Invasive disease by Haemophilus influenzae in Sweden in the era of the H. influenzae type b vaccine

Resman, Fredrik

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Invasive disease by *Haemophilus influenzae* in Sweden in the era of the *H. influenzae* type b vaccine

Aspects of epidemiology, antimicrobial resistance, clinical outcome and bacterial virulence

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by

Fredrik Resman

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Abstract

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In order to answer the initial question, we studied the epidemiology, the clinical burden and antimicrobial resistance of invasive *H. influenzae* in Sweden 1997-2010. Two aspects of the pathogenesis of invasive *H. influenzae* disease were addressed; bacterial binding to the extracellular matrix and the role of complement regulator binding in invasive disease. A case report of a severe invasive *H. influenzae* type f infection, including an examination of contributing host factors, is also presented.

Our results suggest that invasive disease by *H. influenzae* has not disappeared, but the epidemiology has radically changed. We found no support for serotype replacement in young children. In adults, and especially elderly adults, the incidence of invasive disease by non-typeable *H. influenzae* (NTHi) increased significantly during the study period. The results also suggest an increased incidence of invasive disease by *H. influenzae* type f (Hif), and type f is the most common serotype in Sweden today. Cases of invasive disease by non-type b isolates that occurred in healthy adults were often severe, suggesting the existence of hypervirulent non-type b strains. The β-lactam resistance of invasive *H. influenzae* isolates increased during the study period, due to an increase of β-lactamase negative β-lactam resistant NTHi. A clonal expansion of a β-lactamase negative ampicillin resistant (BLNAR) clone frequently found in invasive disease was suggested.

The ability of *H. influenzae* to bind laminin, and anchor the extracellular matrix through the adhesin Protein E was confirmed. Binding to complement regulators was not higher in invasive as compared to nasopharyngeal NTHi isolates, and thus did not seem central for invasive capacity. Speculation, but no comprehensive conclusion as to what host factors relate to invasive disease by *H. influenzae* in adults was possible.

The results suggest that the vaccination against *H. influenzae* type b remains very effective 20 years after it was introduced, but that continued surveillance of incidence and antimicrobial resistance of invasive *Haemophilus* disease is warranted. The results also suggest that ongoing research should focus on non-typeable *H. influenzae*, which today dominates all types of *H. influenzae* infections.

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Fredrik Resman

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Medical Microbiology
Department of Laboratory Medicine, Malmö
Faculty of Medicine, Lund University, Sweden

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till Elsa, som lever inom oss

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II Hallström T, Resman F, Ristovski M and Riesbeck K. **Binding of Complement Regulators to Invasive Nontypeable *Haemophilus influenzae* Isolates is not Increased Compared to Nasopharyngeal Isolates, but Serum Resistance is Linked to Disease Severity** *J. Clin. Microbiol.* 2010; 48: 921-5

III Resman F, Ristovski M, Ahl J, Forsgren A, Gilsdorf JR, Jasir A, Kaijser B, Kronvall G, and Riesbeck K. **Invasive Disease Caused by *Haemophilus influenzae* in Sweden 1997-2009; Evidence of Increasing Incidence and Clinical Burden of Non-type b Strains** *Clin. Microbiol. Infect.* 2011; 17: 1638-45

IV Resman F, Ristovski M, Forsgren A, Kaijser B, Kronvall G, Medstrand P, Melander E, Odenholt I and Riesbeck K**. Increase of β-lactam Resistant Invasive *Haemophilus influenzae* in Sweden 1997-2010,** *Antimicrob. Agents Chemother*. 2012; 56: 4408-15

V Resman F, Svensjö T, Ünal C, Cronqvist J, Brorson H, Odenholt I and Riesbeck K. **Necrotizing Myositis and Septic Shock Caused by *Haemophilus influenzae* type f in a Previously Healthy Man Diagnosed with an IgG3- and a Mannose-Binding Lectin Deficiency** *Scand. J. Inf. Dis.* 2011; 43: 972-76

ABR Abbreviations

ACCP	American College of Chest Physicians
APC	Antigen-Presenting Cells
BLNAR	β-lactamase Negative Ampicillin Resistant
BLNBR	β-lactamase Negative β-lactam Resistant
BLPACR	β-lactamase Positive Amoxicillin-Clavulanate Resistant
BLPAR	β-lactamase Positive Ampicillin Resistant
C4BP	Complement 4b-Binding Protein
CEACAM-1	Carcinoembryonic Antigen-related Cell Adhesion Molecule-1
cha	cryptic haemophilus adhesin
CLSI	Clinical and Laboratory Standards Institute
COPD	Chronic Obstructive Pulmonary Disease
CVID	Common Variable Immunodeficiency
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
EARS	European Antimicrobial Resistance Surveillance Network
ECM	Extracellular matrix
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FITC	Fluorescein Isothiocyanate
gBLNAR	genomic β-lactamase Negative Ampicillin Resistant
Hap	Haemophilus adhesion and penetration protein
Hia	Haemophilus influenzae type a
hia	Haemophilus influenzae adhesin
Hib	Haemophilus influenzae type b

Hif	Haemophilus influenzae type f
HMW	High Molecular Weight adhesin
Hsf	Haemophilus surface fibrils
ICAM-1	Intercellular Adhesion Molecule-1
Ig	Immunoglobulin
IL-8	Interleukin 8
INR	International Normalized Ratio
LOS	Lipooligosaccharide
MAC	Membrane Attack Complex
MASP	Mannose-Binding Lectin-associated Serine Protease
MBL	Mannose-Binding Lectin
MIC	Minimal Inhibitory Concentration
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing
NAD	Nicotinamid Adenine Dinucleotide
NHS	Normal Human Serum
NTHi	Non-typeable Haemophilus influenzae
PAF	Platelet-Activating Factor
PBP	Penicillin-Binding Protein
PE	Protein E
pIgR	polymeric Immunoglobulin Receptor
PRP	Polyribosyl Ribitol Phosphate
SCCM	Society of Critical Care Medicine
SRGA	Swedish Reference Group for Antibiotics
TIGR	The Institute for Genomic Research
TIRAP	Toll-interleukin-1 receptor domain containing adaptor protein
TXM	Trimethoprim-Sulfamethoxazole

Summary

What is the current status of *Haemophilus influenzae* as an agent of invasive infection in Sweden? *H. influenzae* type b (Hib) used to be a common cause of meningitis, epiglottitis and severe sepsis in young children. In the late 1980's an effective conjugated vaccine against Hib was developed and a dramatic fall in Hib incidence was observed in countries that implemented vaccination. This includes Sweden, where the vaccine was implemented in 1992. Since the mid 1990's, scattered international reports have suggested increasing incidences of invasive disease caused by non-type b isolates of *H. influenzae.* A few of these reports have suggested serotype replacement.

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Our results suggest that invasive disease by *H. influenzae* has not disappeared, but the epidemiology has radically changed. We found no support for serotype replacement in young children. In adults, and especially elderly adults, the incidence of invasive disease by non-typeable *H. influenzae* (NTHi) increased significantly during the study period. The results also suggest an increased incidence of invasive disease by *H. influenzae* type f (Hif), and type f is the most common serotype in Sweden today. Cases of invasive disease by non-type b isolates that occurred in healthy adults were often severe, suggesting the existence of hypervirulent non-type b strains. The β-lactam resistance of invasive *H. influenzae* isolates increased during the study period, due to an increase of β-lactamase negative β-lactam resistant NTHi. A clonal expansion of a β-lactamase negative ampicillin resistant (BLNAR) clone, frequently found in invasive disease, was suggested.

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suggest that ongoing research should focus on non-typeable *H. influenzae*, which today dominates all types of *H. influenzae* infections.

Förenklad svensk sammanfattning

Har allvarliga infektioner orsakade av bakterien *Haemophilus influenzae* försvunnit? En undertyp av *H. influenzae*, typ b (Hib), var tidigare en fruktad orsak till struplocksinflammation, hjärnhinneinflammation och svår blodförgiftning hos små barn. Problemet var så stort att allmän vaccination mot Hib infördes i många länder i början av 1990-talet, bland annat i Sverige. Efter detta har infektioner med Hib nästan försvunnit, och den inledande frågan är därför berättigad. Under de senaste 15 åren har flera internationella rapporter gjort gällande att antalet allvarliga infektioner med *H. influenzae* är på väg att öka igen, men nu orsakat av andra typer än just typ b som vaccinationen är riktad mot.

Det huvudsakliga syftet med detta arbete var att ta reda på om antalet allvarliga infektioner (blodförgiftningar eller hjärnhinneinflammationer) orsakade av *H. influenzae* ökat i Sverige sedan mitten av 1990-talet, vilka bakterieisolat som orsakat dessa infektioner, och vad som kännetecknar de patienter som drabbades. Samtidigt undersöktes utvecklingen av antibiotikaresistens hos de bakteriestammar som orsakar allvarliga infektioner. I laboratoriet undersöktes dessutom två faktorer hos bakterierna som kan bidra till att infektioner blir allvarliga; förmågan att fästa sig fast vid komponenter i den bindväv som finns under slemhinnan i luftvägen, och förmågan att avstyra hotet från människans komplementsystem.

Våra resultat visar att det har skett en ökning av allvarliga infektioner orsakade av *H. influenzae* i Sverige sedan 1997, men att denna ökning har drabbat företrädelsevis äldre vuxna. Hos små barn syns ingen ökning, och den farligaste varianten av bakterien, typ b, är fortsatt mycket ovanlig. Ökningen orsakades istället av *H. influenzae* som saknar kolhydrathölje och som därför är otypbara, samt av isolat av typ f. Vi kunde konstatera att de infektioner som drabbade friska vuxna ofta blev mycket allvarliga. Vi identifierade samtidigt en ökande resistens mot den vanligast använda gruppen av antibiotika, β-laktamantibioika.

Undersökningar i laboratoriet visade att *H. influenzae* kan binda till bindväven under slemhinnan i luftvägen genom ett ytprotein, Protein E. Denna förmåga bidrar troligen till bakteriens möjlighet att lämna slemhinnan och orsaka djupare, mer allvarliga infektioner. Vi visste sedan tidigare att *H. influenzae* binder komplementreglerproteiner för att undvika att bli själv bli offer för komplementsystemet. Vi kunde dock inte hitta

någon skillnad i inbindningen till komplementreglerproteiner mellan bakterier som orsakat blodförgiftning och bakterier som orsakat sjukdom i luftvägen.

Sammantaget visar resultaten att vaccination mot Hib fortsatt mycket framgångsrikt förebygger allvarliga infektioner hos små barn, men att övervakningen av *H. influenzae* bör fortsätta. Bland gruppen icke typbara *H. influenzae* ökar både antalet allvarliga infektioner hos vuxna och andelen isolat med resistens mot antibiotika.

Thesis at a glance

Paper	Objective	Method	Result
Paper I	To determine the interactions of *Haemophilus influenzae* Protein E with the extracellular matrix	A laboratory study of protein- protein interactions	Protein E can simultaneously bind laminin and vitronectin, facilitating the colonization of host tissue.
Paper II	To determine the binding to complement regulators of invasive compared with nasopharyngeal isolates of non-typeable *H. influenzae*	A laboratory study with related patient data.	The binding to complement regulators did not differ between invasive and non-invasive NTHi, but serum resistance was linked to disease severity.
Paper III	To determine the epidemiology and clinical outcome of invasive *H. influenzae* disease in Sweden 1997-2009	A retrospective epidemiological study	An increased incidence of invasive *H. influenzae* was observed, but attributed to non-typeable isolates in individuals above 60 years of age.
Paper IV	To determine the epidemiology, mechanisms and phylogeny of invasive β-lactam resistant *H. influenzae* in Sweden 1997-2010	A retrospective study of epidemiology, phylogeny and resistance mechanisms	An increase of β-lactamase-negative, β-lactam resistant isolates was identified, as well as a clonal expansion of such isolates.
Paper V	A report of a case of severe invasive disease by a *H. influenzae* type f including laboratory studies.	A case report including laboratory studies	The case is presented, and speculations on the impact of the identified IgG3 subclass- and MBL-deficiency of the host are made.

Introduction

A brief medical history of *Haemophilus influenzae*

"Having first covered the fire with care, the man of mighty labors sought repose; but it came not, as it long had been wont to do, to comfort and restore after the many and earnest occupations of the well-spent day. The night was passed in feverish restlessness and pain.... The manly sufferer uttered no complaint, would permit no one to be disturbed in their rest on his account, and it was only at daybreak he would consent that the overseer might be called in, and bleeding resorted to. A vein was opened, but no relief afforded. Couriers were dispatched to Dr. Craik, the family, and Drs. Dick and Brown, the consulting physicians, all of whom came with speed. The proper remedies were administered, but without producing their healing effects; while the patient, yielding to the anxious looks of all around him, waived his usual objections to medicines, and took those that were prescribed without hesitation or remark. The medical gentlemen spared not their skill, and all the resources of their art were exhausted in unwearied endeavors to preserve this noblest work of nature....He spoke, but little. To the respectful and affectionate inquiries of an old family servant, as he smoothed down his pillow, how he felt himself, he answered 'I am very ill'. To Dr. Craik, his earliest companion-in-arms, longest tried and bosom friend, he observed. 'I am dying, sir - but am not afraid to die..."

The quote is an eyewitness recount from the 19-year old George Washington Custis [1] at the deathbed of his adoptive father, the founding father of the United States; George Washington. George Washington died later in the evening from what is believed to have been acute epiglottitis [2]. It cannot be proven, but it can be suspected that George Washington died from an infection by *Haemophilus influenzae*, the dominant cause of epiglottitis prior to the vaccine era.

It will not be possible to say if the link between George Washington and *H. influenzae* is correct, and the bacterium has led scientists to incorrect assumptions before. Although he most likely exhausted the resources of his art in unwearied endeavors, the German physician Richard Pfeiffer made one of the most famous incorrect interpretations of data based upon *H. influenzae*. He frequently observed Gram-negative coccobacilli in sputum samples from patients of the influenza pandemic 1889-1892, and concluded that the bacterium was the cause of influenza [3]. He was credited for the discovery in 1896, and the coccobacillus became known as the Pfeiffer bacillus or *Bacillus influenzae*. Many misinterpretations in medicine have been made and have

been forgotten, but this one is perpetuated in the name of the bacteria, which has not been changed. To be fair, Richard Pfeiffer went on to do pioneering research, as he was the first to identify endotoxins from bacteria, the first to isolate *Micrococcus catharralis* (now *Moraxella catharralis*) and developed an early vaccine against typhoid fever.

Some credit the founder of modern bacteriological technique (and also the teacher of Richard Pfeiffer and the man who first isolated *Mycobacterium tuberculosis*), the German bacteriologist Robert Koch, on first isolating *H. influenzae*. This is partly correct. The observations that Koch made in the summer of 1883 (while on a mission to Egypt to unravel the agent of cholera during a cholera outbreak there) on bacteria from purulent conjunctivitis by travellers to Egypt [4] were on the Koch-Weeks bacillus, or *Haemophilus aegyptius*, the same species that the American ophthalmologist John Elmer Weeks in 1886 separately noted as the cause of 'pink eye' [5]. *H. aegyptius* is a close relative to *H. influenzae* biogroup aegyptius, the cause of Brazilian Purpuric Fever, a severe invasive infection that often includes conjunctival symptoms.

The pandemic influenza of 1918-1920 shook the world, causing at least 40 million deaths worldwide. This sparked intense research activity on the proposed cause of influenza, *Bacillus influenzae*. At this time, the bacterium got its present name [6], although it took some time before it caught on. A publication from the Rockefeller University, New York City, questioned the bacterial doctrine of influenza in 1921. In experiments, bacteria were filtered away from specimens of 1919 influenza patients, and the remaining solution still managed to cause severe disease in animal experiments [7]. One of the challenges that made research on *H. influenzae* in the early 20th century difficult was to isolate it in culture without contamination of other upper airway bacteria. Across the Atlantic, in 1929, Alexander Fleming suggested a solution to the problem in one of the most famous scientific manuscripts of all time: "*On the antibacterial action of cultures of a Penicillium, with a special reference to their use in the isolation of* B. influenzae" [8]. At the time, Fleming believed that the foremost use of his finding was a methodological one. By adding penicillin to agar plates, he could separate *Haemophilus influenzae*, which grew on the plates, from non-pathogenic Gram-positive agents in cultures from the respiratory tract. We now know that penicillin has other uses as well, and influenza has another cause. We also know that penicillin can actually be active against *H. influenzae*, but only at high concentrations.

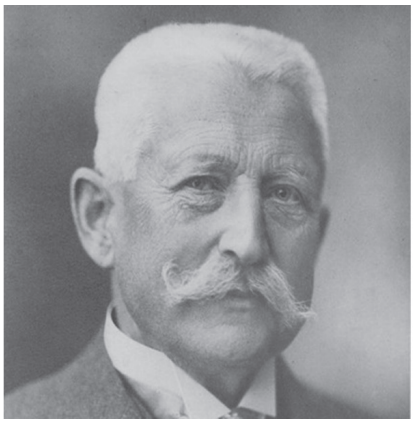



Figure 1. Pictures of Richard Pfeiffer (left) and Margret Pittman (right). Picture of Richard Pfeiffer from the homepage http://historyofvaccines.org courtesy of The college of Physicians of Philadelphia, Philadelphia, Pennsylvania, USA. Picture of Margret Pittman, courtesy of the Office of history, National institutes of Health, Bethesda, Maryland, USA.

The work on *H. influenzae* continued at the Rockefeller University, and in 1931 the 30-year old American bacteriologist Margret Pittman published a paper in which she noted that strains of the "Pfeiffer bacilli" that caused invasive disease had a different appearance on agar plates. She referred to these as S strains (smooth), and found that these strains precipitated with antisera. She separated them from the normal R strains (rough) that did not precipitate. In this pioneering work, Pittman identified two different serotypes that reacted with different antisera; types a and b. [9]. We now know that the "S" strains are encapsulated and that "R" strains are non-encapsulated. The serotypes were later extended to a number of six, a-f. Already in Margret Pittman's manuscript from 1931, serotype b was identified as responsible for almost all cases of "influenza meningitis", the severe cases of disease. In 1933, the influenza virus was isolated [10], and the research activity on *H. influenzae* was rather low until the early 1970's, when rising incidences of invasive disease were observed.

A very different one, but still a major scientific breakthrough came in 1995, when Craig Venter and colleagues at TIGR (the Institute for Genomic Research) fully launched the genomic era. A technique called shotgun sequencing allowed the first full-genome sequence and assembly of a free-living organism. The first organism to be sequenced was an isolate of *Haemophilus influenzae* [11].

Even though the work by Alexander Fleming was the first step towards effective treatment of bacterial infections, the work of Margret Pittman was the first step in the long path towards the development of a *H. influenzae* type b vaccine, which now saves the lives of thousands every year. The "man of mighty labors", George Washington, would probably have endorsed this development judging by his famous quote:

> "*Experience teaches us that it is much easier to prevent an enemy from posting themselves than it is to dislodge them after they have got possession*"

Basic facts on *Haemophilus influenzae*

Bacteriology

Haemophilus influenzae is a Gram-negative, pleomorphic, but generally rod-shaped bacterium. It is a member of the genus *Haemophilus,* which belongs to the *Pasteurellaceae* family. While most members of the *Pasteurellaceae* family are animal pathogens, *H. influenzae* is, to current knowledge, exclusive to humans [12]. It is defined by and named after its *in vitro* growth requirement of blood component factor V (NAD) and an iron source such as factor X (Hemin) [13]. It is a facultative anaerobe, i.e. it can grow with or without oxygen supply. There are six serotypes of encapsulated *H. influenzae* (a-f) which all can be typed using specific antisera. Most isolates have no polysaccharide capsule, and cannot be serotyped (NTHi; non-typeable *H. influenzae*). Based on enzymatic characteristics of bacterial strains, a system of eight different biotypes of *H. influenzae* (I-VIII) was defined by Kilian and followers [14-16].

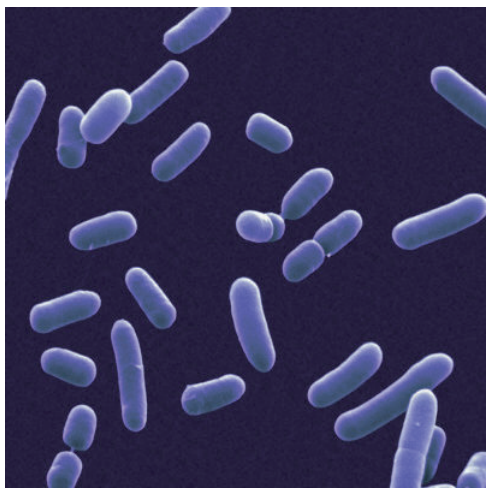


Figure 2. A Scanning electron microscopy (SEM) photograph of *Haemophilus influenzae*, demonstrating the pleomorphic rods © IBL bildbyrå. Reprinted with license.

General clinical characteristics

The spectrum of disease caused by *H. influenzae* is largely dependent on bacterial serotype, but some features are general to the species. *H. influenzae* is exclusive to humans, and rely on effective person-to-person transmission. It is spread through infected respiratory droplets, and isolates have been shown to survive for 18 hours on tissue cloth [17]. *H. influenzae* is with rare exceptions a colonizer of the human pharynx and this colonization is the first step in the pathogenesis of almost all *H. influenzae* infections. One of the rare exceptions is the capacity to colonize the urogenital epithelium and

cause urogenital infections [18]. Recently, *H. influenzae* has been implied as an occasional agent of hepatobiliary infection [19].

