Title: Nasopharyngeal carriage of *Haemophilus haemolyticus* in otitis-prone and healthy children

Running title: *H. haemolyticus* carriage in children with rAOM

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Abstract: *Haemophilus haemolyticus* is often incorrectly categorized as nontypeable *Haemophilus influenzae* (NTHi) upon culture. PCR of 266 ‘NTHi-like’ nasopharyngeal isolates from children with and without recurrent acute otitis media (rAOM) revealed that 11.7% were *H. haemolyticus* and 9.4% gave equivocal results. Children with rAOM were more likely to carry *H. haemolyticus*.

Recent literature has revealed that a substantial number of pharyngeal isolates previously identified as nontypeable *Haemophilus influenzae* (NTHi) are actually the commensal *H. haemolyticus* (2, 3, 7). NTHi and *H. haemolyticus*, particularly non-hemolytic *H. haemolyticus*, cannot be differentiated by standard laboratory tests of cultured organisms as both have hemin (X-factor) and nicotinamide adenine dinucleotide (V-factor) dependence (3). It is important to distinguish between these two species, particularly in pharyngeal carriage studies, as *H. haemolyticus* rarely causes disease whereas NTHi is a major cause of non-invasive mucosal infections such as otitis media and chronic obstructive pulmonary disease (COPD) (4, 8). Molecular differentiation of these two phylogenetically related species (2, 3) is particularly important for studies of new vaccines in which a decrease in pharyngeal colonization is an end-point (5, 6).

The aim of this study was to use PCR to differentiate between NTHi and *H. haemolyticus* isolated from the nasopharynx of children with and without recurrent acute otitis media (rAOM). Nasopharyngeal swabs were collected from 267 children aged 6 to 36 months from 2007 to 2009. All subjects were anaesthetized for either ventilation tube insertion (186 otitis-prone children) or other general surgical procedures (81 otherwise healthy children). Ethics approval was obtained from
Princess Margaret Hospital and informed consent was obtained from parents of participating children before study entry. The nasopharyngeal swabs were stored in skim milk typtone glucose glycerol broth (STGGB) (9), placed on ice and transported to the laboratory within 4 h. Samples were vortexed vigorously for 1 min and stored at -80 °C until analyses. Swabs were cultured using standard microbiology as previously described (9), where two ‘NTHi-like’ colonies were selected and sub-cultured when present. When possible, different NTHi phenotypes (e.g. large/small, grey/yellow) were selected. To distinguish between NTHi and \textit{H. haemolyticus}, colony PCR was conducted on presumed NTHi isolates using a previously published protocol and 16S ribosomal DNA species-specific primers (3). In detail, glycerol stocks of sub-cultured isolates were streaked onto chocolate agar plates and incubated overnight at 37°C, 5% CO2. Three colonies were picked and resuspended in 20µl nuclease-free distilled water. The cell lysate was then used to prepare two separate PCR reactions, one with \textit{H. influenzae} specific 16SrDNA primers and another with \textit{H. haemolyticus} specific 16SrDNA primers. PCR products were run alongside Trackit DNA ladder (Invitrogen, Mulgrave, Australia) on 1% agarose gels at 100V for 80 min. When a 500bp PCR product was observed for only one set of primers, the isolate was determined to be either NTHi or \textit{H. haemolyticus} accordingly. Where a 500bp band was observed for both primer sets or no PCR product was observed, genomic DNA was prepared from these isolates using the Wizard SV gDNA kit (Promega, Alexandria, Australia) and the PCR was repeated on the purified gDNA. If the same results were obtained then the isolates were deemed unidentifiable by 16SrDNA PCR. Further analysis, e.g. DNA sequencing was not undertaken. Pearson Chi-square test was used for statistical analysis using SPSS for Windows version 16.0.
NTHi-like isolates were cultured from nasopharyngeal swabs of 139 children, including 122 otitis-prone children (cases) and 17 healthy children (controls). This gave an ‘NTHi-like’ carriage rate of 65.6% (122/186) for cases and 21.0% (17/81) for controls (P<0.001). Two NTHi-like isolates were obtained from 127 children (n = 254) and only 1 isolate was available from 12 children, resulting in a total of 266 NTHi-like isolates available for molecular analysis. 16SrDNA PCR revealed that 78.9% (n = 210) of isolates were true NTHi, whereas 11.7% (n = 31) were reclassified as *H. haemolyticus*. The remaining 9.4% (n = 25) of the presumptive NTHi isolates gave equivocal results for the 16S rDNA PCR, where neither NTHi nor *H. haemolyticus* status could be given. No characteristic colony morphology was observed for the *H. haemolyticus* 16SrDNA PCR positive isolates to distinguish them from NTHi as descriptions of small, large, mucoid, cream, grey and yellow were given to both NTHi and *H. haemolyticus* colonies. For 19 of the 25 equivocal samples no PCR product was obtained for either 16SrDNA PCR, and for 6 isolates a 500bp PCR product was obtained for the 16SrDNA gene of both NTHi and *H. haemolyticus*. All 25 isolates that were equivocal for the 16SrDNA PCR were found to have the P6 and *hpd* genes, which encode outer membrane proteins expressed by NTHi and *H. haemolyticus* (3, 5).

