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Evaluation of the impact of HIV-1 infection and density of common nasopharyngeal bacterial colonizers in South African children immunized with 7-valent pneumococcal conjugate vaccine

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ABSTRACT

Background: Due to limitations in standard culture methods, the impact of pneumococcal conjugate vaccine (PCV) immunization on nasopharyngeal bacterial carriage density is unclear, including among HIV-infected children.

Methods: The prevalence and density of serotype/serogroup-specific pneumococcal and other nasopharyngeal colonizing bacteria were investigated in archived swabs of HIV-infected and HIV-uninfected, PCV-7 immunized (at 6, 10 and 14 weeks of age) South African children collected at 9 and 16 months of age. During the course of the study, PCV-immunization of children in Soweto was limited to study-participants, as the vaccine had not been introduced into the public immunization program.

Results: At 9 months of age, the prevalence of overall pneumococcal colonization was lower in HIV-infected (58.6%) than HIV-uninfected children (69.9%, p = 0.02), mainly due to lower prevalence of non-vaccine-serotype colonization (27.8% vs. 40%, respectively; p = 0.047). The mean-$\log_{10}$ density of pneumococcal colonization was, however, higher in HIV-infected (4.81 CFU/ml) than HIV-uninfected pneumococcal colonized children (4.44 CFU/ml; p = 0.014); mainly due to higher mean-$\log_{10}$ density of PCV7-serotype colonization (4.21 vs. 3.72 CFU/ml; p = 0.014). No difference in the prevalence or density of overall pneumococci was found at 16 months of age. The prevalence of non-vaccine serotype colonization remained 1.7 fold higher in HIV-uninfected (60.4%) than HIV-infected children (50.9%, p = 0.049). Other differences included a lower prevalence of *H. influenzae* colonization in HIV-infected (42.3% and 56%) than HIV-uninfected children (64.2% and 73.4%) at both 9 and 16 months of age respectively; however, the density of colonization was similar.

Conclusion: Increased carriage density of residual PCV7-serotypes might cause HIV-infected children to have a higher risk of pneumococcal disease. The higher carriage density observed in HIV-infected children could be attributed to a combination of factors, including HIV treatment and impaired host immunity. Additional studies are needed.

1. Background

Approximately 5% of children <5 years of age in South Africa are HIV-infected, with *S. pneumoniae* being the most important cause of invasive bacterial disease (IBD), with the burden being 40-fold greater in HIV-infected compared to HIV-uninfected children even in the era of pneumococcal conjugate vaccine (PCV) immunization [1]. A limited number of studies have investigated the association between HIV-infection status and pneumococcal colonization, with conflicting results on whether HIV-infection is associated with differences in the prevalence of pneumococcal nasopharyngeal colonization in children [2–4]. Further, the effect that HIV-infection has on the prevalence of nasopharyngeal colonization by other bacteria such as *H. influenzae* and *S. aureus* is also unclear. Although no difference in the carriage prevalence of *S. aureus* was observed between HIV-infected and HIV-uninfected South African children [2], others have reported a higher prevalence of colonization in

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HIV-infected children [4–6]. Also, while some studies have reported HIV-infected children to have a higher prevalence of *H. influenzae* colonization [2], others have reported a lower prevalence [4,7] or no difference between HIV-infected and -uninfected children [5].

These studies were, however, limited in having only used routine microbial culture methods recommended by WHO, [8] which usually detects only the dominant colonizing serotype and thus detection of concurrent colonizing serotypes at a lower density is often missed [9]. Further, culture methods are semi-quantitive and thus provide us with limited insight into the density of colonization, they are also laborious and costly [10–13]. Further, the majority of these studies involved PCV non-immunized children, and thus further investigation with more sensitive molecular methods on the association between HIV-infection and pneumococcal carriage prevalence and density in a PCV immunized cohort is warranted. Molecular detection of pneumococci in the nasopharynx has several potential advantages over culture-based methods, including detection of multiple serotypes from a single sample with high sensitivity, as well as quantitative PCR methods being able to measure the density of colonization as well as the relative proportion of the colonizing serotypes [14].

Monitoring pneumococcal carriage post PCV immunization especially in a population with a high HIV-disease burden, and who are at a higher risk of disease, is pertinent in the context of ongoing deployment of PCV-immunization in children, with surveillance of colonization considered an early proxy for disease. In this study, we used a Nanofluidic real-time PCR assay (Fluidigm) to evaluate the effect of HIV-infection on the prevalence and density of nasopharyngeal colonization at 9 and 16 months of age by common, potentially pathogenic bacteria including seven-valent PCV (PCV7)-serotypes, non-PCV7 serotypes, *Haemophilus influenzae*, non-typable *Haemophilus influenzae (*NT*hinf), Staphylococcus aureus,* and *Moraxella catarrhalis.*

2. Material and methods

2.1. Study population

We retrospectively analyzed archived nasopharyngeal swab samples collected from a cohort of HIV-infected and HIV-uninfected infants in South Africa, who had previously been investigated by standard culture methods. Detailed information of the cohort has been described [15]. Briefly, participants included HIV-infected infants with $CD4^+$ T-lymphocytes cells $\geq$25% randomized to initiate ART immediately (ART-Immed); or when clinically and or immunologically indicated as per the WHO recommendations for ART initiation in children (ART-Def), HIV-exposed-uninfected (HEU) and HIV-unexposed-uninfected (HUU) infants who were 6–12 weeks old when enrolled between April 2005 and June 2006. Infants were scheduled to receive three doses of PCV7 (i.e. Prevnar®; Wyeth Vaccines, NJ, USA) at 6, 10 and 14 weeks of age, as well as other recommended childhood vaccines in South Africa, including *H. influenzae* type-b conjugate vaccine (HibCV) [15,16]. During the course of the study, PCV immunization of children in Soweto (birth cohort 28,000 per annum) was limited mainly to study-participants (approximately 600), as the vaccine was only introduced into the public immunization program in May 2009 [17].