Encapsulated *Haemophilus influenzae*

Encapsulated *H. influenzae* are more virulent and more frequently associated with invasive disease than nonencapsulated variants. There are six different serotypes, a-f, but *H. influenzae* type b (Hib) is the most widely known. Invasive infections by Hib were common world-wide prior to the introduction of the conjugated Hib vaccine, and invasive Hib infections are still common in countries where the vaccine has not been implemented [20]. By the end of 2010 this is mainly the case in eastern Europe and Asia [21]. The most common clinical manifestations of invasive Hib disease include pneumonia, meningitis and epiglottitis, and children of ages 3 months - 4 years used to be most commonly affected. Less common manifestations reflect a hematogenous phase, and include cellulitis, arthritis, pericarditis and ostheomyelitis [22, 23]. Hib meningitis can occur through direct spread from the middle ear or through a hematogenous phase [24].

Infections by non-type b encapsulated *H. influenzae* are less well characterized, and have occurred at much lower rates than Hib infections. Some reports indicate that encapsulated *H. influenzae* type a mimic Hib in virulence [25], while infections by encapsulated isolates of type e and f are generally less severe [26-28]. There is very limited information on infections by *H. influenzae* type c and d.

Non-encapsulated *Haemophilus influenzae* – the NTHi

Non-encapsulated isolates of *H. influenzae,* the nontypeable *H. influenzae* (NTHi), are naturally more competent for uptake of external genetic material [29, 30] and prone to transformation. This makes NTHi a heterogeneous group of bacteria, and as a consequence the capacity to cause disease is variable within the group [31, 32]. Although the numbers change with region and season, the pharyngeal carriage rates of NTHi in healthy, pre-school and school children are very high [33-35]. This suggests that most NTHi in the upper respiratory tract are commensal. One of the challenges that *H. influenzae* still poses to a clinician reflects the problem that Richard Pfeiffer encountered in 1892; how to correctly evaluate the significance of an airway culture positive for NTHi during the course of a respiratory infection.

On the other hand, it is clear that NTHi isolates often cause disease, generally upper airway infections such as otitis media, conjunctivitis and sinusitis [36]. NTHi are opportunists, and commonly cause infections in situations where the protection systems in the airway are breached, following viral infections or in patients with Chronic Obstructive Pulmonary Disease (COPD). In COPD patients, *H. influenzae* is the most common bacterial finding in infectious exacerbations [37]. In acute otitis media, NTHi is the second most common pathogen [38]. NTHi can cause occasional cases of inva-

sive disease such as sepsis and meningitis, which was noted already in 1939 [39], but the general view is that this occurs in predisposed individuals with concurrent illness or in the extreme ages of life (neonatal or geriatric) [40, 41]. Urogenital infections by NTHi are rare, but in pregnant women such infections can cause preterm delivery and neonatal sepsis in the newborn. *H. influenzae* has been suggested as the third most common agent of neonatal sepsis [42].

A few studies have proposed a beneficial role for NTHi, since NTHi colonization can outcompete and help clear pneumococcal infections in animal models [43]. *H. influenzae* is not an animal pathogen, but the presence of the bacteria in respiratory tract cultures has been observed to be negatively correlated with the presence of *Streptococcus pneumoniae,* and this provides indirect support for the controversial suggestion [44]. It might be suggested that NTHi is a friend rather than a foe, but is not to be fully trusted.

Host defense systems

On a daily basis, the human body is exposed to microorganisms and pollutants. In all areas of exposure, such as the airway, the skin and the gastrointestinal tract, the tissues are adapted to handle this exposure. To be able to understand the pathogenesis of invasive (and noninvasive) disease by *H. influenzae*, it is necessary to briefly overview the relevant host defense mechanisms in the human respiratory tract. The mechanisms are highly simplified in the text and illustrations (**Figure 3**).

Local and general innate immunity

The mechanical barrier and cilia

The respiratory tract is lined by a pseudostratified columnar epithelium, a single layer of non-aligned cells. The three main cell types are ciliated epithelial cells, goblet cells and basal cells. The epithelium provides mechanical integrity towards microorganisms through tight junctions between cells. A "mucociliary escalator" keeps the airway sterile. In short, goblet cells produce sticky mucus, which organizes on top of the epithelium. Microorganisms and pollutants get stuck in the mucus, and the cilia of the airway propel the mucus towards the pharynx, where it is swallowed or expectorated [45].

Antimicrobial peptides (AMPs)

The respiratory epithelial cells also contribute in immune responses by producing antimicrobial peptides. Many of these peptides are further produced by neutrophils and monocytes that are recruited during inflammation. Lysozymes exert enzymatic activity on the peptidoglycan layer of bacteria and are mainly active against Gram-positive species [46]. Lactoferrin can inactivate IgA protease and degrade the *Haemophilus* adhesion and penetration protein (Hap) of *H. influenzae* and thereby disrupt bacterial adhesion [47]. The

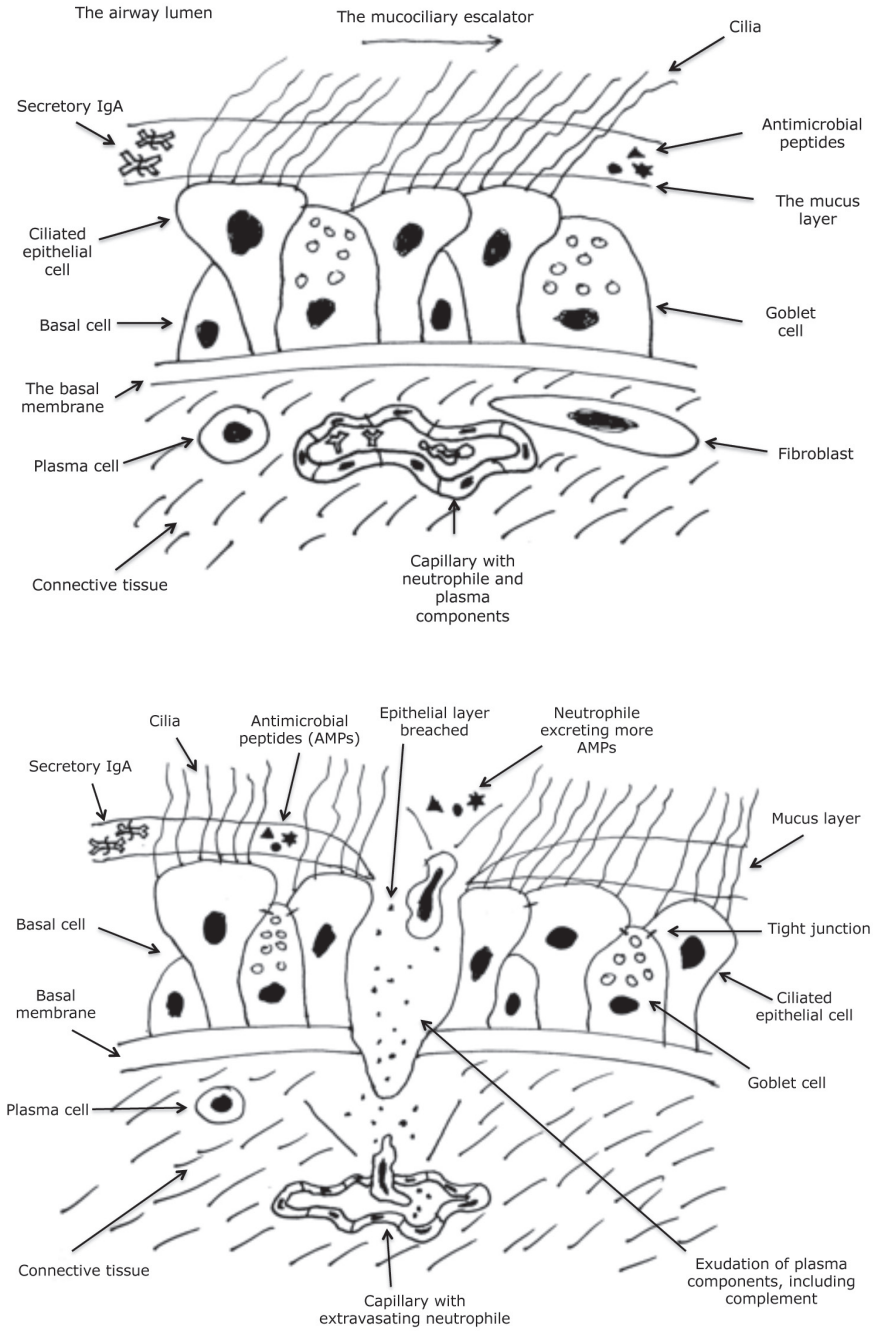


Figure 3. Schematic, simplified sktech of the orgainzation of the airway epithelium, and the components of the local immune defense. In a normal situation (upper sketch) and during tissue breaching and inflammation (lower sketch).

cathelicidin LL-37 permeabilizes the bacterial membranes and inhibits bacterial protein synthesis [48]. β-defensins are cationic and permeabilize anionic bacterial membranes and are chemotactic for dendritic cells and memory T cells. Human β-defensin-2 effectively inhibits growth of respiratory pathogens *in vitro* [46, 49, 50].

Inflammation

Inflammation can be elicited through either direct stimulation of epithelial cells or through phagocytic cells, mainly macrophages. Stimulated epithelial cells activate the immune response through the production of cytokines and chemokines. Neutrophils are recruited from the blood through an IL-8 gradient. When microbes are present, macrophages bind them through specific macrophage receptors and internalize them into phagosomes [51]. Normally a fusion of the phagosomes with lysosomal granules ensues and the microbe is killed. Stimulated macrophages and neutrophils release reactive oxygen intermediates and proteolytic enzymes that recruit more inflammatory mediators, but also potentially damage the epithelium and hamper the mucociliary escalator.

The complement system

The complement system is traditionally a part of serum. During inflammation in the respiratory tract, complement proteins reach the airway through plasma exudation [52]. The complement system is complex, and the following is a simplified description, supported by a schematic illustration (**Figure 4**). The system consists of three pathways that all end with enzymatic cleavage of C3, formation of the Membrane Attack Complex (MAC) and the subsequent lysis of the bacteria. The classical pathway of the complement system is activated by the C1q complex that binds to IgG or IgM antibodies attached to the surface of a pathogen, and is dependent on functional antibodies. The alternative pathway is directly activated by pathogens and is antibody independent. The lectin pathway is a special variant of the classical pathway and is initiated by a human lectin that binds to mannose or other carbohydrates on microbial surfaces. When binding occurs, the mannose binding lectin (MBL) forms a complex with a serine protease (MASP). This complex is structurally similar to the activated C1 complex in the classical pathway and follows this pathway in downstream reactions [53, 54]. The complement system is potentially harmful to the host, and is tightly regulated. The regulatory system can be used by pathogens, and this will be further discussed in subsequent chapters.

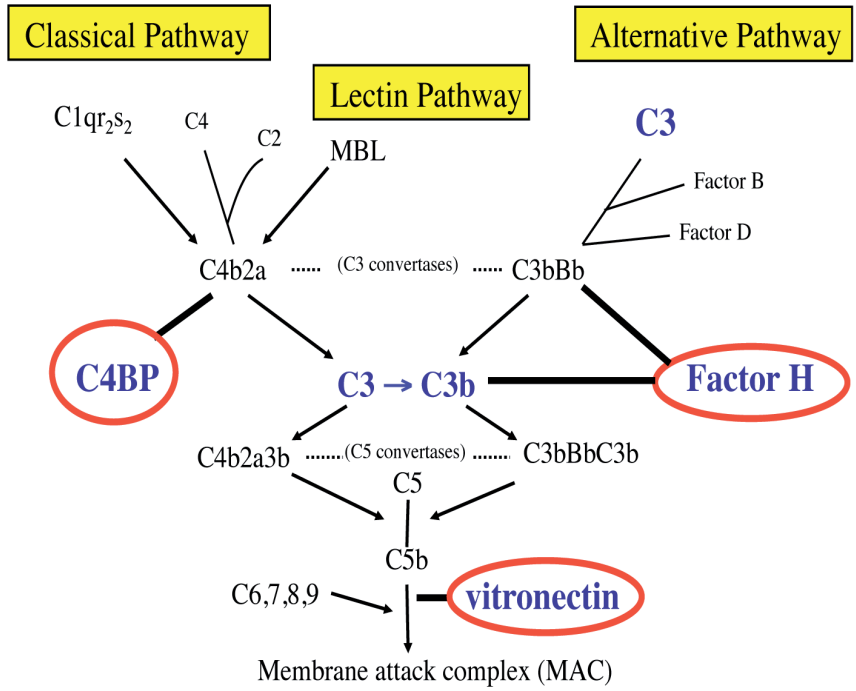


Figure 4. Schematic illustration of the complement system, including a demonstration of the effector sites of the complement regulators included in the study (inside the red rings).

Adaptive immunity

Secretory IgA

IgA is the most abundant immunoglobulin in the body. IgA is present at most mucosal surfaces and function as a specific gatekeeper of the mucosa. In the airway, IgA1 is the dominant subtype. IgAs directed against microbial antigens are produced by nearby plasma cells, bind to the polymeric Ig-receptor (pIgR) on epithelial cells, through which they are passaged and secreted into the airway. Secretory IgA does not active the complement cascade, but agglutinates colonizing bacteria and prevents effective epithelial adherence [55].

Production of specific antibodies

The production of antigen-specific antibodies is an intricate process. Briefly, the process involves antigen-presenting cells (APCs), T-cells and B-cells that mature to plasma cells. Upon primary challenge by an antigen, the production of antibodies with high avidity takes weeks. However, the effector T- and B-cells can differentiate into memory cells, which respond rapidly upon secondary stimulation with the same antigen.

Antibodies, immunoglobulins (Igs) are produced by plasma cells and have two main parts; the highly variable Fab region that binds to the antigen and the Fc region that mediates effector functions such as phagocytosis or complement activation. Five classes of Igs exist; IgA, IgD, IgE, IgG and IgM. IgA and IgG are separated into two and four subclasses respectively. IgG2, and possibly IgG1 antibodies are considered essential for protection against infections with encapsulated bacteria such as *H. influenzae* type b [56]. IgG2-deficiency has been suggested to explain the high incidence and poor vaccine reply in ethnic groups with unusual high incidences of invasive Hib disease [57]. The antibody-mediated protection against NTHi does not seem equally dependent on functional IgG2, and antibodies produced following stimulation by one NTHi isolate offer substantial cross-protection against other NTHi [58].

Pathogenesis of invasive *Haemophilus influenzae* disease

A series of events take place before an invasive infection by *Haemophilus influenzae* is established. The bacterium needs to reach, adhere to, persist in and invade through the respiratory epithelium. Essential factors that *H. influenzae* employs at each step are reviewed here, and are illustrated in **Figure 5**. The mechanisms that are presented are common to encapsulated and nonencapsulated isolates, unless specifically stated.

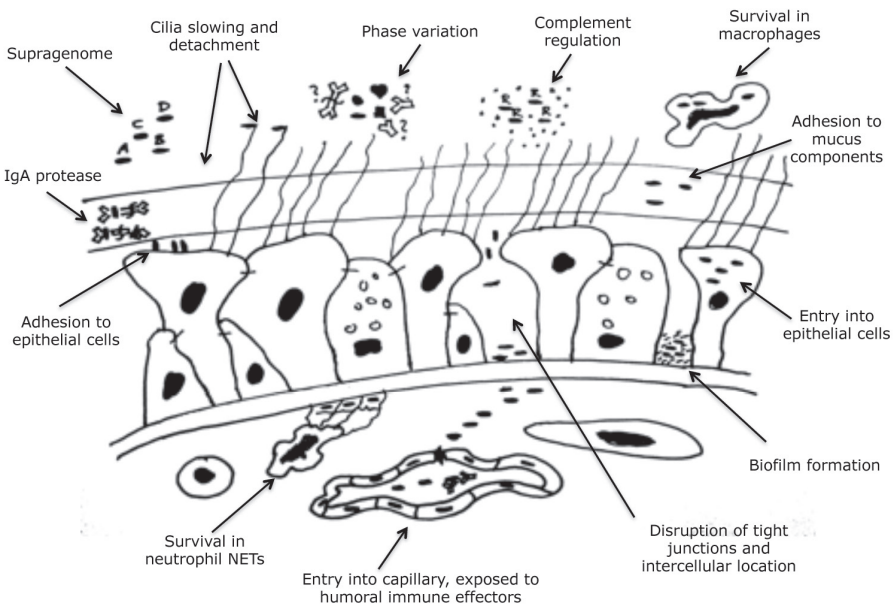


Figure 5. The sketch offers a simplified overview of the mechanisms by which *Haemophilus influenzae* overcomes the immune effectors of the human airway, and demonstrate parts of the pathogenesis of invasive disease by the bacterium.

Step 1: Accessing the airway epithelium

In order to colonize and eventually invade the epithelium, the bacterium first has to access it. The respiratory epithelium is effectively shielded by the mucus layer, which offers mechanical protection and contains active components of the immune defense.

Disrupting the mucociliary escalator

The majority of bacteria that reach the airway are cleared by the mucociliary escalator. In situations when this system is hampered, by cigarette smoke, by viral infections or by diseases such as cystic fibrosis, the risk of colonization and infection by *H. influenzae* is increased. *H. influenzae* itself can affect ciliary beating and cause ciliary detachment, which has been confirmed in cell culture studies [59]. The cilia damage is suggested to be mediated by the lipooligosaccharide (LOS), but the effect is also seen when supernatant culture fluid lacking viable bacteria is added [60]. Histology studies suggest that the effect is mediated by cellular injury to the ciliated cells, and detachment of cilia is only the first step in a series of events, ending in extensive epithelial damage. Mutant strains lacking Protein D do not cause the same extent of damage to cilia [61]. Since Protein D is needed for effective phosphorylcholine acquisition to the LOS [62], the two observations may be linked.

IgA proteases

Secretory IgA1 is the main element of adaptive immunity in the airway, and is secreted in high quantity to the mucus layer coating the epithelium. A factor believed to distinguish pathogenic *Haemophili* is the ability to cleave and degrade secretory IgA1 through IgA1-proteases. This is a trait common to most successful colonizers of the respiratory tract, including *S. pneumoniae* [63]. Two genes coding for IgA1 proteases have been identified in *H. influenzae*, and it is believed that isolates carrying both genes have a higher protease activity, a trait linked to the capacity to cause disease [64, 65].

Step 2: Adhering to the airway epithelium

Another step towards disease is effective attachment to the epithelium. *H. influenzae* attaches to epithelial cells in the upper airway, and preferably to damaged epithelium [66]. A range of different adhesins have been identified and shown to attach to different components of the epithelium. The ones that are best characterized are reviewed here and summarized in **Table 1**.

Major adhesins of Haemophilus influenzae

The genes coding for hemagglutinating pili/fimbiae are present in nearly all Hib isolates, but only on a subset of NTHi. The pili agglutinate erythrocytes through the

Anton antigen [67], but also bind epithelial cells and mucin. The pili are important in the early phase of infection, and help establish adhesion to host cells [68]. However, pili are immunogenic and piliated bacteria are highly susceptible to antibody recognition and killing by normal human serum. Most Hib strains isolated from blood have lost their pili expression [69].

High Molecular Weight adhesins (HMW-1 and HMW-2) are considered as the major non-pilus adhesins of *H. influenzae,* and are present on approximately 75% of NTHi. Despite significant homology, the two HMWs have different ligands. The ligand of HMW-1 is sialylated glycoproteins, while the ligand of HMW-2 is still undefined [70].

Haemophilus surface fibrils (Hsf) are large, non-pilus proteins that are found in most encapsulated *H. influenzae*. Hsf bind to Chang epithelial cells [71] and to vitronectin. Vitronectin, an extracellular matrix component, is also a regulator of the common final pathway of the complement cascade. Vitronectin-binding through Hsf confers serum resistance [72]. *Haemophilus influenzae* adhesin (hia) and cryptic haemophilus adhesin (cha) are two homologs of Hsf. Most NTHi isolates that do not express HMW's express hia. Hia confer binding to respiratory epithelial cells, although the exact ligand is not known [73]. Cha is expressed on isolates of the *H. influenzae* biotype IV cryptic genospecies associated with urogenital colonization and infection. Isolates expressing the cha adhesin can bind to genital as well as respiratory epithelial cells, but the exact ligand is not defined [74].

Haemophilus influenzae adhesion and penetration protein (Hap) adheres to components of the extracellular matrix [75]. The binding domain of Hap is normally released from the cell surface, but can remain cell-associated through the aid of a host antimicrobial peptide, and through this interaction, the bacterial adhesion capacity is increased [76]. P2 and P5 are both major outer membrane proteins expressed on almost all known isolates of *H. influenzae*. They both bind to respiratory mucin [77]. While the lipoprotein P2 is a porin that has adhesive properties and variable, surface-exposed regions [78], P5 is mainly an adhesin and binds to multiple ligands [79]. Both are immunogenic, and assumed essential for *H. influenzae* virulence. Both have been implied as targets for new *H. influenzae* vaccines [80, 81], as is the case for many of the described adhesins. In animal models, immunization with different adhesins has been successful to varying degrees. Since *H. influenzae* is specific to humans, these results have been preliminary. The only available vaccine variant today with potential protective effect against NTHi is based on Protein D.

Protein D is a highly conserved lipoprotein expressed on all tested isolates of *H. influenzae* [82]. Protein D promotes adherence to and internalization into epithelial cells [83]. Immunization with Protein D followed by middle ear and pulmonary challenges in rats led to enhanced clearance of NTHi [84]. Protein D is currently used as a peptide conjugate in an established pneumococcal vaccine, Synflorix®, a vaccine that besides the protection against invasive pneumococcal disease has protective effect against *H. influenzae*-associated acute otitis media. A protective effect against invasive *Haemophilus* infections has not been established [85].

