Vaccine 37 (2019) 296–305

Contents lists available at ScienceDirect

Vaccine

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

Pneumococcal carriage in vaccine-eligible children and unvaccinated infants in Lao PDR two years following the introduction of the 13-valent pneumococcal conjugate vaccine

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ARTICLE INFO

Article history:
Received 6 August 2018
Received in revised form 11 October 2018
Accepted 23 October 2018
Available online 28 November 2018

Keywords:
13-Valent pneumococcal conjugate vaccine
Vaccine impact
Nasopharyngeal pneumococcal carriage
Herd immunity
Lower-middle income country
Antimicrobial resistance

ABSTRACT

Pneumococcal carriage is a prerequisite for disease, and underpins herd protection provided by pneumococcal conjugate vaccines (PCVs). There are few data on the impact of PCVs in lower income settings, particularly in Asia. In 2013, the Lao People's Democratic Republic (Lao PDR) introduced 13-valent PCV (PCV13) as a 3 + 0 schedule (doses at 6, 10 and 14 weeks of age) with limited catch-up vaccination. We conducted two cross-sectional carriage surveys (pre- and two years post-PCV) to assess the impact of PCV13 on nasopharyngeal pneumococcal carriage in 5–8 week old infants (n = 1000) and 12–23 month old children (n = 1010). Pneumococci were detected by quantitative real-time PCR, and molecular serotyping was performed using DNA microarray. Post PCV13, there was a 23% relative reduction in PCV13-type carriage in children aged 12–23 months (adjusted prevalence ratio [aPR] 0.77 [0.61–0.96]), and no significant change in non-PCV13 serotype carriage (aPR 1.11 [0.89–1.38]). In infants too young to be vaccinated, there was no significant change in carriage of PCV13 serotypes (aPR 0.74 [0.43–1.27]) or non-PCV13 serotypes (aPR 1.29 [0.85–1.96]), although trends were suggestive of indirect effects. Over 70% of pneumococcal-positive samples contained at least one antimicrobial resistance gene, which were more common in PCV13 serotypes ($p < 0.001$). In 12–23 month old children, pneumococcal density of both PCV13 serotypes and non-PCV13 serotypes was higher in PCV13-vaccinated compared with undervaccinated children ($p = 0.004$ and $p < 0.001$, respectively). This study provides evidence of PCV13 impact on carriage in a population without prior PCV7 utilisation, and provides important data from a lower-middle income setting in Asia. The reductions in PCV13 serotype carriage in vaccine-eligible children are likely to result in reductions in pneumococcal transmission and disease in Lao PDR.

1. Introduction

Streptococcus pneumoniae (the pneumococcus) is a leading cause of childhood mortality worldwide, causing approximately 826,000 deaths each year [1]. Pneumococcal diseases range from

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https://doi.org/10.1016/j.vaccine.2018.10.077
0264-410X/

mild to severe, including pneumonia, meningitis and sepsis. There are over 95 immunologically distinct capsular types (serotypes). Current paediatric vaccines target 10 or 13 of the most common disease causing serotypes.

Nasopharyngeal carriage is considered a prerequisite for disease [2], and is the primary ecological niche for this human pathogen. Importantly, carriage underpins the powerful herd effects of pneumococcal conjugate vaccines (PCVs) as vaccinated individuals are less likely to carry vaccine serotypes, and therefore less likely to transmit the bacteria to unvaccinated individuals in the community [3]. PCVs result in substantial individual (direct) and herd (indirect) protection against pneumococcal disease [3,4].

PCVs have been introduced nationally into 59 low- and middle-income countries (LMICs) [5]. However, there are few data on vaccine impact in LMIC settings, particularly from Asia. There is also a paucity of data on the indirect effects for children who are too young to be vaccinated [4,6,7], but who have high case fatality rates from pneumococcal disease [8]. Demonstrating vaccine impact helps justify the cost of PCV introduction and maintenance, particularly as countries graduate from Gavi eligibility. It is important to monitor serotype replacement, whereby non-vaccine serotypes become more common in carriage and disease following vaccination, as this may erode vaccine impact over time [9–11]. In many settings, particularly where the pneumococcal disease burden is high, there is insufficient surveillance to measure vaccine impact on pneumococcal disease. Although they have limitations, carriage studies are an efficient and meaningful approach to measure prevalence of pneumococci and demonstrate the biological effect of PCV in a population [12]. Carriage studies may be the only tool available in some settings.

As well as substantially reducing carriage and disease by vaccine-type pneumococci, PCV introduction can reduce antimicrobial resistance (AMR), as vaccine serotypes are more likely to be resistant compared with non-vaccine serotypes [13]. Although there is emerging clinical and experimental evidence for the importance of pneumococcal nasopharyngeal density on the likelihood of disease and transmission [14,15], the effect of PCVs on pneumococcal density is largely unknown [16,17].

The Lao People's Democratic Republic (Lao PDR) has a high child mortality rate of 67/1000 live births and a high burden of childhood pneumonia (https://www.gavi.org/country/lao-pdr/ [Accessed 21 May 2018]). The aim of this study was to describe pneumococcal carriage in children before and after PCV13 introduction in Lao PDR. To do this, we conducted two cross-sectional community carriage surveys (pre- and two years post-PCV13 introduction) in children aged 12–23 months and 5–8 weeks to assess direct and indirect effects. We applied sensitive molecular microbiological approaches [18] to describe overall pneumococcal, PCV13 serotype and non-PCV13 serotype carriage rates and density, and the presence of AMR genes, pre- and post-PCV13 introduction.

2. Methods

2.1. Study site

Lao PDR is a South East Asian lower-middle income country of approximately seven million inhabitants. Lao PDR is ethnically diverse, with 49 government-recognised ethnic groups that compose approximately 34% of the population [19]. In 2012, 22.7% of the population lived below the international poverty line (http://povertydata.worldbank.org/). With support from Gavi, the Vaccine Alliance, Lao PDR introduced PCV13 into the national immunisation program in late 2013, with three doses administered at 6, 10 and 14 weeks of age, with catch-up vaccination to 12 months of age. National coverage of the third dose of PCV13 was estimated at 72% for 2014, 77% for 2015, and 78% for 2016 (http://www.who.int/immunization/monitoring_surveillance/data/lao.pdf). This study was conducted in Vientiane, the capital and largest city in Lao PDR, and the nearby rural Bolikhamxay Province.

