Effect of pneumococcal conjugate vaccine six years post-introduction on pneumococcal carriage in Ulaanbaatar, Mongolia

Supplementary methods

Sample collection and transport

World Health Organization recommended methods were used for the collection, handling and transport of nasopharyngeal samples [1]. Paediatric flocked swabs (Copan Diagnostics) used for sample collection were placed immediately into 1 ml skim milk tryptone glucose glycerol (STGG) medium [2] and refrigerated until transport to the National Center of Communicable Diseases (NCCD) bacteriology laboratory in Ulaanbaatar. At the NCCD samples were vortexed, aliquoted and stored at -80^{o}C within 7 h of collection. Samples were shipped on dry ice to the Murdoch Children's Research Institute in Australia for laboratory testing.

Laboratory procedures

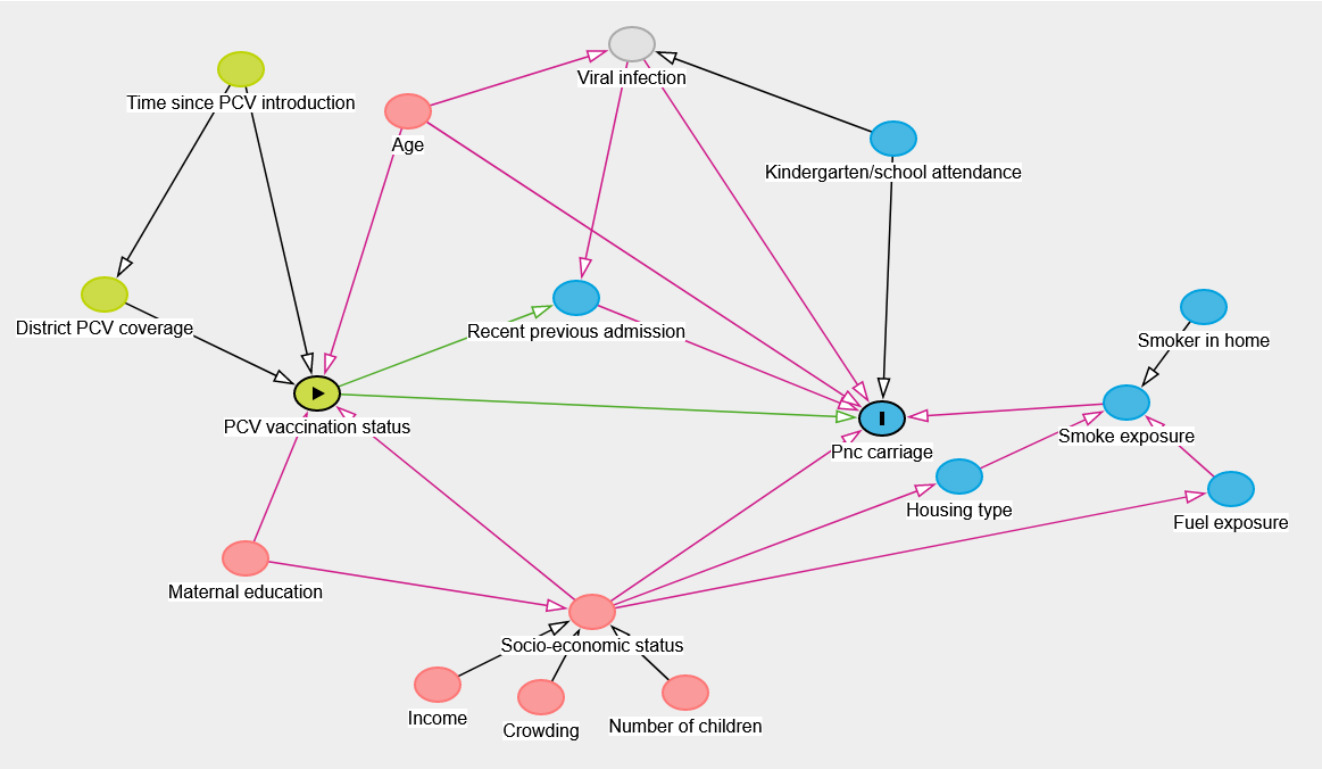
Procedures for pneumococcal detection, quantification, and serotyping has been previously described [3]. DNA was extracted from 100 µl of STGG using the MagNA Pure LC machine (Roche) for 2015 and 2017 samples and the QIAcube HT machine (QIAgen) for samples from the 2022 survey. Real-time quantitative PCR targeting the *lytA* gene (*lytA* qPCR) [4] was then performed using the Stratagene Mx3005 machine for all surveys [5]. Carriage density (in genome equivalents/ml) was calculated by reference to a standard curve prepared from reference isolate genomic DNA [6]. Samples with a *lytA* qPCR cycle threshold value ≤40 were cultured onto horse blood agar containing 5 µg/ml of gentamicin. For all samples with alpha-haemolytic growth, bacteria were harvested and DNA extraction performed followed by molecular serotyping by DNA microarray [7].

