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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 immunization, most *H. influenzae* infections are caused by unencapsulated strains designated nontypeable *H. influenzae* (NTHi) (1). *Haemophilus haemolyticus* is a close relative of NTHi, and both species colonize the human upper respiratory tract. Unlike NTHi, *H. haemolyticus* is rarely associated with invasive infections (7) and is generally considered a commensal organism. However, the two species are difficult to distinguish using a standard microbiological culture or single-plex PCR (8).

Several studies have sought to identify a single gene target for the rapid identification of *H. influenzae* (9–17). Among the suggested targets are genes in the fucose operon of *H. influenzae* (12, 18–20). The operon consists of 4 genes encoding the enzymes fuculokinase (*fucK*), 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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TABLE 1 Primers and probes used for duplex *fucP/hpd#3* qPCR

Target gene	Primer/probe[a]	Sequence (5′ to 3′)	Fragment size (bp)	Reference
fucP	fucP Fwd	GCCGCTTCTGAGGCTGG	68	20
	fucP Rev	AACGACATTACCAATCCGATGG		
	fucP probe[b]	6FAM-TCCATTACTGTTTGAAATAC-BHQ1		
hpd	hpd#3 Fwd	GGTTAAATATGCCGATGGTGTTG	151	16
	hpd#3 Rev	TGCATCTTTACGCACGGTGTA		
	hpd#3 probe[c]	HEX-TTGTGTACACTCCGT/ZEN/TGGTAAAAGAACTTGCAC-3C6		

[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.

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 parainfluenzae* (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), *Alloiococcus 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* PCR (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 boils 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), 15 from MEE (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, thus indicating a *fucP*- or *hpd#3*-negative strain, then the quantity of *H. influ-*

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

PCR	No. of true positives/ total no. of positives[a]	Sensitivity (%) (95% CI[b])	No. of true negatives/ total no. of negatives[c]	Specificity (%) (95% CI)
fucP/*hpd*#3	100/100	100 (96–100)	44/44	100 (92–100)
fucP	83/100	83 (74–90)	44/44	100 (92–100)
hpd#3	99/100	99 (95–100)	44/44	100 (92–100)

[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

Semiquantitative NTHi culture score (no.)[a]	*fucP/hpd*#3 qPCR geometric mean quantity of DNA (pg/μl) (95% CI[b])
+++ (8)	6.58 (−4.13 to 30.13)
++ (18)	1.45 (0.15 to 8.87)
+ (30)	0.18 (0.17 to 2.35)[c]
0 (23)	0.01 (−0.07 to 0.24)[d]

[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 $\geq 10^4$ 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 ($<10^3$ 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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