

A Prospective Study of Respiratory Syncytial Virus Infection Among Children Attending Daycare

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Abstract

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Abstract:

Background: RSV is the most important cause of serious respiratory disease in young children, but transmission among children is not well-characterized. Molecular sequencing of the hypervariable glycoprotein (G) coding portion of the RSV genome allows for description of transmission dynamics.

Methods: We prospectively followed children enrolled in full-time childcare during 3 winter seasons. We collected nasopharyngeal (NP) swabs at enrollment and with illness episodes. The child's caregiver completed a symptom diary. Samples were tested for 12 respiratory viruses by reverse transcriptase polymerase chain reaction (PCR) techniques. We developed a heminested PCR assay to amplify and sequence a region of the RSV G protein. Data were analyzed using GEEs and univariate linear regression models. Phylogenetic analysis was performed using the maximum likelihood method.

Results: RSV was detected in 62 (9%) of episodes. Compared to RSV-negative illness episodes, RSV-positive illness episodes were associated with longer symptom duration, more childcare and work days missed, and increased frequency of health care visits. The median \log_{10} viral copies/mL was 7.50 (2.6-10.0). In 42 (68%) of RSV-positive swabs, another respiratory virus was detected. There was no correlation between RSV viral load and age at illness onset or presence of other respiratory viruses. We observed three RSV outbreaks with a predominance of subtype B in season 1 (21/27) and subtype A in seasons 2 (7/8) and 3 (20/27). In two rooms, 50% of the children had RSV detected within 6 days of the first case, and 75% within 10 days. Sequencing was performed for 27 (44%) of RSV illness episodes. Seven identical strains were isolated in season 1, and 9 identical strains were isolated in season 3. These clusters were distinct from reference sequences, as well as viruses isolated during separate time periods in the childcare facility.

Conclusions: Our study examined the impact and spread of RSV in a multiyear community-based prospective childcare study using molecular techniques. These data demonstrate that RSV is readily

transmitted within enclosed settings, and that it is the most important respiratory viral cause of symptoms and health care utilization among children in the community.

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Section 1: Background

Respiratory syncytial virus (RSV) infection is the most important viral cause of severe respiratory tract infection (RTI) in children¹, and particularly important in young children under two years of age. In a prospective population-based cohort of children in the United States, RSV was associated with 20% of hospitalizations for acute respiratory infections during respiratory season.² Most children who develop RSV infection are previously healthy, though young children who were born prematurely and those with chronic heart and lung disease may have more severe disease³. Among hospitalized infants, higher RSV viral load has been associated with a longer hospitalization but⁴ it is unclear whether co-infection with other respiratory viruses or higher viral load has an impact on disease severity in the outpatient setting⁵⁻⁷.

Although RSV is known to be readily acquired by infants and young children, few studies have examined RSV transmission and disease severity among children in the community using molecular techniques. During epidemics, 98% of children become infected with RSV their first year of life, with frequent reinfections in their second and third year.⁸ Childcare attendance is a risk factor for RSV disease, and at least one-third of children under 5 years of age in the United States are currently enrolled in daily childcare outside the home². A landmark 16-year longitudinal study of children enrolled in childcare published in 1979 showed that RSV was the most frequently isolated viral pathogen in cases of respiratory illness, though a pathogen was isolated in only 31% of cases using culture techniques.⁸ To date, no studies characterizing the molecular epidemiology of RSV infection among children are available.

Antigenic variation is thought to be partially responsible for the ability of RSV to evade the immune response and cause high rates of re-infection⁹. The two main RSV antigenic subtypes, A and B, are further subdivided into genotypes based on variation in the glycoprotein coding sequence. Several genotypes may co-circulate simultaneously within a community during each season^{10,11} and there is a high degree of variability within genotypes. In nosocomial outbreaks, typing of viral strains show both transmission of genetically identical virus as well as presence of multiple distinct co-circulating strains.^{12,13}

We performed a prospective longitudinal cohort study in a community-based childcare setting and identified a respiratory virus by PCR in 67% of illness episodes¹⁴. In this study, we utilized a unique method of stabilizing viral specimens that did not require cold storage, enabling collection and transport from the community setting to the laboratory. Using these specimens and clinical information collected previously, we describe the impact of RSV infection in a childcare setting using modern molecular

virology techniques. We hypothesized that there is spread of unique viral strains within a childcare setting, and that RSV is associated with a greater burden of disease as compared to other respiratory viruses.

Section 2: Methods

Subjects: Children between the age of 6 weeks and 24 months attending full-time childcare in Fort Lewis, WA, were enrolled in a prospective cohort study from 2006 to 2009 as previously described¹⁴. Children with at least 2 of 5 respiratory symptoms, including cough, rhinorrhea, fever, wheezing and nasal congestion, were defined as having an illness episode and underwent a mid-turbinate nasal swab for sample collection at symptom onset and weekly thereafter until symptom resolution. In addition, a child's caregiver completed a daily symptom diary for 10 days following the illness onset. Participating facilities were within Fort Lewis, WA, and located several miles apart, subsequently designed as Facility X, Y, and Z. Children from 6 of 11 rooms in Facility X, 7 of 11 rooms in Facility Y, and 2 of 11 rooms in Facility Z were enrolled in the study as children in those rooms met the appropriate age and attendance requirements. Standard infection control procedures in this daycare were regularly reviewed and documented by daycare administration according to a standard operating procedure, with practices including daily sanitation of all toys through use of bleach, routine hand washing after toileting and diaper changes, and before and after meals, and cleaning of contaminated surfaces with bleach solution. Throughout the study, the research nurse informally noted that all reported sanitation procedures were being observed during visits to the modules¹⁵. IRB approval for the study was obtained from Seattle Children's Hospital and Madigan Army Base.

