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Journal of Clinical Virology 57 (2013) 343–350

Contents lists available at SciVerse ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Molecular epidemiology of respiratory syncytial virus transmission in childcare

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ARTICLE INFO

Article history:
Received 24 September 2012
Received in revised form 10 January 2013
Accepted 16 April 2013

Keywords:
Respiratory syncytial virus
Childcare
Molecular epidemiology
Infection control

ABSTRACT

Background: Respiratory syncytial virus (RSV) is the most important cause of serious respiratory infections in young children. No prior studies using molecular techniques to examine RSV transmission in the community childcare setting have been performed.
Objectives: We seek to characterize the molecular epidemiology of RSV transmission in childcare to evaluate the impact of RSV disease in a community-based population.
Methods: We sequenced RSV-positive nasopharyngeal samples from a prospective longitudinal study of respiratory illnesses among children enrolled in childcare during three winter seasons. Phylogenetic analysis was performed to identify unique viral strains.
Results: RSV was detected in 59 (11%) illnesses. Compared to RSV-negative illnesses, RSV-positive illnesses were associated with longer symptom duration and increased frequency of health care visits. Another respiratory virus was detected in 42 (71%) RSV-positive illnesses. RSV viral load did not differ between RSV-positive illnesses with and without another respiratory virus identified ($P = 0.38$). In two childcare rooms, 50% of the children had RSV detected within six days of the first case. Five (38%) of 13 illness episodes from one childcare room were sequenced and shown to be the same viral strain, suggesting rapid child-to-child transmission within the room over a 16 day period.
Conclusions: RSV is rapidly transmitted within childcare. Childcare facilities may serve as ideal sites for evaluation of new prevention strategies given the high burden of RSV disease in this population and the rapidity of RSV spread between children.

1. Background

Respiratory syncytial virus (RSV) infection is the most important viral cause of severe respiratory tract infection in young children.[1] In the first year of life, 98% of children become infected with RSV, with frequent reinfections in their second and third years.[2,3] The vast proportion of children with RSV are not hospitalized. The burden of RSV disease in the community is substantial, though molecular epidemiology of transmission is not well-characterized.[4–7] In the United States, 41% of children attend childcare,[8] which is a known risk factor for severe RSV disease, and likely serves as an important route of transmission to other attendees and family members.[9,10] The socioeconomic impact of illness associated with childcare attendance includes work days missed and parental stress.[11] A 16-year longitudinal study of childcare attendees in the 1980s using culture techniques showed that RSV was the most frequent viral etiology of respiratory illness.[2,12]

The molecular epidemiology of RSV transmission is characterized by sequencing the hypervariable region of the glycoprotein (G) coding region.[13] Transmission of genetically identical virus as well as the presence of multiple distinct co-circulating strains have been described in nosocomial settings.[14,15] No studies have been performed to characterize RSV disease burden in a community childcare setting using current molecular diagnostics despite its importance as a potential setting for public health interventions, such as vaccines and infection control measures.

Abbreviations: RSV, respiratory syncytial virus; RT-PCR, reverse transcriptase polymerase chain reaction; RTI, respiratory tract infection; NP, nasopharyngeal; GEE, generalized estimating equations.

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http://dx.doi.org/10.1016/j.jcv.2013.04.011

2. Objectives

The goal of our study was to characterize the transmission and clinical impact of RSV in childcare. We hypothesized that the spread of unique viral strains within a childcare setting would be rapid. Rapid transmission was defined as detection of an identical viral strain within a one-week period within the childcare facilities. We further hypothesized that RSV infections in childcare attendees would be associated with increased symptom duration as compared to infections with other respiratory viruses.

3. Methods

3.1. Subjects

Children age 4 weeks–30 months attending full-time childcare on Fort Lewis Army Base, Fort Lewis, Washington, USA were enrolled in a prospective cohort study from February 1, 2006 to April 30, 2008, and from October 28, 2008 to June 30, 2009.[16,17] Informed consent was obtained from caregivers. Children were followed for the presence of respiratory symptoms, and the study nurse was alerted at symptom onset by childcare staff or caregivers. An illness episode was defined as at least 2 of 5 symptoms, including cough, rhinorrhea, fever, wheezing and nasal congestion. Each child had a nasopharyngeal swab collected at symptom onset and weekly thereafter until symptom resolution. A daily symptom diary was completed by the caregiver for 10 days following symptom onset. From February 1, 2006 to May 1, 2006, nasopharyngeal samples were collected only with illness episodes. In children enrolled after May 1, 2006, a sample was also collected at time of enrollment regardless of symptom status.