Assessment of pneumococcal carriage

PCV13 serotypes were defined as those contained in PCV13 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F). All other serotypes, including non-encapsulated pneumococci, were designated as non-PCV13 serotypes [8]. Serotypes 15B and 15C were reported as 15B/C [9], 11F-like was reported as 11A [10], 23B-like as 23B and 33F-like as 33F. A swab that contained both PCV13 and non-PCV13 serotype(s) was considered carriage positive for both regardless of relative abundance or density of each of the serotypes. Samples that were *lytA* qPCR positive (Ct value <35) but could not be serotyped (either culture negative or low DNA yield from culture) were considered pneumococcal positive with an unknown serotype.

The microarray detects ten AMR genes associated with mobile genetic elements. Four genes (*tetM*, *tetK*, *tetO*, *tetL*) encode for tetracycline, one each for chloramphenicol (*cat*), kanamycin (*aphA3*) and streptothricin (*sat4*). Resistance to macrolides are encoded by *mefA* as well as *ermB* and *ermC* genes which also encode resistance to lincosamides and streptogramin B. To determine whether AMR genes were present or not, we only included samples containing a single pneumococcal type with no other species identified.

Supplementary figures

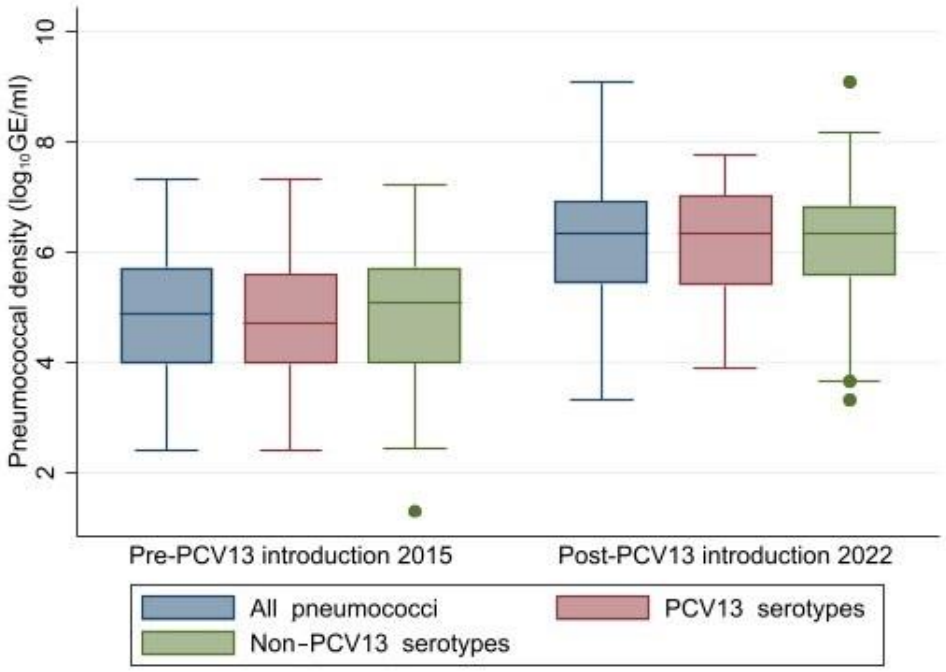


Supplementary Figure S1: Directed acyclic graph (DAG) of the association between PCV13 vaccination status (exposure) and pneumococcal carriage (outcome)

