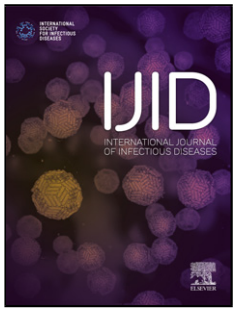

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Pneumococcal carriage, density, and co-colonization dynamics: a longitudinal study in Indonesian infants

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Running title: Longitudinal pneumococcal carriage study

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Highlights

- 85% of infants carried pneumococcus at least once during the first year of life
- Carriage duration longer for the first acquisition compared to subsequent episodes
- Pneumococcal density is affected by antibiotic exposure and respiratory infection
- Pneumococcal density decreases during a carriage episode
- Most infants carried a single serotype at a time

Abstract

Objectives

Nasopharyngeal carriage of Streptococcus pneumoniae underpins disease development and transmission. We examined pneumococcal carriage dynamics, including density and multiple serotype carriage, in Indonesian infants during the first year of life.

Methods

200 healthy infants were enrolled at 2 months of age, with 8 nasopharyngeal swabs collected from enrolment until 12 months of age. Pneumococci were detected using quantitative PCR and serotyped by microarray. Regression models assessed factors influencing pneumococcal carriage and density.

Results

85% of infants carried pneumococci at least once during the study. The median age of first acquisition was 129 days (IQR 41, 216). The median duration of carriage was longer for the first pneumococcal acquisition compared with subsequent acquisitions (151 days vs 95 days, $p < 0.0001$). Of 166 infants who carried pneumococci during the study, the majority (63.9%) carried a single pneumococcal serotype at a time. Pneumococcal carriage density was higher when upper respiratory tract infection symptoms were present, lower during antibiotic usage, decreased with age, and tended to decrease over time during a carriage episode.

Conclusions

The majority of Indonesian infants carry pneumococcus at least once during the first year of life. Pneumococcal carriage is a dynamic process, with pneumococcal density varying during a carriage episode.

Keywords: Streptococcus pneumoniae, pneumococcus, nasopharynx, bacterial carriage, serotypes

Introduction

Streptococcus pneumoniae (pneumococcus) can cause a variety of diseases ranging from otitis media to pneumonia, meningitis, and sepsis, and was responsible for an estimated 317,300 pediatric deaths in 2015.[1] Pneumococci commonly colonize the nasopharynx of young children, with carriage prevalence ranging from 19 – 86%.[2] Carriage is considered an essential first step in the development of pneumococcal disease, and is the source of transmission within human populations.[3] Pneumococcal conjugate vaccines (PCVs) reduce carriage by blocking the acquisition of vaccine-serotype pneumococci, protecting immunized children and resulting in potent indirect effects by reducing transmission to unvaccinated individuals.[4]

Data from longitudinal studies demonstrate that carriage is a dynamic process. The timing of acquisition and clearance is influenced by factors including serotype, age, and previous pneumococcal exposure.[5-7] Young children commonly experience multiple carriage episodes: a study in Thailand reported a median of 7 pneumococcal acquisitions during the first 24 months of life.[6] High pneumococcal density in the nasopharynx is associated with pneumonia in children, and linked to transmission in animal studies.[8, 9] Simultaneous carriage of multiple pneumococcal serotypes is common in children in low- and middle-income countries.[10] Few published longitudinal carriage studies to date examined pneumococcal density or used methods capable of detecting multiple serotype carriage. A detailed investigation of carriage dynamics, including quantitative information on pneumococcal density and detection of co-colonizing serotypes, would improve our understanding of this important process, and provide useful information for estimating and evaluating vaccine effects.

We conducted a longitudinal study in Indonesia, a country with no national PCV program, to examine pneumococcal carriage during the first year of life. Indonesia has a high burden of childhood pneumonia, which is the leading cause of death in children outside the neonatal period, with an estimated incidence of 326 per 1000 children per year in 2015. [11,12] Previous studies have reported pneumococcal carriage prevalence ranging from 43% - 53% in young children in Indonesia.[13-16] The study aim was to determine pneumococcal carriage prevalence and density over time, and investigate the dynamics of carriage, including acquisition, duration, and multiple serotype carriage, using quantitative molecular methods.

Methods

Study population, design, and procedures

This study was conducted in the Bandung region of West Java, Indonesia. Indonesia is a lower-middle-income country consisting of over 17,000 islands, with a population of over 260 million. The estimated case rate of pneumonia in Indonesian children is approximately 5,000 per 100,000 hospital discharges.[17] Childhood mortality varies regionally and between urban and rural settings.[18] Indonesian infants routinely receive BCG, polio, HepB (birth dose), pentavalent (DTP-HepB-Hib), and measles-rubella vaccination as part the Expanded Program on Immunization.

Recruitment was conducted at two health centers, an urban center in Bandung city and a semi-rural center located in the Padalarang district approximately 25 km from Bandung city, from November 2014 – January 2015. Mothers of newborns were identified by community health workers and invited to have their infant participate in the study. Written informed consent was obtained from a parent/guardian prior to any study procedures. Enrolment criteria included age of 8 – 12 weeks, weight >2500 g,

living within the study area and no plans to relocate during the follow-up period, and judged in good health following examination by a pediatrician. Exclusion criteria included moderate/severe illness at the time of screening, axillary temperature ≥ 38°C, antibiotic use within the previous 14 days, prior hospitalization for conditions excluding jaundice, mother with known HIV positive status, and prior or expected PCV immunization.

