Non-typeable *Haemophilus influenzae*, **an under-recognised pathogen.**

A review of the literature.

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SUMMARY

Non-typeable *Haemophilus influenzae* (NTHi) is a major cause of mucosal infections such as otitis media, sinusitis, conjunctivitis and exacerbations of chronic obstructive pulmonary disease. In some geographical regions, there is a strong causal relationship between NTHi and infections of the lower respiratory tract. Over the last 20 years, there has been a steady but constant increase in invasive NTHi disease globally with perinatal infants, young children and the elderly being most at risk. Individuals with underlying co-morbidities are most susceptible and infection is associated with significant mortality. Beta-lactamase production is the predominant mechanism of resistance. However, the emergence and spread of beta-lactamase negative ampicillin resistant strains across many regions of the world is of considerable concern, potentially necessitating alterations to current antibiotic treatment guidelines for community acquired upper and lower respiratory tract infection (LRTI) and potentially increasing morbidity associated with invasive NTHi infections. Standardised surveillance protocols and typing methodologies to monitor NTHi as an emerging pathogen across the world should be implemented. International scientific organisations need to take a lead in raising the profile of NTHi in the clinical and research communities, to document the pathobiology of this microbe as it responds to continued antibiotic and vaccination pressure.

INTRODUCTION

When, over 20 years ago, the *Haemophilus influenzae* type b (Hib) conjugate vaccine was introduced, surveillance recorded the rapid reduction of invasive Hib infections and the near absence of replacement with non-type b encapsulated *H. influenzae*.¹ Following this, interest in *H. influenzae* declined, as demonstrated by the fall in the number of peer-reviewed papers on *H. influenzae* from 18.4 per 1000 bacteriological papers per year in 1991, to just 7 in 2009.²

Non-encapsulated (or non-typeable) *H. influenzae* (NTHi) have never enjoyed the 'star pathogen' status of Hib or *Streptococcus pneumoniae*, despite the fact that NTHi are a common cause of respiratory tract infections both in children and adults. Indeed, to our knowledge, there are few scientific fora with sessions devoted to this microbe. HinMax, a group of clinicians and researchers interested in both *Moraxella* and *H. influenzae*, including NTHi, met in 2008 and in 2011 with 40 and 27 delegates respectively attending and 24 presentations on *H. influenzae*, including NTHi (Hays J. P. personal communication). At the European Congress of Clinical Microbiology and Infectious Diseases in Vienna in 2010, of around 4000 oral and poster presentations, only 11 dealt with NTHi. This apparent lack of interest may be because this pathogen is generally considered to only cause infection in predisposed patients and to be easily treated with beta-lactam antibiotics.

We believe that this lack of focus on NTHi is dangerous, as it facilitates the significant morbidity and increasing mortality associated with NTHi without encouraging the timely development of appropriate interventions. Here, we make the case for heightened surveillance of NTHi because: 1) data are emerging pointing to the increasing importance of NTHi as a pathogen in upper and lower respiratory tract infections,¹¹ 2) there is evidence of an increase in invasive infections caused by NTHi, and 3) epidemiological data from several countries show an increase in the spread of non-beta-lactamase dependent resistance to beta-lactams in NTHi. In an attempt to raise the profile of NTHi, a website has been recently established: <http://nthy-watch.griffith.edu.au/> to provide a single repository of knowledge on the pathogenesis, incidence and treatment of NTHi disease. The European Monitoring Group on Meningococci (EMGM) recently expanded its scope to include *H. influenzae* and in

recognition of the inclusion of *H. influenzae*, the acronym now stands for the European Monitoring Group on Meningitis.

HAEMOPHILUS SPECIES

Haemophilus spp are small fastidious Gram-negative cocco-bacilli that can be speciated by their requirements for X-Factor (haemin) and/or V-Factor (NAD) and other phenotypic characteristics. *H. influenzae* requires both X-Factor and V-Factor. Some strains of *H. influenzae* are encapsulated and can be divided into 6 serotypes (a-f) based on their capsular polysaccharide.¹² NTHi strains cannot be serotyped by conventional type specific anti-serum agglutination. New molecular technologies have allowed a more accurate differentiation between typeable and non-typeable strains by detection of the *cap* locus which encodes the polysaccharide capsule. The *cap* locus consists of 3 regions.¹⁵ Regions 1 and 3 are common to all capsular types and contain highly conserved genes necessary for processing and transport of capsular material to the cell surface.. Region 2 contains type-specific genes involved in capsule biosynthesis. A polymerase chain reaction (PCR)-based method to detect the region 1 *bexB* gene may be used as a proxy for the capsule locus,¹⁹ and can distinguish true NTHi from strains that contain a complete or partial capsule locus. Strains with a partial capsule locus are unable to export polysaccharide to the cell surface and therefore appear to be NTHi as they are non-serotypeable by conventional means.

