

Molecular epidemiology of respiratory syncytial virus illness in children and adults in Seattle, WA over five seasons

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**Abstract**

Molecular epidemiology of respiratory syncytial virus illness in children and adults in Seattle, WA over five seasons

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**Background:** Respiratory syncytial virus (RSV) is the most important cause of pneumonia in children aged <5 years worldwide and can cause severe disease in elderly and high-risk adults. Multiple RSV strains co-circulate and evolve over seasons. We seek to describe the molecular epidemiology and evolution of RSV over five seasons in Seattle, WA, USA.

**Methods:** From 2011 to 2016, subjects aged 6 months and older seeking outpatient care for acute respiratory illness (ARI) at Kaiser Permanente Washington were enrolled in the United States Influenza Vaccine Effectiveness Network (Flu VE Network) and a respiratory swab was collected. Real-time polymerase chain reaction (RT-PCR) was performed to identify and subtype RSV positive samples. A subset of RSV samples were sequenced using primer-amplified next generation sequencing.

Associations between RSV genotype ON1 and severe disease, defined as illness requiring hospitalization or chest imaging in the following 30 days, were assessed using a multivariable logistic regression model.

**Results:** A total of 8,749 individuals were enrolled in the Flu VE Network and RSV was detected in between 11.0 (2015-6) and 16.4 (2012-3) percent of ARI episodes annually. The predominant RSV subtype switched every 1-2 years. RSV-A cumulative incidence was highest in 2015-6 at 13.7 ARI cases per 1,000 population (confidence interval [CI] 11.3, 16.1) and RSV-B incidence was highest in 2014-5 at 15.4 ARI cases per 1,000 (CI 13.6, 17.3). The ratio of RSV-A to RSV-B incidence differed across age groups ( $p < 0.001$ ). Forty of 43 (93%) RSV-A sequenced specimens were the ON1 genotype and the BA genotype represented 40 of 42 (95%) RSV-B specimens. Of 1133 RSV-positive ARIs, 213 (18%) were

categorized as severe disease. Neither RSV subtype A or genotype ON1 was associated with severe disease ( $p > 0.05$ ).

**Conclusion:** There was limited genotype diversity of the RSV genotypes circulating in our outpatient cohort with significant predominance of the ON1 and BA genotypes across all seasons. There was no evidence of an association between molecular subtype or genotype and severe disease. With multiple RSV vaccine candidates in development, understanding the genetic diversity and circulation of RSV viruses within a population is important for analyzing the effects of a vaccine on the evolution and molecular epidemiology of RSV.

## INTRODUCTION

Respiratory syncytial virus (RSV) is the most important cause of pneumonia in children under five years of age resulting in an estimated 33 million RSV infections, 3 million hospitalizations, and 60,000 deaths worldwide in 2015 [1]. While RSV causes more severe disease in infants and young children, adults experience repeated infections throughout life. Usually, adult illness results in mild respiratory symptoms, but can produce severe respiratory disease, especially in elderly and high risk populations, resulting in hospitalization, or even death [2–5]. In temperate regions, RSV follows a seasonal pattern with onset in late fall or early winter and continuing through late spring [6]. Community-based cohorts can provide valuable information about the clinical and molecular epidemiology of RSV, especially if performed over multiple seasons to capture the season-to-season variability in overall and strain-specific measures of occurrence [7].

RSV is categorized into two main antigenically-distinct subtypes, RSV-A and RSV-B, with at least 13 and 20 genotypes existing for subtypes A & B, respectively [8–10]. Subtype and genotype differentiation focuses on the region encoding the attachment (G) protein, the gene product containing greatest genetic divergence. In a given season, one subtype predominates with the dominant subtype alternating in 1-3 year cycles [6]. While multiple genotypes of RSV can circulate in a population simultaneously, generally a single genotype will be predominant in a local epidemic with successive replacement as new genotypes emerge [11]. Previously, genotypes GA2 & NA1 were the predominant genotype globally. However in 2010, genotype ON1, a variant of subtype A containing a unique 72-nucleotide tandem duplication within the C-terminal region of the G-protein, was described in Ontario, Canada and rapidly replaced NA1 as the dominant genotype in reports from Asia, Africa, and Europe [9,12]. The predominant RSV-B genotype since 2005, the Buenos Aires (BA) genotype, also has a 60-nucleotide duplication in a nearby region of the G-gene [13].

RSV-A is generally thought to have a more severe clinical course than RSV-B. However, this finding has not been consistent across studies and is based primarily on analyses of hospitalized infants [14–18]. Similarly, the clinical significance of ON1 remains unclear with discrepant findings between studies [19–

26]. In adults, there is limited sequencing data and no findings on the clinical relevance of specific genotypes [27–30]. There are various novel RSV vaccines and monoclonal antibodies in clinical trials. The genetic diversity of RSV can have a direct impact on the success of these interventions. For example, two mutations in the F protein of the circulating RSV-B strain resulted in the failure to show efficacy of a Fusion (F) protein monoclonal antibody in a phase III clinical trial [31]. Further, it is important to fully characterize the molecular epidemiology over time in both pediatric and adult populations prior to implementation in order to assess the potential effect of the therapy on the evolutionary dynamics of the virus.

Our study aimed to describe the molecular epidemiology of RSV subtypes and genotypes isolated from acute respiratory illness (ARI) in individuals across the age spectrum and to correlate specific molecular types with severe disease.

## **METHODS**

### *Study design and subjects*

Since the 2011-2 influenza season, Kaiser Permanente Washington (KPW), formerly known as Group Health, has performed active, population-based surveillance for influenza among patients aged  $\geq 6$  months seeking care for ARI at KPW outpatient medical centers as part of the United States Influenza Vaccine Effectiveness Network (US Flu VE Network). These episodes are defined as a medically-attended acute respiratory illness (MAARI). Surveillance for respiratory illness occurred at three KPW medical centers in the 2011-2 and 2012-3 seasons, at five centers in the 2013-4 and 2015-6 seasons, and at seven centers in the 2014-5 season. Source populations were defined as all KPW members who receive primary care at one of the surveillance medical centers. Surveillance periods varied by year depending on the duration of influenza season, ranging between 88 days in 2011-2 to 137 days in 2015-6. The results of the US Flu VE study have been published previously and this analysis is limited to the KPW cohort [32,33].

