

# Nasopharyngeal Pneumococcal Density and Evolution of Acute Respiratory Illnesses in Young Children, Peru, 2009–2011

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We examined nasopharyngeal pneumococcal colonization density patterns surrounding acute respiratory illnesses (ARI) in young children in Peru. Pneumococcal densities were dynamic, gradually increasing leading up to an ARI, peaking during the ARI, and decreasing after the ARI. Rhinovirus co-infection was associated with higher pneumococcal densities.

*Streptococcus pneumoniae* commonly colonizes the nasopharynx of young children (1). Nasopharyngeal colonization density is relevant for transmission of bacteria and pathogenesis of pneumococcal diseases (2). Few studies have evaluated the longitudinal relationship between nasopharyngeal pneumococcal density and acute respiratory illnesses (ARIs). We examined the evolution of nasopharyngeal pneumococcal density surrounding ARIs in young children.

## The Study

We performed sequential cross-sectional assessments from a prospective cohort study of Andean children in Peru (3). During 2009–2011, children <3 years of age from the District of San Marcos, Cajamarca, Peru, were assessed for ARIs during weekly household visits. The population was rural and had low incomes and limited access to healthcare (3,4). Use of 7-valent pneumococcal conjugate vaccine (PCV7) started in late 2009. Institutional review boards of Vanderbilt University (Nashville, TN, USA) and the Instituto de Investigación Nutricional (Lima, Peru) approved the study.

An ARI episode was defined as the length of time a child had cough or fever (5,6). If a child was ill during a household visit, we assessed for pneumonia or lower

respiratory tract infection using IMCI-WHO (Integrated Management of Childhood Illness–World Health Organization) criteria (5,7). If the child had an ARI during the preceding 7 days, we collected a nasal swab sample and tested it for respiratory viruses by reverse transcription PCR at Vanderbilt University (6,8–11). Nasopharyngeal swab samples were collected monthly without regard to ARI and tested at Emory University (Atlanta, GA, USA) by using quantitative PCR for pneumococcal density determinations. For this study, we used samples collected in 2009 and 2011, representing periods before and after routine PCV7 use (12) (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/22/11/16-0902-Techapp1.pdf>).

Nasopharyngeal samples were classified according to their collection time surrounding ARIs: peri-ARI periods included pre-ARI (8–14 or 1–7 days before an ARI) and post-ARI (1–7 or 8–14 days after an ARI). Samples outside these periods were considered non-ARI samples. We compared log-transformed pneumococcal nasopharyngeal densities of samples from ARI, peri-ARI, and non-ARI periods by using multivariable quantile regression with robust SEs and adjusting for relevant covariates.

In secondary analyses, we assessed the role of respiratory viruses on pneumococcal density in children with ARIs. Because detection of nonrhinovirus respiratory viruses in nasal swabs was infrequent, we grouped samples into 4 distinct groups: rhinovirus only, rhinovirus and other viruses, other viruses only, and negative for any viruses.

We examined the role of pneumococcal acquisition on pneumococcal density using pneumococci-positive nasopharyngeal samples from children who had a sample collected within the preceding 60 days. Samples were categorized as 1) new colonization if the prior sample was negative, 2) serotype persistence if the prior sample was the same serotype, and 3) serotype replacement if the prior sample was a different serotype. If either serotype was nontypeable or unknown, the pattern was considered undetermined.

We assessed 3,579 nasopharyngeal samples from 833 children: 450 (12.6%) were collected during ARIs, 956 (26.7%) during peri-ARI periods, and 2,173 (57.8%) during non-ARI periods. The median age was 1.39 years. The median duration for ARIs was 8 days (interquartile range [IQR] 5–13 days). According to IMCI-WHO criteria, 33 samples were associated with pneumonia or severe pneumonia (13) (Table).