Childcare centers designated as Facilities X, Y, and Z were located several kilometers apart. Children were enrolled from rooms where full-time care was provided, defined as >20 h/week. These included 6 of 11 rooms in Facility X, 7 of 11 rooms in Facility Y, and 2 of 11 rooms in Facility Z. Infection control procedures were performed and documented according to a standard operating procedure, including daily bleach disinfection of all toys, routine hand washing after toileting and diaper changes and before and after meals, and surface decontamination with bleach.[18] IRB approval was obtained from Seattle Children's Hospital and Madigan Army Medical Center.

3.2. Virologic methods

Nasopharyngeal samples were tested for 13 respiratory viruses by real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay, including RSV, human metapneumovirus, influenza viruses A and B, parainfluenza virus 1–4, adenovirus, human coronavirus Group 1 (229E, N6L 63) and Group 2 (OC43 and HKU1), rhinovirus, and human bocavirus. Subtyping was performed for RSV-positive samples according to previously published protocols.[19,20] RSV-positive samples were sequenced using a hemi-nested PCR protocol targeting the second hypervariable region of the glycoprotein coding region.[21] Complementary DNA (M-MLV Reverse Transcriptase, USB Corp, Cleveland, OH) was amplified with forward primer GAB (5′-YCAYTTTGAAGTGTTCAACTT-3′; G gene, 504–524 nt) and reverse primer FV (5′-GTTATGACACTGGTATACCAACC-3′; F gene, 186–163 nt). The amplification procedure included 2 min at 98 °C, 35 cycles of 98 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 7 min. 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) and 2.5 μL of PCR product with the same protocol as in step 1. Five hundred base pair amplicons were gel-extracted (QIAquick Gel Extraction kit, QIAGEN, Germany) and sequenced (Applied Biosystems 3730XL sequencer). PCR products from some samples were cloned (TOPO TA Cloning Kit, Invitrogen, Grand Island, NY) prior to sequencing. RSV-positive community samples during the same respiratory seasons were sequenced to serve as controls.

3.3. Statistical analysis

Sociodemographic, clinical, and virologic data were analyzed using Stata 10.1 (STATA Corp, College Station, TX). Generalized estimating equations (GEEs) to account for multiple illness episodes per child were used to compare symptom duration (using identity link) and health care visit frequency (using Poisson link) in RSV-positive and RSV-negative illnesses and viral load, symptom duration, and positive sample duration in RSV illnesses with and without another respiratory virus detected. Univariable GEE (using linear link) was used to estimate the influence of clinical characteristics on RSV viral load. Cumulative incidence curves were constructed to describe RSV spread within individual childcare rooms. Rapid transmission was defined as the presence of an identical viral strain within the childcare facility within one week.[16,22]

Nucleotide sequences corresponding to a 205 and 305 base pair region of the G glycoprotein coding region for subtypes A and B, corresponding to nucleotides 5373–5577 for subtype A (GenBank GU591768.1) and nucleotides 5406–5565 for subtype B (GenBank NC_001781.1). These were aligned using Seaview 4.3.[23] Phylogenetic reconstruction was performed using PhyML 3.0 within DIVEIN.[24,25] Neighbor joining trees were drawn using FigTree v1.3.1 using the maximum likelihood method with 100 bootstrap replicates.[26] A Slatkin-Maddison test of compartmentalization of clades was performed to evaluate for sequence diversity.[27] Nucleotide sequences were submitted to GenBank (JX398254–JX398285).

4. Results

4.1. Clinical characteristics

Overall, 255 children were enrolled in the study; 110 (49%) were male. The median age at enrollment was 9 months (range, 1–26 months); median weekly hours of childcare attendance was 40 (range, 20–60 h), median number of siblings was 1 (range, 0–5 siblings), and the median duration of follow up was 210 days (range, 12–811 days). In total, 138 (61%), 72 (32%), and 15 (7%) children were enrolled at Facility X, Y, and Z, respectively. The median age at enrollment was 8, 12, and 5 months at Facility X, Y, Z, respectively. Gender and number of hours of childcare attendance did not differ by facility. Overall, 523 illness episodes occurred in 181 (80%) children with 332 (63%) episodes in Facility X, 175 (28%) in Facility Y, and 16 (3%) in Facility Z (Fig. 1). The median number of illness episodes per child was 3 (range, 1–14). A respiratory virus was detected in 429 (82%) of 523 illnesses. Asymptomatic enrollment samples were collected for 127 (56%) children, and 89 (70%) of these were positive for a respiratory virus, with the most common viruses detected being rhinovirus ($n = 57$; 45%), bocavirus ($n = 26$; 20%), and adenovirus ($n = 18$; 14%).

