Absence of an Important Vaccine and Diagnostic Target in Carriage- and Disease-Related Nontypeable *Haemophilus influenzae*

Heidi C. Smith-Vaughan,a Anne B. Chang,a,b,c Derek S. Sarovich,a Robyn L. Marsh,a Keith Grimwood,a,d,e Amanda J. Leach,a Peter S. Morris,a,f Erin P. Pricea

Menzies School of Health Research, Charles Darwin University, Darwin, Australia; Queensland Children's Medical Research Institute, Queensland University of Technology, Brisbane, Australia; Department of Respiratory Medicine, Royal Children's Hospital, Brisbane, Australia; Department of Infectious Diseases, Royal Children's Hospital, Brisbane, Australia; Queensland Children's Medical Research Institute, The University of Queensland, Brisbane, Australia; Royal Darwin Hospital, Darwin, Australia

**Nontypeable *Haemophilus influenzae* (NTHi)-associated disease is a major health problem globally. Whole-genome sequence analysis identified the absence of *hpd* genes encoding *Haemophilus* protein D in 3 of 16 phylogenetically distinct NTHi isolates. This novel finding is of potential clinical significance, as protein D and *hpd* represent important NTHi vaccine antigen and diagnostic targets, respectively.**

Nontypeable *Haemophilus influenzae* (NTHi)-associated disease represents a major health burden for young children worldwide and, also, for adults with chronic pulmonary disorders. The most commonly reported manifestations relate to chronic middle ear and lung disease, with particularly high rates among Australian Aboriginal children (1, 2). An effective vaccine against NTHi is required, but its development remains a challenge. Nevertheless, an 11-valent prototype pneumococcal-*H. influenzae* protein D conjugated vaccine (PHiD-CV11), relying upon the conserved outer membrane lipoprotein, protein D, as its sole *Haemophilus*-specific antigen, reduced NTHi-associated acute otitis media by 35% (3). The effect of its successor, PHiD-CV10, on NTHi carriage and NTHi-associated middle ear and chronic lung disease (4), however, is still under investigation in several different populations. Interestingly, the data published so far indicate either no or, at best, an inconsistent effect upon nasopharyngeal NTHi colonization in children up to 2 years of age (5, 6).

*H. influenzae* protein D is important for several reasons. First, as described above, it is a potential vaccine antigen. Second, the gene encoding protein D, *hpd*, is a diagnostic target for differentiating NTHi from phenotypically indistinguishable nonhemolytic strains of the respiratory commensal *H. influenzae* (which only rarely acts as a pathogen) (7). Differentiating between these bacteria is important, as it will help to reduce unnecessary antibiotic prescribing for misidentified *H. influenzae* disease. Consequently, since standard microbiological testing cannot distinguish the two species, complementary genotypic testing is necessary. Compared to other methods, a PCR targeting the *hpd* gene provided superior discrimination between *H. influenzae* and *H. haemolyticus*. However, the species cutoff was selected arbitrarily (7), and a more meaningful diagnostic is likely required.

As part of a larger study to investigate the relatedness of common Australian pediatric carriage genotypes and those associated with lower airway infection in Australian Aboriginal children with non-cystic fibrosis bronchiectasis (Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research, approvals 07/63 and 07/85), we performed whole-genome sequencing of 20 *Haemophilus* isolates. These were nasal or nasopharyngeal carriage isolates representing common NTHi genotypes (based on PCR-ribotyping [8]) of over 3,000 isolates) from our previous carriage studies of Northern Territory and Western Australian children. Additionally, NTHi and presumptive *H. haemolyticus* isolates from nasopharyngeal swabs and bronchoalveolar lavage from children with bronchiectasis were selected from children with high airway neutrophilia and clinically significant NTHi infection (>10^4 CFU/ml) in the lower airways (1). A hybrid *de novo* assembly using data from the Ion Torrent and Illumina sequencing platforms was undertaken on one nasopharyngeal NTHi isolate, 60294NP, and subsequently used as a reference for 19 additional genomes generated using Ion Torrent. A neighbor-joining maximum likelihood tree was constructed using 50,172 orthologous single-nucleotide polymorphisms (SNPs) across the 20 genomes (Fig. 1). This method provides a robust measure for strain relatedness for highly recombinogenic bacteria, such as NTHi (9).

