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A PCR–High-Resolution Melt Assay for Rapid Differentiation of Nontypeable *Haemophilus influenzae* and *Haemophilus haemolyticus*

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We have developed a PCR–high-resolution melt (PCR-HRM) assay to discriminate nontypeable *Haemophilus influenzae* (NTHi) colonies from *Haemophilus haemolyticus*. This method is rapid and robust, with 96% sensitivity and 92% specificity compared to the *hpd*#3 assay. PCR-HRM is ideal for high-throughput screening for NTHi surveillance and clinical trials.

Nontypeable *Haemophilus influenzae* (NTHi) is an important cause of respiratory system-related infections, including otitis media (OM), exacerbations of chronic obstructive pulmonary disease, chronic bronchitis, and bronchiectasis (1–3). There are also reports of its increasing importance as a cause of invasive infections in regions with *H. influenzae* type b immunization (4–6). NTHi isolates have colony phenotypes similar to those of nonhemolytic *Haemophilus haemolyticus* (7), a respiratory tract commensal that rarely causes disease (8, 9). Therefore, correct identification of NTHi requires novel methods of discrimination to complement traditional culture techniques (10, 11).

Clinical trials are under way to assess the effect of antibiotics and the 10-valent pneumococcal *H. influenzae* protein D-conjugate vaccine (PHiD-CV; Synflorix) on NTHi nasopharyngeal carriage and infection (for example, NCT01735084 and NCT01174849). An 11-valent precursor to PHiD-CV reduced NTHi-associated acute otitis media by 35% (12). Although a reduction in *H. influenzae* nasopharyngeal carriage was observed following primary and booster vaccination with the 11-valent vaccine (17), no substantial effect has been observed on NTHi carriage in subsequent studies with PHiD-CV, which has 8 of 10 serotypes conjugated to protein D (20, 21). Whether or not PHiD-CV will afford protection against OM or other NTHi infections, particularly in high-risk populations, is yet to be determined.

Given the high rates of misidentification of NTHi as the closely related commensal *H. haemolyticus* by standard culture-based techniques (10, 16), accurate molecular identification of NTHi is essential for surveillance of NTHi-targeted therapies. The development of a molecular tool for NTHi identification that can also identify *H. haemolyticus* will enable a greater understanding of the role of *H. haemolyticus* in carriage and disease. In addition, *H. haemolyticus* also expresses protein D (17), the NTHi antigen used in PHiD-CV. An assay that permits detection of *H. haemolyticus* is therefore important to monitor the impact of PHiD-CV on *H. haemolyticus* colonization. We have previously shown that the *hpd*#3 probe-based real-time PCR (RT-PCR) (13) provided superior NTHi identification compared to other PCR assays (14). We have utilized the discriminatory power of the *hpd* gene to develop a rapid PCR–high-resolution melt (PCR-HRM) assay for high-throughput identification of NTHi and discrimination from *H. haemolyticus*.

Two reference strains (*H. haemolyticus* ATCC 33390 and NTHi 86-028NP [15]), 60 nasopharyngeal carriage isolates from Western Australian children with and without a history of recurrent acute OM (14, 16) (20 NTHi, 19 *H. haemolyticus*, and 21 ambiguous *Haemophilus* isolates by 16S rRNA gene PCR), and 151 isolates from Northern Territory children with bronchiectasis (3) (49 nasopharyngeal, 52 bronchoalveolar lavage, and 50 throat isolates identified as NTHi or not by *hpd*#3 RT-PCR) were used to develop and validate the PCR-HRM assay. The Western Australian isolates were specifically chosen to represent NTHi, *H. haemolyticus*, and ambiguous strains, while the Northern Territory isolates were a sequential selection of clinical isolates selected to validate the method. Isolates were identified by colonial morphology, X and V growth factor dependence, and lack of reaction with capsular antisera using the Phadebact *Haemophilus* coagglutination test, and genomic DNA (gDNA) was isolated as previously described (16). gDNA was eluted in 100 μl sterile water and either used immediately or stored in aliquots at −20°C.