4.2. Clinical and virologic characteristics of RSV illness episodes

RSV was detected in 59 (11%) of the 523 illness episodes, and 3 (2%) asymptomatic enrollment samples. Of the 59 illness episodes, 35 (59%) occurred in children under one year. Compared to the 464 RSV-negative episodes (Table 1), RSV-positive episodes were associated with more days of wheezing ($P < 0.001$), fever ($P < 0.001$), cough ($P < 0.001$), congestion ($P = 0.003$), and an increased percentage of visits to a health care provider ($P < 0.001$) and childcare

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

523 illness episodes	No RSV detected (n = 464) (89%)[a]	RSV detected (n = 59) (11%)[a]	p-Value[b]	RSV-only detected (n = 12)	p-Value[c]
Age at illness (months)	12.2 [6.5]	11.5 [6.7]	0.74	13.0 [7.5]	0.03
Days of wheezing	1.1 [2.5]	2.6 [3.4]	<0.001	2.4 [3.1]	<0.001
Days of fever	1.1 [1.7]	1.7 [1.7]	<0.001	2.9 [1.9]	<0.001
Days of cough	6.5 [3.5]	8.1 [2.4]	<0.001	8.0 [2.1]	0.03
Days of congestion	6.5 [3.7]	7.5 [3.0]	0.003	7.4 [2.8]	0.06
Childcare days missed	1.2 [1.6]	2.6 [7.7]	<0.001	1.5 [1.3]	0.21
Work days missed	1.1 [1.5]	2.5 [7.7]	<0.001	1.1 [0.9]	0.80
Proportion of health care visits per illness episodes [n (%)]	197 (43%)	41 (69%)	<0.001	14 (82%)	<0.001

[a] Numbers are mean [SD] values unless otherwise specified.
[b] Calculated using generalized estimating equations adjusting for multiple illnesses per child; compares RSV-positive and RSV-negative illness episodes.
[c] Calculated using generalized estimating equations adjusting for multiple illnesses per child; compares illness episodes with only RSV detected and RSV-negative illness episodes.

(P < 0.001) and parental work days missed (P < 0.001). The median duration of symptoms was 10 days (range, 4–10 days). The median initial viral load was 7.5 $\log_{10}$ copies/mL (range, 2.6–10.0). Initial RSV viral load was not correlated with age at illness onset (r^2 = 0.03; P = 0.22; data not shown), duration of detection of RSV (r^2 < 0.01; P = 0.73; data not shown) or number of days of symptoms (r^2 = 0.01; P = 0.43; data not shown). There was no relationship between age at illness onset and number of days of symptoms (r^2 < 0.01; P = 0.86; data not shown).

In 42 (71%) RSV-positive episodes, at least one other respiratory virus was detected. The most common were bocavirus (n = 28; 67%), rhinovirus (n = 18; 43%), adenovirus (n = 18; 43%), coronavirus (n = 5; 12%), and parainfluenza type 3 (n = 5; 12%). Duration of symptoms was not significantly different between illness episodes with and without another respiratory virus, though numbers of childcare and work days missed were increased (Table 2). The RSV viral load was not significantly different between RSV-positive episodes with and without another respiratory virus identified (16.1 vs. 17.1 $\log_{10}$ copies/mL; P = 0.38), though duration of detection of RSV (4.9 vs. 1.8 days; P < 0.001) was increased.

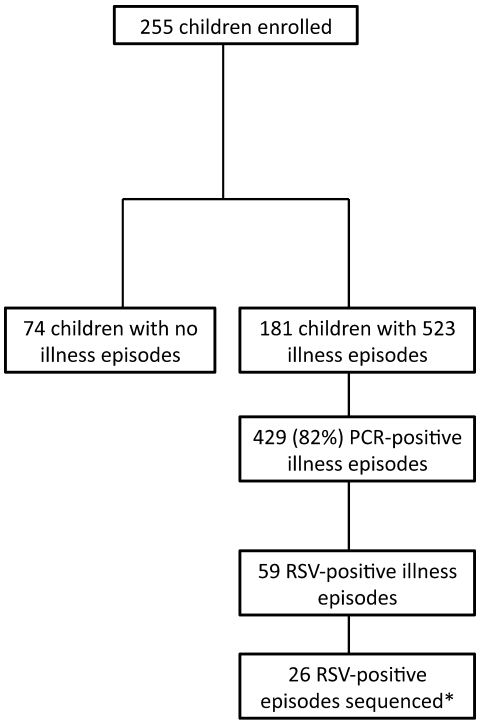

Fig. 1. Flow chart of illness episodes.

Three children had a second illness episode, with a median of 12 months [range, 1–13 months] between illnesss episodes and a median decline in viral load of 8.7 $\log_{10}$ copies/mL [range, 8.2–9.0]. Duration of symptoms was a median of 8 days [range, 5–10 days] for the first illness episode, and 7 days [range, 4–10 days] for the 2nd illness episode.

