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COMMENTARY

# Why we need a vaccine for non-typeable *Haemophilus influenzae*

Marina Cerquetti and Maria Giufrè

Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy

**ABSTRACT**

Nontypeable *Haemophilus influenzae* (NTHi) is increasingly recognized as emerging pathogen. The routine immunization of infants with conjugated vaccines against *H. influenzae* type b (Hib) has greatly reduced the incidence of invasive Hib disease; however a marked change in the predominant invasive serotype from Hib to NTHi has occurred. Localized infections where the role of *H. influenzae* is important, such as otitis media in children and acute exacerbations in chronic obstructive pulmonary disease (COPD) in adults, are almost exclusively associated with NTHi isolates. The implementation of pneumococcal conjugate vaccines has resulted in changes in frequency of nasopharynx colonizing pathogens with an increase of NTHi, although this data is yet under debate.

An effective vaccine against NTHi is not currently available. The major challenge in developing a successful vaccine is the intrinsic heterogeneity of NTHi. *H. influenzae* protein D is used as carrier protein in the licensed 10-valent pneumococcal conjugate vaccine (Synflorix, GlaxoSmithKline), but no robust evidences for protective efficacy against NTHi otitis have been until now obtained. Several other vaccine candidates are under investigations and we hope that significant advancements in vaccine development will be achieved in the next future. Genome-based vaccine strategy might provide an additional useful tool for discovering further vaccine antigens.

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*Haemophilus influenzae* commonly colonizes the upper respiratory tract of both children and adults.[1,2] However, it can act as a pathogen able to cause a wide spectrum of diseases ranging from respiratory tract infections to severe invasive disease, such as meningitis, sepsis, bacteraemic pneumonia and epiglottitis.[3] The species *H. influenzae* comprises capsulated strains (6 capsular types from type a through type f) and non-capsulated strains commonly referred to as non-typeable (non reactive with typing antisera).[4]

Prior to the introduction of vaccination against *H. influenzae* type b (Hib), this capsular type was the leading cause of bacterial meningitis in infants and young children worldwide.[5] Routine immunization of infants with Hib conjugated vaccines has dramatically decreased the incidence of invasive Hib disease in industrialized countries.[6] However, despite the efficacy of Hib conjugate vaccines, invasive disease caused by non-vaccine preventable *H. influenzae* strains continues to occur, although with reduced incidence, mainly sustained by non-typeable *H. influenzae* (NTHi) followed at great distance by other capsular types.[7,8,9]

Considering non-invasive disease due to *H. influenzae*, the upper and lower respiratory tract infections have been associated almost exclusively with NTHi, which is now the most frequently isolated bacterial pathogens in otitis media and sinusitis in children.[2,10] An increasing role for NTHi has been recently documented in episodes of acute exacerbation in adults with chronic obstructive pulmonary disease (COPD).[11,12] NTHi colonization/infection is also quite common in young children with cystic fibrosis, contributing to the disease morbidity.[13,14]

On the basis of the above reasoning, NTHi has been increasingly recognized as emerging pathogen.[9,15,16] An effective vaccine against NTHi is not currently available. The pathogenesis of NTHi, which lacks the polysaccharide capsule, is associated with multiple virulence factors such as lipooligosaccharide (LOS), adhesins, other several surface structures, IgA protease and possibly biofilm formation in chronic and recurrent respiratory tract infections.[17,18,19] No single genotypic or phenotypic trait is characteristic of all strains from disease. The hallmark of NTHi is "heterogeneity" and so far this heterogeneity has been the major obstacle for developing a successful vaccine.

## Impact of the conjugate vaccines against both Hib and *Streptococcus pneumoniae* on carriage of NTHi in children

Colonization of the upper respiratory tract is a prerequisite for developing NTHi disease. Widespread Hib vaccination has decreased or eliminated Hib carriage in both vaccinated and unvaccinated children due to the herd effect.[20] In the present post-Hib vaccination era, NTHi strongly predominates among *H. influenzae* isolates in the oropharynx and nasopharynx of healthy children with carriage rates varying from 10% to more than 60%, depending on the children age.[21,22,23,24] Along with *H. influenzae*, S. *pneumoniae* colonizes the upper respiratory tracts of children and is responsible for mild or severe diseases.