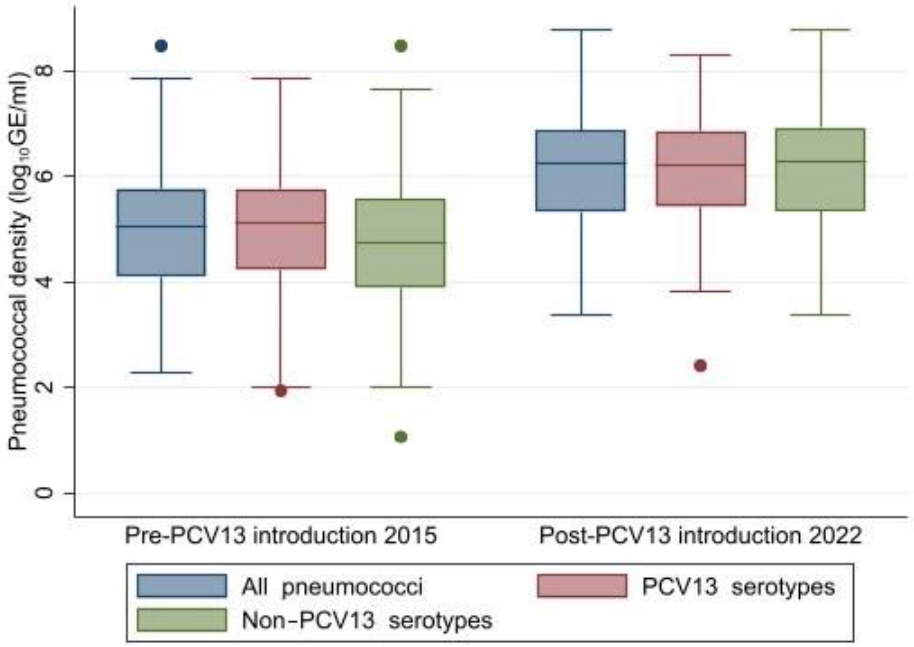
The DAG was used to assist with the identification of potential confounding factors. The green line highlights the causal relationship under investigation and the pink lines highlight potential biasing pathways. The blue variables are ancestors of the outcome, yellow variables ancestors of the exposure and red variables are ancestors of both exposure and outcome. Grey variables represent unobserved variables.

Based on this diagram, we identified that adjusting for housing-type, maternal education, household crowding, number of children under five years of age, household fuel type, and previous admission may block biasing pathways.

