Vaccines for Nontypeable *Haemophilus influenzae*: the Future Is Now

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Infections due to nontypeable *Haemophilus influenzae* result in enormous global morbidity in two clinical settings: otitis media in children and respiratory tract infections in adults with chronic obstructive pulmonary disease (COPD). Recurrent otitis media affects up to 20% of children and results in hearing loss, delays in speech and language development and, in developing countries, chronic suppurative otitis media. Infections in people with COPD result in clinic and emergency room visits, hospital admissions, and respiratory failure. An effective vaccine would prevent morbidity, help control health care costs, and reduce antibiotic use, a major contributor to the global crisis in bacterial antibiotic resistance. The widespread use of the pneumococcal conjugate vaccines is causing a relative increase in *H. influenzae* otitis media. The partial protection against *H. influenzae* otitis media induced by the pneumococcal *H. influenzae* protein D conjugate vaccine represents a proof of principle of the feasibility of a vaccine for nontypeable *H. influenzae*. An ideal vaccine antigen should be conserved among strains, have abundant epitopes on the bacterial surface, be immunogenic, and induce protective immune responses. Several surface proteins of *H. influenzae* have been identified as potential vaccine candidates and are in various stages of development. With continued research, progress toward a broadly effective vaccine to prevent infections caused by nontypeable *H. influenzae* is expected over the next several years.

Vaccines composed of polysaccharide capsule conjugated to protein carriers have virtually eliminated infections caused by encapsulated *Haemophilus influenzae* type b, including meningitis and other systemic infections, in regions of the world where the vaccines are administered widely. However, these conjugate vaccines have no effect on infections caused by nontypeable (nonencapsulated) strains of *H. influenzae*. Common infections caused by nontypeable *H. influenzae* include otitis media in children and lower airway infections (exacerbations) of chronic obstructive pulmonary disease (COPD) in adults. Vaccine development for nontypeable strains of *H. influenzae* presents an entirely different challenge compared to vaccines for encapsulated type b strains for several reasons: (i) nontypeable *H. influenzae* lacks a polysaccharide capsule and thus will require the identification of alternative vaccine antigens; (ii) nontypeable strains demonstrate enormous genetic and antigenic heterogeneity among strains, whereas type b encapsulated strains are generally a clonal population; (iii) the pathogenesis of infections caused by nontypeable *H. influenzae* involves contiguous spread from mucosal surfaces, suggesting that a successful vaccine will require a different immune response from that required to protect from infections by type b strains, which occur through hematogenous dissemination.

This review will assess the current state of vaccine development for nontypeable *H. influenzae*, including the rationale for developing such vaccines, the populations who would benefit from a vaccine, its feasibility, some of the challenges, the antigens under consideration, and a discussion of what is needed to advance vaccine development to prevent infections by nontypeable *H. influenzae*.

**Rationale for a Vaccine for Nontypeable *H. influenzae***

Infections caused by nontypeable *H. influenzae* cause enormous morbidity in two clinical settings: (i) otitis media in children under the age of 6 years and (ii) adults with COPD. The bacterium also causes sinusitis and community-acquired pneumonia in children and adults (1). Nontypeable *H. influenzae* causes pneumonia in children in developing countries, but its role in this infection is not yet well defined (2, 3). Finally, in regions with *H. influenzae* type b vaccination programs, nontypeable strains are now the most common cause of invasive *H. influenzae* infection, although these are far less common than otitis media and exacerbations of COPD (4–7).

Vaccine development efforts are directed primarily toward otitis media and COPD; thus, the discussion of the rationale for nontypeable *H. influenzae* vaccines will focus on these two clinical settings.

**Otitis Media**

Otitis media is the most frequently diagnosed bacterial infection in young children requiring office or clinic visits in the United States. Approximately 70% of children will have at least one episode of otitis media by the age of 3 years (8, 9). Acute otitis media is an inflammation in the middle ear (the cavity between the ear drum and the inner ear) that is characterized by fever and ear pain. The pathogenesis of otitis media involves migration of pathogens from the nasopharynx to the middle ear through the Eustachian tube. Most episodes of bacterial otitis media are triggered by an initial viral upper respiratory tract infection.

Recurrent otitis media is common, with up to 30% of children experiencing 3 or more episodes before the age of 3 years (10). Up to 20% of children experience 4 or more episodes of otitis media...
within a year, and these children are considered otitis prone (11). Otitis media with effusion refers to the presence of fluid in the middle ear without symptoms of acute otitis media. The persistent middle ear effusions associated with recurrent and chronic otitis media and otitis media with effusion cause conductive hearing loss and subsequent delay or impairment in speech and language development (12–14). Thus, preventing otitis media would have a potentially huge impact for the children and families who experience these disorders by preventing these important complications.

The burden of otitis media differs considerably between developing and developed countries. Chronic suppurative otitis media (defined by the World Health Organization as 2 weeks of persistent ear discharge) is a major cause of hearing loss in developing countries, affecting an estimated 65 million to 300 million people globally (15, 16). Socioeconomic conditions (overcrowding, reduced sanitation, and limited access to diagnosis and treatment) and genetic and cultural factors may all play a role in the high rate of early onset of acute otitis media and chronic suppurative otitis media in selected populations (17–19).

