200, as well as all parameters were evaluated with Tracer v.1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Burn-in as the initial 10% of the run length was performed. The consensus trees were visualized with the software FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.6. Phylogeographic analyses

For both phylogeographic analyses (discrete and continuous), Bayesian coalescent-based methods were evaluated with the software BEAST v1.8.3 (Bayesian Evolutionary Analysis by Sampling Trees) (Drummond et al., 2012) provided by The CIPRES Science Gateway (https://www.phylo.org/). Tracer v1.5 was used for the selection of the most suitable molecular clock and demographic models available by Bayes factor, and to analyze all BEAST run logs of the MCMC. The MCMC convergence was evaluated with split frequencies ≤0.01 and an ESS > 200 and a burn-in as the initial 10% of the run length. Tracer was also used to determine the time of the most recent common ancestor (MRCA). The maximum clade credibility trees (MCCT) were inferred by using the Tree-Annotator v1.8.3 and were visualized by FigTree v.1.4.3. Transmission pattern of local circulation clades were analyzed by the software SPREAD v1.0.6 (Spatial Phylogenetic Reconstruction of Evolutionary Dynamics) (Bielejec et al., 2011) and visualized with Google Earth v7.1.7.2606 (https://www.google.com/earth/).

2.7. Statistical analysis

The statistical analysis was performed using Chi-Square Test of the software package InfoStat (v. 2012) (http://www.infostat.com.ar). The statistical significance was defined as $p < 0.05$.

3. Results

Over the entire study period (June 2014–July 2015), a total of 1256 NPA samples were collected. The IFA diagnose confirmed 312 positive cases of respiratory virus, from which 263 (84.29%) resulted RSV positive. Although this study included children up to 14 years of age, most cases belong to patients lower than 2 years of age with 92.82% and 93.85% for the 2014 and 2015 period respectively, and a 93.33% for both periods. Within them, children under 6 months of age represented the 59.11% and 67.21% of the 2014 and 2015 cases respectively, and a 63.16% globally. ALRTI cases caused by RSV were recorded from June to September of 2014 and from April to July of 2015, and included the final portion of the installed outbreak of 2014 and the starting of the 2015 one.

The G Glycoprotein's ectodomain was successfully sequenced in 168 samples (with an average length of 892 nt), from which 56 sequences corresponded to antigenic group A and 112 to antigenic group B. There were 19 and 39 identical sequences for antigenic group A and B, respectively. For the genotyping process, alignments of 662 and 681 nt length were obtained for antigenic group A and B, respectively. The most appropriate nucleotide substitution model for the alignments for both antigenic groups was the general time reversible with gamma distributed rate variation among sites (GTR +G). Phylogenetic analyses were congruent with high statistical support in the three inference methods performed. Fig. 2 shows the Bayesian trees (other trees under request). Some sequences clustered with one known genetic lineage, as is the case of the 56 sequences of the antigenic group A, which belonged to the ON1 lineage, within the GA2 genotype (Fig. 2A); whereas for the antigenic group B, 90 and 21