Nasopharyngeal (NP) swabs were taken at several time intervals, including at 9 and 16 months of age. Swabs were stored in skim milk-tryptone-glucose-glycerol (STGG) transport media at the Respiratory and Meningeal Pathogen Research Unit (RMPRU) in South Africa, as recommended by WHO [18].

2.2. Total nucleic acid extraction

Stored nasopharyngeal swab samples were thawed and processed. Briefly, NP swabs were vortexed for 30 s to release bacteria into the transport media and total nucleic acids were automatically extracted from STGG with the NucliSens ® easyMAG ® extraction system (BioMérieux, Marcy l'Etoile, France) according to manufacturer's instructions. Samples were stored at −20 °C.

2.3. Optimization of the nanofludic real-time PCR system

The optimization of a Fluidigm assay has been described previously in detail, with all the reactions included in the assay optimized according to the MIQE guidelines [9]. Briefly, standard concentrations curves and lower limit of detection (LLD) for each target were determined by amplifying four independent 10-fold serial dilutions of purified genomic DNA extracted from reference strains with the linear dynamic range being 10^1–10^7 copies per standard curve – the number of CFU/ml was estimated from Cq values relative to the standard curve, which is based on the quantitative culture of the control DNA. The LLD was determined by the minimum number of copies of DNA that could be measured repeatedly by the primers in the 10-fold serial dilutions. The correlation coefficient, amplification efficiency, intra-assay variation (repeatability) and inter-assay variation (reproducibility) for each reaction were calculated and a ratio of the difference between the observed copy number and the expected copy number was also calculated to determine the accuracy of each target reaction. All reactions were effective in amplifying their respective targets in the Biomark HD system (Fluidigm), with the efficiency of the reactions ranging from 89% to 105%. Within the linear dynamic range, the correlation coefficients of the reactions were >0.98, and all reactions showed high analytical sensitivities (LLD equivalent >10–100 copies per PCR). Both the inter- and intra-assay variations for all respective reactions were <0.1 standard deviation (SD), while the accuracy ratio was within ±0.1. Standard curves were set up before sample screening and thereafter, every 10 plates with $\leq$1 Cq variation between standards of each run reported.

2.4. Real-time qPCR using the Biomark HD system (Fluidigm)

DNA extracts were quantified for 4 bacterial pathogens, 55 pneumococcal serotypes (16 individual serotypes and 39 serotypes in 13 groups) and 6 serotypes of *H. influenzae* (serotypes a-f) with the Nanofluidic real-time qPCR system as described [19]. Briefly, specific target amplification (STA) was done per manufactures recommendations as the initial step (pre-amplification of DNA) for the Biomark HD system (Fluidigm). The qPCR reactions were then carried out on the STA products with 96.96 dynamic arrays (Fluidigm Corporation, CA, USA) according to the manufacturer's instructions and bacterial loads were quantified using standard curves. Data were analyzed with real-time PCR Analysis Software in the BIOMARK instrument (Fluidigm Corporation, USA). Negative samples were defined as those with Cq values $\geq$35 for each qPCR target.

2.5. Statistical analysis

Pearson X^2 test or student *t*-test was used to compare baseline characteristics between the cohorts. Comparisons and adjusted odds ratios of the prevalence of pneumococcal and bacterial colonization between cohorts were analyzed using logistic regression models adjusted for covariates, including race, gender, study center, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. The associations of nasopharyngeal bacterial colonization between groups were determined by multivariate logistic regression models. For the density of carriage, mean-$\log_{10}$ densities

and 95% CI intervals (95%) of pneumococcal and bacterial concentrations are reported, with associations between groups analyzed using analysis of covariance with adjustment for possible cofounders. Spearman correlations were used to compare the degree of correlation between the mean-$\log_{10}$ densities of each pair of bacteria. Since we did not identify any difference in the prevalence and density of colonization for all bacteria between the ART-immed and ART-Def groups, as well as between the HIV-exposed uninfected and HIV-unexposed uninfected groups in our analysis (Supplementary Table 1), we subsequently analyzed these children as a composite group of HIV-infected and HIV-uninfected children respectively. Statistical analysis was performed with STATA Version 11.0 (StataCorp, Texas, USA). Results were considered significant when the p-value was <0.05.

3. Results

Nanofluidic qPCR analysis was undertaken on 763 (85.1%) of the initial 897 nasopharyngeal swabs collected at either 9 or 16 months of age (Supplementary Fig. 1). Within each cohort, there were no differences in the colonization patterns done by standard culture methodology, between those in whom samples were available and unavailable (Supplementary Figs. 2 and 3). Ninety percent of the children for whom samples were available, were Black-African, and 45.8% were male (Table 1). The mean age at the time of sample collection did not differ between HIV-infected and HIV-uninfected children, being 39.6 (standard deviation; SD 1.5) and 39.3 (SD 3.2) weeks (p = 0.13) and 67.9 (SD 1.9) and 67.7 (1.6) weeks (p = 0.15) at the two sampling time-points, respectively. Co-trimoxazole prophylaxis was expectantly more frequent in HIV-infected children at both 9 months (99% vs. 46.2%; p < 0.001) and 16 months (98.6% vs. 41.4%; p < 0.001) of age. Further, breastfeeding was more common in HIV-uninfected than HIV-infected children at 9 (25.4% vs. 1.4%; p < 0.001) and 16 months of age (23.2% vs. 1.4%%; p < 0.001).