### *Surveillance for MAARI*

Patients  $\geq 6$  months of age who presented with an ARI  $\leq 7$  days duration were identified. From 2011-2, ARI was defined as fever or cough, and from 2012-3 to 2015-6, ARI was defined as cough. Nasal and oropharyngeal swabs were collected from enrolled patients aged  $\geq 2$  years and nasal swabs from those aged  $< 2$  years. Individuals who received influenza antiviral agents for their current illness or enrolled in the study within the previous 14 days were ineligible. Data regarding risk factors for respiratory virus infection were collected as part of the enrollment of the US Flu VE Network surveillance using surveys and ICD codes from KPW from outpatient and inpatient encounters in the previous year. For subjects with MAARI, further data were collected about the clinical course of subjects in the 30 days after their initial outpatient encounter including subsequent hospitalization and imaging. Subjects were categorized by age as of September 1st of each year into seven groups: 6-23 months, 2-9 years, 10-17 years, 18-49 years, 50-64 years, 65-74 years, and 75+ years. The Kaiser Permanente Washington Health Research Institute Institutional Review Board approved this study. A deidentified dataset was shared with University of Washington researchers and the UW Human Subjects Division exempted this analysis from review.

### *Virologic methods*

Respiratory specimens were tested for RSV using a quantitative real-time reverse transcriptase polymerase chain reaction assay (RT-PCR). RSV subtyping was performed as previously described [34]. We performed next generation sequencing (NGS) on a subset of 130 specimens with PCR cycle threshold (CT) values  $< 30$ . From seven age and five season strata, three samples were selected from each, plus 25 randomly selected specimens. Additionally, we performed sequencing of seven specimens from children hospitalized at Seattle Children's Hospital (SCH) and 17 specimens from adults hospitalized at Harborview Medical Center (HMC) during the 2016 to 2018 seasons. In brief, viral RNA was extracted from respiratory samples using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (Qiagen). Viral RNA was reverse transcribed into double stranded cDNA in a two-step process using RSV-A and RSV-B primers as described previously [7]. In brief, RNA was reverse transcribed with forward primers for six amplicons in separate reactions. The resulting cDNA was then

transcribed into double stranded cDNA using reverse primers and following PCR and magnetic bead purification, products were run on 0.6% agarose gel to monitor for amplification success. Specimens with a positive gel band proceeded to library preparation and sequencing at the Fred Hutchinson Cancer Research Center Genomics Core. NGS libraries were constructed with the Nextera kit and sequencing was conducted on an Illumina HiSeq system to achieve approximately 1 million reads per sample with 150 base pair (bp) reads.

### *Phylogenetic analysis*

Paired-end reads were demultiplexed into samples and barcodes were removed. Quality processing included removing primer and adapter sequences, trimming ends for low quality reads (PHRED<20). Processed reads were assembled using SPAdes to generate contigs, which were filtered by length and ordered by aligning to RSV-A or RSV-B reference genomes (accession numbers KU950676 & KU950499) to create a consensus scaffold. Processed reads were remapped to this scaffold to produce a final consensus sequence. This analysis pipeline was adapted from an existing HSV genome pipeline available at GitHub (<https://github.com/proychou/HSV>).

Genotyping analysis was performed based on phylogeny of the full G gene. All included sequences had >95% completeness of the G gene. Phylogenetic analysis of RSV-A and -B were performed separately. One hundred and fifty RSV-A and 120 RSV-B full G gene sequences from GenBank were used as references. We performed multiple sequence alignment of RSV-A and -B separately with study sequences and each respective reference set using MAFFT v6.83 in Geneious 10.2. We phylogenetically reconstructed the aligned sequences using a maximum likelihood HYK85 method with 100 bootstrap replicates [35]. Phylogenetic trees were annotated with metadata in R using package “ggtree” [36]. A study sequence was assigned to the ON1 or BA genotype based on the presence of the characteristic 72 or 60bp duplication, respectively. The central conserved domain (CCD), a target for therapeutic development from amino acids 164 to 186, was evaluated for amino acid sequence changes.

### *Statistical analysis*

We estimated the cumulative incidence of RSV subtypes and genotypes for each influenza season (y) stratified into mutually exclusive groups based on age (a) and number of outpatient MAARI (m), similar to previous analysis of influenza [32]. Every subject for whom a specimen is available (i.e., enrolled in the US Flu VE Network study) received an analytic weight as:

$$\frac{\text{Total number of persons in the stratum } (y,a,m)}{\text{Number of US Flu VE enrollees in the stratum } (y,a,m)}$$

$$\text{Number of US Flu VE enrollees in the stratum } (y,a,m)$$

Outpatient MAARI episodes were captured using ICD codes associated with outpatient visits. Infrequently, individuals enrolled in the Flu VE study had zero MAARI episodes. These study observations were assigned a weight of 1.0 assuming this was their only RSV ARI. Using the analytic weights, we extrapolated from the US Flu VE Network enrollees to the full source population to estimate the total number of medically attended visits due to each virus subtype in each stratum of the population. As the surveillance was targeted to influenza, our surveillance periods did not necessarily cover the full RSV season. Using University of Washington RSV surveillance data, we determined the proportion of cases in the Seattle region that occurred during the study surveillance period (p) [37]. For the number of MAARI episodes associated with each RSV subtype, we scaled up the estimated number of RSV cases by multiplying by 1/p. Similarly, as we sequenced a sampling of study specimens, we scaled up the estimate number of genotypes by the proportion sequenced in each season. Cumulative incidence in each age and/or season stratum for each subtype/genotype was calculated by dividing the estimated number of persons with RSV ARI by the source population of the stratum. Confidence intervals for the cumulative incidence estimates were calculated using bootstrap sampling from the source and sample population with 1,000 repetitions using the “boot” package in R [38].

We correlated clinical outcomes with RSV subtype and genotype. Severe disease was defined as subjects who reported wheezing or shortness of breath at enrollment or experienced subsequent

healthcare encounters related to their illness including hospitalization or chest imaging in the 30 days following the initial encounter. We used a logistic regression model in a multivariate analysis to identify an association between subtype (RSV-A used as reference) or genotype (ON1 used as reference) and severe disease while adjusting for a priori determined variables age group (6 to 23 months, 2-9 years, 10-17 years, 18-64 years,  $\geq 65$  years), any medical comorbidity, season, and sex. Chi-squared test of homogeneity was used to test for statistical difference in the proportion between genotype groups. Statistical significance was determined by a two-sided p value  $< 0.05$ . All statistical analysis was performed in R [39].

## RESULTS

### *Source population & RSV MAARI*

The source populations ranged between 82,266 persons in 2011-12 to 162,633 persons in 2014-5 (Table 1). Compared to the source population, the Flu VE Network enrollees were disproportionately children under the age of nine (18.7 vs. 7.2%). Respiratory swabs were collected from 8,749 MAARI episodes from the 2011-2 through 2015-6 seasons. The general characteristics of the KPW Flu VE cohort are summarized in Tables 1 & 2.

Overall, 1133 (13.0%) swabs were positive for RSV, ranging from 11.0% in 2015-6 to 16.4% in 2012-3. The proportion of swabs positive for RSV was greatest at the extremes of age, at 39.9% in children 6 to 23 months and 19.0% in children 2-9 years, followed by 14.7% in adults  $\geq 75$  years (Figure 1). Across all five seasons, 567 (50.0%) of RSV positive specimens were RSV-A, 547 (48.3%) RSV-B, and 19 (1.7%) untyped. The predominant subtype alternated every 1-2 years (Figure 2A & 2B). The proportion of RSV-positive samples that were RSV-A did not vary meaningfully across the age groups (Figure 2A).

