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Diversity of Nontypeable *Haemophilus influenzae* Strains Colonizing Australian Aboriginal and Non-Aboriginal Children

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Nontypeable *Haemophilus influenzae* (NTHI) strains are responsible for respiratory-related infections which cause a significant burden of disease in Australian children. We previously identified a disparity in NTHI culture-defined carriage rates between Aboriginal and non-Aboriginal children (42% versus 11%). The aim of this study was to use molecular techniques to accurately determine the true NTHI carriage rates (excluding other culture-identical *Haemophilus* spp.) and assess whether the NTHI strain diversity correlates with the disparity in NTHI carriage rates. NTHI isolates were cultured from 595 nasopharyngeal aspirates collected longitudinally from asymptomatic Aboriginal ($n = 81$) and non-Aboriginal ($n = 76$) children aged 0 to 2 years living in the Kalgoorlie-Boulder region, Western Australia. NTHI-specific 16S rRNA gene PCR and PCR ribotyping were conducted on these isolates. Confirmation of NTHI by 16S rRNA gene PCR corrected the NTHI carriage rates from 42% to 36% in Aboriginal children and from 11% to 9% in non-Aboriginal children. A total of 75 different NTHI ribotypes were identified, with 51% unique to Aboriginal children and 13% unique to non-Aboriginal children ($P < 0.0001$). The strain richness (proportion of different NTHI ribotypes) was similar for Aboriginal (19%, 65/346) and non-Aboriginal children (19%, 37/192) ($P = 0.909$). Persistent carriage of the same ribotype was rare in the two groups, but colonization with multiple NTHI strains was more common in Aboriginal children than in non-Aboriginal children. True NTHI carriage was less than that estimated by culture. The Aboriginal children were more likely to carry unique and multiple NTHI strains, which may contribute to the chronicity of NTHI colonization and subsequent disease.

Nontypeable *Haemophilus influenzae* (NTHI) is an opportunistic bacterial pathogen that resides in the human respiratory tract. Infections caused by NTHI include sinusitis, pneumonia, bronchitis, exacerbations of chronic obstructive pulmonary disease (COPD), and otitis media (OM) (1–5). OM is a significant cause of morbidity in Australia, especially for Aboriginal children who experience some of the highest rates of OM in the world (6).

The NTHI population is highly heterogeneous with a genome that is continually modified through recombination, genetic polymorphisms, and phase variation to evade host immunity and overcome antibiotic treatment (7). Identification of the circulating NTHI strains provides insight into the dynamics of NTHI carriage and subsequent infection (8). For example, a European study found that infants and primary caregivers often carry the same NTHI multilocus sequence types, suggesting frequent transmission between individuals in close proximity to one another (9). The carriage of multiple NTHI PCR ribotypes has been suggested to contribute to the chronicity of OM in Australian Aboriginal children (10), and acquisition of a new strain of NTHI is thought to contribute to exacerbations in adults with COPD (11). It has also been shown that host immunity to one NTHI strain does not confer protection against colonization or infection with a different NTHI strain (12). Such data contribute to our understanding of disease burden and can be used to inform vaccine design and guide targeted intervention studies.

Accurate NTHI surveillance is paramount to assessing the potential effect of vaccination with pneumococcal *H. influenzae* protein D conjugate vaccine (PHiD-CV) (13) on NTHI carriage and disease. It is now well established that the respiratory tract commensal bacterium *Haemophilus haemolyticus* can be misidentified as NTHI by standard laboratory methods (14–17). This is due to the loss of the defining "hemolytic" phenotype for many *H. haemolyticus* strains, making them phenotypically indistinguishable from NTHI (16). It is important to use molecular techniques to differentiate NTHI from *H. haemolyticus* for accuracy in NTHI surveillance and vaccine efficacy studies.

