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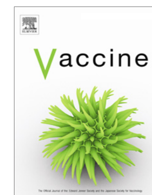

Evaluation of the association of pneumococcal conjugate vaccine immunization and density of nasopharyngeal bacterial colonization using a multiplex quantitative polymerase chain reaction assay

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ABSTRACT

Background: Nasopharyngeal bacterial colonization is a pre-requisite for developing bacterial mucosal and invasive disease. Pneumococcal conjugate vaccine (PCV) immunization of children reduces their risk of colonization by vaccine-serotypes, which could affect the biome of the nasopharynx in relation to colonization by other bacteria. This study evaluated the association of PCV immunization on the prevalence density of nasopharyngeal colonization by common, potentially pathogenic bacteria.

Methods: A multiplex qPCR assay was used to evaluate bacterial nasopharyngeal colonization by 7-valent PCV (PCV7) serotypes, non-vaccine serotypes (NVT), *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis,* and *Neisseria meningitidis* in PCV7-vaccinated and PCV-unvaccinated African children at two time points.

Results: PCV7 vaccination was associated with a higher prevalence of NVT and *H. influenzae* at 9 and 16 months, respectively. While the prevalence of *S. aureus* was higher in PCV7-vaccinated children at 9 months, no difference was found at 16 months. The density of PCV7 serotypes (3.8 vs. 3.4 log_{10}; p = 0.048), NVT (3.6 vs. 3.1 log_{10}; p = 0.018), *H. influenzae* (4.34 vs. 3.86 log_{10}; p = 0.008), *M. catarrhalis* (3.52 vs. 2.98 log_{10}; p < 0.001) and *S. aureus* (4.02 vs. 3.06 log_{10}; p = 0.02) was higher among PCV-vaccinated compared to PCV-unvaccinated children at 9 months, although, this difference diminished at 16 months of age.

Conclusion: The reduction in PCV7-serotype colonization impacted on colonization prevalence and density of other bacterial species of the nasopharynx. The clinical relevance of this needs further exploration in relation to mucosal and invasive disease outcomes, as well as for higher valency PCV vaccines.

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1. Background

Invasive and mucosal disease due to bacterial pathogens such as *Streptococcus pneumoniae, Haemophilus influenzae* and *Staphylococcus aureus* follow on colonization of the pharynx, with bacterial and host factors contributing to the nasopharyngeal colonization patterns of these bacteria [1,2]. Vaccination of children with pneumococcal conjugate vaccine (PCV) reduces the risk of *S. pneumoniae* vaccine-serotypes (VT) nasopharyngeal colonization, but is associated with an increased detection of non-vaccine serotypes (NVT) [3]. The effect on pneumococcal colonization induced by PCV could also affect colonization patterns of other bacteria, including *S. aureus* which has an inverse association with *S. pneumoniae* colonization, particularly with the seven-valent PCV (PCV7) serotypes and *H. influenzae* which is positively associated with pneumococcal colonization [4–6].

Since bacterial nasopharyngeal colonization is the precursor to disease of the upper and lower respiratory tract, carriage analysis in PCV-vaccinated and PCV-unvaccinated children could provide a measure for predicting whether there might be changes in susceptibility to mucosal and systemic bacterial infections in these children as a result of PCV immunization [1]. While a limited number of studies have used quantitative PCR (qPCR) methods to

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investigate bacterial nasopharyngeal colonization [4,7], the majority of studies have focused on non-quantitative culture-based methods as recommended by WHO [8], which may underestimate the prevalence of pneumococcal colonization and do not allow for quantitative evaluation of bacterial colonization or the effect of PCV thereon.

In this study we expand on our previous findings were nasopharyngeal samples from PCV-vaccinated and PCV-unvaccinated children were cultured for *S. pneumoniae*, *H. influenzae* and *S. aureus* using standard culture methods and the dynamics of colonization over the first two years of life were described [9,10]. Here we explore the association between PCV7 immunization and the density of colonization by these bacteria, as well as colonization by *M. catarrhalis, S. pyogenes* and *N. meningitidis* as measured by multiplex qPCR.

2. Results

Quantitative PCR analysis involved 713(83%) of the initial 857 nasopharyngeal swabs collected from children, at two time points [11]. Demographic characteristics of the study cohorts have been reported previously [11].

2.1. Detection of bacterial carriage by culture and qPCR

There was modest concordance between qPCR and culture for the detection of pneumococcus at 9 (*kappa* = 0.59) and 16 months (*kappa* = 0.59) of age. *LytA* qPCR assay was more sensitive than culture in detecting pneumococcus in both PCV7-vaccinated (71% vs. 57%; $p < 0.001$; and 77% vs. 61%; $p < 0.001$) and PCV-unvaccinated (83% vs. 74%; $p < 0.001$; and 81% vs. 68%; $p < 0.001$) children at 9 and 16 months of age, respectively; Table1.

There was modest concordance between qPCR and culture for the detection of *H. influenzae* at 9 (*kappa* = 0.50) and 16 months (*kappa* = 0.61) of age. The qPCR assay was more sensitive than culture in detecting *H. influenzae* in both PCV7-vaccinated (66% vs. 48%; $p < 0.001$; and 72% vs. 54%; $p < 0.001$) and PCV-unvaccinated children (56% vs. 31%; p = <0.001; and 62% vs. 58%; $p = 0.04$) at 9 and 16 months of age, respectively; Table1.