2.2. Study design and participants

Cross-sectional carriage surveys were conducted from November 2013 – February 2014 ("pre-PCV"), and November 2015 – February 2016 ("post-PCV"), to assess pneumococcal carriage at baseline and approximately two years following PCV13 introduction.

Participants were a convenience sample of children living in urban and rural areas; provinces were selected on the basis of access to a laboratory to store the swabs on the day of collection, and status of PCV13 introduction. Urban study participants were identified and recruited from participating maternal and child health centres in Vientiane Capital Province during routine clinic visits. Rural participants were recruited during maternal and child health visits, and visits to surrounding households, in Bolikhamxay Province.

Eligibility criteria were age (5–8 week old infants and 12–23 month old children), an axillary temperature $\leq$37 °C and having lived in the area for at least three consecutive months. Children and infants were excluded from the first survey if they had received any dose of PCV13, and from the second survey if they were aged 5–8 weeks and had received PCV13.

Sample size was determined based on the calculation that 281 participants would detect a 50% reduction, with 90% power at a 5% significance level, in PCV13 serotype prevalence assuming a baseline PCV13 carriage prevalence of 16% [20]. As the true prevalence of pneumococcal carriage in Lao PDR was unknown, the sample size for both age groups was increased to 500 per survey.

The study was conducted according to the protocol approved by the Lao PDR Ministry of Health National Ethics Committee for Health Research (061-NECHR), Western Pacific Regional Office Ethics Review Committee and The Royal Children's Hospital/Murdoch Children's Research Institute Human Research Ethics Committee (HREC 33177A/HREC 33177B). Written informed consent was obtained from the parent or guardian for all participants prior to any study procedures being conducted. To identify potential confounders, the study staff completed a questionnaire documenting potential risk factors for pneumococcal carriage. PCV13 vaccination status was verified by checking the child's national vaccination status card or confirming with health centre records.

2.3. Sample collection and transport

Sample collection, handling and storage were performed according to World Health Organization guidelines [21]. In brief, nasopharyngeal samples were collected using paediatric flocked swabs (Copan Diagnostics) and then placed into 1 ml of skim milk tryptone glucose glycerol medium (STGG). Samples were transported to the Lao-Oxford-Mahosot-Hospital-Wellcome Trust-Research Unit (LOMWRU, Vientiane, Lao PDR) before being vortexed, dispensed into aliquots and stored at ultra-low temperatures. Samples were shipped on dry ice to the Murdoch Children's Research Institute (Parkville, Australia) for laboratory testing.

2.4. STGG DNA extraction and lytA qPCR

To screen for the presence, and to determine the colonisation density, of pneumococci, real-time quantitative PCR targeting the *lytA* gene (*lytA* qPCR) was conducted on the STGG samples. First, genomic DNA was extracted from 100 µl STGG using the MagNA

Pure LC machine (Roche) [22]. *LytA* qPCR [23], with primer and probe concentrations of 100 nM and 200 nM, respectively, was performed on all extracted DNA samples. Final reaction volumes of 25 μl were run on a Stratagene Mx3005 machine using 2 μl template DNA and Brilliant III Ultra-Fast qPCR Master Mix (Agilent Technologies), according to manufacturer's instructions. Reactions were performed in duplicate, with the carriage density (in genome equivalents/ml) calculated using the average cycle threshold (Ct) value, by reference to a standard curve prepared from reference isolate genomic DNA [24]. For these calculations, an assumption was made that each genome has one copy of the *lytA* gene, and that the genome size is 2 Mb. Samples that were *lytA* qPCR positive (Ct value < 35) or equivocal (Ct value 35–40) were cultured for molecular serotyping by microarray.

2.5. Culture, DNA extraction and microarray

50 μl of STGG were cultured on horse blood agar containing 5 μg/ml of gentamicin (Oxoid). DNA was extracted from the harvested growth with the QIAcube HT instrument (Qiagen) and QIAamp 96 DNA QIAcube HT Kit (Qiagen), using a lysis buffer (20 mM Tris/HCl, 2 mM EDTA, 1% v/v Triton, 20 mg/ml lysozyme) and RNase A treatment [18,25]. When only a single α-haemolytic colony grew, it was subcultured prior to DNA extraction for microarray, or in some cases serotyped using latex agglutination [26]. Molecular serotyping by microarray was performed on the extracted DNA using Senti-SPv1.5 microarrays (BUGS Bioscience), as described previously [18]. The microarray data was analysed using a custom web-based software that uses a Bayesian-based model [27].

2.6. Assessment of pneumococcal carriage

Pneumococcal carriage, and the presence and relative abundance of each pneumococcal serotype, was determined by microarray. PCV13 serotypes included serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F. All other serotypes, as well as non-encapsulated pneumococci [28], were considered non-PCV13 serotypes. Serotypes 15B and 15C were reported as 15B/C as these serotypes are known to interconvert [29]. Samples that were *lytA* qPCR positive (Ct value < 35) but not able to be serotyped (either culture negative or low DNA yield from culture) were considered pneumococcal positive, serotype unknown. Samples that were equivocal by *lytA* qPCR (Ct value 35–40) and culture-negative were considered pneumococcal negative. A swab that contained both PCV13 and non-PCV13 serotype(s) was considered positive for both PCV13 and non-PCV13 carriage, irrespective of relative abundance or serotype-specific density. To determine serotype-specific density (genome equivalents/ml), the overall pneumococcal density (as determined by *lytA* qPCR) was multiplied by the per cent relative abundance of each serotype (as determined by microarray).

The microarray detects 10 AMR genes associated with mobile genetic elements, encoding tetracycline (*tetM*, *tetK*, *tetO*, *tetL*), chloramphenicol (*cat*), macrolides (*mefA*), kanamycin (*aphA3*), streptothricin (*sat4*), and *ermB* and *ermC* genes which encode resistance to macrolides including erythromycin, as well as resistance to lincosamides and streptogramin B. To examine the presence of AMR genes, we restricted analysis to samples containing a single pneumococcal type with no other species identified.

2.7. Statistical analysis

Demographic data were double data entered into EpiData version 3.1 databases. The two separate EpiData files were validated, and corrections made as required using the source document (data collection form). Further cleaning was conducted in Stata version 15.1 (StataCorp. 2017. *Stata Statistical Software: Release 15*. College Station, TX: StataCorp LLC). Laboratory data was imported from Excel (Microsoft® Office) to Stata and merged to the demographic Stata file.

Statistical analyses was performed using GraphPad Prism version 7.04 for Windows, (GraphPad Software) and Stata versions 14.2 and 15.1. The Chi-squared test was used to compare categorical data and the Kruskal-Wallis test for continuous data unless otherwise noted.