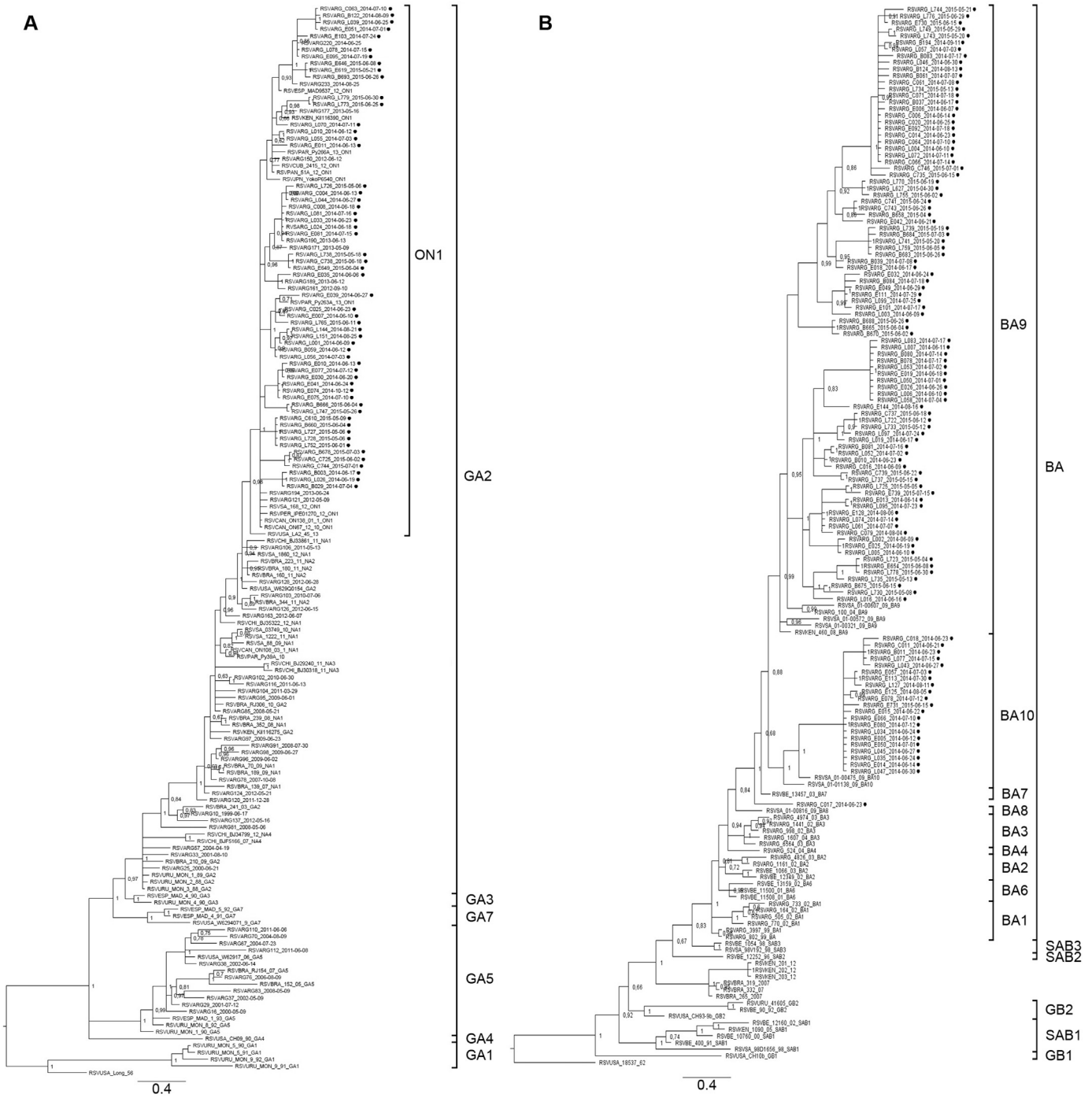

Fig. 2. Bayesian phylogenetic trees. The evolutionary model GTR+G was used for Bayesian reconstructions of RSV antigenic groups A (A) and B (B). In both cases the Bayesian Markov Chain Monte Carlo (MCMC) chains were run for 5E+7 generations, a 5E+4 sample frequency to reach convergence and a 10% burn-in. Genotypes and genetic lineages described by the bibliography are indicated with vertical lines on the right. Node labels represent posterior probabilities (values higher than 0.6 are shown). The scale bar represents nucleotide substitutions per site. Sequences obtained in this study are denoted with a black circle.

sequences clustered with the lineages BA9 and BA10, respectively within the BA genotype. There was only one sequence (RSVARG_C017) from the B antigenic group which clustered with another genetic lineage not mentioned before within the BA genotype (Fig. 2B). These results are shown on Table 1. The genotyping analysis showed co-circulation of the ON1, BA9 and BA10 lineages throughout the analyzed period. The Bayesian analyses showed global, regional and local circulation patterns by their association with different reference sequences. Once the circulating lineages were known, it was interesting to determine if there was an exclusive set of sequences with ancestral origin in Argentina. Thus, we decided to perform phylogeographic analyses to detect the most probable location of the origin that could allow the spread of RSV at the local level.

To determine the correlation between the sequences obtained in this study and reference sequences reported worldwide during the last years (within the detected lineages, ON1, BA9 and BA10), discrete phylogeographic analysis were estimated. Global spatio-temporal diffusion was achieved testing nucleotide substitution models, where the model GTR+G was the most appropriate for both antigenic groups. Analyzing the obtained MCCTs, the MRCA for the ON1 lineage was inferred in October 19th, 2010 located in Panama (Fig. 3A), and for the BA lineages was inferred in July 8th, 1998 in Argentina (Fig. 3B). The

Table 1
Number of RSV positive samples related to their antigenic group and genotypic characteristics (genotypes and genetic lineages), year of collection and hospital's districts where the patients were admitted.