Table 1. Major *Haemophilus influenzae* adhesins

Protein (*gene*)	**Bind to**	**Eukaryotic ligand(s)**	**Presence**	**Reference**
Hemagglutinating pili (*hifA-E*)	Erytrocytes, buccal cells and mucin	AnWj and glyoconjugated receptors	Hib, some NTHi	[67, 86]
HMW 1 *(hmw1)* HMW 2 (*hmw2)*	Chang conjunctival cells	Glycoprotein receptors	75% of NTHi	[87, 88]
Hsf (*hsf*)	Epithelial cells	Vitronectin	Encapsulated *H. influenzae*	[71]
hia (*hia*)	Epithelial cells, Chang cells	?	A subset of NTHi (25%)	[89]
Cha (*cha*)	Vaginal epithelial cells	?	Cryptic genospecies biotype IV	[74]
Hap (*hap*)	Extracellular matrix, epithelial cells	Fibronectin, laminin, collagen IV	Present in all *H. influenzae*	[73, 75]
P2 (*Omp P2*)	mucin	Glycoproteins, sialic acid coated	Present in all *H. influenzae*	[77]
P5 *(Omp P5)*	mucin	ICAM-1, CEACAM-1	Present in all *H. influenzae*	[79, 90]
Protein D (*hpd*)	Mucin, epithelial cells	PAF receptor	Present in all *H. influenzae*	[91]
Protein E (*pe*)	ECM, epithelial cells	Vitronectin, laminin, plasminogen	Present in all *H. influenzae*	[92, 93]

Protein E

Protein E is a low molecular weight lipoprotein that promotes adherence to epithelial cells. Like Protein D, it was first described in Malmö [94]. It is expressed in almost all clinical isolates of *H. influenzae* and is highly conserved in encapsulated as well as non-encapsulated isolates. Upon binding to epithelial cells, Protein E induces an inflammatory response and upregulation of ICAM-1 [95]. Like Hsf, Protein E binds vitronectin, and this interaction is important for resistance to complement-mediated killing by human serum [96].

Step 3: Persisting and evading the host defense

Bacteria and humans have co-evolved over thousands of years. In order to successfully persist in the human host, *H. influenzae* has, like other successful tissue colonizers, developed a number of strategies to evade the host defense effectors of the respiratory tract. It is unclear as to what relevance these respective factors have for the pathogenesis of invasive Hib disease, since Hib isolates are rarely found as pharyngeal colonizers. Invasive disease by Hib in children is suggested to occur by bacterial translocation directly through the epithelial cell layer to capillaries in the nasopharynx without need for concurrent mucosal infection [97].

The supragenome factor

One feature of *H. influenzae*, a feature in common with other effective tissue colonizers, is the existence of a "supragenome". The human airway is colonized with several different isolates of *H. influenzae* at the same time, as demonstrated by studies on colonization dynamics of adult carriers [98]. Since the isolates readily interchange genetic material through horizontal gene transfer and transformation, the group of isolates can adapt to changes in the surrounding microenvironment. This increases the possibility of survival of the group as a whole [99].

Aggregation and biofilm formation

Bacterial aggregation, or flocculation, is a factor believed to promote bacterial population survival. The Hap adhesin is known to promote bacterial aggregation [76].

Most studies suggest that NTHi can form biofilms, a capacity that is considered paramount in recurrent otitis media. The expression of a range of adhesins is necessary for biofilm formation [100], as well as a reduced inflammatory response mediated by modifications of the lipooligosaccharide (LOS) [101, 102]. NTHi in biofilms have demonstrated ability to resist neutrophil killing [103]. However, the capacity of *H. influenzae* to form biofilms has been an issue of controversy [104].

Phase variation

One way to avoid the effectors of adaptive immunity is to modify and vary surface-exposed molecules, a "moving target" strategy. While surface-exposed molecules such as adhesins are necessary for adhesion and colonization, they are often immunogenic and make the bacteria susceptible to recognition and subsequently killing by the adaptive immune response. The surface expression of some such adhesins can be turned on and off. This process is called phase variation, and it is generally reversible. It is made possible by repetitive DNA sequences prone to slipped-strand mutations. *H. influenzae* can vary the surface expression of the lipooligosaccharide (LOS) [105], hemagglutinating pili [106] and the High-Molecular Weight adhesins [107]. The most apparent example of phase variation is the case of hemagglutinating pili in Hib isolates. Even though almost all Hib isolates carry the pili gene cluster, and use the pili to attach to the human airway, hardly any Hib isolate from blood expresses it. To simplify matters, the isolates that are successful at phase varying pili avoid antibody detection in serum, and as a consequence survive in the bloodstream. The LOS can also vary through post-translational modifications such as the addition or subtraction of phosporylcholine [108]. Another process that occurs during disease progression is the antigenic drift of surface-exposed molecules such as the P2 porin [109]. This is also a moving target strategy, but this process is irreversible.

Complement regulation

The complement activity of human serum is crucial in controlling invasive infections by *H. influenzae,* as confirmed by studies using C3-depleted serum [110]. The resistance of the type-b capsule to complement-mediated killing is regarded as one of its central virulence determinants [111]. The complement system in humans is tightly regulated by regulatory proteins. These regulators prevent tissue damage by an excessive activation of the complement cascade. Such regulatory proteins include C4b-binding protein, a regulator of the classical pathway, Factor H, a regulator of the central C3 convertase, and vitronectin, a regulator of the common final pathway (**Figure 4**). Several species of bacteria use this system to their advantage, by adhering to regulatory factors of the complement system and consequently increase their resistance to complement-mediated killing [112]. Different isolates of *H. influenzae* can bind to all of these regulators at varying degrees [72, 96, 113, 114]. Through these interactions, *in vitro* bacterial survival in human serum is increased.

Alteration of the adaptive immune response

The great majority of healthy individuals as well as those with chronic airway disease have strong serum antibody responses to non-typeable *H. influenzae*, and this is believed to be the reason why these bacteria rarely cause invasive infections despite high tissue invasion capacity *in vitro*. Normally, the inflammatory response to NTHi challenge is mediated by Th1 cytokines. However, in inflammation and during chronic conditions such as COPD or bronchiectasis, the macrophage-orchestrated inflammation and the T-cell response to stimulation of a new *H. influenzae* isolate is skewed. Phagocytosis becomes impaired, and the T-cell response can be redirected towards a Th2 response with a different Ig subclass profile [115]. As a consequence, chronic inflammation ensues and the bacteria are less well cleared [116-118].

Step 4: Tissue, cell and bloodstream/meningeal invasion

Whereas the factors that contribute to epithelial colonization and persistence are becoming increasingly clear, it is less clear as to what bacterial and host factors affect invasive disease capacity of *H. influenzae*. Most studies have been performed in the pre-Hib vaccine era, and mainly on invasive Hib disease.

Intra- and intercellular invasion

H. influenzae can "hide" by residing in compartments (inter- and intracellular) where they evade host immune effectors. *In vitro* studies show that *H. influenzae* pass through epithelial cell layers, and reside embedded in or below the epithelium. This capacity is independent of adhesive capacity, presence of fimbriae or a polysaccharide capsule [119], and is suggested to occur through disruption of intercellular tight junctions and

a positioning in-between the bases of epithelial cells (paracytosis) [120]. This positioning shields the bacteria from antibiotics as well as antibody-mediated attacks [121], and has been confirmed in histology studies of bronchial tissue [122]. The ligand/ligands in the intercellular position is not clear. From studies, it seems as though the capacity of paracytosis differs between different isolates of *H. influenzae,* but it is unclear as to what bacterial properties are necessary. Since the process is inhibited by protein synthesis inhibitors, *de novo* protein synthesis seems necessary.

H. influenzae can enter cultured epithelial cells [123] and monocytes [124]. In bronchoscopy-directed bronchial biopsies from patients with chronic bronchitis, the presence of intracellular *H. influenzae* correlated with clinical signs of infectious exacerbation [125]. The implications of intracellular viable *H. influenzae* in macrophages [126] are interesting, and the ability to survive inside macrophages has been correlated to the capacity of invasive disease by Hib [127].

Bloodstream and meningeal interactions

The interactions of *H. influenzae* with endothelial cells have been studied *in vitro*. The capacity to associate with and invade endothelial cells is linked to bacterial concentration, but is inversely related to the presence of a polysaccharide capsule [128]. Studies show that both encapsulated and non-typeable *H. influenzae* can pass through a monolayer of endothelial cells by disrupting endothelial tight junctions [129]. Studies of *H. influenzae* interactions with *in vitro* constructed blood-brain barriers and blood-pulmonary barriers show that *H. influenzae* can, in the presence of complement-inactivated serum, cause cytotoxic effects on these barriers, effects that are inhibited by blocking LOS interactions or removing serum [130].

The role of the polysaccharide capsule

The polysaccharide capsule of encapsulated *H. influenzae* is believed to stabilize the bacteria in the environment and facilitate transmission of bacteria between individuals. Encapsulated isolates adhere to mucus, but *in vitro* studies have shown them to have relatively poor epithelial adhesive and epithelial translocation capacity compared with non-encapsulated counterparts [131]. The primary function of the capsule is to provide a shield against opsonophagocytosis and complement-mediated killing; an effect important in bloodstream infections and during inflammation [110, 132]. The capacity of encapsulated isolates to withstand the humoral defense and allow replication in the bloodstream is superior to NTHi [133, 134].

In a rat model of invasive disease, the virulence of the respective capsule type was assessed by the nasal inoculation of six transformants of *H. influenzae* that were isogenic apart from the capsule locus (a-f). All rats infected with type b capsule transformant developed invasive disease. Half of the rats infected with type a capsule transformant developed invasive disease, and a portion of the rats infected with the type f capsule transformant developed invasive disease, while none of the rats expressing c, d or e

capsule did [135]. When the respective transformants were intraperitoneally injected, all rats that were injected with the type b transformant developed meningitis and sepsis. The same hierarchy of virulence persisted in a study of isogenic capsule mutants in complement-deficient rats, suggesting that the relative virulence of type b isolates is not only dependent on resistance to complement-mediated killing [136]. The studies show that the capsule type is an independent factor of virulence. It was originally believed that the polysaccharide that constitutes the type b capsule, Polyribosyl Ribitol Phosphate (PRP), was especially virulent. In subsequent studies of the capsule gene cluster of type b isolates, it has been established that most invasive type b isolates carry a partial capsule gene duplication that stabilizes a multimer capsule complex [137]. This allows production of a thicker polysaccharide capsule, which is believed to confer improved resistance to phagocytosis and to be the main, but not the exclusive reason for the special virulence of *H. influenzae* type b.

Risk factors for invasive *Haemophilus influenzae* disease

A major risk factor for invasive disease by encapsulated *H. influenzae* is young age. The reason for this is not completely understood, but it seems as though small children do not have the capacity to produce sufficient anti-polysaccharide antibodies [138]. A number of further risk factors for invasive disease by encapsulated *H. influenzae* have been identified. Household crowding, presence of young siblings and day-care attendance have been identified as risk factors related to risk of exposure [139, 140]. The duration of breastfeeding of infants and exposure to cigarette smoke are related to higher risk of disease from normal exposure. Infants that were not breastfed or breastfed for a short duration had a higher risk [141], and infants exposed to cigarette smoke probably have a higher risk, even though results are conflicting [138]. The recognition that individual immunogenetic factors contribute to the relative risk of invasive *H. influenzae* infections was made early [142]. Low levels of IgG2 and IgG4 have been suggested as such factors [57]. Recently, two genetic risk factors associated with Hib vaccine failure have been identified. These are nucleotide polymorphisms in TIRAP, that lead to impaired innate immune response through Toll-like receptor-4, and in IL-10 that lead to a lack of control of local inflammation [143].

Epidemiology of invasive disease by *Haemophilus influenzae*

Before the conjugated Hib-vaccine

Invasive disease by *Haemophilus influenzae* type b used to be common in children 0-4 years of age, although varying incidences between different geographical areas and ethnic groups have been evident. In Scandinavia, the incidence was 30-60 cases/100,000

children < 5 years of age in the 1980's [144-146]. In Great Britain [147], Switzerland [148], Australia [149] and Senegal [150] the incidences were comparable to the ones in Scandinavia. Very high incidences were observed in native populations of Australia [151] and North America [152, 153], while comparatively low incidences were described from Argentina [154] and Hong-Kong [155]. There are few estimates of invasive disease due to non-Hib isolates in children in the pre Hib-vaccine era [156]. From the few studies on invasive *H. influenzae* disease in adults it is clear that Hib was not as dominant in adults as in young children [157, 158].

In Sweden, the incidence of invasive disease by *H. influenzae* in children prior to the Hib vaccine resembled that of other Scandinavian and European countries [145]. Two Swedish pre-vaccine studies include epidemiology on adults. One of these regards only adults, and shows an incidence of 1.1 cases/100,000 individuals [157]. The other study includes all ages, and shows relatively high proportions of invasive cases caused by NTHi and Hif in adults [159].

Vaccination and other interventions

The type b polysaccharide capsule is immunogenic, central for virulence and specific. These traits combine to make it an ideal vaccine candidate. In the 1970's, a polysaccharide vaccine directed against type b *H. influenzae* was tested [160]. The limitations of this vaccine mirrored the epidemiology of Hib disease; small children did not develop protective antibodies using only polysaccharide antigens. In the 1980's, peptide conjugates were added to the polysaccharide vaccine [161], a method that later has been mimicked in the vaccine development for other encapsulated bacteria. Peptide conjugation led to the production of protective anti-polysaccharide antibodies even in small children, and an improved protection upon booster doses regardless of what conjugate was used. The large-scale implementation of conjugated Hib vaccines, starting in the late 1980's, has been extraordinarily successful. A conjugated Hib vaccine was included in the Swedish vaccination program for children in 1992. Following considerable efforts in the 21st century, conjugated Hib vaccines has by the end of 2010 been implemented in 166 countries in the world [21]. However, the vaccine still only reaches about 50% of small children since some densely populated nations (examples of nations include China, India and Russia) have not implemented the vaccine.

Other preventive measures have become all but redundant since the introduction of the conjugated Hib vaccine, but used to include prophylactic antibiotic to siblings and day-care attendants in close proximity to patients [162]. During outbreak situations, the vaccination of pregnant women has been used to protect the newborn [163].

After the conjugated Hib-vaccine

The epidemiology of invasive *H. influenzae* disease has drastically changed since the introduction of the conjugated Hib vaccine. To be more correct, there is abundant evi-

dence that the epidemiology has changed for children < 5 years of age. The evidence of a changing epidemiology among adults is not as compelling. A sharp decline in invasive Hib disease as well as Hib carriage has been seen following implementation of the vaccine [20, 23, 164-166]. Incidence rates of invasive Hib disease plummeted in Sweden as well [167]. The reduced carriage of Hib strains also led to herd immunity with added benefit to non-vaccinated subjects. There was a re-emergence of invasive Hib disease in the early 2000's, primarily in England [168]. It is believed that a combination of host factors [169], bacterial factors [170] and population effects [171] attributed. Apart from this slight "dent", the Hib-vaccine campaign has been a formidable success and invasive Hib disease in the post-Hib vaccine era is very rare [20].

Beginning in the mid 1990's, a few reports have suggested increasing incidences of invasive disease by non-Hib isolates of *H. influenzae*. This has been reported from Canada [172], USA [173], Portugal [174] and England [175, 176], and in one pan-European study [177]. Most of these reports suggest that the increase occurs among individuals with underlying medical conditions, and that the isolates are not clustered (offering less support for the implication of bacterial virulence) [41]. In some reports, an increase of invasive Hif disease is described [27], but Hif seems to resemble NTHi rather than Hib in epidemiology [178]. There are two accounts of invasive disease by *H. influenzae* type a [25, 179], suggesting a virulence capacity that mimics the one of Hib. However, quite a few reports deny any increase of invasive *Haemophilus* disease following implementation of the Hib vaccine [40, 180, 181].

In Sweden, there is comprehensive epidemiology on invasive *Haemophilus* disease until 1994 [167]. There is also a study of the epidemiology of invasive Hib disease 1997-2003, showing no signs of increased incidence [182]. Renewed active surveillance with mandatory reporting of invasive *Haemophilus* disease was started by the Swedish Institute for Communicable Disease Control in 2004, and full surveillance including collection of isolates for capsule typing was started in 2007. There is less information on invasive *H. influenzae* disease in Sweden 1994-2007, a time period where several other countries have reported increasing incidences of non-type b invasive disease.

Antimicrobial resistance of *Haemophilus influenzae*

Antimicrobial resistance testing and breakpoints

Haemophilus influenzae has a wild-type population with a defined range of intrinsic activity of antimicrobial agents [183]. Based on this, susceptibility breakpoints for respective agents have been established. These breakpoints are useful for surveillance, but do not always correlate with clinical outcome of infections. In Sweden, breakpoints are set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and definitely determined by the Swedish Reference Group for Antibiotics (SRGA) and the Nordic committee on Antimicrobial Susceptibility Testing (NordicAST) [184].

Mechanisms of resistance to β-lactam antibiotics

When compared with other Gram-negative bacteria, the outer membrane of *H. influenzae* provides little resistance to intracellular penetration of β-lactams [185]. Due to this, the Minimal Inhibitory Concentration levels (MIC's) for β-lactams are generally lower than for most *Enterobacteraciae*, and as a consequence a more "effective" resistance mechanism is needed for an isolate of *H. influenzae* to become resistant according to breakpoints. In Sweden, as in many other countries, ampicillin is the first choice of treatment for most *H. influenzae* infections. Two mechanisms have been shown to cause clinical resistance to ampicillin and other β-lactams in *H. influenzae*; β-lactamase production and the alteration of Penicillin-Binding Proteins (PBPs) resulting in lowered affinity for β-lactams [186]. A third mechanism; disrupted repression of the acrAB efflux pump, can increase the MIC for ampicillin in isolates with one of the two main resistance mechanisms [187]. Even though only two main mechanisms are described, there is some confusion as how to define isolates with β-lactam resistance. The most commonly used definitions are presented in **Table 2**.

Table 2: Definitions of β-lactam resistant *Haemophilus influenzae*

Acronym	Name	Definition
BLPAR	β-lactamase positive ampicillin resistant	Resistance to ampicillin. Nitrocephine positive.
BLNAR	β-lactamase negative ampicillin resistant	Resistance to ampicillin. Nitrocephine negative.
gBLNAR	genomic β-lactamase negative ampicillin resistant	Any of the following substitutions in PBP-3. Genotype I: Arg517His. Genotype II: Asn526Lys Genotype III: Met377Ile, Ser385Thr, Leu389Phe and Asn526Lys. Nitrocephine negative.
BLPACR	β-lactamase positive amoxicillin-clavulanate resistant	Resistance to amoxicillin-clavulanate. Nitrocephine positive.

BLPAR (β-lactamase positive ampicillin resistant)

Two types of β-lactamases have been identified in *H. influenzae;* TEM and ROB. Both are plasmid-mediated β-lactamases, both confer high-grade resistance to ampicillin, and both are inhibited by β-lactamase inhibitors [188, 189]. Two main types of plasmids that carry β-lactamases in *H. influenzae* are known. Both TEM and ROB β-lactamases can be found on small non-conjugated plasmids, where they generally are the only resistance determinants. TEM can also be found on large plasmids (>10,000bp), which are either integrated in the chromosome or circular [190]. The large plasmids often carry multiple resistance genes, such as for tetracycline or chloramphenicol resistance, and are more common than smaller plasmids.

TEM β-lactamases are more common than ROB. Several different promoters of the *bla*TEM gene have been described [191-193]. The significance and distribution of

these alternative promoters is unknown. The ROB β-lactamase was first described in a meningitis Hib isolate [194]. ROB has been suggested to confer resistance to cefaclor as well as to ampicillin, but new evidence points to that the described cefaclor resistance may be mediated by PBP-alterations in the isolates that carry ROB [195]. ROB is a weaker hydrolyser of nitrocephine than TEM, and there is a risk that ROB-carrying isolates can be misinterpreted.

As noted, it has been suggested that an effective promoter, allowing high levels of transcription of β-lactamase, is necessary to confer ampicillin resistance in *H. influenzae.* This has been proposed as one reason for the relatively delayed emergence of β-lactam resistance in *H. influenzae* [196]. It may also explain the absence of extended spectrum β-lactamases (ESBL). When a transformation of an ESBL gene to an isolate of *H. influenzae* was performed, the isolate did not become cephalosporin resistant according to breakpoints [197]. If this is the general case, the development of ESBL carriage in *H. influenzae* is likely to be less sensitive to selection pressure by broad-spectrum β-lactams. Interestingly, TEM-15 ESBL has been described in strains of *H. parainfluenzae* [198] and this can have future implications for *H. influenzae* since the two *Haemophilus spp.* are known to exchange plasmids [199-201].

BLNAR (β-lactamase negative ampicillin resistant)

In a strict sense, BLNAR isolates are ampicillin-resistant isolates that do not carry a β-lactamase gene. However, the practical definition of BLNAR isolates is more complex. This is not only due to the lack of international consensus on ampicillin resistance breakpoints, which vary from 1 to 4 mg/L in different studies [202-205]. The mechanism of resistance in this group of isolates is the existence of pivotal amino acid substitutions in PBP-3, Penicillin-Binding Protein 3 (**Table 2**). After this definition was established, it became clear that some isolates with such substitutions are not ampicillin resistant according to breakpoints. Isolates that carry key amino acid substitutions, regardless of resistance phenotype, are defined as gBLNAR (genomic BLNAR), a group that overlaps, but does not match the BLNAR group (**Figure 6)**.

