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

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ABSTRACT

Objectives: Nasopharyngeal carriage of *Streptococcus pneumoniae* underpins disease development and transmission. This study was performed to examine pneumococcal carriage dynamics, including density and multiple serotype carriage, in Indonesian infants during the first year of life.
Methods: Two hundred healthy infants were enrolled at 2 months of age. Eight nasopharyngeal swabs were 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: Eighty-five percent of infants carried pneumococci at least once during the study. The median age at first acquisition was 129 days (interquartile range 41–216 days). 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 the 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.

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 paediatric deaths in 2015 (Wahl et al., 2018). Pneumococci commonly colonize the nasopharynx of young children, with carriage prevalence ranging from 19% to 86% (Adegbola et al., 2014). Carriage is considered an essential first step in the development of pneumococcal disease, and is the source of transmission within human populations (Simell et al., 2012). 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 (Mitsi et al., 2016).

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Data from longitudinal studies have demonstrated that carriage is a dynamic process. The timing of acquisition and clearance is influenced by factors including serotype, age, and previous pneumococcal exposure (Hill et al., 2008; Turner et al., 2012; Abdullahi et al., 2012). Young children commonly experience multiple carriage episodes: a study in Thailand reported a median of seven pneumococcal acquisitions during the first 24 months of life (Turner et al., 2012). High pneumococcal density in the nasopharynx is associated with pneumonia in children, and linked to transmission in animal studies (Vu et al., 2011; Short et al., 2012). Simultaneous carriage of multiple pneumococcal serotypes is common in children in low- and middle-income countries (Satzke et al., 2015). Few published longitudinal carriage studies to date have 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.

A longitudinal study was conducted in Indonesia, a country with no national PCV programme, 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 (UNICEF, 2014; McAllister et al., 2019). Previous studies have reported pneumococcal carriage prevalence ranging from 43% to 53% in young children in Indonesia (Soewignjo et al., 2001; Hadinegoro et al., 2016; Farida et al., 2014; Dunne et al., 2018). The aim of this study 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 5000 per 100 000 hospital discharges (Azmi et al., 2016). Childhood mortality varies regionally and between urban and rural settings (Hodge et al., 2014). Indonesian infants routinely receive BCG, polio, HepB (birth dose), pentavalent (DTP–HepB–Hib), and measles–rubella vaccination as part of the Expanded Programme on Immunization.

Recruitment was conducted at two health centres, an urban centre in Bandung city and a semi-rural centre located in the Padalarang district approximately 25 km from Bandung city, from November 2014 to 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 to be in good health following examination by a paediatrician. Exclusion criteria included moderate/severe illness at the time of screening, axillary temperature $\geq$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 demographic characteristics and household information were collected by study staff at the initial visit. Six subsequent visits were conducted monthly, and then a final visit at 12 months of age. Data on upper respiratory tract infection (URTI) symptoms (rhinorrhoea, 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 (Satzke et al., 2013).

The sample size was based on an estimated pneumococcal carriage prevalence of 50% at 5 months of age (Soewignjo et al., 2001). One hundred and twenty participants would estimate carriage prevalence with confidence intervals of $\pm$8.9%. The sample size was increased to 200 to facilitate secondary analyses.

Ethical approval was granted by the Health Research Ethics Committee, Universitas Padjadjaran 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 Padjadjaran, Bandung for aliquoting and storage at −70 °C within 8 h of collection. Pneumococci were detected and serotyped as described previously (Dunne et al., 2018). 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 per millilitre (GE/ml) (Carvalho et al., 2007). 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 and fluorescently labelled, and microarray was conducted using Senti-SP v1.6 (BUGS Bioscience), which reports the identity and relative abundance (%) of all serotypes present within a sample (Satzke et al., 2015). Previously, this method was found to have very high sensitivity, including >93% sensitivity for the detection of secondary serotypes present in low proportions in samples containing multiple serotypes (Satzke et al., 2015). 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 the 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 (Turner et al., 2012; Heinsbroek et al., 2016; Dube et al., 2018; Tigoi et al., 2012). 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). The 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-square test was used to compare categorical data and the *t*-test or analysis of variance (ANOVA) for continuous data, unless noted otherwise. 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% confidence intervals (CI) and an 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 $\geq$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 three-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 age, antibiotic exposure, URTI symptoms, and multiple serotype carriage.