Data on demographics and household information were collected by study staff at the initial visit. Six subsequent visits were conducted monthly, then a final visit at 12 months of age. Data on upper respiratory tract infection (URTI) symptoms (rhinorrhea, cough, or other respiratory symptoms), recent antibiotic usage, breast feeding status, and major illnesses/hospitalizations were collected at follow-up visits. At each visit, a nasopharyngeal swab was collected according to World Health Organization recommendations.[19]

The sample size was based on an estimated pneumococcal carriage prevalence of 50% at 5 months of age.[13] 120 participants would estimate carriage prevalence with confidence intervals of ± 8.9%. The sample size was increased to 200 to facilitate secondary analyses.

Ethics approval was granted from the Health Research Ethics Committee, Universitas Padjadaran Faculty of Medicine, Indonesia (536/UN6.C2.1.2/KEPK/PN/2014) and the Royal Children's Hospital Human Research Ethics Committee, Australia (34124).

Laboratory analyses

Swabs were placed into 1 ml skim milk tryptone glucose glycerol (STGG) medium, kept in a cool box, and transported to the Microbiology Laboratory at the Advanced

Biomedical Laboratory, Faculty of Medicine, Universitas Padjadaran, Bandung for aliquotting and storage at -70°C within 8 hours of collection. Pneumococci were detected and serotyped as previously described.[16] In brief, DNA extracted from STGG was examined by lytA real-time quantitative PCR (qPCR) to detect pneumococci and determine pneumococcal density, reported in genome equivalents/ml (GE/ml).[20] Molecular serotyping by microarray was conducted following a culture-amplification step, in which 50 µl of the neat sample and a 1:10 dilution were plated on selective agar and incubated overnight. The plate containing optimal growth of high-density, distinct colonies was selected for DNA extraction. DNA was fragmented, fluorescently labeled, and microarray conducted using Senti-SP v1.6 (BUGS Bioscience), which reports the identity and relative abundance (%) of all serotypes present within a sample.[10] Previously, this method was found to have very high sensitivity, including >93% sensitivity for the detection of secondary serotypes present in low proportion in samples containing multiple serotypes [10]. Serotype-specific density was determined by multiplying pneumococcal density (determined by qPCR) by the serotype relative abundance (determined by microarray). A representative isolate from each sample was serotyped by latex agglutination/Quellung.

Data analysis

Definitions of pneumococcal carriage episodes were based on identification of an individual serotype, and estimates of dates of acquisition (midpoint between last negative swab and first positive swab) and clearance (midpoint between last positive swab and subsequent negative swab) were consistent with published longitudinal carriage studies.[6, 21-23] For infants carrying pneumococci at enrolment, the date of acquisition was estimated as the midpoint between birth and visit 1. Clinical data

were entered into a database (dBASE software, dBase LLC, Binghamton, NY, USA) then merged with laboratory data and cleaned using Stata version 14.2 (StataCorp, College Station, TX, USA). Statistical analyses were conducted using Stata and GraphPad Prism version 7.03 (GraphPad Software, La Jolla, CA, USA). Pneumococcal density data were $\log_{10}$ transformed prior to analysis. The Chi-squared test was used to compare categorical data and t-test or ANOVA for continuous data unless otherwise noted. Data on carriage prevalence, age at first acquisition, and number of acquisitions were examined overall and by residence type (urban vs semi-rural). All other analyses used overall data. Kaplan-Meier curves were used to examine time to first pneumococcal acquisition and the log-rank test to compare by residence type. Multivariable Cox regression analysis was used to assess whether the following factors were associated with time (in days) to first acquisition: residence type, ethnicity, two or more children <5 years of age in the household, cigarette smoker in the household, breast feeding, parental income, and maternal education. Parametric survival models (Weibull distribution) were used to assess carriage duration; carriage episodes that were ongoing at the final study visit were censored. Carriage episodes that were first detected at the final study visit were excluded from analyses of carriage duration. The log-rank test was used to compare carriage duration for first vs. subsequent acquisitions.

Logistic regression models were used to evaluate relationships between age (months), antibiotic exposure and presence of URTI symptoms (variables selected a priori) and pneumococcal carriage, incorporating Generalized Estimating Equations (GEEs) with robust 95% CIs and unstructured working correlation matrix to account for repeated sampling of individuals. GEE linear regression models were used to investigate associations between overall pneumococcal density and age, antibiotic

exposure, URTI symptoms, and multiple serotype carriage. For longer carriage episodes (serotype detected in ≥ 3 swabs), the detection stages of carriage episode were defined as initial (first detection), middle (subsequent detections), and final (last detection prior to clearance). To examine associations between stage of carriage episode and serotype-specific density, a 3-level linear random intercept model was used, with repeated density measures nested within serotypes and multiple serotypes nested within individuals (to account for repeated sampling and multiple serotype carriage). Covariates in the model included and age, antibiotic exposure, URTI symptoms, and multiple serotype carriage.

Dynamics of pneumococcal carriage were categorized into five observed patterns: 'single serotype carriage' (carriage of a single serotype at a time), 'serotype replacement' (an initial colonizing serotype became outnumbered by a newly acquired serotype and was subsequently cleared), 'serotype dominance' (carriage of an initial colonizing serotype was maintained and newly acquired serotypes were transient), 'stable co-colonization' (two serotypes simultaneously carried across multiple time points, and 'short term co-colonization' (multiple serotype carriage observed at a single time point).