There was also moderate concordance between qPCR and culture for the detection of *S. aureus* at 9 (*kappa* = 0.68) and 16 months (*kappa* = 0.65) of age. Amongst PCV7-vaccinated children, the qPCR method was more sensitive than culture in detecting *S. aureus* at 9 (19% vs. 13%; $p = 0.004$) and 16 months (17% vs. 11%; $p = 0.03$) of age; Table1. While there was no significant difference between the two methods in PCV-unvaccinated children at 9 months of age, the qPCR method was more sensitive than culture for the detection of *S. aureus* in PCV-unvaccinated children at 16 months of age (19% vs. 13%; $p = 0.004$).

M. catarrhalis, *S. pyogenes* and *N. meningitidis* were not tested by culture and thus could not be compared to molecular qPCR; however, their respective prevalence by qPCR were 59% and 54%; 3% and 4%; and 1% and 1% in PCV7-vaccinated; and 61% and 64%: 4% and 6%; and 3% and 2% amongst PCV-unvaccinated children at 9 and 16 months of age, respectively.

The qPCR method demonstrated high sensitivity for all bacteria for which culture was undertaken and used as a referent standard, with 96.8%, 95.6% and 82.3% of culture-positive swabs being positive by qPCR. While it was not possible to accurately calculate the specificity of qPCR due to its superior sensitivity over the gold standard culture method, 90.5%, 94.3% and 97.5% of qPCR negative swabs were also negative by culture for *S. pneumoniae, H. influenzae* and *S. aureus,* respectively. Discordant results between culture and qPCR were strongly associated with the density of carriage, with 88% of culture negative, PCR positive samples having a bacte-

Table 1
Number (%) of bacterial culture and molecular qPCR for the detection of *S. pneumoniae, H. influenzae* and *S. aureus* in nasopharyngeal swabs.

	9 month old children						16 month old children					
	PCV-unvaccinated			PCV7-vaccinated			PCV-unvaccinated			PCV7-vaccinated		
	Culture (−)	Culture (+)	Total	Culture (−)	Culture (+)	Total	Culture (−)	Culture (+)	Total	Culture (−)	Culture (+)	Total
S. pneumoniae												
PCR (−)	27 (15)	4 (2)	31 (17)	45 (26)	5 (3)	50 (29)	32 (17)	4 (2)	36 (19)	39 (24)	2 (1)	41 (25)
PCR (+)	20 (11)	130 (72)	150 (83)	31 (18)	94 (54)	125 (71)	29 (15)	128 (66)	157 (81)	25 (15)	98 (60)	123 (75)
Total	47 (26)	134 (74)	181 (100)	76 (43)	99 (57)	175 (100)	61 (32)	132 (68)	193 (100)	64 (39)	100 (61)	164 (100)
H. influenzae												
PCR (−)	78 (43.1)	2 (1)	80 (44)	59 (34)	4 (2)	63 (36)	68 (35)	5 (3)	73 (37)	42 (26)	4(2)	46 (28)
PCR (+)	47 (26)	54 (30)	101 (56)	32 (18)	80 (48)	112 (66)	14 (7)	106 (55)	120 (62)	33 (20)	85 (52)	118 (72)
Total	125 (69.1)	56 (31)	181 (100)	91 (52)	84 (48)	175 (100)	92 (48)	111 (58)	193 (100)	75 (46)	89 (54)	164 (100)
S. aureus												
PCR (−)	157 (87)	4 (2)	161 (89)	141 (81)	1 (1)	142 (81)	156 (81)	1 (1)	157 (81)	131 (80)	5 (4)	137 (84)
PCR (+)	6 (3)	15 (8)	20 (11)	11 (6)	22 (13)	33 (19)	11 (6)	25 (13)	36 (19)	15 (9)	13 (8)	28 (17)
Total	163 (90)	18 (10)	181 (100)	152 (87)	23 (13)	175 (100)	167 (87)	26 (13)	193 (100)	146 (89)	18 (11)	164 (100)

Table 2
Detection of bacterial carriage by culture, stratified by colonization density measured by quantitative PCR (qPCR).

