Simultaneous identification of Haemophilus influenzae and Haemophilus haemolyticus using real-time PCR

RESEARCH ARTICLE

Future MICROBIOLOGY

Simultaneous identification of *Haemophilus influenzae* and *Haemophilus haemolyticus* using real-time PCR

Erin P Price*[1,2], Tegan M Harris[1], Jessie C Spargo[1], Elizabeth Nosworthy[1], Jemima Beissbarth[1], Anne B Chang[1], Heidi C Smith-Vaughan[‡,1] & Derek S Sarovich[‡,1,2]

Aim: To design a highly specific and sensitive multiplex real-time PCR assay for the differentiation of the pathogen *Haemophilus influenzae* from its nonpathogenic near-neighbor *Haemophilus haemolyticus*. **Materials & methods:** A comparison of 380 *Haemophilus spp.* genomes was used to identify loci specific for each species. Novel PCR assays targeting *H. haemolyticus* (*hypD*) and *H. influenzae* (*siaT*) were designed. **Results & discussion:** PCR screening across 143 isolates demonstrated 100% specificity for *hypD* and *siaT*. These two assays were multiplexed with the recently described *fucP* assay for further differentiation among *H. influenzae*. **Conclusion:** The triplex assay provides rapid, unambiguous, sensitive and highly specific genotyping results for the simultaneous detection of *hypD* and *siaT*, including fucose-positive *H. influenzae* (*fucP*), in a single PCR.

First draft submitted: 16 November 2016; Accepted for publication: 3 February 2017; Published online: 12 June 2017

KEYWORDS
• *fucP* • genotyping
• *Haemophilus haemolyticus*
• *Haemophilus influenzae*
• *hypD* • multiplex
• real-time PCR • *siaT*
• TaqMan • triplex

The Gram-negative bacterium *Haemophilus influenzae* is a frequent commensal of the upper respiratory tract of most healthy children and adults, where it usually resides without incident [1]. Occasionally, this opportunistic pathogen can infect other body sites, leading to potentially life-threatening diseases. Prior to the introduction of the *H. influenzae* serotype B (Hib) vaccine, Hib was a common cause of invasive diseases including bacteraemia, cellulitis, epiglottitis, meningitis and pneumonia, with approximately 0.5% of all American children under 5 years of age being affected [2]. The introduction of the Hib vaccine in1985 has led to substantially lower rates of invasive disease caused by *H. influenzae*.

Unencapsulated or nonserotypeable *H. influenzae* (NTHi) strains have since emerged as an important yet previously overlooked cause of opportunistic disease, particularly in regions where the Hib vaccine has been widely implemented [3]. NTHi is a common pathogen in the lower respiratory tract in chronic obstructive pulmonary disease and bronchiectasis, where it contributes to disease pathology by promoting chronic inflammation [1]. In children with cystic fibrosis, NTHi is an early colonizer that is associated with impaired mucociliary function, thereby increasing the risk of infection by other *H. influenzae* strains or pathogenic species [1,4]. NTHi also causes many other acute and chronic diseases, especially in children, including conjunctivitis, otitis media and sinusitis [5]. In contrast, the closely related species *H. haemolyticus*, which occupies the same niches as *H. influenzae*, is generally considered to be nonpathogenic [4].

Despite its medical importance, correct identification of *H. influenzae* has proven challenging on both the genotypic and phenotypic levels [4,6]. Genetically, NTHi undergoes high levels of lateral gene transfer with other species, usually with other *Haemophilus* spp. but occasionally with other genera [7–10]. The promiscuity of the NTHi genome has greatly impeded prior attempts to specifically target this species using DNA-based methods, most notably when

[1]Child Health Division, Menzies School of Health Research, Darwin, NT, Australia
[2]Centre for Animal Health Innovation, Faculty of Science, Health, Education & Engineering, University of the Sunshine Coast, Sippy Downs, QLD, Australia
*Author for correspondence: Tel.: +61 7 5456 5568; eprice@usc.edu.au
‡Authors contributed equally

Future Medicine part of fsg

attempting to differentiate *H. influenzae* from *H. haemolyticus*. Selective microbiological approaches are also nonspecific, with certain nonhemolytic *H. haemolyticus* strains being indistinguishable from NTHi based on X (hemin) and V (nicotinamide adenine dinucleotide) factor dependency [4]. Accurate identification of NTHi in clinical specimens is important for detection, for guiding appropriate antibiotic administration and for assessing the efficacy of treatments or *H. influenzae*-targeted vaccines [11]. To combat issues with accurate *H. influenzae* identification, we recently used a large-scale comparative genomic approach across 246 *Haemophilus* spp. to delineate *H. influenzae* from other *Haemophilus* spp., with subsequent design of a specific assay for *H. influenzae* [6]. This assay targets *fucP*, a highly conserved gene that encodes for L-fucose permease, a major facilitator superfamily transporter involved in L-fucose uptake [12]. The *fucP* gene resides within the fucose operon [13], which also encodes the multilocus sequencing typing (MLST) 'housekeeping' gene, *fucK* [14]. However, there is a growing body of evidence that certain *H. influenzae* strains lack some, or all, genes within the fucose operon [13,15–17]. For example, a recent study using multilocus sequence analysis across 60 clinical *Haemophilus* spp. strains showed high nucleotide similarity of strains both harboring and lacking the *fucK* locus, suggesting that these strains are genetically related and are in fact the same species [16]. On a phylogenomic level, these 'fuzzy' fucose-negative isolates share a node with 'Clade I' *H. influenzae* [6], a genetically distinct cluster of *H. influenzae* that is nonetheless considered to belong to this species [18].