Hospitalization in the 30 days following initial outpatient MAARI occurred in 13 (1.1%) of PCR-confirmed RSV episodes. Six (46%) of these hospitalizations had a respiratory diagnosis code associated with the admission. Adults over the age of 65 years were most frequently hospitalized, occurring after three percent of RSV outpatient MAARI. Chest imaging was obtained during 218 (18%) RSV MAARI episodes,

including 27% and 44% of adults 65-74 years and over 75 years, respectively, and 26% of individuals with at least one high risk medical condition.

#### *Cumulative incidence of RSV subtypes and genotypes*

RSV-A was the dominant subtype in 2011-2, 2012-3, and 2015-6 (Table 3). The incidence of both RSV-A and RSV-B were highest in children ages 6 months to 2 years peaking at 156 (95% confidence interval [95% CI] 106, 208) RSV-A MAARI cases per 1,000 population in 2012-3 and 147 (95% CI 113, 180) RSV-B MAARI cases per 1,000 population in 2014-5. Among adults, the incidence of both RSV-A and -B subtypes were highest in adults ages 75+ at 13.6 cases per 1,000 population in RSV-A and 13.1 cases per 1,000 population in RSV-B across the five seasons (Table 4). The proportion of RSV-A to RSV-B cases in the source population was not homogenous across age groups ( $p < 0.001$ ; Table 4).

The ON1 and the BA genotype were the predominant RSV-A and B genotypes, respectively, in every season in our cohort. The estimated cumulative incidence of the ON1 and BA genotypes are shown in Table 5. Cumulative incidences of other genotypes were not calculated due to low numbers.

#### *Phylogenetic analysis*

Full G gene sequences were acquired from 85 (65%) of 130 attempted samples, including 43 RSV-A and 42 RSV-B specimens. The ON1 genotype with a 72-nucleotide duplication in the C terminus of the attachment (G) gene represented the predominant RSV-A genotype across all seasons. Three (7%) of all RSV-A specimens (GH130182, GH150016, and GH300066) did not contain the characteristic ON1 genotype, although these sequences fell within the ON1 clade with tree construction (Figure 4). When these samples were re-assembled, they returned contigs that did show the ON1 genotype. Within the ON1 genotype, study sequences fell within four sub-lineages. The ON1.1 cluster contained the majority of Flu VE RSV-A sequences ranging from years 2012 to 2016 and included all age groups. The lineage ON1.2 contained sequences from both children and adults in 2016, as well as, a SCH specimen from 2017. Two study sequences from 2014 and 2016 clustered within the ON1.3 lineage, and two study sequences from 2015-6, plus a SCH sample, fell within the ON1.4 lineage (Figure 4). Lineages ON1.1 &

1.2 contain a series of 2-3 amino acid changes from one of the originally reported ON1 sequences from Ontario (JN257694) including Arg2445Leu, Thr246Pro, and Leu249Ser. All study sequences in lineage ON1.2 contained four additional amino acid changes. Study sequences in lineage ON1.3 all contained three amino acid changes from the Canadian ON1 sequence including Leu274Pro present in many ON1.1 & 1.2 study sequences and Leu298Pro present in ON1.4. There were three additional amino acid changes present in both ON1.4 study sequences. All amino acid changes common to study sequences occurred in the hypervariable regions, most frequently the 2nd hypervariable region.

Forty of the 42 (95%) RSV-B specimens contained the BA 60-bp duplication in the G gene and all aligned within the BA genotype with phylogenetic reconstruction. The RSV-B tree splits into two main clades of BA genotypes with 14 Flu VE study specimens from 2012-5 falling within the first clade. The second clade contains 28 Flu VE study samples from 2012-6, plus seven HMC and five SCH sequences from 2017. Within these two clades additional clustering by year is observed. For example, 10 Flu VE, seven HMC, and four SCH from years 2015-7 cluster together within a branch of the second clade (Figure 5). There was no apparent clustering of RSV-A or -B by age group (Figures 4 & 5). Analysis of amino acid sequence of the CCD of the G protein revealed no nonsynonymous mutations in study sequences of either subtype.

#### *RSV subtypes genotypes and disease severity*

RSV subtype A was not associated with severe disease compared to RSV subtype B in an unadjusted model (Odds ratio [OR] 0.80; 95% CI 0.60, 1.08) or when adjusted for sex, age, and medical comorbidities (OR 0.82; 95% CI 0.60, 1.12). Neither was the ON1 genotype compared to all other genotypes in unadjusted (OR 0.90; 95% CI 0.33, 2.46) or adjusted (OR 0.82; 95% CI 0.29, 2.34) models (Table 6).

## **DISCUSSION**

We used molecular virology techniques to estimate the incidence of RSV subtypes and genotypes associated with medically attended acute respiratory illness in a cohort of children and adults over the

course of five seasons in Seattle, Washington in the United States. To our knowledge, this is the first study to document the molecular epidemiology of RSV across the age spectrum over multiple seasons. We used whole genome sequencing to characterize the RSV genetic heterogeneity over the course of five seasons. The predominant subtype switched in 1-2 year cycles and we observed dominance of the RSV-A ON1 and BA genotypes, as expected. Unexpectedly, our cohort captured limited genetic variability with almost all sequences representing a single genotype per subtype. Our findings provide a baseline for the molecular diversity and epidemiology of RSV in both children and adults prior to the implementation of a RSV vaccine or extended half-life monoclonal antibody [40].

Our estimates of RSV MAARI subtype incidence are comparable to other studies which estimate an overall incidence of RSV MAARI between 14 to 17 cases per 1,000 population [41–43]. In adults, the incidence of RSV increased with age, which is consistent with previous studies [4,41,42]. In children, the incidence is highest in the 6 to 23 month age group and decreases with age [43]. Due to the exclusion of infants <6 months of age, we did not capture the age group with the highest incidence of RSV illness. In Mali, the incidence of symptomatic RSV illness in infants under 6 months was estimated to be 536.8 per 1000 person-years, more than double our estimate of seasonal RSV MAARI in children 6-23 months of age [44]. Few individuals (1.2%) in our cohort developed severe disease requiring hospitalization in the 30 days following the initial MAARI visit. Comparatively, between 6-12% of adults with RSV illness were hospitalized in a similar cohort in Wisconsin versus 3% of adults >65 years in our study [41,42]. Those who did require hospitalization more frequently had a high risk medical condition, which is consistent with previous studies. Similarly, a meta-analysis of industrialized countries, estimated that 14% of RSV ARI episodes resulted in hospitalization in adults >65 years of age [5]. The differences in the proportion of RSV MAARI resulting in hospitalization is unclear. It does not appear to be due to differences in medical comorbidities, but could be related to the inclusion of individuals presenting to the emergency department in the Wisconsin studies, as well as the institutional structure of KPW as a Health Maintenance Organization.