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**Table.** Demographic characteristics for children from whom nasopharyngeal swab samples were collected during different periods surrounding ARIs, Peru, 2009–2011\*

Characteristic	Period of sample collection						Total, N = 3,579
	Non-ARI, n = 2,173	Pre-ARI		Current ARI, n = 450	Post-ARI		
		8–14 days, n = 211	1–7 days, n = 222		1–7 days, n = 332	8–14 days, n = 191	
No. children†	765	186	189	320	262	172	833
<b>Demographics</b>							
Median age, y, at sample collection	1.45	1.36	1.37	1.23	1.25	1.37	1.39
Male	50.5	56.4	52.3	52.9	49.4	48.2	51.1
Attend daycare equivalent	7.8	7.1	8.6	4.2	6.6	5.2	7.1
<b>Patient's home</b>							
Traditional stoves for cooking	63.1	73.5	63.5	61.1	68.7	62.8	64.0
Running water	24.7	17.5	17.6	24.0	18.7	27.2	23.3
Sewer or septic tank	21.1	18.5	18.5	20.4	22.3	22.5	20.9
Electricity	42.3	34.6	37.4	38.7	40.7	41.9	40.9
<b>Season and year at sample collection</b>							
Fall 2009	4.4	8.1	6.8	9.8	6.0	6.8	5.7
Winter 2009	19.3	25.1	27.5	30.0	23.2	25.7	22.2
Spring 2009	23.0	19.0	17.1	19.3	23.2	19.9	21.8
Fall 2011	24.6	25.1	24.3	21.6	22.6	25.1	24.1
Winter 2011	28.7	22.8	24.3	19.3	25.0	22.5	26.2
<b>Altitude, m, of residence</b>							
1,976–2,321, quartile 1	25.4	26.5	23.9	23.8	24.7	26.7	25.1
2,322–2,644, quartile 2	25.1	23.2	24.3	23.8	25.6	26.7	24.9
2,645–2,861, quartile 3	24.6	19.9	25.7	29.3	24.7	25.7	25.0
2,862–3,803, quartile 4	24.9	30.3	26.1	23.1	25.0	20.9	24.9

\*Data are %, except for no. children. ARIs, acute respiratory illnesses; non-ARI, a period outside the pre-ARI, current ARI, and post-ARI periods.

†No. children who contributed samples during each period.

Overall, 36.7% of nasopharyngeal samples were from children who had received  $\geq 2$  PCV7 doses and were considered vaccinated. Approximately 5.0% of samples were from children who had received aminopenicillins, cotrimoxazole, chloramphenicol, or furazolidone within the 7 days preceding sample collection.

Quantitative PCR detected *S. pneumoniae* in 68.9% of nasopharyngeal samples; 78.9% of ARI and 65.3% of non-ARI samples were positive ( $p = 0.06$ ). Unadjusted log-transformed pneumococcal densities varied by ARI periods (online Technical Appendix).

Adjusted analyses showed that densities peaked during ARIs. In post hoc adjusted comparisons, densities were higher during the 1–7 days pre-ARI ( $p < 0.0001$ ), ARI ( $p < 0.0001$ ), 1–7 days post-ARI ( $p < 0.0001$ ), and 8–14 days post-ARI ( $p = 0.007$ ) than during the non-ARI period (Figure 1).

Of 450 ARI nasopharyngeal samples, 435 (97%) had corresponding nasal swab samples available for identification of respiratory viruses; 299 (68.7%) tested positive for at least 1 virus. Rhinovirus, which was detected in 44.6% (194/435) of samples, was the most common virus (online Technical Appendix). The median log-transformed pneumococcal densities of 299 virus-positive samples and 136 virus-negative samples were not significantly different (4.73 vs 3.94, respectively; adjusted  $p = 0.06$ ).