**CONTACT** Marina Cerquetti marina.cerquetti@iss.it Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy.

During the past decade, the pneumococcal conjugate vaccines (PCV) have been introduced and several reports have demonstrated the shift toward non-vaccine pneumococcal serotypes in both carriage and disease. Whether the implementation of the pneumococcal vaccination has influenced the colonization dynamic of *H. influenzae* and *S. pneumoniae* in the nasopharynx resulting in an increase of NTHi is a controversial issue. Some studies reported a significant rise in NTHi carriage associated with pneumococcal vaccination, but other investigations did not observe such rise.[24,25,26,27] Contrasting results may be related to several factors including differences in time intervals between the introduction of pneumococcal vaccination and performing the study in different settings, the size of the samples comprising unvaccinated children at the time of the study, and so on.[24,25,26,27] Best agreement is observed with respect to the impact of pneumococcal vaccination on NTHi as a pathogen causing otitis media: the presence of NTHi has been found significantly increased in middle ear fluid of children during episodes of acute otitis media after PCV-7 implementation.[28,29] Certainly, the impact of the new conjugate vaccines on the dynamic of colonization by pathogens sharing the same ecological niche is a moving complex picture that has yet to be clearly defined, but as far NTHi seems to take advantage.

## Invasive *H. influenzae* disease in the era of Hib conjugate vaccines

The epidemiology of the invasive *H. influenzae* disease has changed substantially as result of the widespread immunization of infants with Hib conjugate vaccines. Prior to the introduction of Hib vaccines, NTHi strains were a minor cause of invasive disease, especially affecting adults. Nowadays, the predominant invasive serotype has been changed from Hib to NTHi.[7,8,10,30] Invasive NTHi disease occur across all age groups and account for 77% of all notified invasive *H. influenzae* cases, in Europe.[31] Considering clinical presentations, NTHi account for the majority of cases of septicemia (81.1%), meningitis (61.7%) and septicemic pneumonia (82.2%).[31] Notably, NTHi disease has the highest serotype-specific case fatality rate (CFR, 12%) compared to other capsular types.[31] No clear evidence of serotype replacement has been reported in most studies, although some investigations supported this phenomenon.[8,32,33,34] According to the European Center for Disease Prevention and Control (ECDC) annual epidemiological report 2014, although there was an upward trend in invasive NTHi isolates, data was too scarce to draw firm conclusions on serotype replacement.[35] Recently, neonatal invasive NTHi infections have captured the attention of the researches in the field. In particular, early-onset neonatal NTHi disease (within 48 h of birth), often presenting with septicemia, has been found strongly associated with premature birth.[36]

Actually, the disease should be considered as a mother-infant infection, since it can affect the pregnant woman, the fetus and the neonate. According to Collins et al.,[36] the overall incidence of early-onset neonatal invasive disease was 4.1/100,000 in England and Wales, but the incidence increased exponentially with prematurity reaching the value of 342/100,000 in infants born at < 28 weeks gestation.[36] Further epidemiological data are needed to investigate the burden of neonatal NTHi disease in other countries. The need for an effective preventive strategy before or during pregnancy has been underlined by Collins et al.[36] Identification of specific microbial traits associated with neonatal disease is required for creating a targeted vaccine. A "cryptic genospecies" of *H. influenzae* (also referred to as "*H. quentini*") has been supposed to be involved in urogenital, neonatal and mother-infant infections.[37] However, recent reports demonstrated that several different clones of NTHi were responsible for most neonatal invasive infections.[38] Therefore no common shared genotype was associated with perinatal infections and the development of a possible targeted vaccine will face the problem of strain heterogeneity.

## Non-invasive *H. influenzae* infections

Both upper and lower respiratory tract infections represents a huge burden of disease and public health problem. We will only mention the major respiratory diseases where the role of *H. influenzae* is important: acute otitis media (AOM), COPD and cystic fibrosis (CF).

