Quantitative PCR confirms culture as the gold standard for detection of lower airway infection by nontypeable Haemophilus influenzae in Australian Indigenous children with bronchiectasis

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Quantitative PCR confirms culture as the gold standard for detection of lower airway infection by nontypeable *Haemophilus influenzae* in Australian Indigenous children with bronchiectasis {REVISED}

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Abstract

Correlation was observed between quantitative PCR and semi-quantitative culture for definition of *Haemophilus influenzae* infection in bronchoalveolar lavage specimens from 81 children with bronchiectasis. However, qPCR data correlated less well with airway neutrophilia, and supports continued use of culture as the gold standard for defining *H. influenzae* lower airway infection.

Keywords

Airway cellularity; Bronchiectasis; Estimates of infection; *Haemophilus haemolyticus*; Lower airway infection; Nontypeable *Haemophilus influenzae*;

Nontypeable *Haemophilus influenzae* (NTHi) is frequently cultured from the lower airways of children with bronchiectasis (Hare et al., 2010). However, culture-based phenotypic identification methods cannot reliably differentiate NTHi from nonhemolytic strains of the primarily commensal *Haemophilus haemolyticus*. Previously, we used a probe-based PCR targeting the protein D gene (*hpd*#3) (Binks et al., 2012; Wang et al., 2011) to differentiate *H. influenzae* and *H. haemolyticus* isolated from bronchoalveolar lavage (BAL) specimens from Australian Indigenous children with bronchiectasis (Hare et al., 2012a). Of 84 BAL specimens tested, 30 (36%) were culture-positive (defined by semi-quantitative growth scores) for NTHi infection. At least one of four isolates collected from these positive specimens was confirmed as *H. influenzae* by *hpd*#3 PCR. However, 27% of culture-positive BAL specimens (8/30) had at least one phenotypic NTHi isolate test negative by *hpd#3* PCR, indicating *H. haemolyticus* (Hare et al., 2012a).

Co-detection of these species raised doubts about previous culture-based estimates of infection attributed to *H. influenzae*. Semi-quantitative counts are made on primary plates where *H. influenzae* and *H. haemolyticus* colonies cannot be differentiated, potentially leading to overestimation of *H. influenzae* density. We thus expanded on our previous work by using the *hpd*#3 PCR quantitatively (*hpd*#3 qPCR) to further define the presence of NTHi in paediatric bronchiectasis. For 81 of the original 84 BAL specimens we compared: (i) the semi-quantitative NTHi culture-based definition with *hpd*#3 qPCR estimation of *H. influenzae* infection; and (ii) both measures of bacterial density with total and differential cell counts to gauge the airway inflammatory response to infection.

BAL specimens were collected from children (63% male) aged 5-to-155 months undergoing bronchoscopy between July 2007 and August 2010. Fifty-three children had received antibiotics in the 2-weeks preceding bronchoscopy. BAL specimens were collected, stored and processed (10µL aliquots plated on selective media) as described previously (Hare et al., 2010). Growth on primary plates was quantified as: 0=no colonies, 1=1-20 colonies, 2=21-50 colonies, 3=51-100 colonies, 4=>100 colonies within primary inoculum only, 5=growth extending into first streak zone, 6=growth in second streak zone, 7=growth in third streak zone. Phenotypic NTHi was identified by colonial morphology, X and V growth factor requirement, and failure to react with *H. influenzae* types a-f antisera (Phadebact®, Bactus AB, Sweden). Lower airway infection was defined by semi-quantitative growth scores ≥4, which correlate with $>10^4$ colony-forming units (CFU)/mL BAL fluid (Hare et al., 2010).

DNA was extracted from 100µL of BAL specimen using QIAamp columns (QIAgen, Australia) with enzymatic pre-treatment as detailed elsewhere (Smith-Vaughan et al., 2006). The qPCR assay included 5 DNA standards (*H. influenzae* reference isolate ATCC 19418) ranging from 10 to 100,000 genome copies per reaction and was performed as described previously (Binks et al., 2012) using published *hpd#3* primer and probe concentrations (Wang et al., 2011) with TaqMan® Universal PCR Master Mix reagents (Applied Biosystems). All samples, standards and 'no template' controls were run in duplicate and required to differ by less than 0.5 cycles. Standard curve correlation coefficients were >0.99. The qPCR efficiency was ≥0.86. The lower limit of detection was 10 cells (genome copies)/reaction. The lower limit of quantification (LOQ) was 100 cells/reaction ($2.5x10^4$ cells/mL). Raw data were analysed using Rotor-Gene 6000 Series Software 1.7.

Total and differential cell counts (reflecting lower airway inflammation) were determined using unfiltered BAL specimens as described previously (Pizzutto et al., 2012). Statistical analyses were performed using nonparametric tests in Stata version 10. The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research approved the study (HREC 07/63).