Assessment of two NTHi-like isolates per child (n = 110/122 cases and 17/17 controls) revealed that for otitis-prone children, 70% (n = 77) of the time both isolates were NTHi, 3.6% (n = 4) of the time both isolates were *H. haemolyticus*, and on 14 occasions (12.7%) the cases were positive for one of each species. Of the 17 healthy controls with 2 NTHi-like isolates, 82.4% (n = 14) of both isolates were NTHi, 1 child was positive for *H. haemolyticus* only, and 1 child positive for each species (Table 1).
The proportion of children from which neither isolate could be distinguished as NTHi nor *H. haemolyticus* was similar for cases and controls (4.5% versus 5.9% respectively). Overall, when all 266 isolates were assessed (including the 12 individual isolates from the cases) we found that otitis-prone children were colonized with *H. haemolyticus* more often than healthy controls (20.5% [n=25] versus 11.8% [n=2]). Defining true NTHi isolates by 16SrDNA PCR resulted in a drop in reported NTHi carriage rates in our cohort from 65.6% to 55.9% for otitis-prone children and 21.0% to 18.5% for healthy children.

This study describes the largest collection, to date, of culture-identified NTHi nasopharyngeal carriage isolates that have been retested for *H. haemolyticus* by molecular methods. The proportion of *H. haemolyticus* identified in the nasopharyngeal swabs from otitis-prone children is similar to that in a previous report, where retrospective molecular analysis of NTHi isolates from nasopharyngeal swabs showed that 27% (12/44) of NTHi isolates were actually nonhaemolytic *H. haemolyticus* (3). Murphy et al. (3) also reported a 10% rate for equivocal 16SrDNA PCRs, which was similar to our study (9.4% of isolates could not be distinguished). The fact that we found NTHi and *H. haemolyticus* together in over 12% of children (Table 1) by only assessing 2 colonies suggests that *H. haemolyticus* carriage may be even greater than anticipated. *H. haemolyticus* was not identified in middle ear effusion collected in our study from children with rAOM (data not shown), supporting previous reports (3) and the view that *H. haemolyticus* is not a cause of otitis media. Studies investigating the colonisation dynamics of NTHi and *H. haemolyticus* in otitis-prone children are warranted to fully assess the proportion of NTHi and *H. haemolyticus* carriage in the same child over time.
With the introduction of a pneumococcal vaccine (PHiD-CV) that is potentially efficacious against NTHi otitis media (6), it is crucial that NTHi carriage and disease isolates are assessed at the molecular level, and not solely by culture. This will provide a more definitive measure of the impact of PHiD-CV on NTHi carriage and otitis media. NTHi isolates from the initial POET (pneumococcal otitis efficacy trial) study using an 11-valent PHiD-CV prototype (6) have recently been re-examined by PCR and immunoblot. By reclassifying 29 NTHi isolates as *H. haemolyticus*, reduction of NTHi nasopharyngeal carriage following vaccination in the POET study changed from 41.4% to 36.7% (5). This was not a significant change to the overall impact of the vaccine on NTHi carriage, however the population assessed had generally low pre-vaccination NTHi carriage rates of ~15% (5). In contrast, over 50% of the otitis-prone children in our study carried NTHi suggesting that an NTHi-targeted vaccine may have an even greater impact on NTHi carriage in such populations. This will require close molecular surveillance.

It is important to acknowledge that *H. haemolyticus* also expresses Protein D (5), the immunogenic NTHi protein component of PHiD-CV (1), and may therefore also be eliminated from the nasopharynx after PHiD-CV vaccination. Potential elimination of such commensals from the polymicrobial environment of the nasopharynx requires close monitoring as this may create a niche for pathogenic bacteria. Without molecular analysis of nasopharyngeal isolates both pre- and post-vaccination, the impact of PHiD-CV (or any other NTHi-targeted vaccine) cannot be accurately determined.
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References


Table 1: Differentiation of NTHi and *H. haemolyticus* by 16SrDNA PCR of presumed NTHi nasopharyngeal isolates from otitis-prone and healthy children.

<table>
<thead>
<tr>
<th>16SrDNA PCR results</th>
<th>CASES* (%)</th>
<th>CONTROLS* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>110</td>
<td>17</td>
</tr>
<tr>
<td>Both NTHi</td>
<td>77 (70.0)</td>
<td>14 (82.4)</td>
</tr>
<tr>
<td>NTHi and Hh</td>
<td>14 (12.7)</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Both Hh</td>
<td>4 (3.6)</td>
<td>0</td>
</tr>
<tr>
<td>NTHi and equivocal</td>
<td>6 (5.5)</td>
<td>0</td>
</tr>
<tr>
<td>Hh and equivocal</td>
<td>4 (3.6)</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Both equivocal</td>
<td>5 (4.5)</td>
<td>1 (5.9)</td>
</tr>
</tbody>
</table>

* cases are children with rAOM; *controls are healthy age-matched children; *Total number of children with two NTHi-like isolates collected, children with only 1 ‘NTHi-like’ isolate are excluded; NTHi, nontypeable *H. influenzae*; Hh, *H. haemolyticus*.