During ARI, the median log-transformed pneumococcal densities varied among virus groups: virus-negative (3.94, IQR 0.00–5.67;  $n = 136$ ), nonrhinovirus (4.49, IQR 3.12–5.48;  $n = 105$ ), rhinovirus-only (4.91, IQR

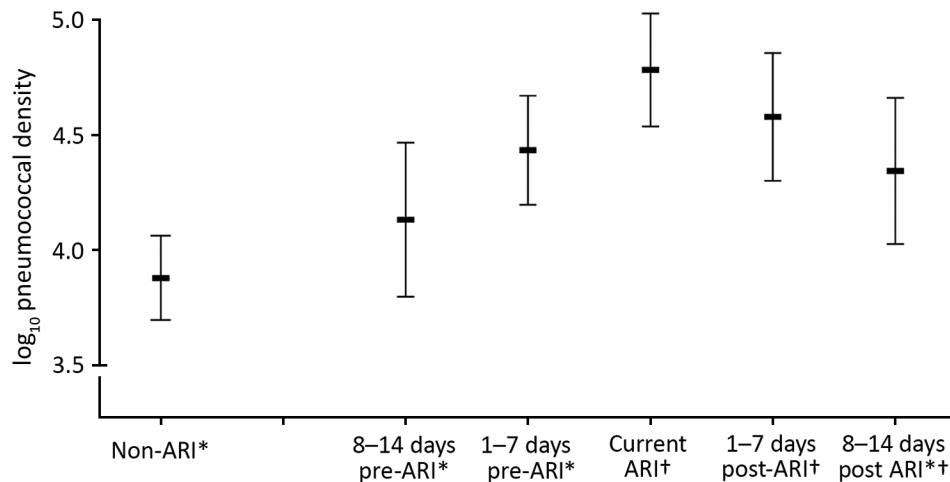
3.43–6.23;  $n = 147$ ), and rhinovirus detected with other viruses samples (5.03, IQR 3.28–6.53;  $n = 47$ ). In multivariable analyses, the only significant difference was between rhinovirus-only and virus-negative samples ( $p = 0.02$ ) (Figure 2).

For the colonization patterns assessment, 2,479 (69.3%) nasopharyngeal samples had another sample collected  $\leq 60$  days before the current sample; the median time between samples was 28 days. The median log-transformed pneumococcal densities among samples that represented new colonizations (5.14, IQR 3.56–6.24;  $n = 411$ ), serotype replacement (5.49, IQR 4.53–6.44;  $n = 322$ ), and serotype persistence (5.79, IQR 4.82–6.47;  $n = 489$ ) were compared. In multivariable analysis, serotype-replacement ( $p = 0.005$ ) and serotype-persistence ( $p = 0.0003$ ) samples had higher density than new colonization samples. The difference between serotype replacement and serotype persistence was not significant ( $p = 0.2$ ).

## Conclusions

Our findings demonstrate a dynamic evolution of pneumococcal densities before, during, and after ARI episodes among young children. We observed a gradual increase in pneumococcal density leading up to an ARI episode, peak density during symptomatic ARI, and a decrease in density post-ARI to levels similar to those in baseline non-ARI periods.

Our observations of higher densities during ARI than non-ARI episodes align with those in studies from Vietnam



**Figure 1.** Estimated median pneumococcal densities with 95% CIs (vertical bars) by acute respiratory illness (ARI) period. Estimates derived from a quantile regression model that accounted for sex, age, daycare attendance, electricity, water supply, housing materials, kitchen type, smokers at home, vaccination, antimicrobial drug use, season, and altitude of residence. Asterisk indicates significantly different from ARI samples; dagger indicates significantly different from non-ARI samples.

and South Africa (14,15) and complement those assessments by illustrating the dynamic evolution of pneumococcal densities and the role of virus co-infections and pneumococcal colonization patterns. Unlike other studies that focused on hospitalized children, our community-based study showed relatively modest variations in nasopharyngeal pneumococcal density.