Results

A total of 200 infants were enrolled in the study, with participant characteristics shown in Table 1. During the study period, there was one participant death (respiratory failure due to severe bronchopneumonia plus suspected congenital rubella syndrome) and two drop-outs, and three participants missed swab collection visits. Eight participants were hospitalized with a diagnosis of pneumonia.

A total of 1575 nasopharyngeal swabs were collected. Four swabs were excluded due to technical issues. The overall pneumococcal carriage prevalence was 22.0% (44/200; 95%CI 16.5 – 28.4) at enrolment and increased to 68.4% (62/196; 95%CI 61.4 – 74.8) at 12 months of age. Pneumococcal carriage rates are shown by site and age in Figure 1. At 2 and 7 months of age, pneumococcal carriage prevalence was higher in semi-rural infants compared with those from urban areas (p = 0.01 and p = 0.045, respectively).

Most (169/198, 85.4%) of infants carried pneumococci at least once during the study period. The median age of first acquisition was 129 days (IQR 41, 216). Figure 2 depicts time to first pneumococcal acquisition for urban and semi-rural infants (p = 0.064). None of the potential risk factors evaluated were associated with time to first acquisition, however there was some evidence that higher parental income was associated with delayed first acquisition (p = 0.055, Supplementary Table 1). The number of pneumococcal acquisitions per child during the study period ranged from 0 to 5, with a median of 1 (IQR 1, 2) overall, 1 for urban infants (IQR 1, 2), and 2 (IQR 1, 3) for semi-rural infants (p = 0.061, Mann-Whitney test). A total of 318 pneumococcal acquisitions occurred during 70,429 days at risk, an acquisition rate of 0.0045 per day (95%CI 0.0040, 0.0050), or 0.14 per month (95%CI 0.12, 0.15).

Antibiotic usage during the study period was reported at least once for 54/198 (27.3%) of study participants. Thirty-seven swabs were collected from infants currently taking oral antibiotics (13 of which [35%] were positive for pneumococcus), and an additional 17 infants took antibiotics within 14 days of swab collection. Overall, 170 (85.6%) study participants had URTI symptoms during at least one study visit, and 364 (23.1%) of swabs were collected from infants with current URTI symptoms. Current or recent antibiotic usage was negatively associated with

pneumococcal carriage whereas presence of URTI symptoms was positively associated (Table 2).

Pneumococci identified during the study period belonged to 46 capsular serotypes and 4 genetic variants of non-encapsulated pneumococci.[24] Thirty of 650 (4.6%) lytA-positive samples were culture negative and therefore not serotyped. Serotypes 6B, NT2 (a non-encapsulated lineage), 19F, 23F, 34, and 15B/C were the most common (Figure 3). All serotype 11A pneumococci identified were typed as 11F-like variants by microarray.[25] 122 (38.4%) of carriage episodes were due to serotypes included in PCV13. The carriage prevalence of PCV13 serotypes, non-PCV13 serotypes, and non-encapsulated pneumococci over time is shown in Supplementary Figure 1. Carriage prevalence of PCV13 serotypes increased from 9.1% at enrolment to 35.1% at 12 months.

Overall, the median duration of carriage was 132 days (IQR 77, 217), ranging from 28 to 328 days. Four infants carried the same pneumococcal serotype for the entire study duration. The median carriage duration for the first pneumococcal acquisition was 158 days (IQR 84, 260), compared with 95 days (IQR 56, 161) for subsequent acquisitions ($p = 0.0013$, log-rank test). Pneumococcal density ranged from 2.33 to 8.76 $\log_{10}$ GE/ml, with a mean of 6.04 $\log_{10}$ GE/ml (95%CI 5.96, 6.11). Pneumococcal density is shown by age and site in Supplementary Figure 2. Density did not differ by site ($p = 0.694$, t-test) and there was some evidence that density differed by age ($p = 0.066$, one-way ANOVA). Regression analysis demonstrated that in pneumococcal carriers, density was higher when URTI symptoms were present and decreased with age, and there was some evidence that density was lower in infants currently on antibiotics (Table 3).

Multiple serotype carriage was observed in 98 samples (two serotypes n = 93, three serotypes n = 5), and increased with age (Supplementary Figure 1). Fifty-nine of 198 (29.8%) participants had multiple serotype carriage observed at least once. Multiple serotype carriage was associated with higher overall pneumococcal density (Table 3). During longer carriage episodes (defined as detected in ≥ 3 swabs), serotype-specific density tended to decrease over time, with mean density higher at initial detection (6.36 $\log_{10}$ GE/ml [95%CI 6.18, 6.53]) compared with the final detection prior to clearance (5.71 $\log_{10}$ GE/ml [95%CI 5.47, 5.95] (Figure 4). In a multi-level model including URTI symptoms, antibiotic use, multiple serotype carriage, and age, density at final detection was 0.62 $\log_{10}$ GE/ml lower at final detection compared with initial detection (Table 4).

We examined carriage dynamics of the 166 participants who carried pneumococci at least once after excluding three with no serotyping data. A representative example of each pattern is shown in Figure 5. Single serotype carriage was the most common (n = 106, 63.9%), followed by serotype replacement (n = 11, 6.6%) and serotype dominance (n = 10, 6.0%). Stable co-colonization and short term co-colonization were each observed in 8 (4.8%) participants. For 23 (13.8%) participants, no pattern was determined as multiple serotype carriage was only observed at the final swab collection.