Carriage prevalence of overall pneumococci, PCV13 serotypes, and non-PCV13 serotypes were determined for the pre- and post-PCV periods. For each age group, prevalence ratios were calculated by comparing the post-PCV13 period with the pre-PCV13 period. The association between overall carriage and potential confounders shown in Table 1 were examined initially via univariable logistic regression. Those considered associated with overall carriage ($p < 0.1$) were included in multivariable log-binomial regression models to estimate adjusted prevalence ratios. Month of swab collection was also included in the adjusted models. If log-binomial models did not converge, Poisson models with robust 95% confidence intervals (CI) were used [30]. Results are reported as prevalence ratios (PR) and adjusted PR (aPR) with 95% CIs. Reductions in PCV13-serotype carriage were calculated as $(1 - \text{aPR}) * 100\%$.

Bacterial density data were $\log_{10}$ transformed prior to analysis to remove skewness, and results reported as $\log_{10}$ genome equivalents/ml ($\log_{10}$ GE/ml). As some density datasets were not normally distributed, non-parametric methods were used for analysis. Density data were examined by survey period (pre- and post-PCV13) for both age groups. To examine potential direct effects of PCV13 on pneumococcal density, median carriage densities were calculated for vaccinated (2 or 3 doses of PCV13) and undervaccinated 12–23 month old children (0 or 1 dose of PCV13), and compared using quantile regression. To adjust for potential confounders, a multivariable quantile regression model included variables identified as associated with overall pneumococcal density by univariable analysis ($p < 0.1$).

3. Results

Characteristics of the study participants (n = 2010) are shown in Table 1. Several characteristics, including exposure to cigarette and cooking fuel smoke, having symptoms of an upper respiratory tract infection (URTI), and antibiotic use in the proceeding fortnight were significantly different between the two survey periods. In the post-PCV13 period, 90% of participants aged 12–23 months had received PCV13. None of the 5–8 week old infants were vaccinated.

We determined the pneumococcal carriage status of 2009 children; one swab was excluded for technical reasons. Table 2 shows the carriage prevalence and prevalence ratios for overall pneumococcal carriage pre- and post-PCV13 introduction.

Serotyping results were obtained from 660 of 668 pneumococcal-positive samples. A total of 749 pneumococci were identified. These included 637 capsular pneumococci belonging to 41 different capsular serotypes, and 112 non-encapsulated pneumococci from five different genetic lineages, with 98% belonging to the NT2 lineage [28]. Eleven of the PCV13 serotypes were detected (serotypes 1 and 5 were not identified).

Following PCV13 introduction (Table 2), there were no significant changes in the carriage prevalence of PCV13 serotypes or non-PCV13 serotypes in 5–8 week old infants, although the expected trends of decreasing PCV13 serotype carriage and increasing non-PCV13 serotype carriage were observed. In 12–23 month old children, carriage prevalence of PCV13 serotypes

Table 1
Characteristics of study participants in cross-sectional community pneumococcal carriage surveys, performed pre- and two years post-PCV13, in children in Lao PDR, by age group and time period.

5–8 week old infants	Pre-PCV13 N = 498[a]	Post-PCV13 N = 502	P value
Median age in weeks (IQR)[b]	6.7 (6.4–7.1)	6.7 (6.6–7.0)	0.475
Female sex, n (%)		n = 501	
	242 (48.6)	248 (49.5)	0.774
Ethnicity, n (%)		n = 501	
Lao Loum	480 (96.4)	485 (96.8)	0.714
Minority groups	18 (3.6)	16 (3.6)	
Residential location, n (%)			
Urban	460 (92.4)	469 (93.4)	0.515
Rural	38 (7.6)	33 (6.6)	
Two or more children <5 years in household, n (%)	195 (39.2)	215 (42.8)	0.238
Exposure to household cigarette smoke, n (%)	n = 497	n = 500	
	201 (40.4)	166 (33.2)	0.018
Symptoms of URTI[c], n (%)	97 (19.5)	71 (14.1)	0.024
Antibiotic use in previous fortnight, n (%)	n = 502		
	24 (4.8)	5 (1.0)	<0.001
Poverty[d], n (%)	31 (6.2)	31 (6.2)	0.974
Born by vaginal delivery, n (%)	410 (82.3)	352 (70.1)	<0.001
Breastfeeding at time of survey, n (%)		n = 501	
	410 (82.3)	435 (86.8)	0.049
Main source of cooking fuel, n (%)			
Electricity	88 (17.7)	57 (11.4)	0.005
Charcoal	256 (51.4)	255 (50.8)	0.847
Wood	65 (13.0)	99 (19.7)	0.004
Gas	89 (17.9)	90 (17.9)	0.981
Kerosene	0 (0.0)	1 (0.2)	0.319
12–23 month old children	Pre-PCV13 N = 503	Post-PCV13 N = 507	P value
Median age in months (IQR)	16.6 (14.7–20.0)	16.1 (14.6–20.3)	0.808
Female sex, n (%)	250 (49.7)	276 (54.4)	0.132
Ethnicity, n (%)			
Lao Loum	474 (94.2)	489 (96.4)	0.095
Minority groups	23 (5.8)	18 (3.6)	
Residential location, n (%)			
Urban	250 (49.7)	290 (57.2)	0.017
Rural	253 (50.3)	217 (42.8)	
Two or more children <5 years in household, n (%)	167 (33.2)	182 (35.9)	0.368
Exposure to household cigarette smoke, n (%)	n = 502	n = 505	0.736
	222 (44.2)	218 (43.2)	
Symptoms of URTI, n (%)	373 (74.2)	264 (52.1)	<0.001
Previously vaccinated with PCV13[e], n (%)		n = 502	<0.001
	0 (0.0)	453 (90.2)	
Antibiotic use in previous fortnight, n (%)	n = 501	n = 506	<0.001
	231 (46.1)	174 (34.4)	
Poverty[d], n (%)	34 (6.8)	19 (3.8)	0.032
Born by vaginal delivery, n (%)	426 (84.7)	405 (79.9)	0.045
Breastfeeding at time of survey, n (%)	n = 502		
	410 (81.7)	398 (78.5)	0.207
Main source of cooking fuel, n (%)	n = 501		
Electricity	48 (9.6)	47 (9.3)	0.866
Charcoal	254 (50.7)	228 (45.0)	0.069
Wood	123 (24.5)	154 (30.4)	0.038
Gas	76 (15.2)	77 (15.2)	0.994
Kerosene	0 (0.0)	1 (0.2)	0.320

[a] Unless otherwise specified.
[b] IQR = Interquartile range.
[c] URTI = upper respiratory tract infection symptoms (includes any of the following: ear discharge, runny nose, and/or cough at time of survey).
[d] Rural poverty <196412.8 LKP/week; urban poverty <221391.1 LKP/week) [51].
[e] received at least 2 doses of PCV13 prior to survey.

was significantly lower in the post-PCV13 period, while there was only a slight, non-significant increase in carriage prevalence of non-PCV13 serotypes.