The magnitude of subtype predominance varied from year to year. In the 2011-2 season, the incidence of RSV-A was 13-fold that of RSV-B whereas, in 2013-4 RSV-B predominated over RSV-A by 1.4 fold. Similar patterns of alternating subtype predominance have been observed in South Africa and Argentina, although with an overall predominance of subtype A during and in the decade prior to our study. [30,45]

The proportion of RSV-A to RSV-B incidence was statistically different across age groups with a generally lower proportion of RSV-A in pediatric patients, especially the 10-17 year age group. This suggests that there may be different subtype populations circulating in pediatric compared to adult community-dwelling populations. In an analysis of the molecular epidemiology of RSV in pediatric and adults surveillance specimens from the United States in 2016-7, the proportion of RSV-B samples were higher in individuals over 6 years compared to children 1-2 years [29]. It was hypothesized that this was due to pre-existing immunity to RSV-A in the older age groups. Our data may be consistent with this hypothesis as the increase in the proportion of RSV-B incidence was specifically observed in the 10-17 year age group. Additionally, we observed an increase in the proportion of RSV-A in the 18-49 year age group with lower proportions in subsequent adult age groups, including 65+ years. This may be an artifact of low incidence among this age group, or could represent waning immunity to RSV-A. Upon phylogenetic analysis, there was no obvious clustering of study sequences by age group suggesting that, while there may be a subtype predominance by age, similar genotypes circulate across age groups. Further analysis of the relationship between genotype and age was limited as we sequenced only a subset of the overall study RSV-positive population.

We observed limited viral genotype diversity in our study cohort with almost all sequences falling within the ON1 or BA genotypes. Moreover, the minority of Flu VE study sequences lacking the ON1 or BA duplications, clustered within the ON1 or BA clades, suggesting that they may represent these genotypes. Furthermore, when reassembled, some contigs were found containing the ON1 variant, which could also may point to a mixed infection. This warrants further investigation through a deeper analysis of variants, validation by PCR or targeted resequencing of this region. Alternatively, resequencing the viral genome with a longer read format such as 2 x 300 may also allow better recovery of these duplicated regions. We expected to observe a significant proportion of non-ON1 genotypes in the RSV-A specimens in the 2011-

2 season as the ON1 genotype was first detected in 2010 and globally, NA1 and GA2 were still circulating in this season [11,12,30]. However, evolutionary analysis of the ON1 genotype estimates its emergence earlier, between 2005 and 2009, and ON1 may have established predominance in Seattle earlier than in other regions [12,46]. However, this may also be a consequence of our sequencing selection strategy in which we only sequenced a subset of specimens with high viral load.

Within the ON1 genotype, we observed multiple lineages as reported in previous studies [12,46,47].

These lineages were characterized by season with one lineage representing the majority of sequences from 2012-4 and another lineage dominating in the 2015-6 season. These two lineages, representing the majority of subtype A study sequences, contained shared amino acid changes differentiating them from the originally reported ON1 genotype in 2011 from Ontario, Canada. It is possible that these lineages arose separately as previous analyses suggest that the ON1 duplication may arise from multiple separate duplication events [21,45]. In our RSV-B analysis, the BA genotype dominated, as expected, with clustering of Flu VE study sequences by season, but not age group. Two sequences did not contain the BA duplication but clustered within the BA genotype suggesting spontaneous deletion of the duplication, a mixed population, or failure to capture the duplication due to short reads. In contrast, a Kenyan study reported three sequences from 2012 that did not contain the BA duplication yet clustered with non-BA genotypes suggesting long persistence despite the overwhelming predominance of the BA genotype over the past decade [48].

Understanding the genetic diversity of RSV in the community is important prior to the development of vaccines, prophylactic monoclonal antibodies, and therapeutics. A prophylactic agent, used widely or in high-risk populations, has the potential to interact with the molecular epidemiology of RSV. This study helps to establish a baseline for the epidemiology of RSV across the age spectrum, especially in adults where data has previously been limited compared to children. Notably, we did not observe evidence of different RSV genotypes circulating in adults versus children. Moreover, our limited number of samples from hospitalized individuals (HMC and SCH specimens) appears to be closely related to the Flu VE specimens from outpatient settings. This implies that from viral genetic perspective, we would expect

similar efficacy of viral-specific agents across age groups if similar RSV strains circulate in children and adults. In turn, the genetics of RSV can have a significant impact on the efficacy of prophylactic agents as demonstrated by the failure to show efficacy of a prophylactic monoclonal antibody during a recent phase III trial due to two mutations in the circulating RSV subtype B strain [31]. Moreover, rare mutations in the RSV F protein have been associated with escape from palivizumab and are reported at a low prevalence in the general population [49–51]. We will evaluate the evolution of and minor variants in F gene in a separate analysis. However, the CCD, a highly conserved region of the G gene considered a promising target for prophylactic and therapeutic agents, was conserved in all our sequences, as expected [31]. A previous study described conservation of G protein epitopes to murine monoclonal antibodies despite the sequence diversity in the G gene with no clear relationship between genetic relatedness and antibody reactivity [52]. This suggests that positive selection of mutations is not strongly immune-driven, rather due to the high mutation rate in combination with other selective processes. Finally, once a RSV-specific therapeutic is available, identifying and establishing the epidemiology of resistance mutations in pre-implementation specimens will be important for evaluating the development and persistence of resistance. A similar process has been observed in influenza where mutations conferring resistance to oseltamivir, baloxavir, and amantadine/rimantadine have emerged, threatening our ability to treat influenza infection [53–55].

Another important consideration in RSV molecular epidemiology is whether viral strain influences clinical outcomes. This could be due to strain differences in virulence or lack of pre-existing population immunity to new strains. In our analysis of the association between molecular viral subtype or genotype and severe disease, we defined severe disease as requiring chest imaging or hospitalization. We did not find an association between subtype A or genotype ON1 and severe disease. This suggests that disease severity is more dependent upon host and environmental factors than viral subtype or genotype in our study. Previous studies have found contradictory findings with some reporting ON1 causes more severe disease [22,23], while others report the opposite [19,20,24,25], or no significant difference [21,26]. These were performed in pediatric, primarily hospitalized cohorts, as opposed to our cohort of outpatient MAARI across the age spectrum. However, our findings also may be related to our definition of severe disease.

Given the low frequency of hospitalization in our ambulatory cohort, the designation of severe disease was driven by the requirement for chest imaging. However, we did not have data on chest imaging findings or symptoms to support a diagnosis of lower respiratory tract disease. While we adjusted for high risk medical conditions and age, which may predispose an individual to receive chest imaging, there is likely residual confounding in our model.