Etiology of otitis media. With the widespread use of pneumococcal conjugate vaccines, which dramatically alter nasopharyngeal colonization patterns, the etiology of otitis media is undergoing continuing changes. The 3 primary bacterial pathogens that cause otitis media are nontypeable H. influenzae, Streptococcus pneumoniae, and Moraxella catarrhalis. Since 2000, most children in the United States have received the 7-valent pneumococcal conjugate vaccine, and this has led to a reduction of otitis media and nasopharyngeal colonization by the 7 vaccine serotypes, resulting in “replacement” by nonvaccine pneumococcal serotypes, nontypeable H. influenzae, and M. catarrhalis. These changes in nasopharyngeal colonization patterns are resulting in changes in the distribution of pathogens that cause otitis media and in an increasing role for nontypeable H. influenzae, which is now the most common cause of acute otitis media and recurrent otitis media according to several recent studies (20–24). The introduction of the 13-valent pneumococcal conjugate vaccine and the increasing use of the 10-valent pneumococcal vaccine with H. influenzae protein D as the carrier molecule in ~40 countries where it is licensed will undoubtedly cause additional changes in nasopharyngeal colonization patterns and the distribution of pathogens causing otitis media (25). These patterns must be monitored carefully.

In clinical practice, otitis media is managed empirically, and so the etiology of individual cases of otitis media is rarely known by those clinicians managing such patients. The gold standard for determining the etiology of acute otitis media has been culture of middle ear fluid obtained by tympanostomy. While studies vary, reasonable current estimates of etiology of acute otitis media based on culture report that nontypeable H. influenzae and pneumococcus each cause 25 to 35% of episodes and M. catarrhalis causes 10 to 20%.

Implications of biofilms in etiology and clinical trial design. As knowledge of the pathogenesis of otitis media has advanced and the central role of biofilms in the course of the disease has become apparent, it is clear that culture of middle ear fluid does not tell the whole story regarding the etiology of otitis media (26). A biofilm is a community of bacteria encased in a matrix. Biofilms show a reduced growth rate, a distinct transcriptome, and increased resistance to effectors of innate and acquired immunity and to the action of antimicrobial agents, compared to planktonic bacteria. Effusions recovered from the middle ear are often sterile by culture but contain abundant pathogens in the form of a biofilm. Hall-Stoodley et al. (27) showed that sterile effusions (by culture) from children with chronic otitis media and otitis media with effusion contained bacterial biofilms based on fluorescent in situ hybridization and confocal scanning laser microscopy. Furthermore, PCR demonstrated the presence of DNA, and live-dead fluorescent stain showed abundant viable bacteria despite negative culture. Another important observation from this study was the presence of a mixed microbial etiology for some of the effusions from children with chronic otitis media or otitis media with effusion.

Studies from several centers have demonstrated by PCR the presence of DNA from H. influenzae, S. pneumoniae, and M. catarrhalis in sterile middle ear fluids (28–30). Rayner et al. (31) further demonstrated the presence of mRNA, indicating the presence of viable bacteria, in effusions that were PCR positive but sterile by culture. A recent PCR study that examined middle ear effusions from 207 consecutive children who were undergoing tympanostomy placement demonstrated the presence of DNA from otitis media pathogens in 87% of children with acute otitis media and in 51% of children with otitis media with effusion (32).

Thus, the recognition of the role of biofilms in multiple forms of otitis media has important implications in understanding the etiology of otitis media and in designing rational vaccine development strategies. Relying solely on cultures of middle ear fluids as the endpoint in clinical trials of otitis media will assess only a subset of otitis media, i.e., those that are culture positive. The design of trials to assess the impact of vaccines in preventing otitis media should take into account the role of biofilms and culture-negative otitis media. Table 1 summarizes potential benefits resulting from a vaccine that will prevent otitis media caused by nontypeable H. influenzae.

**TABLE 1 Rationale for vaccine development for nontypeable \( \text{Haemophilus influenzae} \)**

| Infection          | Population to vaccinate | What vaccine will accomplish |
|--------------------|-------------------------|-----------------------------|
| Otitis media       | All children beginning at 2 mo of age | Prevention of: |
|                    |                         | Acute otitis media          |
|                    |                         | Recurrent otitis media      |
|                    |                         | Chronic otitis media        |
|                    |                         | Otitis media with effusion related to bacterial biofilm formation |
|                    |                         | Chronic suppurative otitis media |
| Exacerbation of COPD | Adults with COPD         | Reductions in: |

Exacerbation | Prevention of: |

Acute exacerbations of COPD |

Chronic airway colonization |

Reductions in: |

Excess antibiotic use |

Health care costs

**INFECTIONS IN COPD**

COPD afflicts ~24 million Americans and is the third most common cause of death in the United States and the world (33, 34). The course of the disease is characterized by intermittent exacerbations that result in enormous morbidity, including lost work...
time, doctor’s office and clinic visits, emergency room visits, hospital admissions, and respiratory failure requiring mechanical ventilation. Bacteria cause approximately half of the exacerbations, with nontypeable *H. influenzae* as the most common bacterial cause (35). Antibiotic therapy results in faster recovery and fewer complications in selected exacerbations, and so many exacerbations are treated with antibiotics. A vaccine to prevent exacerbations would result in a major benefit in reducing morbidity and reducing the use of antibiotics in this population.

*H. influenzae* also plays a more subtle role in the course and pathogenesis of COPD. The organism is absent in healthy airways but colonizes the lower airways of adults with COPD, even during clinically stable periods. The bacteria release highly inflammatory antigens (e.g., lipooligosaccharide, outer membrane protein P6, peptidoglycan fragments, and other antigens) into the airways, contributing to airway inflammation, which is a hallmark of COPD (36). Symptoms of COPD parallel airway inflammation, suggesting that eradication of nontypeable *H. influenzae* in the airways, for example, by a vaccine, could reduce airway inflammation and thus have a beneficial effect on the course of COPD (37). Table 1 summarizes the potential benefits of a vaccine that would prevent exacerbations and eradicate airway colonization by nontypeable *H. influenzae* in adults with COPD.