Twenty-nine of the 81 BAL specimens (36%) had phenotypic NTHi growth scores ≥4 indicating lower airway infection. Typeable *H. influenzae* strains were found in three specimens; all had concurrent phenotypic NTHi infection. Using qPCR, 40/81 (49%) specimens had *H. influenzae* infection defined as $\geq 10^5$ cells/mL (Abdeldaim et al., 2010). Semi-quantitative growth scores correlated well with $\log_{10}$ qPCR bacterial load estimates (Figure); Spearman's rank correlation coefficient [r_s] was 0.66 ($P<0.001$) for the 47 specimens with quantifiable PCR results. Of the 34 BAL specimens either *H. influenzae* PCR-negative or positive below the LOQ ($<2.5 \times 10^4$ cells/mL), 33 had NTHi growth scores of 0 or 1 indicating negative or very low colony counts (<20 colonies/10µL, $<2 \times 10^3$ CFU/mL).

Twenty-seven of the 29 specimens with NTHi growth scores ≥4 had lower airway infection confirmed by qPCR ($\geq 10^5$ cells/mL), including all specimens with concurrent *H. influenzae* and *H. haemolyticus* detection. Thirty-nine specimens were negative for *H. influenzae* infection by both culture (growth score <4) and qPCR ($<10^5$ cells/mL). The remaining 15 specimens had discordant culture and qPCR results, including 13 qPCR-positive, culture-negative (growth score <4) specimens

(Table 1 and Figure). Six of these 13 children had received antibiotics within the previous 2-weeks.

Airway cellularity data were available for 71 (69 with neutrophil data) of the 81 children with BAL culture and qPCR results (Table 2). Total cell counts, neutrophil numbers and neutrophil percentages correlated with both semi-quantitative growth scores (r_s 0.45, 0.48 and 0.41 respectively, all $P<0.001$) and $\log_{10}$ qPCR values (r_s 0.48 [P=0.002], 0.38 [P=0.020] and 0.31 [P=0.057] respectively). Not unexpectedly (Kapur et al., 2012), airway cellularity measures were significantly higher in children with culture and qPCR-defined *H. influenzae* infection than children without infection by these measures (Table 2). In contrast, airway cellularity was similar between those with *H. influenzae* infection defined solely by qPCR and those infection-negative by both qPCR and culture; inflammatory indices were low in both groups (Table 2).

Several factors may lead to PCR-positive, culture-negative results, such as the presence of non-viable bacteria and the fact that CFU/mL does not invariably correspond to cells/mL since several bacteria may aggregate and generate one colony (Abdeldaim et al., 2010). Additionally, culture sensitivity may be negatively influenced by commensal bacteria numbers and/or antibiotic treatment before sampling (Kais et al., 2006). In our study, most specimens with culture and qPCR-defined infection had commensal bacteria (mainly alpha-hemolytic streptococci) growth scores ≥4, suggesting the presence of commensal organisms did not impede NTHi recovery. Consistent with our earlier report (Hare et al., 2012b), we also found that recent antibiotics were not associated with reduced culture evidence of NTHi lower airway infection. While qPCR had greater sensitivity than culture, the

cellularity data raise questions over the clinical relevance of PCR-positive but culture-negative results.

In conclusion, all BAL specimens with concurrent NTHi and *H. haemolyticus* detection had culture-defined NTHi infection confirmed by *H. influenzae* qPCR. qPCR is consistently reported as more sensitive than culture and is an attractive method for *H. influenzae* quantification. However, the lack of airway inflammation in BAL specimens with *H. influenzae* infection defined by qPCR but not culture suggests that reliance on qPCR data alone may lead to overestimation of lower airway infection rates. While PCR remains useful for determining *H. influenzae* presence and density, our data support continued used of culture as the gold standard for diagnosing NTHi lower airway infection.