Discussion

At 12 months of age, 68% of Indonesian infants carried pneumococcus, with a median age of acquisition at 129 days. These findings reflect of a setting of moderate pneumococcal carriage intensity in comparison to longitudinal studies conducted in other areas. In Finland, carriage prevalence peaked at 28% at 18

months of age, and in the US, the mean age of acquisition was six months.[26,27] In high carriage intensity settings such as The Gambia, Kenya, South Africa, Malawi, and a refugee camp in Thailand, median age at first pneumococcal acquisition ranged from 33 to 63 days.[5, 6, 21-23] In these settings, pneumococcal carriage prevalence peaked by six months, for example reaching 64% in South Africa and 80% in Thailand, whereas in Indonesia carriage prevalence continued to rise until 12 months of age.[6, 22] We previously reported a pneumococcal carriage prevalence of 64% in children aged 12-24 month olds in the Bandung region, suggesting that carriage rates stabilize after 12 months in this population.[16] Carriage prevalence tended to be higher in infants living in semi-rural areas than urban residents as observed in some, but not all, other studies.[28,29] Previously, we did not observe differences in pneumococcal carriage between urban and semi-rural Indonesian children aged 12-24 months, suggesting that differences diminish with age.[30]

Data from Indonesia could inform models for pneumococcal vaccine trials in similar settings, particularly in Asia, where PCV introduction has lagged behind compared to other regions.[31,32] Data on serotypes causing invasive pneumococcal disease in Indonesia are extremely limited, a major knowledge gap for this country. We therefore were not able to correlate carriage data with serotypes causing disease. Modeling approaches have used carriage data to predict serotypes causing invasive disease and vaccine impact and may be an avenue for future investigation.[33,34]

We found that duration of carriage was longer for initial pneumococcal acquisitions compared to subsequent acquisitions, regardless of serotype. Although maturation of the immune system with age likely contributes to this effect, it is recognized that pneumococcal colonization is immunizing.[35] Epidemiologic evidence suggests that both serotype-specific and serotype-independent immunity are generated by

pneumococcal carriage. In Bangladesh, acquisition rates of the four most common serotypes were lower in children who had previously experienced carriage of a heterologous serotype, suggestive of serotype-independent immunity.[36] In Thailand, the interval to reacquisition of common serotypes was longer in children who had previously carried either homologous or heterologous serotypes, and carriage duration of serotypes 14 and 19F was shorter when reacquired following homologous carriage.[6] Serotype 6B, which was the most commonly acquired serotype in the current study, was not among the ten most common serotypes carried by children aged 12-24 months in Bandung, suggesting that carriage of serotype 6B during infancy may generate a serotype-specific immune response that is protective in later childhood.[16] A study in Israeli toddlers found that children who previously carried serotypes 14 and 23F had a reduced risk of subsequent carriage of those serotypes, and protection correlated with increased levels of serotype-specific antibodies.[37] Further investigation of the immune responses induced by carriage, particularly those that contribute to serotype-independent immunity, could help in the design of non-capsular pneumococcal vaccines.

High pneumococcal density in the nasopharynx has been linked to pneumococcal pneumonia and invasive pneumococcal disease, and investigated as a potential diagnostic tool.[38,39] Our study provides new insights into pneumococcal density during a carriage episode, highlighting the fact that carriage is a dynamic process, affected by multiple factors including antibiotic exposure and URTIs, as well as the presence of co-colonizing pneumococcal serotypes. We found that multiple serotype carriage was associated with higher overall pneumococcal carriage density by lytA qPCR, but lower serotype-specific density in comparison to carriage of a single pneumococcal serotype.

We demonstrated that density during longer carriage episodes tends to decrease over time. This observation is consistent with a mouse model of pneumococcal carriage, in which mice inoculated with a high dose of pneumococci display decreasing pneumococcal density in the nasopharynx prior to clearance.[40] Bacterial characteristics may also affect colonization density: a cross-sectional study reported that pneumococcal density varies by serotype.[41] However, studies that rely on a single sample to evaluate pneumococcal density should be aware of the limitations of that approach, particularly if they do not adjust for potential confounding.

The main limitation to our study was the sampling time frame, with monthly intervals between swab collection, and a ~4 month gap prior to collection of the final swab at 12 months of age. Short carriage episodes that occurred between sampling points would not have been detected, so pneumococcal acquisition was likely underestimated. As the first swab was collected at two months of age, we were unable to determine an accurate date of acquisition for the 44 participants who carried pneumococcus at enrolment. Longitudinal carriage studies conducted in The Gambia and South Africa collected swabs at birth and twice a month thereafter, and are therefore a better source of data on pneumococcal carriage in early infancy.[5,22]

Wider sampling time frames bias estimates of carriage duration upwards, so this may in part explain why the median carriage duration in our study (131 days) was longer than those reported in studies with more frequent sampling from The Gambia (84 days), Kenya (31 days), Thailand (31 days), and South Africa (30 days).[5-7, 22] The ability of our methods to detect secondary serotypes present in lower abundance (as opposed to conventional methods that typically detect a single

serotype per sample) would have increased the sensitivity of detection and also contributed to longer estimates of carriage duration. We did not evaluate serotype-specific differences in carriage duration due to small numbers, however differences have been reported in studies from Thailand and The Gambia.[5,6] Serotype, antimicrobial resistance, and prophage were found to be associated with carriage duration in a large pneumococcal genomics study.[42]