**VACCINES TO REDUCE ANTIMICROBIAL RESISTANCE**

Widespread use of antimicrobial agents is causing a global crisis in antibiotic resistance (38, 39). Otitis media is the most common reason for children in industrialized countries to receive antimicrobial therapy, and children play an important role in transmission of resistant isolates into the community (40). Infections in COPD are one of the most common indications for antimicrobial therapy in adults. Thus, antimicrobial therapy for infections in these two clinical settings (otitis media in children and exacerbations of COPD in adults) plays an important role in driving global antimicrobial resistance. Because up to 70% of all children experience at least one episode of otitis media and up to 20% of children experience recurrent otitis media, the microbiome of a substantial proportion of the population is exposed to the antimicrobial agents used to treat otitis media.

The availability of a vaccine to prevent otitis media and exacerbations of COPD will need to treat these infections with antimicrobial agents. Indeed, the rate of antibiotic-resistant invasive pneumococcal infections has decreased in young children and older adults with the widespread use of pneumococcal conjugate vaccines (41). Palmu et al. (42) performed an innovative cluster randomized controlled trial using data from a national registry of antibiotic purchases to show that vaccination of every 5 children with the 10-valent pneumococcal *H. influenzae* protein D conjugate vaccine prevented one antibiotic purchase. A growing body of evidence supports the concept that vaccines to prevent otitis media and exacerbations of COPD will have a broad impact in reducing antimicrobial use, an important contributor to the global burden of antimicrobial resistance (43, 44).

**FEASIBILITY OF A VACCINE FOR NONTYPEABLE *H. influenzae***

Pneumococcal conjugate vaccines, particularly the 10-valent pneumococcal *H. influenzae* protein D conjugate vaccine, represent a proof of principle of the feasibility of a vaccine to prevent otitis media caused by pneumococcus and by *H. influenzae* (25, 45). In a prospective clinical trial, 4,968 infants were randomized to receive the protein D conjugate vaccine or hepatitis A vaccine as a control. The vaccines were administered at the ages of 3, 4, 5, and 12 to 15 months. Children were followed for episodes of otitis media and underwent tympanocentesis and culture of middle ear fluid for clinically documented otitis media. The primary endpoint of the study was protective efficacy against the first episode of acute otitis media caused by pneumococcal vaccine serotypes. A secondary endpoint was protective efficacy against a first episode of otitis media caused by nontypeable *H. influenzae*. During the follow-up period, an efficacy of 36% for preventing a first episode of *H. influenzae* otitis media was observed (45). While improvement in this level of efficacy is needed, the observation that immunization with a surface protein of *H. influenzae* induces protection from otitis media in children is important in establishing the feasibility of vaccines to prevent otitis media caused by *H. influenzae*.

An important mechanism by which pneumococcal conjugate vaccines induce protection is through reduction of nasopharyngeal colonization by the pneumococcus (46–48). The question of whether the protein D pneumococcal conjugate vaccine also reduces nasopharyngeal colonization by *H. influenzae* was addressed by van den Bergh et al. (49), who performed a randomized controlled trial comparing the effect of the 7-valent pneumococcal conjugate vaccine (which does not contain an *H. influenzae* antigen) with the effect of the protein D pneumococcal conjugate vaccine on nasopharyngeal colonization by *H. influenzae*. The protein D pneumococcal conjugate vaccine had no differential effect on *H. influenzae* nasopharyngeal colonization compared to the 7-valent vaccine in children up to 2 years of age. The strengths of this study included the randomized controlled study design, high follow-up rate, assessment of colonization at multiple time points, use of molecular methods in addition to culture to detect bacterial density, and the inclusion of a 7-valent pneumococcal vaccine control group. Based on this study, "herd immunity" to *H. influenzae* infection is not likely to be observed with widespread use of the protein D pneumococcal conjugate vaccine, but further studies are needed.

**CORRELATES OF PROTECTION**

For a vaccine antigen to be effective, it must induce a protective immune response in the human host. Thus, an important consideration in vaccine development is identifying a correlate of protection. Several animal models have contributed important information on immune responses to *H. influenzae*, including pulmonary clearance and nasopharyngeal colonization models in rats and mice. The chinchilla model of otitis media is widely regarded as the best animal model for studying otitis media. The model is used extensively by multiple research groups and has played a key role in elucidating mechanisms of pathogenesis of *H. influenzae* otitis media and in assessing and prioritizing *H. influenzae* vaccine antigens (10, 50–55).

Novotny et al. (56) demonstrated that passive immunization of chinchillas with serum from children who were immunized with protein D pneumococcal conjugate vaccine conferred 34% protection against otitis media, closely paralleling the level of protection (36%) observed in the clinical trial described above (45). This important study provided a line of evidence that protection from otitis media in the chinchilla model predicts protection from otitis media in humans.
media in humans. The study also provided evidence that protection is at least partially antibody mediated.

In addition to the chinchilla model, serum bactericidal assays are potentially useful as guides in identifying potentially protective antigens. The presence of serum bactericidal antibodies to a strain of nontypeable H. influenzae is associated with protection from otitis media due to that strain (57, 58). Therefore, antigens that induce bactericidal antibodies are potentially promising vaccine candidates.