Given that the *fucP* assay excludes fucose-negative *H. influenzae*, we used our previously described comparative genomics approach [6] to design a new TaqMan assay that encompasses both *fucP*-positive and *fucP*-negative *H. influenzae* strains. We also employed this same approach to develop a *H. haemolyticus*-specific assay. These two novel assays were combined with the existing *fucP* TaqMan assay in a triplex format for the simultaneous, specific and sensitive detection of fucose-positive and fucose-negative *H. influenzae*, and *H. haemolyticus*.

Methods

• Ethics statement

Ethics approval for this study was obtained as detailed previously [6].

• *Haemophilus* genomes

A total of 380 *Haemophilus* spp. genomes were available for this study: 316 *H. influenzae* (145 generated in the current study, 19 unpublished and 152 published elsewhere [6,18–34]), 59 *H. haemolyticus* (44 generated in the current study, one unpublished and 14 published elsewhere [6,26,28,35–36]), and four *Haemophilus parainfluenzae* (current study). A summary of the *Haemophilus* genomes is listed in **Supplementary Table 1**. Of the *H. influenzae* isolates, 23 lack the L-fucose permease (*fucP*) locus, 283 are NTHi, 13 are capsulated (or derived from a capsulated strain) and 20 have insufficient information to determine capsular status.

• Bacterial isolates for PCR specificity testing

Genomic DNA from 104 *Haemophilus* spp. isolates (27 *H. haemolyticus*, 65 *fucP*-positive *H. influenzae*, 10 *fucP*-negative *H. influenzae*, one *H. parahaemolyticus* and one *H. parainfluenzae*) and 38 non-*Haemophilus* isolates representing 33 different clinically relevant species **(Table 1 & Supplementary Table 1)** was examined using the triplex PCR assay. DNA extraction,

Table 1. Summary of triplex assay performance for *Haemophilus influenzae* and *Haemophilus haemolyticus* identification.

Species	No. isolates tested (PCR; *in silico*)†	No. *fucP*-positive‡	No. *siaT*-positive	No. *hypD*-positive	Detection rate (no. false positives; no. false negatives)
H. influenzae	316 (75; 256)	292	316	0	*fucP*: 92% (0; 24) *siaT* and *hypD*: 100% (0; 0)
H. haemolyticus	59 (27; 38)	0	0	59	All assays: 100% (0; 0)
Other species	37, 325 (40; 37, 287)	0	0	0	All assays: 100% (0; 0)

†Where available, assay performance was determined both by PCR and *in silico* methods.
‡The *fucP* assay differentiates between fucose-negative and fucose-positive *H. influenzae*.