We assessed published DNA sequences for the gene encoding protein D, *hpd*, from NTHi and *H. haemolyticus* (13) and identified a hypervariable region (bp 580 to 1010) with potential discriminatory power. This region was amplified from the gDNA of 31 diverse strains using primers *hpd*1 fwd (5′-CAAAGTGTTGAAAAAATATGGCTATGA-3′) and *hpd*1 rev (5′-GTTGCACCTGATTTATTCAATAATGC-3′). Amplicons were then sequenced by the Australian Genome Research Facility using primers *hpd*2 fwd (5′-TTGCCTGGTTTAGATTGTTC-3′) and *hpd*2 rev (5′-GTTCAATTAGTGGCTTATACGG-3′) and standard DNA sequencing techniques (18). The *hpd*1 amplification primers were used for sequencing in cases where *hpd*2 sequencing reactions failed.

Alignment of our and all published *hpd* sequences identified polymorphic regions suitable for differentiating NTHi from *H. haemolyticus*. PCR primers were designed to amplify a 50-bp product containing two nucleotide polymorphisms for differentiation of NTHi (A and T) from *H. haemolyticus* (G and G) using high-resolution melt (HRM) technology (Table 1). Degeneracies

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TABLE 1 Variant NTHi and *H. haemolyticus* sequences amplified by *hpd* HRM primers

Identifier	Species	50-bp sequence (5′ to 3′)	% GC	Curve
Hi86	NTHi	ATGGATTTGAAATTAGTTCAATTA**ATT**GCTTATACAGATTGGAAAGAAAC	26	A
H17	NTHi	ATGGATTTGAAATTAGTTCAATTA**ATT**GCTTATACAGACTGGAAAGAAAC	28	B
H4	*H. haemolyticus*	ATGGATTTGAAATTAGTTCAATTA**GTG**GCTTATACAGATTGGAAAGAAAC	30	C
33390	*H. haemolyticus*	ATGGATTTGAAATTAGTTCAATTA**GTG**GCTTATACGGATTGGAAAGAAAC	32	D
H9	*H. haemolyticus*	ATGGATTTGAAATTAGTTCAATTA**GTG**GCTTATACTGACTGGCAAGAAAC	34	E
H12	*H. haemolyticus*	ATGGATTTGAAACTAGTTCAATTA**GTG**GCTTATACGGATTGGAAAGAAAC	34	E
H47	*H. haemolyticus*	ATGGATTTGAAATTAGTTCAATTA**GTG**GCTTATACTGACTGGCATGAAAC	34	E
H16	*H. haemolyticus*	ATGGATTTGAAACTAGTTCAATTA**GTG**GCTTATACTGACTGGCATGAAAC	36	F
Forward primer	*hpd* HRM FWD	ATGGATTTGAAATTAGTTCAATTA		
Reverse primer	*hpd* HRM REV	CGAATATGHCTRACCKTWCTTTG		

in the reverse primer were necessary to ensure *hpd* amplification in both NTHi and *H. haemolyticus*. The BLAST function (NCBI) was used to rule out primer sequence similarity with other species of respiratory bacteria.

Real time-PCR and the HRM assay were performed on the Rotorgene 6000 system (Corbett Life Science). Reaction mixtures contained 5 μl of 2× SensiMix SYBR green (Bioline), 100 nM (each) primer, and 1 μl of gDNA in a total volume of 10 μl. To reduce sample preparation time, the PCR-HRM method was also tested using 1 μl of a colony boil preparation rather than gDNA. Two to three *Haemophilus* colonies from a subset of isolates were placed into 200 μl sterile water, heated at 100°C for 10 min, placed on ice, and centrifuged, and 1 μl of supernatant was used in the PCR-HRM assay. Cycling conditions were 50°C for 2 min, 95°C for 2 min, and then 40 cycles of 95°C for 15 s, 66°C for 15 s, and 70°C for 30 s. The HRM assay was carried out from 65°C to 80°C in 0.1°C increments for 2 s each. Raw HRM curves were normalized in the region of 64.5 to 79°C prior to analysis using the Rotorgene 6000 software v1.7. All samples were run in duplicate and were required to be within 0.5 cycles.