Pneumococcal density by qPCR (CFU/ml)	Number culture positive/number in density category by PCR (%)				
	9 month old children		16 month old children		Total
	PCV-unvaccinated	PCV7-vaccinated	PCV-unvaccinated	PCV7-vaccinated	
S. pneumoniae					
Undetected	4/31 (13)	5/50 (10)	4/36 (11)	2/41 (5)	15/159 (9)
$0–10^2$	1/2 (50)	0/1	0/2	0/3	1/8 (13)
$>10^2–10^3$	14/20 (70)	5/16 (31)	8/17 (47)	6/14 (43)	33/67 (49)
$>10^3–10^4$	31/38 (82)	11/21 (52)	33/43 (77)	17/21 (81)	92/123 (75)
$>10^4–10^5$	40/46 (87)	26/30 (87)	45/51 (88)	31/37 (84)	142/164 (87)
$>10^5–10^6$	34/34 (100)	33/37 (89)	31/33 (94)	34/38 (89)	132/142 (93)
$>10^6–10^7$	9/9 (100)	16/17 (94)	9/9 (100)	6/6 (100)	40/41 (98)
$>10^7–10^8$	1/1 (100)	3/3 (100)	2/2 (100)	4/4 (100)	10/10 (100)
Total	134/181 (74)	99/175 (57)	132/193 (68)	100/164(61)	465/713 (65)
H. influenzae					
Undetected	2/80 (3)	4/63 (6)	5/73 (7)	4/46 (9)	15/262 (6)
$0–10^2$	0/10	0/5	–	0/1	0/16
$>10^2–10^3$	5/22 (23)	5/14 (36)	5/9 (56)	2/6 (33)	17/51 (33)
$>10^3–10^4$	12/22 (55)	13/23 (57)	12/16 (75)	9/17 (53)	46/78 (59)
$>10^4–10^5$	15/20 (75)	28/34 (82)	23/27 (85)	17/25 (68)	83/106 (78)
$>10^5–10^6$	19/24 (79)	27/29 (93)	36/38 (95)	20/27 (74)	102/118 (86)
$>10^6–10^7$	3/3 (100)	7/7 (100)	25/25 (100)	28/33 (85)	63/68 (93)
$>10^7–10^8$	–	–	5/5 (100)	9/9 (100)	14/14 (100)
Total	56/181 (31)	84/175 (48)	111/193 (58)	89/164 (54)	340/713 (48)
S. aureus					
Undetected	4/161 (2)	4/145 (3)	1/157 (1)	6/137 (4)	15/598 (3)
$0–10^2$	–	–	–	0/1	0/3
$>10^2–10^3$	5/9 (56)	6/13 (50)	9/16 (56)	4/13 (31)	24/51 (54)
$>10^3–10^4$	4/6 (67)	3/5 (60)	4/6 (67)	7/12 (58)	18/29 (71)
$>10^4–10^5$	2/2 (100)	3/4 (75)	4/5 (80)	–	9/11 (82)
$>10^5–10^6$	3/3 (100)	4/5 (80)	6/7 (86)	1/1 (100)	15/16 (94)
$>10^6–10^7$	–	2/2 (100)	2/2 (100)	–	4/4 (100)
$>10^7–10^8$	–	1/1 (100)	–	–	–
Total	18/181 (10)	23/175 (13)	26/193 (13)	18/164 (11)	85/713 (12)

CFU/ml, colony forming units per millimetre as measured by qPCR. PCV7, seven-valent pneumococcal conjugate vaccine. Values are number culture positive/number in density category by qPCR (%).

rial load of $<10^5$ CFU/ml, Table 2. Similarly, 93.3% of PCR negative, culture positive samples were scored as "scant" (<5colonies/plate). This trend was observed across all bacterial pathogens, with the overall concordance between culture and PCR being 95.3%, 89.5% and 95% for *S. pneumoniae, H. influenzae* and *S. aureus* respectively when estimated PCR loads were $>10^5$CFU/ml.

2.2. Prevalence of nasopharyngeal bacteria as determined by qPCR

At least one bacterial species was detected in 93.8% (669/713) of all infant, with 76.2% (543/713) of individuals being co-colonized with two or more species. At 9 months of age, overall pneumococcal colonization prevalence was lower in PCV7-vaccinated (71.4%) than PCV-unvaccinated (82.9%), Fig. 1. The net reduction in carriage was due to a lower prevalence of PCV7-serotype colonization in PCV7-vaccinated (36%) than PCV-unvaccinated children (62%). However, there was a corresponding higher colonization prevalence of non-vaccine serotype among the PCV7-vaccinated children (40% vs. 33.7%). By 16 months of age, there was no difference in overall pneumococcal colonization between the two cohorts. This was due to the magnitude of lower prevalence of PCV7-serotype colonization in PCV7-vaccinated children (32.9%) than PCV-unvaccinated children (51.8%), being largely offset by a higher colonization prevalence of non-vaccine serotype among the PCV7-vaccinated children (62.2% vs. 37.8%), Fig. 1.

In contrast, *H. influenzae* colonization prevalence was higher in PCV7-vaccinated than PCV-unvaccinated children (66.3% vs. 55.8%) at 9 months of age; and at 16 months of age (72% vs. 62%), Fig. 1. Further, *S. aureus* colonization prevalence was higher in PCV7-vaccinated (18.9%) compared to PCV-unvaccinated children (11.1%) at 9 months of age; however, no difference was identified at 16 months of age. No significant differences in the prevalence of colonization at either 9 or 16 months of age were evident for *M. catarrhalis, S. pyogenes* or *N. meningitidis* in respect to PCV7-vaccination status.

Logistic regression models demonstrated *S. pneumoniae* and *H. influenzae* to be positively associated with each other at 9 and 16 months of age in PCV7-vaccinated ($p < 0.001$ and $p = 0.002$, respectively) and PCV-unvaccinated children ($p = 0.02$ and $p = 0.003$, respectively; Table3), with associations being independent of PCV7-serotype group. *S. pneumoniae* and *M. catarrhalis* colonization were found to be positively associated with each other in both PCV7-vaccinated ($p = 0.01$ and $p = 0.02$) and PCV-unvaccinated children ($p = 0.05$ and $p = 0.03$) at 9 and 16 months of age, respectively. These associations were independent of PCV7-serotype group at 9 months of age, but were driven by the PCV7-serotype group in both PCV7-vaccinated ($p = 0.02$) and PCV-unvaccinated children ($p = 0.02$) at 16 months of age. *H. influenzae* and *M. catarrhalis* were found to be positively associated with each other in PCV7-vaccinated ($p = 0.023$ and $p = 0.019$) and PCV-unvaccinated children ($p < 0.001$ and $p = 0.003$) at 9 and 16 months of age, respectively. The low prevalence of *S. pyogenes* and *N. meningitidis* colonization limited comparisons from being made in relation to association of colonization by other bacteria.