Serological capsular typing is prone to misinterpretation. Satola et al.²⁰ reported discrepancies between serological typing and PCR-based capsular typing for 17.5% of 360 invasive isolates of *H. influenzae*: 48 strains reported as encapsulated by serological typing were NTHi by PCR ; 8 NTHi by serotyping were capsulated; 6 capsulated by serotyping were a different type by PCR and 2 capsulated by serotyping were capsule-deficient (Hib-minus) strains. For this reason, molecular typing is the preferred method for accurate identification of NTHi.

There are a number of methods for sub-typing strains of *H. influenzae*, including biotyping,²³ outer membrane protein (OMP) subtyping,²⁴ and multilocus sequence typing (MLST).²⁵ The highly clonal nature of Hib strains renders subtyping useful only in epidemiological studies where a strain of an infrequent subtype is involved. In contrast, NTHi exhibit a wider

variety of phenotypes and genotypes and subtyping can be useful when investigating nosocomial transmission, which has been reported in nursing home and respiratory ward settings.^{26,28} The preferred method for subtyping is MLST (www.mlst.net). However there are no reliable methods to differentiate commensal and disease-causing NTHi strains, although there is some evidence that bacterial IgA1 protease activity is significantly higher in NTHi strains from symptomatic patients compared to NTHi isolated from throat swabs of asymptomatic individuals.³⁰ Similarly, there are currently no data to indicate whether different strains of NTHi are involved in mucosal and invasive disease.

Haemophilus influenzae biogroup aegyptius is a distinct NTHi strain. For more than a century, this organism was known to cause seasonal epidemics of acute purulent conjunctivitis, especially in warmer climates.^{31, 32} In 1984, a virulent clone of *H. influenzae* biogroup aegyptius emerged in Brazil as the cause of an acute febrile illness in children with high fatality rates.³⁴ This infection, subsequently known as Brazilian Purpuric Fever, caused a series of outbreaks and sporadic cases almost exclusively in Brazil over the next decade but has virtually disappeared since then.

A second X- and V-factor requiring *Haemophilus* species, *H. haemolyticus*, is frequently found in the upper respiratory tract, and has long been regarded as a commensal. Microbiologists relied on the production of zones of beta-haemolysis on horse blood agar to differentiate *H. haemolyticus* from NTHi. Recent observations have indicated that 10-40% of *H. haemolyticus* strains are non-haemolytic.³⁵ PCR-based methods for detecting protein D and fuculose kinase, which are both positive for NTHi,³⁷ matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS),³⁸ and fluorescence in situ hybridisation (FISH),³⁹ have been reported to facilitate the differentiation of NTHi from non-haemolytic *H. haemolyticus*. However, Binks et al.⁴⁰ found that no single gene target could unequivocally differentiate NTHi from *H. haemolyticus*. They recommended the use of an *hpd#3* probe-based real time PCR assay as the best currently available method. A scheme including *hpd*- and *iga*- based PCR assays and standard microbiological methods has been recommended to improve the identification of *H. haemolyticus*.⁴¹

NTHi AS A CAUSE OF NON-INVASIVE INFECTIONS

The global burden of NTHi non-invasive infections such as otitis media (OM), sinusitis and conjunctivitis, exacerbations of chronic obstructive pulmonary disease (COPD) and non-bacteraemic pneumonia is immense. These infections are by nature mucosal and often poly-microbial. As a consequence, the role of NTHi in the pathogenesis of these infections may be under-reported. The development of new and sensitive PCR technology will be invaluable in understanding the aetiology and pathology of these infections. It may then be possible to determine whether or not “infection strains” of NTHi exist at mucosal surfaces and their relationship to invasive disease.