4.3. Molecular epidemiology of RSV episodes

RSV episodes occurred from November to April in the three successive winter seasons of the study. RSV subtype B predominated in Season 1 with 21 (78%) of 27 episodes, while RSV subtype A predominated in Seasons 2 and 3 with 7 (88%) of 8 episodes and 20 (74%) of 27 episodes, respectively [Fig. 2A and B (1, 8, and 6 episodes in Seasons 1, 2, and 3, respectively, were not included due to occurrence outside of the time interval illustrated)]. We sequenced samples from 25 (42%) of 59 illness episodes as well as one nasopharyngeal sample collected from an asymptomatic child at enrollment. Episodes that were sequenced were more likely to have higher viral loads (P < 0.001), but did not differ by date of collection (P = 0.71), implying that the inability to sequence a subset of samples was due to limited template rather than sample degradation. Clusters of identical RSV strains were detected in childcare samples from the same seasons and within rooms, which were distinct from six community controls, eight reference sequences obtained from GenBank, and viruses isolated during different time periods in the same childcare facilities. Phylogenetic trees constructed for subtype A (Fig. 3) and subtype B viruses (Fig. 4) showed evidence of compartmentalized clades for subtype A (P < 0.001), but insufficient variation within subtype B strains to distinguish clades.

4.4. Classroom-level transmission

We identified clusters of RSV episodes in rooms Y11 (Subtype B, Season 1) and X3 (Subtype A, Season 3). During both clusters, 50% of enrolled children in each room were infected within six days of the first case (Fig. 5). In total, 8 of 12 children in Y11 and 8 of 13 children in X3 developed RSV over a 16-day period. Samples from two episodes 14 days apart in Y11 contained the same RSV strain, as did 5 episodes in X3 that spanned the entire 16-day period. These results suggest that child-to-child transmission of one viral strain was likely responsible for these clusters of RSV illness. A third cluster of seven RSV episodes was identified in room X3 during Season 1, consisting of two subtype A and five subtype B viruses. Sequencing of the two subtype A episodes showed identical viral strains, while sequencing of three subtype B episodes showed two different viral strains, indicating that multiple viral strains co-circulated simultaneously in the childcare room (Fig. 6).

Table 2
Comparison of clinical and virologic characteristics of RSV-positive illness episodes with and without another respiratory virus detected.

59 illness episodes	RSV only (n = 17) (29%)[a]	RSV and another respiratory virus (n = 42) (71%)[a]	p-Value[b]
Age at illness (months)	13.0 [7.5]	10.7 [6.1]	0.34
Days of wheezing	2.4 [3.1]	2.7 [3.5]	0.03
Days of fever	2.1 [1.9]	1.6 [1.6]	0.16
Days of cough	8.0 [2.1]	8.2 [2.5]	0.87
Days of congestion	7.4 [2.8]	7.6 [3.1]	0.93
Childcare days missed	1.5 [1.3]	3.1 [9.1]	<0.001
Work days missed	1.1 [0.9]	3.0 [9.1]	<0.001
Proportion of health care visits per illness episodes [n (%)]	14 (82%)	27 (64%)	0.20
Viral load ($\log_{10}$ copies/mL)	17.1 (0.8)	16.1 (0.7)	0.38
Days of RSV detection	1.8 (0.6)	4.9 (1.3)	<0.001

[a] Numbers are mean [SD] values unless otherwise specified.
[b] Calculated using generalized estimating equations adjusting for multiple illnesses per child.

4.5. Facility-level transmission

In Season 1, we identified four subtype B episodes with identical strains in rooms Y11 and X3 from November 13 to December 8. At the same time, three subtype A episodes with identical strains were identified in rooms X3 and Y2. These two facilities were located several kilometers 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 different facilities. However, sibling pairs who were not enrolled in the study or parents, many of whom worked on Fort Lewis, may also have transmitted RSV. In Season 3, eight subtype A episodes with

