Duplex Quantitative PCR Assay for Detection of Haemophilus influenzae That Distinguishes Fucose- and Protein D-Negative Strains

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We have developed a specific Haemophilus influenzae quantitative PCR (qPCR) that also identifies fucose-negative and protein D-negative strains. Analysis of 100 H. influenzae isolates, 28 Haemophilus haemolyticus isolates, and 14 other bacterial species revealed 100% sensitivity (95% confidence interval [CI], 96% to 100%) and 100% specificity (95% CI, 92% to 100%) for this assay. The evaluation of 80 clinical specimens demonstrated a strong correlation between semiquantitative culture and the qPCR (P < 0.001).

Haemophilus influenzae is an important human respiratory tract pathogen that causes otitis media, acute exacerbations of chronic obstructive pulmonary disease, and chronic bronchitis and sinusitis, in addition to invasive diseases such as meningitis and bacteremia (1–6). Since the introduction of global H. influenzae serotype b (1–6). Since the introduction of global H. influenzae serotype b immunization, most H. influenzae infections are caused by unencapsulated strains designated nontypeable H. influenzae (18–20). The operon consists of 4 genes encoding the enzymes fuculokinase (fucP), fucose permease (fucP), fuculose phosphate aldolase (fucA), and fucose isomerase (fucI). H. haemolyticus does not possess the fucose operon, and hence these genes are potential markers for specific H. influenzae identification. The fucP gene was recently demonstrated to be a highly specific discriminatory target for H. influenzae identification based on a large comparative genomics study of 338 NTHi and 116 related Haemophilus isolates (20). However, 5% of the H. influenzae isolates in the Price et al. study were found to be missing the fucP gene (20). Other studies have confirmed the presence of H. influenzae strains that are missing all, or part of, the fucose operon (21–23). It has been suggested that these fucose-negative isolates are a distinct cluster of H. influenzae (20, 23). While still regarded as NTHi, these variant strains would not be detected by a fucP PCR assay.

Another established NTHi target is the hpd gene, which encodes the surface lipoprotein, protein D. This protein is conserved in H. influenzae and is a component of the 10-valent pneumococcal nontypeable H. influenzae protein D conjugate vaccine (PHiD-CV) (24). H. haemolyticus also possesses an hpd gene, but it is variable enough from that of H. influenzae to be used as a discriminatory molecular target (17). A primer pair designated hpd#3, specific for H. influenzae hpd, is sensitive and specific for the identification of H. influenzae (16, 25). However, studies have since identified clinical NTHi isolates lacking protein D (17, 26), which are not identified with an hpd-based assay.

The purpose of this study was to develop a specific quantitative PCR (qPCR) assay to quantify H. influenzae in nasopharyngeal swabs (NPS) and middle ear effusions (MEE) for use in future otitis media studies, including the assessment of the impact of immunization with PHiD-CV on NTHi carriage and disease. For this, we have duplexed the fucP and hpd#3 gene targets to give an H. influenzae-specific qPCR that also detects variant fucP- or hpd-negative strains.

Specific primers and probes were used to amplify a 68-bp fragment of the fucP gene and a 151-bp fragment of the hpd gene in H. influenzae (Table 1). Both primers were previously described for single-plex qPCRs (16, 20); however, probes were modified for compatibility in the duplex assay (Table 1). Real-time qPCR was conducted on the CFX96 real-time PCR detection system (Bio-Rad, CA, USA). The reaction mix consisted of 5 μl of 2× SensiMix II Probe No-ROX (Bioline, Alexandria, NSW, Australia), 1,000 nM each primer and probe (Integrated DNA Technologies, Baulkham Hill, NSW, Australia) diluted in molecular-grade water (Sigma-Aldrich, Castle Hill, NSW, Australia), and 1 μl of sample, to a total volume of 10 μl. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. A standard curve was generated for each run using serial dilutions of genomic DNA (2000 pg to 0.02 pg) from the reference strain NTHI 86-028NP, originally isolated from the nasopharynx of a child with otitis media (27, 28). All samples were run in duplicate. The duplex fucP/hpd#3 qPCR assay consistently had an efficiency of 90 to 110% and a limit of quantification (LOQ) of 0.0125 pg for fucP and hpd#3, which is equivalent to 6 copies of H. influenzae DNA (corresponding to a limit of detection of a quantification cycle [Cq] value of 35).