The sequenced isolates are listed in Table 1. For the children assigned identifications (ID) 1 to 5, nasal NTHi isolates representing five common genotypes were sequenced. One PCR-ribotype each was performed for isolates from an Aboriginal child (ID 3) and four non-Aboriginal children attending three different child care centers in 2001 in Darwin, Australia (10). The nasopharyngeal NTHi isolate representing another common genotype is from an Aboriginal child (ID 6) who was enrolled in a randomized controlled trial of antibiotics for acute otitis media in 2003 (11). The children assigned ID 7 to 16 were Aboriginal children who underwent bronchoscopy at Royal Darwin Hospital between 2008 and 2010 (1), and they provided 14 *Haemophilus* isolates, including NTHi isolates from bronchoalveolar lavage (BAL) specimens (ID 7 to 12), two NTHi nasopharyngeal isolates...

Received 3 October 2013 Returned for modification 31 October 2013 Accepted 16 November 2013 Published ahead of print 27 November 2013

Editor: M. F. Pasetti

Address correspondence to Heidi C. Smith-Vaughan, heidi.smith-vaughan@menzies.edu.au.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.
doi:10.1128/CVI.00632-13
with contemporaneously paired BAL fluid isolates (ID 13 and 14), and similarly, two pairs of BAL fluid and nasopharyngeal isolates identified as presumptive *H. haemolyticus* based on an hpd-targeted PCR assay (ID 15 and 16). The *Haemophilus* isolates were identified by colony morphology and X and V factor dependence, and all failed to react with capsular antisera using the Phadebact Haemophilus coagglutination test. Although concurrent colonization/infection with multiple genotypes is common in Aboriginal (8) and non-Aboriginal Australian children, only one colony from each specimen was sequenced in this project.

The two pairs of BAL fluid and nasopharyngeal isolates identified as presumptive *H. haemolyticus* clustered with NTHi using an orthologous SNP phylogeny (Fig. 1, ID 15 and 16) and were found to have been misidentified due to the absence of the hpd gene. Screening of the remaining genomes revealed that a further nasal NTHi isolate lacked hpd (Fig. 1, ID 3). These findings confirmed negative PCR results using hpd#3 primers (7). Overall, 5 of 20 NTHi genomes from 3 of 16 unrelated children lacked hpd. Furthermore, the orthologous SNP phylogeny demonstrated that the three independent isolates were phylogenetically unrelated (Fig. 1) and, thus, did not represent expansion of a single hpd-negative clone. The hpd-negative isolates were detected in 2001 and 2009, and none were detected in children who had received PHiD-CV10 (Table 1).

To our knowledge, this is the first report on the absence of hpd in clinical NTHi strains. Despite involving only a small number of isolates, our findings have implications for hpd-based diagnostic tests currently recommended for differentiating between NTHi and *H. haemolyticus*. Our findings also identify a potential limitation in the efficacy of PHiD-CV11 (3) and PHiD-CV10 (5) against NTHi-related carriage and disease. They highlight the challenges inherent in developing single-target NTHi diagnostics and vaccines, given the high degree of heterogeneity in the NTHi population. Screening NTHi populations for hpd in diverse patient groupings and geographic settings will provide information on whether alternative NTHi diagnostic tests are needed to predict the likely effectiveness of PHiD-CV10 for NTHi-related disease. The significance of the absence of hpd for NTHi pathogenicity and host immune responses also remains to be discovered.