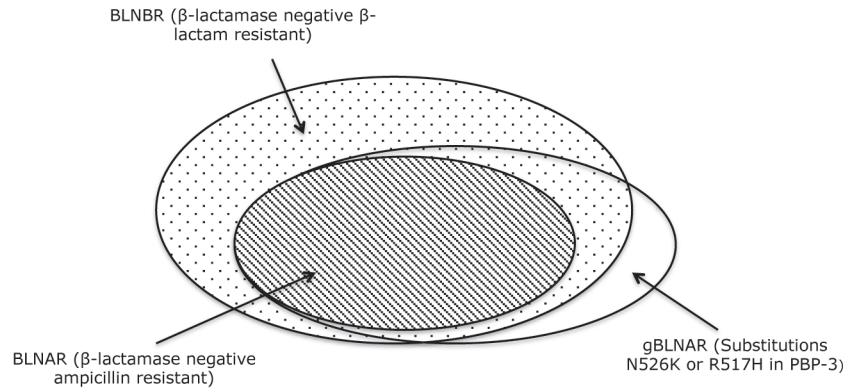


Figure 6. Schematic illustration of how the different groups of β-lactamase negative isolates of *Haemophilus influenzae* with β-lactam resistance are interrelated. The BLNAR isolates are a subset of the BLNBR and of the gBLNAR group. It is unclear how many of the gBLNAR isolates are outside the BLNBR group.

The conservative interpretation of the BLNAR definition only considers resistance to ampicillin. Unlike BLPAR isolates, resistance phenotypes resulting from PBP-3 alterations vary considerably. BLNAR isolates are often resistant to early-generation cephalosporins, such as loracarbef, cefaclor and cefuroxime. Since many gBLNAR isolates are not ampicillin resistant according to resistance breakpoint, some argue that cefaclor resistance may be a better way of defining the group, defying the logic of the name [206]. Today the BLNAR definition often implies concurrent cephalosporin resistance. The appearance of imipenem-resistant *H. influenzae* isolates in clinical settings where carbapenems are widely used is worrying in its own right, but also adds confusion since the imipenem resistance is suggested to be related to substitutions in PBP-3 as well [207, 208]. We suggest using the umbrella definition BLNBR (β-lactamase negative β-lactam resistant) for isolates with resistance to any β-lactam and no evidence of β-lactamase (**Figure 6**).

Even if the name is sometimes misleading, BLNAR phenotypes are almost always related to mutations in the *ftsI* gene, leading to amino acid substitutions in Penicillin binding Protein 3 (PBP-3). These mutations lower the affinity of β-lactams to PBP-3 [203, 209-211]. The amino acid substitutions need to be adjacent to the active sites of PBP, but since different β-lactams likely bind at different sites, the resulting phenotypes reflect varying combinations of substitutions. A number of gBLNAR genotype subdivisions based on amino acid substitution patterns have been suggested. The currently most used subdivision is based on the classification by Dabernat [204], which relates to a subdivision in an earlier Japanese study [203]. One of the two following substitutions is considered necessary for gBLNAR definition; Arg517His or Asn526Lys. However, as stated in a review of the subject; substitutions at 24 different positions in PBP-3 have been associated with the BLNAR phenotype, the number of substitutions in a single

BLNAR isolate can range from one to eleven, and finally, no single substitution is present in all BLNAR isolates [186].

BLPACR

If the other names are not confusing enough, the term BLPACR (β-lactamase positive amoxicillin-clavulanate resistant) is surely the least logical of the acronyms used to portray different phenotypes of *H. influenzae* with resistant to β-lactams. The term is used to describe isolates that carry a β-lactamase and have substitutions in PBP-3 simultaneously. The only real difference between BLNAR (which in many cases are amoxicillin-clavulanate resistant) and BLPACR isolates is the MIC for penicillin and ampicillin, which due to the concurrent β-lactamase is generally very high [212].

Mechanisms of resistance to non β-lactam antibiotics

The recommended first-line antibiotic for most infections by *H. influenzae* is ampicillin. In some situations, such as the occurence of ampicillin resistance, allergy to antibiotics or clinical factors, treatment with ampicillin or other β-lactam antibiotics is not possible or recommended. Non β-lactam antibiotics that can be relevant for treatment of *H. influenzae* infections include folic acid metabolism inhibitors, tetracyclines and fluoroquinolones. *H. influenzae* has intrinsic resistance to macrolide and ketolide agents. These agents provide poor clearance in otitis media [213] and are not recommended for use in *H. influenzae* infections.

Resistance to folic acid metabolism inhibitors trimethoprim and sulfamethoxazole can be mediated by different mechanisms. The enzymes dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) both reduce reactions leading to the production of tetrahydrofolate, which is essential for DNA replication and cell survival. Trimethoprim and sulfamethoxazole inhibit the production of DHFR and DHPS, respectively, and thereby interfere with the production of tetrahydrofolate. The main mechanism of trimethoprim resistance is the production of an alternate DHFR that is not targeted by trimethoprim, but is still functional in producing dihydrofolate [214]. The alternative DHFRs are normally carried on plasmids [186]. Two mechanisms have been described for sulfamethoxazole resistance in *H. influenzae*; production of *sul2*, which encodes a plasmid-carried, alternative DHPS that is not targeted by sulfamethoxazole, or an alteration of *folP*, the chromosomal gene encoding for DHPS, with the same effect [215].

Tetracycline resistance in *H. influenzae* is almost exclusively mediated by an efflux pump coded by *tet(b),* and the *tet(b)* gene is often co-located with β-lactamases on large plasmids. The gene *tet(M),* coding for a ribosomal protection protein has been described in *H. Ducreyi* where it is not located on plasmids [216].

Fluoroquinolones exert their antimicrobial effect by affecting DNA replication. Resistance to fluoroquinolones occurs by mutations in the genes encoding DNA gyrase or topoisomerase IV. Since only point mutations are needed for resistance development,

resistant isolates are believed to be selected during the use of oral fluoroquinolones [217]. As fluoroquinolones are not recommended for use in children, the main carriers of *H. influenzae* isolates, fluoroquinolone resistance in *H. influenzae* is relatively uncommon.

Epidemiology of antimicrobial resistance of *Haemophilus influenzae*

It is challenging to assess any global trend in the epidemiology of antimicrobial resistance of *H. influenzae.* There is substantial variation in resistance epidemiology in different areas and trends seem to vary just as much. The latest assessment on global trends suggested 30% ampicillin resistance in North America but no signs of increase, and around 15-20% resistance but increasing trends in Europe and South America [218]. In that study Asian countries were not included. An illustration of the general trends in the world is given in **Figure** 7.

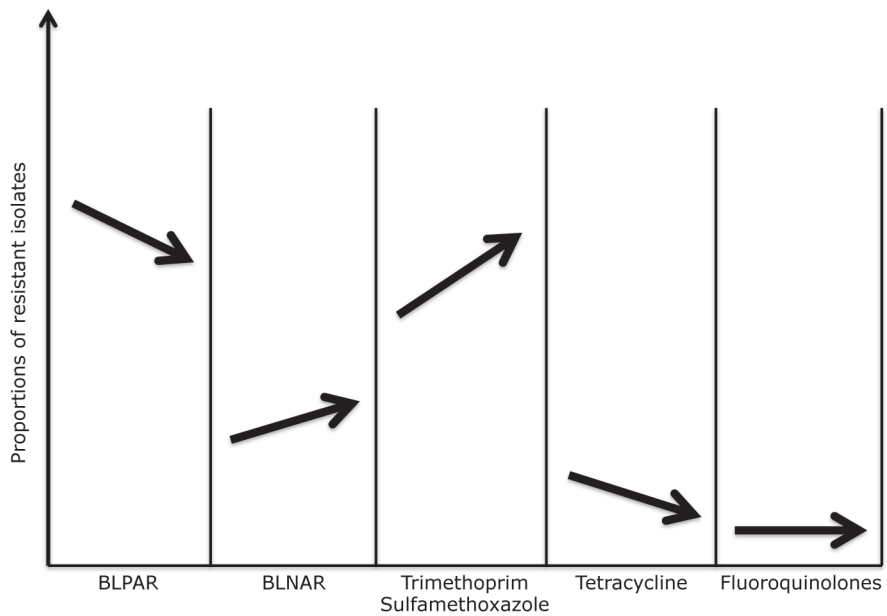


Figure 7. An illustration of worldwide levels and trends of antimicrobial resistance in *Haemophilus influenzae*. While BLPAR (β-lactamase positive ampicillin resistant) isolates have a downward trend from a high level, BLNAR (β-lactamase negative ampicillin resistant) isolates have an upward trend from a low level etc..

β-*lactam antibiotics*

The first description of an isolate of *H. influenzae* with resistance to ampicillin was published in 1974 [219]. From the early 1970's, the proportion of *H. influenzae* with resistance to β-lactams increased until the mid 1990's. Substantial variation in different

geographical areas has been evident, with incidences varying from a few percent in regions with low incidence to >50% in regions with high incidence [220]. *H. influenzae* is not included in the EARS-net surveillance program [221], and resistance epidemiology data is based on individual reports. BLPAR isolates have dominated until the 2000's, and TEM-carrying isolates have been more common than ROB-carrying ones [189]. In the past decade the numbers of β-lactamase producing strains have leveled out or even decreased in some areas with high incidence, such as North America and Spain [222, 223].

BLNAR isolates are very common in Japan and its neighboring countries, with reports of incidences of around 50-60% [224, 225]. These numbers stand out compared to the rest of the world. In North America and Europe, BLNAR incidences vary greatly from different studies and time periods. One multi-center European study showed stable rates [226], and another multi-center study showed BLNAR incidences from 2-20% in 2002 [202]. A Polish study encompassing isolates from 2002-2004 showed a BLNAR incidence of 13% [227]. Interestingly, one recently published study from Spanish hospitals showed all but diminished incidences of BLNAR isolates [228]. One issue that may contribute to the variation in BLNAR incidences across the world is the varying methodology and definitions that exist.

Non β-lactam antibiotics

In *H. influenzae*, resistance to fluoroquinolones is still uncommon, though resistant isolates can pose a clinical problem [186, 220, 229, 230]. One report encompassing isolates from varying regions of the world suggests that the proportion of isolates with tetracycline resistance is declining, and that incidences remain below 5% in all regions studied [220]. Increasing incidences of resistance to trimethoprim-sulfamethoxazole (TXM) in *H. influenzae* is a clinical problem, since TXM is one of the few treatment alternatives to β-lactams in small children. In the SENTRY surveillance study of 1997-2001, regional differences were obvious as the incidence of TXM resistance was 20% in Europe and North America and 40% in Latin America [218]. Two European observations of TXM resistance in invasive *H. influenzae* isolates reflect varying epidemiology. In England the incidence of TXM resistance is increasing from a low level (5%) [231], while in Spain the incidence of TXM resistance is decreasing from a very high level (50%) [191].

Swedish resistance epidemiology

In Sweden, yearly surveillance of antimicrobial resistance in nasopharyngeal *H. influenzae* has been carried out by the Swedish Institute for Communicable Disease Control [232]. The surveillance is based on 100 consecutive nasopharyngeal *H. influenzae* from each laboratory in Sweden in every year. From this surveillance (**Figure 8**), it is clear that the proportion of BLPAR isolates has increased in the last decade and is 18% in 2011. The proportion of BLNAR isolates has been low, although there are no data from 2010 or 2011. The incidence of tetracycline and fluoroquinolone resistance is stable at a

low level. The standout observation from the surveillance data is the marked increased incidence of resistance to trimethoprim-sulfamethoxazole, from levels around 10% in the late 1990's to 24% in 2011.

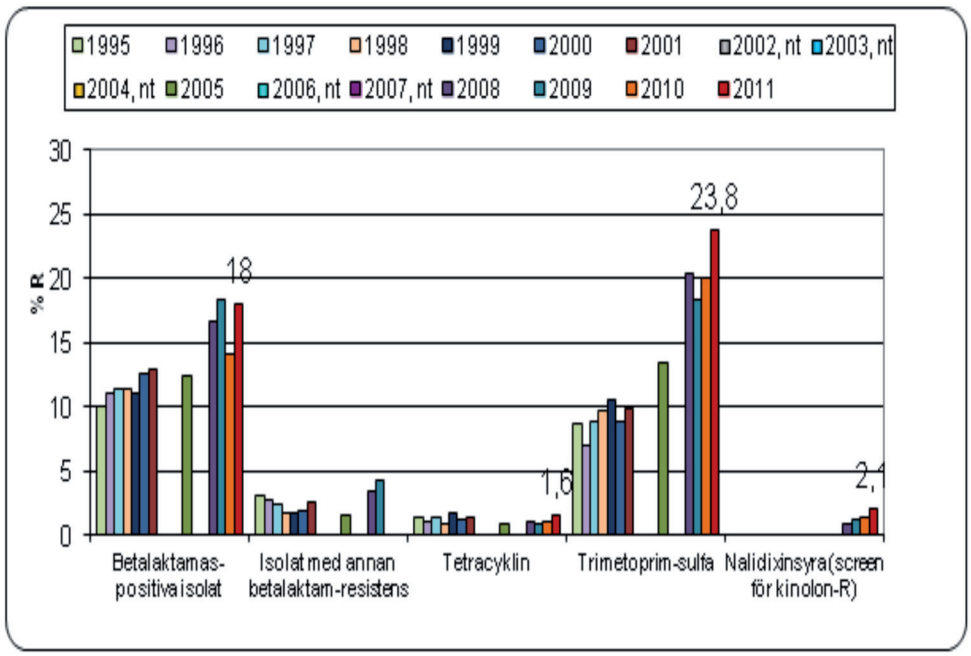


Figure 8. Resistance epidemiology of *Haemophilus influenzae*, Sweden, 1995-2011. Text in Swedish. Translated categories, from left to right: BLPAR, BLNAR, tetracycline resistance, trimethoprim-sulfamethoxazole resistance, fluoroquinolone resistance. Image from Smittskyddsinstitutet, Stockholm. Reprinted with permission.

Antimicrobial resistance of invasive Haemophilus influenzae

There is a marked difference in the epidemiology of invasive and nasopharyngeal isolates of *H. influenzae* in Sweden. The median age of patients with *H. influenzae* isolated from the nasopharynx in upper respiratory infections in Malmö is around 3 years while the median age of patients with invasive isolates is 67 years (unpublished data). It is not known whether this difference in epidemiology affect the epidemiology of antimicrobial resistance. Most epidemiological data of antimicrobial resistance in *H. influenzae* is based on non-invasive isolates. There are exceptions, including studies from Great Britain, Canada and Italy, where the incidences of antimicrobial resistance in invasive isolates were generally equal to or lower than reported resistance incidences from upper respiratory isolates [231, 233, 234].

Aims and objectives

The aims and objectives of this thesis were:

- to investigate the interactions of *Haemophilus influenzae* adhesin Protein E with components of the extracellular matrix.
- to investigate the role of complement regulator-binding and *in vitro* serum resistance of non-typeable *Haemophilus influenzae* causing invasive disease.
- to describe the epidemiology of invasive disease by *Haemophilus influenzae* in Sweden in the post Hib-vaccine era 1997-2009, as well as to investigate the disease severity and host factors related to invasive cases.
- to describe the epidemiology, phylogeny and resistance mechanisms of β-lactam resistant invasive *Haemophilus influenzae* in Sweden 1997-2010.
- to describe a case of severe invasive disease by non-type b *Haemophilus influenzae* as well as to reconstruct bacterial and host factors that could have contributed.

Materials and methods

The methods are described in detail in the respective Papers. This section provides an overview of the methods used, and is in part a repetition of the method sections in the Papers. However, it also provides an elaboration on the definitions on which the methodology is based, and in some parts a brief comparison with alternative methods.

Study designs

Paper I is a based on a laboratory study of clinical isolates of *Haemophilus influenza*e.

Paper II is based on a laboratory "case-control" study of invasive and non-invasive clinical isolates of *Haemophilus influenzae*, including an assessment of sepsis severity of invasive cases.

Paper III is based on a retrospective epidemiological study on invasive *Haemophilus influenzae* disease in southern Sweden 1997-2009, including laboratory studies of the bacterial isolates as well as an assessment of basic epidemiological data, sepsis severity of the cases and immune competence of the patients from Skåne county.

Paper IV is based on a retrospective epidemiological study of antimicrobial resistance in invasive *Haemophilus influenzae* isolates, including a thorough laboratory investigation of β-lactam resistant isolates, and a phylogenetic analysis of resistant isolates.

Paper V is a case report of a severe invasive *Haemophilus influenzae* type f infection, including studies on the patient's immune system and virulence studies of the bacterial isolate.

Study populations

The studies in Papers III-IV are based on clinical cases of invasive disease by *Haemophilus influenzae* (defined as growth of *H. influenzae* in blood or cerebrospinal fluid) 1997-2010, identified at the Clinical microbiology laboratories in Skåne (2 laboratories),

Göteborg (2 laboratories) or Stockholm (Karolinska). In Paper II and the study of sepsis severity in Paper III, only cases from Skåne were included. No other criteria were used. The included laboratories serve densely populated areas of Sweden, with a current total population of approximately 3 million. The geographical regions that are served by the laboratories, as well as the population density of different Swedish regions are shown in **Figure 9**, which is also Supporting information Figure 1 fom paper III.

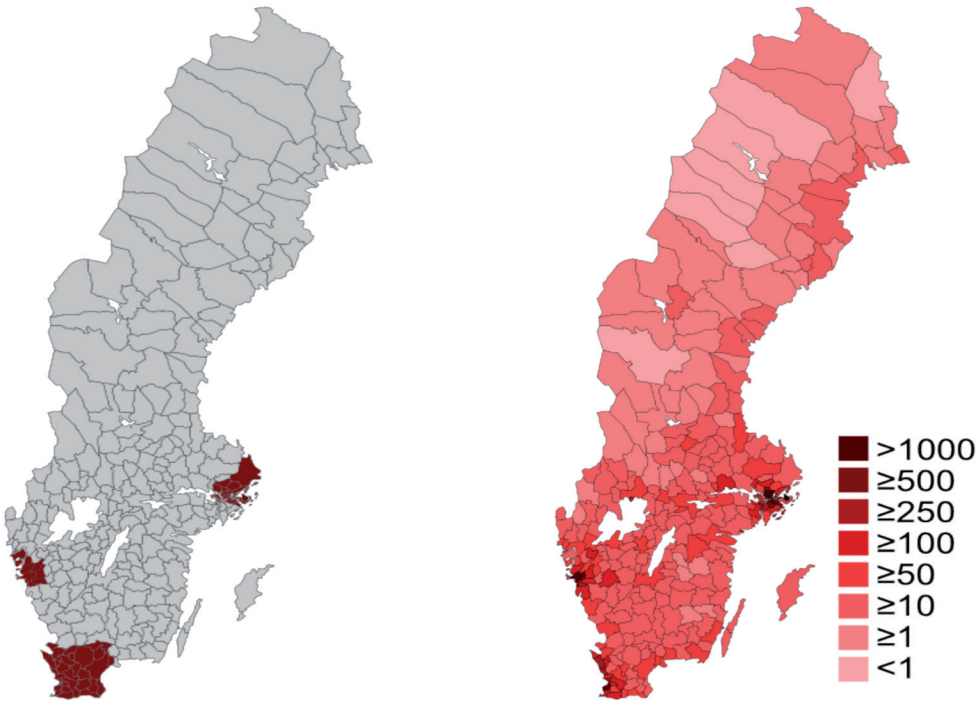


Figure 9. Map of Sweden showing regions included in the study (left) and population density by region (inhabitants/km2) (right). Images based on SWE-Map Kommuner2007.svg by Lokal_Profil, licensed under CC-BY-SA2.5 (Supporting information Figure 1 fom paper III).

Information on the geographical regions served by each laboratory and year was gathered, and the population per geographical region and year was collected from Statistics Sweden. This gave population denominators for each year in the epidemiological analysis in Paper III. Two further estimates were performed: since laboratories served densely populated urban areas, they also served tertiary care units, such as units for neurosurgery or organ transplant patients. These units accept patients from regions outside the defined geographical area. To evaluate the risk that such patients skewed the results we screened all lab referrals for referring unit. Only 11/410 patients were treated at tertiary care units, and at least 6 of these lived within the geographical area. The risk of the remaining 5 patients skewing the results was deemed as minimal.

A second analysis was performed in Paper III. In this analysis each laboratory was assessed separately per study year. If, in a given year in one laboratory, half or more of

the registered invasive isolates had been or could be capsule typed by PCR, then the all isolates from the laboratory for that year were included in the analysis. Consequently the whole population served by the laboratory that year was added to the denominator. If less than half of the isolates could be or had been capsule typed, then all of the isolates from the laboratory, and the corresponding population, were excluded from the analyses for the given year. This procedure resulted in that 17 non-typed isolates were included in the final analysis, and a small number of capsule typed isolates were excluded. The procedure was performed to get comprehensive epidemiological data with reliable, comparable population denominators throughout the study period.

Clinical definitions

Defining sepsis severity

There are several systems available to assess the severity of a septic event. In our study we were limited to retrospective data from medical reports in a population where only a portion of patients were expected to need intensive care monitoring. We applied the ACCP/SCCM consensus definition, which is based on SIRS criteria to assess sepsis severity [235]. The definitions are summarized in **Tables 3a and b**.