3.1. Prevalence of bacterial colonization with respect to HIV infection status

3.1.1. Streptococcus pneumoniae colonization

In the multivariate-adjusted analysis, the prevalence of overall pneumococcal colonization was lower in the composite groups of HIV-infected (58.6%) than HIV-uninfected children (69.9%, p = 0.02; Table 2) at 9 months of age. This was largely due to the lower prevalence of non-vaccine-serotype colonization (27.8% vs. 40%, respectively; p = 0.047), whilst prevalence of PCV7-serotype colonization was similar (37.5% vs. 36%; p = 0.48). Although the serotype-specific analysis was limited by the low numbers of individual serotypes identified within each group; the colonization prevalence of non-vaccine serogroup 15A/B/C/F was 3.3 (8.2% vs. 3.5%; p = 0.029) fold higher in HIV-infected than HIV-uninfected children at 9 months of age (Fig. 1) despite the overall prevalence of non-vaccine serotype colonization being lower in HIV-infected children. By 16 months of age, there was no difference in the overall pneumococcal colonization between the composite groups of HIV-infected and HIV-uninfected children (p = 0.26), although the prevalence of non-vaccine serotype colonization remained 1.7 fold higher in HIV-uninfected (60.4%) than HIV-infected children (50.9%, p = 0.049; Table 2). Other differences included the colonization prevalence of PCV7-serogroup 6A/B being 3.7 fold higher in HIV-uninfected (7.9%) than HIV–infected children (2.3%; p = 0.03; Fig. 2).

3.1.2. Haemophilus influenzae colonization

In the multivariate-adjusted analysis, the prevalence of overall *H. influenzae* colonization was lower in HIV-infected (42.3%) than HIV-uninfected children at 9 months of age (64.2%, p = 0.003; Table 2), largely due to a lower prevalence of NT*hinf* colonization (37.5% vs. 54.8%, p = 0.006). Similarly, the prevalence of overall *H. influenzae* colonization was also lower in HIV-infected (56%) than HIV-uninfected children at 16 months of age (73.4%, p = 0.018), also largely due to a lower prevalence of NT*hinf* colonization (50% vs. 59.2%, p = 0.04).

3.1.3. Other bacterial nasopharyngeal colonizers

In the multivariate-adjusted analysis, there was no difference in the prevalence of *S. aureus* colonization between HIV-infected and HIV-uninfected children at 9 and 16 months of age, respectively (Table 2). The prevalence of *M. catarrhalis* colonization was, however, lower in HIV-infected (43.3%) than HIV-uninfected children (57.8%, p = 0.03) at 9 months of age.

3.2. Association of bacterial colonization between common nasopharyngeal (NP) pathogens stratified by HIV-status

In logistic regression models, the prevalence of *S. pneumoniae* colonization was positively associated with *H. influenzae* colonization in HIV-uninfected (p < 0.001) and HIV-infected (p < 0.001) children at 9 months of age, with positive associations been driven

Table 1
Demographic features at two study visits in HIV-infected and HIV-uninfected, PCV7-vaccinated children.

	9 Month old children			16 Month old children		
	HIV-infected	HIV-uninfected	p-value	HIV-infected	HIV-uninfected	p-value
Available samples for molecular analysis	208	173		218	164	
Mean birth weight (SD) grams	2967(4 8 9)	3092(4 4 8)	0.02	2958(4 5 1)	3126(4 7 6)	0.001
Male: sex (%)	80(45.5)	96 (55.5)	0.001	85(39)	89(54.3)	0.003
Race: Black African (%)	199 (95.7)	148(85.5)	0.001	211(96.8)	132(80.5)	<0.001
: Mixed ancestry (%)	9(4.3)	25(14.5)		7(3.2)	32(19.5)	
Smoking household contact (%)	90(43.3)	79(45.7)	0.49	92(44.4)	64(39)	0.57
Drugs administered: Co-trimoxazole prophylaxis (%)	206(99)	80(46.2)	<0.001	215(98.6)	68(41.4)	<0.001
: Amoxicillin (%)	43(24.86)	70 (33.65)	0.061	34(20.73)	63(28.9)	0.069
: Ampicillin (%)	1(0.58)	2(0.96)	0.673	0	0	
: Fluconazole (%)	0	1(0.48)	0.361	0	0	
: Isoniazid (%)	6(3.47)	8(3.85)	0.845	2(1.22)	8(3.67)	0.138
: Cefotaxime (%)	0	2(0.96)	0.196	1(0.61)	3(1.37)	0.192
: Erythromycin (%)	0	0	–	2(1.22)	1(0.46)	0.404
Mean number of household contacts (SD)	5.1(2.4)	5.3(2.3)	0.53	5.0(1.9)	5.1(2.4)	0.67
Breast feeding (%)	3(1.4)	44(25.4)	<0.001	3(1.4)	38(23.2)	<0.001
Mean age (SD) in weeks at visit	39.6(1.5)	39.3(3.2)	0.23	67.9(1.9)	67.7(1.6)	0.25

Pearson X^2 test or student t-test was used to compare baseline characteristics between the cohorts and demographic features with a p-value <0.2 were included as possible confounders in multivariate analysis.

Table 2
Prevalence of nasopharyngeal (NP) colonizers in HIV-infected and HIV-uninfected, PCV7- vaccinated children.