Fig. 1 shows the carriage prevalence for each of the PCV13 serotypes and the 10 most common non-PCV13 serotypes in both age groups, pre- and post-PCV13 introduction. For 5–8 week old infants, 32/71 (45%) of pneumococci belonged to PCV13 serotypes in the pre-PCV13 survey, compared with 27/90 (30%) post-PCV13 introduction (p = 0.049). The carriage prevalence of serotype 6A was higher pre-PCV13 compared with the post-PCV13 survey (11/495 [2.2%] vs 3/501 [0.6%], p = 0.030), whereas serotype 23A prevalence was lower pre-PCV13 (0/495 [0.0%] vs post-PCV13 4/501 [0.8%], p = 0.046). The most common serotypes in the pre-PCV13 period were 6A (n = 11), 15B/C, 6B, and NT2 (n = 6 each), compared with NT2 (n = 15), 15B/C (n = 12), and 15A, 23F, 6B, and 6C (n = 5 each) in the post-PCV13 period.

For 12–23 month old children, 176/323 (54.5%) of pneumococci belonged to PCV13 serotypes in the pre-PCV13 survey, compared

Table 2
Carriage prevalence and prevalence ratios for pneumococcal carriage (overall, PCV13 serotypes, and non-PCV13 serotypes) for 5–8 week old infants (5–8 wk) and 12–23 month old children (12–23 mo) pre- and two years post-PCV13 introduction.

	Pre-PCV13 prevalence (%) (95% CI)	Post-PCV13 prevalence (%) (95% CI)	Unadjusted prevalence ratio (95% CI)	Adjusted prevalence ratio[a] (95% CI)
All pneumococci[b]				
5–8 wk	14.3 (11.3–17.6)	17.1 (13.9–20.7)	1.20 (0.90–1.60)	1.05 (0.77–1.43)
12–23 mo	55.8 (51.3–60.2)	45.6 (41.2–50.0)	0.82 (0.72–0.92)	0.89 (0.78–1.02)[c]
PCV13 serotypes				
5–8 wk	6.5 (4.5–9.0)	5.2 (3.4–7.5)	0.80 (0.49–1.33)	0.74 (0.43–1.27)
12–23 mo	32.9 (28.8–37.2)	19.8 (16.4–23.6)	0.60 (0.49–0.75)	0.77 (0.61–0.96)[c]
Non-PCV13 serotypes				
5–8 wk	7.7 (5.5–10.4)	12.2 (9.4–15.4)	1.59 (1.08–2.33)	1.29 (0.85–1.96)
12–23 mo	26.9 (23.1–31.1)	30.0 (26.0–34.2)	1.11 (0.91–1.35)	1.11 (0.89–1.38)

[a] The following variables were used to adjust the prevalence ratios for each age group: 5–8 week old (ethnicity, residential location, two or more children under five years in the household, main source of cooking fuel, mode of delivery, poverty, and month of swab collection); 12–23 months old (ethnicity, residential location, two or more children under five years in the household, URTI symptoms, mode of delivery, main source of cooking fuel, and month of swab collection).
[b] Overall carriage prevalence does not necessarily equal the sum of PCV13 serotype and non-PCV13 serotype prevalence. This is due to multiple serotype carriage and/or exclusion of pneumococcal-positive samples for which serotype was not determined from analysis for PCV13 and non-PCV13 serotype carriage.
[c] Log-Binomial models did not converge, so Poisson models with robust 95% confidence intervals (CI) were used.