A

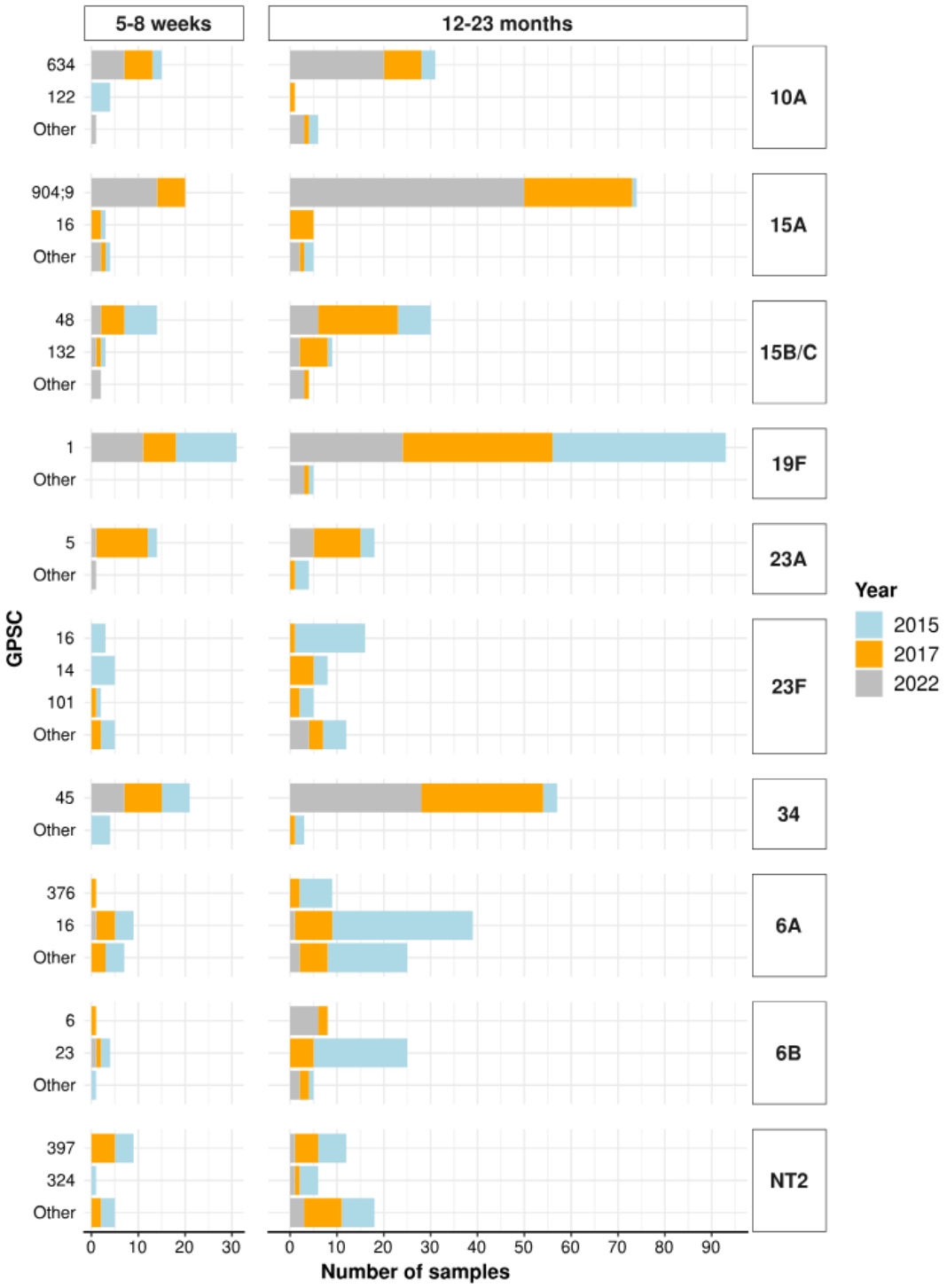


B



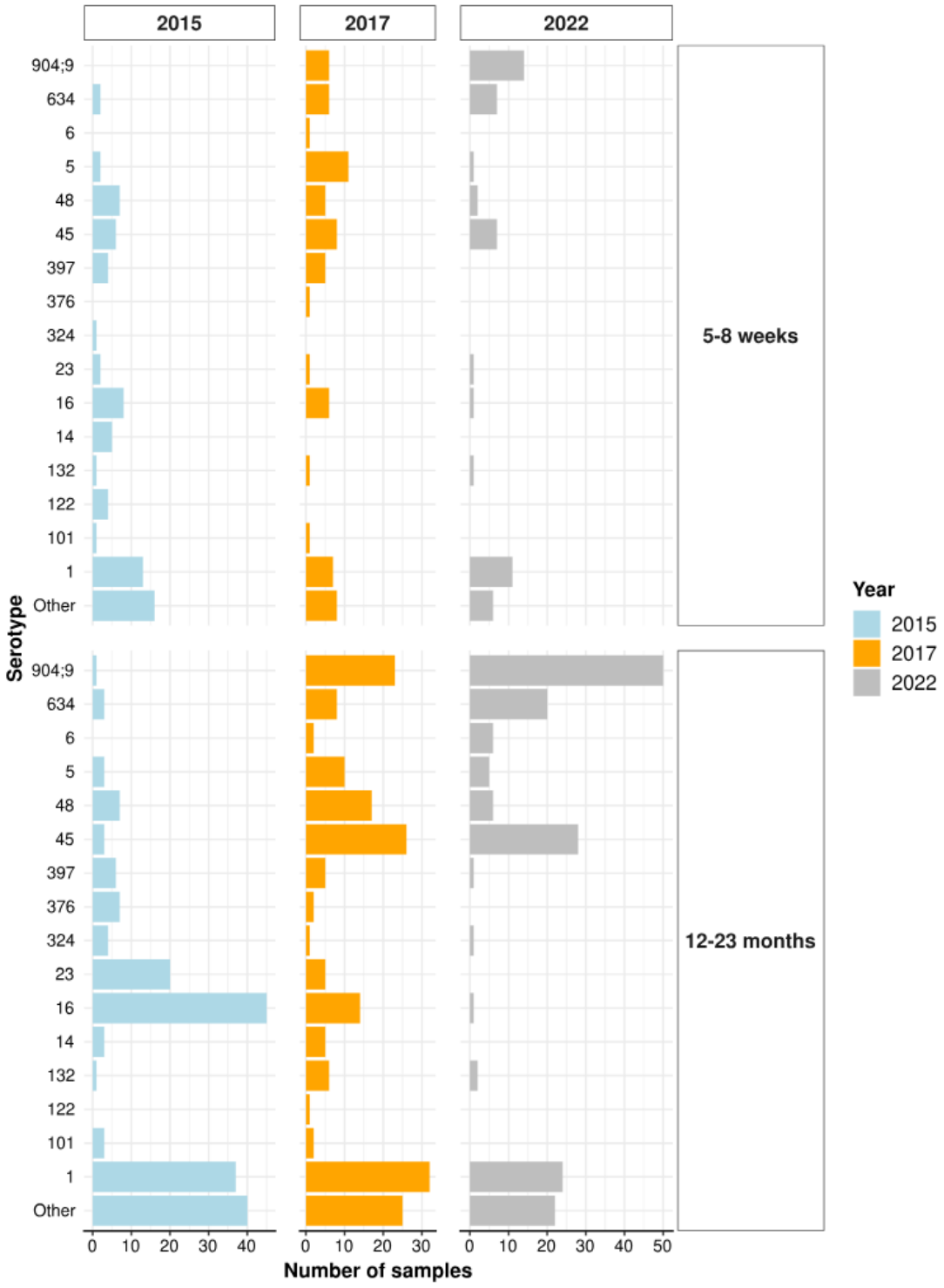
Supplementary Figure S2: Nasopharyngeal pneumococcal carriage density (log_{10} genome equivalents/ml) in (A) 5-8 week old infants (2015 n=131, 2022 n=87) and (B) 12-23 month old children (2015 n=294, 2022 n=241) who were positive for pneumococcal carriage.

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 ($p<0.001$ quantile regression Table S3).



Supplementary Figure S3. Number of samples belonging to each Global Pneumococcal Sequence cluster (GPSC) in the top 10 most common serotypes across all surveys.

GPSCs were inferred for the calls with the highest relative abundance using DNA microarray and were analysed for lineage composition. Bars are coloured by year. Other refers to GPSCs that were found in fewer than five samples for the corresponding serotype. Source data are provided as a Source Data file (5-8 weeks N=190, 12-23 months N=533).



Supplementary Figure S4. Number of samples belonging to each Global Pneumococcal Sequence cluster (GPSC) across all surveys.