	HIV-infected *n* = 208	HIV-uninfected *n* = 173	aOR (95% CI); *p*-value	HIV-infected *n* = 218	HIV-uninfected *n* = 164	aOR (95% CI); *p*-value
Overall *S. pneumoniae*	122(58.6)	121(69.9)	0.58(0.35–0.96); *p* = 0.04	149(68.4)	125(76.2)	0.74(0.45–1.24); *p* = 0.26
S. pneumoniae VT	78(37.5)	63(36)	1.24(0.71–2.18); *p* = 0.48	74(33.9)	55(33.5)	0.85(0.50–1.44); *p* = 0.55
S. pneumoniae NVT	58(27.8)	70(40)	0.59(0.35–1.01);*p* = 0.047	111(50.9)	105(64)	0.65(0.41–1.04); *p = 0.049*
Overall *H. influenzae*	88(42.3)	111(64.2)	0.48(0.29–0.79); *p = 0.003*	122(56)	121(73.4)	0.50(0.31–0.81); *p* = 0.005
H. influenzae A	0	1(0.6)	–	4(1.8)	8(4.9)	0.45(0.12–1.73); *p* = 0.25
H. influenzae B	3(3.4)	2(1.2)	1.56(0.15–15.8); p = 0.7	1(0.5)	1(0.6)	0.49(0.23–8.98); *p* = 0.63
H. influenzae C	1(1)	0	–	0	6(3.7)	–
H. influenzae D	2(2.3)	1(0.9)	2.57(0.22–30.67); *p* = 0.46	2(0.9)	2(1.2)	0.49(0.06–3.98); *p* = 0.51
H. influenzae E	1(1.1)	2(1.2)	0.33(0.02–5.97); *p = 0.45*	1(0.5)	2(1.2)	0.26(0.02–2.99); *p* = 0.28
H. influenzae F	4(4.8)	4(2.3)	3.41(0.43–26.78); *p = 0.24*	5(2.3)	6(3.7)	0.46(0.11–1.61); *p* = 0.21
NT *H. influenzae*	78(37.5)	97(54.8)	0.54(0.32–0.93); *p* = 0.03	109(50)	97(59.2)	0.56(0.53–1.37); *p* = 0.04
M. catarrhalis	90(43.3)	100(57.8)	0.55(0.32–0.94); *p* = 0.03	125(57.3)	92(56.1)	1.04(0.66–1.64); *p* = 0.86
S. aureus	34(16.3)	30(17.2)	0.77(0.36–1.67); *p* = 0.51	31(14.2)	25(15.2)	0.99(0.52–1.94); *p* = 0.99

Adjusted odd ratios (aOR) for bacterial colonization were determined by multivariate logistic regression, controlling for race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. 95% CI, 95% confidence levels. 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, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/12B/12F/44/46, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22A/F, 23A/B, 25A/25F/38, 34/37/17A and 35B). –, too few observations to calculate *p*-value. A *p*-value of <0.05 was considered significant.

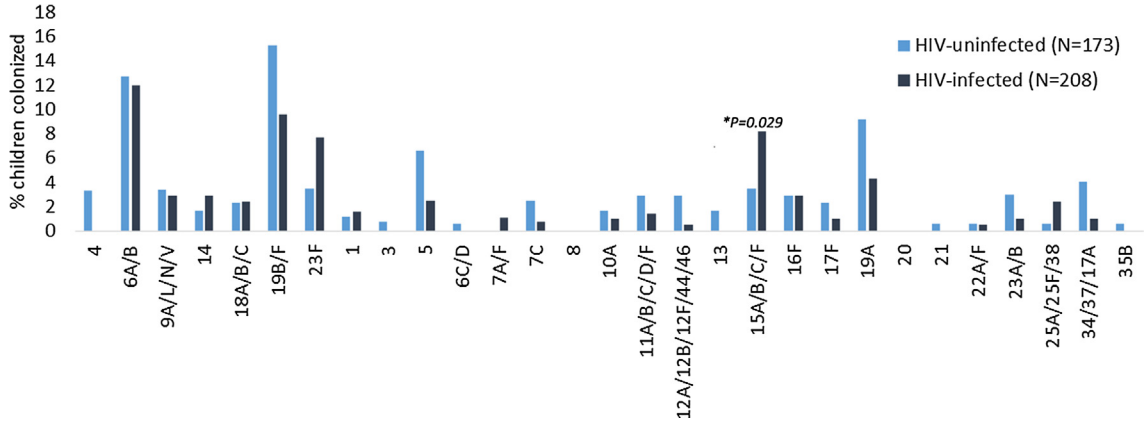

Fig. 1. Prevalence of nasopharyngeal (NP) pneumococcal colonization among the HIV-infected and HIV-uninfected children at 9 months of age *p-values were determined by multivariate logistic regression, controlling for race, gender, study center, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. *p-Values of < 0.05 were considered significant.*