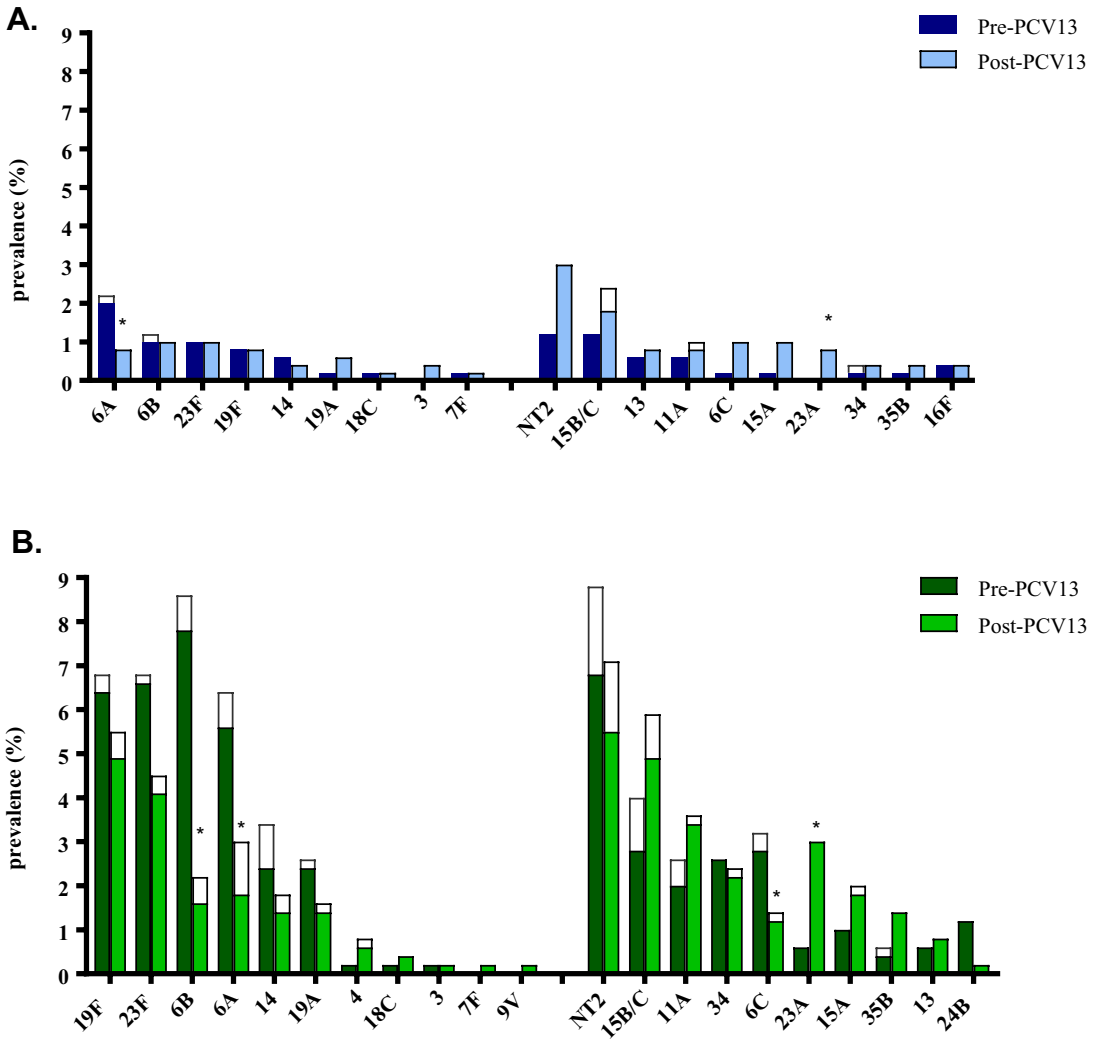

Fig. 1. Carriage prevalence of PCV13 serotypes and the 10 most common non-PCV13 serotypes in 5–8 week old infants (A) and 12–23 month old children (B) before (pre-PCV13) or after (post-PCV13) vaccine introduction. Solid bars indicate carriage that was detected as a single or major (dominant) serotype, open bars indicate carriage that was detected as a minor (second or third) serotype. NT2 = non-encapsulated pneumococci containing *nspA*/*pspK* [28,52]. *$p < 0.05$, chi-squared test.

with 102/265 (38.5%) post-PCV13 introduction ($p < 0.001$). The carriage prevalence of PCV13 serotypes 6A and 6B, and non-PCV13 serotype 6C decreased following PCV13 introduction: for 6A, 32/501 (6.4%) pre-PCV13 compared with 15/504 (3.0%) post-PCV13, $p = 0.010$; for 6B, 43/501 (8.6%) pre-PCV13 compared with 11/504 (2.2%) post-PCV13, $p < 0.001$; for 6C, 16/501 (3.2%)

pre-PCV13 compared with 6/504 (1.2%) post-PCV13, p = 0.030. In contrast, non-PCV13 serotype 23A prevalence increased following PCV introduction (from 3/501 [0.6%] to 15/504 [3.0%], p = 0.004). The most common serotypes identified in this age group pre-PCV13 were NT2 (n = 44), 6B (n = 43), 19F (n = 34) and 23F (n = 34) compared with NT2 (n = 36), 15B/C (n = 30) and 19F (n = 28) post-PCV13.

The majority of pneumococcal-positive samples contained a single serotype (575/660, 87.1%); multiple serotype carriage was relatively uncommon (85/660, 12.9%). Infants aged 5–8 weeks carried a maximum of two serotypes, whereas children aged 12–23 months carried up to three. The proportion of children with multiple serotype carriage did not differ significantly pre- and post-PCV13 introduction. For 5–8 week old infants, the prevalence of multiple serotype carriage was 0.6% (95% CI: 0.1–1.7) pre-PCV13 and 1.0% (95% CI: 0.3–2.3) post-PCV13 introduction (p = 0.488). For 12–23 month old children, the prevalence of multiple serotype carriage was 8.2% (95% CI: 5.9–10.9) pre-PCV13, and 7.1% (95% CI: 5.0–9.7) post-PCV13 introduction (p = 0.535).

AMR genes were common, with 70.8% of samples containing at least one of the 10 AMR genes assessed. PCV13 serotypes were more likely to have at least one AMR gene detected (specifically *tetM*, *cat*, *mefA*, and *ermB*), as well as being more likely to carry ≥ 3 AMR genes compared with non-PCV13 serotypes (Table 3). Non-PCV13 serotypes were more likely to carry *aphA3* than PCV13 serotypes. When examining the pre- and post-PCV13 surveys, we found that the proportion of samples from 5–8 week old infants containing AMR genes did not change. However, in the 12–23 month old children, the proportion of samples containing *cat* decreased whilst *sat4*, *ermB* and *ermC* increased (Table 4).

In children who carried pneumococci, densities for overall pneumococci, PCV13 serotypes, and non-PCV13 serotypes were higher in the post-PCV13 period compared with the pre-PCV13 period for both age groups (Fig. 2). In 12–23 month old children, the density of pneumococci overall, PCV13 serotypes, and non-PCV13 serotypes were higher in PCV13 vaccinated (2 or 3 doses) compared with undervaccinated (0 or 1 dose) children (Table 5). Individual serotype densities were examined for the eight most common serotypes (PCV13 serotypes 19F, 23F, 6B, 6A, and 14, and non-PCV serotypes NT2, 15B/C, and 11A). No differences in median density of individual serotypes were observed between PCV13 vaccinated and undervaccinated children (Supplementary Table).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.10.077.

4. Discussion

There is a paucity of data on the impact of PCVs from Asia, particularly from LMICs. In this study, we describe pneumococcal carriage in children aged 12–23 months and 5–8 week old infants, who are too young to be vaccinated, before and two years after PCV13 introduction in Lao PDR. In children aged 12–23 months, there was a 23% reduction in PCV13 serotype carriage two years after vaccine introduction. Although there was no significant increase in carriage of non-vaccine serotypes, we observed a trend

Table 3
Antimicrobial resistance genes detected by microarray in nasopharyngeal samples from Laotian children aged 5–8 weeks and 12–23 months that contained a single pneumococcal serotype with no other species identified. Detection rate of antimicrobial resistance genes shown for all pneumococci, PCV13 serotypes, and non-PCV13 serotypes.