We did not conduct testing of viruses or detection of other colonizing bacteria such as Haemophilus influenzae that can influence the density and dynamics of pneumococcal carriage.[16, 43,44] The positive association between URTI symptoms and pneumococcal carriage rates and density observed in our study is consistent with several studies reporting increased pneumococcal density during viral URTIs.[8, 45] In children under three years old in rural Peru, pneumococcal densities peaked during acute respiratory illness and were higher in children who tested positive for a respiratory virus, particularly rhinovirus, compared to those who were virus-negative.[46] Interestingly, DeMuri et al. recently demonstrated that pneumococcal densities were higher in American children aged 4 – 7 when a respiratory virus was detected, regardless of whether the children displayed URTI symptoms.[47] HIV status was not determined for mothers or infants in our study, but as the prevalence of HIV in the general population of Indonesia is estimated to be <0.5%, we assume a similar low rate amongst participants.[48]

The laboratory methods utilized in our study enabled discrimination of different pneumococcal colonization patterns. Carriage of a single serotype at a time was most common, in line with epidemiologic data and a mouse model indicating that pneumococcal carriage inhibits acquisition of a second pneumococcal strain.[49,50]

Recently, the competitive advantage of an established pneumococcal strain against newcomers was found to be dependent on the quorum sensing system.[51]

Within-host serotype replacement was observed; this may be due to an immune-mediated reduction in density of the original colonizing serotype that facilitates expansion of a more recently acquired serotype and/or direct competition between pneumococcal strains, which has been observed in experimental models.[52,53]

As multiple serotype carriage was highest at 12 months in our study, it would be interesting to examine co-colonization dynamics in slightly older children. Larger sample sizes would be required to examine the role of serotype in co-colonization dynamics.

Our study provides useful data on pneumococcal carriage during infancy in Indonesia, with relevance to other moderate-intensity carriage settings. Better understanding of how the natural history of pneumococcal colonization affects subsequent pneumococcal carriage episodes may help develop vaccines that can harness the immunizing effects of colonization, and also improve mathematical models of pneumococcal carriage. Our findings highlight the dynamic nature of pneumococcal carriage, particularly the changes in pneumococcal density that can occur during a carriage episode.

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Figure Captions

Figure 1. Pneumococcal carriage prevalence by age for infants living in urban and semi-rural areas. Bars indicate ± 95%CI. * P < 0.05, chi-squared test.

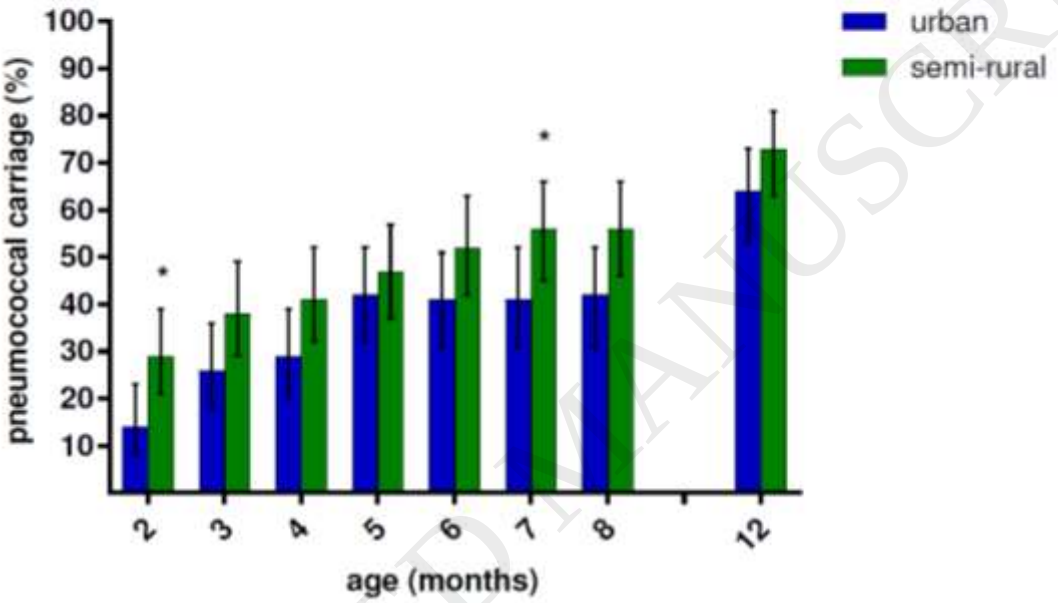


Figure 2. Age (in days) at first pneumococcal acquisition for infants living in urban and semi-rural areas. P = 0.064, log-rank test.