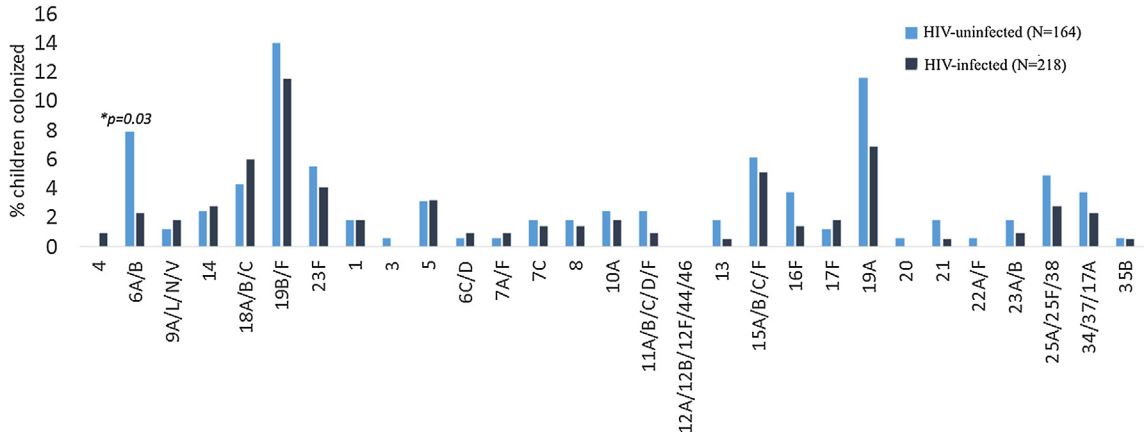

Fig. 2. Prevalence of nasopharyngeal (NP) pneumococcal colonization among the HIV-infected and HIV-uninfected children at 16 months of age *p-values were determined by multivariate logistic regression, controlling for race, gender, study center, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. *p-Values of < 0.05 were considered significant.*

by PCV7-serotypes or non-vaccine serotypes and NT*hinf*. (Table 3). Similarly, a positive association between prevalence of pneumococcal and *H. influenzae* colonization was also observed at 16 months of age in HIV-infected (p = 0.04) and HIV-uninfected (p = 0.036) children; with similar trends observed between PCV7-serotypes or non-vaccine serotypes and NT*hinf;* albeit only significant for non-vaccine serotypes and NT*hinf* in HIV-infected children (p = 0.004).

Positive associations were also observed between colonization of *M. catarrhalis* and pneumococcus in HIV-infected (p < 0.001)

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

	9 Month old children		16 Month old children	
	HIV-infected aOR (95% CI); *p*-value	HIV-uninfected aOR (95% CI); *p*-value	HIV-infected aOR (95% CI); *p*-value	HIV-uninfected aOR (95% CI); *p*-value
S. pneumoniae & M. catarrhalis	4.14(2.22–7.75); $p < 0.001$	4.59(2.24–9.43); $p < 0.001$	3.65(1.98–6.73); $p < 0.001$	1.69(0.79–3.60); $p = 0.17$
VT *S. pneumoniae* & *M. catarrhalis*	2.06(1.08–3.90); $p = 0.028$	3.20(1.41–7.23); $p = 0.005$	1.77(0.93–3.37); $p = 0.08$	1.94(0.91–4.11); $p = 0.082$
NVT *S. pneumoniae* & *M. catarrhalis*	2.40(1.21–4.74); $p = 0.012$	2.03(1.04–3.95); $p = 0.037$	2.98(1.53–5.80); $p = 0.001$	1.34(0.70–2.59); $p = 0.37$
S. pneumoniae & S. aureus	0.65(0.39–1,13); $p = 0.056$	0.48(0.37–1.23); $p = 0,18$	0.55(0.25–1.22); $p = 0.14$	0.94(0.05–5.30); $p = 0.08$
VT S. pneumoniae & S. aureus	0.45(0.22–1.95); $P = 0.73$	0.16(0,03–0.52); $P = 0.07$	0.38(0.13–1.16); $p = 0.09$	1.10(0.39–3.08); $p = 0.86$
NVT *S. pneumoniae* & *S. aureus*	0.99(0.28–3.84); $P = 0.84$	0.54(0.09–3.56); $p = 0.65$	0.76(0.31–1.85); $p = 0.54$	1.94(0.77–4.89); $p = 0.16$
S. pneumoniae & H. influenzae	4.41(2.33–8.33); $p < 0.001$	4.36(2.10–9.07); $p < 0.001$	1.83(1.01–3.30); $p = 0.04$	2.37(1.06–5.28); $p = 0.036$
VT *S. pneumoniae* & *H. influenzae*	3.37(1.75–6.49); $p < 0.001$	2.98(1.27–6.99); $p = 0.012$	1.39(0.75–2.59); $p = 0.29$	1.50(0.65–3.47); $p = 0.34$
NVT *S. pneumoniae* & *H. influenzae*	4.2(2.10–8.39); $p < 0.001$	2.75(1.34–5.62); $p = 0.006$	2.95(1.54–5.66); $p = 0.001$	1.89(0.89–4.04); $p = 0.1$
S. pneumoniae & NThinf	2.99(1.62–5.52); $p < 0.001$	3.11(1.52–6.35); $p = 0.002$	1.56(0.86–2.81); $p = 0.14$	1.09(0.50–2.38); $p = 0.82$
VT *S. pneumoniae & NThinf*	2.36(1.24–4.48); $p = 0.009$	3.90(1.68–9.07); $p = 0.002$	1.13(0.61–2.09); $p = 0.69$	1.08(0.42–1.82); $p = 0.72$
NVT *S. pneumoniae & NThinf*	3.0(1.52–5.91); $p = 0.001$	2.49(1.26–4.90); $p = 0.008$	2.47(1.32–4.61); $p = 0.004$	1.34(0.69–2.61); $p = 0.39$
VT and NVT *S. pneumoniae*	0.53(0.24–1.21); $p = 0.13$	1.23(0.58–2.50); $P = 0.59$	0.73(0.36–1.47); $p = 0.38$	0.75(0.36–1.56 = 5); $p = 0.43$
M. catarrhalis & S. aureus	0.48(0.18–1.22); $p = 0.12$	1.09(0.41–2.89); $p = 0.87$	0.45(0.20–1.0); $p = 0.052$	1.79(0.67–4.78); $p = 0.25$
M. catarrhalis & H. influenzae	2.82(1.57–5.08); $p = 0.001$	2.01(1.06–3.81); $p = 0.03$	1.74(1.0–3.03); $p = 0.051$	0.97(0.47–2.0); $p = 0.94$
M. catarrhalis & NThinf	2.36(1.30–4.26); $p = 0.005$	1.65(0.88–3.09); $p = 0.11$	1.87(1.06–3.27); $p = 0.03$	1.03(0.53–2.0); $p = 0.92$
S. aureus & H. influenzae	1.35(0.57–3.22); $p = 0.49$	0.99(0.37–2.64); $p = 0.98$	1.18(0.53–2.61); $p = 0.69$	0.73(0.27–1.97); $p = 0.54$
S. aureus & NThinf	1.71(0.72–4.09); $p = 0.22$	1.07(0.41–2.82); $p = 0.89$	1.29(0.59–2.84); $p = 0.52$	1.06(0.41–2.75); $p = 0.9$