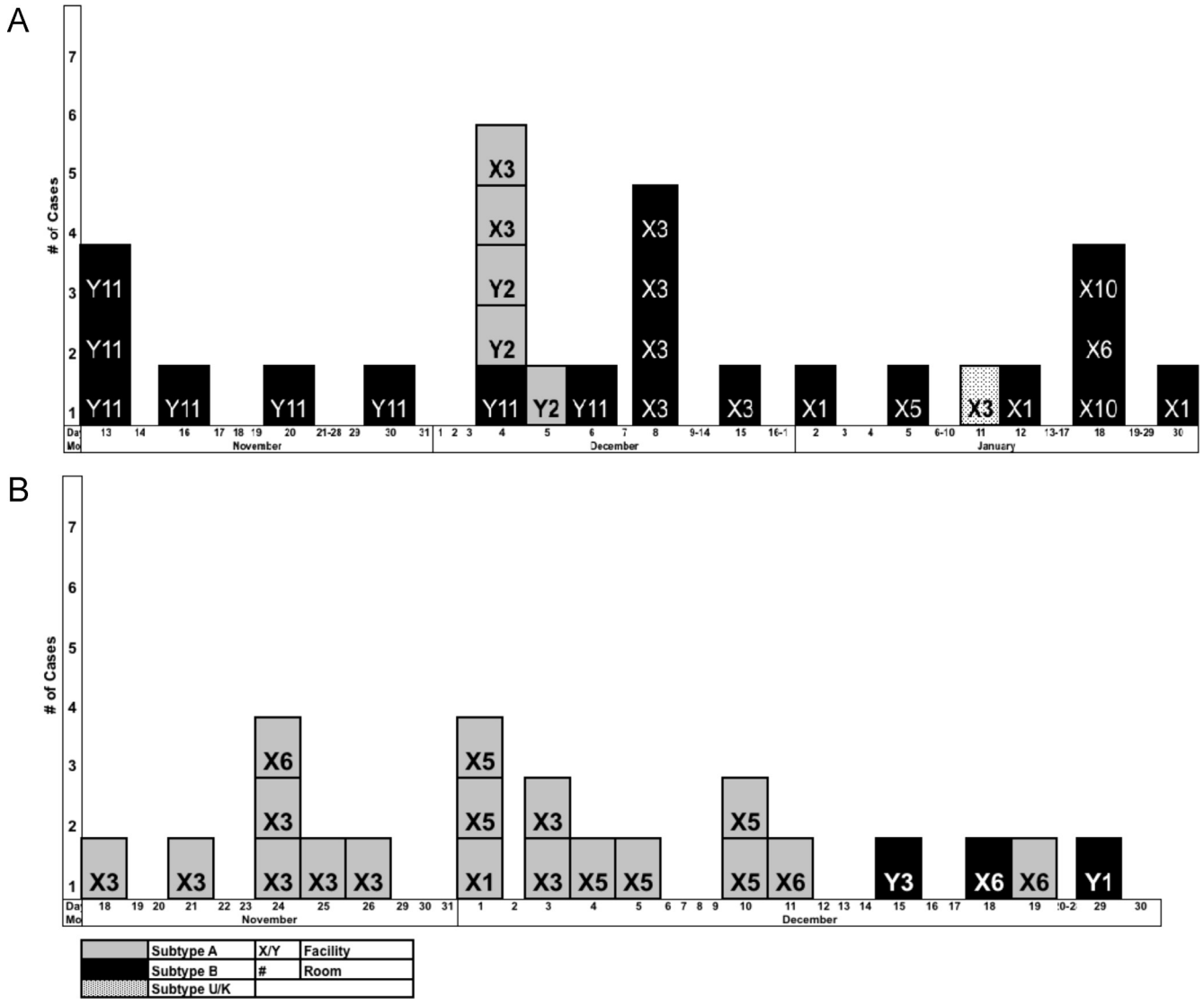

Fig. 2. Epidemic curve of RSV spread within childcare rooms by subtype in Season 1 (2A) and Season 3 (2B) from November to January. The y-axis is the number of episodes. The x-axis represents time. Each box represents one episode. The numbers and letters within each box represent the facility and room number of the child. One, eight, and six episodes in seasons 1, 2, and 3, respectively, were not included due to occurrence outside of the time period shown.