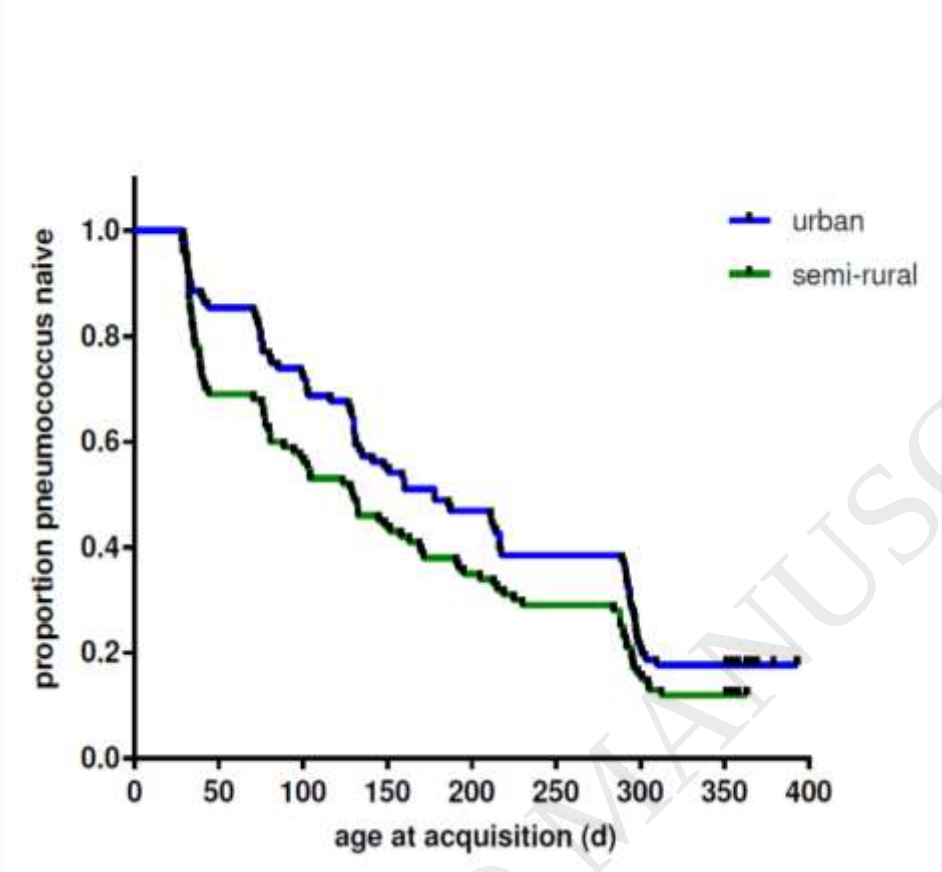


Figure 3. Pneumococcal carriage episodes by serotype. PCV13 serotypes are shown in red (* indicates the three serotypes not included in PCV10), non-PCV13 capsular serotypes are shown in black, and non-encapsulated pneumococci (categorized into genetic variants [22]) are shown in grey. ‘Other PCV13’ consists of serotypes 1 and 7F (n = 2, each). ‘Other NVT’ consists of serotypes 8, 45, 17F, 23B (n = 3 each), 38, 39, 18A, 19B, 28F, 35F, 7C (n = 2 each), and 40, 10F, 12F, 15F, 17A, and 28A (n = 1 each). ‘Other NESp’ consists of non-encapsulated lineages NT4b (n = 2) and NT2/NT3b (n = 1).

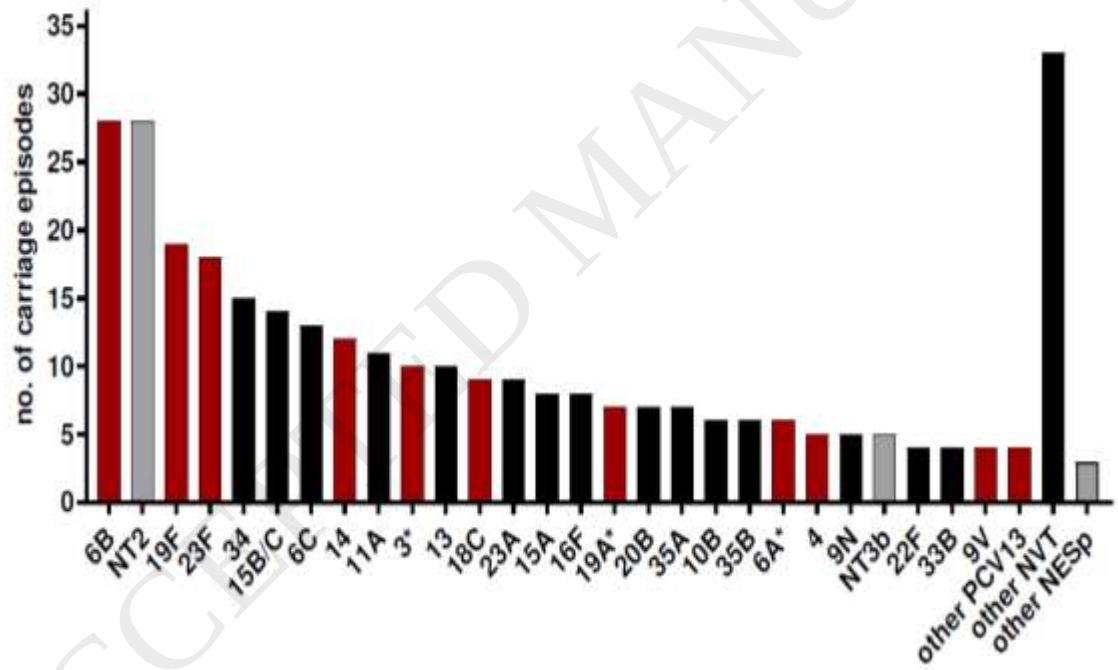


Figure 4. Serotype-specific pneumococcal carriage density (in log_{10} genome equivalents/ml) shown by stage of carriage episode, for episodes that were detected in ≥ 3 swabs. Detection stages defined as initial detection, middle (subsequent detections), and final (last detection prior to clearance). Bars indicate mean ± 95% CI. $p < 0.0001$, one-way ANOVA.