2.3. PCV vaccination and density of bacterial nasopharyngeal carriage

At 9 months of age pneumococcal colonization density was higher in PCV7-vaccinated (4.68 $\log_{10}$ CFU/ml) than PCV-unvaccinated (4.28 $\log_{10}$ CFU/ml; $p = 0.007$) children; Fig. 2a. This

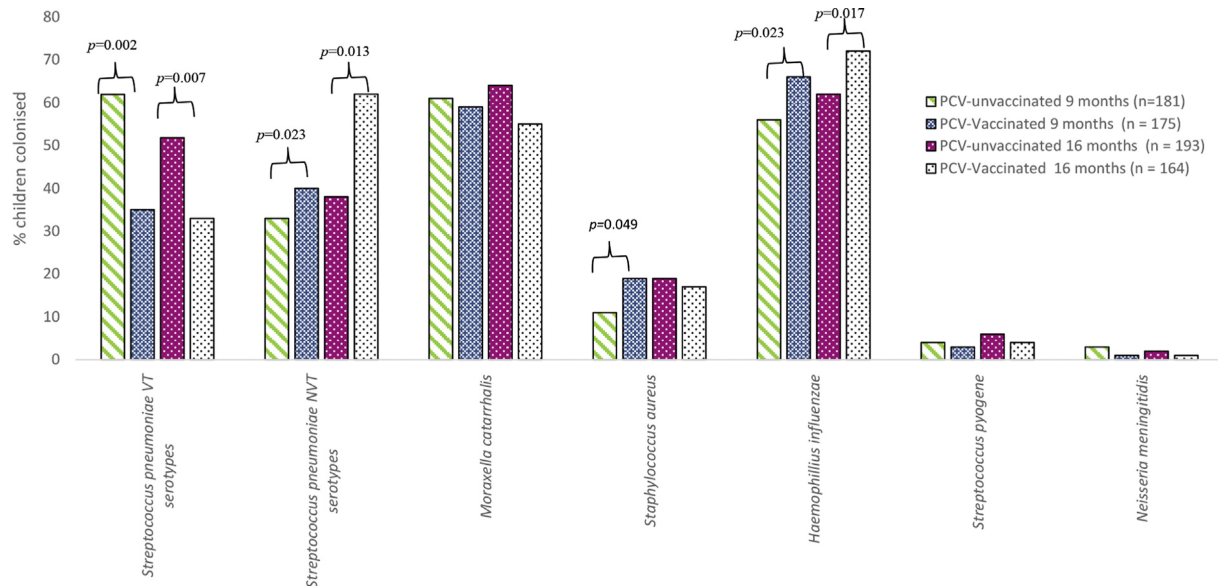

Fig. 1. Prevalence of nasopharyngeal (NP) bacterial colonization in PCV7-vaccinated and PCV-unvaccinated, HIV-uninfected children as measured by molecular qPCR. p-values were determined by multivariate logistic regression, controlling for race, smoking household contact, co-trimoxazole use, day care attendance and mean age at time of sample collection, by use of generalised estimating equations. PCV7, seven-valent pneumococcal conjugate vaccine. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 14, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, 34/37/17A). *p-Values of <0.05 were considered statistically significant.

Table 3
Association of nasopharyngeal bacterial colonization as determined by quantitative molecular PCR among PCV7-vaccinated and PCV-unvaccinated children.

Bacterial association	9 month old children				16 month old children			
	PCV7-vaccinated (*n* = 175)		PCV-unvaccinated (*n* = *181*)		PCV7-vaccinated (*n* = *164*)		PCV-unvaccinated (*n* = *193*)	
	aOR (95% CI)	*p*-value *	aOR (95% CI)	*p*-value *	aOR (95% CI)	*p*-value *	aOR (95% CI)	*p*-value *
S. pneumoniae & H. influenzae	4.53 (2.07–9.9)	<0.001	4.38 (2.6–5.17)	0.02	2.61 (1.15–5.94)	0.002	1.54 (0.71–3.33)	0.03
VT *& H. influenzae*	1.24 (0.51–3.01)	0.63	1.08 (0.48–2.47)	0.85	1.18 (0.48–2.86)	0.72	1.28 (0.69–2.37)	0.44
NVT *& H. influenzae*	2.05 (0.84–5.02)	0.06	0.94 (0.49–1.81)	0.86	2.64 (1.09–6.39)	0.03	1.25 (0.64–2.44)	0.52
S. pneumoniae & M. catarrhalis	2.66 (1.32–5.33)	0.01	1.33 (0.05–4.18)	0.05	1.92 (0.87–4.25)	0.02	2.36 (1.07–5.23)	0.03
VT *& M. catarrhalis*	0.45(0.23–1.02)	0.06	1.09 (0.47–2.55)	0.84	2.25 (1.15–5.54)	0.02	2.18 (1.16–4.11)	0.02
NVT *& M. catarrhalis*	1.78 (0.79–3.98)	0.16	0.83 (0.38–1.8)	0.63	1.41 (0.69–2.9)	0.35	2.46 (1.19–5.04)	0.01
S. pneumoniae & *S. aureus*	0.71 (0.28–1.82)	0.47	0.81 (0.244–2.67)	0.73	1.54 (0.47–5.06)	0.48	0.53 (0.22–1.3)	0.16
VT & *S. aureus*	0.39 (0.14–1.07)	0.07	0.50 (0.16–1.59)	0.24	0.77 (0.27–2.19)	0.63	0.73 (0.33–1.6)	0.43
NVT & *S. aureus*	1.34 (0.5–3.61)	0.55	2.59 (0.84–7.99)	0.1	1.42 (0.55–3.64)	0.47	0.94 (0.4–2.21)	0.9
S. pneumoniae & *S. pyogenes*	1.03 (0.06–17.79)	0.98	ND		ND		ND	
VT & *S. pyogenes*	2.72 (0.22–33.1)	0.43	0.37 (0.08–1.65)	0.19	ND		ND	
NVT & *S. pyogenes*	1.26 (0.11–15.01)	0.87	2.67 (0.57–12.39)	0.21	ND		ND	
S. pneumoniae & N. meningitidis	ND		ND		ND		ND	
H. influenzae & M. catarrhalis	1.69 (0.86–3.3)	0.049	5.56 (2.81–11)	<0.001	2.25 (0.99–5.09)	0.049	2.26 (1.19–4.29)	0.01
H. influenzae & S. aureus	1.5 (0.61–3.69)	0.38	1.26 (0.43–3.66)	0.67	0.87 (0.33–2.26)	0.77	0.67 (0.31–1.44)	0.31
H. influenzae & S. pyogenes	1.57 (0.14–17.83)	0.52	2.79 (0.55–14.14)	0.22	ND		ND	
H. influenzae & N. meningitidis	0.3 (0.02–5.28)	0.41	2.98 (0.23–38.53)	0.4	ND		ND	
M. catarrhalis & S. aureus	0.61 (0.27–1.4)	0.24	0.34 (0.11–1.06)	0.06	1.45 (0.54–3.88)	0.46	0.62 (0.27–1.4)	0.25
M. catarrhalis & S. pyogenes	2.82 (0.24–32.72)	0.41	0.17 (0.03–0.92)	0.06	ND		ND	
M. catarrhalis & N. meningitidis	ND		0.13 (0.01–1.68)	0.12	ND		ND	
S. aureus & S. pyogenes	4.38 (0.56–34.23)	0.16	0.9 (0.09–8.56)	0.92	2.98 (0.47–18.87)	0.25	ND	
S. aureus and N. meningitidis	ND		ND		ND		ND	