This study has several limitations. First, our cohort was recruited from outpatient MAARI encounters and did not fully capture the breadth of severity, including less severe disease that did result in care seeking behavior or those with more severe disease who presented directly for inpatient care. Lacking data on the two extremes may limit our ability to detect differences in disease severity between RSV genotypes. Also, we likely did not completely represent the diversity of RSV genotypes circulating in the region as we may not have captured certain RSV strains that were more prevalent in an inpatient setting. Additionally, we only sequenced a subset of RSV-positive specimens with higher viral load, which may be associated with specific RSV genotypes [56]. Moreover, as the Flu VE Network study was designed to evaluate influenza vaccination, the study dates did not always align with the RSV season. While we attempted to adjust for this in our incidence calculations, we may have missed genotypes circulating outside of our study dates. Lastly, our cohort does not include infants <6 months as this age group is not surveyed in the Flu VE Network. Many infants experience their first RSV infection during the first six months of life and they are the most vulnerable to severe disease. The exclusion of this group limits our ability to fully capture the burden of RSV and specific viral genotypes in the community.

In conclusion, using a closed population in Kaiser Permanente of Washington, we were able to combine next generation sequencing with epidemiologic methods to estimate the population incidence of specific subtypes and genotypes resulting in outpatient MAARI. Many other studies estimating RSV epidemiology rely on cohorts of hospitalized patients or do not include molecular virology data. We also report sequencing data across age groups, including adults >65 years. While this population is at high risk for severe RSV infection, there is limited RSV sequencing data from this demographic. Additionally, our study included five seasons allowing us to track changes in the incidence of subtypes and patterns in the

genetic lineages over time which is important for establishing a baseline of the RSV molecular epidemiology and evolution prior to the licensure of a prophylactic RSV agent.

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## TABLES

**Table 1.** Source and study populations from Kaiser Permanente of Washington and Flu VE Network study, seasons 2011-12 through 2015-6

Age category	Source population: KPW members					Study population: Flu VE Network				
	2011 - 2012	2012 - 2013	2013 - 2014	2014 - 2015	2015 - 2016	2011 - 2012	2012 - 2013	2013 - 2014	2014 - 2015	2015 - 2016
6 – 23 mos	938	783	1269	1572	1405	104	102	121	217	100
2 - 9 yrs	5741	4978	6855	9537	8117	169	114	150	365	196
10 - 17 yrs	5662	4836	7017	10486	8382	101	88	84	262	141
18 - 49 yrs	35949	34851	48704	63759	48938	509	485	649	944	685
50 - 64 yrs	21767	21009	28848	40766	31015	234	238	369	530	346
65 - 74 yrs	8384	9298	13439	22444	19405	88	102	169	318	233
75 + yrs	6440	6506	8995	14069	11622	54	69	86	198	129
<b>Total</b>	<b>84881</b>	<b>82261</b>	<b>115127</b>	<b>162633</b>	<b>128884</b>	<b>1259</b>	<b>1198</b>	<b>1628</b>	<b>2834</b>	<b>1830</b>

Abbreviations: Kaiser Permanente of Washington (KPW), Influenza Vaccine Efficacy Network (Flu VE Network)

**Table 2.** Characteristics of all MAARI and RSV-positive MAARI episodes in Flu VE Network study participants

Characteristic	All MAARI (n=8749)		RSV-positive MAARI (n=1133)	
	N	%	N	%
Season				
2011 - 2012	1259	14	142	13
2012 - 2013	1198	14	196	17
2013 - 2014	1628	19	227	20
2014 - 2015	2834	32	367	32
2015 - 2016	1830	21	201	18
Age category				
6 – 23 mos	644	7	257	23
2 - 9 yrs	994	11	189	17
10 - 17 yrs	676	8	49	43
18 - 49 yrs	3272	37	257	23
50 - 64 yrs	1717	20	183	16
65 - 74 yrs	910	10	119	11
75 + yrs	536	6	79	7
Gender, male	3874	44	528	47
High risk conditions, any	3320	38	414	37
Chronic lung disease	1728	20	229	20
Cardiovascular disease	1142	13	147	13
Diabetes mellitus	665	8	76	7
Immunosuppression	58	0.7	4	0.4
Other*	1330	15	156	14
Received influenza vaccination	4758	54	684	60

\*Other conditions include malignancy, kidney disease, liver disease, blood disorders, cerebrovascular disease, and high risk neurological or musculoskeletal conditions

Abbreviations: Influenza Vaccine Efficacy Network (Flu VE Network), Medically attended acute respiratory illness (MAARI), Respiratory syncytial virus (RSV)

**Table 3.** Cumulative incidence by 1,000 people of RSV subtypes A & B by season with 95% confidence intervals

Season	RSV-A			RSV-B		
	Cases per 1000 people	LCL	UCL	Cases per 1000 people	LCL	UCL
2011 - 2012	11.8	9.5	14.1	0.9	0.3	1.5
2012 - 2013	13.1	10.6	15.7	6.1	4.4	7.8
2013 - 2014	7.4	5.8	9.0	10.4	8.5	12.3
2014 - 2015	2.4	1.7	3.2	15.4	13.6	17.3
2015 - 2016	13.7	11.3	16.1	3.6	2.3	4.9

Abbreviations: Lower confidence limit (LCL), Respiratory syncytial virus (RSV), Upper confidence limit (UCL)

**Table 4.** Cumulative incidence by 1,000 people of RSV subtypes A & B by age category across all five seasons with 95% confidence intervals

Age category	RSV-A			RSV-B			Incidence rate ratio RSV-A to -B
	Cases per 1000 people	LCL	UCL	Cases per 1000 people	LCL	UCL	
6 – 23 mos	96.9	81.1	113.4	90.6	74.4	105.8	1.07
2 - 9 yrs	25.5	20.3	30.8	24.0	18.7	29.3	1.06
10 - 17 yrs	3.8	1.9	5.7	5.5	3.3	7.6	0.69
18 - 49 yrs	4.9	4.1	5.8	4.1	3.3	4.9	1.20
50 - 64 yrs	6.6	5.2	8	6.4	5.1	7.8	1.03
65 - 74 yrs	10.1	7.4	12.8	9.3	6.8	11.6	1.09
75 + yrs	13.6	9.2	18.1	13.1	8.8	17.4	1.04

Abbreviations: Lower confidence limit (LCL), Respiratory syncytial virus (RSV), Upper confidence limit (UCL)

**Table 5.** Cumulative incidence per 1,000 subjects of RSV genotypes by season.

Genotypes	2011-2012		2012-2013		2013-2014		2014-2015		2015-2016	
	Cases per 1000 people	95% CI	Cases per 1000 people	95% CI	Cases per 1000 people	95% CI	Cases per 1000 people	95% CI	Cases per 1000 people	95% CI
RSV-A ON1	12.4	4.9, 19.8	9.5	2.6, 16.6	6.3	-0.9, 13.6	0.5	-0.4, 1.5	13.6	4.9, 22.6
RSV-B BA	1.3	-0.6, 3.2	9.6	2.6, 16.5	9.9	1.7, 18.2	16.7	7.9, 25.3	3.1	-1.8, 7.3

Abbreviations: confidence interval (CI), respiratory syncytial virus (RSV)

**Table 6.** Logistic regression model associating viral subtype and genotype with severe disease, defined as illness resulting in chest radiologic imaging or hospitalization in 30 days following MAARI episode.

