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%, 63/346) 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
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 were reached, stored, and cultured for respiratory bacteria as previously described (19). By age 2 months, non-Aboriginal children experiencing OM between 10 and 14 months of age (19), the relative richness of NTHI isolated in the Aboriginal children and from 11.1% to 9.4% (RD, 4.4 to 0.8%; 95% CI 5.7% [95% CI 11.7 to 0.2%]; 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 Aboriginal...
TABLE 1 *Haemophilus* isolates derived from the nasopharynges of Aboriginal and non-Aboriginal children assessed in this study

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

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

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 nasopharynges 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
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
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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