Adjusted odds ratios (aORs) for bacterial associations were determined by multivariate logistic regression, controlling for race, smoking house hold contact, co-trimoxazole use, day care attendance and mean age at time of sample collection, and colonization by other bacteria by use of generalised estimating equations.

* p-values of <0.05 were considered statistically significant. ND, not done as too few observations available for analysis. CI, confidence intervals. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 14, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, 34/37/17A).

was driven by an increase in carriage density, albeit lower prevalence of colonization of PCV7-serotypes, with densities of 3.8 $\log_{10}$ CFU/ml vs. 3.4 $\log_{10}$ CFU/ml respectively ($p = 0.048$) and by an increase in NVT serotypes, with density of 3.6 $\log_{10}$ CFU/ml vs. 3.1 $\log_{10}$ CFU/ml, respectively ($p = 0.018$). No difference in density of colonization between the PCV7-vaccinated and -unvaccinated groups was found at 16 months of age; Fig. 2b. Scatter plots illustrating the difference in bacterial density have been included as a Supplementary Fig.

A similar trend was noted for the density of the other bacteria at 9 months of age, where the density of colonization was higher in PCV7-vaccinated than PCV-unvaccinated children for *H. influenzae* (4.34 vs. 3.86 $\log_{10}$ CFU/ml; $p = 0.008$), *M. catarrhalis* (3.52 vs. 2.98 $\log_{10}$ CFU/ml; $p < 0.001$) and *S. aureus* (4.02 vs. 3.06 $\log_{10}$ CFU/ml;

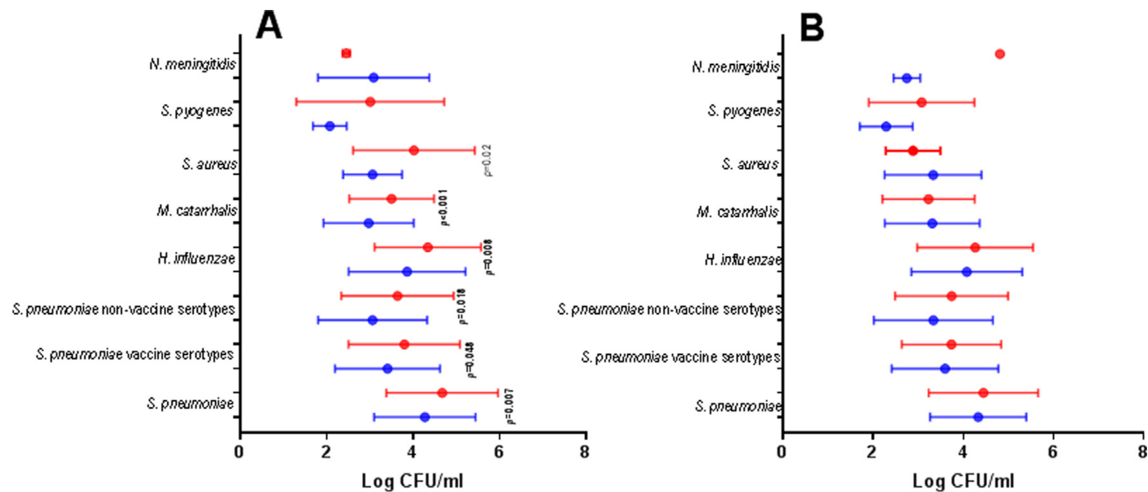

Fig. 2. Density of nasopharyngeal bacterial carriage in PCV7-vaccinated and PCV-unvaccinated, HIV-unifected children as measured by quantitative molecular qPCR at a) 9 months of age and b) 16 months of age. For density of carriage mean densities and 95% CI intervals (95% CI) of pneumococcal and bacterial mean concentrations were calculated following $\log_{10}$ transformations on data and compared with multivariate analysis controlling for race, smoking house hold contact, co-trimoxazole use, day care attendance and mean age at time of sample collection. PCV7, seven-valent pneumococcal conjugate vaccine. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, 34/37/17A). Blue lines represent PCV-unvaccinated children, while red lines represent PCV7-vaccinated children. *p-values of <0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Correlation of density of nasopharyngeal bacterial colonizers as determined by quantitative molecular PCR among PCV7-vaccinated and PCV-unvaccinated children.