Virologic methods: Immediately after nasopharyngeal samples were collected, swabs were inserted in lysis buffer, rinsed in the transport media and discarded, and the viral lysis buffer stored at room temperature for up to 7 days prior to refrigeration and transport to the University of Washington Virology Laboratory.¹⁶ Nucleic acid extraction and testing for 12 respiratory viruses, including RSV, using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) techniques were performed as described previously¹⁷. RSV subtypes A and B were identified in RSV positive samples using a duplex real time RT-PCR assay targeting the RSV polymerase gene, which uses consensus forward and reverse primers and type specific TaqMan probes¹⁶. For sequencing of the RSV positive samples, we designed a hemi-nested PCR protocol targeting the second hypervariable region of the G-protein using a modification of a previously published protocol¹⁸. Complementary DNA was obtained using the M-MLV Reverse Transcriptase (USB) protocol, and was amplified with forward primer GAB - 5'YCAAYTTTGAAGTGTCAACTT 3' (G gene, 504–524 nt) and reverse primer FV -

5'GTTATGACACTGGTATACCAACC 3' (F gene, 186-163 nt). The reaction mixture contained 5 uL of cDNA, 2 U Velocity DNA Polymerase (Bioline), 10 uL 5X Hi-Fi Buffer, 1.25 uL of 10 uM dNTP, and 1 uL each of 20 uM forward and reverse primers for a total volume of 50 uL. The amplification procedure included 2 minutes at 98°, 35 cycles of denaturation at 98° for 30 seconds, annealing at 55° for 1 minute, and extension at 72° for 1 minute, followed by a final extension at 72° for 7 minutes. A hemi-nested PCR was then performed using M13-tagged forward primer GAB and reverse primer F1AB - 5' CAACTCCATTGTTATTTGCC 3' (F gene, 3–22 nt) using 2.5 uL of PCR product with the same reaction mixture and cycling protocol as in step 1. Both cDNA synthesis and PCR were performed with multiple negative and positive controls, and in segregated environments for pre- and post-amplification procedures. Amplified products that showed the correct size (500 base pairs) were extracted from the gel (QIAquick Gel Extraction kit, QIAGEN) and submitted for sequencing at the University of Washington DNA sequencing facility (Applied Biosystems 3730XL sequencer). To increase the sequencing yield in a subset of samples with low viral loads, the gel-excised RSV G gene PCR products were cloned using the TOPO TA kit (Invitrogen). Cloning steps included ligation, transformation of competent E. coli cells, plating and picking white colonies, making plasmid preps, and checking the plasmid inserts by restriction endonuclease digestion and gel electrophoresis. Clones with the appropriate size insert were sent for sequencing using the M13 forward and reverse primers as described above. Finally, to determine whether the strains circulating in the childcare were similar to community strains, we also sequenced samples from patients with RSV infection seen at Seattle Children's Hospital and the Seattle Cancer Care Alliance (SCCA) from 2006-2009. Nucleotide sequence editing was performed using Sequencher 4.10.1.

Phylogenetic analysis: Nucleotide sequences for RSV A & B were aligned using Seaview 4.3.3¹⁹. Phylogenetic reconstruction was performed using DIVEIN²⁰. Neighbor joining trees were drawn to scale with bars indicating 0.01 and 0.005 nucleotide substitutions for Subtypes A & B, respectively, using FigTree v1.3.1. Evolutionary distances were calculated using the maximum likelihood method, with 100 bootstrap replicates. Reference sequences from GenBank as well as community controls were included in the comparison. A Slatkin-Maddison test of compartmentalization of clades was performed to evaluate for sequence diversity²¹.

Statistical analysis: Sociodemographic, clinical, and virologic data were analyzed using Stata 10.1 (STATA Corp, College Station, Texas). Symptom duration in RSV-positive and RSV-negative illness episodes were compared using generalized estimating equations (GEEs) with a Poisson link. Comparisons of the occurrences of health care visits were performed using GEEs with a binomial link. Log transformation was performed on viral loads to centralize distributions. Univariable GEE linear regression

was used to calculate beta coefficients between RSV viral load and clinical characteristics of the illness episodes. GEEs were used to compare viral load (Gaussian link), symptom duration, and duration of positive swabs (Poisson link) in RSV illness episodes with and without another respiratory virus isolated. Cumulative incidence curves were constructed to describe the spread of RSV within individual rooms in the childcare facilities.