Table 3a Definitions of sepsis severity

Bacteremia	Sepsis	Severe Sepsis	Septic shock
Positive blood culture but not fulfillment of SIRS criteria	SIRS – 2 of the following 4 criteria fulfilled: • Fever (Body temperature >38 or <36°C) • Tachycardia (Heart rate > 90 bpm) • Hyperpnea (>20 breaths/min) • White blood cell count >12x10^9/l or <4x10^9/l	Sepsis + newly acquired organ failure according to the definitions in **Table 3b**	Persisting hypotension/ hypoperfusion and organ failure after adequate initial resuscitation

Table 3b Definitions of newly acquired organ failure

Organ	Definition of organ failure
Circulation	Hypotension (systolic blood pressure < 90mmHg) Hypoperfusion (elevated P-lactate)
Kidney	Oliguria despite resuscitation or an elevation of P-Creatinine of > 45µmol/l
Respiratory	PaO_2 < 7.0 kPa or PaO_2 < 5.6 kPa for a primary lung infection.
Coagulation	Platelet count < 100x10^9/L or a spontaneous INR > 1.5
CNS	Confusion or altered mental state
Liver	S-bilirubin > 45µmol/l

Defining immune deficiency

In Paper III, we studied the incidence of immune deficiency among patients with invasive *H. influenzae* disease. In most epidemiological studies of infections, all concurrent medical conditions are included in the analysis, regardless whether they result in reduced immune competence or not. This means that older adults are often registered with underlying conditions for which the relative risk of infection is unclear. In the present study, we aimed to include only conditions that clearly contribute to reduced immune competence. The definition used is presented in **Table 4**.

Table 4. Definition of immune deficiency in the study (Supporting information Table 1 fom paper III).

Category		**Examples of diseases and conditions**
Primary immune deficiency		Mainly genetic disorders including T and B lymphocyte and phagocytic cell defects and complement deficiences
Secondary immune deficiency	Acquired immune deficiency	HIV, haematologic malignancies such as leukemia, lymphoma, multiple myeloma and active solid tumors
	Chronic disease	End-stage liver or renal disease, severe COPD, end-stage heart disease, and dysregulated diabetes mellitus
	Iatrogenic conditions	Medication and treatment such as steroids, immunomodulatory drugs, chemotherapy, radiation therapy, and organ transplants

Determining antimicrobial susceptibility

Several methods to determine the antimicrobial resistance of *H. influenzae* exist. While the disk diffusion method is sensitive [236], perhaps too sensitive, an excellent correlation between Etests and the golden standard method suggested by CLSI, the broth microdilution method, has been confirmed [237, 238]. In the Swedish (and now Nordic) testing protocol for β-lactam resistance in *H. influenzae* [184], sorting of resistant isolates is not based on ampicillin, but penicillin (phenoxymethylpenicillin for the length of our study) and cefaclor discs. Isolates with resistance to penicillin are tested for β-lactamase production using the nitrocephine test. This testing regimen has excellent sensitivity, but slightly poorer specificity, in identifying BLNAR isolates [239]. The testing procedure is illustrated in **Figure 10**. In Paper IV, this technique was used as screening for β-lactam resistance, with two modifications. In the case of penicillin resistance, an Etest for ampicillin was performed on all saved isolates. Furthermore, all nitrocephine negative isolates with resistance to a β-lactam were defined as BLNBR (β-lactamase negative, β-lactam resistant). From this group, the BLNAR and gBLNAR were subsets, identified according to ampicillin MIC and PBP-3 sequence, respectively.

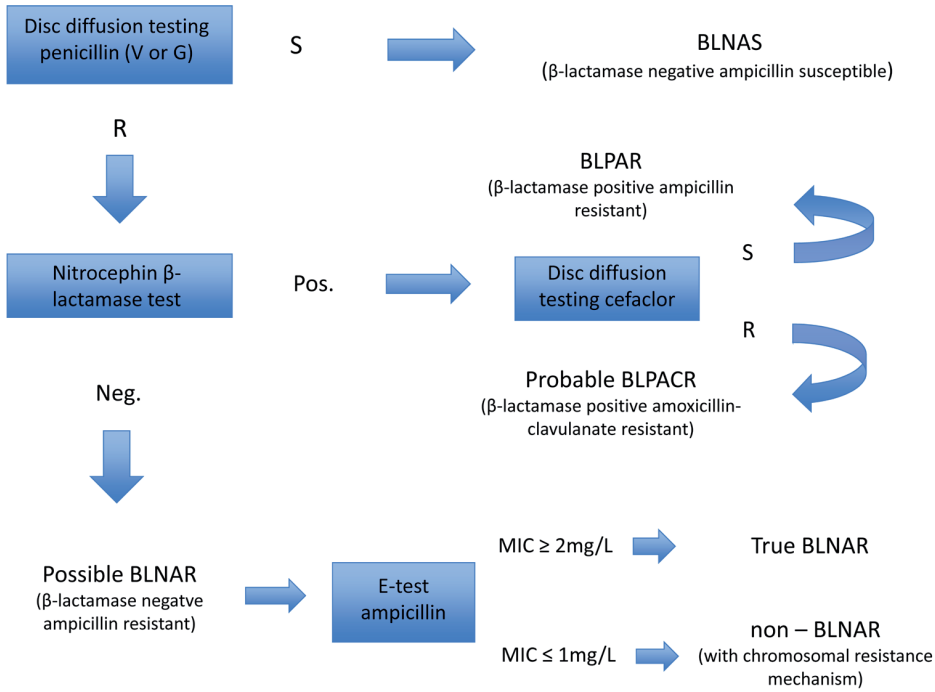


Figure 10. Testing scheme for β-lactam resistant variants of *Haemophilus influenzae* in Sweden, and the base for resistance screening in Paper IV. R=resistant according to breakpoints, S= susceptible according to breakpoints.

Laboratory methods

Construction of mutants

In paper I, three different mutants of *Haemophilus influenzae* 3655 were used. NTHi3655 is an otitis media strain originally from Missouri, USA with a Multi Locus Enzyme Electrophoresis (MLEE) profile close to a Hib cluster [240], and virulence in an experimental otitis model that resembles encapsulated isolates [241]. The end regions of the genes encoding for Hap (*hap*) and Protein E (*pe*) were amplified as cassettes with the introduction of restriction enzymes and uptake sequences [242]. The resulting PCR products for *pe* and *hap* were cloned into plasmid vectors containing kanamycin and chloramphenicol resistance gene cassettes, respectively. The NTHi3655 isolate was transformed using starvation media (M-IV), and transformed bacteria not expressing Hap or PE were selected using culture media supplemented with kanamycin or chloramphenicol. For the double mutant, NTHi3655Δ*pe* was transformed with the method described for *hap* above. double mutants were selected using growth medium complemented with both antibiotics.

Binding assays

Five different binding assays were performed in the studies leading up to Papers I and II. In a direct protein-protein binding assay, the extracellular matrix proteins laminin, vitronectin, fibrinogen and fibronectin (Paper I) as well as complement regulators factor H and C4b binding protein (C4BP) (Paper II) were labeled with radioactive ^{125}Iodine. After the labeled proteins were incubated with bacteria and centrifuged, binding was measured in a gamma counter as the portion of radioactivity obtained in the bacterial pellet from the radioactivity in the whole sample (pellet and supernatant).

In Paper I, binding of bacteria to immobilized laminin was assessed by coating of glass slides with laminin, followed by incubation with bacteria. The result was evaluated by Gram-staining and visual judgment by microscopy.

In Paper I, the dynamic interaction of PE and laminin was tested using a flow-chamber system (surface-plasmon resonance; Biacore®, where laminin was immobilized on a chip, and thereafter PE$^{22\text{-}160}$ was injected at different concentrations. Data analysis was performed using Biacore software.

To identify the different binding sites of PE in paper I, microtiter plates were coated with different peptide PE-fragments, and laminin and vitronectin was added. Binding was detected using anti-laminin and anti-vitronectin antibodies conjugated with secondary HRP-conjugated antibodies, and measured as the absorbance at the correct wavelength.

In Paper II, to assess whether NTHi bound complement regulators directly from human serum, bacteria were incubated with pooled normal human serum (NHS). The binding was tested using specific antibodies against vitronectin, C4BP and factor H conjugated with secondary HRP-conjugated antibodies.

Transmission electron microscopy

Transmission electron microscopy was used in Paper I to assess the binding of gold-labeled laminin to wildtype and mutant NTHi3655, and to assess the binding of gold-labeled PE$^{22\text{-}160}$ to laminin.

Serum resistance assays

Serum bactericidal assays to assess survival in media complemented with human serum were performed in Papers II and V. Pooled human serum from healthy donors (n=5) was used at a concentration of 5% and added to defined concentrations of bacteria suspended in media that optimize complement activity. Alternative bactericidal assays, using buffers that are less optimized for complement survival, have been used by others [243], allowing bactericidal assays at 40% serum.

Flow cytometry

In Papers II and V, flow cytometry analyses were performed. In Paper II, flow cytometry was used to assess the binding of IgG to NTHi isolates using anti-human IgG

antibodies and FITC-conjugated secondary antibodies. In Paper V, FITC-conjugated anti-MBL antibodies were used to assess binding of Mannose-Binding Lectin (MBL) to different isolates of *H. influenzae*. FITC and biotin-conjugated antibodies directed against specific human-Ig subclasses were also used, to determine the binding of Ig subclasses from the patient's serum and pooled human serum from healthy donors to the Hif myositis strain as well as to the well characterized controls MinnA (Hib) and NTHi3655 (NTHi).

Polymerase Chain Reaction (PCR)

Polymerase chain reaction to detect and/or to sequence genes was used in Papers III, IV and V. The PCRs were performed with the primers and conditions given in respective cited reference unless otherwise stated. In Papers III-V, capsule typing was performed by PCR, since this mode of typing has demonstrated higher specificity as compared with typing with antisera [244]. Screening was performed with primers detecting a part of a gene necessary for capsule transport, *bexA* [245], but since variation in *bexA* has been found, supplementary screening with *bexB* PCR was carried out [246]. For *bex*-positive isolates, sequential *cap* PCRs ensued until the capsule type was defined [247]. In Paper III, a PCR to exclude the strict commensal *Haemophilus haemolyticus,* which can be non-hemolytic and indistinguishable from *H. influenzae*, was performed [248]. A slightly modified protocol was used, with an initial PCR using the primer pair 16S3' and 16SNor. In Paper IV, PCR was used to detect *bla*TEM and *bla*ROB genes [249], to detect and sequence the *ftsI*-gene coding for PBP-3 [250] and to detect and sequence the seven MLST alleles [251].

Epidemiological, statistical and phylogenetic methods

In Paper II, a Mann-Whitney U-test was used to compare invasive and nasopharyngeal *H. influenzae* isolates, as well as to compare invasive isolates from cases of sepsis with isolates from cases of severe sepsis. The method was used due to non-parametric data and small data sets.

In Papers III and IV, the incidence of invasive *H. influenzae* disease and the proportions of variants of β-lactam resistant invasive *H. influenzae* were examined. In both materials, time series of yearly incidence numbers were used. In both materials, whole years were considered as continuous variables for two reasons. Firstly, population statistics were available for full years only, making the analysis in paper III possible only for full years. Secondly, there is a seasonal variation in invasive and noninvasive *H. influenzae* disease [252], which is compensated for by using full years.

In Paper III, we first investigated the incidence per capita 1997-2009 of all invasive *H. influenzae*. We also determined the incidences per capita per serotype. A trend test using regression analysis was performed, without a priori knowledge or expectations

on the dataset. The data was plotted and the relations were approximated as linear, and thus a linear regression was performed. In hindsight, it can be discussed whether a linear model was the best assumption for the *H. influenzae* type f subset. Giving the data from 2010, which became available after publication, a third degree polynomial regression may have been more appropriate. It is important to note that the significances and confidence intervals given from a trend test show the stability and significance of the trend only, and not the rate of change. The rate of change is, when using a linear model, given by the constant.

In Paper III, an alternative way to study the effect of age on the epidemiology of invasive *H. influenzae* disease would have been to apply age-standardization to the dataset. However, the epidemiological studies of invasive disease by *H. influenzae* with which we wish to compare have indicated that the risk does not increase with age; in fact individuals of young age have been at the highest risk. Therefore, age-standardization risks a loss of valuable information.

In Paper IV, we investigated the yearly proportions 1997-2010 of all β-lactam resistant invasive *H. influenzae* as well as the individual phenotypes (BLNBR and BLPAR). In this material, two factors differed from the material in Paper III. Instead of an outcome variable of incidence per capita, the dependent variable was binomial (resistant/non-resistant) and we had an a priori knowledge from Paper III that the absolute numbers of isolates were not evenly distributed throughout the study period. Due to this, we performed logistic regressions on the respective datasets. After testing and plotting the respective datasets, it was clear that two of the variables were not linear. The data were fitted and the dataset of all resistant isolates was best fitted as a quadratic equation (a second degree polynome). The dataset of BLNBRs was equally well fitted as a second or third degree polynome. On these two datasets, quadratic logistic regressions were performed.

In Paper IV, a phylogenetic analysis was performed using concatenated Multi Locus Sequence Typing (MLST)-sequences. A jModeltest was performed to assess the ideal nucleotide substitution model for the dataset [253]. Many different methods have been used for phylogenetic analysis and outbreak detection of *H. influenzae*. An early evolutionary clustering analysis of isolates was performed using MLEE [240, 254], identifying two major groups of encapsulated *H. influenzae* from different evolutionary paths. However, MLEE has also been shown to separate clusters of NTHi causing disease from carrier isolates [255]. Concatenated MLST sequences offer a comparable separation of encapsulated isolates to MLEE [256]. The question still remains whether MLST offers enough resolution for the heterogeneous NTHi, and whether a common substitution model can be assumed for all seven concatenated sequences. A comprehensive review of molecular typing techniques for respiratory pathogens, including *H. influenzae*, was recently published [257].

Ethical considerations

An ethical permit to collect and present data from medical reports of individual patients with invasive *H. influenzae* in the county of Skåne was sought and granted by the Regional Ethical Review Board in Lund (2009/536). The permit allowed collection of basic data such as age and gender, but also clinical indicators of sepsis severity, starting antibiotic and time of hospital stay. This data was used for the analyses in Papers I-IV. Oral as well as written permission was granted from the patient in Paper V. The patient was demonstrated the manuscript in person, including the figures and informed on all the results of the study, before submission.

Results and Discussion

The detailed results are presented in the respective Papers and will only be summarized here as a basis for discussion.

Bacterial and host factors related to invasive disease of *Haemophilus influenzae* (Papers I, II and V)

Binding of NTHi to the extracellular matrix (Paper I)

The interactions of the recently described adhesin of *Haemophilus influenzae*, Protein E (PE), with epithelial cells as well as with the extracellular matrix component and complement regulator vitronectin have been established in earlier work [95, 96]. The experiments in Paper I suggest that PE binds to further components of the extracellular matrix. As stated in the introduction, the *Haemophilus* adhesion and penetration protein (Hap) is known to bind to extracellular matrix components, including laminin. As expected, the NTHi3655Δ*hap*, lacking the *hap* gene, bound less laminin than the wild-type NTHi3655 (**Figure 11**). However, the NTHi3655Δ*pe*, lacking the *pe* gene, also bound less laminin. In the NTHi3655Δ*pe*/*hap*, the laminin-binding was even further reduced. The results suggest that PE is involved in laminin-binding, but the difference between the double mutant and the respective single mutant was small. The results also show that laminin-binding still occurs in the double mutant, suggesting the existence of additional laminin-binding proteins in *H. influenzae*.

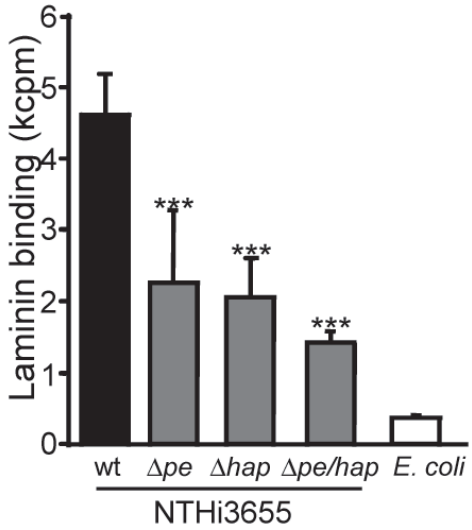
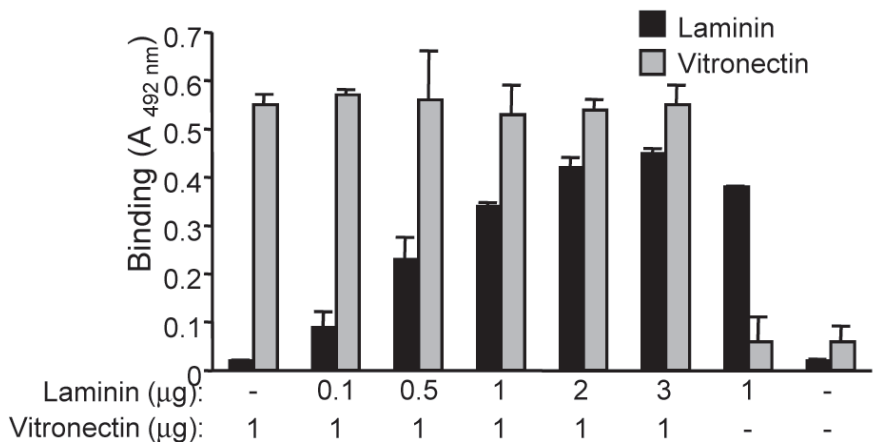


Figure 11. The laminin-binding of the wildtype and the respective mutants of NTHi 3655. The binding is reduced to approximately the same extent in NTHi3655Δ*hap* and NTHi3655Δ*pe*. The binding is slightly further reducced in the double mutant. From paper I, © Oxford University Press. Reprinted with permission.

From the testing with different PE peptide fragments, the laminin-binding site was identified in the N-terminal region (amino acids 41-68), which is distinct from the vitronectin-binding site (amino acids 84-108). The addition of increasing amounts of free laminin did not affect the capacity to bind vitronectin, suggesting that PE can bind vitronectin and laminin concurrently (**Figure 12**). This is important, considering that vitronectin-binding through Protein E has been shown to increase serum resistance of *H. influenzae*, a trait that theoretically would be favorable outside the respiratory tract.



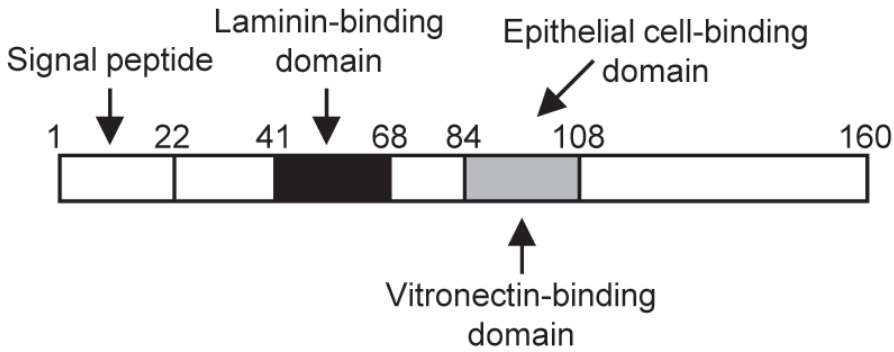


Figure 12. Recombinant Protein E can bind vitronectin and laminin at the same time. The upper graph shows that vitronectin binding is not affected by increasing concentrations of laminin, and the lower figure displays the distinct binding sites for laminin and vitronectin. From Paper I © Oxford University Press. Reprinted with permission.

What is the relevance of binding to the extracellular matrix in invasive *H. influenzae* disease? Matrix components that are rich in laminin, such as the basement membrane, are not exposed in the human airway during normal conditions. In situations when *H. influenzae* infections frequently occur, during viral infections or in COPD patients, the epithelial barrier is often breached and the basement membrane is exposed. Tissue biopsies have shown a changed histology of the respiratory epithelium in patients with COPD, including thicker, less compliant matrix structures and increased laminin presence [258, 259]. Furthermore, it is clear from *in vitro* experiments and tissue biopsies that NTHi pass through epithelial cell layers through transcytosis or disruption of tight junctions, and persist in-between the basal parts of epithelial cells or below the basement membrane [119, 120]. In this localization, binding to the basement membrane as well as to other matrix components is likely to be important. Binding to components of the extracellular matrix is utilized by a range of microbial human pathogens [260]. Interestingly, two of the four invasive isolates of the study were poor laminin-binders. It may be coincidental, but it cannot be ruled out that low capacity to bind laminin could be related to invasive capacity. It is possible to speculate that these isolates have high affinity for other extracellular matrix components reducing the need to bind laminin, or that a reduced expression of laminin-binding adhesins may lead to impaired antibody recognition by the host and increased survival in the bloodstream, but this is mere speculation.

Recently, the role of Protein E as a multi-faceted adhesin has been further elucidated. The crystal structure has been solved [260, 261], indicating that the laminin and vitronectin binding sites are exposed, which is necessary for the interactions. PE also binds plasminogen, a regulator of the coagulation cascade and a component involved in innate immunity [262]. This interaction occurs through the same peptide fragment that binds laminin, again suggesting surface exposure. Furthermore, NTHi3655Δ*pe* shows impaired bacterial aggregation capacity, suggesting a link to the Hap autotransporter.

Aim: to investigate the interactions of *Haemophilus influenzae* adhesin Protein E with components of the extracellular matrix.

Conclusion: Protein E binds the extracellular matrix component laminin. The binding site is close to the N-terminal of the Protein, and is different from the site involved in vitronectin- and epithelial cell-binding. Protein E can bind laminin and vitronectin concurrently. It is believed that the adhesion capacity to laminin and other components of the extracellular matrix is important for colonization during inflammation.