Otitis Media (OM) is the most common childhood disease for which medical assistance is sought, affecting >75% of children aged <3 years.⁴² The predominant bacterial pathogens in OM are *S. pneumoniae*, NTHi and *M. catarrhalis*, with *S. pneumoniae* and NTHi being cultured from the middle ear in 55-95%, depending on geographical location.⁴³⁻⁵¹ In both the United States (USA) and Australia, pneumococcal conjugate vaccination (Prevenar®, Wyeth Pharmaceuticals, USA) has resulted in a higher proportion of cases with NTHi isolated from middle ear fluid, from 41% in 1992-1998 to 56% in 2000-2003.^{43, 52} With the widespread use of higher-valency pneumococcal conjugate vaccines offering protection against more *S. pneumoniae* serotypes, it is critical to monitor changes in the bacterial aetiology of OM and disease severity. The prevalence of NTHi clearly increases in children with antimicrobial therapy failure,⁵³ in those suffering from bilateral OM,⁴⁷ and in those with recurrent acute OM (AOM),^{43, 55} indicating that NTHi OM may be more resistant to treatment and more chronic in nature. **It has been demonstrated that NTHi is able to form biofilms in the middle ear and exist in a poly-microbial community.**⁵⁶ **This may partly explain, resistance to antibiotic treatment and the recurrent and chronic nature of NTHi OM. A number of options are available for the development of a vaccine against NTHi OM.**⁹ Studies with a prototype 11-valent pneumococcal conjugate vaccine in which the pneumococcal polysaccharides were conjugated to the *H. influenzae* derived protein D, demonstrated 35.3% efficacy against AOM caused by NTHi.⁴⁹ This vaccine formulation also reduced NTHi carriage in the nasopharynx.⁵⁸ However, the descendant licensed 10-valent vaccine does not appear to effect nasopharyngeal NTHi colonisation or bacterial load in healthy young children.⁵⁹

Bacterial conjunctivitis is more common in children than in adults and NTHi is responsible for 44-68% of cases,⁶⁰⁻⁶⁵ compared with ~25% in adults.⁶⁶ Conjunctivitis-otitis syndrome (COS) is often accompanied by AOM and the predominating causative agent in the conjunctiva and the middle ear is NTHi of the same strain.^{60, 66, 69} Between 20-70% of children who present with bacterial conjunctivitis also have AOM. Given the high NTHi prevalence in bacterial conjunctivitis and the strong COS association, these children are most likely to also present with NTHi AOM. Conversely, since the introduction of Prevenar® in the USA, an increase in the proportion of NTHi AOM has been observed,⁴³ , which is also likely to increase the incidence of NTHi conjunctivitis. Surveillance studies should be undertaken to determine the impact of pneumococcal vaccination on COS prevalence.

Bacterial sinusitis is a common complication of childhood viral upper respiratory tract infection, and the fifth most common diagnosis for which antibiotics are prescribed in children in the USA.⁷¹ In children with chronic sinusitis, *S. pneumoniae* and *H. influenzae* predominate.⁷⁶ The introduction of Prevenar™ in the USA saw an increase in the proportion of nasopharyngeal NTHi colonisation in children with sinusitis, from 25% to 41% from 1997 to 2005.⁷⁷

Exacerbations of COPD, persistent bacterial bronchitis and cystic fibrosis: COPD is one of the leading causes of death globally with disease burden expected to increase significantly.⁷⁸ There is now extensive evidence demonstrating that NTHi colonisation of the airways is a major cause of airway inflammation and tissue damage, particularly during acute exacerbations.⁷⁹ Firstly, NTHi is a major pathogen colonising damaged airways in subjects with COPD,⁵ and when present, predominates.⁶ There is evidence that acute exacerbations of COPD can be attributed to the acquisition of 'exacerbation' isolates of NTHi.⁸¹ During acute exacerbations, NTHi detection in bronchus brush biopsies increases from 33% in subjects with stable COPD to 87% in subjects with exacerbations.⁸¹ Secondly, oral immunisation with an enteric killed whole-cell vaccine of subjects with severe COPD significantly reduces the number and severity of acute exacerbations.^{83,84} Thirdly, the detection of specific NTHi IgE antibodies in subjects with COPD suggests that an IgE-mediated hypersensitivity to NTHi antigens contributes to the reversible airway obstruction and bronchial hyper-reactivity often observed in these subjects.⁸⁵