Section 3: Results

Clinical characteristics of study participants

Overall, 225 children were enrolled in the study over 3 seasons from February 1, 2006 to April 30, 2008, and from October 28, 2008 to June 30, 2009, and followed until age 30 months. The median age at enrollment was 9 months [range: 1-26]. The median duration of follow up was 210 days [range: 12-811]. Of the total children enrolled, 110 (49%) were male, median weekly hours of childcare attendance was 40 [range 20-60], and median number of siblings was 1 [range: 0-5]. In total, 138 (61%) children were enrolled at Facility X, 72 (32%) at Facility Y, and 15 (7%) at Facility Z. The median age at enrollment was 8 months at Facility X, 12 months at Facility Y, and 5 months at Facility Z. Gender and number of hours of childcare attendance did not differ by facility. Out of 657 total episodes recorded, 134 were asymptomatic enrollment episodes and 523 were illness episodes. Overall, 1118 nasopharyngeal samples were collected. Asymptomatic enrollment swabs were collected for 127 (56%) children, and 89 (70%) of these were positive for a respiratory virus (**Figure 1**). Illness episodes occurred in 181 (80%) children, with a median number of 3 illness episodes per child [range: 1-14]. A respiratory virus was detected by PCR in 429 illness episodes (82%). Overall, 332 (63%) episodes occurred in Facility X, 175 (28%) in Facility Y, and 16 (3%) in Facility Z.

Clinical and virologic characteristics of RSV illness episodes

RSV was detected in 62 (9%) episodes. Of these, 59 (95%) were symptomatic illness episodes. In comparing RSV-positive and RSV-negative illness episodes, RSV-positive episodes were associated with more days of fever, wheezing, rhinorrhea, and cough and an increased percentage of visits to a health care provider and childcare and parental work days missed (**Table 1**). Among RSV-positive illness episodes, the median duration of RSV detected by PCR was 1 day [range 1- 47], though the majority of children (n=47; 76%) had only one swab obtained. The median duration of symptoms was 10 days [range: 4-10]. The median initial viral load was 7.5 log₁₀ copies/mL [range: 2.6-10.0] for all RSV-positive illness episodes. (**Table 2**). For the 10 episodes where more than one swab was collected, the median decline in viral load from the first to the last positive swab was 7.5 log₁₀ copies/mL [range: 1.5-9.4].

Initial RSV viral load was not correlated with age at illness onset ($r^2=0.03$, p-value 0.22, **Figure 2**), duration of detection of RSV by PCR ($r^2<0.01$, p-value 0.73, data not shown) or number of days of symptoms ($r^2=0.01$, p-value 0.43, data not shown). Furthermore, there was no relationship between age at illness onset and number of days of symptoms ($r^2<0.01$, p-value 0.86, data not shown).

In 42 (67%) episodes, another respiratory virus was detected. The most common respiratory viral co-infections detected were bocavirus (n=28, 67%), rhinovirus (n=18, 43%), adenovirus (n=18, 43%), coronavirus (n=5, 12%), and parainfluenza type 3 (n=5, 12%). Notably, the RSV viral load was not significantly different between RSV-positive illness episodes with and without another respiratory virus identified (p-value 0.63, **Figure 3**). However, the mean duration of symptoms and of positive swabs were both increased in illness episodes with another respiratory virus present (9.0 vs. 8.5; p-value 0.02 & 4.9 vs. 1.8, p-value <0.001, respectively).

Three children in the study had a second RSV illness episode during the study. The time between the first and second episode ranged from 12-13 months, and decrease in viral load between the first and second episode ranged between 7.5-8.7 \log_{10} copies/mL. All 3 children were infected with a subtype B strain in the first episode, and a subtype A strain in the second.

RSV was detected in two sibling pairs, both of whom were in the same childcare room. In one sibling pair, RSV was detected simultaneously in both children. In the other sibling pair, RSV was detected one week apart. Sequencing results from one of the sibling pairs showed identical viral strains in both children.

RSV subtyping and molecular sequencing analysis

RSV illness episodes occurred between November to April in the 3 successive winter seasons of the study. There were 27 RSV-positive illness episodes from November 2006 to March 2007, 8 RSV-positive illness episodes from January 2008 to February 2009, and 27 RSV-positive illness episodes from November 2008 to March 2009. Overall, RSV subtype B predominated in the 2006-2007 season with 21 (78%) of 27 episodes, while RSV subtype A predominated in 2007-2008 with 7 (88%) of 8 episodes and 2008-2009 with 20 (74%) of 27 episodes (**Figure 4**).

We successfully sequenced 27 (44%) RSV-positive episodes, including 18 subtype A and 9 subtype B viral strains. Illness episodes that were sequenced were more likely to have higher viral loads (median \log_{10} copies/mL viral load: 8.3; range: 5.9-10.0) as compared to those where sequences were not obtained (median: 6.6; range: 0-9.3). A greater proportion of subtype A illness episodes [18/32 (56%)] were

sequenced as compared to subtype B illness episodes [9/29 (31%)]. We found clustering of identical viral strains during seasons and within rooms. These clusters of identical viral strains were distinct from 6 community controls, 6 reference sequences obtained from GenBank, as well as viruses isolated during separate time periods in the childcare. We sequenced samples from patients seen at Seattle Children's Hospital and the Seattle Cancer Care Alliance as community controls. Phylogenetic trees were separately constructed for subtype A and subtype B viruses (**Figures 5, 6**). There was evidence of compartmentalized clades for subtype A (p-value <0.001), but insufficient variation within subtype B strains to distinguish clades.