The 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).

Table 1
Characteristics of study participants at enrolment.[a]

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)	2.1 (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)
$\leq$ Regional minimum salary[b]	159 (79.5)	73 (74)	86 (84)
> Regional minimum salary	39 (19.5)	24 (24)	15 (15)
Number of children <5 years old 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)

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

Results

A total of 200 infants were enrolled in the study. Characteristics of the participants are 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 of the infants (169/198, 85.4%) carried pneumococci at least once during the study period. The median age at first acquisition was 129 days (interquartile range (IQR) 41–216 days). Figure 2 depicts the 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 material Table S1). 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 per day), or 0.14 per month (95% CI 0.12–0.15 per month).

Antibiotic usage during the study period was reported at least once for 54/198 (27.3%) study participants. Thirty-seven swabs were collected from infants currently taking oral antibiotics, 13 (35%) of which were positive for pneumococcus; 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 the swabs were collected from infants with current URTI symptoms. Current or recent antibiotic usage was negatively associated with pneumococcal carriage, whereas the presence of URTI symptoms was positively associated (Table 2).

Pneumococci identified during the study period belonged to 46 capsular serotypes and four genetic variants of non-encapsulated pneumococci (Salter et al., 2012). 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 (Manna et al., 2018). One hundred and twenty-two (38.4%) of the 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 material Figure S1. 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 days), ranging from 28 to 328 days. Four infants carried the same pneumococcal serotype for the entire study duration. The

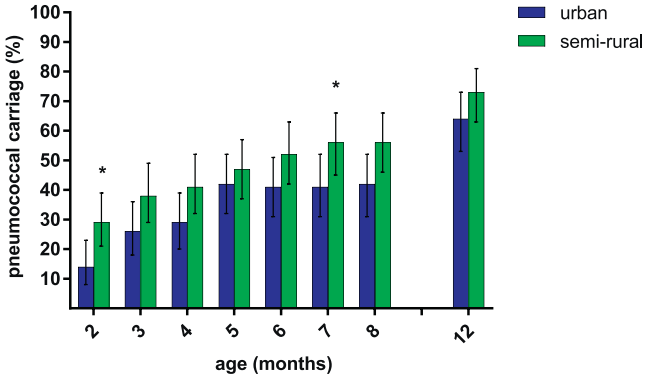

Figure 1. Pneumococcal carriage prevalence by age for infants living in urban and semi-rural areas. Bars indicate ±95% confidence interval; *$p < 0.05$, Chi-square test.

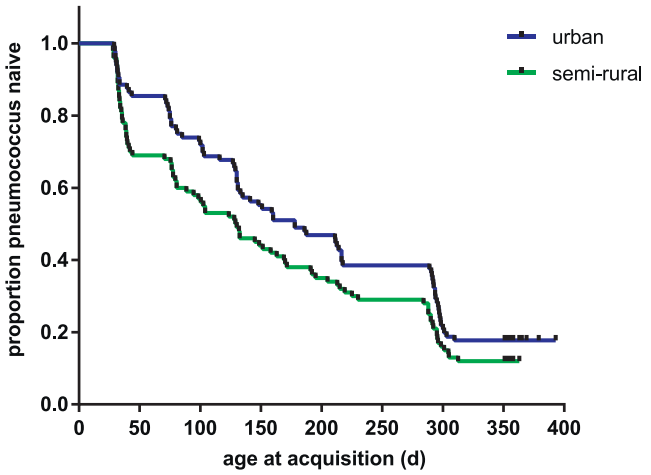

Figure 2. Age (in days) at first pneumococcal acquisition for infants living in urban and semi-rural areas; $p = 0.064$, log-rank test.

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 ($n = 1571$).