GPSCs were inferred for the calls with the highest relative abundance using DNA microarray and were analysed for lineage composition. Bars are coloured by year. Other refers to GPSCs that were found in fewer than 20 samples (for all data). Source data are provided as a Source Data file (5-8 weeks N=190, 12-23 months N=533).

Supplementary Tables

Supplementary Table S1: Carriage prevalence and prevalence ratios for pneumococcal carriage (all, PCV13 serotypes, and non-PCV13 serotypes) for 5-8 week old infants and 12-23 month old children for 2022 carriage survey compared with 2015 and 2017 carriage surveys.

	Carriage prevalence 2015 (%) (95% CI)	Carriage prevalence 2017 (%) (95% CI)	Carriage prevalence 2022 (%) (95% CI)	Unadjusted prevalence ratio 2022 vs 2017 (95% CI)	Adjusted prevalence ratio[a] 2022 vs 2017 (95% CI)	Unadjusted prevalence ratio 2022 vs 2015 (95% CI)	Adjusted prevalence ratio[a] 2022 vs 2015 (95% CI)
All pneumococci[b]							
5–8 week old	28.4 (24.3 - 32.8)	24.0 (20.3 - 28.0)	17.4 (14.2 - 21.0)	0.73 (0.57 - 0.93)	0.75 (0.58 - 0.96)	0.61 (0.48 - 0.78)	0.67 (0.52 - 0.85)
12–23 month old	60.1 (55.6 - 64.4)	55.9 (51.4 - 60.3)	48.2 (43.7 - 52.7)	0.86 (0.76 - 0.97)	0.85 (0.76 - 0.96)	0.80 (0.71 - 0.90)	0.81 (0.72 - 0.91)
PCV13 serotypes							
5–8 week old	12.9 (10.0 - 16.3)	6.3 (4.3 - 8.9)	4.0 (2.5 - 6.1)	0.64 (0.37 - 1.11)	0.63 (0.35 - 1.12)	0.31 (0.19 - 0.51)	0.33 (0.20 - 0.56)
12–23 month old	42.2 (37.8 - 46.7)	19.7 (16.3 - 23.5)	12.5 (9.7 - 15.8)	0.64 (0.47 - 0.85)	0.65 (0.48 - 0.90)	0.30 (0.23 - 0.38)	0.29 (0.22 - 0.38)
Non-PCV13 serotypes							
5–8 week old	16.2 (12.9 - 19.9)	17.4 (14.2 - 21.0)	13.1 (10.3 - 16.4)	0.75 (0.56 - 1.01)	0.80 (0.59 - 1.08)	0.81 (0.59 - 1.10)	0.88 (0.65 - 1.21)
12–23 month old	26.4 (22.6 - 30.6)	40.2 (35.9 - 44.7)	36.9 (32.6 - 41.3)	0.92 (0.78 - 1.07)	0.89 (0.76 - 1.05)	1.39 (1.16 - 1.68)	1.36 (1.12 - 1.65)

[a] The following variables were used to adjust the prevalence ratios in each group: housing type (formal or informal), maternal education, household crowding (greater than three people per room), number of children under five years of age, household fuel type, and previous admission.

[b] All 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.

Supplementary Table S2: Vaccine serotypes detected as major and minor serotypes by age group and survey year.

	Vaccine serotypes detected as:	Survey years		
		2015	2017	2022
		n (%)	n (%)	n (%)
Infants (5-8 weeks)	Major serotypes	55 (93)	28 (90)	18 (90)
	Minor serotypes	3 (5)	2 (7)	1 (5)
	Both major and minor serotypes	1 (2)	1 (3)	1 (5)
	Total	206 (100)	98 (100)	62 (100)
Toddlers (12-23 months)	Major vaccine serotypes	177 (86)	80 (82)	55 (89)
	Minor vaccine serotypes	17 (8)	16 (16)	7 (11)
	Both major and minor serotypes	12 (6)	2 (2)	0 (0)
	Total	59 (100)	31 (100)	20 (100)

Supplementary Table S3: Median density and quantile regression analysis of all pneumococci, PCV13 serotypes and non-PCV13 serotypes in pre-PCV13 (2015) and post-PCV13 (2017 and 2022) period in children who were pneumococcal carriers.