In Season 1, we found 4 identical subtype B strains in rooms Y11 and X3 from November 13 to December 8. At the same time, 3 identical subtype A strains were identified in rooms X3 and Y2. These two facilities were located several miles apart, making it less likely that direct inoculation from child to child was the mode of transmission. No RSV-positive sibling pairs enrolled in the study were located in two different facilities. However, sibling pairs who were not enrolled in the study or parents of children, of whom many worked on Fort Lewis may have transmitted RSV to each other and between facilities. In Season 3, 7 identical subtype A strains were detected in rooms X3, X5, and X6 from November 18 to December 5, suggesting transmission between rooms within Facility X (**Figure 6**).

We identified clusters of RSV illness episodes in two separate rooms: Y11 (Subtype B, Season 1) and X3 (Subtype A, Season 3). During both illness clusters, 50% of enrolled children in the room were infected within 6 days of the first case (**Figure 7**). In room Y11, 8 of 12 children enrolled in the study developed RSV illness over the course of 16 days. We sequenced samples from 2/11 illness episodes in Y11 and found that these two were identical strains. Eight of 13 children enrolled in room X3 had an RSV illness episode within a 16 day period. We sequenced samples from 7 of 8 illness episodes from this outbreak and found that 3 separate subtype A viral strains were co-circulating during this outbreak. A third cluster of 7 RSV illness episodes was identified in room X3 (Season 1) consisting of 2 subtype A & 5 subtype B viruses. Sequencing of 2/2 subtype A illness episodes showed identical viral strains, while sequencing of 3/5 subtype B illness episodes showed 2 different viral strains.

Section 4: Discussion

In our study, RSV was the most important respiratory virus in young children attending childcare based on symptom duration, disease severity, and visits to health care providers. Using novel methods of specimen collection and molecular sequencing, we were able to demonstrate high rates and rapid transmission of identical RSV strains in children attending the same room in the childcare setting. We

demonstrated that over 50% of children in individual classrooms became infected within 6 days of the first case during both subtype A and subtype B outbreaks. Sequencing show that more than one viral strain may circulate in a room simultaneously during outbreaks, though the clustering of identical viral strains suggests transmission within the facility as well. These findings demonstrate that routine infection control measures are insufficient to prevent transmission in childcare settings. No association was found between initial RSV viral load and age, symptom duration, PCR-positive swab duration, or presence of other respiratory viruses. We did find, however, that the presence of another respiratory virus was associated with greater duration of symptoms and increased days of RSV-positive swabs.

Our findings are consistent with national and international data showing that RSV is the main cause of hospitalization for respiratory illness in young children²² and highlights the substantial burden of RSV disease among children in the community who do not seek medical attention or require hospitalization. RSV viral load in hospitalized infants has been shown to predict duration of hospitalization²³ and studies of experimentally RSV-infected adults show that peak viral load correlates with symptom score and that peak viral load and symptoms occurred 5-7 days after inoculation with RSV²⁴. We did not find this to be the case in our study, and suggest that the criteria used to characterize disease severity in the inpatient setting may not be applicable to our outpatient population.

The role of respiratory viral co-infection remains controversial. Prior to our study, the impact of respiratory viral co-infection on RSV disease severity has only been examined in the inpatient setting. Two studies examining the presence of single versus multiple respiratory viruses in hospitalized children in Seattle, WA and in Sao Paolo, Brazil did not find increased disease severity with multiple viruses detected^{5,25}. Further, a study performed in Alaska Native children hospitalized with pneumonia did not show that presence of multiple viruses contributed to disease severity²⁶. This is in contrast to data among hospitalized infants showing that co-infection with RSV and human metapneumovirus is associated with increased disease severity as compared to RSV alone, as measured by intensive care unit admission.²⁷

Our study showed simultaneous circulation of multiple strains in an outbreak setting, as well as predominance of one viral strain. This is consistent with prior data from RSV outbreaks in the bone marrow transplant setting, where predominance of one viral strain suggests nosocomial transmission^{28,29}. Viral transmission occurs by inoculation of secretions from hands or inhalation of large particulate aerosols into the nose, and correlates directly with degree and intensity of contact with large droplets or fomites.^{30,31} It is evident that prevention of transmission in this environment may be problematic. Infection control measures such as hand-washing and sanitation of surfaces can reduce transmission, and

were utilized according to current guidelines in our childcare facilities. The results from our study, however, imply that policies to restrict symptomatic children from childcare attendance should be more heavily emphasized as a method to decrease RSV transmission.

Strengths:

In our study, we had the unique ability to use clinical and nasopharyngeal samples from a prospective 3 year study of children enrolled in childcare to characterize the impact of RSV infection in a community-based setting. We collected and transported swabs from a community setting to a molecular virology laboratory. We obtained detailed clinical and sociodemographic information from caregivers and health care providers for each illness episode, and were able to correlate these findings with the virologic characteristics of RSV illness episodes. We were also able to obtain weekly swabs until illness resolution, and to obtain asymptomatic nasopharyngeal swabs upon enrollment into the study in a subset of the study participants. Further we collected detailed information about the location of the children within the childcare facility at the time of their illness episodes, and were able to correlate this with the illness episode data to create an epidemic curve of RSV outbreaks over 3 seasons at the childcare facility. Using a molecular sequencing technique that we developed, we then identified viral strains and characterized transmission dynamics of identical viral strains over the course of 3 seasons.