Adjusted odd ratios (AOR) for bacterial colonization were determined by multivariate logistic regression, controlling for race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. 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, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/12B/12F/44/46, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22A/F, 23A/B, 25A/25F/38, 34/37/17A and 35B). NT, non-typable *H. influenzae*. 95% CI, 95% confidence levels. A *p*-value of < 0.05 was considered significant.

and HIV-uninfected ($p < 0.001$) children at 9 months of age, significant for PCV7-serotypes and non-vaccine serotypes. By 16 months of age, this association was only significant in HIV-infected children ($p < 0.001$), and mainly due to non-vaccine serotypes ($p = 0.001$). Similar positive trends between colonization of *M. catarrhalis*, overall pneumococcal, PCV7-serotypes, and non-vaccines serotypes were still evident in HIV-infected and HIV-uninfected children at 16 months of age. Further, colonization of *M. catarrhalis* and *H. influenzae* were found to be positively associated with each other in both HIV-uninfected ($p = 0.03$) and HIV-infected ($p = 0.001$) children at 9 months of age; however, by 16 months of age, this association was no longer significant. In addition, a positive association was found between prevalence of *M. catarrhalis* and NT*hinf* colonization in HIV-infected children at both 9 ($p = 0.005$) and 16 ($p = 0.03$) months of age, respectively.

3.3. HIV-status and mean-log$_{10}$ densities of bacterial nasopharyngeal carriage

The mean-log$_{10}$ density of overall pneumococcal colonization was higher in HIV-infected (4.81; 95%CI: 4.59–5.03, CFU/ml) than HIV-uninfected colonized infants (4.44; 95% CI: 44.24–4.63, CFU/ml) at 9 months of age, $p = 0.014$; Table 4, despite the lower overall prevalence of colonization in HIV-infected children. Also, the mean-log$_{10}$ density of PCV7-serotype colonization was higher in HIV-infected (4.21 CFU/ml) than HIV-uninfected colonized children (3.72 CFU/ml; p = 0.04). There were no differences in the mean-log$_{10}$ density of overall pneumococcus between HIV-infected and HIV-uninfected colonized children by 16 months of age.

Although there was no difference in mean-log$_{10}$ density of overall *H. influenzae* colonization between HIV-infected and HIV-uninfected infants at 9 months of age ($p = 0.18$; Table 4); a higher mean-log$_{10}$ density of colonization was observed at 16 months of age in HIV-uninfected (4.95; 95% CI; 4.73–5.17, CFU/ml) compared to HIV-infected children (4.32; 95%CI: 4.07–4.57, CFU/ml); which was largely due to the higher mean-log$_{10}$ density of NT*Hinf* in HIV-uninfected children (5.0 vs. 4.23; $p < 0.001$). No difference in the mean-log$_{10}$ density of *S. aureus* colonization was found between the groups.

Spearman correlation coefficients showed the overall mean-log$_{10}$ densities of *S. pneumoniae, H. influenzae* and *M. catarrhalis* to be positively associated with each other in both HIV-infected and HIV-uninfected colonized children at both 9 and 16 months of age, with associations by PCV7-serotype, non-vaccine serotype and NTh*inf* (Table 5). Further, a negative association between mean-log$_{10}$ densities of *S. pneumoniae* and *S. aureus* was observed in HIV-infected colonized children at 9 months of age.

4. Discussion

This study investigated the impact of HIV-1 infection and the prevalence and density of bacterial carriage of common nasopharyngeal colonizers such as *S. pneumoniae, H. influenzae* and *S. aureus* in a PCV7-immunized cohort, using a quantitative molecular Nanofluidic real-time qPCR method. The density of overall pneumococcal carriage was found to be higher in HIV-infected children despite a lower carriage prevalence, which could possibly explain the higher disease burden in HIV-infected compared to HIV-uninfected children even in the era of PCV-immunization [16]. While there were some differences in the baseline characteristics of HIV-infected and HIV-uninfected children including a higher administration rate of co-trimoxazole prophylaxis (CoT) in HIV-infected children and a higher rate of breastfeeding in HIV-uninfected children, the effect on pneumococcal carriage prevalence and density remained unchanged even after adjusting for these possible confounders. Nevertheless, antibiotic usage including CoT prophylaxis, in general, is higher in HIV-infected children and thus should be considered when interpreting the findings of our study especially since a recent study in Zambia showed CoT to increase the risk of colonization with CoT-resistance pneumococci within 6 weeks of starting prophylaxis [20]. Also, other studies have also reported resistance as high as 50–80% in HIV-infected children which is predominantly found in vaccine-type pneumococci [21–23]. Antimicrobial exposure in HIV-infected children can thus decrease their risk of non-vaccine serotype pneumococci (due to their sensitivity to antimicrobials) and further retain their CoT-resistant vaccine pneumococci which were consistent with our findings. Nevertheless, it is also plausible that the PCV induced vaccine effect [24] was more pronounced in HIV-infected children

Table 4
Density of nasopharyngeal bacterial carriage stratified by HIV status.