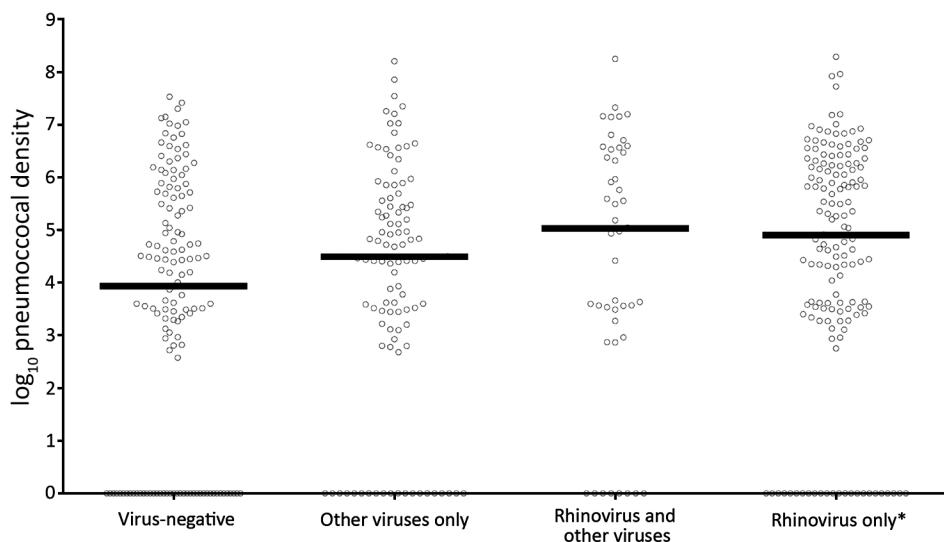
Rhinovirus detection was associated with increased pneumococcal density during ARI. Although we observed an even higher median pneumococcal density in samples co-infected with rhinovirus and other respiratory viruses, the number of observations was small and statistical power to demonstrate significant differences was limited.

Compared with new colonization in our study, serotype persistence and replacement were associated with higher pneumococcal density. Because many new colonizations might ultimately succumb to host mechanisms and fail to establish stable colonization (2), the observed lower densities might reflect a decline of pneumococcal populations

as clearance evolved. Nevertheless, although statistically significant, the differences in density were relatively modest, and we cannot establish the precise time of colonization or clearance in our samples.

Our study has several limitations. ARI identification depended on the presence of cough or fever, which are subjective but widely used for routine ARI surveillance (5–7). Because our study used household-based rather than health facility-based surveillance, severe disease was infrequent, precluding detailed assessments of disease severity. Due to small numbers, we could not study serotype-specific pneumococcal densities. In addition, because the study was conducted in rural communities of Peru, caution is warranted when extrapolating our findings to other settings.

Our findings demonstrated that, among young children, nasopharyngeal pneumococcal density started increasing before the onset of ARI symptoms, peaked during symptomatic ARI, and decreased after symptoms subsided.



**Figure 2.** Pneumococcal densities of current acute respiratory illness samples subdivided by reverse transcription PCR detection of respiratory viruses. Each circle represents a single bacterial density measurement. The median for the samples of each subgroup is represented by a gray horizontal line. Asterisk indicates significantly different from virus-negative samples.

Rhinovirus co-infection, serotype persistence, and serotype replacement were associated with increased nasopharyngeal pneumococcal density. Nasopharyngeal pneumococcal density is dynamic surrounding ARI episodes and likely driven by complex virus–bacteria–host interactions.

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# Nasopharyngeal Pneumococcal Density and Evolution of Acute Respiratory Illnesses in Young Children, Peru, 2009–2011

## Technical Appendix

### Expanded Description of Methods

#### Study Population

Our prospective cohort study (RESPIRA-PERU) (1,2) was designed to examine the epidemiology and etiology of acute respiratory illness (ARI) in children <3 years old in the District of San Marcos, Cajamarca, Peru. The local population is mostly low income, living in rural communities with limited access to healthcare (1). The 7-valent pneumococcal conjugate vaccine (PCV7) was initially introduced into the communities in late 2009, and uptake slowly increased throughout the study period. This study was approved by the institutional review boards of Vanderbilt University (Nashville, TN, USA) and the Instituto de Investigacion Nutricional (Lima, Peru).