Limitations:

We were limited in our ability to obtain regular respiratory virus sampling in asymptomatic participants. Children may become infected with respiratory viruses and remain totally or minimally asymptomatic. In a subset of children who had nasal swabs obtained at enrollment independent of clinical symptoms, we found a substantial minority of children had respiratory viruses detected by PCR. Many of these viruses obtained in asymptomatic children at enrollment were rhinovirus, bocavirus, and adenovirus¹⁴. However, RSV infection is less likely to be or at least to remain asymptomatic, particularly among young children experiencing their 1st or 2nd RSV infection. In addition, obtaining frequent NP swabs over months of time would likely have interfered with recruitment and retention in this study. Because we only captured data when a child became symptomatic and weekly thereafter, it is possible that we did not obtain peak viral load data. Further, we examined the duration of symptoms as opposed to daily symptom severity. The lack of relationship between age and symptom duration was unexpected in our study. Because we only captured 10 days of symptom data, we may not have captured information on children who may have been more likely to have symptoms lasting greater than 10 days. We noted that a subset of children attending childcare had continued respiratory symptoms lasting for weeks to months indicating that a time of cessation of respiratory symptoms in this population is not simple to detect. We were unable to obtain

sequencing results from RSV detected in 35 of 62 episodes. We note that our ability to sequence viruses from clinical samples, including those with relatively low levels of virus, is consistent with other studies reporting on detection and characterization of respiratory syncytial virus.²⁸

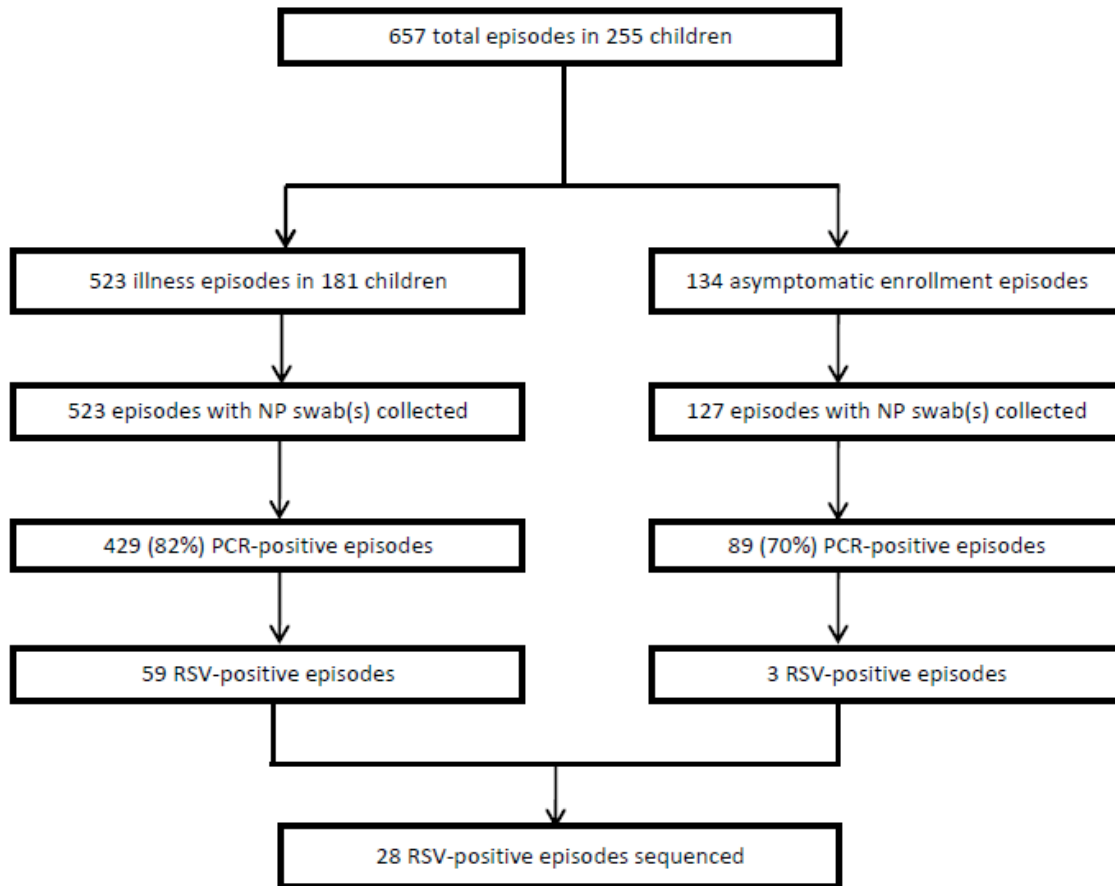
Clinical and public health implications:

In conclusion, molecular virologic techniques were used to characterize RSV infections in children attending full time childcare. We demonstrated that RSV was associated with greater duration of symptoms and more frequent health care utilization than other respiratory viruses. We also show that multiple strains circulate rapidly within a childcare setting despite use of routine infection control procedures and restriction of symptomatic children from daycare attendance.

Further studies may also be performed in this setting evaluating the role of staff in disease transmission through collection of clinical data and nasopharyngeal swabs in symptomatic childcare providers. It is also important to evaluate the impact of more stringent infection control measures on RSV disease transmission. The use of monoclonal antibody prophylaxis with palivizumab in high-risk infants should also be re-addressed to include consideration of childcare attendance as a risk factor for disease acquisition.

Finally, this study highlights the need for an RSV vaccine to prevent child-to-child transmission. Multiple vaccine candidates are in development, and childcare facilities may serve as vaccine trial sites given the high burden of RSV disease in this population, and the rapidity of RSV spread between children.