Based on all available sequences for the 50-bp amplicon of the *hpd* gene and the assumption that GC content defines the melting temperature (19), 6 different melt curves were predicted (Table 1, curves A to F). The PCR-HRM assay was conducted on all 31

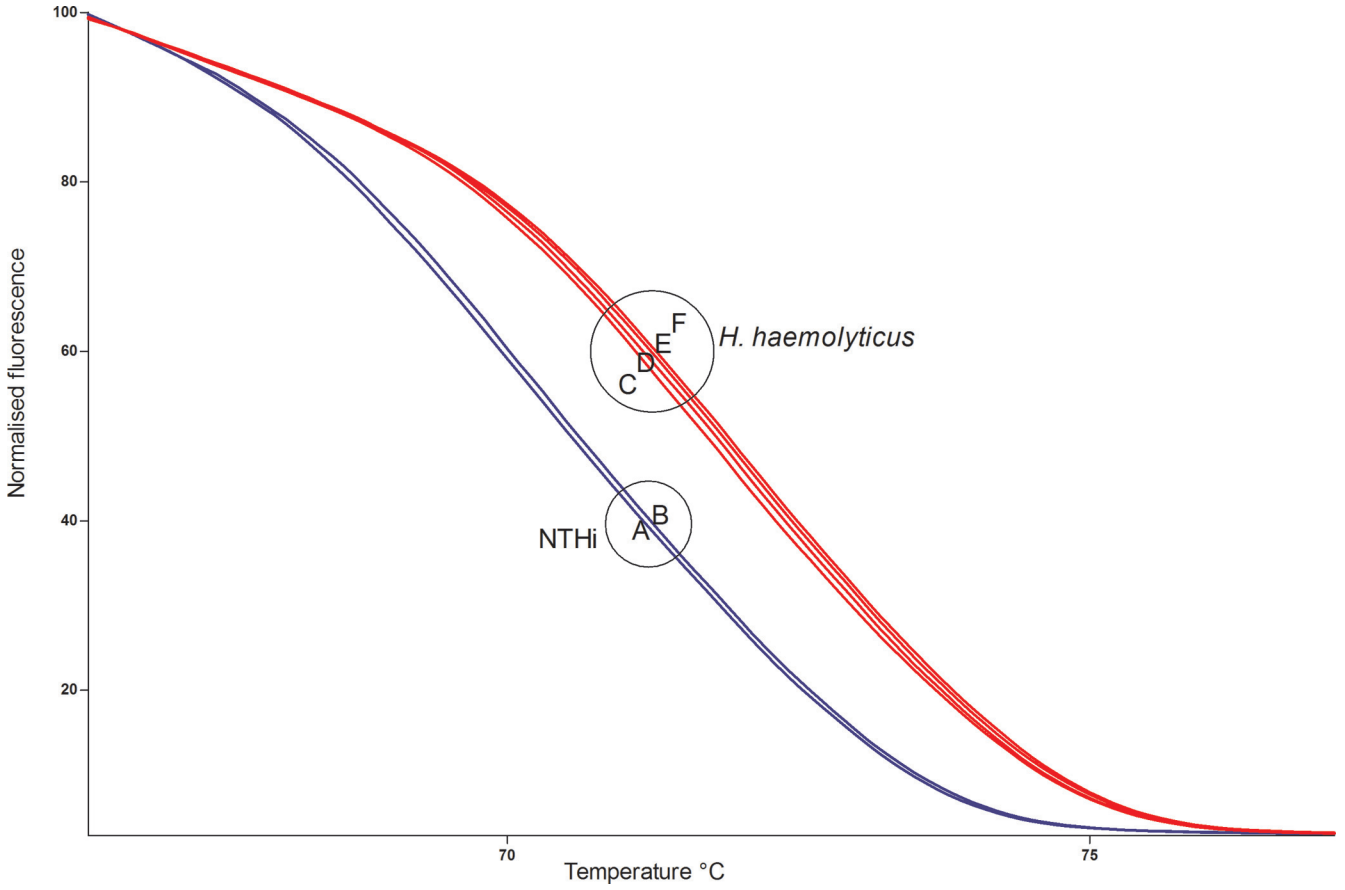

FIG 1 Normalized PCR-HRM curves of NTHi and *H. haemolyticus* variants. NTHi amplicons encompass an internal ATT and melt earlier (blue) than do *H. haemolyticus* amplicons, which encompass an internal GTG (red). A to F relate to curve types in Table 1.