Bacterial correlation	9 month old children				16 month old children			
	PCV7-vaccinated (*n* = 175)		PCV-unvaccinated (*n* = 181)		PCV7-vaccinated (*n* = 164)		PCV-unvaccinated (*n* = 193)	
	Rho	*p*-value *	*Rho*	*p*-value *	*Rho*	*p*-value *	*Rho*	*p*-value *
S. pneumoniae & H. influenzae	0.338	<0.001	0.4	<0.001	0.309	0.0025	0.46	<0.001
VT *& H. influenzae*	0.085	0.59	0.287	0.78	0.1472	0.24	0.2321	0.16
NVT *& H. influenzae*	0.152	0.27	0.09	0.61	−0.0858	0.58	0.3095	0.026
S. pneumoniae & M. catarrhalis	0.333	0.002	0.4488	0.005	0.252	0.0035	0.343	0.0013
VT *& M. catarrhalis*	0.423	0.016	0.3186	0.006	0.0857	0.48	0.2106	0.24
NVT *& M. catarrhalis*	0.251	0.15	0.0791	0.61	0.1239	0.4	0.1619	0.34
S. pneumoniae & *S. aureus*	−0.489	0.84	0.28	0.31	0.192	0.38	−0.063	0.74
VT & *S. aureus*	0.7364	0.07	−0.3143	0.54	0.432	0.21	0.151	0.54
NVT & *S. aureus*	0.086	0.78	0.033	0.93	−0.373	0.19	0.611	0.34
S. pneumoniae & *S. pyogenes*	ND		ND		0.143	0.76	0.360	0.15
VT & *S. pyogenes*	ND		ND		0.867	0.33	0.1	0.83
NVT & *S. pyogenes*	ND		ND		ND		0.31	0.46
S. pneumoniae & N. meningitidis	ND		ND		ND		ND	
H. influenzae & M. catarrhalis	0.360	0.048	0.19	0.03	0.2523	0.04	0.343	0.0013
H. influenzae & S. aureus	−0.139	0.56	−0.351	0.29	−0.159	0.54	−0.165	0.48
H. influenzae & S. pyogenes	−0.4	0.60	0.1	0.87	0.679	0.093	−0.077	0.81
H. influenzae & N. meningitidis	ND		ND		ND		ND	
M. catarrhalis & S. aureus	0.286	0.30	0.519	0.15	0.412	0.11	0.24	0.32
M. catarrhalis & S. pyogenes	ND		0.5	0.67	ND		0.436	0.18
M. catarrhalis & N. meningitidis	ND		ND		ND		ND	
S. aureus & S. pyogenes	ND		ND		ND		ND	
S. aureus and N. meningitidis	ND		ND		ND		ND	

Bacterial correlations were determined by spearman's coefficients. ND, not done as too few observations available for analysis. PCV7, seven-valent pneumococcal conjugate vaccine. VT, vaccine serotypes/serogroups (4, 6A/B, 9A/L/N/V, 18A/B/C, 19B/F and 23F). NVT, non-vaccine serotypes/serogroups (1, 3, 5, 6C/D, 7C, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B, 34/37/17A). *p*-values of <0.05 were considered significant.

p = 0.02). There was, however, no difference in density of colonization between cohorts at 16 months of age. No significant differences in density of colonization were observed for *S. pyogenes* and *N. meningitidis* between PCV7-vaccinated and PCV-unvaccinated children.

Spearman correlation coefficients showed the overall densities of *S. pneumoniae* and *H. influenzae* to be weakly correlated in PCV7-vaccinated (*rho* = 0.338 and *rho* = 0.309) and PCV-unvaccinated children (*rho* = 0.4 and 0.464) at 9 and 16 months, respectively. These correlations were independent of PCV7–serotype group; Table4. A weak positive correlation was found between *S. pneumoniae* and *M. catarrhalis* densities in PCV7-vaccinated (*rho* = 0.333) children at 9 months, while a moderate correlation was found between *S. pneumoniae* and *M. catarrhalis* densities in PCV-unvaccinated (*rho* = 0.45) children. These correlations were evident for vaccine serotypes in both cohorts (*rho* = 0.423 for PCV7-vaccinated and *rho* = 0.3186 for PCV-unvaccinated). A similar trend was noted at 16 months, with a weak positive correlation between the densities in both PCV7-vaccinated (*rho* = 0.25) and PCV-unvaccinated(*rho* = 0.343)

children. These correlations were, however, independent of PCV7-serotype group. A weak correlation was also identified between densities of *H. influenzae* and *M. catarrhalis* in PCV-vaccinated children ($rho = 0.36$ and $rho = 0.2523$) and PCV-unvaccinated ($rho = 0.19$, $p = 0.03$ and $rho = 0.343$) at 9 and 16 months, respectively. These associations were independent of PCV7–serotype group. No significant correlations with density of colonization were found between other bacteria.