Figure 1: Flow chart of enrollment and illness episode data



*PCR: Polymerase chain reaction; NP: Nasopharyngeal

Table 1: Comparison of characteristics of illness episodes with and without RSV detected

Mean [SD] (n=523)	RSV detected	No RSV detected	p-value*
Number of illness episodes (% of total)	59 (11%)	464 (89%)	--
Age at illness (months)	11.5 [6.7]	12.2 [6.5]	0.74
Days of wheezing	2.6 [3.4]	1.1 [2.5]	<0.001
Days of fever	1.7 [1.7]	1.1 [1.7]	<0.001
Days of cough	8.1 [2.4]	6.5 [3.5]	<0.001
Days of congestion	7.5 [3.0]	6.5 [3.7]	0.003
Childcare days missed	2.6 [7.7]	1.2 [1.6]	<0.001
Work days missed	2.5 [7.7]	1.1 [1.5]	<0.001
Proportion of health care visits per illness episodes	41 [69%]	197 (43%)	<0.001

*Calculated using generalized estimating equations adjusting for multiple illnesses per child

Table 2: Virologic characteristics of 62 episodes where RSV was detected

Variable (n=62)	Median	Range
Duration of positive swabs (samples collected weekly)	1 day	1-47
Viral load	7.5 log ₁₀ copies/mL	1.5-9.4
# with coinfection	42 (68%)	
Months to 2 nd episode (n=3)	12 mos	1-13
Decline in viral load from 1 st to 2 nd episode (n=3)	8.7 log ₁₀ copies/mL	8.2-9

Figure 2: Scatterplot of initial RSV viral load by child's age at illness onset

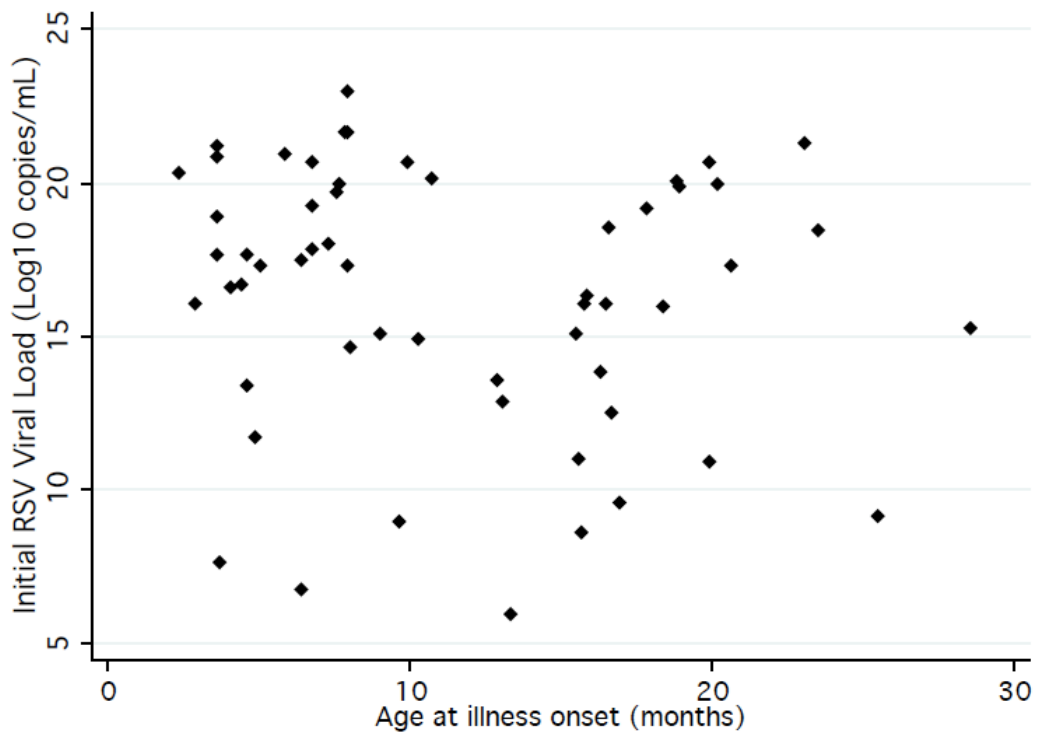


Figure 3: Scatterplot comparing initial RSV viral load in illness episode with and without another respiratory virus detected.