AOM is the most common bacterial infection in childhood. Several studies have shown that the *S. pneumoniae*, NTHi (virtually all *H. influenzae* isolates from AOM are nontypeable) and *Moraxella catarrhalis* are the most frequent pathogens found in the middle ear fluid of children with AOM, with NTHi causing 25–35% of the AOM episodes.[2,10] The role of NTHi seems to be relevant especially in recurrent otitis media where NTHi is the most common pathogen.[2] Furthermore, the quite high prevalence of ampicillin resistant NTHi isolates (both $\beta$-lactamase producers and non-$\beta$-lactamase producers) might complicate treatments and management of AOM infection.[16] As above-mentioned, the implementation of pneumococcal conjugate vaccines has resulted in a predominance of NTHi in middle ear fluids of children suffering for AOM (not only in recurrent AOM) in comparison with that detected before 7-valent-PCV introduction when *S. pneumoniae* predominantly grew.[28,29]

COPD is characterized by airway inflammation and irreversible airflow obstruction. Patients with COPD suffer for episodes of acute exacerbations that contribute to the progression of the disease. Currently, the role that bacteria play in pathogenesis of COPD has gained importance and acute exacerbations have been associated with the acquisition of a new strain of NTHi and/or other pathogens, such as *S. pneumoniae* and *Moraxella catarrhalis*, the so called "exacerbation isolates."[12,39] Of note, the differential expression of specific IgA proteases by NTHi has recently been suggested to influence the propensity of strains to cause exacerbations and to persist in respiratory epithelial cells in COPD.[40]

Several pathogens contribute to morbidity in patients with CF but the prevalence of the different species changes with the age of patients.[13] NTHi colonizes/infects the respiratory tract of more than 20% CF patients early in childhood (with or without *Staphylococcus aureus*), initiating the inflammatory process distinctive of the disease.[13,14] Colonization with multiple NTHi strains is common, however persistence of the same strain for a period ranging from 1 month to 24 months has been observed in about one third of patients.[14] It has been suggested that

differential capability to form biofilms may contribute to strain persistence.[19]

## State of the art of vaccine development for NTHi

The development of an effective vaccine against NTHi to be used for immunization of population groups at risk of NTHi infection would be valuable. A vaccine capable of preventing various NTHi infections such as neonatal invasive disease, AOM in infants and children and episodes of acute exacerbation in adults with COPD should be the ultimate goal. However, this purpose seems difficult to achieve at present. Since the "heterogeneity" of NTHi isolates, even recovered from the same infection site, the identification of immunogenic antigens according to the classical procedures (antigens conserved among all isolates and capable to induce production of protective antibodies against both invasive and non-invasive NTHi disease) is far from becoming reality. A number of promising vaccine candidates including major and minor outer-membrane proteins and lipooligosaccharide have been reported in the literature, but none has found application in a vaccine currently available, with the only exception of *H. influenzae* protein D.[41] *H. influenzae* protein D is a highly conserved lipoprotein that is exposed on the *H. influenzae* cell surface and plays a role in pathogenesis of NTHI infection.[42] It is included in the currently available 10-valent pneumococcal conjugate vaccine (Synflorix, GlaxoSmithKline) as the carrier protein for pneumococcal capsular polysaccharides. The vaccine is indicated for active immunization against invasive disease, pneumonia and otitis media caused by *S. pneumoniae* in infants and children. Partial protection (35.3%) against otitis due to NTHi was observed in an early efficacy trail (the Pneumococcal Otitis Efficacy Trial, POET) with a 11-valent pneumococcal protein D-conjugate vaccine.[43] However, no robust evidence supporting the indication for protective efficacy against NTHi otitis was obtained for the licensed 10-valent pneumococcal protein D-conjugate vaccine and further studies are in progress.[44]