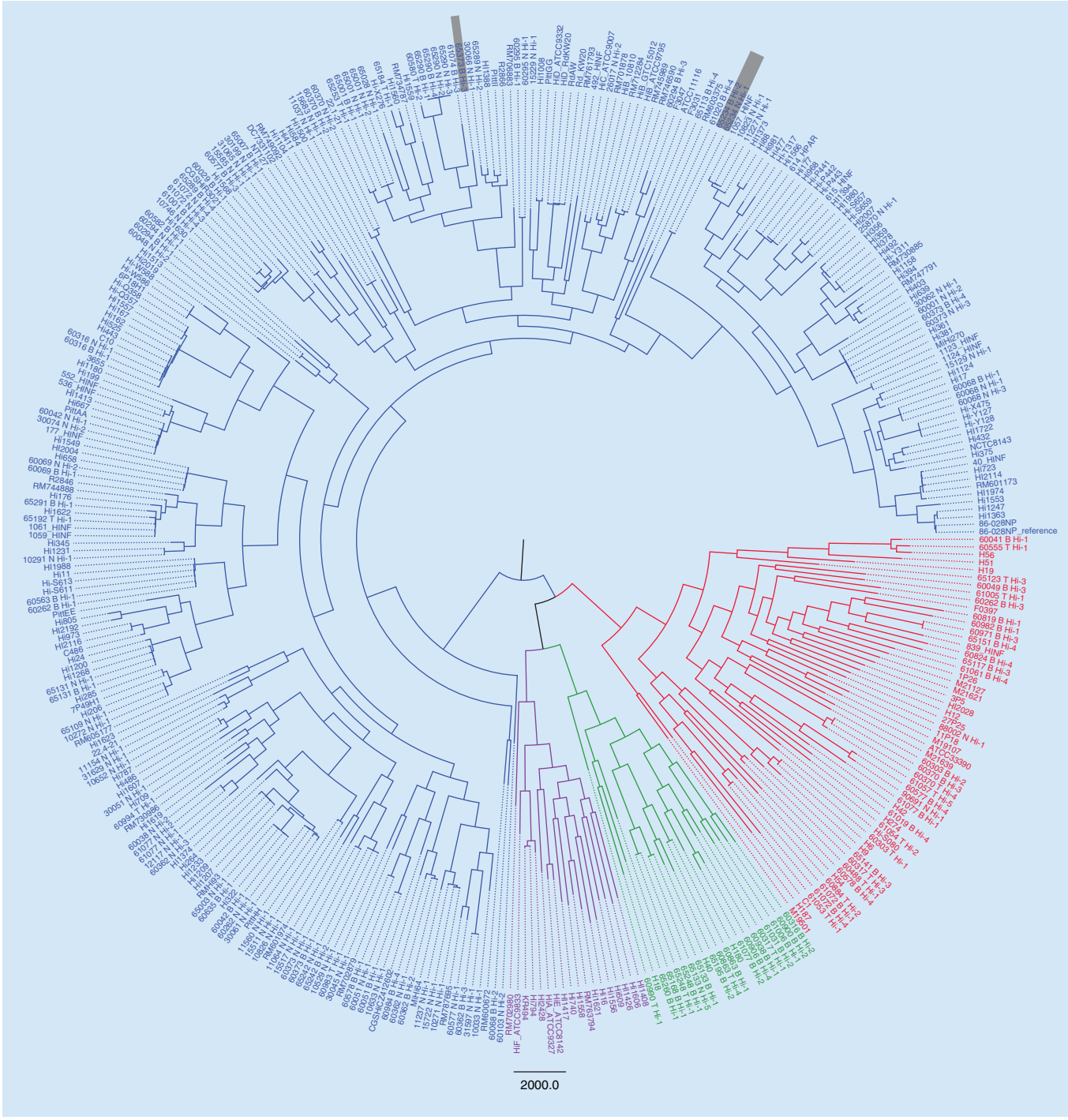

Figure 1. Phylogenomic analysis of *Haemophilus influenzae* and *Haemophilus haemolyticus*, and assay specificity.
A midpoint-rooted maximum parsimony tree was reconstructed based on 56,187 biallelic, orthologous single-nucleotide polymorphisms found among 373 *H. influenzae* and *H. haemolyticus* genomes **(Supplementary Table 1)**. The *hypD* assay differentiates *H. haemolyticus* (red) from all other species and the *siaT* assay differentiates *H. influenzae* (blue), 'Clade I' *H. influenzae* (purple) and fucose operon-negative *H. influenzae* strains (green) from all other species. The *fucP* assay [6] was designed to differentiate fucose operon-positive *H. influenzae* (blue) and 'Clade I' *H. influenzae* (purple) [18] from all other species. However, we uncovered three isolates in the 'conventional' *H. influenzae* clade (grey shading) that are *fucP*-negative by PCR and *in silico*. Consistency index = 0.10.

quantification and dilution for PCR was carried out as previously detailed [6].

• Identification of *H. haemolyticus* & *H. influenzae*-specific loci

The comparative genomics tool SPANDx v2.7 [37] was used to perform phylogenetic reconstruction of 375 *H. influenzae* and *H. haemolyticus* genomes, using default settings. The *H. parainfluenzae* genomes were excluded from phylogenetic analysis due to relative genetic dissimilarity. Four isolates (839_HINF, C1, F0397 and 614_HPAR) were reassigned their original species designation based on our phylogenetic analysis, which confidently placed them as belonging to either *H. haemolyticus* (839_HINF, C1, F0397) or *H. influenzae* (614_HPAR; **Figure 1**). Following phylogenetic reconstruction, SPANDx was re-run with the inclusion of the *H. parainfluenzae* genomes to identify genetic loci specific to *H. influenzae*, including the *fucP*-negative clade, and to *H. haemolyticus*, as described previously [6]. Reads were mapped against the closed reference genome *H. influenzae* 86–028NP (GenBank ref: NC_007146) [22] and the draft *H. haemolyticus* genome M19501 (GenBank ref: NZ_AFQN00000000) [35].