We have previously described nasopharyngeal colonization (18) and OM (19, 20) in Aboriginal and non-Aboriginal children living in the Kalgoorlie-Boulder area of the Goldfields in Western Australia as part a prospective longitudinal cohort study known as the Kalgoorlie Otitis Media Research Project (KOMRP) (21). Aboriginal children in the KOMRP had higher NTHI carriage rates than non-Aboriginal children (42.0% versus 11.1%) in the first 2 years of life. Rates of OM were highest for Aboriginal children, and the peak prevalence of OM occurred earlier with 70% experiencing OM between 5 and 9 months of age compared with 40% of

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non-Aboriginal children experiencing OM between 10 and 14 months of age (19). By age 2 months, *H. influenzae* was isolated at least once from 27% of Aboriginal children compared with 6% of non-Aboriginal children (18). Early carriage of NTHI was associated with an increased risk of subsequent OM (20).

In the present study, we investigated further the disparity in NTHI carriage between Aboriginal and non-Aboriginal children. We used 16S rRNA gene PCR to determine the true number of NTHI isolates and PCR ribotyping to determine their genetic relatedness. As there is no cross-protective immunity to colonization or disease caused by different NTHI strains, we hypothesized that the higher NTHI carriage rate observed in Aboriginal children is associated with greater strain richness (number of different ribotypes).

MATERIALS AND METHODS

Study population. The KOMRP study population has been well described (18, 21). Briefly, 100 Aboriginal and 180 non-Aboriginal children living in the Kalgoorlie-Boulder region were recruited into the study soon after birth between April 1999 and January 2003. A total of 1,559 nasopharyngeal aspirates (NPAs) were obtained from study participants (504 from the Aboriginal children and 1,045 from the non-Aboriginal children) at 1 to 3 weeks, at 6 to 8 weeks, and again at 4, 6, 12, 18, and 24 months of age. Twenty-seven percent of the Aboriginal and 48% of the non-Aboriginal children had 7 NPAs collected (18). Fifty-one percent of the Aboriginal children and 66% of the non-Aboriginal children completed 2 years of follow-up (21).

Laboratory methods. (i) Culture and storage. The NPAs were collected, stored, and cultured for respiratory bacteria as previously described (18). Ten microliters of each sample was inoculated onto the following media: horse blood agar, chocolate agar containing bacitracin (300 mg/liter), vancomycin (5 mg/liter) and clindamycin (0.96 mg/liter), and colistin nalidixic acid blood agar plates (Oxoid, SA, Australia). The NTHI isolates were identified by the colony morphology, the requirements for X (hemin) and V (β-NAD) factors, and the lack of reaction with capsular antisera using the Phadebact *Haemophilus* test (MKL Diagnostics, Stockholm, Sweden). For each NPA cultured, two apparent NTHI colonies were selected where available, including any that were morphologically distinct. The isolates were subcultured and stored in brain heart infusion broth (PathWest Media, WA, Australia) containing 20% glycerol (Sigma-Aldrich, Castle Hill, Australia) at −80°C.

(ii) PCR to differentiate between NTHI and other *Haemophilus* spp. Isolates were streaked onto chocolate agar plates and grown overnight in a humidified incubator at 37°C with 5% CO_2. A single bacterial colony was lysed in 20 μl of sterile molecular-grade water by vigorous pipetting (Invitrogen, Life Technologies, VIC, Australia). A 16S rRNA gene colony PCR specific for NTHI and all *Haemophilus* spp. was carried out on all lysates as described previously (16). PCRs were conducted on a LightCycler system (Bio-Rad) and included an NTHI 86-028NP reference strain positive control and a nuclease-free water negative control. Ten microliters of PCR products were run on a 10% electrophoresis gel with a TrackIt DNA ladder (Invitrogen) at 100 V for 80 min. The products were deemed positive with detectable band fluorescence and a correct size of 500 bp.