	OR (95% CI)	*p*-Value	aOR (95% CI)	*p*-Value
Antibiotic exposure[a]	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

OR, odds ratio; aOR, adjusted odds ratio; CI, confidence interval; URTI, upper respiratory tract infection.
[a] Current and/or within the previous 14 days.

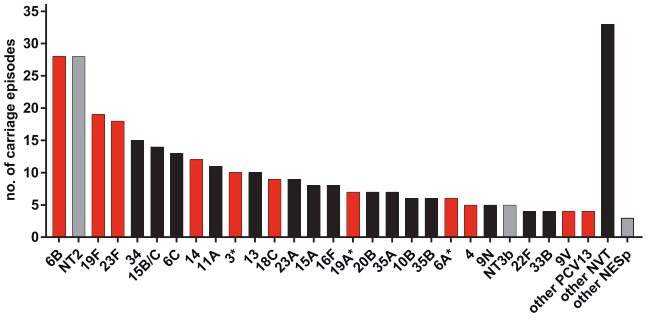

Figure 3. Pneumococcal carriage episodes by serotype. PCV13 serotypes are shown in red (* indicates the three serotypes not included in PCV10), non-PCV13 capsular serotypes are shown in black, and non-encapsulated pneumococci (categorized into genetic variants (Salter et al., 2012) are shown in grey. '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 ($n = 1$).

median carriage duration for the first pneumococcal acquisition was 158 days (IQR 84–260 days), compared with 95 days (IQR 56–161 days) 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 $\log_{10}$ GE/ml). Pneumococcal density is shown by age and site in Supplementary material Figure S2. 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 material Figure S1). Fifty-nine of 198 participants (29.8%) 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 $\log_{10}$ GE/ml) compared with the final detection prior to clearance (5.71 $\log_{10}$ GE/ml, 95% CI 5.47–5.95 $\log_{10}$ GE/ml) (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 compared with initial detection (Table 4).

The carriage dynamics of the 166 participants who carried pneumococci at least once were examined 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 eight (4.8%) participants. For 23 (13.8%) participants, no pattern was determined, as multiple serotype carriage was only observed at the final swab collection.

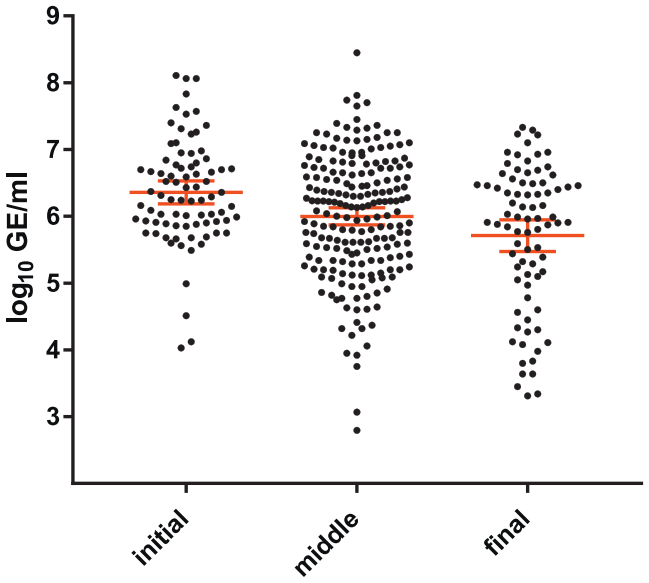

Figure 4. Serotype-specific pneumococcal carriage density (in $\log_{10}$ genome equivalents/ml) shown by stage of carriage episode, for episodes that were detected in ≥3 swabs. Detection stages defined as initial detection, middle (subsequent detections), and final (last detection prior to clearance).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

Discussion

At 12 months of age, 68% of Indonesian infants carried pneumococcus, with a median age at acquisition of 129 days. These findings reflect 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 USA, the mean age at acquisition was 6 months (Leino et al., 2001; Gray et al., 1980). 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 (Hill et al., 2008; Turner et al., 2012; Heinsbroek et al., 2016; Dube et al., 2018; Tigoi et al., 2012). In these settings, pneumococcal carriage prevalence peaked by 6 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 (Turner et al., 2012; Dube et al., 2018). We previously reported a pneumococcal carriage prevalence of 64% in children aged 12–24 months old in the Bandung region, suggesting that carriage rates stabilize after 12 months in this population (Dunne et al., 2018). Carriage prevalence tended to be higher in infants living in semi-rural areas than in urban residents, as observed in some, but not all, other studies (Hanieh et al., 2014; Nisar et al., 2018). 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 (Fadlyana et al., 2018).