• *hypD* & *siaT* real-time PCR assay design

Due to high sequence diversity among both *H. influenzae* and *H. haemolyticus* genomes, we chose the TaqMan™ minor groove binding probe (MGB; Applied Biosystems, CA, USA) format for species detection. The MGB moiety raises the melting temperature of the probes, enabling the design of shorter probes that mitigate issues of potential sequence diversity in longer probes. TaqMan MGB probes also exhibit high sensitivity and specificity in a background of high noise, such as clinical specimens [38], and the availability of nonfluorescent quenchers and multiple fluorogenic detector dyes permits multiplexing [39]. To detect *H. haemolyticus*, the following primers and TaqMan MGB probe were designed: hypD- F (5′-GGCA ATCAGATGGTTTACAACG), hypD-R (5′-CAGCTTAAAGYAAGYAGTGAATG; Macrogen Inc., Geumcheon-gu, Seoul, Rep. of Korea) and hypD-Probe (5′-VIC-CCACAACGAGAATTAG-MGBNFQ; Applied Biosystems). For *H. influenzae* detection, siaT-For (5′-AATGCGTGATGCTGGTTATGAC) and siaT-Rev (5′-AAGAGTTTTGCGATAGA TTCATTGG) were used in combination with siaT-Probe (TET-AGAAGCAGCAGTAATT-MGBNFQ). The *siaT* and *hypD* assays generate 138 bp and 187 bp amplicons, respectively. The 68 bp *fucP* assay, which uses a 6FAM™-labelled TaqMan probe (Applied Biosystems) for increased specificity, was included with *hypD* and *siaT* in a triplex assay format for the simultaneous detection of *H. influenzae* (including fucose operon-negative *H. influenzae*) and *H. haemolyticus* in a single reaction.

Amplicon specificity for *hypD* and *siaT* was confirmed using microbial discontiguous MegaBLAST analysis ([40]; performed 8 February 2016) across 3992 complete microbial genomes, 29,229 draft microbial genomes, 2078 complete plasmid genomes and 1988 complete bacteriophage genomes (total: 37,287 genomes). A nonredundant nucleotide (nr/nt) collection BLAST search was also conducted to determine amplicon specificity in nonmicrobial genomes.

• Triplex real-time PCR conditions

PCRs were performed using an ABI PRISM® 7900HT instrument (Life Technologies, VIC, Australia) in 384-well optical plates (Life Technologies). Following optimization in singleplex format, the three assays were merged into a triplex format. Each reaction contained 0.25 µM of *fucP*, 0.3 µM *siaT* and 0.15 µM *hypD* primers, 0.1 µM of the *fucP*, 0.2 µM of the *siaT* and 0.2 µM of the *hypD* TaqMan MGB probes, 1 µl genomic DNA, 1× Platinum® PCR SuperMix (Life Technologies), 0.5 µM ROX™ reference dye (Life Technologies) and molecular-grade water, to a total volume of 5 µl. All samples were run in duplicate, and all runs contained positive and no-template controls. Thermocycling was conducted as previously described [6]. The FAM, TET and VIC channels were used for simultaneous fluorescence detection of *fucP*, *siaT* and *hypD*, respectively.

• Limit of quantification for the triplex real-time PCR

To determine the lower limit of quantitation (LOQ) for the *fucP*, *siaT* and *hypD* assays in the triplex format, we performed a standard curve analysis comprising 16 replicates tested across 1:10 serial dilutions, with the template concentrations ranging from 2 to 2×10^{-8} ng. *H. influenzae* ATCC 49247 was used as the control for *fucP* and *siaT*, and *H. haemolyticus*

ATCC 33390 the control for *hypD*. LOQ values were determined for each assay based on previously described criteria [41], with minor modifications. Briefly, replicates at a given dilution with a cycles to threshold (C_T) standard deviation (σ) of ≥0.8 were considered to exceed the LOQ, with one or more amplification failures also deemed a LOQ failure. The upper LOQ value was not determined due to the unlikelihood of encountering such high-pathogen DNA concentrations in clinical specimens.

Results & discussion

• Phylogenomic analysis of *Haemophilus* spp. for species determination

To accurately delineate *Haemophilus* spp., we first constructed a genome-wide phylogenetic tree. We have previously employed this approach across 63,447 core genome, biallelic single-nucleotide polymorphisms (SNPs) identified among 246 *Haemophilus* spp. genomes [6]. In the current study, we expanded our dataset to include recently published *H. influenzae* and *H. haemolyticus* genomes. This updated analysis identified 56,187 core genome, biallelic SNPs among 375 *Haemophilus* spp. genomes, and again revealed a clear delineation between *H. influenzae* and *H. haemolyticus* **(Figure 1)**. Fewer SNPs were found in this re-analysis due to the reduction in the core genome as a consequence of the additional 127 taxa. We used this phylogeny to reclassify species designations for strain 839_HINF from *H. influenzae* to *H. haemolyticus*, strains C1 and F0397 from *Haemophilus* spp. to *H. haemolyticus* and strain 614_HPAR from *H. parainfluenzae* to *H. influenzae* **(Supplementary Table 1)**. The species misclassification of these strains by the original authors [28] exemplifies the difficulties associated with accurate speciation of *Haemophilus* spp.