Antimicrobial resistance gene	Encodes resistance to	Detected in all pneumococci (N = 519) n (%)	Detected in PCV13 serotypes (N = 236) n (%)	Detected in non-PCV13 serotypes (N = 283) n (%)	P value[a]
tetM	Tetracycline	305 (58.8)	199 (84.3)	106 (37.5)	<0.001
tetK	Tetracycline	47 (9.1)	16 (6.8)	31 (11.0)	0.099
tetO	Tetracycline	1 (0.2)	0 (0.0)	1 (0.4)	0.361
tetL	Tetracycline	1 (0.2)	1 (0.4)	0 (0.0)	0.273
cat	Chloramphenicol	83 (16.0)	64 (27.1)	19 (6.7)	<0.001
mefA	Macrolides	127 (24.5)	81 (34.3)	46 (16.2)	<0.001
aphA3	Kanamycin	20 (3.8)	4 (1.7)	16 (5.6)	0.020
sat4	Streptothricin	16 (3.1)	4 (1.7)	12 (4.2)	0.095
ermB	Erythromycin	159 (30.6)	87 (36.8)	72 (25.4)	0.005
ermC	Erythromycin	22 (4.2)	6 (2.5)	16 (5.6)	0.080
Any antimicrobial resistance gene		368 (70.9)	207 (87.7)	161 (56.9)	<0.001
≥3 antimicrobial resistance genes		119 (22.8)	78 (33.0)	41 (14.5)	<0.001

[a] PCV13 serotypes vs non-PCV13 serotypes.

Table 4
Antimicrobial resistance genes detected by microarray in nasopharyngeal samples from Laotian children containing a single pneumococcal serotype with no other species identified, shown pre- and post-PCV13 introduction by age group.

	5–8 week old infants			12–23 month old children		
Antimicrobial resistance gene	Pre-PCV13 (N = 62) n (%)	Post-PCV13 (N = 72) n (%)	P value	Pre-PCV13 (N = 219) n (%)	Post-PCV13 (N = 166) n (%)	P value
tetM	37 (60)	34 (47)	0.150	131 (59.8)	103 (62.0)	0.657
tetK	22 (36)	16 (22)	0.089	4 (1.8)	5 (3.0)	0.446
cat	13 (21)	7 (10)	0.069	46 (21.0)	17 (10.2)	0.005
mefA	8 (13)	15 (21)	0.225	61 (27.8)	43 (25.9)	0.670
aphA3	7 (11)	5 (7)	0.380	2 (0.9)	6 (3.6)	0.066
sat4	7 (11)	3 (4)	0.118	1 (0.5)	5 (3.0)	0.045
ermB	15 (24)	22 (31)	0.411	55 (25.1)	67 (40.4)	0.001
ermC	7 (11)	8 (11)	0.974	1 (0.5)	6 (3.6)	0.022
Any antimicrobial resistance gene	48 (77)	46 (64)	0.088	154 (70.3)	120 (72.3)	0.673
≥3 antimicrobial resistance genes	19 (31)	21 (29)	0.852	42 (19.2)	37 (22.3)	0.454

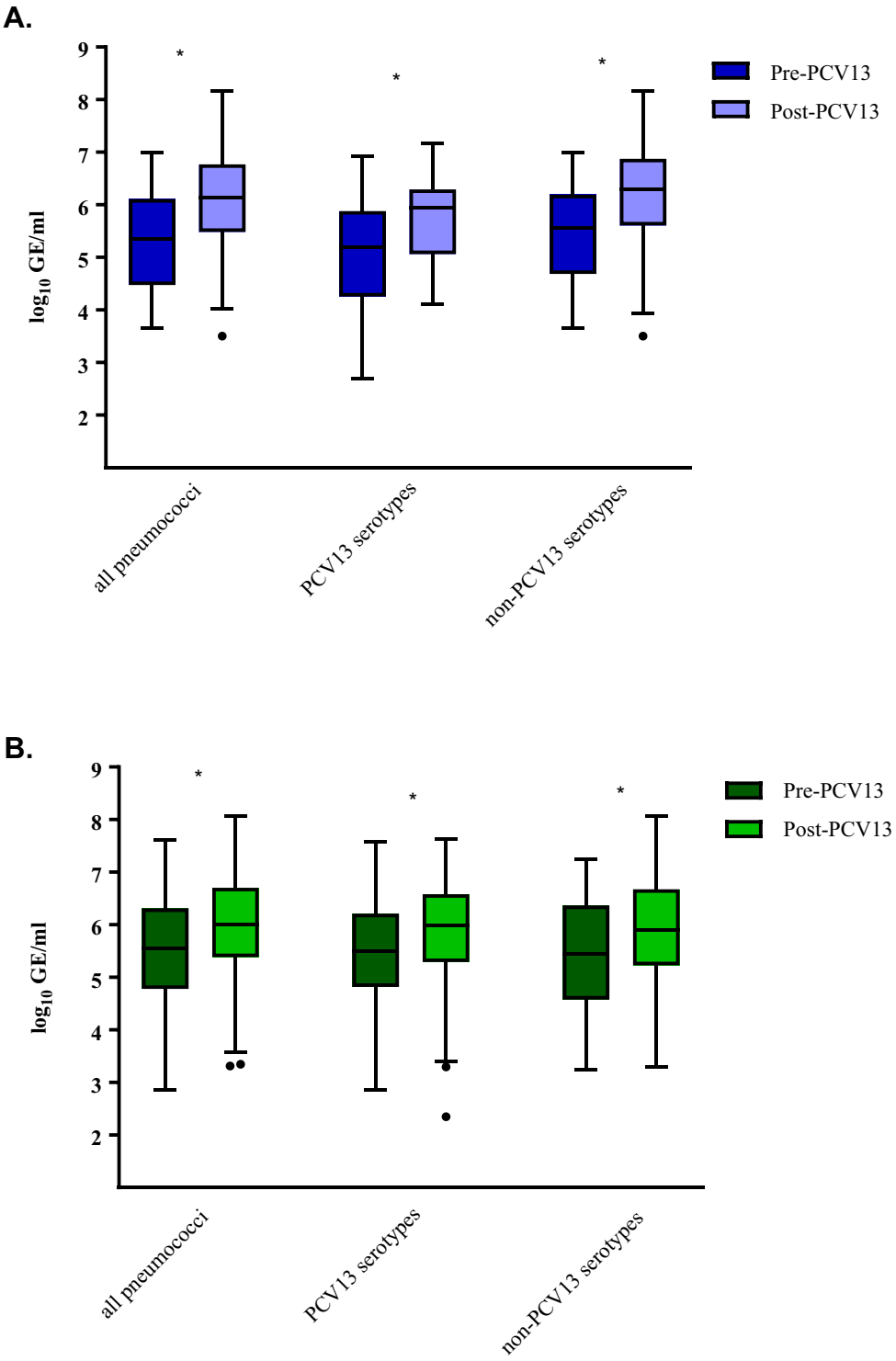

Fig. 2. Nasopharyngeal carriage density (log_{10} genome equivalents/ml) of all pneumococci, PCV13 serotypes, and non-PCV13 serotypes in 5–8 week old infants (A) and 12–23 month old children (B). Boxes depict interquartile range (IQR) with a central line at the median, and whiskers extend 1.5 times IQR past the quartiles. Values outside whiskers plotted as individual points. For both age groups, the median density of all pneumococci, PCV13 serotypes, and non-PCV13 serotypes was higher post-PCV13 compared with pre-PCV13 introduction. *$p < 0.01$, Mann-Whitney test.

indicating that serotype replacement in carriage is beginning to occur as we would expect [10,31–33].