	N§	Median density (IQR)*	Unadjusted coefficient (95% CI)†	P value^	Adjusted coefficient (95% CI)‡	P value^
Children 5-8 weeks						
All pneumococci						
Pre-PCV13 2015	131	4.88 (3.98 - 5.72)	Reference		Reference	
Post-PCV13 2017	120	6.06 (5.41 - 6.69)	1.17 (0.75 - 1.58)	<0.001	1.30 (0.91 - 1.70)	<0.001
Post-PCV13 2022	87	6.35 (5.43 - 6.93)	0.73 (0.52 - 0.95)	<0.001	0.82 (0.58 - 1.06)	<0.001
PCV13 serotypes						
Pre-PCV13 2015	59	4.71 (3.98 - 5.61)	Reference		Reference	
Post-PCV13 2017	31	5.97 (5.46 - 6.52)	1.26 (0.62 - 1.91)	<0.001	1.53 (0.97 - 2.08)	<0.001
Post-PCV13 2022	20	6.36 (5.40 - 7.04)	0.76 (0.35 - 1.18)	<0.001	0.71 (0.34 - 1.08)	<0.001
Non-PCV13 serotypes						
Pre-PCV13 2015	74	5.10 (3.99 - 5.72)	Reference		Reference	
Post-PCV13 2017	86	6.12 (5.33 – 6.78)	1.12 (0.63 - 1.60)	<0.001	1.16 (0.63 - 1.69)	<0.001
Post-PCV13 2022	65	6.35 (5.57 - 6.85)	0.69 (0.45 - 0.94)	<0.001	0.74 (0.46 - 1.02)	<0.001
Children 12-23 months						
All pneumococci						
Pre-PCV13 2015	294	5.04 (4.10 - 5.76)	Reference		Reference	
Post-PCV13 2017	279	5.79 (5.08 - 6.51)	0.75 (0.48 - 1.02)	<0.001	0.73 (0.46 - 1.01)	<0.001
Post-PCV13 2022	241	6.23 (5.33 - 6.87)	0.59 (0.46 - 0.73)	<0.001	0.58 (0.44 - 0.72)	<0.001
PCV13 serotypes						
Pre-PCV13 2015	206	5.11 (4.23 - 5.75)	Reference		Reference	
Post-PCV13 2017	98	5.75 (4.83 - 6.47)	0.64 (0.27 - 0.74)	0.001	0.64 (0.24 - 1.03)	0.002
Post-PCV13 2022	62	6.20 (5.42 - 6.85)	0.54 (0.34 - 0.74)	<0.001	0.58 (0.36 - 0.79)	<0.001
Non-PCV13 serotypes						
Pre-PCV13 2015	129	4.74 (3.89 - 5.57)	Reference		Reference	

Post-PCV13 2017	200	5.75 (5.07 - 6.51)	1.00 (0.65 - 1.36)	<0.001	1.03 (0.69 - 1.38)	<0.001
Post-PCV13 2022	183	6.28 (5.34 - 6.91)	0.77 (0.58 - 0.95)	<0.001	0.83 (0.64 - 1.02)	<0.001

*Density reported in $\log_{10}$ genome equivalents/ml and interquartile range (IQR). †Coefficient is the difference in medians as determined by quantile regression, reported with 95% confidence intervals (CI). ^Two-sided p-values determined by quantile regression. Each post-PCV13 year was compared with the pre-PCV13 year using separate quantile regression models. ‡Adjusted for housing type (formal or informal), maternal education, household crowding (greater than three people per room), number of children under five years of age, household fuel type, and previous admission. §Number of pneumococcal carriers.