Genotypes	Unadjusted OR	Unadjusted 95% CI	Adjusted OR*	Adjusted 95% CI*
RSV-A subtype**	0.80	0.60, 1.08	0.82	0.60, 1.12
RSV-A ON1***	0.90	0.33, 2.46	0.82	0.29, 2.34

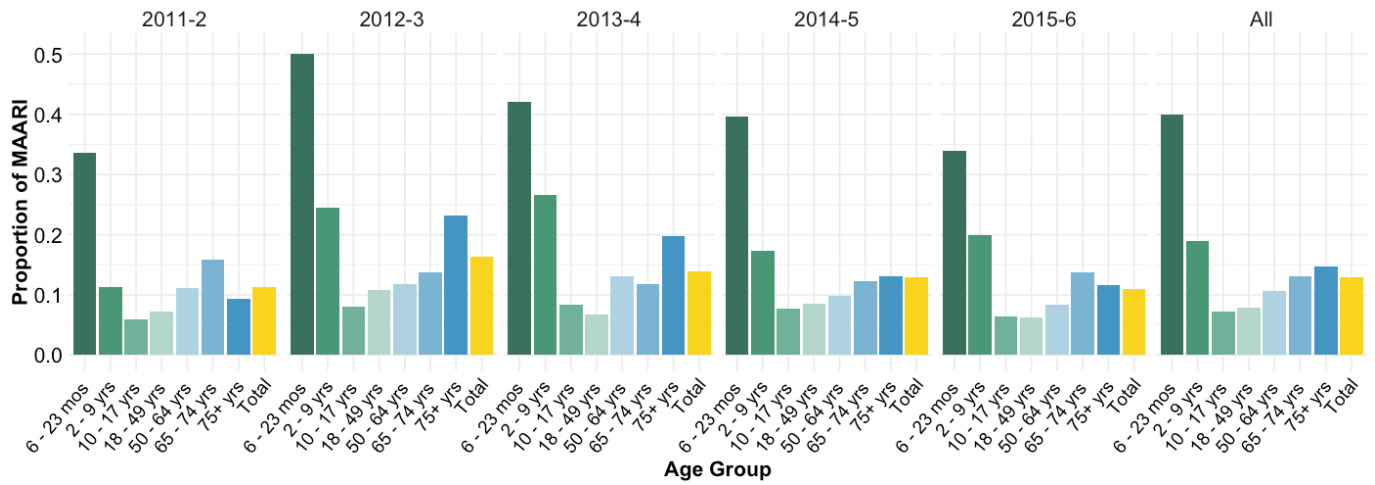
\*Adjusted for sex, age group, any medical comorbidity

\*\*N=1133

\*\*\*N=85

Abbreviations: confidence interval (CI), medically attended respiratory illness (MAARI), respiratory syncytial virus (RSV)

## FIGURES



**Figure 1.** Proportion of MAARI episodes positive for RSV by RT-PCR, categorized by age group and season.

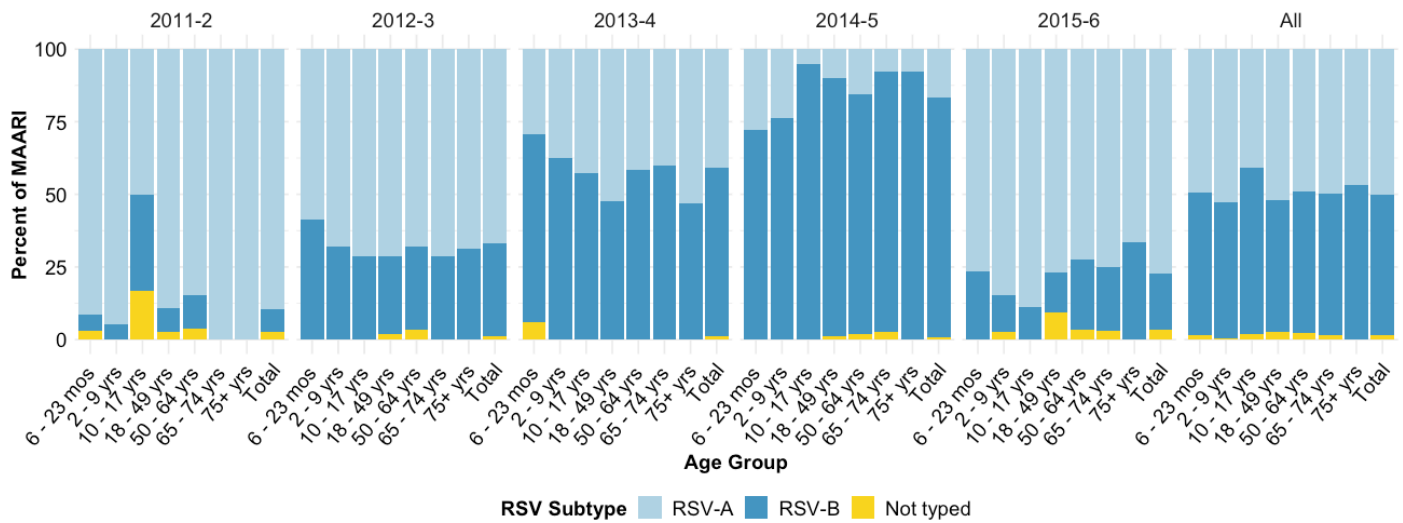


Figure 2A. Proportion of RSV MAARI episodes by subtype, categorized by age group and season.