Data from Indonesia could inform models for pneumococcal vaccine trials in similar settings, particularly in Asia, where PCV introduction has lagged behind other regions (Tricarico et al., 2017; Cai et al., 2018). Data on serotypes causing invasive pneumococcal disease in Indonesia are extremely limited, a major knowledge gap for this country. It was therefore not possible to correlate carriage data with serotypes causing disease. Modelling approaches have used carriage data to predict serotypes causing invasive disease and vaccine impact and may be an avenue for future investigation (Weinberger et al., 2011; Flasche et al., 2015).

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[a] (95% CI)	p-Value	Adjusted coefficient (95% CI)	p-Value
Current antibiotic use[b]	−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

CI, confidence interval; URTI, upper respiratory tract infection.
[a] 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 $\log_{10}$.
[b] 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[a] (95% CI)	*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[b]	−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

URTI, upper respiratory tract infection; CI, confidence interval.
[a] Coefficient is the difference in means ($\log_{10}$ genome equivalents/ml). See Table 3 footnote for further information on how to interpret the coefficient.
[b] Antibiotic use at the time of swabbing.

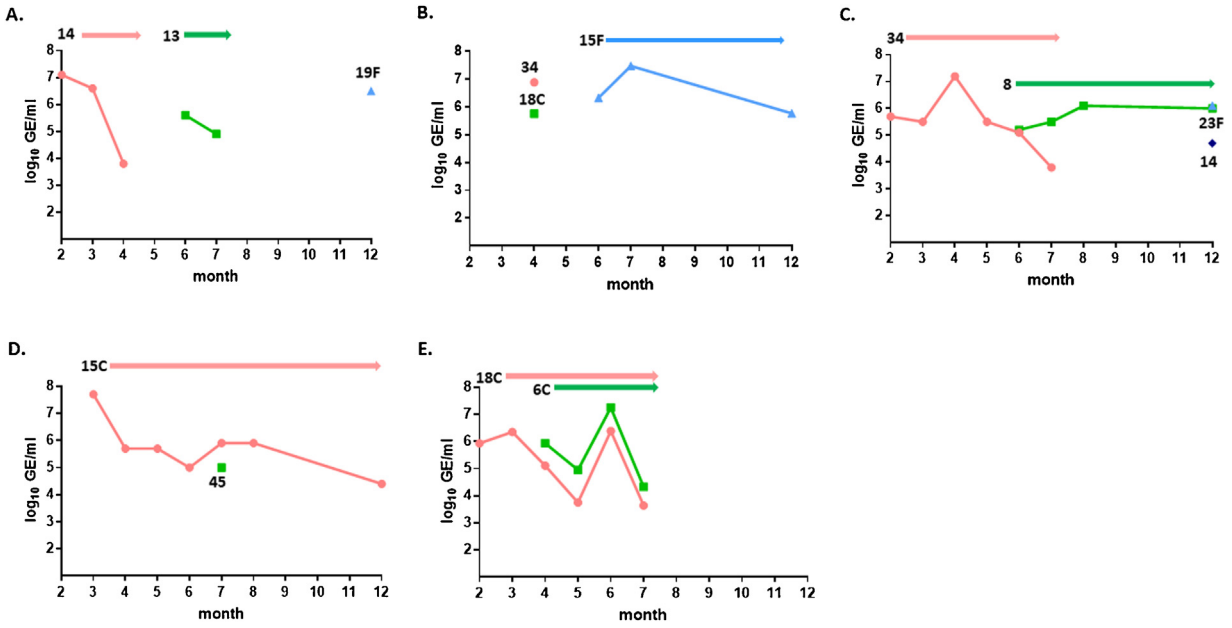

Figure 5. Patterns of pneumococcal carriage dynamics. Representative examples, each from an individual study participant, are shown for the following patterns: (A) single serotype carriage; (B) short-term co-colonization; (C) serotype replacement; (D) serotype dominance; (E) stable co-colonization. Serotype-specific 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 dark blue.