Persistent bacterial bronchitis (PBB) is an underdiagnosed clinical presentation which is most common in <5 year-olds and is characterised by chronic cough.^{86, 87} Culture of cough swabs obtained from children with PBB were positive for *H. influenzae* from 81% of samples tested,⁸⁸ and most of these were NTHi. Broncho-alveolar lavage (BAL) samples from children with PBB have confirmed NTHi as the major causative agent in Greece and the United Kingdom (UK).⁸⁹ Importantly, a recent Australian study of indigenous children with bronchiectasis confirmed by molecular typing that the majority of phenotypic NTHi isolates from both BAL and nasopharynx were PCR-positive for protein D and, therefore, not *H. haemolyticus*.⁹⁰ **In young children with cystic fibrosis, NTHi is often isolated from the respiratory tract^{91, 92} and in up to 30% of sputum samples.⁹¹ NTHi is thought to set up the inflammatory processes that permit *Pseudomonas aeruginosa* colonisation.^{93, 94}** Recent studies have supported this concept and demonstrated the presence of NTHi in biofilms on airway epithelium with increased antimicrobial resistance.^{95, 96}

Lower respiratory tract infections (LRTI): The role of NTHi as a causative agent of childhood pneumonia has recently been reviewed.⁹⁷ There are substantial technical difficulties in obtaining reliable samples to determine the bacterial aetiology of LRTI. Trans-thoracic fine needle aspiration is the most reliable method but this procedure, which is only performed in the presence of consolidation, is not routinely used. Therefore, other less direct diagnostic approaches have been used, including blood, sputum and BAL cultures. In the early 1980's in Papua New Guinea, NTHi clearly predominated in trans-thoracic fine needle aspiration cultures.⁹⁸ Studies considered to be technologically sound and conducted outside of Papua New Guinea have not demonstrated a similar NTHi predominance, but have reported positive lung aspirate cultures and molecular typing for NTHi in 15-40% of cases.⁹⁹⁻¹⁰² **In children with bacteraemic pneumonia, positive blood cultures for NTHi have been reported at rates between 2 - 10%.^{98, 104-106} However, blood culture detection of *H. influenzae* may vary between isolates and medium.¹⁰³ New quantitative real-time PCR technology using serum samples will significantly aid the aetiological diagnosis of pneumonia.¹⁰⁷** Sputum and BAL cultures from children with LRTI are often criticised because they are contaminated with nasopharyngeal microflora. However, if these samples are collected according to standardised protocols, they provide valuable information on microbial colonisation of the lower airways.^{108, 110} Good quality sputum and BAL cultures have reported the presence of

NTHi in 20-94% of samples collected from children with community-acquired pneumonia.¹¹³⁻¹¹⁵

NTHi AS A CAUSE OF INVASIVE INFECTIONS

Prior to routine vaccination, Hib was the commonest cause of invasive *H. influenzae* disease, occurring predominantly in healthy <5 year-olds, for whom it was the commonest cause of bacterial meningitis.¹¹⁶ **In England and Wales, 90% of invasive *H.influenzae* infections were due to Hib, 10% due to NTHi and other serotypes accounted for <1%.¹¹⁷ Less than a quarter (23%) of NTHi infection occurred in <5 years-olds, 62% in adults, and 39% in >65 year-olds. NTHi incidence varied by age, ranging from 14.9/100,000 in neonates to 0.39/100,000 in >65 year-olds. Bacteraemia without a focus of infection was the predominant clinical presentation (37%), followed by pneumonia (27%) and meningitis (12%).**

The introduction of the Hib conjugate vaccine into national immunisation programmes in the early 1990's led to rapid and sustained reduction in Hib disease across all age groups through a combination of direct and indirect (herd) protection.¹¹⁶ . **One of the concerns regarding the long-term consequences of Hib vaccination is the possible occurrence of replacement disease caused by other *H. influenzae* strains. As Hib conjugate vaccination reduces pharyngeal carriage, there is a theoretical possibility that other *H. influenzae* strains may take its place and, in turn, cause more invasive disease.**

Most European countries have an established national *H. influenzae* surveillance system, albeit with varying surveillance and diagnostic methodologies and case definitions.¹¹⁸