Supplementary Table S4: Antimicrobial resistance (AMR) genes detected by microarray in nasopharyngeal samples from healthy Mongolian children aged 5-8 weeks and 12-23 months of age.* Detection rate of antimicrobial resistance genes shown for all pneumococci, PCV13 and non-PCV13 serotypes.

AMR gene	Encodes resistance to	Overall pneumococci (N = 545) n (%)	PCV13 serotypes (N = 262) n (%)	Non-PCV13 serotypes (N = 283) n (%)	p-value†
tetM	tetracycline	407 (74.7)	241 (92.0)	166 (58.7)	<0.001
tetK	tetracycline	42 (7.7)	16 (6.1)	26 (9.2)	0.18
tetO	tetracycline	0 (0.0)	0 (0.0)	0 (0.0)	N/A
tetL	tetracycline	1 (0.2)	1 (0.4)	0 (0.0)	0.30
cat	chloramphenicol	126 (23.1)	86 (32.8)	40 (14.1)	<0.001
mefA	macrolides	195 (35.8)	141 (53.8)	54 (19.1)	<0.001
aphA3	kanamycin	5 (0.9)	3 (1.1)	2 (0.7)	0.59
sat4	streptothricin	5 (0.9)	3 (1.1)	2 (0.7)	0.59
ermB	erythromycin	306 (56.1)	189 (72.1)	117 (41.3)	<0.001
ermC	erythromycin	44 (8.1)	21 (8.0)	23 (8.1)	0.96
Any antimicrobial resistance gene		436 (80.0)	247 (94.3)	189 (66.8)	<0.001
Multiple antimicrobial resistance genes**		206 (37.8)	156 (59.5)	50 (17.7)	<0.001

*Only samples that contained a single pneumococcal serotype with no other species identified were included in the analysis.

†p-values compared VT versus NVT serotypes using two-sided chi-squared test.

**≥3 antimicrobial resistance genes detected

Supplementary Table S5: Antimicrobial resistance (AMR) genes detected by microarray in nasopharyngeal samples from healthy Mongolian children, shown pre-PCV13 (2015) and post-PCV13 (2022) introduction by age group.*

	5-8 week old infants			12-23 month old children		
AMR gene	**Pre-PCV13 (N = 124) n (%)**	**Post-PCV13 (N = 28) n (%)**	**p-value†**	**Pre-PCV13 (N = 239) n (%)**	**Post-PCV13 (N = 157) n (%)**	**p-value†**
tetM	87 (70.2)	19 (67.9)	0.81	198 (82.8)	109 (69.4)	0.002
tetK	32 (25.8)	7 (25.0)	0.93	4 (1.7)	3 (1.9)	0.86
tetO	1 (0.8)	0 (0.0)	0.63	0 (0.0)	0 (0.0)	N/A
tetL	1 (0.8)	0 (0.0)	0.63	1 (0.4)	0 (0.0)	0.42
cat	49 (39.5)	5 (17.9)	0.03	65 (27.2)	10 (6.4)	<0.001
mefA	49 (39.5)	14 (50.0)	0.31	98 (41.0)	44 (28.0)	0.008
aphA3	2 (1.6)	4 (14.3)	0.002	1 (0.4)	0 (0.0)	0.42
sat4	2 (1.6)	3 (10.7)	0.02	1 (0.4)	0 (0.0)	0.42
ermB	57 (46.0)	14 (50.0)	0.70	159 (66.5)	86 (54.8)	0.02
ermC	31 (25.0)	4 (14.3)	0.22	13 (5.4)	0 (0.0)	0.003
Any antimicrobial resistance gene	101 (81.4)	22 (78.6)	0.73	210 (87.9)	111 (70.7)	<0.001
Multiple antimicrobial resistance genes**	63 (50.8)	16 (57.1)	0.54	103 (43.1)	33 (21.0)	<0.001

*Only samples that contained a single pneumococcal serotype with no other species identified were included in the analysis.

†p-values compared pre-PCV13 to post-PCV13 period using two-sided chi-squared test.

**≥3 antimicrobial resistance genes detected

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