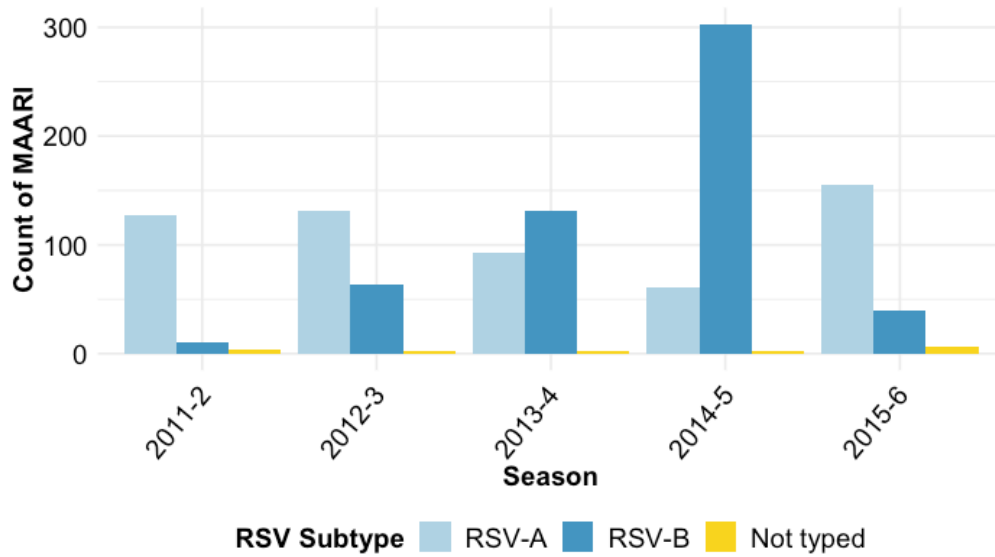
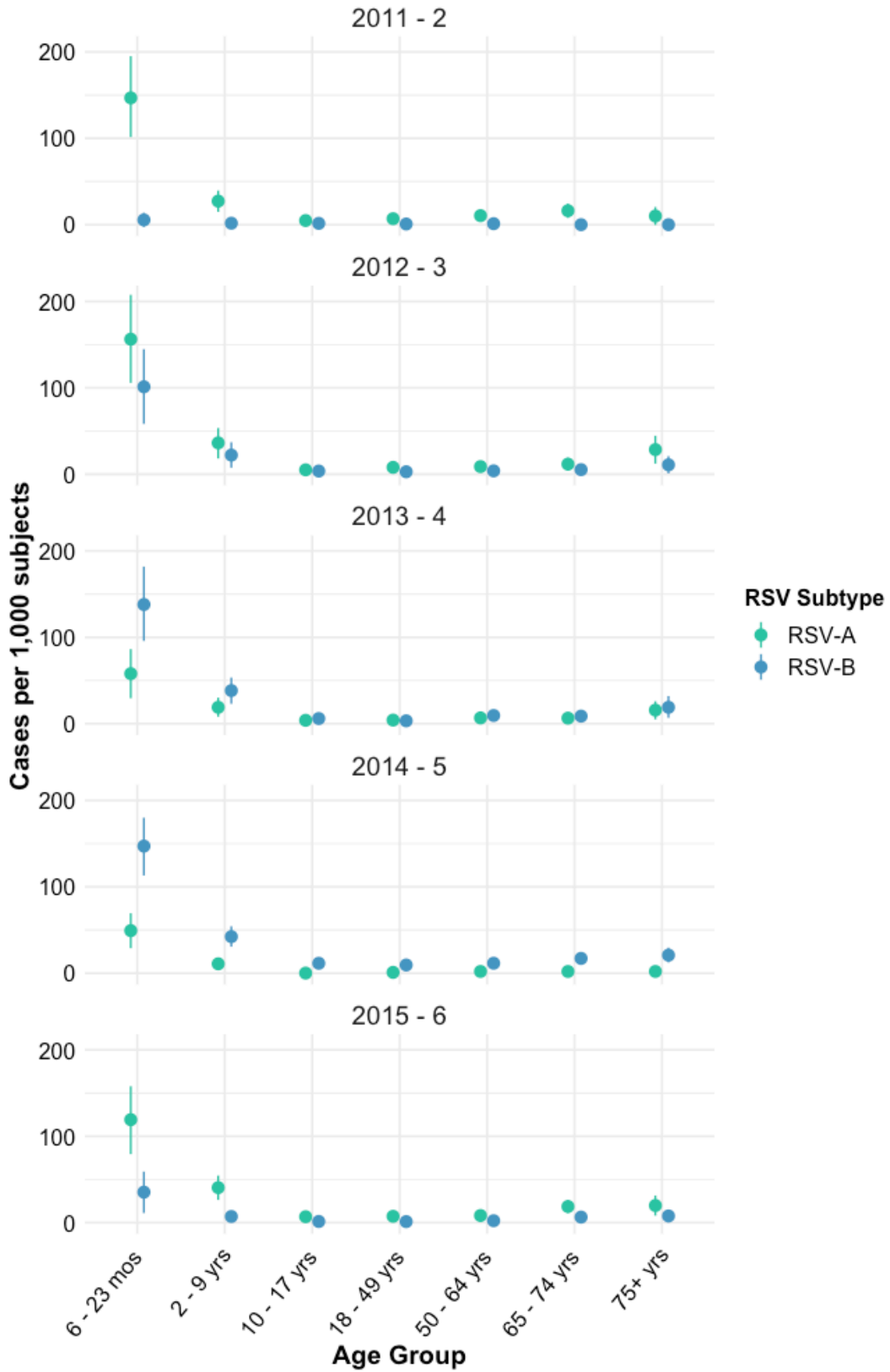
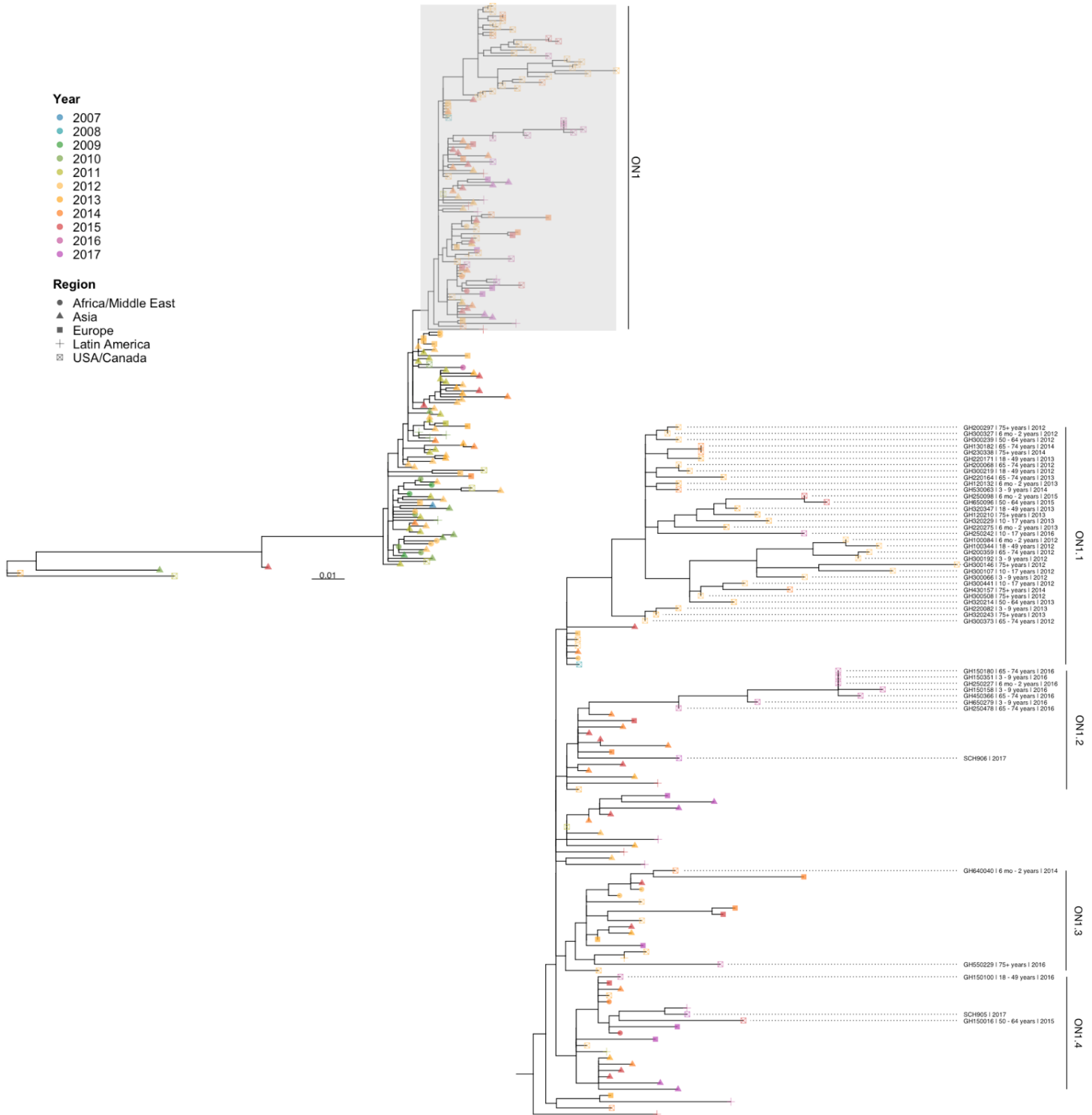