	Antigenic group A		Antigenic group B				**Total**		
	GA2 genotype		BA genotype						
	ON1		BA9		BA10				
	2014	2015	2014	2015	2014	2015	2014	2015	**2014–2015**
Berazategui	4	4	11	7	1	0	16	11	**27**
Florencio Varela	4	4	9	6	2	0	15	10	**25**
Ezeiza	14	3	14	3	10	1	38	7	**45**
Lanús	14	9	21	19	7	0	42	28	**70**
Total	**36**	**20**	**55**	**35**	**20**	**1**	**111**	**56**	**167**

ON1 sequences obtained in this study gathered in two different clades with exclusive origin in Argentina and related to each other. A small one originated in December 21st, 2013 that share a common ancestor in the past with reported sequences from Spain (ON1-2, blue clade in Fig. 3A), and a large one originated in September 28th, 2011 (ON1-1, yellow clade in Fig. 3A). For the analyzed antigenic group B sequences, we also found two different clades with a common ancestor in Argentina. One clade of BA10 lineage (green in Fig. 3B) was originated in April 14th, 2011 and shared a common ancestor in the past with reported sequences from Paraguay, and the other clade of BA9 lineage was originated in March 08th, 2005 (red on Fig. 3B). For ON1-1 and BA9 clades, only sequences with exclusive local circulation were considered in the subsequent analyses. In summary, on both MCCTs, we identified clades that were feasible for analyzing the viral transmission patterns, hence they were considered for continuous phylogeographic analyses. The results of these analyses are shown through the diffusion dynamic plots on Fig. 4 and the interactive animation in Supplementary files KML 1–4. They demonstrated that there was a spatio-temporal transmission between local strains. In the center of the analyzed area (between Lomas de Zamora and Lanús districts), a common hot spot for all the analyzed clades was found, where all the MRCAs were located. In all cases, the intermediate ancestors spread following a centrifugal dispersion pattern, moving north, southeast and southwest. Since it was interesting the finding that the MRCAs of all genetic clades (independently analyzed) spread from the same place, we were interested in analyzing the beginning of each outbreak per clade, per year. Thus, we analyzed in the cases which were possible the transmission pattern of each clade considering, on the one hand, only the sequences of 2014 and, on the other, those of 2015 (Fig. 5). For the 2014 period, the location of the MRCA for the ON1-1, BA9 and BA10 clades was not different from the one inferred in the analyses that considered both years simultaneously (Fig. 5A and C and; Supplementary files KML 5–7), while in 2015 period, the inferred locations of the MRCAs for the ON1-1 and BA9 clades were slightly relocated to the north, closer to Lanús district (Fig. 5B and D; Supplementary files KML 8 and 9). For ON1-2 (2014–2015) and BA10 (2015) lineages, the low number of sequences impeded their analysis.

Considering that the characteristic spreading inferred for RSV could be attributed to some features in the studied population, we analyzed the population density of the region and the survey form obtained from each patient. Initially, we found that the central and northern districts of the VI Sanitary Region (where the location for all MRCAs were estimated) have the highest population density as shown on Fig. 1, with Lanús district as the most populated.

Afterwards, we evaluated if there was a statistical correlation between the population density and the risk of overcrowding (according to the inhabitants in a household and number of rooms from each patient's house). All children that lived in houses with more than 3 persons per room were classified as overcrowded as was determined in Argentina in the document "Unsatisfied basic-needs methodology" reported by the Provincial Bureau of Statistics (Ministry of Economy of the Buenos Aires Province, 2016) and by UNICEF (https://www.unicef.org/argentina/spanish/monitoreo_Pobreza_Completo.pdf). The nine districts of the analyzed region were classified into three zones with low, medium or high population density (Fig. 1 and Table 2). In order to determine if there was a different amount of overcrowded or not overcrowded children geolocalized in the three mentioned zones, a statistical analysis was performed. We found no significant differences among the different population densities (p=0.7815) (Table 2).