Another approach is to develop a vaccine formulation including multiple NTHi adhesins capable to prevent nasopharyngeal colonization. This might be a winning strategy for the prevention of NTHi diseases, also considering that NTHi carriage has increased following implementation of pneumococcal conjugate vaccines. Several adherence factors that are immunogenic have been considered as potential vaccine candidates including Hap, HMW1 and HMW2 and Hia proteins and P5 fimbrin.[17] An updated list of NTHi candidate vaccine antigens including adhesins has been recently reported by Murphy.[41] Some adhesins, such as HMW1/HMW2 proteins (the major adhesins of NTHi), after being considered as vaccine components have been partially discarded because they have a phase-variable expression. However, a recent study demonstrated that Hia adhesin (nearly expressed by all NTHi strains lacking HMW adhesins) is also subject to reversible switching of gene expression and that selection of Hia expression is a crucial factor in NTHi initial colonization of the nasopharynx, suggesting that the position of phase-variable adhesins among vaccine components might be re-evaluated.[45] Interestingly, a new model of human nasopharyngeal colonization with NTHi has been recently described, providing researchers with a system for evaluating both new vaccine candidates and vaccine formulations.[46]

Whole-genome sequencing has been successfully applied in the field of vaccinology for designing protein-based vaccines such as the multicomponent vaccine against meningococcal capsular group B.[47] Recently, several authors investigated the genome-based population structure of NTHi providing an in-deep characterization of NTHi diversity.[48,49] Whether the availability of genomic data in combination with bioinformatics tools could help in the identification of new conserved protective proteins is a question that requires further studies to be addressed. In a recent study, synthetic peptides corresponding to predicted high conserved surface exposed regions of NTHi previously identified by genomic analysis were used to rise antisera that conferred passive protection in a rat model of NTHi bacteremia, providing proof of principle that peptides might be components of a vaccine against NTHi.[50]

Finally, we would like to spend a few words about the development of a vaccine for preventing mother-infant invasive NTHi infection to be used during pregnancy. As above mentioned, no strain features associated with disease have been recognized until now and significant scientific efforts should be directed toward identifying potential candidate vaccine antigens. Moreover, since the recipients of the vaccine are pregnant women, additional safety studies are required.

## Conclusions

In industrialized countries, the impact of the routine use of Hib conjugate vaccines together with the implementation of the pneumococcal conjugate vaccines have resulted in epidemiological changes in both colonization and disease by *H. influenzae*. NTHi has become an emerging pathogen. The rationale for a vaccine against NTHi is based on firm foundations. Since NTHi infections require a significant burden of antimicrobial use, an additional value of effective NTHi vaccines is to reduce antimicrobial use that can result in reducing global antimicrobial resistance. In the process of research and development of effective NTHi vaccines, the challenge is the intrinsic "heterogeneity" of the microorganisms. In recent years, several efforts have been made to find potential conserved NTHi antigens and several candidates are currently under investigations. We hope that significant progress in the development, production and clinical testing of effective vaccines against NTHi will be achieved in the next future.

## Abbreviations

| | |
|---|---|
| AOM | acute otitis media |
| CF | cystic fibrosis |
| COPD | chronic obstructive pulmonary disease |
| Hib | *H. influenzae* type b |
| NTHi | Nontypeable *Haemophilus influenzae* |
| PCV | pneumococcal conjugate vaccines. |

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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## References

[1] Lemon KP, Klepac-Ceraj V, Schiffer HK, Brodie EL, Lynch SV, Kolter R. Comparative analyses of the bacterial microbiota of the human nostril and oropharynx. MBio 2010; 22:1. pii:e00129-10; PMID:20802827.