During 1992-2006, national *H. influenzae* surveillance data from 14 European and other countries (www.eu-ibis.org) that routinely serotyped all *H. influenzae* isolates identified a 3.5% (95% confidence interval (CI), 2.1-5.2%) year-on-year annual increase in invasive non-type b *H. influenzae* disease, mainly due to NTHi, which accounted for 97% of cases.¹¹⁸ In 2007, surveillance was transferred to the European Centre for Disease Prevention and Control (www.ecdc.eu). In 2008 and 2009, invasive NTHi disease incidence in 29 reporting countries was 0.41/100,000 and 0.36/100,000 respectively, with higher rates in Sweden (1.78/100,000 and 1.58/100,000) and Norway (1.58/100,000 and 1.48/100,000).^{119,120} **Not**

all countries, however, have reported an increase in invasive NTHi following routine Hib vaccination. In Italy, for example, even though the proportion of invasive *H. influenzae* cases caused by NTHi increased from 28% of 220 isolates in 1997-2002, to 73% of 78 isolates in 2007- 2009, the incidence remained similar (0.037 and 0.032 per 100,000).¹²⁴

In the US, 80% of invasive *H. influenzae* infections in 1989 were caused by Hib compared with 17% by NTHi, whereas by 2008, Hib was responsible for only 3% compared with 68% for NTHi.¹²¹ During 1999-2008, NTHi incidence was 0.99/100,000, with the highest incidence in <1 –year-olds (5.87/100,000) and ≥65 year-olds (4.09/100,000).¹²¹ In Canada, Hib incidence in 2011 was 0.11/100,000 and 0.75/100,000 for non-type b disease.¹²³

A number of factors may be responsible for the small but steady increase in invasive NTHi infections following routine Hib vaccination and it is likely that they have all contributed to some extent. The evidence for strain replacement following Hib vaccination is limited.¹³⁵ In England and Wales, for example, a 3.4% year-on-year increase in invasive NTHi disease was reported in children during 1994-2008, but the resurgence of invasive Hib disease during 2000-2003, eight years after the introduction of routine Hib vaccination, had no impact on invasive NTHi disease epidemiology.¹²⁵ On the other hand, it is well-described that invasive NTHi disease has a predilection for vulnerable populations such as premature infants, older adults, the immunosuppressed, individuals with malignancy and those with chronic cardiovascular, respiratory and other conditions.¹²⁵ Improvements in survival rates may, therefore, have increased the population of at-risk individuals for invasive NTHi and other infections. Alternatively, the observed increase may be a consequence of any combination of (i) increased recognition by clinicians of the spectrum of disease caused by NTHi, (ii) changes in clinical practice (e.g. number of blood cultures taken), (iii) laboratory testing (e.g. increased sensitivity of modern, automated blood culturing systems), and/or (iv) more complete reporting of laboratory-confirmed infections to national surveillance programmes. In particular, the transition from slide agglutination to PCR-based methods has significantly improved the accuracy of serotyping results. Like many other bacterial infections, the highest incidence of invasive NTHi disease occurs at the extremes of age. Neonatal NTHi infections are well described, with an incidence of 1.6 to 4.9 per 1,000 live births¹⁴⁰⁻¹⁴² and account for about 5% of all neonatal invasive bacterial infections.^{140, 141} The infection develops rapidly (usually

within 24 hours of birth) and follows a fulminant course with a significant case fatality, particularly among premature infants. The organism is known to colonise the female genital tract and it has been proposed that an ascending infection may result in both maternal and foetal infection, resulting in septicaemia, increased complications during labour and preterm delivery. A number of observational studies have resorted higher rates of invasive NTHi disease in pregnancy (ref: Gkentzi, Slack, Ladhani, 2012). Variant strains of NTHi isolated from the genito-urinary tract were first described by Albritton et al.¹⁴⁸ and further studied by Quentin et al.¹⁴⁹ They reported that most of these strains were biotype IV and described them as a “cryptic genospecies of Haemophilus biotype IV”. The name *Haemophilus quentini* has been proposed for these strains. These strains are distinguishable from typical strains of NTHi by multilocus enzyme electrophoresis, 16S rRNA gene sequence and DNA hybridisation.¹⁵⁰ Subsequent studies however, have reported more diverse *H. influenzae* biotypes causing female genital tract infections^{143, 151-152}, highlighting the need for further research on perinatal NTHi infections. “*H. quentini*” has also been isolated from routine urine and urethral cultures in adult males¹⁵⁴, though the significance is unknown.