4. Discussion

The sequencing of the G gene's ectodomain allowed the subtyping and genotyping of the clinical samples collected in a population that has not been studied before, and therefore it allowed the identification of the circulating genotypes and lineages of RSV in the analyzed area during the studied period. Although the age of the analyzed patients was extended to 14 years old, children under six months were found to be the most susceptible to suffer severe infections, requiring hospitalization. Therefore, this should be the target population to be protected against severe infections. Nevertheless, it would be interesting to analyze the RSV impact on ambulatory patients with ALRTI and whether the most susceptible age group is different from the ones of hospitalized patients. In addition, it would also be interesting to determine if the onset of the outbreak in the ambulatory population is earlier than that of the hospitalized patients which could therefore explain the transmission chains in the entire population.

We found a predominance of B antigenic group over A in both studied periods, which differs from what was reported in Buenos Aires City in 2014 (Viegas et al., 2016). However, we cannot ruled out that the other antigenic group could have predominated during the first part of the outbreak (not analyzed in this study) and even could have changed the prevailing antigenic group in the entire outbreak.

Discrete phylogeographic analyses showed that some strains were related to ancestors reported at neighboring or transatlantic countries, which were introduced to Argentina, and others samples related to ancestors with local circulation. The continuous phylogeographic analysis of the clades with exclusive origin in Argentina showed that the MRCA origin for all lineages was located in the center of the analyzed area between Lomas de Zamora and Lanús districts. Interestingly, those districts show the highest population density. The individual analysis of the clades from the 2014 period resulted in non-significant variation of the inferred MRCAs location. The analyses of the ON1-1 and BA9 clades for the 2015 period showed a slight move to north, their MRCAs approached to the Lanús district, and since the samples from 2015 belong to the onset of an outbreak, the reason for that movement could support that the beginning of any new outbreak would be linked to highly populated areas. Outbreaks of infections are more frequent at high population densities given that the viral