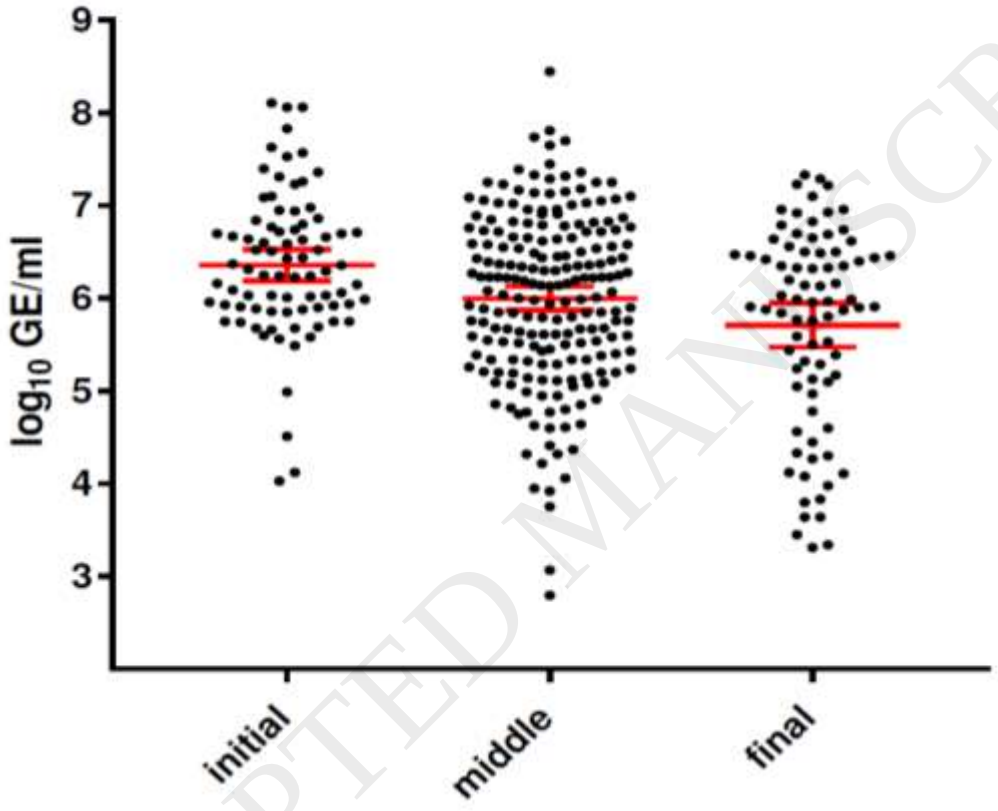
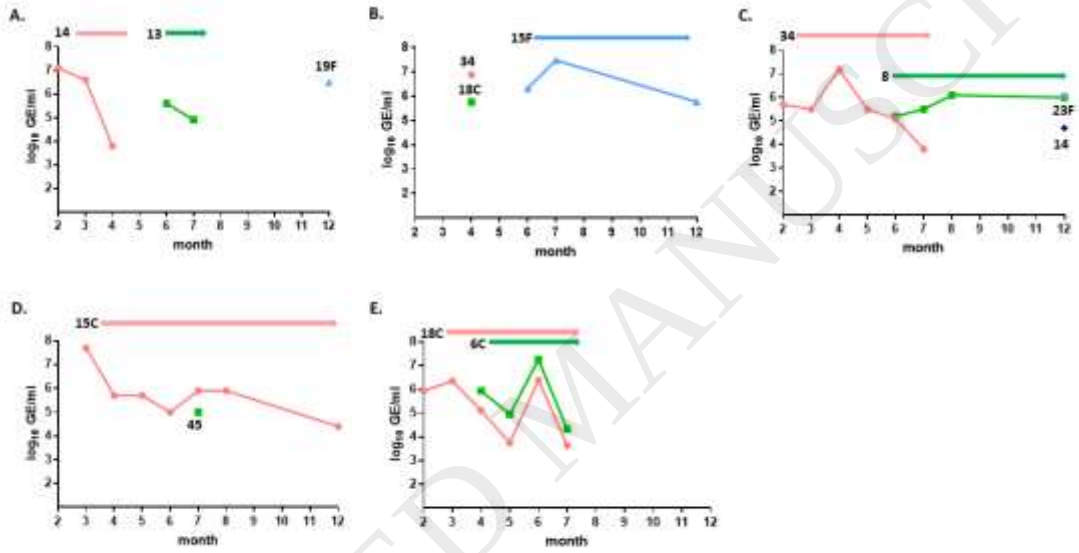


Figure 5. Patterns of pneumococcal carriage dynamics. Representative examples, each from an individual study participant, are shown for the following patterns: A. Single serotype carriage; B. Short-term co-colonization; C. Serotype replacement; D. Serotype dominance; E. Stable co-colonization. Serotype-specific pneumococcal carriage density (in $\log_{10}$ genome equivalents/ml) is shown over time. Serotypes are labelled on the graph, with the first acquisition shown in pink, second acquisition in green, third in light blue, and fourth in navy.



Tables

Table 1. Characteristics of study participants at enrolment.