Serum resistance and complement evasion (Paper II)

As stated in the introduction, one of the main mechanisms in the pathogenesis of invasive Hib disease is the ability of Hib isolates to withstand complement-mediated killing by human serum. *In vitro* studies suggest that NTHi isolates actually more readily than Hib isolates pass through epithelial and endothelial cell layers [128, 131], but are very sensitive to complement mediated-killing. By this reasoning, it is an attractive hypothesis that the relative resistance to complement-mediated killing of different NTHi isolates could be important for invasive disease capacity. In this direct comparison of the *in vitro* serum resistance and complement regulator-binding of 21 NTHi isolates from blood with 21 NTHi isolates from the upper respiratory tract, we found no immediate support for this hypothesis. The average serum resistance between the two groups of isolates did not differ (**Figure 13)**, nor did their capacity to bind complement regulators.

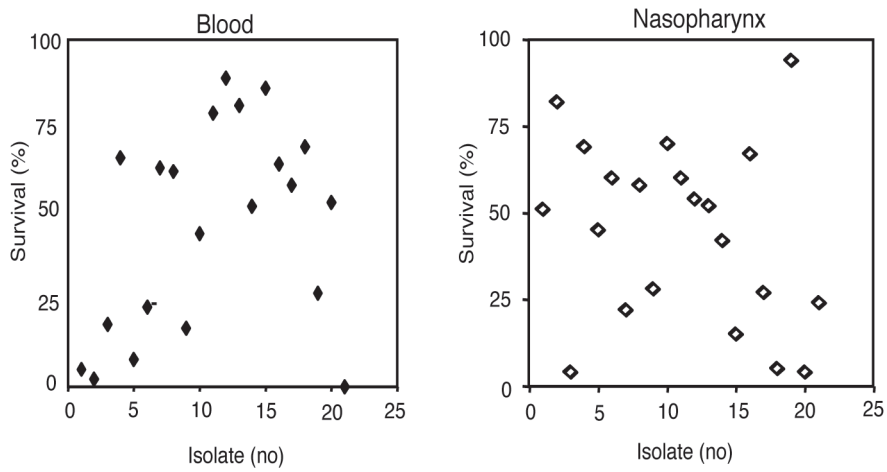


Figure 13. Survival after 20 mins of exposure to 5% human serum of invasive (left) and nasopharyngeal (right) NTHi isolates. There is great interindiviual variation between isolates in both groups, but no general difference between the two groups. Paper II © American society for Microbiology. Reprinted with permission.

The sepsis severity of each case, as well as evidence of immune deficiency of the respective patient was registered in 17 out of 21 cases of invasive NTHi disease. The cases could be roughly sorted into two categories: Five of the six patients with evidence of immune deficiency (of which four primarily were related to impaired humoral immunity) presented with mild sepsis, while most healthy subjects presented with severe sepsis. The findings implicate a prominent role of bacterial virulence in the cases of severe sepsis. Even though the numbers were small, the serum resistance was significantly higher for bacterial isolates in cases with severe sepsis according to ACCP/SCCM criteria than in cases with mild clinical presentation. Since higher serum resistance would offer increased survival in the bloodstream, further spread and more severe disease, the findings are reasonable.

Two reasons for the lack of difference in serum resistance or complement regulator binding between nasopharyngeal and blood isolates may be suggested. Firstly, the high proportion of individuals with impaired immunity may have skewed the results. Secondly, the nasopharyngeal isolates of the study are not merely carrier strains, but isolates from patients with upper respiratory infections. *H. influenzae* often cause persistent inflammation, neutrophil attraction and plasma exudation in the airway during disease [32, 263, 264]. Binding to complement regulators and consequently serum resistance would be favorable for bacterial persistence during inflammation, which is the main "objective" for *H. influenzae,* as an obligate parasite of the human airway. It has been demonstrated that complement and complement regulators are present in the middle ear during acute otitis media [265].

It is not entirely clear as to which mechanisms contribute the most to serum resistance in *H. influenzae*. Recently, a study was presented that focused on the relative contributions of molecular mechanisms [266], and that analysis implicated two loci which both modify the phospholipid content of the bacterial outer membrane. From the results in Paper II, it was possible to make a preliminary correlation between the binding level to the respective complement regulator and the serum resistance of each isolate. The correlations of the Factor H- and vitronectin-binding with serum resistance levels were poor, but the correlation was slightly better for C4BP-binding. In an analysis of a serum-resistant NTHi [243], the serum resistance was mediated by a delay in the synthesis of C3b even though the binding of immunoglobulins to the bacteria was normal. Though uncertain, this could implicate an important role for C4BP, it being a regulator of the formation of C3b by the classical (Ig-dependent) complement pathway [112]. Preliminary data suggest that C4BP-binding by *H. influenzae* is a trait mainly of NTHi and not of encapsulated isolates [113].

Aim: to investigate the role of complement regulator-binding and *in vitro* serum resistance of non-typeable *Haemophilus influenzae* causing invasive disease.

Conclusion: Invasive isolates of *H. influenzae* did not have higher *in vitro* binding levels to complement regulators Factor H, vitronectin or C4b-binding Protein compared with nasopharyngeal *H. influenzae* from respiratory infections. The level of resistance

to complement-mediated killing by human serum did not differ between the groups either. The results suggest a role for complement regulation in mucosal *H. influenzae* infections. The results may be affected by the fact that several of the patients with invasive disease had evidence of impaired humoral immunity. In the group of invasive cases, high levels of serum resistance were seen in isolates from patients in good health, and were linked to severe disease presentation.

Host and bacteriological factors in a case of invasive *Haemophilus influenzae* type f disease (Paper V)

While papers I and II offer insights into bacterial factors related to invasive disease by non-type b *H. influenzae*, Paper V offers a ghastly account of a case of necrotizing myositis caused by an encapsulated *H. influenzae* type f (Hif). Soft tissue infections by Hib have been infrequently described [22, 267]. We investigated the myositis isolate, the Hif KR 494. The capacity of the isolate of epithelial cell adhesion, invasion and transmigration was average compared to Hib and NTHi reference strains, as expected from work by others [131]. The biofilm forming capacity was low, which was expected as well [100]. We found no evidence of an IS1016-*bexA* deletion specifically related to highly virulent Hib and Hia isolates [137, 268]. This was also expected, since the Hif capsule locus lacks flanking insertion elements [269]. Finally, the serum resistance was comparable to that of Hib and NTHi controls. Conclusively, we could not identify any particular trait of virulence besides the type f capsule, which is normally not associated with aggressive disease in healthy individuals.

The patient was 70-years old on presentation to hospital. He was in good health, with no medical conditions but coxarthrosis. In interviews after the septic event, the patient denied being prone to infections, respiratory or others. The patient had suffered from a sore throat and coughing in the weeks prior to the septic event, following a family visit. A family member had symptoms of a respiratory infection during the visit, and subsequently developed pneumonia. However, this infection was treated in another region of Sweden, and no cultures were drawn prior to treatment. Thus, the significance of this infection is difficult to ascertain.

Approximately six months after recovery from myositis, lab tests were performed and the patient was diagnosed with a Mannose-Binding lectin (MBL)–deficiency, genotype LPB/LPB with 10% function of the lectin pathway. MBL-deficiencies affect approximately 10% of the population, and even though MBL binds to several microorganisms [270], its role in the pathogenesis of infections is unclear [271]. It has been suggested that the MBL genotype and MBL-levels in serum are separate risk factors, and that low MBL-levels are associated with disease severity and mortality in infections, for instance in pneumococcal infections [272]. MBL polymorphisms are not associated with increased nasopharyngeal colonization with *H. influenzae*, but with other encapsulated agents such as pneumococci [273]. *In vitro* studies have suggested a weak MBL-binding to Hib isolates, and virtually no binding to non-type b *H. influenzae* [271]. The *in vitro*

binding of recombinant MBL to KR494 and Hib/NTHi controls in our study was weak and inconsistent, and consequently, there is little support for a major role of MBL in *H. influenzae* infections.

The test results of the patient also revealed an IgG3-deficiency and high levels of IgG4. Even though relevant biobanks were contacted, we could not access patient serum from before the septic event. It is therefore unclear whether the observed Ig-subclass distribution was constitutional or acquired. Even though it is age-dependent, the central Ig-subclass that binds polysaccharide antigens is believed to be IgG2, and studies have proved this to be true for Hib, with an added role of IgG1 [56, 274]. The production of protective anti-polysaccharide antibodies is dependent on both B and T-cells and is in small children elicited by the conjugated vaccine in contrast to a plain polysaccharide vaccine [275, 276]. Low levels of IgG2 and IgG4 have been suggested to explain the high incidence of invasive Hib disease in Native American populations [57], and the protective effect of breastfeeding on invasive Hib infections has been linked to a sustained increased production of IgG2 antibodies [277]. IgG3-deficiency is the most commonly found Ig-subclass disorder, and it is in some individuals linked to recurrent upper respiratory tract infections and asthma bronchiale while others remain healthy [278]. IgG3-deficiency is not, unlike Common Variable Immune Deficiency (CVID), associated with increased carriage of *H. influenzae* [279], and individuals with IgG3 deficiency readily develop protective polysaccharide antibodies upon vaccination with a conjugated Hib vaccine [280]. Taken together, there is no support from the literature that IgG3-deficiency is a major risk factor for invasive infections by encapsulated *H. influenzae*.

We showed in a flow cytometry assay that patient serum had an increased IgG2 binding to KR494 compared with pooled normal human serum (NHS), indicating an IgG2 response upon infection by the encapsulated bacterium. The IgG3 binding of the patient's serum was lower than the binding of NHS to all tested isolates, including KR494. Patient serum IgG4 bound to KR494, while there was no IgG4 binding from NHS. In a case control study of children with and without a history of invasive Hib disease, elevated anti-PRP IgG4 levels were seen in patients with a history of invasive Hib disease, while anti-PRP IgG2 levels were comparable in the two groups [281]. There was no difference in total IgG4 levels. IgG4 antibodies may reflect a history of invasive disease, while IgG2 antibodies are found also following mucosal infections by encapsulated *H. influenzae*. Since IgG3-deficiency is often combined with a compensatory elevation of IgG4 levels, it can be hypothesized that the high IgG4 levels of our patient, if present prior to the septic event, elicited an elevated immune response upon polysaccharide stimulation, leading to massive inflammation and severe disease.

Aim: to describe a case of severe invasive disease by non-type b *Haemophilus influenzae* as well as to reconstruct bacterial and host factor that could have contributed.

Conclusion: The case report shows the disease capacity of a non-type b isolate of *H. influenzae*. No evident bacterial virulence factor apart from the type f polysaccharide

capsule was identified. The significance of the observed MBL- and IgG3-deficiency of the patient in the presented case is unclear.

Epidemiology of invasive disease by *Haemophilus influenzae* in the post-Hib vaccine era (Paper III)

Epidemiology

The study of the epidemiology of invasive disease by *Haemophilus influenzae* in southern Sweden 1997-2009 yielded interesting results. Arguably, the most important and unambiguous result of the study is that the incidence of invasive infections by *H. influenzae* type b (Hib) is low and stable in Sweden in the post-Hib vaccine era. In 2004, the Hib incidence was higher than in other years (n=10). After a review of the data from this year, the cases were from different geographical areas, and 7 out of 10 cases occurred in adults that were presumably non-vaccinated. There was very little suggestion of vaccine-escape mutant Hib strains described in other settings [170]. Only 6 of the individuals with invasive Hib disease during the 13 years of the study were born after 1992 and were 4 months of age or older, suggesting that they had been offered the conjugated Hib vaccine. Of these 6, 2 were 5 months of age and not fully vaccinated. No further investigation of the 6 cases was possible within the ethical permit, since Hib vaccine failure was not the main issue of the study. It is nonetheless clear that Hib vaccine failure is very rare, and the data supports the conclusion from a Swedish study suggesting that there currently is no need for a further Hib vaccine booster in Sweden [282].

Our study identified an increased incidence of invasive *H. influenzae* /100.000 individuals 1997-2009 (**Figure 14A)**. In the analysis that included capsule typed isolates only, this increase was found to be due to NTHi isolates, with a contribution of serotype f (Hif) isolates towards the end of the study (**Figure 14B)**. The increasing incidence of invasive NTHi was seen throughout the study period, and also suggested for 2010 (Paper IV).

The finding that NTHi isolates dominate in the post-Hib vaccine era is in line with observations from other countries [173, 177]. In the period following the publication of Paper III, other authors have raised the question of serotype replacement and increasing incidences of invasive *H. influenzae.* Some studies suggest increases [283, 284], but even more repudiate such suggestions [285-287].

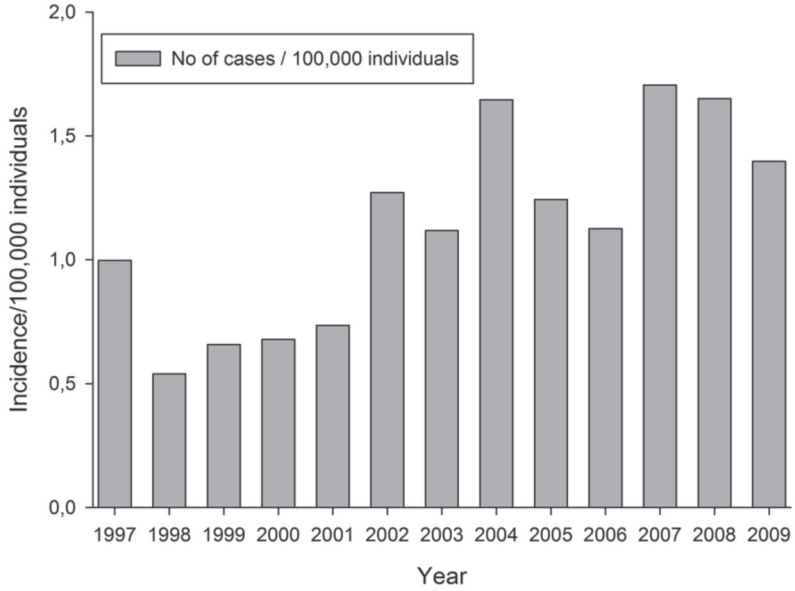


Figure 14A. Invasive disease by *Haemophilus influenzae*/100.000 individuals in Sweden 1997-2009, regardless of serotype. An increase during the study period is seen. Paper III © Wiley & sons Ltd. Reprinted with permission.

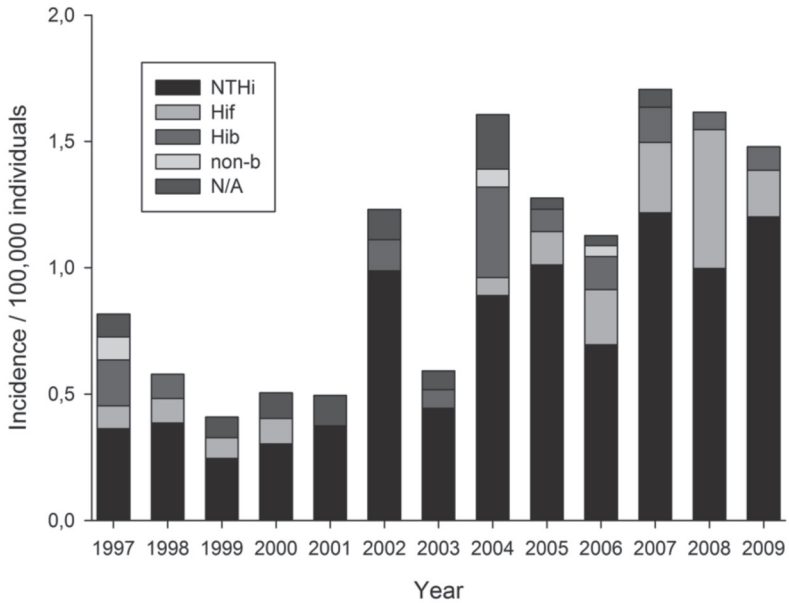


Figure 14B. Invasive disease by *Haemophilus influenzae*/100.000 individuals in Sweden 1997-2009, sorted by serotype. An increase of NTHi and Hif isolates is identified. Paper III © Wiley & sons Ltd. Reprinted with permission.

The epidemiology of Hif isolates is more difficult to assess. A trend test, assuming a linear relation of data, showed a significant increase of Hif isolates in our study. From the limited absolute number of Hif isolates, it is hard to tell whether the findings represent an ongoing increase, a natural variation or a clonal outbreak of Hif isolates with a culmination in 2008. In 2008, the 16 identified Hif isolates were evenly distributed throughout the geographical regions of the study. The data from 2010 (not shown) suggest that the increase of Hif isolates has leveled out. Hif is the most common encapsulated variant in our study, which is true for most countries with Hib-vaccination programs today [27, 177, 178]. However, Hif isolates accounted for almost 20% of cases in a small study of adult invasive *Haemophilus* disease in Sweden already in the pre-Hib vaccine era [159].

Age used to be a distinct predisposing factor for invasive disease by *H. influenzae* in the pre-Hib vaccine era, and age still is important **(Figure 15)**. While invasive Hib disease mainly affected children from 3 months to 4 years of age, invasive non-type b disease mainly affects individuals at the extreme ends of life; neonates and elderly adults. The incidence /100.000 individuals > 80 years is about 5-10-fold higher than for the age group 20-60 years throughout the study. Increased incidence of sepsis by many agents is seen with high age. Even though the immune system changes with high age [288], the incidence likely has little to do with age per se. It is rather a reflection that a higher proportion of elderly individuals have immune impairment of some sort.

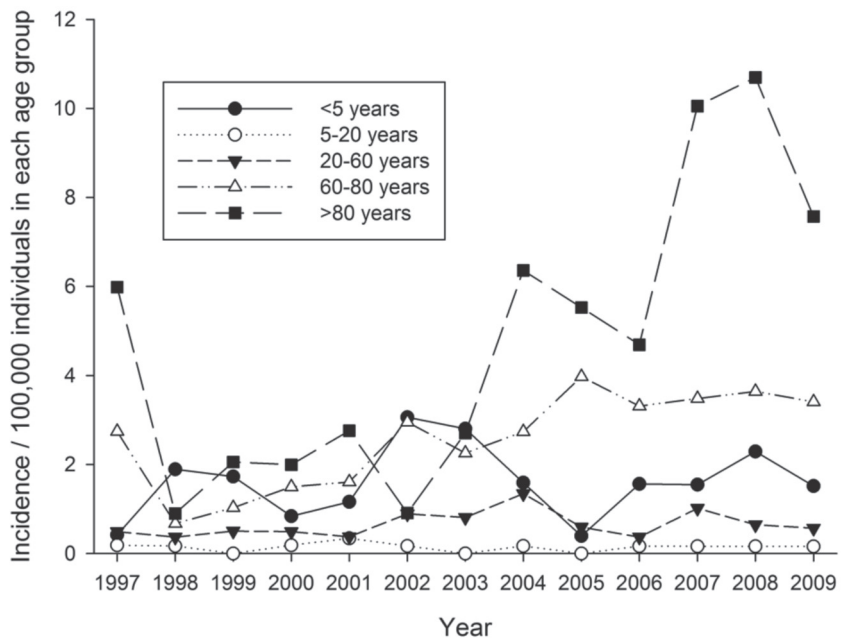


Figure 15. Incidence of invasive *Haemophilus influenzae* disease/100.000 individuals of each age group. Note the increasing incidences in both age groups > 60 years. Paper III © Wiley & sons Ltd. Reprinted with permission.

The observed increase in invasive infections with *H. influenzae* may have several reasons. The most straightforward explanation is that the finding represents an actual increase. It is important to consider other alternatives. The efficacy and technique for handling blood cultures continuously improves. Furthermore, the number of cultures arriving to the laboratories indicates that the frequency of drawing blood from patients with infectious diseases such as pneumonia has increased in the past decade. Treatment with agents that affect susceptibility to invasive infections increases, leaving more people vulnerable to opportunistic invasive infections. The incidence numbers of invasive *Haemophilus* disease suggest that Sweden has a high incidence compared to other European countries [177]. Whether this reflects differences in disease burden, in diagnostic efficacy or in surveillance accuracy is difficult to tell.

Clinical manifestations, host and bacterial aspects

We assessed the sepsis severity of each case of invasive *H. influenzae* disease in Skåne county. Even though data were often incomplete or missing, and even though only objective accounts of criteria were included, 47% of the cases of NTHi sepsis and 59% of the cases of Hif sepsis were defined as severe sepsis or septic shock. Assumed as opportunists, the proportion of severe cases by NTHi and Hif was surprisingly high. Almost 20% of invasive NTHi cases were treated in an intensive care unit and 7% met the criteria of septic shock. Among the group of invasive Hif cases, 36% needed intensive care, while 14% met the criteria of septic shock. The patients with NTHi and Hif invasive disease that needed intensive care typically presented with severe pneumonia, but cases of Hif and NTHi epiglottitis and meningitis were also observed. Among the 13 invasive Hib cases, 10 (77%) met the criteria of severe sepsis and 7 (54%) needed intensive care.

A total of 38% of patients with invasive NTHi disease and 32% of patients with invasive Hif disease met the study criteria of immune deficiency. Conditions affecting humoral immunity such as myeloma and lymphatic leukemia were commonly found. This underlines the importance of humoral immunity to control invasive *Haemophilus* disease, a suggestion that is in line with theoretical findings [58, 133], and has recently been confirmed by others [289, 290].

Based on the material, cases of invasive non-b *H. influenzae* disease can roughly be separated into two categories (**Table 5)**. The first category includes the elderly and patients with reduced immune competence. In this category patients often present with mild sepsis, but the 1-year-mortality is high. In such cases, host factors seem central, and the *H. influenzae* isolates can be regarded as true opportunists. The other category typically includes healthy subjects with severe disease presentation, but with very low rates of case mortality. Here, the impact of bacterial virulence seems central. The same pattern was seen in paper II, albeit on a smaller scale.

Table 5: The two general categories of invasive disease by non-type b *Haemophilus influenzae* observed in the study (and paper II).