**APPROACH TO VACCINE DEVELOPMENT FOR NONTYPEABLE H. INFLUENZAE**

Many research groups are taking the approach of identifying surface proteins that are conserved among strains as vaccine antigens (10, 59). A challenge to this approach is the enormous genetic heterogeneity among strains of nontypeable H. influenzae, resulting in sequence heterogeneity of many surface antigens (60–64). For example, the P2 porin protein, the most abundant protein on the bacterial surface, contains several surface loops that show sequence differences among strains, raising the possibility that P2 may not induce broadly reactive immune responses.

**Characteristics of an ideal vaccine antigen for nontypeable H. influenzae.**

(i) **Surface exposure.** As noted above, in vitro and in vivo evidence indicates that humoral immunity is important for protection from nontypeable H. influenzae infection (56, 57). Antibodies induce protective responses by blocking adherence, directing complement-mediated killing, or opsonizing for killing, all of which require binding to the bacterial surface.

(ii) **Sequence conservation among strains.** A conserved antigen will induce a response that protects against all or most strains. A related strategy to identifying conserved surface proteins is identification of conserved regions of abundantly expressed surface molecules. For example, the conserved regions of the P2 porin protein may represent an effective approach (65, 66). Similarly, the lipooligosaccharide molecule is a prominent surface antigen that displays antigenic variability among strains. However, a detoxified form of lipooligosaccharide utilizing relatively conserved regions of the molecule has shown promise in animal models (67, 68).

(iii) **Phase variation.** A vaccine antigen must be expressed by the bacterium during infection or colonization in the human host. A surface molecule that is expressed when grown in vitro but whose expression is shut off under in vivo conditions would not be an effective vaccine antigen.

(iv) **Immunogenicity of the antigen.** In order for an antigen to be effective, it must be capable of inducing an immune response in the target population. Otitis-prone children will benefit most from a vaccine to prevent otitis media; however, it is not yet possible to predict which children are otitis prone. Thus, a vaccine for otitis media would need to be immunogenic in infants, because an episode of otitis media in the first year of life is a risk factor for recurrent otitis media. Therefore, many authorities recommend universal vaccination for otitis media beginning in the first 2 months of life once effective vaccines are available (69, 70). Multiple protein vaccines used in routine immunization of infants reliably induce protective immune responses, providing evidence that surface proteins of H. influenzae will be immunogenic.

(v) **Induction of protective responses.** As discussed above, serum bactericidal antibodies and protection in the chinchilla model are reasonable correlates of protection. Each vaccine antigen will require testing in rigorous clinical trials as the definitive test of efficacy.

**CANDIDATE VACCINE ANTIGENS**

Table 2 lists candidate vaccine antigens that have many of the characteristics of vaccines noted above and are in various stages of development. This list reflects data that are available from publications in peer-reviewed journals and in the public domain. Additional antigens may be under consideration, and additional data on the antigens in Table 2 may be known but not yet widely available.

Vaccines for nontypeable H. influenzae are an active area of research. A search of clinicaltrials.gov using the search terms “vaccine” and “Haemophilus influenzae” revealed 226 registered clinical trials. A total of 25 of those involve administration of noncapsular H. influenzae antigens in vaccine formulations designed for preventing infections caused by nontypeable H. influenzae. Of those 25, 3 are actively recruiting, 2 are registered but not yet recruiting, 18 have been completed, and 2 were terminated. Many of the completed trials involve(d) testing of protein D formulations.

### Table 2: Haemophilus influenzae vaccine antigens under study

| Antigen                                | Molecular mass (kDa) | Function                                         | Reference(s) |
|----------------------------------------|----------------------|--------------------------------------------------|--------------|
| *Haemophilus* adhesin protein (Hap)     | ~155                 | Adhesin                                          | 71–73        |
| HMW1, HMW2                              | ~120–125             | Adhesins                                         | 74–76        |
| *H. influenzae* adhesin (Hia)           | ~115                 | Adhesin                                          | 77, 78       |
| D15 protein                             | ~80                  | Predicted nucleotidyltransferase                 | 79, 80       |
| HtrA                                    | ~46                  | Heat shock protein                               | 81           |
| P2 porin                               | 36–42                | Porin protein                                    | 65, 66, 82   |
| Lipoprotein D                           | ~42                  | Glycerophosphodiester phosphodiesterase         | 45, 56, 83   |
| P5 fimbrin                              | 27–35                | Adhesin, OMP A-like protein                      | 51, 84–86    |
| P4 protein                              | ~30                  | Acid phosphatase                                 | 87–89        |
| Protein F                               | ~30                  | Adhesin, ABC transporter                         | 90–92        |
| OMP 26                                  | ~26                  | Skp family of translocation proteins             | 85, 93–95    |
| P6 protein                              | ~16                  | Peptidoglycan-associated lipoprotein            | 96–102       |
| Protein E                               | ~16                  | Adhesin, binds IgD                               | 103–106      |
| PilA (type IV pilus)                    | ~14                  | Adhesin, transformation                          | 51, 107–109  |
| Detoxified lipooligosaccharide          | 3–5                  | Endotoxin                                        | 110–113      |
SUMMARY AND FUTURE DIRECTIONS

This is a dynamic and exciting time for the development of vaccines to prevent infections caused by nontypeable *H. influenzae*, in particular, otitis media in children and exacerbations in adults with COPD. A vaccine to prevent infections in these clinical settings would result in prevention of an enormous global morbidity, reduced mortality related to COPD, and billions of dollars in health care savings. The early success of protein D in preventing episodes of otitis media provides a path forward to assess additional *H. influenzae* antigens as vaccines. Several conserved surface proteins are in various stages of development as vaccine antigens. Several of these antigens are ready for preclinical and early clinical testing as vaccines for nontypeable *H. influenzae*. Investment in such studies is needed to advance the development of these important vaccines.