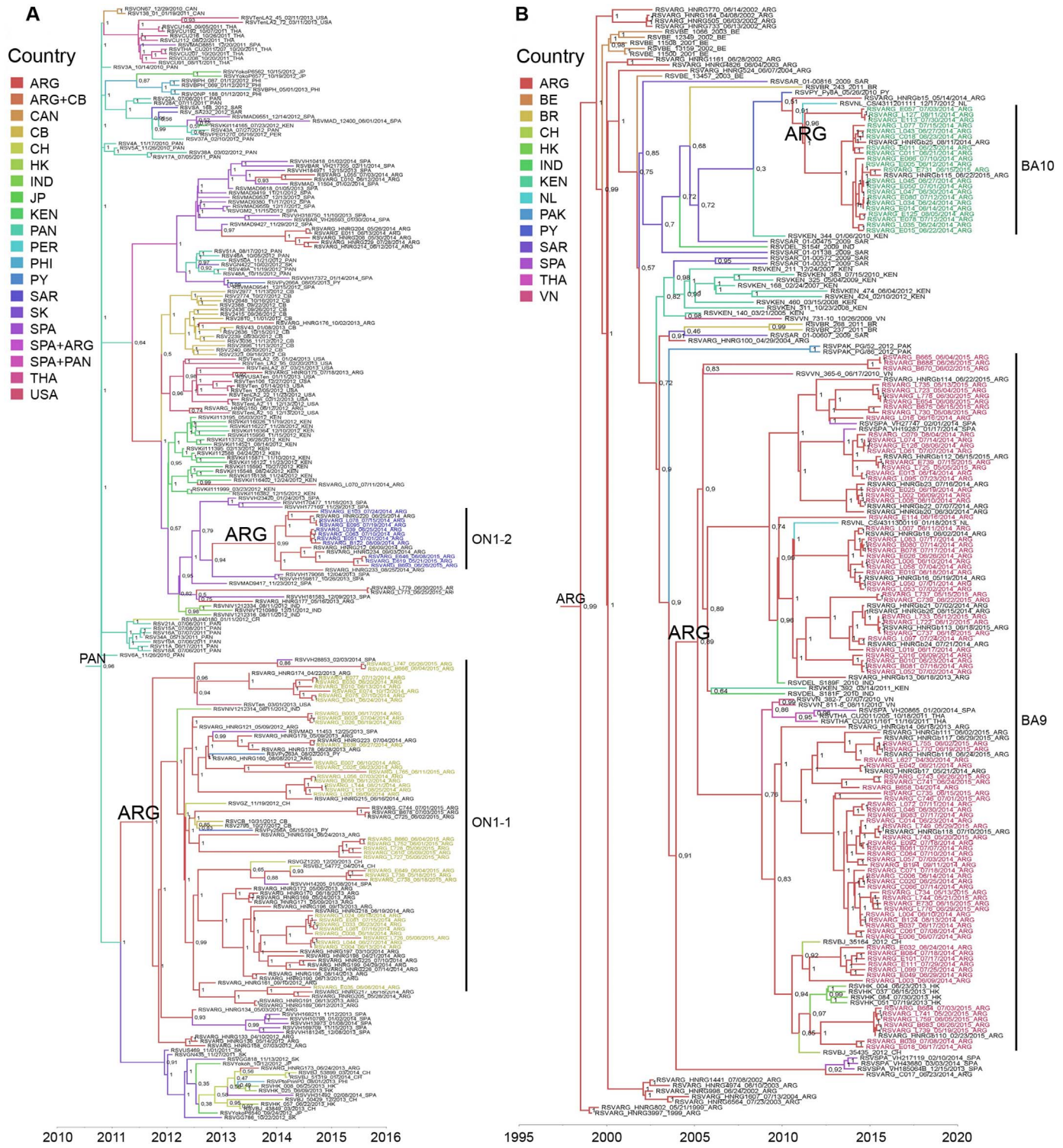

Fig. 3. Maximum Clade Credibility Tree (MCCT) discrete phylogeographic results. Trees for RSV antigenic group A ON1 lineage (A) and antigenic group B, BA9 and BA10 lineages (B) were obtained with the evolutionary model GTR+G. Lognormal relaxed clock (uncorrelated) model as the molecular clock. Bayesian Skyline was the most suitable demographic model for the antigenic group A, and the GMRF Bayesian Skyride for the B one. Branches are denoted in color by the location of the reported sequences, and the acronym indicates their country of origin. Node labels represent the probability support for that inference. Scale axis represents years. Local sequences names are highlighted: yellow for ON1-1; blue for ON1-2; red for BA9 clade and green for BA10 clade. Acronyms: ARG Argentina, BE Belgium, BR Brazil, CAN Canada, CB Cuba, CH China, HK Hong Kong, IND India, JP Japan, KEN Kenya, NL Nederland, PAK Pakistan, PAN Panama, PY Paraguay, PER Peru, PHI Philippines, PY Paraguay, SAR South Africa, SK South Korea, SPA Spain, THA Thailand, USA United States, VN Vietnam.

transmission is favored especially in the case of respiratory diseases. It is important to mention that the sampling process conducted by all physicians from different participating hospitals was continuous during all the analyzed period and the number of samples collected in this study did not present bias of collection. Therefore, the high number of samples from Lanús could support the phylogeographic results, indeed the outbreak would begin there and expand to other districts as was observed in the continuous analyses, in which diverse intermediate