[2] Murphy TF, Faden H, Bakaletz LO, Kyd JM, Forsgren A, Campos J, Virji M, Pelton SI. Nontypable *Haemophilus influenzae* as a pathogen in children. Pediatr Infect Dis J 2009; 28:43-8; PMID:19057458; http://dx.doi.org/10.1097/INF.0b013e318184dba2

[3] Turk DC. The pathogenicity of *Haemophilus influenzae*. J Med Microbiol 1984; 18:1-16; PMID:6146721; http://dx.doi.org/10.1099/00222615-18-1-1

[4] Pittman M. Variation and type specificity in the bacterial species *Haemophilus influenzae*. J Exp Med 1931; 53:471; PMID:19869858; http://dx.doi.org/10.1084/jem.53.4.471

[5] Wenger JD, Hightower AW, Facklam RR, Gaventa S, Broome CV. Bacterial meningitis in the United States, 1986: report of a multistate surveillance study. J Infect Dis 1990; 126:1316-23; PMID:2230261; http://dx.doi.org/10.1093/infdis/162.6.1316

[6] Peltola M. Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. Clin Microbiol Rev 2000; 13:302-17; PMID:10756001; http://dx.doi.org/10.1128/CMR.13.2.302-317.2000

[7] Ladhani S, Slack MP, Heath PT, von Gottberg A, Chandra M, Ramsay ME, European Union Invasive Bacterial Infection Surveillance participants. Invasive *Haemophilus influenzae* disease, Europe, 1996-2006. Emerg Infect Dis 2010; 16:455-63; PMID:20202421; http://dx.doi.org/10.3201/eid1603.090290

[8] Adam HJ, Richardson SE, Jamieson FB, Rawte P, Low DE, Fisman DN. Changing epidemiology of invasive *Haemophilus influenzae* in Ontario, Canada: Evidence for herd effects and strain replacement due to Hib vaccination. Vaccine 2010; 28:4073-8; PMID:20398617; http://dx.doi.org/10.1016/j.vaccine.2010.03.075

[9] Langereis JD, de Jonge MI. Invasive disease caused by nontypeable *Haemophilus influenzae*. Emerg Infect Dis 2015; 21:1711-8; PMID:26407156; http://dx.doi.org/10.3201/eid2110.150004

[10] Agrawal A, Murphy TF. *Haemophilus influenzae* infections in the *H. influenzae* type b conjugate vaccine era. J Clin Microbiol 2011; 49:3728-32; PMID:21900515; http://dx.doi.org/10.1128/JCM.05476-11

[11] Simpson JL, Baines KJ, Horvat JC, Essilfie AT, Brown AC, Tooze M, McDonald VM, Gibson PG, Hansbro PM. COPD is characterized by increased detection of *Haemophilus influenzae, Streptococcus pneumoniae* and a deficiency of Bacillus species. Respirology 2016; 21: 697-704; PMID:26781464; http://dx.doi.org/10.1111/resp.12734

[12] Chin CL, Manzel LJ, Lehman EE, Humlicek AL, Shi L, Starner TD, Denning GM, Murphy TF, Sethi S, Look DC. *Haemophilus influenzae* from patients with chronic obstructive pulmonary disease exacerbation induce more inflammation than colonizers. Am J Respir Crit Care Med 2005; 172:85-91; PMID:15805181; http://dx.doi.org/10.1164/rccm.200412-1687OC

[13] Harrison F. Microbial ecology of the cystic fibrosis lung. Microbiology 2007; 153:917-23; PMID:17379702; http://dx.doi.org/10.1099/mic.0.2006/004077-0

[14] Cardines R, Giufrè M, Pompilio A, Fiscarelli E, Ricciotti G, Di Bonaventura G, Cerquetti M. *Haemophilus influenzae* in children with cystic fibrosis: Antimicrobial susceptibility, molecular epidemiology, distribution of adhesins and biofilm formation. Int J Med Microbiol 2012; 302:45-52; PMID:22001303; http://dx.doi.org/10.1016/j.ijmm.2011.08.003

[15] Van Eldere J, Slack MP, Ladhani S, Cripps AW. Non-typeable *Haemophilus influenzae*, an under-recognised pathogen. Lancet Infect Dis 2014; 14:1281-92; PMID:25012226; http://dx.doi.org/10.1016/S1473-3099(14)70734-0

[16] Gilsdorf JR. What the pediatrician should know about non-typeable *Haemophilus influenzae*. J Infect 2015; 71:S10-4; PMID:25917803; http://dx.doi.org/10.1016/j.jinf.2015.04.014