3. Discussion

In this study a quantitative PCR method was established and used to examine the effect that PCV has on common bacterial nasopharyngeal colonizers in PCV7-vaccinated and PCV-unvaccinated children. Our analysis was in agreement with culture based methods, and showed a decrease in the carriage prevalence of pneumococcus and a corresponding increase in the carriage prevalence of *H. influenzae* associated with PCV7 vaccination [12]. Quantitative PCR, however, generated a higher yield of detection and allowed us to investigate the association of PCV immunization on differences in density of carriage. This included a higher overall density of *H. influenzae* in PCV7-vaccinated infants, which could possibly explain the moderate effects that PCV has on all-cause otitis media, even with vaccine efficacy of 50–80% in reducing vaccine-type pneumococcal colonization [13–15]. This is further corroborated by studies from USA which showed an increase in incidence of *H. influenzae* acute otitis media following on routine PCV immunization [16].

Although there are conflicting reports on whether PCV vaccination in children is associated with an increase in *S. aureus* colonization studied by culture methods, the findings from our study align to that from The Netherlands where a temporary increase in prevalence of *S. aureus* colonization was identified following PCV7 during infancy, but which was similar to PCV-unvaccinated children in the 2nd year of life [17–19]. These differences might be due to natural changes in *S. aureus* colonization, waning prevalence in older children, driven by interactions with other colonizing bacteria, and host factors such as age-dependent maturation of the immune system. The qPCR method also allowed quantification and showed a temporary increase in the density of *S. aureus*, raising concerns of the effect PCV7-vaccination may have on both *S. aureus* carriage and disease, which might also contribute to increase in *S. aureus* otitis media cases [20] as well as bacteremic pneumonia (unpublished data-personal correspondence S. Madhi from randomized, placebo-controlled trial of 9-valent PCV in South Africa). Furthermore, a study from the US showed a temporal increase in *S. aureus* empyema associated with PCV-immunization [21]. Although two cross sectional studies, one in primary care visiting children from Massachusetts and the other in children from France with otitis media reported no change the prevalence of colonization by *S. aureus* following PCV immunization [22,23] these studies were limited in that they included children of varying ages and therefore the temporary effect of PCV on *S. aureus* carriage could have been missed.

While some studies have shown the carriage prevalence of *M. catarrhalis* to either decrease [24] or increase after PCV in infancy [25,26], our analysis was in agreement with several other studies that showed the colonization prevalence to remain constant after PCV immunization [4,17,18]. Further, the qPCR method allowed for quantification and showed a temporary increase in the density of *M. catarrhalis* after PCV immunization which might explain the reported increase in otitis media caused by *M. catarrhalis* that is associated with PCV immunization [27].

Although the efficacy of PCV in reducing vaccine-type colonization and associated with an increase in NVT colonization has been well documented [28–30]; the findings from our study expand on these findings and suggest that after the decrease in vaccine-serotype colonization, momentum is directed by both non-vaccine type serotypes and other bacterial species to colonize the vacant niche; and thus newly established bacteria-bacteria interactions may impact on clinically important nasopharyngeal bacterial colonization dynamics.

Our findings are in line with other studies, in that positive associations with respect to prevalence were reported between pneumococcus, *H. influenzae* and *M. catarrhalis* [4–6]. Whilst these observations may well be associated with non-bacterial factors such the presence of respiratory viruses which may cause an ubiquitous increase in bacterial outgrowth in mucosal secretions, these common observations could also be associated with the quality of nasopharyngeal swab that was collected, and as such, justifies further research into these interactions.

Limitations of our study included that nasopharyngeal swabs do not favour the isolation of *S. aureus* and *S. pyogenes* in which anterior nares and throat swabs are better for detection, respectively [31,32]. The prevalence of these bacteria could have thus been underestimated. Furthermore, the *S. pneumoniae* typing qPCR method was not able to discriminate between all serotypes within their respective serogroup.

In conclusion, there is a natural balance between pneumococci and other co-colonizing bacterial species. Interfering with this may lead to disequilibrium within the host, which could affect susceptibility to disease from other serotypes and bacteria species. On-going surveillance, especially on the implication of such changes in the nasopharyngeal biome on particularly mucosal and invasive disease is warranted.

4. Material and methods

4.1. Study population

Archived nasopharyngeal swab samples collected from PCV-unvaccinated and PCV7-vaccinated cohorts of HIV-uninfected children from Soweto, South Africa were retrospectively analysed. Detailed information of the cohorts has been described [33,34]. Briefly, the PCV7-vaccinated cohort was enrolled between April 2005 and June 2006 and included 125 HIV-exposed-uninfected (HEU) and 125 HIV-unexposed infants, all between 6 and 12 weeks old at enrolment. These infants received three doses of PCV7 (Prevnar®) at 6, 10 and 14 weeks [34,35]. From January 2007 through to October 2007 251 PCV7 naïve infants, including 125 HEU and 126 HIV-unexposed infants were enrolled in a separate pneumococcal carriage study [33]. During both studies, pneumococcal immunization of children in Soweto (birth cohort 28,000 per annum) was limited mainly to study-participants (approximately 600 children), as PCV7 was only introduced into the pubic immunization program in May 2009 [36].