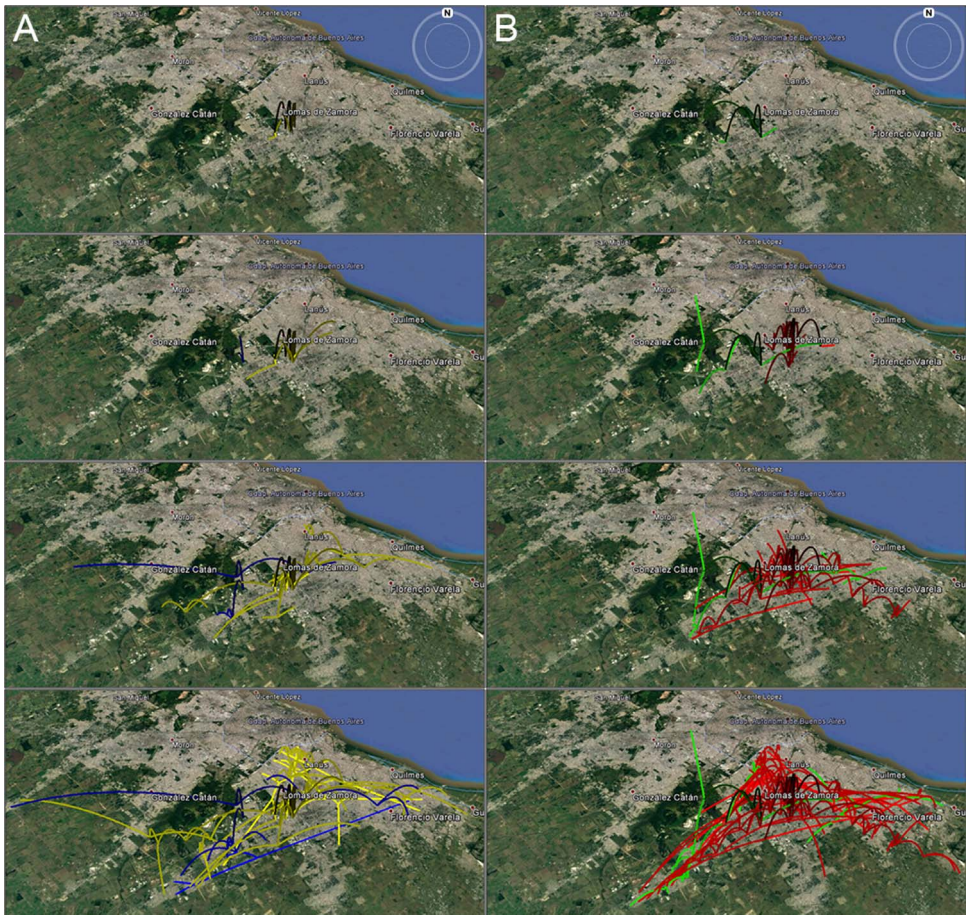

Fig. 4. Diffusion dynamic plot of continuous phylogeographic analysis. (A) RSV antigenic group A, ON1 lineage. The selected demographic models for ON1-1 and ON1-2 clades were Bayesian skyline and GMRF Bayesian skyride respectively. Continuous trait models were Homogenous Brownian and Gamma RRW for the small and the large clades respectively. MCMC were run for 2E+08 generations for both to reach convergence. (B) RSV antigenic group B, BA9 and BA10 lineages. GMRF Bayesian skyride was the demographic model with the Homogenous Brownian as the Continuous trait model for both clades. MCMC were run for 4E+08 generations to reach convergence. A gradient from dark to lighter color was used to represent the transition of ancestors to the samples obtained in this work, where the color of each clade shown in Fig. 3 was maintained. The KML files for visualization in Google Earth are available as supplementary files (KML files 1–4). The maps are based on satellite images made available in Google Earth (http://earth.google.com).