	9 month old children HIV-infected*n* = 208	HIV-uninfected*n* = 173	*p*-value	16 month old children HIV-infected*n* = 218	HIV-uninfected*n* = 164	*p*-value
S. pneumoniae	4.81(4.59–5.03)	4.44(4.24–4.63)	0.04	4.49(4.26–4.72)	4.39(1.18–4.61)	0.89
VT S. pneumoniae	4.21(3.12–5.11)	3.72(3.45–3.99)	0.04	3.60(3.30–3.89)	3.61(3.30–3.93)	0.93
NVT S. pneumoniae	3.48(3.16–3.79)	3.29(3.03–3.54)	0.35	3.45(3.09–3.81)	3.34(3.09–3.59)	0.42
M. catarrhalis	3.55(3.33–3.77)	3.27(3.06–3.48)	0.42	3.49(3.29–3.69)	3.24(3.04–3.44)	0.24
H. influenzae	5.0(4.72–5.29)	4.73(4.49–4.97)	0.08	4.32(4.07–4.57)	4.95(4.73–5.17)	<0.001
NT*H. influenzae*	4.92(4.62–5.23)	4.71(4.45–4.97)	0.07	4.23(3.97–4.50)	5.0(4.75–5.24)	<0.001
S. aureus	3.93(3.44–4.42)	4.03(3.43–4.63)	0.39	2.21(1.44–3.0)	3.03(2.71–3.34)	0.82

For density of carriage c mean-$\log_{10}$ densities and 95% CI intervals (95% CI) of pneumococcal and bacterial mean concentrations were calculated and compared with multivariate analysis controlling for race, gender, study centre, birth weight, co-trimoxazole prophylaxis administered and breastfeeding. 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, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/B/F, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22A/F, 23A/B, 25A/25F/38, 34/37/17A and 35B).m NT*hinf*, non-typable *H. influenzae*.. A *p*-value < 0.05 was considered significant.

Table 5
Correlation of mean-$\log_{10}$ density of nasopharyngeal bacterial colonizers as determined by Nanofluidic real-time qPCR Stratified by HIV-status.

	9 Month old children		16 Month old children	
	HIV-infected *n* = 208	HIV-uninfected *n* = 173	HIV-infected *n = 218*	HIV-uninfected *n = 164*
	Rho; *p*-value			
S. pneumoniae & M. catarrhalis	0.336; $p = 0.005$	0.271;$p = 0.013$	0.308; $p = 0.002$	0.321; $p = 0.005$
VT *& M.catarrhalis*	0.255; $p = 0.05$	0.30; $p = 0.03$	0.372; $p = 0.018$	0.389; $p = 0.03$
NVT *& M. catarrhalis*	0.462; $p = 0.01$	0.255; $p = 0.11$	0.239; $p = 0.05$	0.391; $p = 0.012$
S. pneumoniae & *S. aureus*	−0.659; $p = 0.01$	0.0964; p=0.0.73	0.007; $p = 0.97$	0.139; $p = 0.36$
VT & *S. aureus*	0.143; $p = 0.76$	0.2; $p = 0.75$	0.371; $p = 0.46$	NP
NVT & *S. aureus*	−0.714; $p = 0.11$	0.143; $p = 0.76$	0.201; $p = 0.52$	NP
S. pneumoniae & H. influenzae	0.329; $p = 0.01$	0.422; $p < 0.001$	0.441; $p < 0.001$	0.368; $p < 0.001$
VT *& H. influenzae*	0.22; $p = 0.02$	0.262; $p = 0.01$	0.419; $p = 0.01$	0.552; $p < 0.001$
NVT *& H. influenzae*	0.328; $p = 0.03$	0.272; $p = 0.03$	0.287; $p = 0.05$	0.381; $p = 0.005$
S. pneumoniae & NThinf	0.27; $p = 0.04$	0.363; $p = 0.002$	0.421; $p = p < 0.001$	0.262; $p = 0.024$
VT *&* NThinf	0.112; $p = 0.56$	0.221; $p = 0.24$	0.408; $p = 0.025$	0.429; $p = 0.03$
NVT *&* NThinf	0.244; $p = 0.21$	0.313; $p = 0.052$	0.259; $p = 0.1$	0.359; $p = 0.21$
M. catarrhalis & S. aureus	−0.428; $p = 0.34$	−0.518; $p = 0.08$	0.007; $p = 0.97$	0.259; $p = 0.1$
M. catarrhalis & H. influenzae	0.266; $p = 0.04$	0.344; $p = 0.004$	0.237; $p = 0.04$	0.502; $p < 0.001$
M. catarrhalis & NThinf	0.296; $p = 0.05$	0.342; $p = 0.01$	0.288; $p = 0.02$	0.514; $p = 0.001$
S. aureus & H. influenzae	−0.084; $p = 0.8$	0.147; $p = 0.65$	NP	0.036; $p = 0.9$
S. aureus & NThinf	−0.084; $p = 0.79$	0.054; $p = 0.87$	NP	0.016; $p = 0.96$

Bacterial correlations were determined by spearman's coefficients. 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, 7A/F, 7C, 8, 10A, 11A/B/C/D/F, 12A/12B/12F/44/46, 13, 15A/B/C/F, 16F, 17F, 19A, 20, 21, 22A/F, 23A/B, 25A/25F/38, 34/37/17A and 35B). NT*hinf*, non-typable *H. influenzae.* NP, study not powered to calculate AOR and *p*-value due to too few observations. A *p*-value <0.05 was considered significant.

due to impaired host immune response, allowing for residual invasive vaccine serotypes to be carried at a higher carriage density. Additional studies investigating the immunological responsiveness and IGA levels in HIV infected children with respect to colonization density could provide clarity.