Characteristic		Total (n = 200) N (%)	Urban (n = 98) N (%)	Semi-rural (n = 102) N (%)
Sex				
	Male	115 (57.5)	64 (65)	51 (50)
	Female	85 (42.5)	34 (35)	51 (50)
Age (months)				
	Median (IQR[2])	2.1 (2.0, 2.3)	2.0 (2.0, 2.2)	2.2 (2.0, 2.5)
Weight (kg)				
	Median (IQR)	5.2 (4.8, 5.8)	5.2 (4.8, 5.8)	5.2 (4.8, 5.8)
Height (cm)				
	Median (IQR)	58 (57, 60)	58 (56, 60)	58 (57, 59)
Weight-for-length Z score				
	Median (IQR)	-0.4 (-1.2, 0.2)	-0.4 (-1.2, 0.1)	-0.4 (-1.2, 0.4)
Ethnicity				
	Sundanese	162 (81.0)	68 (69)	94 (92)
	Javanese	35 (17.5)	29 (30)	6 (6)
	Other	3 (1.5)	1 (1)	2 (2)
Paternal education				
	Elementary school	23 (11.5)	6 (6)	17 (17)
	Junior high school	53 (26.5)	16 (16)	37 (37)
	Senior high school	99 (49.5)	59 (60)	40 (39)
	University	25 (12.5)	17 (17)	8 (8)
Maternal education				
	None	1 (0.5)	1 (1)	0 (0)
	Elementary school	23 (11.5)	4 (4)	19 (19)
	Junior high school	51 (25.5)	15 (15)	36 (35)
	Senior high school	105 (52.5)	66 (67)	39 (38)
	University	20 (10.0)	12 (12)	8 (8)
Parental monthly income				
	Declined to answer	2 (1.0)	1 (1)	1 (1)
	≤ Regional minimum salary[3]	159 (79.5)	73 (74)	86 (84)
	> Regional minimum salary	39 (19.5)	24 (24)	15 (15)
Number of children < 5y in the household				
	1	109 (54.5)	39 (39)	70 (69)

	2	64 (32.0)	39 (39)	25 (24)
	3	18 (9.0)	13 (13)	5 (5)
	4	9 (4.5)	7 (7)	2 (2)
Cigarette smoker in the household				
	No	57 (28.5)	36 (37)	21 (21)
	Yes	143 (71.5)	62 (63)	81 (79)
Breast feeding at 12 months				
	No	19/198 (9.6)	9/96 (9)	10/102 (9.8)
	Yes	179/198 (90.4)	87/91 (94)	92/102 (90.2)

[1]Chi-squared test for categorical values, Mann-Whitney test for age, t-test for weight and height.
[2]IQR= interquartile range
[3]Regional minimum salary rates for 2014 were 2,000,000 IDR/month for urban and 1,738,476 IDR/month for semi-rural participants.

Table 2. Logistic regression analysis of factors associated with pneumococcal carriage in Indonesian infants during the first year of life. Models incorporate Generalized Estimating Equations to account for repeated sampling. (n = 1,571)

	OR (95%CI)[1]	P value	Adjusted OR (95%CI)	P value
Antibiotic exposure[2]	0.53 (0.33, 0.85)	0.009	0.44 (0.25, 0.78)	0.004
URTI symptoms	1.25 (1.06, 1.48)	0.010	1.21 (1.01, 1.46)	0.041
Age (months)	1.21 (1.17, 1.26)	<0.001	1.22 (1.17, 1.27)	<0.001

[1]OR = odds ratio
[2]current and/or within the previous 14 days

Table 3. Linear regression analysis of factors associated with pneumococcal carriage density in pneumococcal-positive samples (n = 680). Models incorporate Generalized Estimating Equations to account for repeated sampling.

	Unadjusted coefficient (95%CI)[1]	P value	Adjusted coefficient (95%CI)	P value
Current antibiotic use[2]	-0.56 (-1.07, -0.05)	0.031	-0.74 (-1.51, 0.03)	0.059
URTI symptoms	0.27 (0.10, 0.44)	0.002	0.31 (0.15, 0.46)	<0.001
Age (months)	-0.03 (-0.06, -0.01)	0.010	-0.04 (-0.06, -0.01)	0.005
Multiple serotype carriage	0.18 (0.00, 0.37)	0.051	0.26 (0.06, 0.46)	0.011

[1]Coefficient is the difference in means (log_{10} genome equivalents/ml). For example, infants on antibiotics at the time of swabbing had a mean pneumococcal density 0.56 log_{10} lower than those not currently using antibiotics, whereas infants with URTI symptoms had a mean pneumococcal density 0.27 log_{10} higher than those without URTI symptoms. For each month increase in age, the mean pneumococcal density decreased by 0.03 log_{10}.

[2]Antibiotic use at the time of swabbing.

Table 4. Multivariable analysis of serotype-specific pneumococcal density during longer carriage episodes (detected in ≥ 3 swabs). The three-level model accounts for repeated sampling and multiple serotype carriage, and included detection stage, age, antibiotic exposure, URTI symptoms, and multiple serotype carriage. (n = 364)

	Unadjusted coefficient (95%CI)[1]	P value	Adjusted coefficient (95%CI)	P value
Stage of carriage episode				
Initial	reference		reference	
Middle	-0.36 (-0.59, -0.13)	0.002	-0.35 (-0.60, -0.09)	0.008
Final	-0.65 (-0.92, -0.38)	<0.001	-0.62 (-0.99, -0.25)	0.001
URTI symptoms	0.06 (-0.16, 0.28)	0.594	0.18 (-0.04, 0.40)	0.106
Current antibiotic use[2]	-0.90 (-1.65, -0.15)	0.019	-0.96 (-1.70, -0.23)	0.010
Multiple serotype carriage	-0.30 (-0.56, -0.05)	0.020	-0.27 (-0.53, -0.02)	0.033
Age (months)	-0.09 (-0.14, -0.04)	0.001	-0.01 (-0.09, 0.07)	0.792

[1]Coefficient is the difference in means (log_{10} genome equivalents /ml). See Table 3 footnote for further information on how to interpret the coefficient.

[2]Antibiotic use at the time of swabbing.