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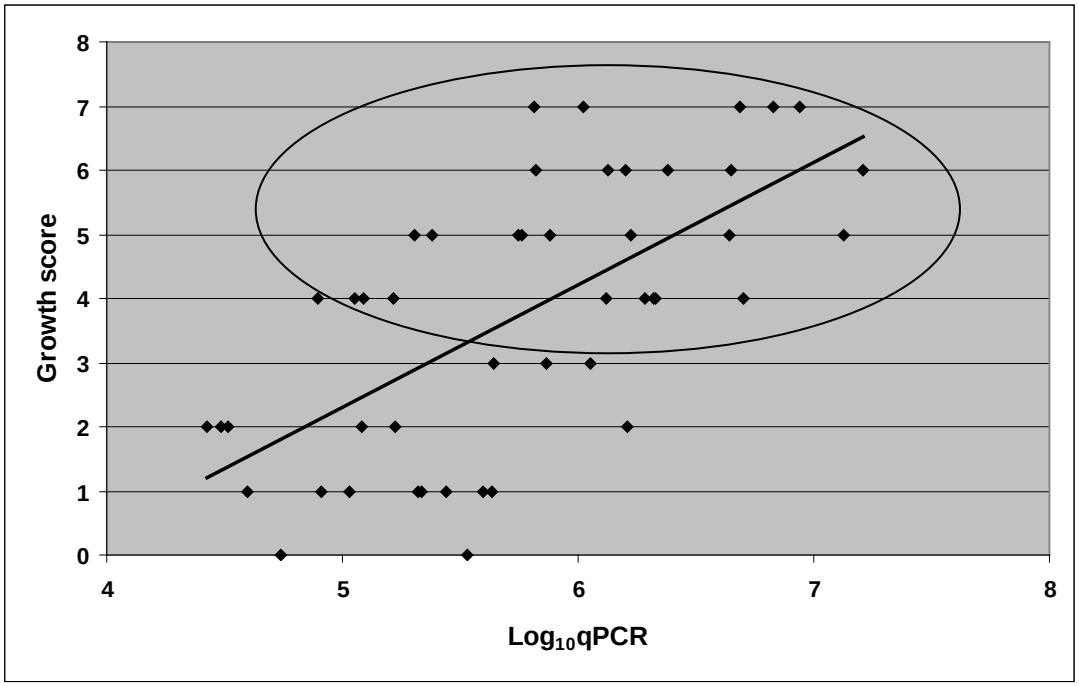
Table 1: *Haemophilus influenzae* lower airway infection in Australian Indigenous children defined by semi-quantitative culture and quantitative PCR

	qPCR $\geq 10^5$ cells/mL		
Growth score ≥4	**Negative**	**Positive**	**Total**
Negative	39	13	52
Positive	2	27	29
Total	41	40	81

Using culture as the gold standard, qPCR sensitivity is 93% and specificity 75%.

Figure: *Haemophilus influenzae* lower airway infection in Australian Indigenous children defined by semi-quantitative culture and quantitative PCR



Scatter plot with linear trend line showing culture-based semi-quantitative growth scores of phenotypic nontypeable *Haemophilus influenzae* (NTHi) and $\log_{10}$ *Haemophilus influenzae hpd*#3 qPCR results from 47 bronchoalveolar lavage (BAL) specimens with a quantifiable specific PCR result. The oval encompasses specimens with lower airway infection as determined by semi-quantitative culture (score ≥4) and qPCR ($\geq 10^5$ cells/mL).

Table 2: Airway cellularity data for Australian Indigenous children with and without *Haemophilus influenzae* lower airway infection[a]

Culture-defined NTHi infection	**Negative (growth score <4)**	**Positive (growth score ≥4)**	**P-value[b]**
Median (IQR) TCC (x 10^6 cells/L)	N=49 350 (240, 570)	N=22 810 (460, 1210)	P < 0.001
Median (IQR) neutrophils (x 10^6 cells/L)	N=48 30 (10, 120)	N=21 250 (100, 740)	P = 0.002
Median (IQR) neutrophil percentage	N=47 11 (6, 31)	N=22 35 (9, 62)	P = 0.022
qPCR-defined Hi infection	**Negative (<10^5 cells/mL)**	**Positive (≥10^5 cells/mL)**	**P-value[b]**
Median (IQR) TCC (x 10^6 cells/L)	N=38 340 (200, 580)	N=33 650 (370, 1020)	P = 0.003
Median (IQR) neutrophils (x 10^6 cells/L)	N=37 30 (10, 140)	N=32 120 (30, 600)	P = 0.005
Median (IQR) neutrophil percentage	N=36 10 (3, 32)	N=33 21 (11, 59)	P = 0.015

qPCR-defined Hi infection for NTHi culture negative BALs	**Culture negative (growth score <4) qPCR negative (<10^5 cells/mL)**	**Culture negative (growth score <4) qPCR positive (≥10^5 cells/mL)**	**P-value**[b]
Median (IQR) TCC (x 10^6 cells/L)	N=36 340 (190, 560)	N=13 370 (290, 570)	P = 0.497
Median (IQR) neutrophils (x 10^6 cells/L)	N=35 30 (10, 140)	N=13 50 (20, 100)	P = 0.190
Median (IQR) neutrophil percentage	N=34 10 (2, 31)	N=13 13 (11, 28)	P = 0.171

BAL, bronchoalveolar lavage; Hi, *Haemophilus influenzae*; IQR, interquartile range; NTHi, nontypeable Hi; qPCR, quantitative PCR; TCC, total cell count

[a] Exclusion of 21 BAL samples with growth scores ≥4 for respiratory pathogens other than Hi did not alter these findings

[b] Two-sample Wilcoxon rank-sum (Mann-Whitney) test