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The *fucP/hpd* #3 qPCR assay was evaluated on 144 bacterial isolates comprising 94 NTHi, *H. influenzae* serotypes a to f (ATCC 9006, ATCC 10211, ATCC 9007, ATCC 9332, ATCC 8142, ATCC 9833), 28 *H. haemolyticus* (including ATCC 33390), 2 *Haemophilus para-influenzae* (including ATCC 33392), 2 *Haemophilus parahaemolyticus* (ATCC 10014, ATCC 49700), *Staphylococcus aureus* (ATCC 25923), *Aggregatibacter aphrophilus* (ATCC 7901), *Moraxella catarrhalis* (ATCC 25138), *Streptococcus pneumoniae* (NCTC 7466), *Pseudomonas aeruginosa* (ATCC 27853), *Neisseria meningitidis* (ATCC 13090), *Neisseria lactamica* (ATCC 23970), *Escherichia coli* (ATCC 11775), *Alloccocus otitidis* (ATCC 51267), *Streptococcus oralis*, *Streptococcus mitis*, and *Streptococcus pyogenes* (ATCC 19615). All NTHi and *H. haemolyticus* isolates were X factor- and V factor-dependent and were identified as either *NTHi*, *H. haemolyticus*, or fucose-negative NTHi by 16S (29), high-resolution melt *hpd* qPCR (17), *fucP* PCR, or whole-genome sequencing (20). The bacterial isolates were cultured on suitable plate media for 24 h, and then 2 to 3 colonies were resuspended into 200 μL of RNase-free water (Sigma-Aldrich) and prepared as colony boils as previously described (17). The colony boïes were diluted 1:10 in RNase-free water for use in the PCR assay. Of the 94 clinical NTHi isolates identified using standard microbiology techniques, 41 were isolated from NPS (30), 33 from blood, 1 from cerebrospinal fluid, and 4 from unknown clinical sites (kindly supplied by Daniel J. Morton).

Genomic DNA preparations from 80 clinical specimens (67 NPS and 13 MEE), of which 57 were culture positive for NTHi, were evaluated to compare *H. influenzae* quantification using the duplex *fucP/hpd* #3 qPCR assay with culture results (see Table 3). Collection, storage, and culture of the NPS and MEE used in this study have been described (30). Prior to DNA extraction, the specimens were thawed, vortexed thoroughly for 30 s, and centrifuged at 13,000 × g in DNA IQ spin baskets (Promega) to ensure maximum DNA recovery. The swabs were then removed from the NPS specimens, and the remaining material was centrifuged at 13,000 × g for 7 min. The supernatant was discarded, and DNA was extracted from the pellet using enzymatic extraction and the QIAamp DNA minikit (Qiagen) as previously described (31).

The duplex *fucP/hpd* #3 qPCR was found to be 100% sensitive (95% CI, 96% to 100%), with all of the 100 *H. influenzae* isolates positive for at least one of the two genes (Table 2). When individual targets only were assessed, 83% of the *H. influenzae* isolates were positive for *fucP* and 99% were positive for the *hpd* gene (Table 2). Calculation of the specificity of the duplex *fucP/hpd* #3 qPCR was based on the 44 non-*H. influenzae* strains, where all 44 isolates were negative for both target genes, demonstrating 100% specificity (95% CI, 92% to 100%) for *H. influenzae* detection (Table 2).

Of the 57 culture-positive specimens, 55 were positive in the *fucP/hpd* #3 qPCR assay (Table 3). Of the 23 specimens that were culture negative, 2 were positive by the *fucP/hpd* #3 qPCR. Assuming culture to be the current gold standard for NTHi detection, the sensitivity of the *fucP/hpd* #3 duplex assay for NTHi detection in clinical specimens was 96% (95% CI, 88% to 100%), and the specificity was 91% (95% CI, 72% to 99%). When a specimen was positive for both qPCR targets, the geometric mean DNA concentrations (picograms per milliliter) for *fucP* and *hpd* #3 were calculated (although the DNA concentrations were usually similar for both targets). If one target gene was below the LOQ, this did not affect the quantity of *H. influenzae* culture-positive specimens that were less than the LOQ for both targets. Specimens in which both targets were not detected were assigned half of the LOQ for statistical analyses.

### TABLE 1 Primers and probes used for duplex *fucP/hpd* #3 qPCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer/probe</th>
<th>Sequence (5’→3’)</th>
<th>Fragment size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fucP</em></td>
<td><em>fucP</em> Fwd</td>
<td>GCCGCTTCTGAGGCCTGG</td>
<td>68</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td><em>fucP</em> Rev</td>
<td>ACCGACATTACAAATCGATGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>fucP</em> probe</td>
<td>6FAM-TCCATTACTGTGGAAATAC-BHQ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>hpd</em></td>
<td><em>hpd</em> Fwd</td>
<td>GGTAAATAGCCGATGTTGTTG</td>
<td>151</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td><em>hpd</em> Rev</td>
<td>TGCATCTTTACGACCGTGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>hpd</em> probe</td>
<td>HEX-TTGTGTACACTCGGT/ZEN/TGGATAAGAACTTGCGAC-3C6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Fwd, forward; Rev, reverse.