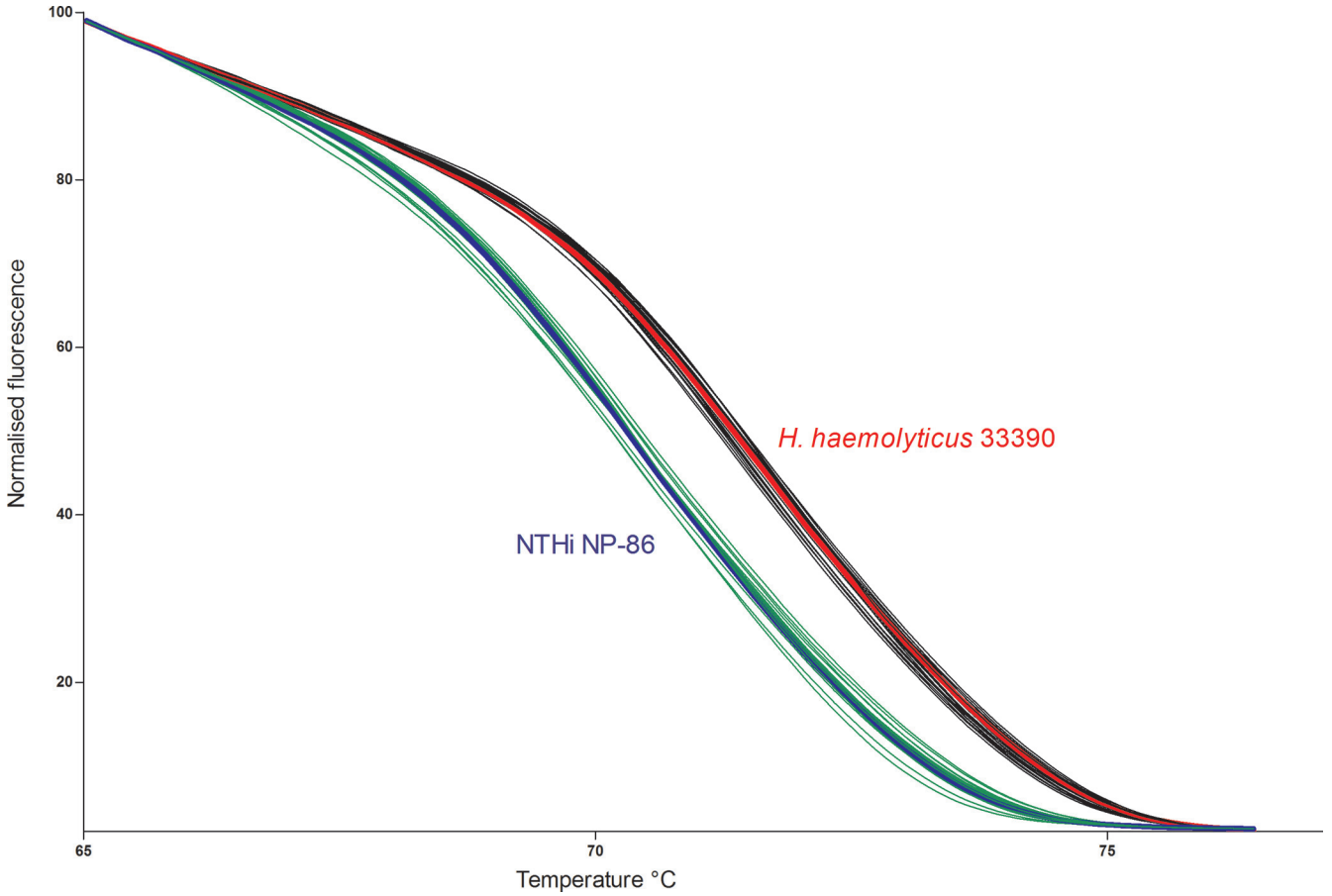

FIG 2 Separation of PCR-HRM curves from 8 *H. haemolyticus* (black) and 9 NTHi (green) isolates. All *H. haemolyticus* isolates align with the reference *H. haemolyticus* strain ATCC 33390, shown in red, and NTHi isolates align with NTHi reference strain 86-028NP, shown in blue. Duplicate reactions are displayed.