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REFERENCES

1. Agrawal A, Murphy TF. 2011. *Haemophilus influenzae* infections in the *H. influenzae* type b conjugate vaccine era. J Clin Microbiol 49:3728–3732. http://dx.doi.org/10.1128/JCM.05476-11.

2. Hausdorff WP, Dagan R. 2008. Serotypes and pathogens in paediatric pneumonia. Vaccine 26(Suppl 2):B19–B23. http://dx.doi.org/10.1016/j.vaccine.2008.05.033.

3. Howie SR, Morris GA, Tokarz R, Ebruke BE, Machuka EM, Ideh RC, Chimah O, Secka O, Townsend J, Dione M, Oluwalana C, Njie M, Jallow M, Hill PC, Antonio M, Greenwood B, Briese T, Mulloolland K, Corrao T, Lipkin WI, Adegbole RA. 2014. Etiology of severe childhood pneumonia in The Gambia, West Africa, determined by conventional and molecular microbiological analyses of lung and aspirate samples. Clin Infect Dis 59:682–685. http://dx.doi.org/10.1093/cid/ciu384.

4. Livorsi DJ, Macneil JR, Cohn AC, Bareta J, Zansky S, Petit S, Gershman K, Harrison LJ, Lyndfeld R, Reingold A, Schaffner W, Thomas A, Coronado F, Zell ER, Mayer LW, Clark TA, Messonnier NE. 2011. Ten years of Hib vaccination in Italy: prevalence of non-encapsulated *Haemophilus influenzae* among invasive isolates and the possible impact on antibiotic resistance. Vaccine 29:3857–3862. http://dx.doi.org/10.1016/j.vaccine.2011.03.059.

5. Giufre M, Cardines R, Caporalli MG, Accogli M, D’Ancona F, Cerqueti M. 2011. Ten years of Hib vaccination in Italy: prevalence of non-encapsulated *Haemophilus influenzae* among invasive isolates and the possible impact on antibiotic resistance. Vaccine 29:3857–3862. http://dx.doi.org/10.1016/j.vaccine.2011.03.059.

6. MacNeil JR, Cohn AC, Farley M, Mair R, Baumbach J, Bennett N, Gershman K, Harrison LJ, Lyndfeld R, Petit S, Reingold A, Schaffner W, Thomas A, Coronado F, Zell ER, Mayer LW, Clark TA, Messonnier NE. 2011. Current epidemiology and trends in invasive *Haemophilus influenzae* disease: United States, 1989-2008. Clin Infect Dis 53:1230–1236. http://dx.doi.org/10.1093/cid/cit735.

7. Dworkin MS, Park L, Borchardt SM. 2007. The changing epidemiology of invasive *Haemophilus influenzae* disease, especially in persons ≥85 years old. Clin Infect Dis 44:810–816. http://dx.doi.org/10.1086/511861.

8. Auinger P, Lanphear BP, Kalkwarf HJ, Mansour ME. 2003. Trends in otitis media among children in the United States. Pediatrics 112:514–520. http://dx.doi.org/10.1542/peds.112.3.514.

9. Teele DW, Klein JD, Rosner B, The Greater Boston Otitis Media Study Group. 1989. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. J Infect Dis 160:83–94. http://dx.doi.org/10.1093/infdis/160.1.83.

10. Pelton SI, Pettigrew MM, Barenkamp SJ, Godfroid F, Grijalva CG, Leach AJ, Patel J, Murphy TF, Selak S, Bakaletz LO. 2013. Panel 6: vaccines. Otolaryngol Head Neck Surg 148:E90–E101. http://dx.doi.org/10.1177/0194599812466535.

11. Faden H. 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur J Pediatr 160:407–413. http://dx.doi.org/10.1007/s0043100100754.

12. Baldwin RL. 1993. Effects of otitis media on child development. Am J Otol 14:601–604.
going tympanostomy tube placement had multiple bacterial pathogens. BMC Pediatr 12:87. http://dx.doi.org/10.1186/1471-2431-12-87.

35. Lopez AD, Shibuya K, Rao C, Mathers CD, Hansell AL, Held LS, Schmid V, Buist S. 2006. Chronic obstructive pulmonary disease: current burden and future projections. Eur Respir J 27:397–412. http://dx.doi.org/10.1183/09031936.06.00025006.

36. Mannino DM, Buist AS. 2007. Global burden of COPD: risk factors, prevalence, and future trends. Lancet 370:765–773. http://dx.doi.org/10.1016/S0140-6736(07)61380-4.

37. Sethi S, Murphy TF. 2008. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. N Engl J Med 359:2355–2365. http://dx.doi.org/10.1056/NEJMc0800333.

38. Berenson CS, Murphy TF, Wrona CT, Sethi S. 2005. Outer membrane protein Ps of nontypeable Haemophilus influenzae is a potent and selective inducer of human macrophage proinflammatory cytokines. Infect Immun 73: 2728–2735. http://dx.doi.org/10.1128/IAI.73.5.2728-2735.2005.