In infants too young to be vaccinated, there was no significant change in carriage of PCV13 serotypes or non-PCV13 serotypes. However, the proportion of pneumococci that belonged to PCV13 serotypes was lower in the post-PCV13 period, providing some early suggestive evidence for indirect effects of PCV13 in this age group. Similar to the 12–23 month olds, an increasing trend in non-PCV13 serotype carriage was observed. Further studies will be needed to ascertain when indirect effects that result in a significant decrease in PCV13 serotype carriage prevalence will be observed in this age group in Lao PDR.

This is one of the first studies to describe population based nasopharyngeal carriage effects following PCV13 introduction,

Table 5
Median density and quantile regression analysis of overall pneumococci, PCV13 serotypes, and non-PCV13 serotypes in PCV13 vaccinated (2 or 3 doses) and undervaccinated (0 or 1 dose) 12–23 month old children who were pneumococcal carriers.

	Median density (IQR)[a]	Unadjusted coefficient (95% CI)[b]	P value	Adjusted coefficient (95% CI)[c]	P value
Overall pneumococci					
Undervaccinated (304)[d]	5.60 (4.82–6.37)	Reference		Reference	
PCV13 vaccinated (205)	6.00 (5.40–6.68)	0.43 (0.20–0.67)	<0.001	0.50 (0.26–0.74)	<0.001
PCV13 serotypes					
Undervaccinated (176)	5.54 (4.84–6.28)	Reference		Reference	
PCV13 vaccinated (89)	5.97 (5.30–6.54)	0.42 (0.09–0.75)	0.012	0.40 (0.07–0.73)	0.017
Non-PCV13 serotypes					
Undervaccinated (149)	5.47 (4.65–6.35)	Reference		Reference	
PCV13 vaccinated (135)	5.92 (5.23–6.69)	0.44 (0.12–0.77)	0.007	0.54 (0.20–0.87)	0.002

[a] Density reported in $\log_{10}$ genome equivalents/ml and interquartile range (IQR).
[b] Coefficient is the difference in medians as determined by quantile regression, reported with 95% confidence intervals (CI).
[c] Adjusted for ethnicity, URTI symptoms, and mode of delivery.
[d] Number of pneumococcal carriers shown in parentheses.

without prior PCV7 introduction. The only published data in this context is from Greenland, where PCV13 was introduced using a 2 + 1 schedule with catch-up for children <23 months. In children aged 0–6 years, a carriage study conducted in 2013 found an adjusted odds ratio of 0.44 (p = 0.01) for PCV13 serotype carriage compared with that of children sampled one year post-PCV13 introduction in 2011 [34]. There are five published studies examining population-based effects of the introduction of PCV10 (without prior PCV7 introduction) on nasopharyngeal carriage. PCV10 was introduced in Kenya in 2011 in a 3 + 0 schedule. Cross-sectional carriage studies conducted in the Kilifi region, where a catch-up campaign included children 12–59 months, found that two years post-introduction there was a 64% decline in PCV10 serotype carriage in children <5 years and a 66% decline in people >5 years of age [31]. In Brazil, a 91% reduction in PCV10 serotype carriage was measured in 12–23 month old children approximately three years following introduction using a 3 + 1 schedule with catch-up vaccination for children <23 months of age [33]. PCV impact on carriage was assessed in children attending day-care centres in Iceland following PCV10 introduction using a 2 + 1 schedule without catch-up [32]. Approximately 2–4 years after PCV10 introduction, there was a 94% and 56% reduction of PCV10 serotype carriage in vaccinated children aged <4 years, and older children (3.5–6.3 years) who were not eligible for vaccination, respectively. In Fiji, three years after PCV10 was introduced using a 3 + 0 schedule (with no catch-up) there was a 44% and 66% decline in PCV10 carriage for 5–8 week old unvaccinated infants and 12–23 month old children, respectively [22]. Recently, Sigauque et al. reported a 41% decline in PCV10 serotype carriage in HIV-uninfected children in Mozambique two years after introduction using a 3 + 0 schedule without catch-up [35].

The reductions in vaccine-type carriage that were observed in Lao PDR were smaller than observed for Fiji, Iceland, Brazil and Kenya. Differences in baseline demographics, vaccination schedule, catch-up campaign used, PCV coverage and the number of years post-PCV introduction are likely to contribute to differences in the magnitude of reductions in vaccine-serotype carriage.

Of the >95 pneumococcal serotypes identified globally to date, 41 were represented in this study. We also identified several putative serotype variants (including the 11F-like variant described previously [36]), which will be characterised further and used to inform future carriage and disease surveillance. Non-encapsulated pneumococci were common in our study population, especially the NT2 lineage that is common in other settings [37–39]. Carriage prevalence of PCV13 serotypes 6A and 6B was lower post-PCV13 in 12–23 month old children, and 6A also decreased in the 5–8 week old infants. Serotype 23A increased in both age groups, and has been associated with serotype replacement in invasive pneumococcal disease and otitis media following PCV13 introduction in other settings [40,41].

The effect of PCV on multiple serotype carriage is an open question. Valente et al. [42,43] found evidence of a decline in multiple serotype carriage with PCV introduction in Portugal. However, consistent with Brugger et al. [44] and our recent study in Fiji [22], we did not find evidence for an effect of PCV on the proportion of children who carried more than one serotype in Lao PDR. Interestingly, there was a significant decrease in the proportion of pneumococci that were PCV13 serotypes (and a corresponding increase in the proportion of pneumococci that were non-PCV13 serotypes) for both age groups in Lao PDR, suggesting that changes in pneumococcal serotype distribution may be a more sensitive measure of vaccine impact in the early years post-PCV introduction, even in the context of low multiple serotype carriage.