[17] Rao VK, Krasan GP, Hendrixson DR, Dawid S, St Geme JW 3rd. Molecular determinants of the pathogenesis of disease due to non-typable *Haemophilus influenzae*. FEMS Microbiol Rev 1999; 23:99-129; PMID:10234841; http://dx.doi.org/10.1111/j.1574-6976.1999.tb00393.x

[18] Erwin AL, Smith AL. Nontypeable *Haemophilus influenzae*: understanding virulence and commensal behavior. Trends Microbiol 2007; 15:355-62; PMID:17600718; http://dx.doi.org/10.1016/j.tim.2007.06.004

[19] Swords WE. Nontypeable *Haemophilus influenzae* biofilms: role in chronic airway infections. Front Cell Infect Microbiol 2012; 2:97. doi: 10.3389/fcimb.2012.00097; PMID:22919686.

[20] Mohle-Boetani JC, Ajello G, Breneman E, Deaver KA, Harvey C, Plikaytis BD, Farley MM, Stephens DS, Wenger JD. Carriage of *Haemophilus influenzae* type b in children after widespread vaccination with conjugate *Haemophilus influenzae* type b vaccines. Pediatr Infect Dis J 1993; 12:589-93; PMID:8346003; http://dx.doi.org/10.1097/00006454-199307000-00009

[21] Puig C, Marti S, Fleites A, Trabazo R, Calatayud L, Liñares J, Ardanuy C. Oropharyngeal colonization by nontypeable *Haemophilus influenzae* among healthy children attending day care centers. Microb Drug Resist 2014; 20:450-5; PMID:24716536; http://dx.doi.org/10.1089/mdr.2013.0186

[22] Oikawa J, Ishiwada N, Takahashi Y, Hishiki H, Nagasawa K, Takahashi S, Watanabe M, Chang B, Kohno Y. Changes in nasopharyngeal carriage of *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* among healthy children attending a day-care centre before and after official financial support for the 7-valent pneumococcal conjugate vaccine and *H. influenzae* type b vaccine in Japan. J Infect Chemother 2014; 20:146-9; PMID:24582389; http://dx.doi.org/10.1016/j.jiac.2013.10.007

[23] Giufrè M, Daprai L, Cardines R, Bernaschi P, Ravà L, Accogli M, Raponi M, Garlaschi ML, Ciofi degli Atti ML, Cerquetti M. Carriage of *Haemophilus influenzae* in the oropharynx of young children and molecular epidemiology of the isolates after fifteen years of *H. influenzae* type b vaccination in Italy. Vaccine 2015; 33:6227-34; PMID: Can't; http://dx.doi.org/10.1016/j.vaccine.2015.09.082

[24] Bosch AA, van Houten MA, Bruin JP, Wijmenga-Monsuur AJ, Trzciński K, Bogaert D, Rots NY, Sanders EA. Nasopharyngeal carriage of *Streptococcus pneumoniae* and other bacteria in the 7th year after implementation of the pneumococcal conjugate vaccine in the Netherlands. Vaccine 2016; 34:531-9; PMID:26667610; http://dx.doi.org/10.1016/j.vaccine.2015.11.060

[25] Camilli R, Vescio MF, Giufrè M, Daprai L, Garlaschi ML, Cerquetti M, Pantosti A. Carriage of *Haemophilus influenzae* is associated with pneumococcal vaccination in Italian children. Vaccine 2015; 33:4559-64; PMID:26190092; http://dx.doi.org/10.1016/j.vaccine.2015.07.009

[26] van Gils EJ, Veenhoven RH, Rodenburg GD, Hak E, Sanders EA. Effect of 7-valent pneumococcal conjugate vaccine on nasopharyngeal carriage with *Haemophilus influenzae* and *Moraxella catarrhalis* in a randomized controlled trial. Vaccine 2011; 29:7595-8; PMID:21893151; http://dx.doi.org/10.1016/j.vaccine.2011.08.049