4.2. Multiplex qPCR methods

DNA from control strains for *S. pneumoniae* serotypes [11], *H. influenzae* (ATCC 33533), *M. catarrhalis* (ATCC 25238), *S. aureus*, (ATCC 25923), *N. meningitides* (ATCC 19424) and *S. pyogenes* (ATCC 19615) were used to optimize PCR assays and as positive controls.

Stored swab were thawed and total nucleic acids were automatically extracted using the NucliSens® easyMAG® extraction system according to manufacturer instructions. Similarly, total nucleic acids were extracted from pneumococcal, *S. aureus, M. catarrhalis, S. pyrogenes* and *N. meningitidis* reference strains. Extracted nucleic acids were stored at −20 °C.

The sequences of oligonucleotide primers and dye-labelled MGB probes for pneumococcal serotyping have been described [11]. The sequences for *H. influenzae, M. cattarrhalis, S. aureus, N. meningitidis* and *S. pyogenes* were taken from previously published sequences, or designed with the ABI primer express software package; Supplementary TableS1. All primer and probe sequences were tested *in silico* using NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and were specific to a broad range of their respective intended species. Primer/probe sets were tested against a positive control strains, as well as clinical isolates that had previously been identified positive by culture, and were successful in amplifying their respective targets. Further, all primers where tested against a number of non-template controls (NTC), with no amplification being detected.

4.2.1. Primer optimisation, standard curves and quantification of the real-time PCR assays

All qPCR were optimized as previously described [11] with the exception of DNA for pneumococcus and other bacteria being harvested from exponential phase cultures at an OD_{600} of 0.1 and 1.0 respectively. The performance of the serotyping assays have been described [11]. All bacteria targets showed good sensitivity and specificity with their respective primer and probe pairs with the limit of detection equivalent to 10 copies per PCR. Furthermore, the efficiency of these assays was 91–96%. Within the linear dynamic range, the correlation coefficients(r^2) of all the assays were 0.99. All primer pairs and probes were tested with genomic DNA from all pneumococcal and bacterial controls, as well as other bacterial species including *Escherichia coli, Klebsiella pneumonia, Salmonlella* spp., Bordetella species and *Neisseria lactamica* and no cross reactions occurred. In addition, both the inter-assay variation (repeatability) and inter-assay variation (reproducibility) for all assays was <0.1, while the accuracy for all assays was within ±0.1. Bacterial targets were duplexed (Supplementary Table S2) and paired reactions were tested to ensure that the quantification cycle (Cq) values did not shift by >1Cq value, and that sensitivity and specificity remained the same as for single-plex reactions.

4.2.2. Real-time PCR multiplex assay

Target DNA was pre-screened for pneumococcus, *H. influenzae*¸ *M. catarrhalis, S. aureus*, *S. pyogenes and N. meningitidis.* All samples positive for pneumococcus (Cq < 35) were regarded as positive for Streptococci and further molecularly serotyped for PCV7 serotypes/groups (4, 6A/B, 9A/L/N/V, 14, 18A/B/C, 19B/F and 23F) and non-vaccine serotypes/groups (1, 3, 4, 5, 6C/D, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 23A/B and 34/37/17A) as described [11]. Duplex PCR assays were carried out in 25μL reactions containing universal TaqMan master mix (Applied Biosystems), 0.25 μM of each probe and 0.25 μM of each primer, except *S. auerus* reverse primer (0.13 μM). DNA was amplified with the 7500 Real-Time PCR System (Applied Biosystems) using the following cycling parameters: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15sec and 60 °C for 1 min. Amplification data was analysed with the Applied Biosystems 7500 software with manually defined threshold. Negative samples were defined as those with Cq values ≥35 for each bacterial species. All samples negative for all tested bacteria were tested for the human GAPDH target to confirm the efficiency of the DNA extraction, with all qPCR negative samples being positive for GAPDH.

4.3. Statistical analysis

Pearson X^2 test or student *t*-test was used to compare baseline characteristics between the cohorts. Concordance between the qPCR and culture methods was measured using kappa statistics. Comparisons of prevalence of bacterial colonization between cohorts were analysed using multiple logistic regression models adjusted for race, passive smoke exposure, day care attendance, co-trimoxazole usage, and mean age in weeks at sample collection; adjusted odd ratios (aOR) were calculated. Colonization density data were presented as colony forming units (CFU)/ml and log mean densities. Confidence intervals (95% CI) of bacterial density were calculated following $\log_{10}$ transformation, using analysis of covariance to adjust for possible covariates. To assess whether colonization by one bacterial species was associated with colonization by another, logistic regression models with generalised estimating equations (GEE) were used. Spearman correlations were used to compare the degree of correlation between densities of each pair of bacteria. Results were considered significant at a *p*-value of <0.05. Statistical analysis was performed with STATA Version 11.0.

5. Ethics

Ethical approval for the original two studies was obtained from the Medical Human Research Ethics Committee (HREC) of the University of Witwatersrand (Vaccinated cohort: HREC:040704, also registered under Clinical trials number NCT00099658, and the PCV-unvaccinated cohort: HREC:050705). Approval for further testing of samples was obtained from the HREC (M120972). Written, informed consent was obtained from the parents/guardians of the study participants at the time of enrolment.

Conflict of interest

The authors declare no conflict of interest.

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Authors contributions

C.P.O., P.V.A., and S.A.M., conceived and designed the study. C.P.O. and M.C.N. was responsible for data curation and analysis. Funding acquisition was undertaken by S.A.M. C.P.O wrote the first draft of the manuscript under supervision of SAM, and all authors where involved in reviewing and editing the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.04.068.

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