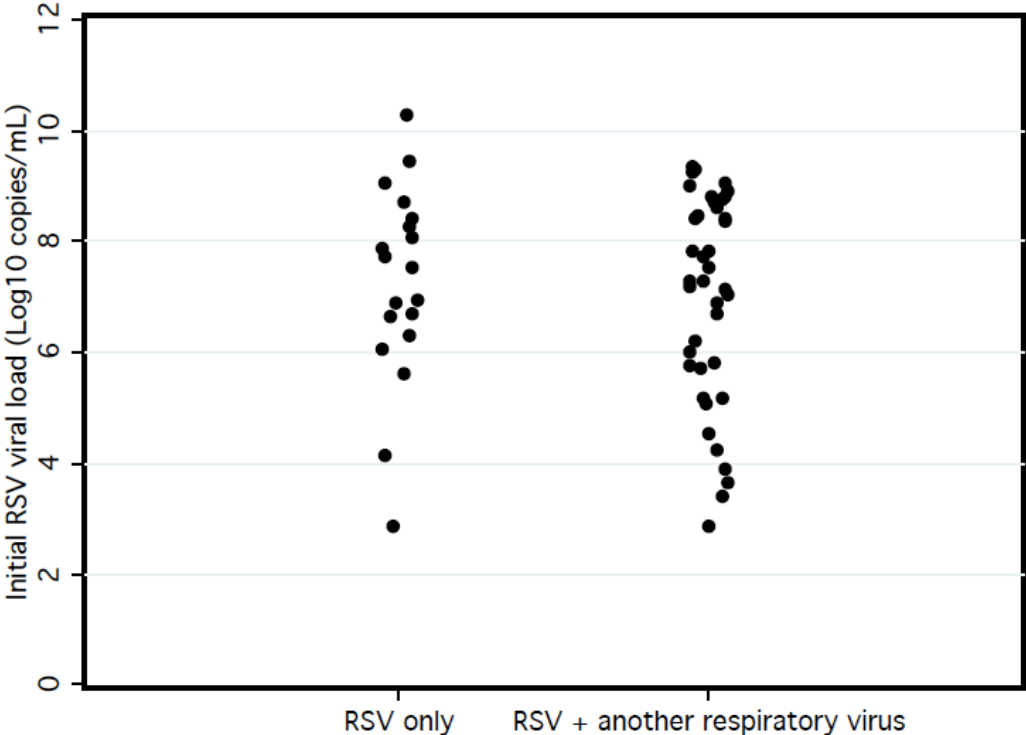


Figure 4: Epidemic curve of RSV spread within childcare rooms by subtype in Season 1 (top) and Season 3 (bottom). The y-axis is the number of illness episodes. The x-axis represents time. Each box represents one illness episode. The numbers and letters within each box represent the facility and room number of the child at the time of their illness episode.

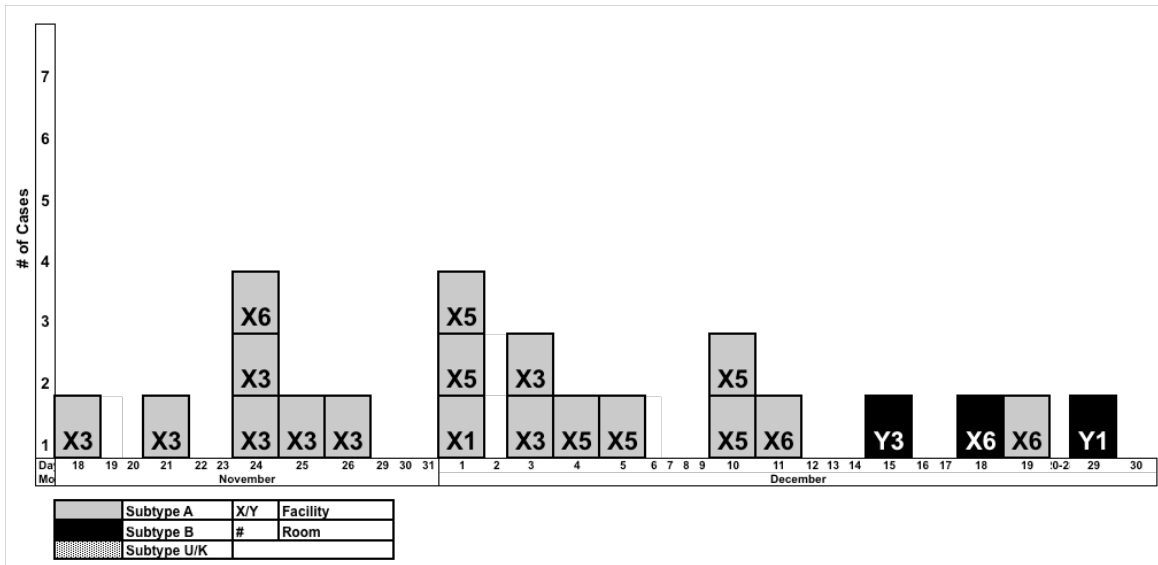
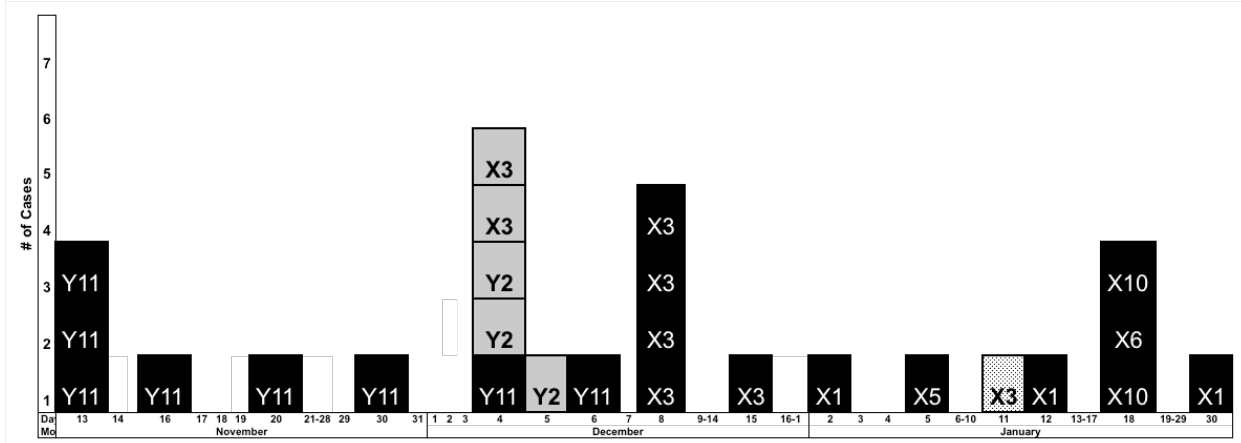