[27] Spijkerman J, Prevaes SM, van Gils EJ, Veenhoven RH, Bruin JP, Bogaert D, Wijmenga-Monsuur AJ, van den Dobbelsteen GP, Sanders EA. Long-term effects of pneumococcal conjugate vaccine on nasopharyngeal carriage of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis*. PloS one 2012; 7(6):e39730; PMID:22761879; http://dx.doi.org/10.1371/journal.pone.0039730

[28] Casey JR, Pichichero ME. Changes in frequency and pathogens causing acute otitis media in 1995–2003. Pediatr Infect Dis J 2004; 23:824-28; PMID:15361720; http://dx.doi.org/10.1097/01.inf.0000136871.51792.19

[29] Wiertsema SP, Kirkham LA, Corscadden KJ, Mowe EN, Bowman JM, Jacoby P, Francis R, Vijayasekaran S, Coates HL, Riley TV, et al. Predominance of nontypeable *Haemophilus influenzae* in children with otitis media following introduction of a 3 + 0 pneumococcal conjugate vaccine schedule. Vaccine 2011; 29: 5163-70; PMID:21621576; http://dx.doi.org/10.1016/j.vaccine.2011.05.035

[30] Giufrè M, Cardines R, Caporali MG, Accogli M, D'Ancona F, Cerquetti M. Ten years of Hib vaccination in Italy: prevalence of non-

encapsulated *Haemophilus influenzae* among invasive isolates and the possible impact on antibiotic resistance. Vaccine 2011; 29:3857-62; http://dx.doi.org/10.1016/j.vaccine.2011.03.059

[31] European Centre for Disease Prevention and Control. Surveillance of invasive bacterial diseases in Europe, 2012 Stockholm: ECDC; 2015.

[32] MacNeil JR, Cohn AC, Farley M, Mair R, Baumbach J, Bennett N, Gershman K, Harrison LH, Lynfield R, Petit S et al. Current epidemiology and trends in invasive *Haemophilus influenzae* disease-United States, 1989-2008. Clin Infect Dis 2011; 53:1230-6; PMID:22080119; http://dx.doi.org/10.1093/cid/cir735

[33] Dworkin MS, Park L, Borchardt SM. The changing epidemiology of invasive *Haemophilus influenzae* disease, especially in persons ≥65 years old. Clin Infect Dis 2007; 44:810-6; PMID:17304452; http://dx.doi.org/10.1086/511861

[34] Berndsen MR, Erlendsdóttir H, Gottfredsson M. Evolving epidemiology of invasive Haemophilus infections in the post-vaccination era: results from a long-term population-based study. Clin Microbiol Infect 2012; 18:918-23; PMID:22070637; http://dx.doi.org/10.1111/j.1469-0691.2011.03700.x

[35] European Centre for Disease Prevention and Control. Annual epidemiological report 2014 – Vaccine-preventable diseases – invasive bacterial diseases Stockholm: ECDC; 2015.

[36] Collins S, Litt DJ, Flynn S, Ramsay ME, Slack MP, Ladhani SN. Neonatal Invasive *Haemophilus influenzae* Disease in England and Wales: Epidemiology, Clinical Characteristics, and Outcome. Clin Infect Dis 2015; 60:1786-92; PMID:25784720; http://dx.doi.org/10.1093/cid/civ194

[37] Quentin R, Martin C, Musser JM, Pasquier-Picard N, Goudeau A. Genetic characterization of a cryptic genospecies of *Haemophilus* causing urogenital and neonatal infections. J Clin Microbiol 1993; 31:1111-6; PMID:8099082.