After the neonatal period, the incidence of NTHi invasive disease is low and relatively stable. In countries with established Hib vaccination programmes, the median age at onset of invasive *H. influenzae* disease has now shifted from early childhood to late adulthood because most invasive infections are now due to NTHi, which has a median age at disease onset of around 60 years.¹¹⁸ Most invasive NTHi cases in children (40% to 70%) and adults (60% to 80%) occur in individuals with underlying co-morbidities, particularly chronic respiratory disease and impaired immunity,^{137, 141, 155-159} although a recent Swedish study reported co-morbidity in only 38% of cases.¹²⁰ Interestingly, this study found disorders of B cell immunity such as chronic lymphatic leukaemia and multiple myeloma to be particularly common among patients with invasive NTHi, suggesting an important role for humoral immunity.¹²⁰ The authors also speculated that the age-related decline in B-cell function may also explain the increasing incidence of invasive NTHi with age.¹²⁰

The clinical presentation of invasive NTHi disease varies with age. The vast majority of neonatal cases develop septicaemia without a focus. Clinical presentation with meningitis is more common in older infants and children and then declines with age. The most common

clinical presentation overall is pneumonia, which increases with age and occurs mainly in older adults who often have underlying respiratory tract co-morbidities.^{124, 158} Other clinical presentations such as epiglottitis (characteristic of invasive Hib disease), bone and joint infections, as well as skin and soft tissue infections, have been reported but are uncommon, although cholecystitis appears to be a particular feature of invasive NTHi disease.^{124-126, 158} It is important to emphasise that, as in neonates, NTHi can cause serious morbidity in older age groups. The aforementioned Swedish study, for example, reported that 48% of 101 invasive NTHi cases had severe sepsis or septic shock and 20% required intensive care.¹²⁰

Reported case fatality ratio (CFR) for invasive NTHi disease range between 12-22%,^{122, 125, 155, 157, 158, 160} and is associated with age, clinical presentation, presence of co-morbidity and duration of follow-up after infection. In Europe, compared with Hib, the age-adjusted odds ratio for death from invasive NTHi disease was 2.4 (95% CI, 1.9–3.1, $P < 0.0001$) overall, 3.3 (95% CI, 1.5–7.5; $P = 0.004$) for pneumonia and 3.3 (95% CI, 1.5–7.5; $P = 0.004$) for bacteraemia, with no significant difference observed for meningitis. CFR also increases with longer follow-up periods after the initial NTHi infection, suggesting that the pathogen may particularly be targeting those with poor health who, even if they survive their infection, will subsequently succumb to their underlying illness. In the Swedish study, for example, the 28-day CFR was 8%, but 1-year CFR increased to 29%.¹²⁰

DEVELOPMENT OF RESISTANCE IN NTHI

In *H. influenzae* and NTHi, beta-lactamase production is still the dominant resistance mechanism against beta-lactams. The beta-lactamase prevalence in NTHi differs widely worldwide. Over the last decade, studies have reported percentages of beta-lactamase - positive NTHi between 10% and 25% in most countries (South-Africa, Europe, USA, Canada, Central and South-America).¹⁶¹⁻¹⁶⁶ In some regions, beta-lactamase -positive NTHi represent up to 55% of NTHi (Taiwan, Vietnam, Japan, South-Korea).¹⁶⁷⁻¹⁷¹ The beta-lactamase found in *H. influenzae* is usually a plasmid-encoded class A serine beta-lactamase; most often a TEM-1, rarely ROB-1, although regional differences exist.¹⁷² It is surprising that although mutations have appeared in the promoter region of these genes, no TEM-type extended-spectrum beta-lactamases (ESBLs) have yet been detected in *H. influenzae*, in contrast to

other Gram-negatives. However, the possibility of broader spectrum beta-lactamases emerging in *H. influenzae* cannot be excluded. ESBL-type beta-lactamases have been found in *H. parainfluenzae*, a potential source of DNA for transformation of *H. influenzae*.¹⁷³