• Identification of loci specific for *H. influenzae* & *H. haemolyticus*

Our genomics approach was used to identify a sialic acid transporter (*siaT*; encoded by *NTHI0234* in 86–028NP) that was present in all 316 *H. influenzae* genomes, but not in *H. haemolyticus* or *H. parainfluenzae*. Similarly, we identified a hydrogenase formation protein (*hypD*, encoded by *GG9_RS01545* in *H. haemolyticus* M19501, contig 15) that was present in all 59 *H. haemolyticus* genomes yet absent in *H. influenzae* and *H. parainfluenzae*.

• *In silico* & PCR specificity for *hypD* & *siaT*

Nonredundant nucleotide BLAST (nr/nt) results for the *hypD* amplicon revealed 100% primer and probe specificity for *H. haemolyticus* strains, with no other microbial species giving significant hits. For *siaT*, BLAST revealed 100% primer and probe specificity for *H. influenzae*, with the next closest hit, *H. haemolyticus*, having three SNPs in the forward primer, and a minimum of three SNPs in the *siaT* TaqMan probe and reverse primer. Triplex PCR testing across a panel of 143 *Haemophilus* and non-*Haemophilus* DNA samples demonstrated expected amplification of these templates in 100% of cases, with only *H. haemolyticus* amplifying with the *hypD* probe and *H. influenzae* amplifying with the *siaT* probe. Those strains lacking the fucose operon according to whole-genome sequencing were negative with the *fucP* assay; all fucose operon-positive isolates were positive for *fucP*. No other templates amplified with the triplex PCR **(Table 1 & Supplementary Table 1)**.

• LOQ of the triplex assay

The lower LOQ values for the *fucP*, *siaT* and *hypD* assays in the triplex format were 2×10^{-5} ng, 2×10^{-5} ng and 2×10^{-4} ng, respectively. These values correspond to approximately ten genome equivalents for *fucP* and *siaT*, and approximately 100 genome equivalents for *hypD*.

• Reclassification of a 'fuzzy' fucose operon-negative *Haemophilus* sp. as *H. influenzae*

Other researchers [13,15–16] have reported that certain *H. influenzae* isolates may lack *fucK*, one of the seven genes used in the *H. influenzae* MLST scheme [14]. The absence of this gene has historically confounded *H. influenzae* speciation attempts such as the probable incorrect assignment of *fucK*-negative *H. influenzae* strains as *H. haemolyticus* [42]. The *fucK* gene is encoded in the same operon as *fucP*; thus, isolates lacking *fucK* largely, if not exclusively, also lack *fucP* [6]. On the genome level, most *fucP*-negative *H. influenzae* strains reside in a distinct, genetically distant lineage that separates them from conventional *fucP*-positive *H. influenzae* strains by several thousand SNPs [6]. This genomic dissimilarity between *fucP*-negative and conventional *fucP*-positive strains previously led us to also propose that *fucP*-negative strains, while closely related to *H. influenzae*, might comprise a novel 'fuzzy' *Haemophilus* species [6]. However, this distance

is similar to that observed between conventional *H. influenzae* and Clade I *H. influenzae*, a lineage reported by De Chiara *et al.* in their comparison of 97 *H. influenzae* genomes [18], at the time the largest comparative genomic analysis of *H. influenzae*. We have since shown that Clade I *H. influenzae* and *fucP*-negative strains share a node [6], implying that they have shared ancestry. These 'fuzzy' isolates should therefore be considered fucose-negative *H. influenzae*. Although it is unfortunate that the developers of the *H. influenzae* MLST scheme inadvertently chose a variably present gene for their scheme, our genomic approach confirms the suspicion that many in the Pasteurellaceae research community have had for some time regarding the existence of fucose-negative *H. influenzae* strains. Redesign of the existing *H. influenzae* MLST scheme to incorporate a conserved seventh locus in *H. influenzae* and *H. haemolyticus* in place of *fucK* will lead to greater uptake of this highly useful genotyping scheme globally.

The novel *siaT* assay developed in the present study should be used to identify all *H. influenzae* strains. Although we can no longer recommend the use of the *fucP* (or *fucK*) gene for identification of *H. influenzae*, this assay still has utility for differentiating *fucP*-positive and *fucP*-negative *H. influenzae*, and for confirming *siaT*-positive isolates and clinical specimens. Future studies focused on the relative abundance of *fucP*-positive and *fucP*-negative *H. influenzae* strains may uncover previously undetected associations with certain types of NTHi-associated disease. In addition, the inclusion of the *hypD* assay in the triplex PCR enables unambiguous detection and assignment of suspected *H. haemolyticus* strains.

