Title: Culture and PCR detection of *Haemophilus influenzae* and *Haemophilus haemolyticus* in Australian Indigenous children with bronchiectasis.

Running title: *Haemophilus* species in the upper and lower airways.

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ABSTRACT

A PCR for protein D (hpd#3) was used to differentiate nontypeable *Haemophilus influenzae* (NTHi) from *Haemophilus haemolyticus*. While 90% of nasopharyngeal and 100% of lower airway specimens from 84 Indigenous Australian children with bronchiectasis had phenotypic NTHi isolates confirmed as *H. influenzae*, only 39% of oropharyngeal specimens with phenotypic NTHi had *H. influenzae*. The nasopharynx is therefore the preferred site for NTHi colonization studies, and NTHi is confirmed as an important lower airway pathogen.
Nontypeable *Haemophilus influenzae* (NTHi) colonizes the upper airways where it is an important cause of otitis media (10). It is also isolated frequently from the lower airways of adults with chronic obstructive pulmonary disease (COPD) (8) and children and adults with bronchiectasis (3,4).

Accurate identification of NTHi is important since non-hemolytic strains of the closely-related (primarily commensal) *Haemophilus haemolyticus* (Hh) may be misidentified as NTHi by phenotypic methods used in most clinical microbiology laboratories (9). Molecular-based detection techniques have revealed that 12-27% of phenotypic NTHi nasopharyngeal isolates from healthy and otitis-prone children were Hh (5,9), while Hh was not identified in middle ear fluid from children with acute otitis media (5). In contrast, Hh comprised 40% of phenotypic NTHi sputum isolates from adults with COPD (9).

These findings raise important questions over NTHi as a true lower respiratory pathogen. We therefore investigated the proportion of Hh amongst phenotypic NTHi isolates in the upper and lower airways of Indigenous Australian children with bronchiectasis, a population with high rates of NTHi colonization and associated respiratory disease (2,3,12).

The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research approved the study. Following written, informed parent/guardian consent, paired nasopharyngeal swabs and bronchoalveolar lavage (BAL) fluid specimens were collected from a convenience sample of 84 (62% male) Indigenous children aged 5 to 155 (median 27) months.
undergoing routine diagnostic evaluation following radiographic confirmation of bronchiectasis at the Royal Darwin Hospital between July 2007 and December 2010. Oropharyngeal swabs were also collected from 56 of these children. Specimens were stored and cultured using standard microbiologic methods (3). Lower airway infection was defined by a semi-quantitative growth score of ≥4, which correlated with >10⁴ colony-forming units (cfu)/mL BAL fluid as determined by serial dilution and quantitative counts (3).

Phenotypic NTHi isolates were identified by morphology and requirement for X and V growth factors. Up to 4 colonies (including any with differing morphology) were isolated from each culture positive specimen and tested using PCR. Confirmation of phenotypic NTHi isolates was performed using defined DNA extraction methods (12) and a Taqman®-based, real-time PCR assay targeting protein D (hpd#3), which accurately discriminates *H. influenzae* (Hi) and Hh isolates (1,13). Phenotypic NTHi isolates returning negative PCR results were considered to be Hh, since the only other X and V factor dependent *Haemophilus* species (*H. aegyptius*, an important cause of conjunctivitis) has a different appearance, requires additional growth factors, and is unlikely to be cultured from these sites (6).

The table shows the numbers, proportions and distribution of phenotypic NTHi, *hpd#3* PCR confirmed Hi, presumptive Hh and concurrent Hi and Hh in the 224 specimens collected from the upper and lower airways. A total of 214 isolates from 108 phenotypic NTHi-positive specimens (an average of 1.8 colonies isolated per nasopharyngeal and oropharyngeal swab and 2.4 colonies per BAL specimen) were tested. Most nasopharyngeal (87%) and BAL fluid (88%) isolates were confirmed as
Hi, but almost two-thirds (65%) of oropharyngeal isolates were presumptive Hh. Hi and Hh were isolated concurrently from 10% (4/42) nasopharyngeal swabs, 17% (6/36) oropharyngeal swabs, and 27% (8/30) BAL fluid cultures from children with phenotypic NTHi carriage or lower airway infection.

This study shows that, similar to healthy and otitis-prone Western Australian children (5), most phenotypic NTHi nasopharyngeal isolates from Indigenous children with bronchiectasis were confirmed as Hi. In contrast, many apparent NTHi isolates from oropharyngeal swabs were Hh in our study population. Previous oropharyngeal culture-based studies of Hi may have therefore over-estimated NTHi carriage and our data instead support the nasopharynx as the preferred site for Hi carriage studies in children. Such studies are important to monitor antimicrobial resistance and detect changes in pharyngeal biota that may result from antibiotic administration or vaccination.

Quantifying pathogens in BAL fluid helps adjust for upper airway contamination during bronchoscopy. Our finding that 100% of BAL specimens with phenotypic NTHi lower airway infection (>10⁴ cfu/mL BAL fluid) were PCR-positive for Hi confirms NTHi as a lower airway pathogen. However the role of Hh is unclear. Prior studies report that Hh is rarely found in sterile sites, including middle ear fluid, or associated with clinically-defined infections (5,7,9,11). While our findings suggest that Hh has a propensity for the oropharynx, without specific molecular detection and quantification we can not determine whether its presence in lower airway cultures represents upper airway contamination or a pathogenic role.
In conclusion, we have used Hi-specific PCR to reaffirm the importance of NTHi as a lower airway pathogen in Australian Indigenous children with bronchiectasis. In addition, we have shown that the nasopharynx rather than the oropharynx is the preferred site for NTHi carriage studies in this population.

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Table: Culture and PCR results for phenotypic NTHi isolates from nasopharyngeal and oropharyngeal swabs and bronchoalveolar lavage fluid specimens from 84 Australian Indigenous children with bronchiectasis.

<table>
<thead>
<tr>
<th></th>
<th>Number of specimens</th>
<th>Number (%) specimens with phenotypic NTHi</th>
<th>Number (%) specimens with phenotypic NTHi confirmed by PCR as Hi</th>
<th>Number (%) specimens with presumptive Hh</th>
<th>Number (%) specimens with concurrent Hi and presumptive Hh</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP swab</td>
<td>84</td>
<td>42 (50)</td>
<td>38 (45)</td>
<td>8 (10)</td>
<td>4 (5)</td>
</tr>
<tr>
<td>OP swab</td>
<td>56</td>
<td>36 (64)</td>
<td>14 (25)</td>
<td>28 (50)</td>
<td>6 (11)</td>
</tr>
<tr>
<td>BAL fluid</td>
<td>84</td>
<td>30* (36)</td>
<td>30 (36)</td>
<td>8 (10)</td>
<td>8 (10)</td>
</tr>
<tr>
<td>Total</td>
<td>224</td>
<td>108 (48)</td>
<td>82 (37)</td>
<td>44 (20)</td>
<td>18 (8)</td>
</tr>
</tbody>
</table>

*NP = nasopharyngeal; OP = oropharyngeal; BAL = bronchoalveolar lavage

Hi = *Haemophilus influenzae*; NTHi = nontypeable Hi; Hh = *Haemophilus haemolyticus*

* Specimens with phenotypic-NTHi lower airway infection (>10^4 cfu/mL BAL fluid)