The genus-specific 16S rRNA gene PCR (16) was conducted on all isolates to identify all *Haemophilus* spp., including *H. haemolyticus*, in combination with the species-specific NTHI 16S rRNA gene PCR to positively identify the true NTHI isolates. Subtraction of the total positive 16SrRNA gene NTHI isolates from the total positive 16SrRNA gene *Haemophilus* isolates was used to determine the proportion of misidentified *Haemophilus* spp. Where both PCRs for an isolate were negative, genomic DNA was extracted from overnight growth (Wizard QV extraction system, Promega, VIC, Australia), and the PCRs were repeated. The *H. haemolyticus*-specific 16S rRNA gene PCR (16) regularly failed to amplify the positive control DNA and was therefore not used for analysis of the non-NTHI isolates.

(iii) PCR ribotyping. PCR ribotyping was conducted to assess the genetic diversity of the NTHI isolates. Two colonies selected as described above were PCR ribotyped from each NTHI-positive NPA. The total genomic DNA was extracted from single colonies by lysis of bacteria embedded in agarose at the bottom of the microtiter wells followed by extraction with proteinase K (22). The technique was described previously (23). Briefly, approximately 100 ng DNA was amplified in a 20-μl reaction mixture consisting of PC2 buffer (24), 0.2 mM deoxynucleoside triphosphates, 4 pmol each of 16SG primer and 5S primer. and 0.12 μl *Taq*/*Pfu* (15 U *Taq* polymerase and 2.5 U *Pfu* DNA polymerase in 16 μl). The cycling conditions were 25 times at 94°C for 10 s and 68°C for 8 min. To the resulting PCR product, 1.5 U of HaeIII was added, and the mixture was incubated at 37°C for 2 h. The restriction fragments were separated by electrophoresis on a 1.5% agarose gel in 1× TAE buffer (4 mM Tris acetate, 0.2 mM EDTA [pH 8.0]) containing 0.5 μg ethidium bromide/ml. PCR ribotypes were assigned based on restriction fragment lengths (0.4 kb to 1.2 kb) following alignment to a 2-log DNA ladder (New England BioLabs) and an in-house standard. To aid pattern recognition, the images were divided into 16 predetermined sections.

Statistical methods. Statistical analyses were conducted using GraphPad Prism version 5.02 (GraphPad Inc., CA). A two-tailed chi-square test with Yates' correction was used to compare the changes in the carriage rates for non-Aboriginal children. A two-tailed Fisher's exact test was used in all other instances where the proportion differences were compared. Stata version 13 was used to calculate the risk difference (RD) with 95% confidence intervals (CIs) for the change in carriage rates. P values of <0.05 were considered significant.

Ethics approvals. Ethics approval was granted by the Western Australian Aboriginal Health Information and Ethics Committee (WAAHIEC), the Northern Goldfields Health Service and Nursing Education Ethics Committee in Kalgoorlie, the Princess Margaret Hospital Ethics Committee, and the Confidentiality of Health Information Committee of the Health Department of Western Australia.

RESULTS

The proportion of NTHI misidentification was similar in culture-defined isolates from Aboriginal and non-Aboriginal children. A total of 595 culture-defined NTHI isolates were assessed (378 isolates from 81 Aboriginal children and 217 from 76 non-Aboriginal children). The overall proportion of misidentified NTHI defined by 16S rRNA gene PCR in this study was 9.5%, and the proportion misidentified did not differ significantly between groups (8.5% in the Aboriginal group and 11.5% in the non-Aboriginal group; $P = 0.248$) (Table 1). Removal of misidentified *Haemophilus* spp. reduced the actual NTHI carriage rates from 42.0% to 36.3% (RD, −5.7% [95% CI −11.7 to 0.2]; $P = 0.0708$) in the Aboriginal children and from 11.1% to 9.4% (RD, −2% [95% CI −4.4 to 0.8]; $P = 0.1950$) in the non-Aboriginal children (Table 1).

