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# Culture and PCR Detection of *Haemophilus influenzae* and *Haemophilus haemolyticus* in Australian Indigenous Children with Bronchiectasis

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**A PCR for protein D (hpd#3) was used to differentiate nontypeable *Haemophilus influenzae* (NTHI) from *Haemophilus haemolyticus*. While 90% of nasopharyngeal specimens 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 nonhemolytic strains of the closely related (primarily commensal) *Haemophilus haemolyticus* may be misidentified as NTHI by phenotypic methods used in most clinical microbiology laboratories (9). Molecular detection techniques have revealed that 12 to 27% of nasopharyngeal isolates from healthy and otitis-prone children, which were initially identified by classical phenotypic methods as NTHI isolates (here referred to as phenotypic NTHI isolates), were actually *H. haemolyticus* (5, 9), while *H. haemolyticus* was not identified in middle ear fluid samples from children with acute otitis media (5). In contrast, *H. haemolyticus* comprised 40% of phenotypic NTHI sputum isolates from adults with COPD (9).

These findings raise important questions about NTHI as a true lower respiratory pathogen. We therefore investigated the proportion of *H. haemolyticus* isolates among 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 who were 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 semiquantitative growth score of $\geq 4$, which correlated with $>10^4$ CFU/ml BAL fluid, as determined by serial dilution and quantitative counts (3).

Phenotypic NTHI isolates were identified by morphology, requirement for X and V growth factors, and failure to react with Phadebact (Bactus AB, Sweden) antisera specific for *H. influenzae* type b or types a and c through f. 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 discriminates between *H. influenzae* and *H. haemolyticus* isolates (1, 13). Phenotypic NTHI isolates returning negative PCR results were considered to be *H. haemolyticus*, 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).

Table 1 shows the numbers, proportions, and distribution of phenotypic NTHI, hpd#3 PCR-confirmed *H. influenzae*, presumptive *H. haemolyticus*, and concurrent *H. influenzae* and *H. haemolyticus* 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 *H. influenzae*, but almost two-thirds (65%) of oropharyngeal isolates were presumptive *H. haemolyticus*. *H. influenzae* and *H. haemolyticus* were isolated concurrently from 10% (4/42) of nasopharyngeal swabs, 17% (6/36) of oropharyngeal swabs, and 27% (8/30) of BAL fluid cultures from children with phenotypic NTHI carriage or lower-airway infection.

This study shows that, similar to healthy and otitis-prone Western

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**TABLE 1** 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[a]

| Isolate source | No. of specimens | No. (%) of specimens with: | | | |
|---|---|---|---|---|---|
| | | Phenotypic NTHI | Phenotypic NTHI confirmed by PCR as *H. influenzae* | Presumptive *H. haemolyticus* | Concurrent *H. influenzae* and presumptive *H. haemolyticus* |
| NP swab | 84 | 42 (50) | 38 (45) | 8 (10) | 4 (5) |
| OP swab | 56 | 36 (64) | 14 (25) | 28 (50) | 6 (11) |
| BAL fluid | 84 | 30[b] (36) | 30 (36) | 8 (10) | 8 (10) |
| Total | 224 | 108 (48) | 82 (37) | 44 (20) | 18 (8) |

[a] NP, nasopharyngeal; OP, oropharyngeal; BAL, bronchoalveolar lavage; NTHI, nontypeable *Haemophilus influenzae*.
[b] Specimens with phenotypic NTHI lower-airway infection ($>10^4$ CFU/ml BAL fluid).

Australian children (5), most phenotypic NTHI nasopharyngeal isolates from Indigenous children with bronchiectasis were confirmed as *H. influenzae*. In contrast, many apparent NTHI isolates from oropharyngeal swabs were *H. haemolyticus* in our study population. Previous oropharyngeal culture-based studies of *H. influenzae* may have therefore overestimated NTHI carriage, and our data instead support the nasopharynx as the preferred site for *H. influenzae* 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 ($>10^4$ CFU/ml BAL fluid) were PCR positive for *H. influenzae* confirms NTHI as a lower-airway pathogen. However, the role of *H. haemolyticus* is unclear. Prior studies report that *H. haemolyticus* 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 *H. haemolyticus* has a propensity for the oropharynx, without specific molecular detection and quantification, we cannot determine whether its presence in lower-airway cultures represents upper-airway contamination or a pathogenic role.

In conclusion, we have used *H. influenzae*-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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