• Fucose operon-negative *H. influenzae* comprise several lineages

We identified three 'conventional' *H. influenzae* isolates from our Australian isolate collection that lack *fucP* (isolates 65373 BAL Hi-3, 65234 BAL Hi-2 and 65234 NP Hi-1; **Figure 1**). This finding demonstrates that there is an imperfect association between the *fucP*-negative *H. influenzae* and 'conventional' *H. influenzae* clades, a phenomenon that has also been reported by LaCross *et al.* [17]. The small number of *fucP* 'failures' highlights the inherent difficulties associated with *Haemophilus* spp. identification, and consolidates our previous assertion that no single genetic target is likely to be 100% diagnostic for a given species due to high levels of active recombination occurring within and among strains [6]. Nevertheless, we show that, for now at least, the *siaT* and *hypD* assays provide 100% specificity for *H. influenzae* and *H. haemolyticus* across our dataset. Given the high risk of eventually identifying *siaT hypD* false positives and false negatives, the development of further species-specific assays for *H. influenzae* and *H. haemolyticus* is encouraged. Future *H. influenzae* or *H. haemolyticus* studies should use such redundancy for accurate species determination, with comparative phylogenomic analysis used as the 'gold standard' method for species assignment wherever possible.

Conclusion

We have used comparative genomics to identify robust species-specific markers for *H. influenzae* (*siaT*) and its closest near-neighbor, *H. haemolyticus* (*hypD*). These assays were tested across a panel of 143 *Haemophilus* and non-*Haemophilus* spp., where they demonstrated 100% specificity and sensitivity. Multiplexing these newly developed assays with the previously developed *fucP* assay allowed further differentiation among *H. influenzae*. The lower LOQ was determined as ten genome equivalents for *H. influenzae* templates (*siaT* and *fucP*) and 100 genome equivalents for *H. haemolyticus* (*hypD*). This triplex assay is recommended for studies where accurate speciation of *H. influenzae* and *H. haemolyticus* is required; the *fucP* assay can be omitted where the identification of fucose-negative *H. influenzae* strains is not sought. One recognized limitation of this study is that we only tested the triplex assay on DNA extracted from purified colonies. Future work should focus on examining the robustness and sensitivity of these assays in the clinical setting and on clinical specimens from various regions.

Future perspective

Comparative genomics is rapidly advancing our understanding of infectious diseases. As next-generation sequencing technologies improve, these methods will see even greater implementation in laboratories worldwide. Nevertheless, PCR-based methods remain an attractive option for sensitive, specific and rapid species identification due to their relatively low cost, speed and ease-of-use. Direct testing of clinical samples for the presence of *H. influenzae* and

H. haemolyticus will provide further insights into the contribution of these bacteria in human infectious diseases and will enable the use of more targeted, narrow-spectrum antibiotics.

Acknowledgements

The authors thank A Leach (Menzies School of Health Research), A Keil (PathWest Laboratory Medicine Western Australia) and LA Kirkham (University of Western Australia) for previously providing isolates for genome sequencing. The authors are also grateful to all researchers in the Pasteurellaceae community who have generously made their genome data publicly available.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb-2016-0215

Financial & competing interests disclosure

This project was funded by the Channel 7 Children's Research Foundation (awards 13699 and 151068). Clinical specimens used in this project were partly funded by the Australian National Health and Medical Research Council (NHMRC; awards 1023781 and 1042601). EP Price is funded by a USC Research Fellowship, TM Harris is funded by a NHMRC Centre of Research Excellence in Lung Health of Aboriginal and Torres Strait Islander Children Fellowship (1040830) and AB Chang is funded by an NHMRC Practitioner Fellowship (1058213). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

SUMMARY POINTS

- The Gram-negative bacterium *Haemophilus influenzae* is an important yet often-overlooked opportunistic pathogen that can cause invasive disease, and is frequently associated with exacerbations of chronic respiratory diseases such as chronic obstructive pulmonary disease.
- Accurate identification of *H. influenzae* cannot be performed using microbiological methods alone as it can be phenotypically indistinguishable from its nonpathogenic relative *Haemophilus haemolyticus*.
- Although molecular (DNA-based) assays provide the promise of rapid, specific and cost-effective *H. influenzae* detection, they currently suffer from a lack of specificity and/or sensitivity as they were either designed in the pregenomics era or on small genome datasets, which is insufficient for accurate identification of highly recombining genera such as *Haemophilus*.
- Next-generation whole-genome sequencing of large *Haemophilus* spp. strain collections has been essential for the accurate delineation of *H. influenzae* and *H. haemolyticus*; however, this methodology is still expensive and time-consuming, and more rapid methods are needed for clinic or pathology laboratory settings.
- We used genomic data for 316 *H. influenzae* and 59 *H. haemolyticus* isolates to identify genetic loci specific for each of these species, followed by design of highly specific TaqMan probe real-time PCR assays for their rapid detection.
- The *H. influenzae*-specific *siaT* and *H. haemolyticus*-specific *hypD* assays provide highly accurate (100% sensitivity and specificity) identification of these species, with inclusion of the *fucP* assay allowing differentiation among *H. influenzae* strains.
- This triplex assay is suitable for the rapid and inexpensive identification of *H. influenzae* and *H. haemolyticus*, including fucose-negative *H. influenzae*, but its performance remains to be assessed on complex clinical specimens like sputa or bronchoalveolar lavage.
- Improved recognition of this pathogen as an etiologic agent in myriad communicable diseases will lead to more effective antibiotic stewardship, and ultimately, better patient treatment and outcomes.