sequenced isolates, and curves representing each of the 6 unique sequence types are shown in Fig. 1. The largest temperature demarcation occurred between NTHi and *H. haemolyticus* isolates, as would be predicted by the central A-to-G and T-to-G single nucleotide polymorphisms (SNPs). The PCR-HRM assay was then conducted on gDNA of the remaining 180 isolates (29 Western Australian and 151 Northern Territory isolates). Figure 2 shows the melt curves from 19 representative isolates tested (including the 2 reference strains). One hundred sixty-one of the 180 isolates clustered with the melt curves for either NTHi or *H. haemolyticus* reference strains. Isolates were classified as NTHi or *H. haemolyticus* based on the reference strain with which they clustered. Compared with the *hpd*#3 RT-PCR assay, the PCR-HRM assay had 96% sensitivity and 92% specificity (Table 2). This was based on the assumption that for microbiologically identified *Haemophilus* isolates, a positive *hpd*#3 RT-PCR assay confirmed *H. influenzae* and that an *hpd*#3 RT-PCR-negative result identified presumptive *H. haemolyticus*. The PCR-HRM assay also allowed the clear classification of strains that were previously ambiguous by 16S rRNA gene PCR. A comparison of the PCR-HRM assay on gDNA versus colony boils demonstrated that both DNA preparation methods worked equally well; the colony boil method is now standard for our studies.

TABLE 2 Comparison of sensitivity and specificity for molecular detection of suspected *H. influenzae* colonies[a]

Assay for *H. influenzae* detection	TP/TP + TN	% sensitivity	95% CI	TN/TN + FP	% specificity	95% CI
hpd#3 assay	134/138	97.1	92–99	48/54	88.8	77–95
hpd HRM assay	134/140	95.7	90–98	48/52	92.3	81–97

[a] Abbreviations: TP, number of true positives; TN, number of true negatives; FP, number of false positives; CI, confidence interval.

Nineteen *Haemophilus* isolates (4 ambiguous Western Australian and 15 Northern Territory isolates that were all X and V factor dependent) could not be amplified and were also negative by the *hpd*#3 RT-PCR assay. The failure to amplify suggests significant variation or absence of the *hpd* gene. While this is a limitation of the current assay, the 4 ambiguous Western Australian isolates could not be classified using other PCR targets (*ompP2*, *ompP6*, *lgtC*, 16S rRNA, *fucK*, and *iga* [11]) and are a reminder that complete species differentiation by PCR will likely remain elusive.

Design of a conserved reverse primer downstream from the chosen discriminatory SNPs was not possible for amplification of both *Haemophilus* species. Therefore, a degenerate primer which could add complexity to the amplicon melt profile was designed; that is, amplicon variation may widen the area at which species-specific curves fall, potentially obscuring species differentiation. To gauge this potential effect, we conducted the PCR-HRM assay on representatives of each amplicon variant identified from the

sequencing. There was 1°C separation between the highest melt curve for the NTHi variants (74.6°C) and the lowest melt curve for *H. haemolyticus* variants (75.6°C). Furthermore, as shown in Fig. 1 and 2, the dichotomous separation of isolates by HRM was almost completely explained by the GC differences of the internal SNPs with seemingly little influence by GC variation in the primer region. In short, variation in the primer region had little effect on the melting temperature and no effect on assay outcome. This may be the result of a primer binding bias within the degenerate primer pool despite the stringency of the relatively high annealing temperature used for this assay. Furthermore, despite the potential for overlap in GC content theoretically afforded by the degenerate primer, this has not been observed.

NTHi has a significant role in morbidity, and thus, accurate surveillance is required for determining vaccine efficacy (i.e., the impact of Synflorix) and monitoring antibiotic resistance. The *hpd#3* RT-PCR assay (13) is useful for discriminating *H. influenzae* from closely related *Haemophilus* species and can be applied to swabs directly. However, in the context of routine microbiological identification of NTHi, the PCR-HRM assay is rapid (colony boil preparations), accurate, and inexpensive (no probe required) and has potential for high-throughput analysis. It also has the added advantage of identifying *H. haemolyticus*. In summary, our PCR-HRM assay allows discrimination of NTHi from *H. haemolyticus* and has excellent concordance with the *hpd#3* RT-PCR assay.

Nucleotide sequence accession numbers. The *hpd* sequences were deposited in GenBank under accession numbers KF048057 to KF048087.

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