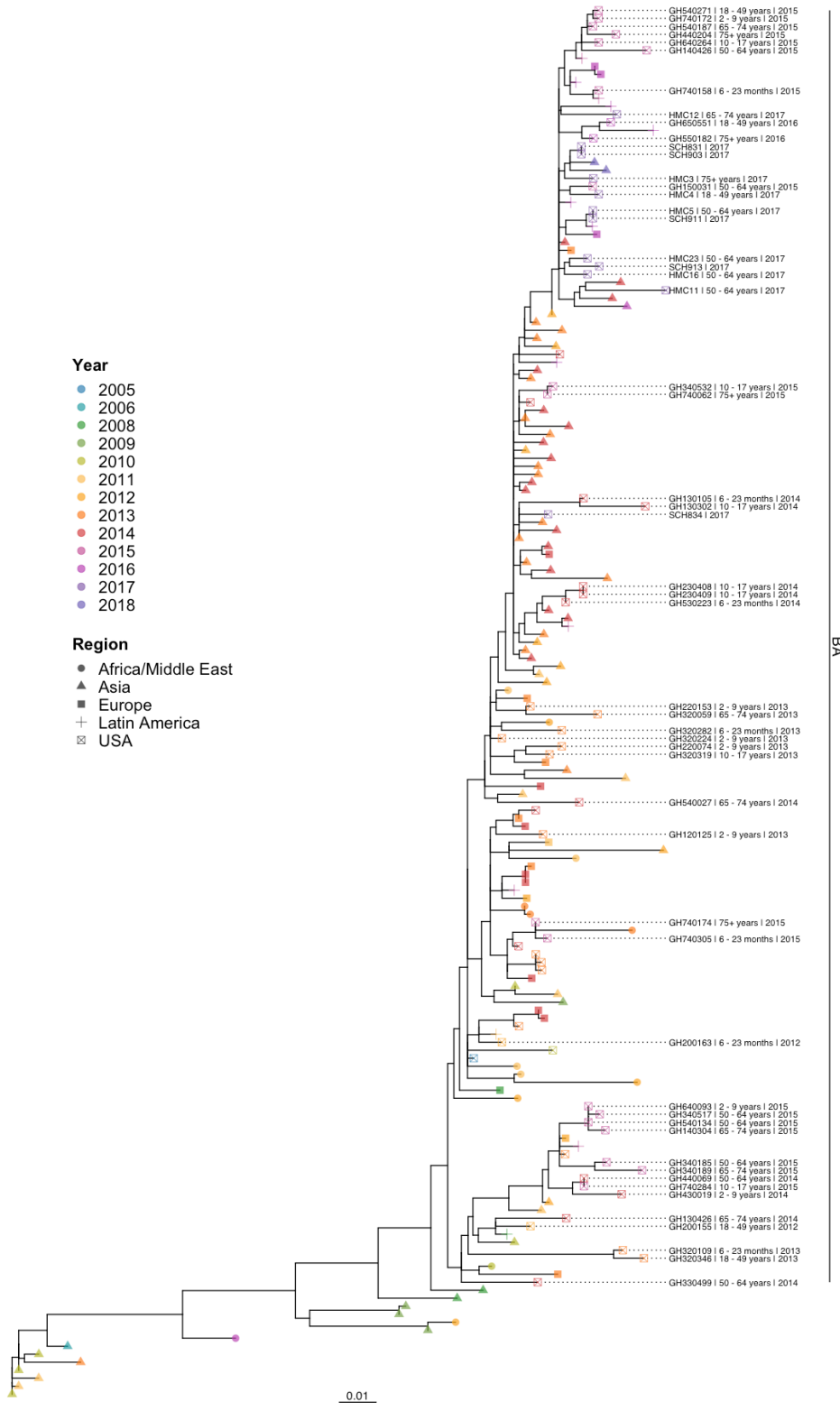
Figure 2B. Count of RSV MAARI episodes by season.



**Figure 3.** Estimated cumulative incidence of RSV-A and RSV-B medically attended acute respiratory illness cases per 1,000 individuals in source population by season and age group.



**Figure 4.** (Top) Maximum likelihood phylogenetic tree with 100 bootstrap replicates of RSV-A study and reference sequences from G gene analysis. Symbol represents region of specimen origin and color represents year of collection. ON1 lineage (highlighted in top) is shown below main tree with labels of ON1 lineages containing study sequences. Flu VE Network specimens and sequences from hospitalized children at SCH are labeled with ID, year of collection, and age of individual.



**Figure 5.** Maximum likelihood phylogenetic tree with 100 bootstrap replicates of RSV-B study and reference sequences from G gene analysis. Symbol represents region of specimen origin and color represents year of collection. Flu VE Network specimens, and sequences from hospitalized adults at HMC and hospitalized children at SCH are labeled with ID, year of collection, and age of individual.