Strain richness is proportional to carriage rates in Aboriginal and non-Aboriginal children. PCR ribotyping did not distinguish NTHI from other *Haemophilus* spp., with 16 shared PCR ribotypes. Of the 538 true NTHI isolates, as defined by 16S rRNA gene PCR and henceforth referred to as NTHI, 525 were available for PCR ribotyping. A total of 75 different PCR ribotypes were identified. Although 65 NTHI ribotypes were identified from the Aboriginal children and 37 NTHI ribotypes were identified from the non-Aboriginal children (Fig. 1), the relative richness of NTHI strains (number of PCR ribotypes/number of true NTHI isolates) for both populations was similar (65/346 [18.7%] for the Aborig-

TABLE 1 *Haemophilus* isolates derived from the nasopharynxes of Aboriginal and non-Aboriginal children assessed in this study[a]

Sample	No. (%) of *Haemophilus* isolates in:		
	Aboriginal children	Non-Aboriginal children	*P* value (proportion difference)
Children carrying culture-defined NTHI on any visit	81/100 (81.0)	76/180 (42.2)	<0.0001
Swabs with presumptive NTHI isolates/total swabs (initial carriage rate)	212/504 (42.0)	117/1045 (11.1)	<0.0001
NTHI-like isolates derived from swabs	378	217	
True NTHI/viable isolates analyzed by PCR	346/378 (91.5)	192/217 (88.5)	0.248
Other haemophili (proportion of misidentification)	32/378 (8.5)	25/217 (11.5)	0.248
Swabs with PCR-confirmed NTHI isolates/total swabs (true carriage rate)	183/504 (36.3)	98/1045 (9.4)	<0.0001
True NTHI isolates available for PCR ribotyping	346	192	
Richness, i.e., no. of ribotypes/no. of true NTHI isolates available for PCR ribotyping	65/346 (18.7)	37/192 (19.3)	0.909
No. of different NTHI ribotypes/total no. of all ribotypes	65/75	37/75	<0.0001
Proportion of unique ribotypes	38/75 (50.7)	10/75 (13.3)	<0.0001
Proportion of unique ribotypes/total individual ribotypes	38/65 (58.4)	10/37(27.0)	0.004
No. of swabs with >1 NTHI PCR ribotype	37/183 (20.2)	8/98 (8.1)	0.010

[a] Where possible, two isolates were collected and stored from each NPA swab.

inal children versus 37/192 [19.3%] for the non-Aboriginal children; $P = 0.909$) (Table 1).

Aboriginal children carried more unique NTHI strains, were more likely to be colonized with two different NTHI strains, and displayed PCR ribotype profiles different from those of non-Aboriginal children. Thirty-eight (50.7%) of the 75 different PCR ribotypes were unique to the Aboriginal children, whereas 10 (13.3%) were unique to the non-Aboriginal children ($P < 0.0001$) (Table 1). The Aboriginal children were 2.5 times more likely to be carrying two different PCR ribotypes than the non-Aboriginal children (20.2% versus 8.1%, respectively; $P = 0.010$) (Table 1). The most common ribotypes for the Aboriginal children were 3 ($n = 36$), 8 ($n = 19$), 13 ($n = 19$), 4 ($n = 14$), 94 ($n = 14$), 15 ($n = 13$), and 46 ($n = 12$) compared with 4 ($n = 25$), 20 ($n = 23$), 3 ($n = 16$), 14 ($n = 13$), and 8 ($n = 10$) for the non-Aboriginal children (Fig. 1).

Persistence of the same PCR ribotype was rare within the first 2 years of life for Aboriginal and non-Aboriginal children. Figure 2 illustrates carriage dynamics for the 81 Aboriginal and 76 non-Aboriginal children (Table 1, row 1) with identification of the same PCR ribotype illustrated by a connecting line (continuous for the next visit and broken for a later visit). In most cases, NTHI carriage cleared or was replaced with a different NTHI ribotype by the next examination. However, the same ribotype was observed at successive examinations in 5 instances for the Aboriginal children and 2 for the non-Aboriginal children. "Hidden carriage" or recolonization with the same strain at a later visit was apparent in 5 children, all of whom were Aboriginal. The persistent/reacquired ribotype was different for 5 of 9 Aboriginal children (child no. 14, 17, 43, 45, and 74) but the same for the two non-Aboriginal children (child no. 32 and 37).