39. Desai H, Eschberger K, Wrona C, Grove L, Agrawal A, Grant B, Yin J, Parameswaran GI, Murphy T, Sethi S. 2014. Bacterial colonization increases daily symptoms in patients with chronic obstructive pulmonary disease. Ann Am Thorac Soc 11:303–309. http://dx.doi.org/10.1513/AnnalsATS.201310-350OC.

40. Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, Huovinen P, Jacoby GA, Kishony R, Kreiswirth BN, Kutter E, Lerner SA, Levy S, Lewis K, Lomovskaya O, Miller JH, Mobashery S, Piddock LJ, Projan JS, van den Bergh MR, Veenhoven RH, Sanders EA. 2013. Effects of the 10-valent pneumococcal nontypable 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/cit392.

41. Novotny LA, Clements JD, Bakaleto LO. 2013. Kinetic analysis and evaluation of the mechanisms involved in the resolution of experimental nontypeable Haemophilus influenzae-induced otitis media after transcutaneous immunization. Vaccine 31:3417–3426. http://dx.doi.org/10.1016/j.vaccine.2012.10.033.

42. Novotny LA, Clements JD, Bakaleto LO. 2011. Transcutaneous immunization as preventative and therapeutic regimens to protect against experimental otitis media due to nontypeable Haemophilus influenzae. Musco Immunol 44:56–467. http://dx.doi.org/10.1016/j.ijporl.2009.11.006.

43. Figueira MA, Ram S, Goldenstein R, Hood DW, Moxon ER, Pelton SI. 2007. Role of complement in defense of the middle ear revealed by restoring the virulence of nontypeable Haemophilus influenzae siaB mutants. Infect Immun 75:325–333. http://dx.doi.org/10.1128/IAI.01354-06.

44. Bakale PO, Pelton SI, Liu Z. 2005. Experimental acute otitis media due to nontypeable Haemophilus influenzae: comparison of high and low azithromycin doses with placebo. Antimicrob Agents Chemother 49:2194–2199. http://dx.doi.org/10.1128/AAC.49.7.2194-2199.2002.

45. Armbuster CE, Hong W, Pang B, Weimer KE, Junea RA, Turner J, Swords WE. 2010. Indirect pathogenicity of Haemophilus influenzae and Moraxella catarrhalis in polymicrobial otitis media occurs via interspecies quorum sensing. mBio 1(3):e00102-10. http://dx.doi.org/10.1128/mBio.00102-10.

46. Hong W, Mason K, Jurcisek J, Novotny L, Bakaleto LO, Swords WE. 2007. Phosphorylcholine decreases early inflammation and promotes the establishment of stable biofilm communities of nontypeable Haemophilus influenzae strain 86-028NP in a chinchilla model of otitis media. Infect Immun 75:938–943. http://dx.doi.org/10.1128/IAI.01691-06.

47. Novotny LA, Jurcisek JA, Godfroid F, Poolman JT, Denedo PA, Bakaleto LO. 2006. Passive immunization with human anti-protein D antibodies induced by polysaccharide protein D conjugates protects chinchillas against otitis media after intranasal administration. Vaccine 24:8404–8411. http://dx.doi.org/10.1016/j.vaccine.2006.03.021.

48. Faden H, Bernstein J, Brodsky LS, Stanievich J, Krystofik D, Shuff C, Hersh H, Ogura PL. 1989. Otitis media in children. I. The systemic immune response to nontypeable Haemophilus influenzae. J Infect Dis 160:999–1004.

49. Shurin PA, Pelton SI, Tazer IB, Kasper DL. 1980. Bacterialid antibody and susceptibility to otitis media caused by nontypeable strains of Haemophilus influenzae. J Pediatr 97:364–369. http://dx.doi.org/10.1016/S0022-3476(80)80182-X.

50. Murphy TF. 2009. Current and future prospects for a vaccine for nontypeable Haemophilus influenzae. Curr Resp Dis Rep 11:177–182. http://dx.doi.org/10.1007/s11908-009-0027-1.

51. Shen K, Antalis P, Gladitz J, Sayeed S, Ahmed A, Yu S, Hayes J, Johnson S, Dice B, Dopico R, Keefe R, Janto B, Chong W, Goodwin J, Wadowsky RM, Erdos G, Post JC, Ehrlich GD, Hu FZ. 2005. Identification, distribution, and expression of novel genes in 10 clinical isolates of nontypeable Haemophilus influenzae. Infect Immun 73:3479–3491. http://dx.doi.org/10.1128/IAI.73.7.3479-3491.2005.

52. Hogg JS, Hu FZ, Janto B, Boissay R, Hayes J, Jekke R, Post JC, Ehrlich GD. 2007. 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:R103. http://dx.doi.org/10.1186/gb-2007-8-6-r103.

53. Erwin AL, Sandstedt SA, Bonthuis PJ, Geelhoed JL, Nelson KL, Unrath WC, Diggie MA, Theodore MJ, Pleatman CR, Mothershed EA, Sacchi CT, Mayer LW, Gilford JR, Smith AL. 2008. Analysis of genetic relatedness of Haemophilus influenzae isolates by multilocus sequence typing. J Bacteriol 190:1473–1483. http://dx.doi.org/10.1128/JB.01207-07.

54. Eutsey RA, Hiller NL, Earl JP, Janto BA, Dahlgren ME, Ahmed A,
Loosmore SM, Yang YP, Oomen R, Shortreed JM, Coleman DC, Klein MH. 1998. The Haemophilus influenzae HtrA protein is a protective antigen. Infect Immun 66:899–906.