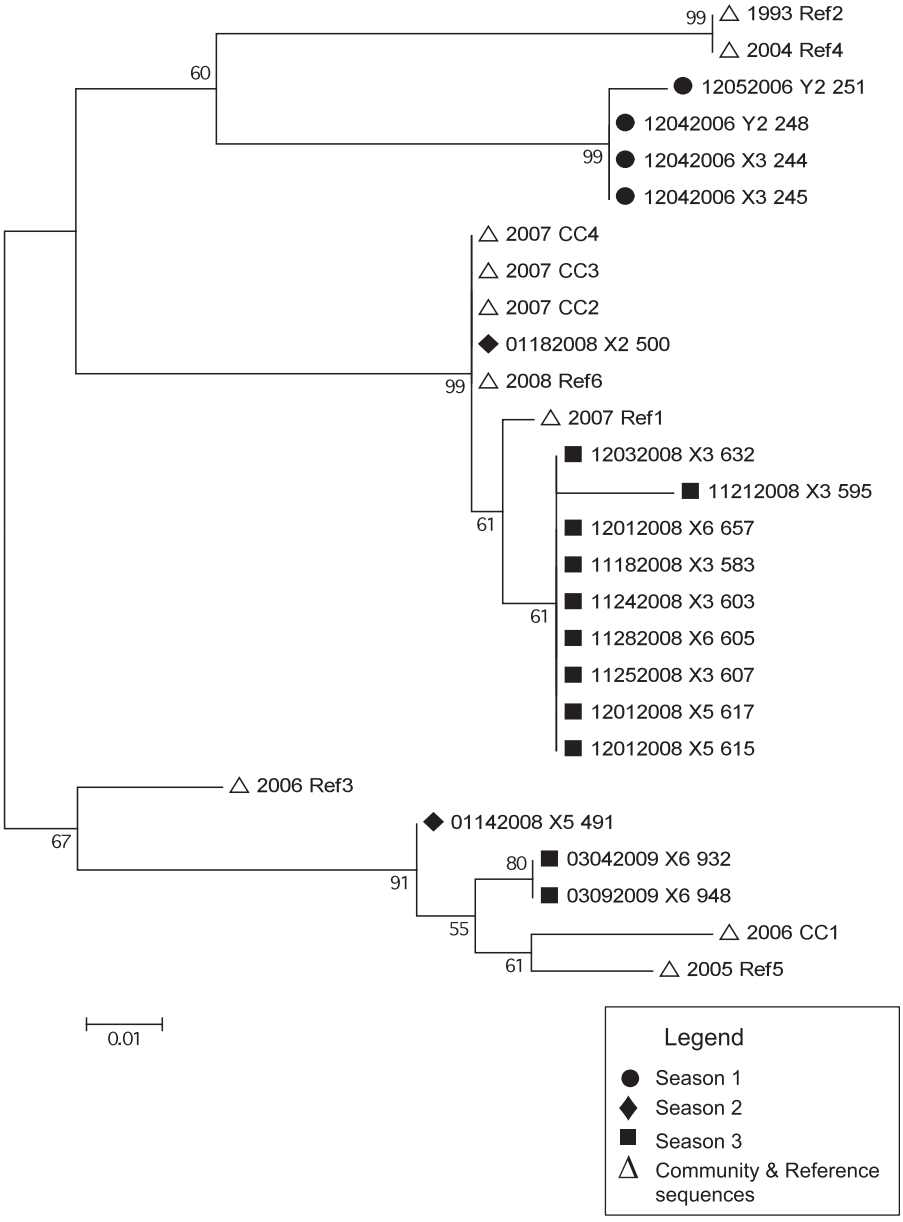

Fig. 3. Phylogenetic trees were constructed using 17 subtype A viral strains detected in 3 seasons compared to community controls and GenBank sequences. The samples are identified by date followed by location and patient identifier. GenBank sequences are identified by year of sample followed by "Ref." 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 100 bootstrap replicates.

identical strains were detected in Facility X in rooms X3, X5, and X6 from November 18 to December 5, suggesting transmission both within and between rooms within Facility X during Season 3, with temporal and geographic clustering of two additional clusters of identical strains in December and March (Fig. 6).

RSV was also detected in two sibling pairs, both in the same childcare room. In one sibling pair, RSV was detected simultaneously in both children. Sequencing results from this pair showed identical viral strains in both children. In the other sibling pair, RSV was detected one week apart, though these samples were not sequenced.

5. Discussion

Our study is the first to describe the molecular epidemiology of RSV transmission in childcare, a potential target of infection control interventions in the community. Among childcare attendees in our study, RSV-positive illness episodes were associated with greater symptom duration, number of parental work and childcare days missed, and visits to health care providers than illness episodes without RSV detected. Using molecular sequencing, we documented high rates of infection and transmission of identical RSV strains in childcare. Within one week of the index case, we detected an identical viral strain in another child in the same facility. This same strain was detected in 7 additional children in the facility within a 16-day period.

RSV viral load in hospitalized infants may predict duration of hospitalization and studies of experimentally RSV-infected adults show that peak viral load correlates with symptom score.[28,29] We did not find a relationship between viral load and symptom duration. The criteria used to characterize disease severity in the inpatient setting may not be applicable to our outpatient population, or we may have captured both primary and secondary RSV cases, potentially impacting viral load.

The significance of respiratory viral co-infection remains controversial. We found no relationship between co-infection and RSV