DISCUSSION

We hypothesized that we would detect greater genetic diversity in NTHI strains from Aboriginal children than in those from non-Aboriginal children who have less NTHI-related disease. This was based on evidence that the mucosal immune response to NTHI colonization is strain specific with little or no cross-protection against colonization with a different NTHI strain (12). Therefore, exposure to more strains might mean a greater risk of colonization or infection. However, we observed that there was no difference in the relative richness of NTHI strains colonizing the Aboriginal children compared with those colonizing the non-Aboriginal children when we accounted for the increased NTHI carriage rates in the Aboriginal children.

The NTHI strains isolated from the nasopharynxes of the Aboriginal children were different from those isolated from the non-Aboriginal children, with >50% of the strains unique to the Aboriginal children compared to only 13% of strains unique to the non-Aboriginal children. This demonstrates a lack of NTHI strain transmission between the two populations. Whether these unique strains are better adapted to colonization and, therefore, disease requires further investigation. Additionally, we cannot ignore the environmental factors that lead to a propensity for NTHI carriage (and OM) in Aboriginal children such as overcrowding and exposure to tobacco smoke (20, 25).

We found that Aboriginal children were more likely to carry multiple NTHI strains than their non-Aboriginal counterparts. We have previously reported that Aboriginal children can be colonized with multiple NTHI ribotypes (10) and are more likely to be colonized with multiple species of OM-causing bacteria than their non-Aboriginal counterparts, which contributes to the risk of developing OM (26). Carriage of multiple NTHI strains has been observed in non-Aboriginal populations with COPD or OM (27–29). The density of bacterial carriage has also been directly related to disease outcome, with higher NTHI loads in the nasopharynx of Aboriginal children leading to increased risk of developing suppurative OM (30). In addition to carriage of multiple bacterial species, carriage of multiple strains of the same species is likely to contribute to the increased bacterial burden and disease risk in Aboriginal children. Indeed, increased numbers of circulating NTHI strains provide a larger gene pool, ultimately increasing the opportunity for recombination events that provide a selective advantage for the pathogen. Further studies into the microbial ecology of NTHI and other otopathogens are warranted.

We rarely observed persistent NTHI carriage in Aboriginal or non-Aboriginal children in the first 2 years of life. This is in contrast to adults with COPD, who can be persistently colonized with the same NTHI strain, and a different population of Aboriginal infants who were found to be persistently colonized with the same ribotype over 3-month periods (10). If we had characterized more