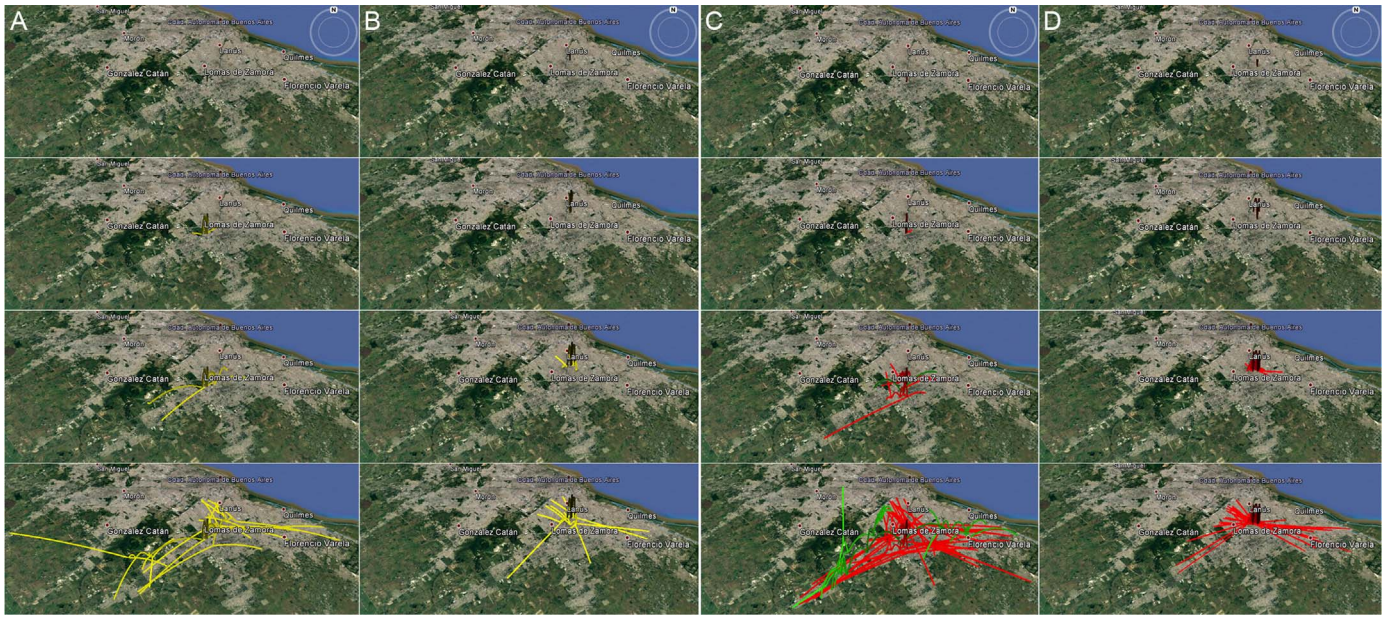

Fig. 5. Diffusion dynamic plot of the individual continuous phylogeographic analyses per clade per year. Transmission patterns of the ON1-1 clade for 2014 (A) and for 2015 (B), and for antigenic group B, BA9 and BA10 lineages for 2014 (C) and only the BA9 clade for 2015 (D). The colors and gradients of each clade of Figs. 3 and 4 were maintained. The KML files for visualization in Google Earth are available as supplementary files (KML files 5–9). The maps are based on satellite images made available in Google Earth (http://earth.google.com).

Table 2
Number of children with positive RSV samples classified according to their overcrowding characteristics and to their location zone by population density.

Zone's population density (People/Km^2)	District	Not overcrowded (< 3 inhabitants/ room)	Overcrowded (> 3 inhabitants/ room)
High (5000.1–15000.0)	Lomas de Zamora, Avellaneda, Lanús	37	33
Medium (1000.1–5000.0)	Esteban Echeverría, Almirante Brown, Quilmes, Florencio Varela, Berazategui	46	40
Low (100.0–1000.0)	Ezeiza	43	31

Statistical analysis: χ^2 (0.493) and p-value (0.781).

ancestors have spread to different directions: northbound, southeast and southwest. In this sense, the idea that the outbreak begins in the most populated area and then is distributed centrifugally to other areas could describe the social characteristics of a region, the connections of public transport and relations among communities, as well as the architecture of the connection among the districts that comprise the sanitary region analyzed (Read et al., 2012). In fact, the division of the territory into sanitary regions was carried out with the aim of organizing health system and being able to make decisions in the prevention and treatment of the most important diseases that impact to public health. The data provided in this work allowed us to trace a map of vulnerability of ALRTI caused by RSV in the analyzed region, which in the future could be implemented as a model for other health regions or for other transmissible infections.

We supposed that the viral transmission pattern could be linked to certain overcrowding characteristics of the analyzed population, but we did not find any significant differences in the number of residents living in overcrowded conditions among the three geographic areas defined by population density. However, as the overcrowding was similar in each of the defined areas, the high population density should be considered as a risk factor for transmission. On the other hand, we cannot exclude that other social characteristics, such as number of smokers, type of home heating systems, structural characteristics of the dwellings could be related to the transmission pattern described. Further analyses considering those characteristics are needed to correlate socio-epidemiological features and RSV transmission patterns.

Since there are diverse vaccination strategies under study and many of them are about to be approved, in the very near future, we will have the possibility to prevent severe infections caused by this virus. In this sense, several studies have attempted to describe the patterns of RSV transmission from the patient's point of view, with the objective of determining the target population to be vaccinated. In the United States, Yamin et al. have shown that targeting of children younger than five years of age is highly efficient per dose, and is also the most effective strategy to reduce RSV in young children and older adults across all states and transmission settings. Nevertheless, they analyzed the possibility to vaccinate the rest of the population to decrease the number of cases (although with substantially lower efficiency) and they proposed a cost-effectiveness analysis to specific states and transmission settings (Yamin et al., 2016). Considering the viral circulation patterns described in this study -where each viral outbreak begins in certain areas with high population density- health systems should evaluate vaccination at all ages in those areas, while in lower density population areas only susceptible children should be vaccinated.

5. Conclusion

In this study, it was possible to identify viral transmission pathways through the analysis of RSV's genetic perspective from two consecutive outbreaks in a limited geographical region. Considering this study as a precedent of the transmission dynamics of RSV in a population, it will be useful to describe patterns of social mixing behavior at the individual level and to contribute to the knowledge of the dynamics of infection at the population level. These results suggest that future cost-effectiveness analyses of RSV vaccination should be tailor-made for specific population and to specific regions.

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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.virol.2017.04.030.

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