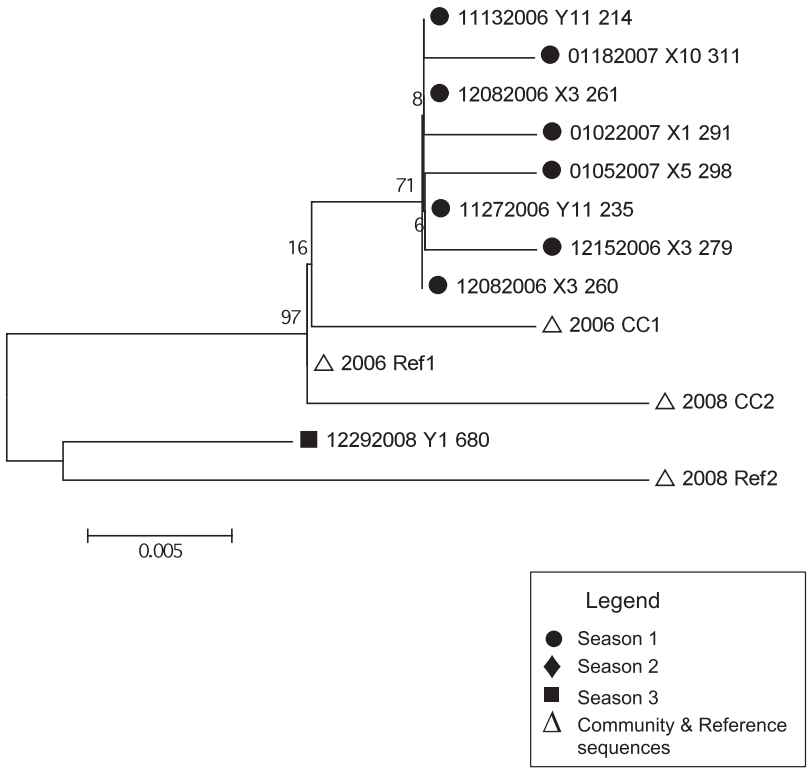

Fig. 4. Phylogenetic trees were constructed using 9 subtype B viral strains detected in 3 seasons compared to community controls and GenBank sequences. The samples are identified by date followed by location and patient identifier. GenBank sequences are identified by year of sample followed by "Ref." 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 −423.6 with 100 bootstrap replicates.

viral load or symptom duration. Four prior studies did not find increased RSV disease severity among hospitalized children when multiple viruses were detected.[30–33] This is in contrast to a study showing that co-infection with RSV and human metapneumovirus was associated with increased disease severity compared to RSV alone, as measured by intensive care unit admission.[34] No children were co-infected with RSV and human metapneumovirus in our study.

We documented simultaneous circulation of multiple RSV strains in an outbreak setting with predominance of one viral strain within a 16-day period among 9 childcare attendees in the same facility. We showed that within one week of the first case, another child had an identical strain detected, suggesting rapid child-to-child transmission within the facility. Further, we demonstrated that once RSV was detected in a childcare room, over 50% of children in the room became infected within a week. While there was clustering of identical viral strains within rooms and facilities, we also found that several unique viral strains circulated in a room simultaneously during an outbreak. This is consistent with data from outbreaks in bone marrow transplant settings,[35,36] but has not been previously investigated in the childcare setting, an important source of community-acquired respiratory viral infections. Viral transmission correlates directly with degree and intensity of hand or nasal inoculation with large droplets or fomites,[37,38] activities which are likely to be frequent within childcare. Although infection control measures including handwashing and sanitation of surfaces were utilized according to current guidelines in our facilities,[39–41] our results imply that RSV is readily transmitted in childcare facilities despite these routine measures.

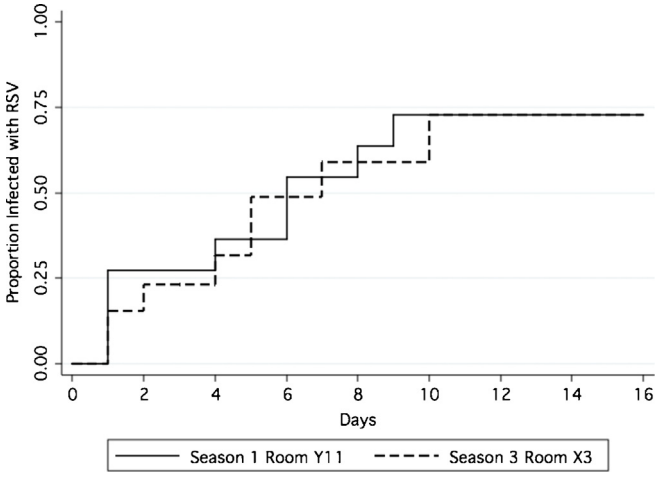

Fig. 5. Cumulative incidence curves of RSV spread in two childcare rooms in Season 1 and Season 3.

This study has some limitations. Children may shed and transmit RSV after they become asymptomatic, and we did not perform regular sampling in asymptomatic participants.[42] Although we captured ten days of symptom data, younger children may experience symptoms lasting longer. Further, we were not able to use a preceding asymptomatic period as criteria for a new illness episode. Many children enrolled in our childcare facilities were never symptom-free, particularly when considering cough and rhinorrhea. Each new illness episode that was close in timing to a prior illness episode was reviewed by the study staff and a consensus decision was made to decide whether this should be classified as a new episode versus a continuation of a prior illness. In general, children who developed new symptoms, as opposed to worsening of previous symptoms, were classified as having a new illness episode. Finally, we sequenced RSV from only 42% of episodes, though our ability to sequence viruses from clinical samples is consistent with other studies characterizing RSV transmission.[35]