It was found in this study that the duration of carriage was longer for initial pneumococcal acquisitions than for 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 (Jochems et al., 2017). Epidemiological 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 (Granat Simo et al., 2009). In Thailand, the interval to reacquisition of common serotypes was longer in children who had previously carried either homologous or heterologous serotypes, and the carriage duration of serotypes 14 and 19F was shorter when reacquired following homologous carriage (Turner et al., 2012). Serotype 6B, which was the most commonly acquired serotype in the current study, was not among the 10 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 (Dunne et al., 2018). A study in Israeli toddlers found that children who had 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 (Weinberger et al., 2008). 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 (Baggett et al., 2017; Brotons et al., 2017). The present 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. It

was 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.

This study 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 displayed decreasing pneumococcal density in the nasopharynx prior to clearance (Neill et al., 2014). Bacterial characteristics may also affect colonization density: a cross-sectional study reported that pneumococcal density varies by serotype (Rodrigues et al., 2016). 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 of this study was the sampling time frame, with monthly intervals between swab collection and an approximate 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 2 months of age, it was not possible 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 (Hill et al., 2008; Dube et al., 2018).

Wider sampling time frames bias estimates of carriage duration upwards, so this may in part explain why the median carriage duration in this 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) (Hill et al., 2008; Turner et al., 2012; Abdullahi et al., 2012; Dube et al., 2018). 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 (Hill et al., 2008; Turner et al., 2012). Serotype, antimicrobial resistance, and prophage were found to be associated with carriage duration in a large pneumococcal genomics study (Lees et al., 2017).

No testing of viruses or detection of other colonizing bacteria such as *Haemophilus influenzae* that can influence the density and dynamics of pneumococcal carriage was conducted (Dunne et al., 2018; Chien et al., 2013; Lewnard et al., 2016). The positive association between URTI symptoms and pneumococcal carriage rates and density observed in this study is consistent with several studies reporting increased pneumococcal density during viral URTIs (Vu et al., 2011; Morpeth et al., 2018). In children under 3 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 (Fan et al., 2016). Interestingly, DeMuri et al. recently demonstrated that pneumococcal densities were higher in American children aged 4–7 years when a respiratory virus was detected, regardless of whether the children displayed URTI symptoms (DeMuri et al., 2018). HIV status was not determined for mothers or infants in this study, but as the prevalence of HIV in the general population of Indonesia is estimated to be <0.5%, a similar low rate amongst participants was assumed (Januraga et al., 2018).

The laboratory methods utilized in this study enabled discrimination of different pneumococcal colonization patterns. Carriage of a single serotype at a time was most common, in line with epidemiological data and a mouse model indicating that pneumococcal carriage inhibits the acquisition of a second pneumococcal strain (Auranen et al., 2010; Lipsitch et al., 2000). Recently, the competitive advantage of an established pneumococcal strain against newcomers was found to be dependent on the quorum sensing system (Shen et al., 2019).

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 (Dawid et al., 2007; Wu et al., 2017).

As multiple serotype carriage was highest at 12 months in this 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.

This 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 in the development of vaccines that can harness the immunizing effects of colonization, and also improve mathematical models of pneumococcal carriage. The study 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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Conflict of interest

St George's, University of London, UK (SGUL), but not JH, has received funding from GSK, Sanofi Pasteur and Pfizer for research conducted by JH as an SGUL employee. JH is co-founder, board member and shareholder of BUGS Bioscience, a not-for-profit spin-out company of SGUL, but JH receives no personal income from this activity. EMD, CN, CS, and EKM have received research funding from Pfizer.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.06.024.

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