#### Weekly Household Visits

In the RESPIRA-PERU study, local field workers were trained to interview parents about respiratory signs and symptoms of respiratory illness based on the Integrated Management of Childhood Illness (IMCI-WHO) protocol (3,4). For this study, an ARI episode was defined as the period of time a child had either cough or fever (5,6). If ill at the time of the weekly household visit, the presence of IMCI-WHO pneumonia danger signs (inability to drink or breastfeed, persistent vomiting, convulsions, lethargy, unconsciousness, stridor, severe malnutrition) or signs of lower respiratory tract infection (tachypnea, audible wheezing, chest retractions, grunting, nasal flaring, stridor, or cyanosis) was assessed (4,5).

## **Respiratory Samples**

Nasal swabs were collected for each ARI episode (through 7 days after symptom resolution), and processed as previously described (6,7). Samples were shipped to Vanderbilt University for detection of influenza viruses, respiratory syncytial virus, human metapneumovirus, rhinovirus, adenovirus, and parainfluenza viruses by real-time reverse transcription PCR (RT-PCR) (6,8–10). Monthly nasopharyngeal (NP) samples were collected according to WHO guidelines (11,12), transported in 1 mL of skim milk-tryptone-glucose-glycerine (STGG) medium and frozen in the media at  $-70^{\circ}\text{C}$ . Analyses at Emory University (Atlanta, GA < USA) included culture and DNA extraction from *Streptococcus pneumoniae* isolates and NP specimens using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA). Colonization density was determined using a targeted *lytA* gene real-time quantitative PCR (qPCR)(13,14). Pneumococcal colonization density (CFU/mL) was quantified using purified genomic DNA from *S. pneumoniae* reference strain TIGR4 and serially diluted 10-fold to prepare standards ( $4 \times 10^0$  to  $4 \times 10^6$  CFU) (14). Standards were run along with DNA from NP samples in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) using the software Bio-Rad CFX manager (14). Pneumococcal serotyping was performed by multiplex PCR (13,15,16). We used samples collected in 2009 and 2011, representing the time periods before and after routine use of PCV7 in the study communities, respectively (17).

## **ARI and Peri-ARI Periods**

The duration of an ARI episode was defined as the number of days a child had either cough or fever (5,6). We defined peri-ARI periods including 8–14 days pre-ARI, 1–7 days pre-ARI, 1–7 days post-ARI and 8–14 days post-ARI. Samples collected outside the above categories were classified as non-ARI. When a sample met criteria for >1 category because it was collected between 2 ARIs, it was classified relative to the closest ARI.

## **Patterns of Pneumococcal Serotype Acquisition**

A secondary analysis included all pneumococcal-positive samples in which there was a previous NP sample within 60 days. When the previous sample was qPCR negative, the current sample was classified as new colonization. When the previous sample was qPCR positive, the current sample was classified as persistence if the serotype was the same, or as replacement if a different serotype was detected. If either serotype was nontypeable or unknown, then the pattern was considered undetermined. Unknown refers to those samples for which the serotyping

reactions were inconclusive. Some samples that are *lytA* positive may contain insufficient pneumococcal material for the multiplex PCR reactions to test positively for any serotype.

### **Statistical Analyses**

We compared pneumococcal NP densities during ARI, peri-ARI, and non-ARI periods. Bacterial densities were presented on a logarithmic scale. To retain the samples with zero density, we applied a  $\log_{10}(x+1)$  transformation, where  $x$  represents the measured density. The median density was chosen as the most appropriate measure of central tendency as it is more robust to extreme values. Due to the persistent non-normal distribution of the transformed values, we used multivariable quantile regression to investigate the relationship between the log-transformed pneumococcal densities, periods of observation and relevant covariates. These covariates included sex, age, daycare attendance, electricity, water supply, housing materials, kitchen type, smokers at home, vaccination, antimicrobial drug use, season, and altitude. We used a restricted cubic spline function to allow for nonlinear effects of age in the model. Since a child could contribute >1 sample for our analyses, we accounted for this correlation of observations by calculating robust SEs using the Huber-White Sandwich variance estimator. The median pneumococcal densities for comparison groups were calculated using adjusted predictions from the respective quantile regression models, while integrating the influence of other model parameters. For post hoc comparisons of adjusted densities between the non-ARI and the ARI periods, we conservatively applied a Bonferroni correction to the type I error (5 comparisons, and a corrected  $p < 0.01$  to define statistical significance).