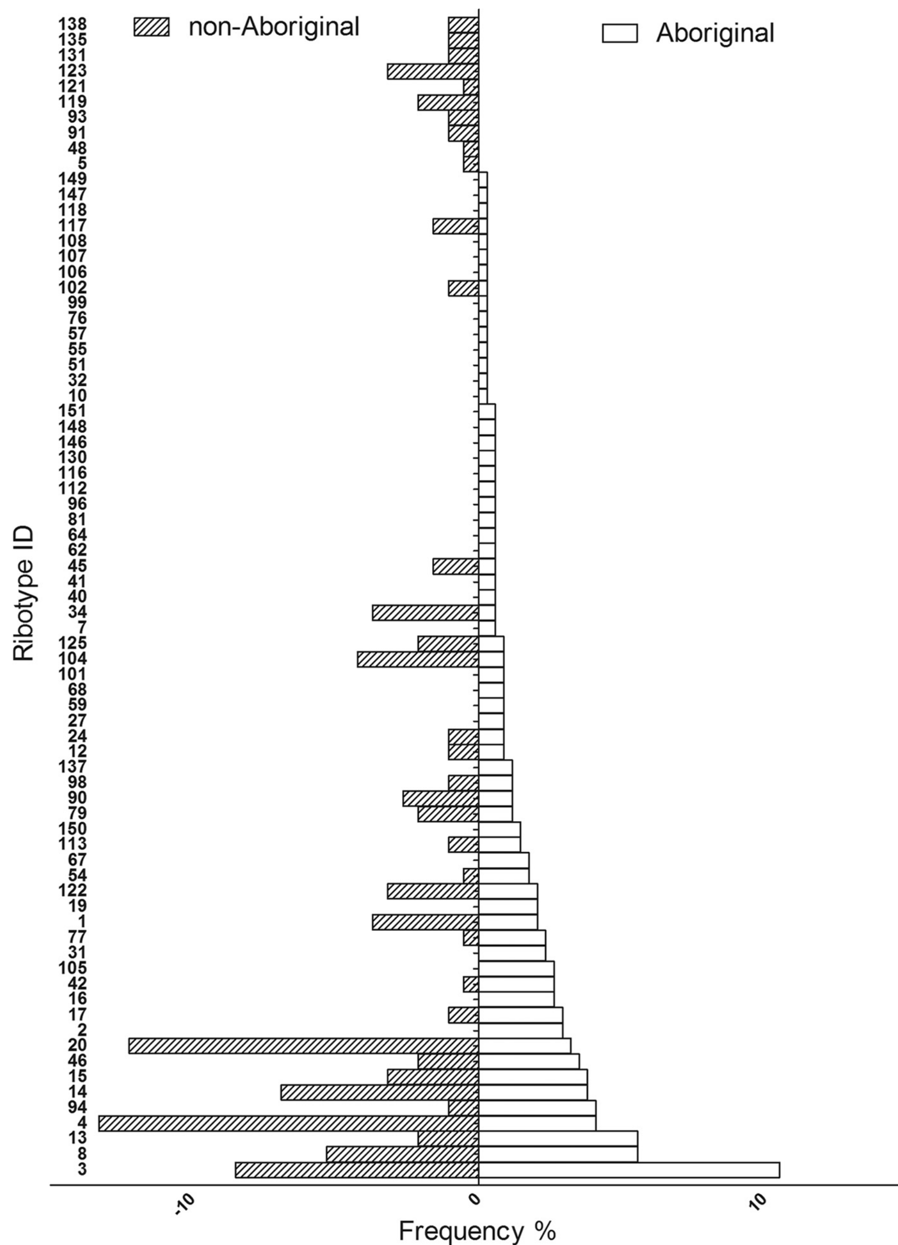

FIG 1 Comparison of NTHI ribotype profiles for the Aboriginal and non-Aboriginal children.

than 2 isolates per nasopharyngeal aspirate and conducted more frequent swabbing, then it is likely that the strain richness would have been greater and strains present in low density might have been detected. However, a modeling study of acquisition and loss of carriage of *H. influenzae* strains in Aboriginal children estimated that each NTHI strain was carried for 137 days but only detected on 37% of occasions. Furthermore, the study determined that an average of 1.5 strains was identified by routine typing of four colonies per nasopharyngeal swab (31).

PCR ribotyping is a rapid and cost-effective tool to understand pathogen diversity and identify outbreaks (32). However, this method does not distinguish NTHI from other *Haemophilus* species, such as *H. haemolyticus*, which can share the same PCR ribotype. The proportion of misidentified NTHI isolates in our study (9.5%) is comparable to that found in previous studies using 16S rRNA gene PCRs (16, 17). Although identification of true NTHI isolates remains a challenge (33), it is important for surveillance of NTHI carriage, disease, and vaccine efficacy studies.

A limitation of this study is the difference in complete follow-up of children to 2 years of age (21). An increase in complete follow-up for Aboriginal children would result in an increased number of *Haemophilus* isolates and more unique ribotypes; however, these are unlikely to alter the strain richness, which is proportional to the carriage rate. More complete follow-up for Aboriginal children may reveal an increase in persistent carriage of the same strain.