References

1 King P. *Haemophilus influenzae* and the lung (*Haemophilus* and the lung). *Clin. Transl. Med.* 1(1), 10 (2012).

2 Cochi SL, Broome CV, Hightower AW. Immunization of US children with *Hemophilus influenzae* type b polysaccharide vaccine. A cost-effectiveness model of strategy assessment. *JAMA* 253(4), 521–529 (1985).

3 Van Eldere J, Slack MP, Ladhani S, Cripps AW. Nontypeable *Haemophilus influenzae*, an under-recognised pathogen. *Lancet Infect. Dis.* 14(12), 1281–1292 (2014).

4 Murphy TF, Brauer AL, Sethi S, Kilian M, Cai X, Lesse AJ. *Haemophilus haemolyticus*: a human respiratory tract commensal to be distinguished from *Haemophilus influenzae*. *J. Infect. Dis.* 195(1), 81–89 (2007).

5 Murphy TF, Faden H, Bakaletz LO *et al.* Nontypeable *Haemophilus influenzae* as a pathogen in children. *Pediatr. Infect. Dis. J.* 28(1), 43–48 (2009).

6 Price EP, Sarovich DS, Nosworthy E *et al.* *Haemophilus influenzae*: using comparative genomics to accurately identify a highly recombinogenic human pathogen. *BMC Genomics* 16, 641 (2015).

7 Binks MJ, Temple B, Kirkham LA *et al.* Molecular surveillance of true nontypeable *Haemophilus influenzae*: an evaluation of PCR screening assays. *PLoS ONE* 7(3), e34083 (2012).

8 Mccrea KW, Xie J, Lacross N *et al.* Relationships of nontypeable *Haemophilus influenzae* strains to hemolytic and nonhemolytic *Haemophilus haemolyticus* strains. *J. Clin. Microbiol.* 46(2), 406–416 (2008).

9 Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A. Role of inter-species recombination of the *ftsI* gene in the dissemination of altered penicillin-binding-protein-3-mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J. Antimicrob. Chemother.* 69(6), 1501–1509 (2014).

10 Kroll JS, Wilks KE, Farrant JL, Langford PR. Natural genetic exchange between *Haemophilus* and *Neisseria*: intergeneric transfer of chromosomal genes between major human pathogens. *Proc. Natl Acad. Sci. USA* 95(21), 12381–12385 (1998).

11 Pickering J, Richmond PC, Kirkham LA. Molecular tools for differentiation of nontypeable *Haemophilus influenzae* from *Haemophilus haemolyticus*. *Front. Microbiol.* 5, 664 (2014).

12 Dang S, Sun L, Huang Y *et al.* Structure of a fucose transporter in an outward-open conformation. *Nature* 467(7316), 734–738 (2010).

13 Ridderberg W, Fenger MG, Norskov-Lauritsen N. *Haemophilus influenzae* may be untypable by the multilocus sequence typing scheme due to a complete deletion of the fucose operon. *J. Med. Microbiol.* 59(Pt 6), 740–742 (2010).

14 Meats E, Feil EJ, Stringer S *et al.* Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. *J. Clin. Microbiol.* 41(4), 1623–1636 (2003).

15 Shuel ML, Karlowsky KE, Law DK, Tsang RS. Nonencapsulated or nontypeable *Haemophilus influenzae* are more likely than their encapsulated or serotypeable counterparts to have mutations in their fucose operon. *Can. J. Microbiol.* 57(12), 982–986 (2011).

16 De Gier C, Kirkham LA, Norskov-Lauritsen N. Complete deletion of the fucose operon in *Haemophilus influenzae* is associated with a cluster in multilocus sequence analysis-based phylogenetic group II related to *Haemophilus haemolyticus*: implications for identification and typing. *J. Clin. Microbiol.* 53(12), 3773–3778 (2015).

17 Lacross NC, Marrs CF, Gilsdorf JR. Population structure in nontypeable *Haemophilus influenzae*. *Infect. Genet. Evol.* 14, 125–136 (2013).

18 De Chiara M, Hood D, Muzzi A *et al.* Genome sequencing of disease and carriage isolates of nontypeable *Haemophilus influenzae* identifies discrete population structure. *Proc. Natl Acad. Sci. USA* 111(14), 5439–5444 (2014).

19 Atack JM, Srikhanta YN, Fox KL *et al.* A biphasic epigenetic switch controls immunoevasion, virulence and niche adaptation in nontypeable *Haemophilus influenzae*. *Nat. Commun.* 6, 7828 (2015).

20 Garmendia J, Viadas C, Calatayud L *et al.* Characterization of nontypable *Haemophilus influenzae* isolates recovered from adult patients with underlying chronic lung disease reveals genotypic and phenotypic traits associated with persistent infection. *PLoS ONE* 9(5), e97020 (2014).