*b* Probe was modified with a black hole quencher (BHQ-1) instead of the TaqMan MGB.

*c* Probe was modified with a ZEN internal quencher instead of the BHQ internal quencher.

### TABLE 2 Sensitivity and specificity for identification of *H. influenzae* isolates with duplex *fucP/hpd* #3 qPCR

<table>
<thead>
<tr>
<th>PCR</th>
<th>No. of true positives/ total no. of positives</th>
<th>Sensitivity (%) (95% CI)*</th>
<th>No. of true negatives/ total no. of negatives</th>
<th>Specificity (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fucP</em></td>
<td>100/100</td>
<td>100 (96–100)</td>
<td>44/44</td>
<td>100 (92–100)</td>
</tr>
<tr>
<td><em>fucP</em></td>
<td>83/100</td>
<td>83 (74–90)</td>
<td>44/44</td>
<td>100 (92–100)</td>
</tr>
<tr>
<td><em>hpd</em> #3</td>
<td>99/100</td>
<td>99 (95–100)</td>
<td>44/44</td>
<td>100 (92–100)</td>
</tr>
</tbody>
</table>

*a* Calculations include all *H. influenzae* strains.

*b* 95% CI, the 95% exact binomial confidence interval.

*c* Calculations include all non-*H. influenzae* strains.

### TABLE 3 Comparison of NTHi culture and *fucP/hpd* #3 qPCR quantification in clinical specimens

<table>
<thead>
<tr>
<th>Semiquantitative NTHi culture score (no.)</th>
<th><em>fucP/hpd</em> #3 qPCR geometric mean quantity of DNA (pg/μL) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>++ + (8)</td>
<td>6.58 (~4.13 to 10.33)</td>
</tr>
<tr>
<td>+ + (18)</td>
<td>1.45 (0.15 to 8.87)</td>
</tr>
<tr>
<td>+ (30)</td>
<td>0.18 (0.17 to 2.35)</td>
</tr>
<tr>
<td>0 (23)</td>
<td>0.01 (~0.07 to 0.24)</td>
</tr>
</tbody>
</table>

*a* One culture-positive specimen was excluded from analysis as a semiquantitative culture was not recorded.

*b* 95% CI, 95% confidence interval.

*c* Includes 2 *H. influenzae* culture-positive specimens that were less than the LOQ for both targets. Specimens in which both targets were not detected were assigned half of the LOQ for statistical analyses.

*d* Two of the culture-negative specimens were qPCR positive.
enzae in the sample was based on the positive gene only. The cultures for 79/80 specimens were semiquantitatively scored as +, ++, and ++++, which corresponded to <10^3, 10^3 to 10^4, and ≥10^4 CFU/ml. A strong correlation between NTHi densities determined by duplex qPCR and semiquantitative culture of the specimens was observed, as determined by the Spearman rho (r = 0.825; P < 0.001).

Within the 57 H. influenzae qPCR-positive specimens, 5 (9%) were negative for the fucP gene and 4 (7%) were negative for the hpd gene (all from NPS specimens). Duplex qPCR analysis of “culture-defined” NTHi isolates from 8 of the 9 specimens (1 was culture negative) identified to contain variant H. influenzae strains revealed a 50% concordance between isolate identification and specimen identification, with isolates from 3 out of 5 specimens identified as fucP-negative NTHi and 1 out of 3 specimens as hpd-negative NTHi. This concordance would probably increase if 2 isolates were examined per specimen. Molecular analysis of 2 culture-defined NTHi isolates from each of the 2 culture-positive (<10^5 CFU/ml) but qPCR-negative specimens confirmed that the isolates were H. influenzae and both targets were present. It is surprising that the qPCR did not identify H. influenzae in these 2 specimens, but possibly the H. influenzae density was very low. The 2 culture-negative/qPCR-positive specimens were from children who were currently taking or had recently received antibiotics.

As the duplex qPCR can distinguish variant H. influenzae strains within clinical specimens, it is suitable for use in determining the frequency at which fucose-negative and protein D-negative H. influenzae strains occur within populations. Such an assay is useful for determining the clinical relevance of these variant strains and, in the case of the hpd-negative isolates, for determining whether there is selective pressure from the PHID-CV vaccine. Development of a rapid and reliable method for distinguishing H. influenzae from related species is important for surveillance and diagnosis of H. influenzae disease. A single-target PCR assay cannot reliably identify H. influenzae. We have therefore developed a duplex fucP/hpd#3 qPCR assay with high sensitivity and specificity that can accurately quantify H. influenzae in clinical samples. This fucP/hpd#3 qPCR has the potential to play an important role in clinical diagnostics and carriage surveillance of H. influenzae in addition to evaluation of preventative therapies for NTHi disease.

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