Further, the HIV-infected children included in this study were initiated on antiretroviral treatment immediately upon diagnosis of their HIV-infection status or within the first year of life. This might also explain the lower carriage prevalence of overall pneumococcus in the HIV-infected cohort as PCV has been shown to induce a more durable and functional antibody response in individuals on ART at the time of vaccination compared to ART-naïve individuals, independently of the baseline CD4+ cell count [24]. The possible effects of ARTs on pneumococcal carriage density are however unknown and the clinical relevance of this warrants further studies as the temporary increase in pneumococcal carriage density observed in this study might explain why invasive pneumococcal disease remains high in HIV-infected individuals despite ART usage [25].

In addition, we also identified that carriage prevalence and density of *H. influenzae* to be higher in HIV-uninfected children, which was consistent with our previous findings by culture [7] and to another study done in Tanzania [4], albeit differing from another South African study which reported that the prevalence of *H. influenzae* was higher in HIV-infected children [2,26]. The increase in the carriage prevalence and density driven by NT*hinf* was most likely due to the low detection rate of the serotypes a-f. Differences observed in the prevalence of pneumococcal and *H. influenzae* colonization between the PCV-vaccinated HIV-infected and HIV-uninfected children in our study, may be due to receipt of CoT prophylaxis in HIV-infected children. Previous studies have reported a lower prevalence of *S. pneumoniae* colonization in HIV-infected participants on CoT prophylaxis which in turn could result in a lower prevalence of *H. influenza* which acts synergistically with *S. pneumoniae* colonization [7]. Also while we found no change in the carriage prevalence or density of *S. aureus*, other studies from South Africa [5,6] and Tanzania [4] found the carriage prevalence of *S. aureus* to be higher in HIV-infected children. Differences in the findings from these studies could possibly be attributed to the different PCV-vaccination status of the children; however, additional studies in PCV-vaccinated cohorts with more sensitive molecular methods are still needed to clarify these associations.

Positive associations with respect to prevalence were found between pneumococcus, *H. influenzae* and *M. catarrhalis* in both HIV-infected and HIV-uninfected children, with associations being driven by PCV7-serotypes, non-vaccine serotypes, and NT*hinf.* This finding is in contrast to other studies that found these associations in HIV-negative children alone [2]. The HIV-infected children in our study, however, differed from previous studies on bacterial colonization interactions in that children received antiretroviral treat-

ment and were thus relatively immunocompetent. Further, no association between pneumococcus and *S. aureus* prevalence was found in either group which is consistent with findings from another study in South Africa [6]. Although this was a colonization study and we are unable to measure whether IBD from one bacteria is associated with IBD from another, colonization studies are increasingly been advocated as a proxy for measuring the efficacy of PCV immunization on reducing disease. It is plausible that the host, including adaptive immune factors, plays a role in the likelihood of IBD on NP carriage and may contribute to the complex bacterial-bacterial interactions of potentially pathogenic bacteria and possibly commensal bacteria in the nasopharynx, particularly HIV-infected who are at an increased risk of nasopharyngeal acquisition due to deficiencies in their immunological mechanisms [27,28].

Our study was limited in that the Fluidigm method was not optimized to discriminate between all serotypes within their respective serogroups. Further, the study was not adequately powered to evaluate the difference in carriage density of serotypes/serogroups as the sample size of detected minor serotypes was too small. Nasopharyngeal swabs do not favor the isolation of *S. aureus* in which anterior nares are better for detection [29]. The prevalence of these bacteria could have thus been underestimated and the analysis on *S. aureus* prevalence needs to be interpreted with caution. Lastly, only a selection of NP organisms was analyzed and thus analysis at a microbiome level may be advantageous. DNA-based microbiota density estimation methods could be useful in investigating nasopharyngeal microbial bacteria, especially since this approach allows for both host and microbial factors to be investigated across a diverse set of microorganisms and could be used to describe microbiota density changes in disease [30].

Since NP colonization is the precursor to disease of the upper and lower respiratory tract, analysis of the NP tract flora can provide a simple means to signal changes, which could inform future trends in the long term effectiveness of these vaccines, especially in children with HIV infection who have a higher disease burden. The ability of the Fluidigm instrument to simultaneously detect multiple pneumococcal serotypes and bacterial pathogens at a low carriage density allowed us to gain a better understanding of the impact of HIV-infection and nasopharyngeal bacterial carriage. In our study we showed the carriage density of overall pneumococcus to be higher in HIV-infected children, despite the lower carriage prevalence. This might explain the higher invasive disease burden in HIV-infected compared to HIV-uninfected children as a high nasopharyngeal density of *Streptococcus pneumoniae* has previously been shown to be associated with invasive pneumococcal pneumonia [31]. The higher carriage density observed in HIV-infected children could be attributed to a combination of factors, including HIV treatment and impaired host immunity. Additional studies are needed to fully understand the impact of HIV-infection on the density of common nasopharyngeal bacterial colonizers.

Ethics approval

Ethical consent for the initial enrolment of the cohort 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]. Approval for further testing of samples was obtained from the HREC (M140907). Written, informed consent had been obtained from the parents/guardians of all the study participants at the time of initial enrolment.

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Author's contributions

C.P.O., P.V.A., and S.A.M. conceived and designed the study. C.P.O. was responsible for data curation and analysis. C.P.O wrote the first draft of the manuscript, and all authors were involved in reviewing and editing the manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.12.033.

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