In Asia, antibiotic use is often unregulated and AMR is very common [45,46], including for pneumococci. For example, nearly 60% of the 2184 pneumococci isolated from patients in 11 Asian countries were resistant to at least three antibiotic classes [47]. Consistent with this, we found that over 70% of pneumococcal-positive samples contained at least one AMR gene, and that over 40% of the 12–23 month old children had reported antibiotic use in the preceding fortnight. Data on the presence of AMR genes obtained by microarray can be used to provide an ecological snapshot of resistance genes in pneumococcal populations. PCV13 serotypes were in general more likely to harbour resistance genes compared with non-PCV13 serotypes, suggesting that Lao PDR may be a setting where PCV introduction would be expected to reduce the prevalence of AMR [13], although AMR genes were also common in non-PCV13 serotypes. However, changes in the prevalence of AMR genes following PCV13 introduction was not necessarily reflective of their distribution prior to vaccine introduction. In particular, we observed an increase in *ermB*, which encodes resistance to erythromycin, following PCV13 introduction in spite of the fact that PCV13 serotypes were more likely to carry *ermB* than non-PCV13 serotypes. This did not appear to be related to an increase in a specific *ermB* harbouring clone. However, *ermB* was commonly found in non-PCV13 serotypes 23A, 15A, and 11A, all of which became relatively more common in the post-PCV13 survey. Other reasons that could contribute to changes in AMR gene prevalence may include changes in antibiotic utilisation, or possibly represent an increase in another colonising species carrying AMR genes that is present in the samples (and grows on selective agar) but is not detected by microarray. Interestingly, our recent study in Fiji also found that *ermB* prevalence increased post-PCV introduction [22]. Analysis of AMR genes was limited to those included on the microarray, and some antibiotics of clinical importance with more complex mechanisms of resistance, such

as penicillin, were not assessed. Examining the clinical relevance and impact of PCV on AMR requires phenotypic, and potentially genotypic, testing.

There are few data on the impact of PCVs on pneumococcal density, and results to date have been contradictory. In a randomised-controlled trial, American Indian children given PCV7 were less densely colonised than those receiving the control meningococcal vaccine [16]. Roca et al. reported a cluster-randomised trial in The Gambia where vaccine serotype density was reduced in vaccinated and partially-vaccinated villages; however the density of non-vaccine serotypes also declined, a finding difficult to explain in the context of a PCV effect. Dagan et al. [17] also used semi-quantitative methods and found that PCV13-vaccinated Israeli children carried the six additional serotypes at the same density as children vaccinated with PCV7. Recently, Olwagen et al. [48] found that HIV uninfected 9 month old infants in South Africa who were PCV7-vaccinated had higher carriage density compared with unvaccinated infants from a separate study, however this observation was consistent for both PCV7 and non-PCV7 serotypes, and densities no longer differed when children were 16 months of age.

In observational studies, Hanke et al. [49] found that PCV7 vaccination did not impact overall pneumococcal density (by qPCR) in Peruvian children colonised with a PCV7 serotype, but density was higher in vaccinated children colonised by a non-PCV7 serotype. In our Fiji cross-sectional study, we found that both vaccine serotype and non-vaccine serotype density was lower in vaccinated compared with unvaccinated children aged 12–23 months, however the effect may be temporal rather than vaccine-related [22]. In Mozambique, density of serotypes 11A, 19A, and 19F were compared pre- and post-PCV10 introduction and no differences were observed [35]. In Lao PDR, we found that pneumococcal density increased post-PCV introduction. Given that both PCV13 and non-PCV13 density increased, we do not ascribe these changes to PCV. We could not identify any field, clinical or laboratory changes that could explain this observation, and so hypothesise that it may be due to temporal variation and/or secular trends. Although high pneumococcal density has been shown to be associated with respiratory infections in children, we and others have shown that there is considerable variability in pneumococcal carriage density, even in healthy children [14,22,50]. Current evidence suggests that carriage density is not important in PCV impact studies, as no clear links between PCV and pneumococcal density have been demonstrated. However, further research to elucidate the respective roles of pneumococcal carriage, density in the nasopharynx, and serotype in the development of pneumococcal disease is warranted.

Key strengths of this study are that we used sensitive molecular methods [18,21] to measure prevalence and density of pneumococcal carriage, in a lower-middle income setting where vaccine impact data are sparse. Carriage is a practical and biologically relevant end-point, but does not measure the impact of PCV on disease. Carriage surveys are able to be implemented quickly, and in settings without prior surveillance data. This was valuable in Lao PDR where we were able to roll out carriage surveys alongside vaccine introduction to provide baseline data. For programmatic reasons, the baseline survey was conducted in the four months following vaccine introduction. However, there were no PCV vaccinees in the first survey, and this is a period of time unlikely to result in any herd effects that could confound baseline results. Relatively few participants belonged to minority ethnic groups, and in the 5–8 week age group, over 90% of participants were urban. This may limit generalisability of our findings to the whole Lao PDR population. Nevertheless, our study provides important supportive evidence of the effects of PCV13 on carriage in Lao PDR; declines in PCV13 serotype carriage are likely to translate into declines in disease caused by PCV13 serotypes. We expect that serotype replacement in carriage in Lao PDR will become more prominent over time. Ongoing surveillance is required.

This study provides evidence of PCV impact following introduction in a lower-middle income country in Asia. This is important, as there are very little available data from this region despite the high burden of disease. Our results are consistent with the large body of evidence from high-income settings, and growing evidence from low- and middle-income settings, that PCV use results in declines in vaccine serotype carriage in both vaccinated and unvaccinated individuals. Our data, together with other on-going studies (including those funded by Gavi, the Vaccine Alliance), will provide a stronger evidence base for PCV introduction and maintenance in the region.

Acknowledgements

The project was funded by Gavi, the Vaccine Alliance, and the World Health Organization Western Pacific Regional Office, with support from the Victorian Government's Operational Infrastructure Support Program. FR was supported by a NHMRC Early Career and TRIP Fellowships. CS was supported by a NHMRC Career Development Fellowship (1087957) and a veski Inspiring Women Fellowship. EFGN holds an Australian Government Research Training Scholarship. We thank Chansay Pathammvong and Phounphenghack Kongxay (Ministry of Health, Lao PDR); Alejandro Ramirez-Gonzalez and Rita Reyburn (World Health Organization); Jana Lai, Kathryn Bright and Kathryn Stanhope (MCRI).

Competing interests

JH: 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.

KG: SGUL sub-contract KG to BUGS Bioscience as an SGUL employee, but KG receives no personal income from this activity. CS and EMD received the Robert Austrian Research Award in Pneumococcal Vaccinology, which was funded by Pfizer but awarded by ISPPD.

All the other authors have no declarations of competing interests to report.

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