More worrying however, are data on the emergence and spread of beta-lactamase independent beta-lactam resistant strains. These strains are called BLNAR, for beta-lactamase negative ampicillin-resistant strains. BLNAR can be identified by their resistance to ampicillin. Different breakpoints for ampicillin resistance have been used (1, 2 or 4 µg/ml, reviewed by Tristram et al¹⁷⁴) and the term BLNAI is also used, for beta-lactamase negative ampicillin-intermediately-resistant strains. Another way to define these strains is on the basis of resistance-inducing mutations in the *ftsI* gene. The *ftsI* gene encodes a transpeptidase enzyme called penicillin-binding protein 3 (PBP3), which is involved in septum formation of dividing cells. Based on the type of mutations, BLNAI/R are subdivided into genotypes I, II (low gBLNAR) and III (high gBLNAR).¹⁷⁵⁻¹⁷⁷ These different genotypes are also linked to different minimum inhibitory concentration (MIC) values; genotype I/II strains usually have ampicillin MIC values between 0.5 and 2 µg/ml and genotype III strains have MICs ranging between 1.0 and 16 µg/ml, often combined with reduced susceptibility to cephalosporins (see table 2). There are also BLPACR, for beta-lactamase positive amoxy-clavulanate-resistant strains. These strains combine beta-lactamase production with the presence of PBP3 mutations. Compared to BLNAR with the same PBP3 mutations but without a beta-lactamase, these BLPACR strains have higher ampicillin MICs and similar amoxy-clavulanate and cephalosporin MICs (table 2). BLPACR can again be subdivided based on the specific *ftsI* mutations.

In Japan, surveillance studies on respiratory pathogens in children and adults have documented a marked increase of these BLNAI/R. Prior to 1984, no BLNAI/R were isolated; in the 1990's there was a slow increase, but from 2000 onwards, BLNAI/R increased rapidly from 15% to around 50% in 2007 and almost 40% in 2009.^{171, 178-183} Notably, type III strains are gradually replacing the type I/II strains. These type III strains can behave as clones that appear to spread among the population.^{178, 184} Similar observations were made for BLPACR strains.¹⁸⁵ In a 2007 review on *H. influenzae*,¹⁷⁴ the authors describe BLNAR as genotypically diverse with only a few instances of clonal spread. Since then, more reports of clonal spread have been published. Does this herald the emergence of clones with high beta-lactam MIC

values and epidemic characteristics? The rapid increase in *S. pneumoniae* resistance in the 1990's was also due to the worldwide spread of resistant clones.

A high prevalence of BLNAR strains has also been reported from neighbouring regions such as Taiwan, Vietnam, South-Korea.^{167, 169, 170} Most countries in Europe and the USA have reported beta-lactamase in 5% to 25% of NTHi isolates and few or no BLNAR strains. In Europe, an exception is Spain, where higher levels of BLNAI/R and the presence of BLNAR (genotype III-like) exhibiting characteristics of clonality, with increased MIC values for cefuroxime (up to 16 µg/ml) and cefotaxime (up to 4 µg/ml), have been reported.^{186, 187} These BLNAR strains were isolated primarily from cases of conjunctivitis. Reports also indicate higher and increasing levels of BLNAI/R in Poland, France and Portugal, with increasing prevalence of genotype III-like strains.¹⁸⁸⁻¹⁹² In Sweden, a significant increase in beta-lactam-resistant invasive *H. influenzae* mainly due to beta-lactamase-negative isolates was observed from 2007 onwards. One cluster of BLNAR genotype IIb isolates was identified and it included isolates from all geographical areas.¹⁹³ A preliminary report also suggests and increase in BLNAI in Canada starting in 2000.^{194, 195} For many parts of the world, little or no data on BLNAI/R are available. Likely because regular surveillance of NTHi is lacking or microbiological work-up of NTHi is limited to a nitrocefin test for beta-lactamase production. In addition, currently accepted resistance breakpoints of >1 µg/ml or >2 µg/ml as determined by the Clinical and laboratory Standards Institute (CLSI) and EUCAST, respectively, for beta-lactamase negative *H. influenzae* probably led to an underestimation of the prevalence of BLNAR. This is also the case for BLPACR; the CLSI breakpoint of ≥8 µg/ml for co-amoxycylav is likely to miss many strains in which the MIC value is increased to only 2 µg/ml. The EUCAST breakpoint of >1 µg/ml is more likely to pick up these strains. The further spread of non-beta-lactamase dependent beta-lactam resistant strains, and in particular the genotype III BLNAR and BPLACR, has important implications for treatment. This is not only the case for strains with ampicillin MIC values of 4 µg/ml or more. Clinical data suggest that current dosages of aminopenicillins will not guarantee clinical success in certain types of infections, such as chronic or recurrent OM, if MIC values are 1 or 2 µg/ml. Changing to other classes of antibiotics offers only a partial solution. *H. influenzae* is intrinsically resistant to macrolide-lincosamide-streptograminB agents including ketolides.¹⁹⁶⁻¹⁹⁸ Even for strains with low macrolide MIC values, the outcome in AOM for

treatment with macrolides has been shown not to differ from placebo.¹⁹⁹ *H. influenzae* is rarely resistant to quinolones but their use in children is not yet approved. It is also commonly accepted that the use of quinolones in paediatric respiratory infections would significantly enhance selective pressure for the emergence and selection of resistant *S. pneumoniae* and *H. influenzae*. In conclusion, a further spread of these BLNAR and BLPACR could make it necessary to change the current treatment guidelines for community-acquired upper and lower respiratory tract infections.