General clinical types of non-Hib invasive disease	**Type 1**	**Type 2**
Patient	Age > 80years, premature infant or reduced immune competence	Healthy individual < 80years of age
Clinical presentation	Mild sepsis	Often severe sepsis
1-year mortality	High (25-35%)	Low (0-5%)
Site of infection	Generally pneumonia	Often pneumonia, but also meningitis, epiglottitis and soft-tissue infections
Proportion	60% of NTHi cases, 40% of Hif cases	40% of NTHi cases, 60% of Hif cases
Impact of host factor	High?	Average?
Impact of bacterial virulence	Low?	High?

The majority of patients with invasive non-Hib disease presented with pneumonia and bacteremia (70%). Other presentations included meningitis and epiglottitis, but also cellulitis, myositis, cholecystitis, salpingitis and neonatal sepsis. While urogenital infections, and consequently neonatal sepsis by NTHi have been described before [42], clinical cases with gastrointestinal presentations are distinctly uncommon. A small case series of such infections was recently published [19]. In our material, two cases with suspected or proven gastrointestinal focus were found; in one of the cases the only type e isolate was identified, and in the other an NTHi was isolated.

While studies have shown that Hif isolates are genetically homogenous [289, 291, 292], NTHi are heterogeneous. Attempts to clarify what virulence factors of NTHi that contribute to invasive disease have been made, and such attempts have given clues but not a complete picture [87, 293]. Suggestions of traits associated with invasive NTHi disease include presence of the IS1016 insertion element and biotypes I and V [294, 295]. If the polysaccharide capsule mainly contributes to protection from the host humoral defense and persistence in blood, then bacterial factors that contribute to serum resistance and resistance to opsonophagocytosis could be central also for NTHi. Since our material allows separation of invasive NTHi cases in two categories, with a subset of cases where bacterial virulence seems central, the material is suitable for further studies on NTHi invasive capacity.

Aim: to describe the epidemiology of invasive disease by *Haemophilus influenzae* in Sweden in the post Hib-vaccine era 1997-2009, as well as to investigate disease severity and host factors related to invasive cases

Conclusion: We found no support for serotype replacement of invasive Hib disease in small children, and Hib vaccine failure was very rare. However, we identified an increasing incidence of invasive *H. influenzae* disease in Sweden in the past decade. The increase occurred in adults, mainly elderly adults, and was attributed to non-typeable *H. influenzae* and to a smaller extent to Hif isolates. The patients with invasive non-type b *H. influenzae* disease surprisingly often presented with severe sepsis and need

for intensive care treatment. The cases could roughly be separated in two categories; mild clinical disease in patients with evidence of immune deficiency or severe clinical disease in patients of good health. This suggests circulation of hypervirulent non-type b isolates of *H. influenzae*. Host factors that contributed to risk of invasive *H. influenzae* disease included extreme age (neonatal or >80 years of age) and disorders of humoral immunity.

Antimicrobial resistance of invasive *Haemophilus influenzae* in Sweden (Paper IV)

Epidemiology

In Paper IV, the epidemiology and the mechanisms of antimicrobial resistance in invasive *Haemophilus influenzae* in Sweden are addressed. Non-typeable isolates (NTHi) dominate (Paper III), and almost all antimicrobial resistance observed in the material is seen in NTHi isolates. However, it is important to note that not all isolates had been capsule typed or were available for capsule typing. Among the encapsulated isolates, five Hif and four Hib isolates were β-lactam resistant, and two of each serotype carried a β-lactamase. The only exception to this general finding was seen regarding tetracycline resistance. Of the seven isolates that were tetracycline resistant, five were encapsulated.

A significant increase in the proportion of β-lactam resistant invasive *H. influenzae* was seen in the period 1997-2010. In the final years of the study 30% of invasive isolates were β-lactam resistant and more than 20% of isolates had ampicillin MIC > 2 mg/L. There was no significant increase in the proportion of BLPAR isolates, but a significant increase of β-lactamase negative, β-lactam resistant isolates (denoted BLNBR) (**Figure 16**). Of these isolates, about half were true BLNAR or gBLNAR. The observations are in contrast with the Swedish surveillance of nasopharyngeal isolates where the incidence of BLPAR isolates increased during the same time period, and the incidence of BLNBR isolates remain below 5% in all study years, even though no data is available from 2010 or 2011 (**Figure 8**). The incidence of isolates with resistance to trimethoprim-sulfamethoxazole did not increase in our material of invasive isolates, which also contrasts to the findings from the surveillance of nasopharyngeal isolates. Resistance to tetracycline was uncommon, as was resistance to fluoroquinolones, but the majority of fluoroquinolone-resistant isolates were seen in the period 2007-2010. The resistance epidemiology differed very little between the contributing regions. One exception was the incidence of fluoroquinolone and imipenem resistance, which both were concentrated to one geographical region.

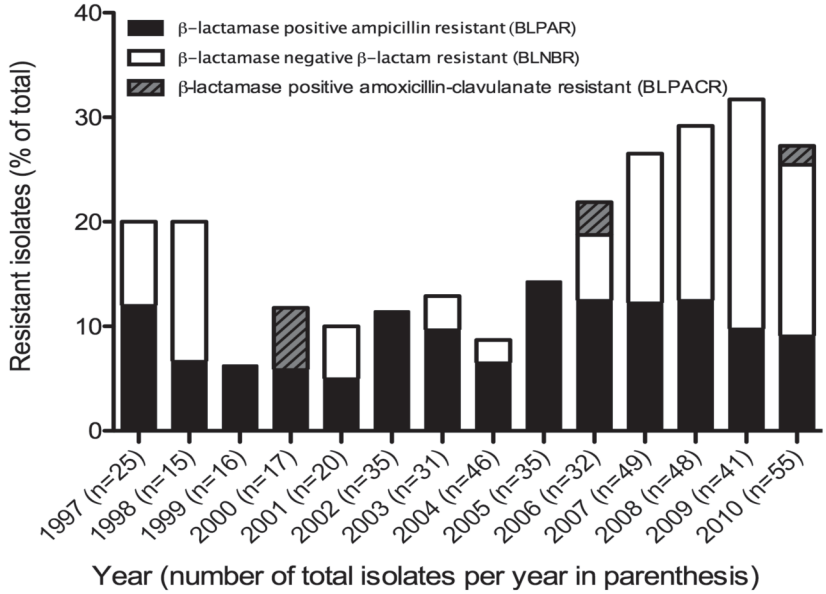


Figure 16. The proportions of different phenotypes of β-lactam resistant invasive *Haemophilus influenzae* in Southern Sweden 1997-2010. An increase of the total proportion is evident 1999-2009, and and increase of β-lactamase negative isolates is evident from 2006 and onwards. Paper IV © American Society for Microbiology. Reprinted with permission.

It is believed that selection pressure by oral antibiotics leads to increased incidences of BLNAR isolates. The widespread use of oral cephalosporins in respiratory tract infections of children in Japan, a strategy uncommon in other parts of the world, is suggested as the reason behind the unparalleled high incidence of BLNAR isolates in Japan. Cephalosporins have high affinity to PBP-3 in *H. influenzae*, and exert their effect mainly through PBP-3 [203]. Due to this, single mutations can lead to cephalosporin resistance, making cephalosporins apt selectors of resistant isolates. *In vitro* trials have shown that the capacity of cefprozil, an oral cephalosporin, to select resistant *H. influenzae* is higher than of amoxicillin-clavulanate [296]. Ampicillin has high affinity to both PBP-1, 3 and 4, and is not considered as apt at selecting resistant isolates [203]. In our material, all isolates with cephalosporin resistance, identified by cefaclor or loracarbef discs, were susceptible to third-generation cephalosporins suggesting variations in affinity to PBP within the cephalosporin group. The reason for the higher incidence of invasive BLNBR isolates compared to the numbers in the nasopharyngeal surveillance is not known. A link between PBP-3 mutations and invasive capacity has been suggested [297], but the finding may reflect a skewed dataset towards metropolitan areas.

Oral cephalosporins are not widely used in Sweden, and antibiotic prescription patterns have not changed dramatically in the last decade. An alternative explanation to the increase of BLNBR isolates is the expansion of a resistant clone. To address this possibility, a phylogenetic analysis of available BLNBR isolates was performed, using

concatenated MLST-sequences (**Figure 17**). We identified seven BLNAR isolates that clustered in the analysis. All of these isolates had identical PBP-3 sequences, genotype IIb, and all geographical regions were represented in the cluster. The data support the notion of a limited clonal expansion. We have recently included isolates from an outbreak of invasive NTHi disease in a nursing home in Blekinge, Sweden. We found that the isolates from this outbreak were all BLNAR genotype IIb, and clustered with the BLNAR cluster in Paper IV in a subsequent MLST analysis (data not shown). This provides further support of a clonal expansion of a BLNAR NTHi clone with unusual high invasive capacity.

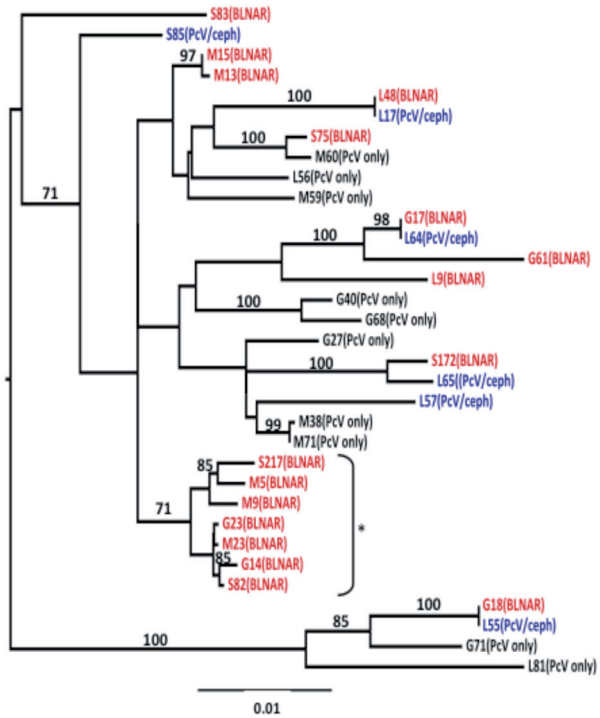


Figure 17. A dendrogram based on concatened MLST sequences of invasive BLNBR isolates. True BLNARs are in red text, isolates with cephalosporin resistance are in blue text, and isolates with sole penicillin resistance are in black text. The asterisk marks the cluster of BLNAR isolates, which is significantly separated from the other isolates. Paper IV © American Society for Microbiology. Reprinted with permission.

Resistance mechanisms

Of the 33 nitrocephine-positive isolates that were available for further testing, a corresponding β-lactamase could be identified in 30. Since 29 were TEM-1 and one was ROB, the TEM domination was confirmed [189]. Three nitrocephine-positive isolates remained negative for TEM and ROB with the primers used in the material. There are known variations in the promoter region of TEM-1, where the standard forward

TEM-1 primer is positioned [249], and it is known that certain TEM-1 variants go undetected by standard PCR primers [298]. One isolate with a hitherto unidentified β-lactamase also displayed cephalosporin resistance, although no mutations in PBP-3 indicating gBLNAR could be identified. This warrants further investigation.

Two different variants of *bla*TEM-1 were seen, identified by two different sizes of *bla*TEM PCR products using the same primer pair. Sequencing showed that the difference was due to a deletion in the promoter region, *bla*TEM-1 P(del) previously described in southern Europe [192]. Isolates with the TEM-1 P(del) variant were more common than isolates with wild-type promoter, but no consistent differences in the MICs for ampicillin were seen.

It may seem awkward to monitor the resistance to penicillin in *H. influenzae* isolates, since oral penicillin is not effective for treatment of *H. influenzae* infections. Disc diffusion testing with penicillin is a sensitive method to detect both BLPAR and BLNAR isolates, and offers superior surveillance as compared with ampicillin. There has been a change of method for penicillin resistance detection of *H. influenzae* issued by EUCAST in 2011. The new method has not been used in Paper IV. The new method suggests screening for β-lactam resistance using PcG disks in Mueller-Hinton media. The method offers excellent identification of isolates with established key PBP-3 mutations [299].

Not surprisingly, all studied isolates with a true BLNAR phenotype (MIC for ampicillin ≥ 2mg/L) had gBLNAR-defining substitutions in PBP-3, but not all gBLNAR isolates were ampicillin resistant. This is consistent with observations from others [186]. However, we identified a group of isolates with penicillin resistance that did not have substitutions in PBP-3 defining them as gBLNAR. Seven of these isolates had wild-type PBP-3. The finding suggests that other mechanisms than amino acid substitutions in PBP-3 are involved in β-lactam resistance of *H. influenzae.* These mechanisms may also explain the imperfect correlation between PBP-3 mutations and resistance phenotype.

What other mechanisms could be involved? Three possibilities can be suggested; i) a change in membrane permeability to β-lactam antibiotics; ii) an increased efflux of β-lactams by efflux pumps; or iii) alterations of other Penicillin-Binding Proteins. The outer membrane of *H. influenzae* is generally more permeable to β-lactams than other Gram-negative bacteria [185]. This may be due to the high expression of porins in the outer membrane. There is evidence from other Gram-negative bacteria that the down-regulation of surface porins can affect resistance levels to β-lactams [300, 301]. Furthermore, one study has shown that the disrupted repression of the acrAB efflux pump leading to increased efflux of antibiotics can increase the MIC for ampicillin [187]. Many studies have confirmed the lowered affinity for ampicillin in isolates with altered PBP-3, while the role of alterations in PBP-4 is less clear [203, 209, 210]. It seems as though alterations in PBP-4 is not an independent factor leading to ampicillin resistance, but it cannot be ruled out that it can contribute [211]. It has been suggested that imipenem susceptibility is little affected by PBP-3 alterations, and that imipenem has a primary affinity to PBP-2 [209]. PBP-2 has also been implied in some cases of ampicillin resistance [210].

Aim: to describe the epidemiology, phylogeny and resistance mechanisms of β-lactam resistant invasive *Haemophilus influenzae* in Sweden 1997-2010

Conclusion: The proportion of invasive *H. influenzae* isolates with resistance to β-lactam antibiotics increased during the period 1997-2010. The increase was attributed to isolates that did not carry β-lactamases, and presumably have chromosomal resistance mechanisms. The increase of such isolates was especially evident in the final 5 years of the study, and is alarming since these isolates have capacity for resistance to a broad range of β-lactams. In a portion of these isolates, no alterations in PBP-3 could be found, suggesting that alternative resistance mechanisms are involved. A phylogenetic analysis of the resistant isolates without β-lactamase production identified a possible clonal expansion of a highly resistant BLNAR clone, a cluster that encompassed isolates from all included geographical areas.

Concluding remarks and future perspectives

In this year, 2012, the conjugated vaccine against *Haemophilus influenzae* type b has been a part of the Swedish national vaccination program administered to children for 20 years. The vaccine has been extraordinarily successful. The results in this thesis show that there is still little, if any, evidence of serotype replacement of invasive *H. influenzae* disease or Hib vaccine failure in Sweden. However, the results in this thesis also suggest that continued surveillance is called for. We identified continued scattered cases of invasive Hib disease. This confirms that Hib isolates still circulate in Sweden, and that future lower vaccine coverage rates may lead to a rebound of invasive Hib disease. Importantly, we identified an increased incidence of invasive non-type b *H. influenzae* (NTHi) disease in adults during the past decade, mainly by non-typeable isolates but also by *H. influenzae* type f. A surprisingly high proportion of these cases had a severe clinical presentation and affected individuals that were otherwise in good health. The results indicate the existence of hypervirulent isolates of non-type b *H. influenzae* in Sweden. Laboratory studies suggested that the *in vitro* resistance to complement-mediated killing by human serum was higher in isolates associated with cases of severe sepsis. The indication was further supported by the identification of a clonal expansion of invasive BLNAR isolates, which recently was implied in an outbreak of invasive disease. Moreover, the proportion of β-lactam resistant invasive *H. influenzae* isolates has increased in the past decade, an increase that has been accentuated in the past few years, and that is not related to β-lactamase production.

Continued research efforts will focus on NTHi isolates, which now dominate all types of infections by *H. influenzae* in Sweden, and is the group with the highest proportion of β-lactam resistance. Future research plans based on the results of this thesis include continued investigations on the virulence factors that contribute to the invasive capacity of non-type b isolates. The novel categorization of invasive non-type b isolates, separating truly opportunistic isolates from isolates with enhanced virulence should offer improved opportunity to identify such mechanisms. Further plans also include an investigation of alternative mechanisms that may contribute to β-lactam resistance in *H. influenzae*.

Perspectives that are further downstream and not directly related to the results of this thesis include studying the interrelationship of the different pathogens in the respira-

tory tract including further investigations of the potential beneficial role of NTHi, as well as an assessment of alternative techniques, such as MALDI-TOF, to separate different isolates of *H. influenzae* with varying capacity to cause disease.

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Paper I

MAJOR ARTICLE

Haemophilus influenzae Protein E Binds to the Extracellular Matrix by Concurrently Interacting With Laminin and Vitronectin

Teresia Hallström,[1,4] Birendra Singh,[1] Fredrik Resman,[1] Anna M. Blom,[2] Matthias Mörgelin,[3] and Kristian Riesbeck[1]

[1]Medical Microbiology and [2]Medical Protein Chemistry, Department of Laboratory Medicine Malmö, Lund University, Skåne University Hospital, Malmö, Sweden; [3]Section of Clinical and Experimental Infectious Medicine, Department of Clinical Sciences, Lund University; and [4]Department of Infection Biology, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute, Jena, Germany

Nontypeable *Haemophilus influenzae* (NTHi) causes otitis media and is commonly found in patients with chronic obstructive pulmonary disease (COPD). Adhesins are important for bacterial attachment and colonization. Protein E (PE) is a recently characterized ubiquitous 16 kDa adhesin with vitronectin-binding capacity that results in increased survival in serum. In addition to PE, NTHi utilizes *Haemophilus* adhesion protein (Hap) that binds to the basement-membrane glycoprotein laminin. We show that most clinical isolates bind laminin and that both Hap and PE are crucial for the NTHi-dependent interaction with laminin as revealed with different mutants. The laminin-binding region is located at the N-terminus of PE, and PE binds to the heparin-binding C-terminal globular domain of laminin. PE simultaneously attracts vitronectin and laminin at separate binding sites, proving the multifunctional nature of the adhesin. This previously unknown PE-dependent interaction with laminin may contribute to NTHi colonization, particularly in smokers with COPD.

Haemophilus influenzae is an important human-specific pathogen that can be classified according to the presence of a polysaccharide capsule [1]. The encapsulated strains cause invasive disease, whereas the unencapsulated and hence nontypeable *H. influenzae* (NTHi) are mainly found in local upper and lower respiratory tract infections, albeit an increased incidence of invasive disease has been observed also by NTHi the last 5–10 years [2]. NTHi is after *Streptococcus pneumoniae* the most common bacterial pathogen in upper and lower respiratory tract infections and causes acute otitis media, sinusitis, and bronchitis [3–5]. In addition, NTHi is often found in patients with chronic obstructive pulmonary disease (COPD), both during stable disease as colonizers and during exacerbations [6].

An initial step in NTHi pathogenesis is adherence to the mucosa, basement membrane, and the extracellular matrix (ECM). The ECM of mammals comprises 2 main classes of macromolecules: the fibrous proteins that have both structural and adhesive functions (eg, laminin, collagens, and elastin) and the glycosaminoglycans that are linked to proteins in the form of proteoglycans [7]. The ECM stabilizes the physical structure of tissue and is involved in regulating eukaryotic cell adhesion, differentiation, migration, proliferation, shape, and structure. Bacterial interactions with the ECM play important roles in colonization of the host, and the ECM is not necessarily exposed to pathogens under normal circumstances. However, after tissue damage due to a mechanical or chemical injury or a bacterial–viral coinfection through the activity of toxins and lytic enzymes, the pathogen may gain access to the ECM.

Laminins are a family of heterotrimeric, cruciform-shaped glycoproteins of ~400–900 kDa consiting of an

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Correspondence: Kristian Riesbeck, MD, PhD, Medical Microbiology, Department of Laboratory Medicine Malmö, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden (kristian.riesbeck@med.lu.se).

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α, β, and γ chain [8]. There are different α, β, and γ chains, which combine into different laminin isoforms. The major role of laminin for the epithelium is to anchor cells to the basal membrane. Several pathogens bind laminin, including *H. influenzae*, *Yersinia enterocolitica*, *Mycobacterium tuberculosis*, and *Leptospira interrogans* [9–13]. *Moraxella catarrhalis* is another pathogen that via ubiquitous surface proteins (Usp) A1 and A2 interacts with laminin, and this interaction may play an important role in *M. catarrhalis* infection during exacerbations in patients with COPD [14].

NTHi expresses a number of surface structures that influence the process of adherence and colonization. Both pilus and nonpilus adhesins of *H. influenzae* have displayed adherence to ECM proteins. *Haemophilus* adhesion and penetration protein (Hap) is an adhesin that binds fibronectin, laminin, and collagen I [15]. Hap is ubiquitous among *H. influenzae* isolates and mediates adhesion to respiratory cells, invasion, and bacterial aggregation [16, 17]. In addition to Hap, Protein E (PE) is a low molecular weight (16 kDa) outer membrane lipoprotein with adhesive properties [18, 19]. PE induces a proinflammatory epithelial cell response resulting in an increased interleukin 8 (IL-8) secretion and intercellular adhesion molecule 1 (ICAM-1) upregulation that leads to an enhanced neutrophil adherence to epithelial cells [18]. The adhesive PE domain is located within the central part of the molecule (amino acids 84–108). In addition, PE binds vitronectin, and this interaction is important for attachment and for survival of NTHi in human serum [20]. We recently analyzed a large series of clinical NTHi isolates ($n = 186$), encapsulated *H. influenzae* strains, and culture collection strains [21]. PE was expressed in >98% of all NTHi independently of the growth phase, and was highly conserved in both NTHi and encapsulated *H. influenzae* (96.9%–100% identity without the signal peptide). The epithelial cell-binding region in the central part of the PE molecule (PE^{84-108}), which also binds to human vitronectin [20], was completely conserved supporting a significant biological function [21].