Nemeth MF, Yi K, Karabatsos N, Murphy TF. 2001. Antibodies to loop 6 of the P2 porin protein of nontypeable Haemophilus influenzae are bactericidal against multiple strains. Infect Immun 69:773–778. http://dx.doi.org/10.1128/IAI.69.2.773-778.2001.

Forsgren A, Riesbeck K, Janson H. 2008. Protein D of Haemophilus influenzae: a protective nontypeable H. influenzae antigen and a carrier for pneumococcal conjugate vaccines. Clin Infect Dis 46:726–731. http://dx.doi.org/10.1086/527296.

Novotny LA, Juricsek JA, Pichichero ME, Bakaletz LO. 2000. Epitope mapping of the outer membrane protein P5-homologous fimbrin adhesin of nontypeable Haemophilus influenzae. Infect Immun 68:2119–2128. http://dx.doi.org/10.1128/IAI.68.4.2119-2128.2000.

Kyd JM, Cripps AW, Novotny LA, Bakaletz LO. 2003. Efficacy of the 26-kilodalton outer membrane protein and two P5 fimbrin-derived immunogens to induce clearance of nontypeable Haemophilus influenzae from the rat middle ear and lungs as well as from the chinchilla middle ear and nasopharynx. Infect Immun 71:4691–4699. http://dx.doi.org/10.1128/IAI.71.8.4691-4699.2003.

Bakaletz LO. 2001. Peptide and recombinant antigens for protection against bacterial middle ear infection. Vaccine 19:2323–2328. http://dx.doi.org/10.1016/S0264-410X(00)00522-3.

Hotomi M, Ikeda Y, Suzumo M, Yamauchi K, Green BA, Zlotnick GW, Billal DS, Shimada J, Fujihara K, Yamanaka N. 2005. A recombinant P4 protein of Haemophilus influenzae induces specific immune responses biologically active against nasopharyngeal colonization in mice after intranasal immunization. Vaccine 23:1294–1300. http://dx.doi.org/10.1016/j.vaccine.2004.08.042.

Green BA, Vasquez ME, Zlotnick GW, Quigley-Reape G, Swarts JD, Green I, Cowell JL, Bluestone CD, Doyle WJ. 1993. Evaluation of mixtures of purified Haemophilus influenzae outer membrane proteins in protection against challenge with nontypeable H. influenzae in the chinchilla otitis media model. Infect Immun 61:1950–1957.

Green BA, Farley JE, Quinn-Dey T, Deich RA, Zlotnick GW, Green I, Cowell JL, Bluestone CD, Doyle WJ. 1993. High-molecular-weight outer membrane proteins of nontypeable Haemophilus influenzae: biologic activity of anti- e-serum and cloning and sequencing of the structural gene. Infect Immun 59:3191–3198.

Jalalvand F, Su YC, Morgenlin M, Brant M, Hallgren O, Westergren-Thorsson G, Singh B, Riesbeck K. 2013. Haemophilus influenzae protein F mediates binding to laminin and human pulmonary epithelial cells. J Infect Dis 207:803–813. http://dx.doi.org/10.1093/infdis/jis754.

Jalalvand F, Littorin N, Su YC, Riesbeck K. 2014. Impact of immunization with protein F on pulmonary clearance of nontypeable Haemophilius influenzae. Vaccine 32:2261–2264. http://dx.doi.org/10.1016/j.vaccine.2014.02.082.

Su YC, Jalalvand F, Morgenlin M, Blom AM, Singh B, Riesbeck K. 2013. Haemophilus influenzae acquires vir trotetraconin via the ubiquitous protein F to subvert host innate immunity. Mol Microbiol 87:1245–1266. http://dx.doi.org/10.1111/mmi.12164.

Kyd JM, Cripps AW. 1998. Potential of a novel protein, OMP26, from nontypeable Haemophilus influenzae to enhance pulmonary clearance in a rat model. Infect Immun 66:2272–2278.

Riedmann EM, Lubitz W, McGrath J, Kyd JM, Cripps AW. 2011. Effectiveness of engineering the nontypeable Haemophilus influenzae antigen OMP 26 as an S-layer fusion in bacterial ghosts as a mucosal vaccine delivery. Hum Vaccin 7(Suppl):99–107. http://dx.doi.org/10.4161/hv.7.0.14569.

Riedmann EM, Kyd JM, Smith AM, Gomez-Gallego S, Jalava K, Cripps AW, Lubitz W. 2003. Construction of recombinant S-layer protein (sRsba) and their expression in bacterial ghosts: a delivery system for the nontypeable Haemophilus influenzae antigen OMP 26. FEMS Immunol Med Microbiol 37:185–192. http://dx.doi.org/10.1016/S0928-441X(03)00067-0.

Green BA, Metcalf BJ, Quinn-Dey T, Kirkle DH, Quataert SA, Deich RA. 1990. A recombinant non-fatty acylated form of the Hi-PAL (P6) protein of Haemophilus influenzae elicits biologically active antibody against both nontypeable and type b H. influenzae. Infect Immun 58:3272–3278.

Hotomi M, Yamanaka N, Shimada J, Suzumo M, Ikeda Y, Sakai A,
Arai J, Green B. 2002. Intranasal immunization with recombinant outer membrane protein P6 induces specific immune responses against non-typeable *Haemophilus influenzae*. Int J Pediatr Otorhinolaryngol 65: 109–116. http://dx.doi.org/10.1016/S0165-5876(02)00076-9.