We conducted secondary analyses to assess the role of respiratory viruses on pneumococcal density, restricting the analysis to samples collected during ARI or post-ARI periods. Given that rhinoviruses were commonly detected but other viruses were less frequently detected, samples were classified into 4 mutually exclusive groups: 1) positive for rhinovirus only, 2) positive for rhinovirus and other viruses, 3) positive for other viruses only; and 4) negative for any viruses. Another secondary analysis examined the role of the previously described patterns of pneumococcal serotype acquisition on pneumococcal density. For all secondary analyses, we used multivariate quantile regression models to account for covariates. Stata® version 14.0 was used for all statistical analyses (StataCorp, College Station, TX, USA).

## Expanded Description of Results

### Summary of Enrollment and Surveillance in Parent RESPIRA-Peru Study

Enrollment and surveillance in the RESPIRA-Peru study have been described elsewhere (1). In brief, during May 2009–September 2011, a total of 892 children were enrolled, 55,661 household visits were scheduled, 89% were executed (i.e., field workers reached their target households), and 79% were successfully completed (i.e., information was collected during the visit). A total of 4,655 nasal and 10,722 NP swabs were collected. Collection of NP swabs was completed in 88% of scheduled collections.

### Additional Details on Pneumococcal Density Assessments

The unadjusted log-transformed pneumococcal densities are shown for each period in Technical Appendix Figure. In unadjusted analysis, the median NP density during the non-ARI periods (3.71, IQR 0.00–5.59) was statistically lower than the 1–7 days pre-ARI (4.42, IQR 2.72–5.86;  $p < 0.001$ ), the ARI period (4.49, IQR 3.04–5.95;  $p < 0.001$ ), and 1–7 days post-ARI (4.48, IQR 0.00–5.79;  $p = 0.002$ ) periods.

In the multivariable quantile regression model that examined the association between ARI study periods and pneumococcal density, season was significantly associated with median pneumococcal density. Spring (October, November) 2009, Fall (May, June) 2011, and Winter (July, August, September) 2011 were associated with elevated density compared to Fall 2009 and Winter 2009. Housing characteristics (including running water, electricity, and sewage system), PCV7 vaccination, health facility visits, antimicrobial drug use and the presence of smokers in the home did not have statistically significant associations with median pneumococcal density.

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**Technical Appendix Table 1.** Viral detections in nasal swab samples\*