**TABLE 1** Details on the 20 analyzed NTHi isolates from 16 Australian children

| Child ID | Age (yr) | Site of isolation* | Yr of isolation | Clinical status | No. of doses of PHiD-CV10 | hpd gene |
|----------|----------|--------------------|----------------|----------------|--------------------------|----------|
| 1        | 1.6      | Nasal cavity       | 2001           | Asymptomatic carriage | 0            | +        |
| 2        | 4.0      | Nasal cavity       | 2001           | Otitis media with effusion | 0            | +        |
| 3        | 1.4      | Nasal cavity       | 2001           | Asymptomatic carriage | 0            | −        |
| 4        | 2.5      | Nasal cavity       | 2001           | Asymptomatic carriage | 0            | +        |
| 5        | 4.4      | Nasal cavity       | 2001           | Asymptomatic carriage | 0            | +        |
| 6        | 1.5      | Nasopharynx        | 2003           | Acute otitis media | 0            | +        |
| 7        | 2.5      | BAL fluid          | 2009           | Bronchiectasis   | 0            | +        |
| 8        | 4.1      | BAL fluid          | 2009           | Bronchiectasis   | 0            | +        |
| 9        | 6.8      | BAL fluid          | 2009           | Bronchiectasis   | 0            | +        |
| 10       | 0.4      | BAL fluid          | 2010           | Bronchiectasis   | 2            | +        |
| 11       | 1.7      | BAL fluid          | 2010           | Bronchiectasis   | 1            | +        |
| 12       | 1.8      | BAL fluid          | 2008           | Bronchiectasis   | 0            | +        |
| 13       | 4.2      | BAL fluid          | 2008           | Bronchiectasis   | 0            | +        |
| 14       | 4.2      | Nasopharynx        | 2008           | Bronchiectasis   | 0            | +        |
| 15       | 2.0      | BAL fluid          | 2009           | Bronchiectasis   | 0            | +        |
| 16       | 2.0      | Nasopharynx        | 2009           | Bronchiectasis   | 0            | +        |
| 17       | 1.1      | BAL fluid          | 2009           | Bronchiectasis   | 0            | +        |
| 18       | 1.1      | Nasopharynx        | 2009           | Bronchiectasis   | 0            | +        |
| 19       | 2.1      | BAL fluid          | 2009           | Bronchiectasis   | 0            | +        |
| 20       | 2.1      | Nasopharynx        | 2009           | Bronchiectasis   | 0            | +        |

* BAL, bronchoalveolar lavage.
ACKNOWLEDGMENTS

This work was supported by Channel 7 Children’s Research Foundation (grant number 13699) and the Australian National Health and Medical Research Council (grants 1023781, 1040830, and 1024175 to H.C.S.-V., 1020561 to A.J.L., 1034703 to R.L.M., and 545216 to A.B.C.).

We thank the families who participated in these studies. We also thank the Menzies Ear Health Research Team, Respiratory Team, and Child Health Laboratory Team, particularly Gabrielle McCallum, Jemima Beissbarth, Kim Hare, and Elizabeth Nosworthy, for clinical swabs, clinical data, and laboratory support.

REFERENCES

1. Hare KM, Grimwood K, Leach AJ, Smith-Vaughan H, Torzillo PJ, Morris PS, Chang AB. 2010. Respiratory bacterial pathogens in the nasopharynx and lower airways of Australian indigenous children with bronchiectasis. J. Pediatr. 157:1001–1005. http://dx.doi.org/10.1016/j.jpeds.2010.06.002.

2. Leach A, Wood Y, Gadil E, Stubbs E, Morris P. 2008. Topical ciprofloxin versus topical framycetin-gramicidin-dexamethasone in Australian aboriginal children with recently treated chronic suppurative otitis media: a randomized controlled trial. Pediatr. Infect. Dis. J. 27:692–698. http://dx.doi.org/10.1097/INF.0b013e318126ca9d.