21 Giufrè M, De Chiara M, Censini S *et al.* Whole-genome sequences of nonencapsulated *Haemophilus influenzae* strains isolated in Italy. *Genome Announc.* 3(2), E00110–E00115 (2015).

22 Harrison A, Dyer DW, Gillaspy A *et al.* Genomic sequence of an otitis media isolate of nontypeable *Haemophilus influenzae*: comparative study with *H. influenzae* serotype d, strain KW20. *J. Bacteriol.* 187(13), 4627–4636 (2005).

23 Hogg JS, Hu FZ, Janto B *et al.* Characterization and modeling of the *Haemophilus influenzae* core and supragenomes based on the complete genomic sequences of Rd and 12 clinical nontypeable strains. *Genome Biol.* 8(6), R103 (2007).

24 Langen H, Takács B, Evers S *et al.* Two-dimensional map of the proteome of *Haemophilus influenzae*. *Electrophoresis* 21(2), 411–429 (2000).

25 Mussa HJ, Vanwagoner TM, Morton DJ, Seale TW, Whitby PW, Stull TL. Draft genome sequences of eight nontypeable *Haemophilus influenzae* strains previously characterized using an electrophoretic typing scheme. *Genome Announc.* 3(6), (2015).

26 Ormerod KL, George NM, Fraser JA, Wainwright C, Hugenholtz P. Comparative genomics of non-pseudomonal bacterial species colonising paediatric cystic fibrosis patients. *PeerJ* 3, e1223 (2015).

27 Power PM, Bentley SD, Parkhill J, Moxon ER, Hood DW. Investigations into genome diversity of *Haemophilus influenzae* using whole genome sequencing of clinical isolates and laboratory transformants. *BMC Microbiol.* 12, 273 (2012).

28 Roach DJ, Burton JN, Lee C *et al.* A year of infection in the intensive care unit: prospective whole genome sequencing of bacterial clinical isolates reveals cryptic transmissions and novel microbiota. *PLoS Genet.* 11(7), e1005413 (2015).

29 Smith-Vaughan HC, Chang AB, Sarovich DS *et al.* Absence of an important vaccine and diagnostic target in carriage- and disease-related nontypeable *Haemophilus influenzae*. *Clin. Vaccine Immunol.* 21(2), 250–252 (2014).

30 Strouts FR, Power P, Croucher NJ *et al.* Lineage-specific virulence determinants of *Haemophilus influenzae* biogroup aegyptius. *Emerg. Infect. Dis.* 18(3), 449–457 (2012).

31 Su YC, Hörhold F, Singh B, Riesbeck K. Complete genome sequence of encapsulated *Haemophilus influenzae* type f KR494, an invasive isolate that caused necrotizing myositis. *Genome Announc.* 1(5), E00470–E00513 (2013).

32 Vanwagoner TM, Morton DJ, Seale TW *et al.* Draft genome sequences of six nontypeable *Haemophilus influenzae* strains that establish bacteremia in the infant rat model of invasive disease. *Genome Announc.* 3(5), E00899–E00915 (2015).

33 Whitby PW, Seale TW, Morton DJ, Stull TL. Antisera against certain conserved surface-exposed peptides of nontypeable *Haemophilus influenzae* are protective. *PLoS ONE* 10(9), e0136867 (2015).

34 Zhang L, Xie J, Patel M *et al.* Nontypeable *Haemophilus influenzae* genetic islands associated with chronic pulmonary infection. *PLoS ONE* 7(9), e44730 (2012).

35 Jordan IK, Conley AB, Antonov IV *et al.* Genome sequences for five strains of the emerging pathogen *Haemophilus haemolyticus*. *J. Bacteriol.* 193(20), 5879–5880 (2011).

36 Post DMB, Ketterer MR, Coffin JE *et al.* Comparative analyses of the lipooligosaccharides from nontypeable *Haemophilus influenzae* and *Haemophilus haemolyticus* show differences in sialic acid and phosphorylcholine modifications. *Infect. Immun.* 84(3), 765–774 (2016).

37 Sarovich DS, Price EP. SPANDx: a genomics pipeline for comparative analysis of large haploid whole genome re-sequencing datasets. *BMC Res. Notes* 7, 618 (2014).

38 Afonina IA, Reed MW, Lusby E, Shishkina IG, Belousov YS. Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. *Biotechniques* 32, 940–949 (2002).

39 Mackay IM. Real-time PCR in the microbiology laboratory. *Clin. Microbiol. Infect.* 10, 190–212 (2002).

40 BLAST. http://blast.ncbi.nlm.nih.gov/

41 Price EP, Dale JL, Cook JM *et al.* Development and validation of *Burkholderia pseudomallei*-specific real-time PCR assays for clinical, environmental or forensic detection applications. *PLoS ONE* 7(5), e37723 (2012).

42 Anderson R, Wang X, Briere EC *et al.* *Haemophilus haemolyticus* isolates causing clinical disease. *J. Clin. Microbiol.* 50(7), 2462–2465 (2012).