[38] Giufrè M, Cardines R, Accogli M, Cerquetti M. Neonatal Invasive *Haemophilus influenzae* Disease and Genotypic Characterization of the Associated Strains in Italy. Clin Infect Dis 2015; 61:1203-4; PMID:26123935; http://dx.doi.org/10.1093/cid/civ516

[39] Sethi S, Evans N, Grant BJ, Murphy TF. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. N Engl J Med 2002; 347:465-71; PMID:12181400; http://dx.doi.org/10.1056/NEJMoa012561

[40] Murphy TF, Kirkham C, Jones MM, Sethi S, Kong Y, Pettigrew MM. Expression of IgA Proteases by *Haemophilus influenzae* in the Respiratory Tract of Adults With Chronic Obstructive Pulmonary Disease. J Infect Dis 2015; 212:1798-805; PMID:25995193; http://dx.doi.org/10.1093/infdis/jiv299

[41] Murphy TF. Vaccines for Nontypeable *Haemophilus influenzae*: the Future Is Now. Clin Vaccine Immunol 2015; 22:459-66; PMID:25787137; http://dx.doi.org/10.1128/CVI.00089-15

[42] Janson H, Melhus A, Hermansson A, Forsgren A. Protein D, the glycerophospho-diester phosphodiesterase from *Haemophilus influenzae* with affinity for human immunoglobulin D, influences virulence in a rat otitis mode. Infect Immun 1994; 62:4848-54; PMID:7927765.

[43] Prymula R, Peeters P, Chrobok V, Kriz P, Novakova E, Kaliskova E, Kohl I, Lommel P, Poolman J, Prieels JP, et al. 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 2006; 367:740-8; PMID:16517274; http://dx.doi.org/10.1016/S0140-6736(06)68304-9

[44] Leach AJ, Wigger C, Hare K, Hampton V, Beissbarth J, Andrews R, Chatfield M, Smith-Vaughan H, Morris PS. Reduced middle ear infection with non-typeable *Haemophilus influenzae*, but not *Streptococcus pneumoniae*, after transition to 10-valent pneumococcal non-typeable *H. influenzae* protein D conjugate vaccine. BMC Pediatr 2015; Oct 19;15:162; http://dx.doi.org/10.1186/s12887-015-0483-8

[45] Atack JM, Winter LE, Jurcisek JA, Bakaletz LO, Barenkamp SJ, Jennings MP. Selection and Counterselection of Hia Expression Reveals a Key Role for Phase-Variable Expression of Hia in Infection Caused by Nontypeable *Haemophilus influenzae*. J Infect Dis 2015; 212:645-53; PMID:25712964; http://dx.doi.org/10.1093/infdis/jiv103

[46] Winokur P, Chaloner K, Doern G, Ferreira J, Apicella M. Safety and immunological out- comes following human inoculation with nontypeable *Haemophilus influenzae*. J Infect Dis 2013; 208:728-38; PMID:23715660; http://dx.doi.org/10.1093/infdis/jit238

[47] Palumbo E, Fiaschi L, Brunelli B, Marchi S, Savino S, Pizza M. Antigen identification starting from the genome: a "Reverse Vaccinology" approach applied to MenB. Methods Mol Biol 2012; 799:361-403; PMID:21993656; http://dx.doi.org/10.1007/978-1-61779-346-2_21

[48] De Chiara M, Hood D, Muzzi A, Pickard DJ, Perkins T, Pizza M, Dougan G, Rappuoli R, Moxon ER, Soriani M et al. Genome sequencing of disease and carriage isolates of nontypeable *Haemophilus influenzae* identifies discrete population structure. Proc Natl Acad Sci U S A 2014; 111:5439-44; PMID:24706866; http://dx.doi.org/10.1073/pnas.1403353111

[49] Eutsey RA, Hiller NL, Earl JP, Janto BA, Dahlgren ME, Ahmed A, Powell E, Schultz MP, Gilsdorf JR, Zhang L et al. Design and validation of a supragenome array for determination of the genomic content of *Haemophilus influenzae* isolates. BMC Genomics 2013; 14:484; PMID:23865594; http://dx.doi.org/10.1186/1471-2164-14-484

[50] Whitby PW, Seale TW, Morton DJ, Stull TL. Antisera Against Certain Conserved Surface-Exposed Peptides of Nontypeable *Haemophilus influenzae* Are Protective. PLoS One 2015; 10:e0136867; PMID:26390432; http://dx.doi.org/10.1371/journal.pone.0136867