GENERAL CONCLUSION

Improved surveillance of *H. influenzae* is needed to follow the trends described here. National reference centres could play an important role by ensuring comprehensive and standardised surveillance of all cases of invasive *H. influenzae* disease; collection of epidemiological data on cases, including demographic details, underlying co-morbidities, risk factors and outcome of the infection; accurate identification of the organism based on molecular typing methods; antimicrobial resistance rates and mechanisms of resistance of invasive disease isolates. Respiratory isolates could also be monitored to follow trends in antimicrobial resistance. A substantial number of pharyngeal isolates previously identified as NTHi are actually *H. haemolyticus*.³⁶ Discrimination between these two can be challenging. New PCR technology and MALDI-TOF may offer a solution to this problem.²⁰¹ The low rates of NTHi infections observed in some countries probably reflect the low proportion of strains referred to reference laboratories and highlight the potential for improving case ascertainment. At the international level, cooperation and genetic comparison of strains between countries through reference centres would help to document the spread of resistant clones. International scientific organisations should take the lead in establishing networks and study groups. At a more practical level, an international consensus on the breakpoints for ampicillin resistance and the inclusion of ampicillin susceptibility testing in the routine work-up of *H. influenzae* are needed. The novel disk diffusion method to detect beta-lactam resistance in *H. influenzae* issued by the EUCAST (www.eucast.org) in 2011 will hopefully help in harmonising and improving the detection of these strains. Another issue that needs to be resolved is whether sampling from the nasopharynx is a valid alternative for sampling from the middle ear.

The increasing presence of NTHi in difficult-to-treat respiratory infections and the high level of resistance observed in these strains make it necessary to include these isolates in surveillance. Further studies are required to assess whether the recently licensed 10-valent pneumococcal conjugate vaccine (Synflorix,[®] GSK, Belgium) that includes Protein D from *H. influenzae* as a carrier protein might protect against serious infection in high-risk groups. Ongoing clinical studies will determine the efficacy of this vaccine against NTHi OM. It is also important to define the driving factors underlying the observed trends. Several factors have been put forward. Routine use of the pneumococcal conjugate vaccine has been suggested to increase nasopharyngeal colonisation with NTHi. This may explain the increased prevalence of NTHi in respiratory infections and provides increased opportunity for gene exchange and emergence of resistance. In Japan, the use of oral cephalosporins is thought to be driving the spread of BLNAR and BPLACR. This is in contrast to Europe and the USA, where amoxicillin (or amoxicillin-clavulanate in cases of suspected *H. influenzae* involvement) is used as a first-line therapy for community-acquired respiratory infections. A generalised spread of BLNAR and BLPACR is of considerable concern, as it might threaten the efficacy of our current first line empirical treatment of respiratory tract infections.

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Conflicts of Interest

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Table 1 Predominant diseases caused by NTHi and their burden

Disease	Burden	Ref
Non-invasive disease		
Otitis media	55-95% of cases in children	43-51
Bacterial conjunctivitis	44-68% of cases in children 25% of cases in adults	60-65 66
Bacterial sinusitis	41% of cases in children	77
Exacerbations of COPD	>90% during an acute exacerbation	6, 80-81
Persistent bacterial bronchitis	81% of cases in children	88
Cystic fibrosis	Up to 30% of sputum samples	91
Lower respiratory infections	BAL: 20-94% of cases with community acquired pneumonia ¹ Lung Aspirate: 15-40% of cases with pneumonia Blood culture 2-10% of cases with bacteriemic pneumonia	113-115 99-102 98, 104-106
Invasive Disease²		
Neonatal infections (septicaemia and meningitis)	1.6 – 4.9 per 100 live births	140-144

¹ Geographically variable² Usually associated with underlying co-morbidity (40-70% in children; 60-80% in adults)Epiglottitis and bone, joint, skin and soft tissue infections have been reported but at a low incidence.¹²⁴⁻¹²⁶