Figure 5: Phylogenetic tree constructed using 18 subtype A viral strains detected in the 3 respiratory seasons in childcare compared to reference sequences from community controls and GenBank sequences. The patient samples are identified by date followed by location and illness episode code. GenBank sequences are identified by "Ref." Community controls are identified by the year of the sample followed by "CC." The numbers at the nodes are the bootstrap values. The log likelihood value is -416.0 with bootstrap with 100 replicas.



Figure 6: Phylogenetic tree constructed using 9 subtype B viral strains detected in the 3 respiratory seasons in childcare as compared to reference sequences from community controls and GenBank sequences. The log likelihood value is -423.6.

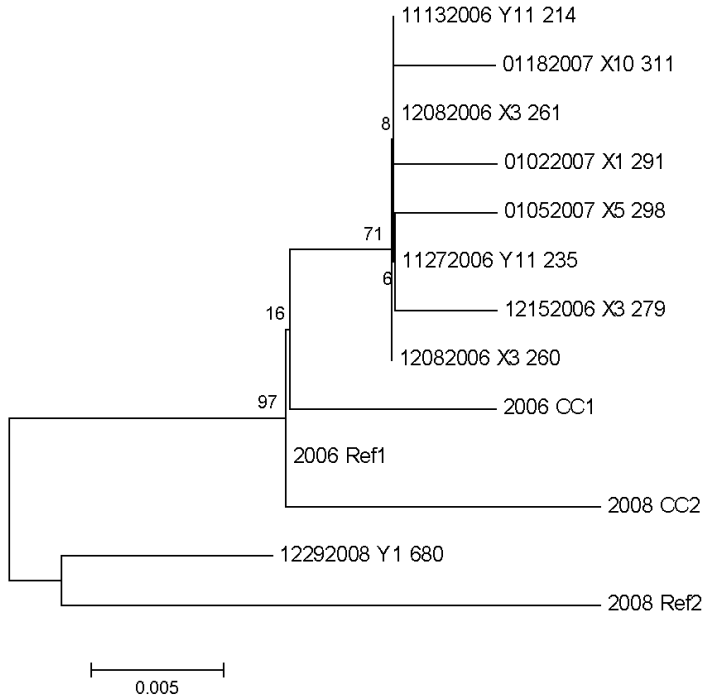
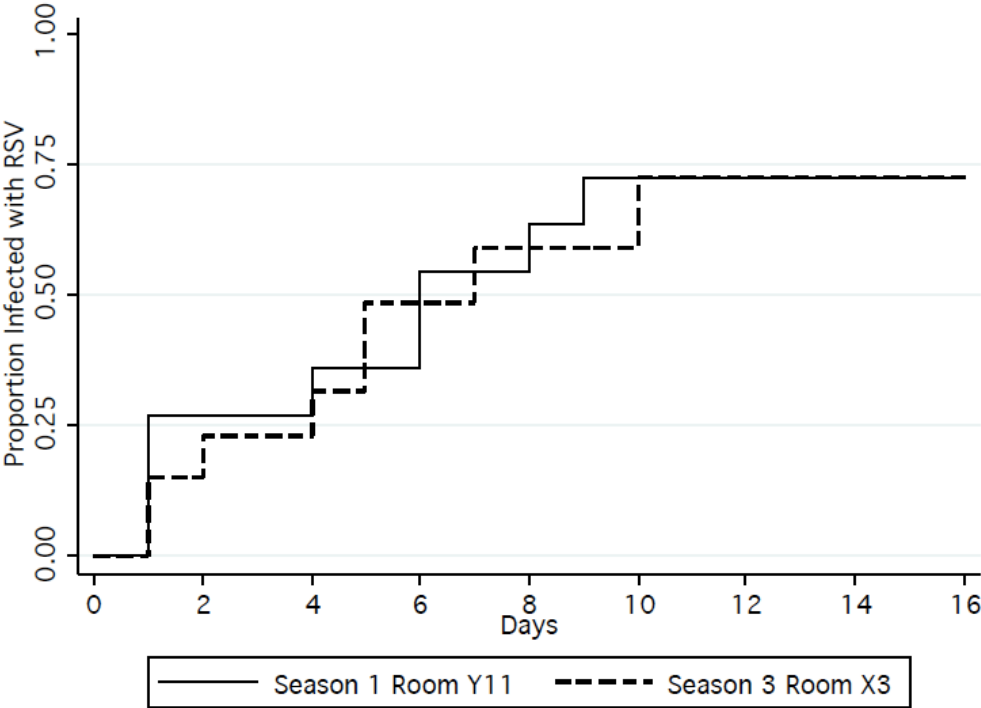


Figure 7: Cumulative incidence curves of RSV spread in two childcare rooms in Season 1 and Season 3.



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