In summary, Aboriginal and non-Aboriginal children in the KOMRP were colonized with different NTHI ribotypes. Although

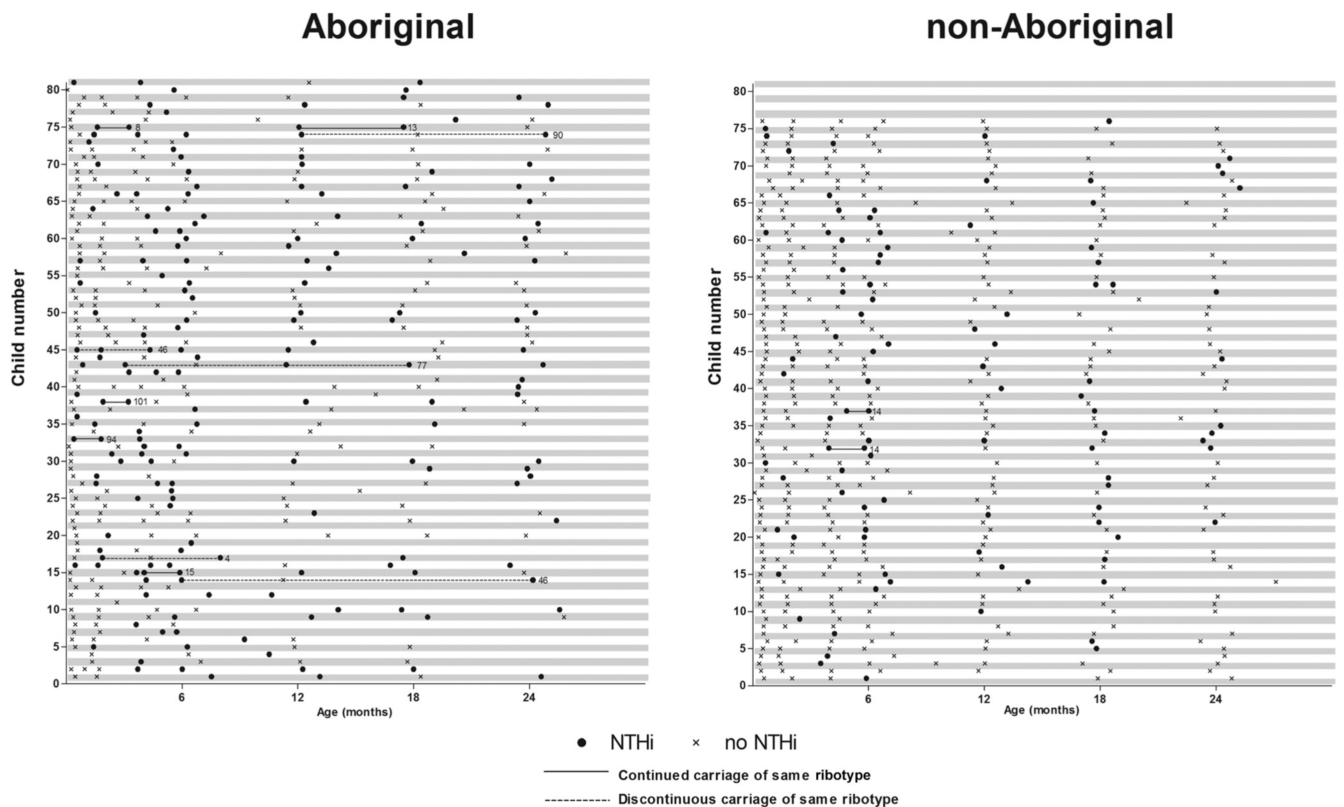

FIG 2 Dynamics of NTHI carriage in 81 Aboriginal children and 76 non-Aboriginal children over the first 2 years of life. The longitudinal carriage of the same PCR ribotype is illustrated by a continuous line for adjacent visits and a broken line for a later visit. Continued carriage or reacquisition of the same ribotypes (indicated by PCR ribotype identification numbers next to carriage points) was infrequent.

more NTHI ribotypes were isolated from Aboriginal children, the proportions of NTHI strains that were different ribotypes (strain richness) were similar in Aboriginal and non-Aboriginal children. Aboriginal children were more likely to carry unique and multiple NTHI strains, which may contribute to the chronicity of NTHI colonization and subsequent disease in Aboriginal children. Whether particular NTHI strains have a greater propensity to cause disease than others remains to be determined.

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