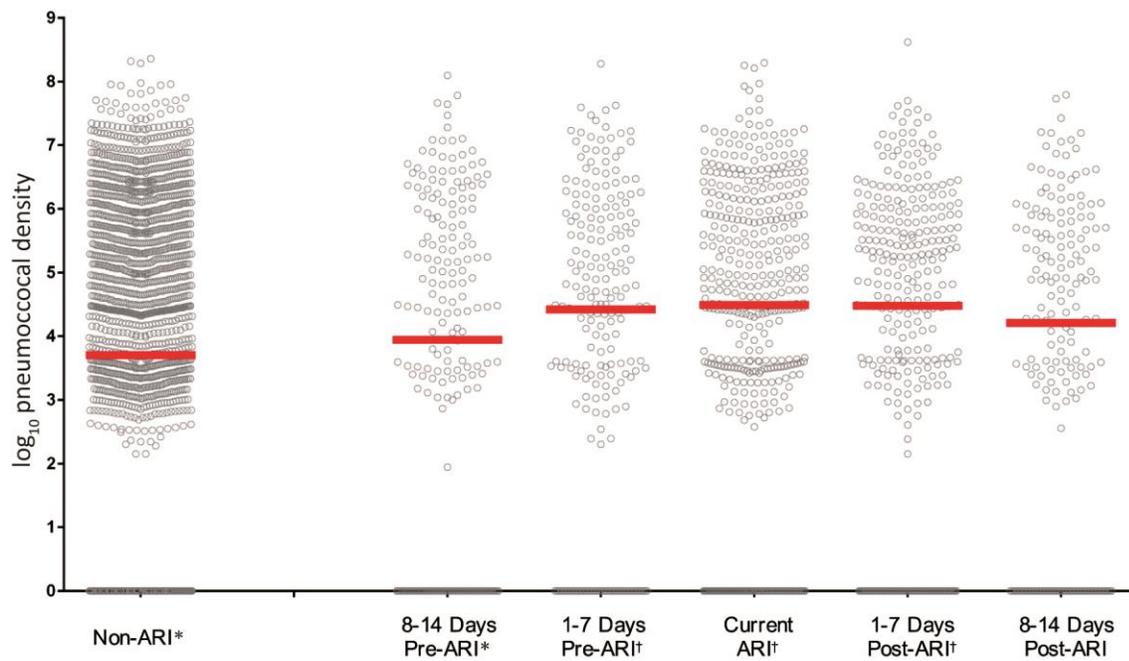
Virus	No.	Nasal swab samples, %
Rhinovirus	257	51.2
Adenovirus	84	16.7
Influenza	46	9.2
Parainfluenza 1–3	42	8.4
Respiratory syncytial virus	40	8.0
Human metapneumovirus	33	6.6

\*Some samples had >1 virus detected.

**Technical Appendix Table 2.** Pneumococcal serotype detections in NP swab samples\*

Serotype	No.	NP swab samples, %
1	4	0.2
10A	87	4.3
10F/10C/33C	14	0.7
11A	102	5.0
11A/11D	53	2.6
12F	2	0.1
13	51	2.5
14	49	2.4
15A	38	1.9
15A/15F	22	1.1
15B	37	1.8
15B/15C	31	1.5
15C	38	1.9
16F	15	0.7
17F	26	1.3
18A	1	0.1
18A/18B/18C	8	0.4
18A/18B/18C/18F	8	0.4
18C	9	0.4
19A	61	3.0
19F	159	7.8
2	1	0.1
20	6	0.3
21	16	0.8
22A	3	0.2
22F	16	0.8
22F/22A	8	0.4
23A	25	1.2
23B	57	2.8
23F	138	6.8
24A/24B/24F	6	0.3
25F	1	0.1
28A	7	0.3
3	29	1.4
31	6	0.3
33A/33F/37	8	0.4
33B	7	0.3
33B/33D	2	0.1
33F	10	0.5
34	22	1.1
35A	16	0.8
35A/35C/42	18	0.9
35B	29	1.4
35C	1	0.1
35F	27	1.3
35F/47F	21	1.0
38/25F	7	0.3
39	3	0.2
4	16	0.8
5	2	0.1
6A	42	2.1
6A/6B	51	2.5
6A/6B/6C	69	3.4
6B	111	5.4
6C	127	6.2
6D	5	0.2
7B/7C/40	11	0.5
7C	27	1.3
7F	5	0.2
8	3	0.2
9A	3	0.2
9A/9V	13	0.6
9L/9N	10	0.5
9N	1	0.1
9V	12	0.6
NT	229	11.2
Total	2,042	100

\*When serotyping reactions were unable to differentiate between  $\geq 2$  serotypes, the multiple serotypes are reported. NP, nasopharyngeal.



**Technical Appendix Figure.** Pneumococcal densities for each acute respiratory illness (ARI) period. Each circle represents a single bacterial density measurement. The median for the samples of each period is represented by a red line. \*Significantly different from ARI samples. †Significantly different from non-ARI samples.