3. Prymula R, Peeters P, Chrobok V, Kriz P, Novakova E, Kaliskova E, Kohl I, Lommel P, Poomajjanakun J, Pries JP, Schuerman L. 2006. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. Lancet 367:740–748. http://dx.doi.org/10.1016/S0140-6736(06)68304-9.

4. O’Grady KA, Grimwood K, Crits-Christoph P, Mulholland EK, Morris P, Torzillo PJ, Wood N, Smith-Vaughan H, Revell A, Wilson A, Van Asperen AP, Richmond P, Thornton R, Rablin S, Chang AB. 2013. Does a 10-valent pneumococcal-Haemophilus influenzae protein D conjugate vaccine prevent respiratory exacerbations in children with recurrent protracted bacterial bronchitis, chronic suppurative lung disease and bronchiectasis protocol for a randomised controlled trial. Trials 14:282. http://dx.doi.org/10.1186/1745-6215-14-282.

5. van den Bergh MR, Spijkerman J, Swinnen KM, Francois NA, Pascal TG, Borys D, Schuerman L, Ijzerman EP, Bruin JP, van der Ende A, Veenhoven RH, Sanders EA. 2013. Effects of the 10-valent pneumococcal nontypeable Haemophilus influenzae protein D-conjugate vaccine on nasopharyngeal bacterial colonization in young children: a randomized controlled trial. Clin. Infect. Dis. 56:e30–e39. http://dx.doi.org/10.1093/cid/cis922.

6. Prymula R, Habib A, Francois N, Borys D, Schuerman L. 2013. Immunological memory and nasopharyngeal carriage in 4-year-old children previously primed and boosted with 10-valent pneumococcal nontypeable Haemophilus influenzae protein D conjugate vaccine (PHID-CV) with or without concomitant prophylactic paracetamol. Vaccine 31:2080–2088. http://dx.doi.org/10.1016/j.vaccine.2013.01.044.

7. Binks MJ, Temple B, Kirkham LA, Wiertsema SP, Dunne EM, Richardson PC, Marsh RI, Leach AJ, Smith-Vaughan HC. 2012. Molecular surveillance of true nontypeable Haemophilus influenzae: an evaluation of PCR screening assays. PLoS One 7:e34083. http://dx.doi.org/10.1371/journal.pone.0034083.

8. Smith-Vaughan HC, McBroom J, Mathews JD. 2001. Modelling of endemic carriage of Haemophilus influenzae in Aboriginal infants in Northern Australia. FEMS Immunol. Med. Microbiol. 31:137–143. http://dx.doi.org/10.1111/j.1574-695X.2001.tb00510.x.

9. Pearson T, Giffard P, Beckstrom-Sternberg S, Auerbach R, Hornstra H, Kool A, Price EP, Glass MB, Leadem B, Beckstrom-Sternberg JS, Allan GF, Foster JT, Wagner DM, Okinaka RT, Sim SH, Pearson O, Wu Z, Chang J, Kaul R, Hoffmaster AR, Brettin TA, Robison RA, Mayo M, Gee JE, Tan P, Currie BJ, Keim P. 2009. Phylogeographic reconstruction of a bacterial species with high levels of lateral gene transfer. BMC Biol. 7:278. http://dx.doi.org/10.1186/1741-7007-7-278.

10. Smith-Vaughan HC, Byun R, Nadkarni M, Jacques NA, Hunter N, Halpin S, Morris PS, Leach AJ. 2006. Measuring nasal bacterial load and its association with otitis media. BMC Ear Nose Throat Disord. 6:10. http://dx.doi.org/10.1186/1747-6815-6-10.

11. Morris PS, Gadir G, McCallum GB, Wilson CA, Smith-Vaughan HC, Torzillo P, Leach AJ. 2010. Single-dose azithromycin versus seven days of amoxicillin in the treatment of acute otitis media in Aboriginal children (AATAAC): a double blind, randomised controlled trial. Med. J. Aust. 192:24–29.