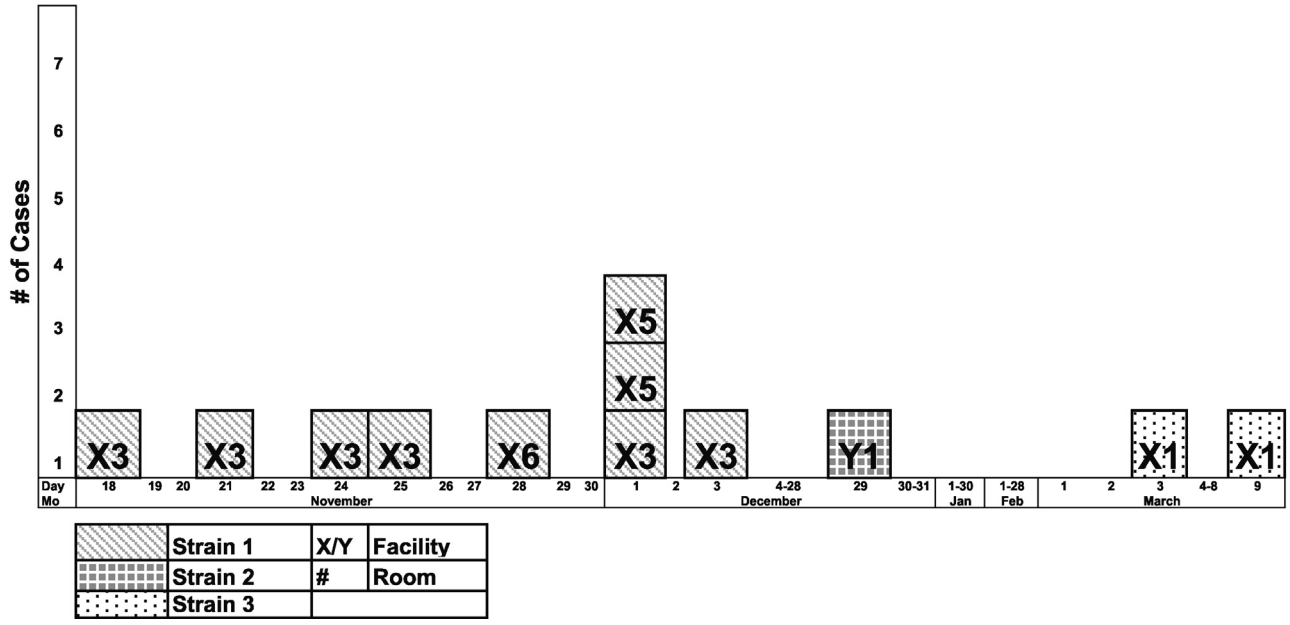

Fig. 6. Epidemic curve of RSV spread within childcare rooms by strain for the 12 sequenced samples from November to March in Season 3. The *y*-axis is the number of episodes. The *x*-axis represents time. Each box represents one episode. The numbers and letters within each box represent the facility and room number of the child. The pattern of the box corresponds to the viral strain. Three unique viral strains were detected during Season 3.

Our study shows that the childcare setting is a significant source of rapid child-to-child RSV transmission, and that once acquired, RSV leads to significantly greater symptom duration than other respiratory viruses. These results highlight the significant clinical and socioeconomic impact of RSV in the community, and emphasize that the childcare setting is an important potential site of interventions to reduce the burden of RSV disease. Interventions that reduce RSV transmission in childcare, such as vaccines and enhanced infection control measures, are likely to have a substantial clinical and socioeconomic impact.

Funding

This study was supported by the National Institutes of Health [T32 5T32AI007044-35 (HYC) & K24 AI071113 (AW)] and an investigator-initiated grant from MedImmune, Inc. (JAE).

Competing interest

JAE has received research support from MedImmune, Inc. and Novartis, and serves as a consultant for GlaxoSmithKline and Novavax. All other authors declare no conflicts of interest.

Ethical approval

IRB approval was obtained from Seattle Children's Hospital and Madigan Army Medical Center.

Authors' contributions

HYC and JAE jointly conceived the study. HYC, CR, and JK designed and performed the laboratory portion. HYC, MS, and AM performed the statistical analysis. HYC wrote the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

Prior presentation of results

The findings described here are unpublished but were presented in part as a poster abstract at the Clinical Virology Symposium (Daytona Beach, FL, April 21–25, 2012) and the 2007 RSV International Symposium (Marcos Island, FL, October 27, 2007).

Acknowledgments

The phylogenetic analysis for this study was performed with assistance from the University of Washington Center for AIDS Research (CFAR), an NIH funded program (P30 AI027757), which is supported by the following NIH Institutes and Centers (NIAID, NCI, NIMH, NIDA, NICHD, NHLBI, NIA).

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