Noda K, Kodama S, Umemoto S, Abe N, Hirano T, Suzuki M. 2010. Nasal vaccination with P6 outer membrane protein and alpha-galactosylceramide induces non-typeable *Haemophilus influenzae*–specific protective immunity associated with NKT cell activation and dendritic cell expansion in nasopharynx. Vaccine 28:5068–5074. http://dx.doi.org/10.1016/j.vaccine.2010.05.005.

Kodama S, Hirano T, Noda K, Abe N, Suzuki M. 2009. Identification of a 16,600-dalton outer membrane protein on nontypeable *Haemophilus influenzae* as a target for human serum bactericidal antibody. J Immunol 182:2020–2027. http://dx.doi.org/10.1016/j.vaccine.2010.05.005.

Abe N, Kodama S, Hirano T, Eto M, Suzuki M. 2006. Nasal vaccination with CpG oligodeoxynucleotide induces protective immunity against non-typeable *Haemophilus influenzae* in the nasopharynx. Laryngoscope 116:407–412. http://dx.doi.org/10.1097/01.mlg.0000199740.04730.d4.

Murphy TF, Bartos LC, Rice PA, Nelson MB, Dudas KC, Apicella MA. 1986. Identification of a 16,600-dalton outer membrane protein of nontypeable *Haemophilus influenzae* as a target for human serum bactericidal antibody. J Clin Invest 78:1020–1027. http://dx.doi.org/10.1172/JCI112656.

Abe Y, Murphy TF, Sethi S, Faden HS, Dmochowski J, Harabuchi Y, Thanavala YM. 2002. Lymphocyte proliferative response to P6 of *Haemophilus influenzae* is associated with relative protection from exacerbations of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 165:967–971. http://dx.doi.org/10.1164/ajrccm.165.2.2109009.

Ronander E, Brant M, Janson H, Sheldon J, Forsgren A, Riesbeck K. 2008. Identification of a novel *Haemophilus influenzae* protein important for adhesion to epithelial cells. Microbes Infect 10:87–96. http://dx.doi.org/10.1016/j.micinf.2007.10.006.

Barthel D, Singh B, Riesbeck K, Zipfel PF. 2012. *Haemophilus influenzae* uses the surface protein E to acquire human plasminogen and to evade innate immunity. J Immunol 188:379–385. http://dx.doi.org/10.4049/jimmunol.1101927.

Singh B, Jalalvand F, Morgelin M, Zipfel P, Blom AM, Riesbeck K. 2011. *Haemophilus influenzae* protein E recognizes the C-terminal domain of vitronectin and modulates the membrane attack complex. Mol Microbiol 81:80–98. http://dx.doi.org/10.1111/j.1365-2958.2011.07678.x.

Hallstrom T, Singh B, Resman F, Blom AM, Morgelin M, Riesbeck K. 2011. *Haemophilus influenzae* protein E binds to the extracellular matrix by concurrently interacting with laminin and vitronectin. J Infect Dis 204:1065–1074. http://dx.doi.org/10.1093/infdis/jir459.

Bakaletz LO, Baker BD, Jurcisek JA, Harrison A, Novotny LA, Bookwalter JE, Mungru R, Munson RS, Jr. 2005. Demonstration of type IV pilus expression and a twitching phenotype by *Haemophilus influenzae*. Infect Immun 73:1635–1643. http://dx.doi.org/10.1128/IAI.73.3.1635–1643.2005.

Jurcisek JA, Bookwalter JE, Baker BD, Fernandez S, Novotny LA, Munson RS, Jr, Bakaletz LO. 2007. The PilA protein of non-typeable *Haemophilus influenzae* plays a role in biofilm formation, adherence to epithelial cells and colonization of the mammalian upper respiratory tract. Mol Microbiol 65:1288–1299. http://dx.doi.org/10.1111/j.1365-2958.2007.05864.x.

Novotny LA, Adams LD, Kang DR, Wiet GJ, Cai X, Sethi S, Murphy TF, Bakaletz LO. 2009. Epitope mapping immunodominant regions of the PilA protein of non-typeable *Haemophilus influenzae* (NTHI) to facilitate the design of two novel chimeric vaccine candidates. Vaccine 28:279–289. http://dx.doi.org/10.1016/j.vaccine.2009.08.017.

Sun J, Chen J, Cheng Z, Robbins JB, Battey JF, Gu XX. 2000. Biological activities of antibodies elicited by lipoooligosaccharide-based-conjugate vaccines of nontypeable *Haemophilus influenzae* in an otitis media model. Vaccine 18:1264–1272. http://dx.doi.org/10.1016/S0264-410X(99)00381-3.

Gu X-X, Tsai C-M, Ueyama T, Barenkamp SJ, Robbins JB, Lim DJ. 1996. Synthesis, characterization, and immunologic properties of detoxified lipoooligosaccharide from nontypeable *Haemophilus influenzae* conjugated to proteins. Infect Immun 64:4047–4053.

Hirano T, Hou Y, Jiao X, Gu XX. 2003. Intranasal immunization with a lipoooligosaccharide-based conjugate vaccine from nontypeable *Haemophilus influenzae* enhances bacterial clearance in mouse nasopharynx. FEMS Immunol Med Microbiol 35:1–10. http://dx.doi.org/10.1111/j.1574-695X.2003.tb00642.x.

Gu XX, Rudy SF, Chu C, McCullagh L, Kim HN, Chen J, Li J, Robbins JB, Van Waes C, Battey JF. 2003. Phase I study of a lipoooligosaccharide-based conjugate vaccine against nontypeable *Haemophilus influenzae*. Vaccine 21:2107–2114. http://dx.doi.org/10.1016/S0264-410X(02)00708-5.