NTHi is commonly found in patients suffering from COPD [22]. Smoking is associated with increased incidence and severity of COPD, and a previous study has demonstrated that the laminin layer in the basement membrane is significantly thicker in smokers than in nonsmokers [23]. This increased laminin expression thus may pave the way for laminin-binding respiratory pathogens and explain the increased incidence of NTHi in COPD patients. In this study, we show that NTHi binds soluble and immobilized laminin and that both PE and Hap are involved in this interaction. The specific laminin-binding region was defined within the N-terminal part of the PE molecule and the C-terminal globular domains of laminin are responsible for the binding to PE. Taken together, the PE-dependent adhesion to laminin may be important in NTHi pathogenesis, particularly in lower respiratory tract infections.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

NTHi3655 was a kind gift from R. Munson [24]. The NTHi wild type and mutants were cultured in brain–heart infusion (BHI) liquid broth supplemented with nicotinamide adenine dinucleotide (NAD) and hemin (both at 10 μg/mL), or on chocolate agar plates at 37°C in a humid atmosphere containing 5% CO_2. NTHi3655Δ*pe* was cultured in BHI supplemented with 17 μg/mL kanamycin (Merck), and NTHi3655Δ*hap* was incubated with 3 μg/mL chloramphenicol (Sigma-Aldrich). Both kanamycin and chloramphenicol were used for growth of the NTHi3655Δ*pe/hap*. NTHi were isolated from patients ($n = 19$; Southwest Skåne) with upper and lower airway infections, meningitis, and sepsis (Table 1) in 2007.

Manufacture of Mutant NTHi Strains

NTHi3655Δ*pe* was as previously described [18]. To produce Hap-deficient mutants, the 5′-end of *hap* (accession number: U11024) was amplified as 2 cassettes introducing the restriction enzyme sites *Bam*HI and *Sal*I, or *Sal*I and *Xho*I in addition to specific uptake sequences [25]. The polymerase chain reaction (PCR) products were cloned into pBluescript SK(±), and a chloramphenicol resistance gene cassette was amplified introducing a *Sal*I restriction enzyme site. The product was ligated into the truncated *hap* gene fragment. NTHi3655 and NTHi3655Δ*pe* were transformed according to the M-IV method [26].

Protein Labeling and Direct Binding Assay Using Iodine-125–Labeled ECM Proteins

To analyze binding of NTHi to various ECM proteins, we labeled laminin-1 (from Engelbreth–Holm–Swarm mouse sarcoma; murine laminin shows 82% identity with human laminin [27]), human vitronectin, fibronectin, and fibrinogen (all from Sigma-Aldrich) with iodine using the chloramine T method [28]. The NTHi strains were grown overnight in BHI liquid broth and washed with phosphate-buffered saline containing 1% bovine serum albumin (PBS-1%BSA). We incubated bacteria (2×10^7) with iodine-125 (^{125}I)–labeled ECM proteins or increasing concentrations of ^{125}I-labeled laminin (0–800 kcpm) for 1 hour at 37°C. After incubation, bacteria were either washed with PBS-1%BSA followed by measurement of ^{125}I-labeled laminin bound to the bacteria in a gamma counter or centrifuged (10 000 × g) through 20% sucrose. The sucrose tubes were frozen and cut, and we measured radioactivity in pellets and supernatants in a gamma counter. We calculated binding as amount of bound radioactivity (pellet) vs total radioactivity (pellet plus supernatant). In the competitive binding assay, we added laminin or fibrinogen at increasing concentrations (5–100 μg/mL) to the reactions.

Transmission Electron Microscopy

We used negative staining and transmission electron microscopy (TEM) to analyze binding of gold-labeled laminin to the surface

Table 1. Epidemiological Data and Clinical Diagnoses Related to the Strains of NTHi in the Study

Strain number	Age, y	Gender	Culture site	Clinical diagnosis[a]
KR 248	4	Male	Nasopharynx	Upper airway infection
KR 251	1	Male	Naspoharynx	Lower airway infection
KR 255	3	Female	Nasopharynx	Upper airway infection
KR 258	29	Male	Bronchoalveolar lavage	Pneumonia
KR 266	83	Male	Blood	Meningitis and severe sepsis
KR 269	58	Male	Blood	Pneumonia with sepsis
KR 270	69	Male	Blood	Pneumonia with sepsis
KR 275	60	Female	Blood	Sepsis (unknown origin)
KR 289	2	Male	Nasopharynx	Lower airway infection
KR 314	1	Male	Nasopharynx	Otitis media
KR 315	3	Female	Nasopharynx	Upper airway infection
KR 316	23	Female	Nasopharynx	Lower airway infection
KR 317	0	Female	Nasopharynx	Otitis media
KR 318	8	Male	Nasopharynx	Upper airway infection
KR 319	4	Male	Nasopharynx	Upper airway infection
KR 320	1	Female	Nasopharynx	Lower airway infection
KR 327	5	Male	Nasopharynx	Upper airway infection
KR 343	77	Male	Nasopharynx	Pneumonia
KR 360	1	Female	Nasopharynx	Upper airway infection

[a] Based on the clinical information provided by the referring physician in the cases of nasopharyngeal and bronchoalveolar lavage culture. In the cases of blood cultures, the information is based on a retrospective control of the medical journal (Regional ethical committee for medical research, Lund, Sweden [2009/536]).

of NTHi3655 wild type and mutants and binding of gold-labeled PE to laminin as described [29]. We labeled laminin and recombinant PE^{22-160} with 5 nm colloidal thiocyanate gold [30].

Binding of NTHi to Immobilized Laminin

To analyze binding of NTHi to immobilized laminin, we coated glass slides with 20 μg laminin or BSA, air-dried them at room temperature (RT), washed them twice with PBS, and incubated them with NTHi at late exponential phase (OD_{600} = 0.9) for 2 hours at RT. Thereafter, we washed the slides twice with PBS, and bound bacteria were Gram stained.

Enzyme-Linked Immunosorbent Assay

We coated microtiter plates (F96 Polysorb, Nunc-Immuno Module) with peptide fragments of PE (40 μM) (Innovagen), full-length PE (5–10 μg/mL), or BSA (10 μg/mL) in 0.1 M Tris-HCl (pH 9.0) overnight at 4°C. We washed plates with PBS-0.05% Tween and blocked them for 1 hour at RT with PBS-2%BSA. After washings, we added laminin (5–30 μg/mL) and vitronectin (5–30 μg/mL) in PBS-2%BSA and incubated plates for 1 hour at room temperature. We detected binding with rabbit antilaminin pAb or rabbit antivitronectin pAb and HRP-conjugated anti-rabbit pAb. We washed the wells, developed them with 20 mM tetramethylbensidine or 0.1 M 1,2-phenylenediamine dihydrochloride (OPD, DakoCytomation), and finally measured the absorbance at 450 or 492 nm, respectively. In the competition assay, we incubated PE^{22-160} with laminin (5 μg/mL) that had been preincubated with increasing concentrations of PE^{41-68} (0–300 μg/mL), PE^{84-108} (0–250 μg/mL) or heparin (0–1000 μg/mL).

Surface Plasmon Resonance

The PE–laminin interaction was analyzed using surface plasmon resonance (Biacore 2000). We activated 2 flow cells of a CM5 sensor chip, each with 20 μl of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 0.05 M *N*-hydroxysulfosuccinimide at a flow rate of 10 μl/min, after which we injected laminin (10 μl/mL in 10 mM sodium acetate buffer, pH 4.0) over flow cell 2 to reach 4000 resonance units (RU). We blocked unreacted groups with 20 μl of 1 M ethanolamine (pH 8.5). We prepared a negative control by activating and subsequently blocking the surface of flow cell 1. We studied binding for various concentrations of purified PE^{22-160} in the range of 40–1250 nM in flow buffer (50 mM HEPES, pH 7.5 containing 150 mM NaCl, 3 mM EDTA, and 0.005% Tween-20). We injected protein solutions for 8 minutes during the association phase at a constant flow rate of 15 μl/min and then allowed them to dissociate for 10 minutes. We injected the sample first over the negative control surface and then over immobilized laminin. We subtracted the signal from the control surface. Bound PE^{22-160} was removed during each regeneration step with consecutive injections of 10 μl each of 4 M $MgCl_2$ and 2 mM NaOH. We used BiaEvaluation 3.0 software (Biacore) for data analysis.

Statistics

Results were assessed by the Student *t* test for paired data. $P \leq .05$ was considered statistically significant (*, $P \leq .05$; **, $P \leq .01$; ***, $P \leq .001$).

RESULTS

Clinical Nontypeable *H. influenzae* Isolates Bind Laminin

Several bacterial species have been shown to bind laminin and thereby interact with the ECM [9, 12, 14]. To analyze whether laminin binding is a common feature of NTHi, 19 clinical NTHi isolates and NTHi3655 were tested for binding of ^{125}I-laminin. Of the NTHi strains, 16 significantly bound laminin, whereas 4 were low binders with a variable binding as compared with binding capacity of an *Escherichia coli* laboratory strain (Figure 1*A*). Among all the clinical isolates tested, our virulent model strain NTHi3655 [24] showed the highest laminin binding capacity (38.8% ± 7.1% of added ^{125}I-laminin). Thus, the capacity to bind laminin is shared by most of the clinical NTHi isolates tested.

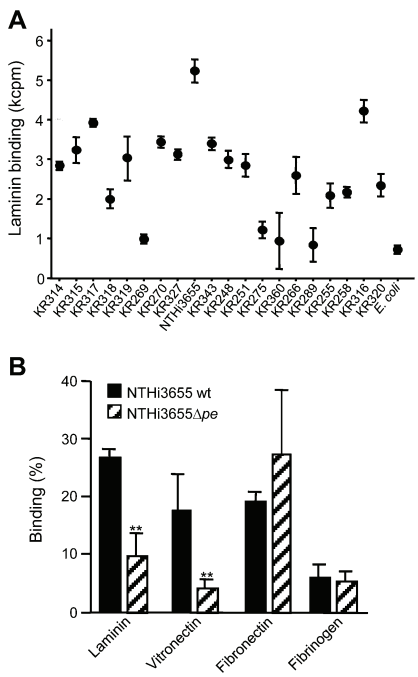

Figure 1. Protein E (PE) plays a major role in nontypeable *Haemophilus influenzae* 3655 (NTHi3655)–dependent binding of soluble laminin. *A*, Binding of laminin to a series of nasopharyngeal nontypeable *H. influenzae* (NTHi) isolates. Bacteria (2×10^7) were incubated with iodine-125 (^{125}I)–labeled laminin. The bound fraction of laminin was measured in a gamma counter. *B*, PE plays a major role in NTHi3655-dependent binding of soluble laminin and vitronectin, whereas other extracellular matrix (ECM) proteins are not bound by PE, as revealed with a wild-type strain (wt) and a PE-deficient mutant. Bacteria (2×10^7) were incubated with various ^{125}I-labeled ECM proteins. Binding was determined as percentage of bound radioactivity vs added radioactivity measured after separation of free and bound ^{125}I-labeled proteins over a sucrose column. The mean values of 3 experiments with duplicates are shown with error bars indicating standard deviation (SD) (**, $P \leq .01$).

PE-Deficient NTHi Shows a Significantly Decreased Binding to Laminin

Because PE is a recently discovered adhesin found in most *H. influenzae* strains [18, 21], we investigated whether PE plays a role in adhesion to the ECM protein laminin. The high-capacity laminin-binding NTHi3655 wild type (Figure 1) was chosen for analysis of binding of the different radiolabeled ECM proteins: laminin, vitronectin, fibronectin, and fibrinogen (Figure 1*B*). In addition, a specific NTHi3655 mutant without PE [19] was included in the analysis. PE-expressing NTHi3655 bound significantly better both iodine-labeled laminin and vitronectin [20] than did the NTHi3655Δ*pe*, suggesting that PE was involved in the NTHi–laminin interaction. In contrast, both NTHi3655 and NTHi3655Δ*pe* bound fibronectin and fibrinogen to a similar extent, proving that PE is not the major bacterial receptor in these interactions but mainly is involved in the interaction with laminin and vitronectin.

PE and Hap Are the Major Laminin-Binding Proteins Expressed by NTHi

Increasing concentrations of iodine-labeled laminin were added to NTHi3655 and we found that bacteria bound laminin in a saturable manner (Figure 2*A*). In contrast, a significantly decreased laminin binding was observed with NTHi3655Δ*pe* as compared with the isogenic NTHi3655 wild-type strain. To test the specificity of the laminin binding to NTHi, bacteria were incubated with increasing concentrations of unlabeled laminin (5–100 μg/mL) with ^{125}I-labeled laminin. Laminin inhibited the binding of ^{125}I-laminin to NTHi3655 in a dose-dependent manner (Figure 2*B*) with >70% inhibited binding at 50 μg/mL of laminin. Because fibrinogen did not bind to PE (Figure 1*B*), this ECM protein was included as a negative control (Figure 2*B*). Thus, the binding between NTHi3655 and laminin was specific, and the PE-deficient NTHi showed a significantly decreased binding to laminin.

Because Hap is an NTHi adhesin that also binds laminin [15], we investigated the role of Hap in relation to PE. To accomplish this, Hap was mutated in NTHi3655 as well as in NTHi3655Δ*pe*. The wild-type NTHi3655 and specific mutants without PE or Hap were analyzed in the direct binding assay using ^{125}I-laminin. We observed a significantly reduced laminin binding with all mutants compared with the wild-type counterpart (Figure 2*C*). Thus, both PE and Hap significantly contributed to the interaction with soluble laminin. *E. coli* was a negative control in these experiments and showed background binding. These interactions were further confirmed by TEM using gold-labeled laminin (Figure 2*D*).

To investigate the attachment of bacteria to immobilized laminin, we applied the different NTHi3655 strains to glass slides coated with laminin. PE- and Hap-expressing NTHi3655

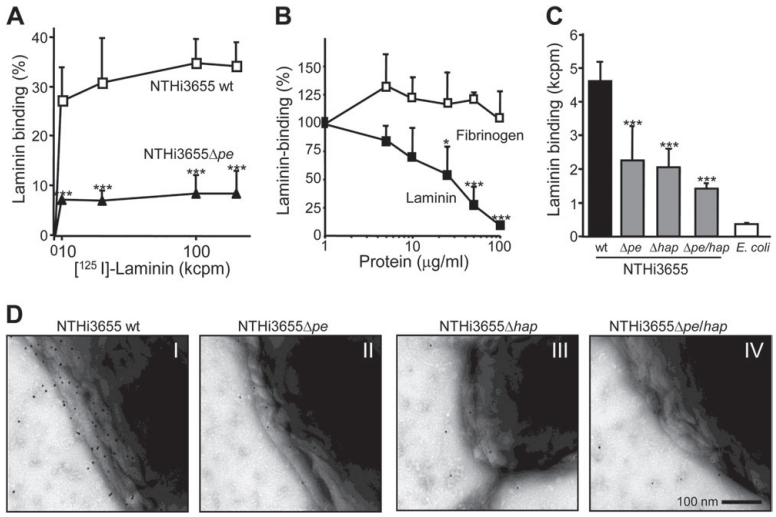

Figure 2. The protein E (PE)–deficient and Hap-deficient mutants show decreased binding to laminin. *A*, PE-expressing nontypeable *Haemophilus influenzae* 3655 (NTHi3655) binds laminin in a saturable manner. *B*, Laminin inhibits the binding of iodine-125 (^{125}I)–labeled laminin to NTHi3655 wild type (wt). *C–D*, The NTHi3655*Δpe* and NTHi3655*Δhap* mutants in addition to the double-mutant NTHi3655*Δpe/hap* show a decreased binding to soluble laminin. In *A–C*, bacteria (2×10^7) were incubated with ^{125}I-labeled laminin (*A, C*), or ^{125}I-labeled laminin with or without increasing amounts of unlabeled laminin or fibrinogen (*B*). *A, B*, Binding was determined as percentage of bound radioactivity vs added radioactivity measured after separation of free and bound ^{125}I-labeled laminin over a sucrose column. The laminin binding of nontypeable *H. influenzae* (NTHi) in the absence of competitor was defined as 100%. The mean values of these experiments with duplicates are shown with error bars indicating standard deviation (SD). Statistical significance of differences was estimated using the Student *t* test. ***, $P \leq .001$, *, $P \leq .05$. In *D*, gold-labeled laminin was used to examine the binding to NTHi. Gold-labeled laminin was incubated with NTHi3655 wild type (wt) (panel I) and corresponding mutants (panels II–IV). The bar in panel IV represents 100 nm.

strains were found to adhere to the laminin-coated glass slides (Figure 3*A*), whereas the PE- or Hap-deficient mutants showed a reduced adherence compared with the wild-type strain (Figure 3*B–D*). *E. coli* bound only weakly to laminin (Figure 3*E*), and NTHi3655 wild type did not adhere to BSA that was included as an additional negative control (Figure 3*F*). Taken together, both Hap and PE were major laminin-binding NTHi proteins as shown in a series of different experimental setups.

The Laminin Binding Region Is Located Within the N-terminal Part of PE (Amino Acids 41–68)

To pinpoint the interaction of PE with laminin, we incubated recombinant PE^{22-160} with increasing concentrations of laminin. PE^{22-160} bound soluble laminin in a dose-dependent manner (Figure 4*A*). When the interaction between PE and laminin was studied using surface plasmon resonance with laminin immobilized on a CM5 chip, we revealed a dose-dependent binding with the affinity constant dissociation constant = 1.54 ± 1.01 μM (Figure 4*B*).

The major laminin-binding region was located within the N-terminal PE^{41-68} and the binding was dose-dependent and saturated (Figure 4*C* and *D*). In addition, PE^{64-108} also bound laminin but with a lower binding capacity (Figure 4*C*). To confirm these findings, we tested PE^{41-68} for its ability to inhibit PE^{22-160}-binding to soluble laminin. PE^{41-68} was able to inhibit this interaction, and 150 μg/mL was required to reduce the binding by 50% (IC_{50}) (Figure 4*E*). PE^{84-108} at low concentrations decreased the binding slightly but at higher concentrations no inhibition of the laminin-binding to PE^{22-160} was detected (Figure 4*F*).

To confirm that laminin and vitronectin bound simultaneously to different parts on the PE molecule, we analyzed concurrent binding. Immobilized PE^{22-160} was incubated with a mixture of laminin and vitronectin at different concentrations followed by separate detection of bound proteins with either antilaminin or antivitronectin pAbs in enzymed-linked immunosorbent assay (ELISA). Laminin did not interfere with the binding of vitronectin to PE^{22-160}, as increasing concentrations of laminin did not affect the vitronectin binding (Figure 5*A*). Similarly, vitronectin did not affect the laminin–PE interaction as increasing concentrations of vitronectin did not quench laminin (Figure 5*B*). In conclusion, PE is a multifunctional adhesin containing 2 separate binding sites; the N-terminal region PE^{41-68} harbored the laminin-binding part of the molecule, whereas the vitronectin-binding region [20] in addition to the adhesive domain [18] were located within PE^{84-108} (Figure 5*C*).

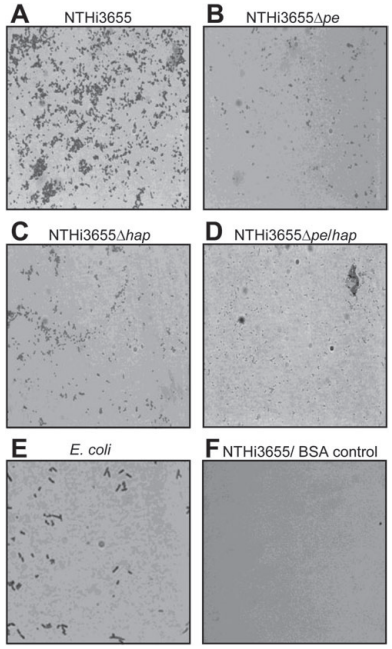

Figure 3. The nontypeable *Haemophilus influenzae* (NTHi) strain 3655*Δpe* and NTHi3655*Δhap* in addition to the double mutant (NTHi3655*Δpe/hap*) show a decreased binding to immobilized laminin. *A*, The NTHi3655 wild type was able to adhere at a high density on laminin-coated glass slides, whereas NTHi3655*Δpe* (*B*), NTHi3655*Δhap* (*C*), and NTHi3655*Δpe/hap* (*D*) mutants adhered significantly less densely. *E*, *Escherichia coli* adhered poorly to immobilized laminin. *F*, NTHi3655 did not bind to bovine serum albumin (BSA) that was included as a negative control. Glass slides were coated with laminin (20 μg) or BSA (20 μg) followed by incubation with bacteria. After several washes, NTHi were Gram stained. Results are shown from a typical experiment out of 3 performed.

PE^{22-160} Interacts With the C-terminal Globular Domains of Laminin

Laminin is a glycoprotein that is composed of an α-, β-, and γ-polypeptide chain joined together through a coiled coil with 1 long and 3 short arms (Figure 6*A*) [31]. Both human and murine laminin-1 contains the α1, β1, and γ1 chains, and the gene encoding for the human and mouse α1 and β1 chains shows an identity of 76 and 93%, respectively [33, 34]. The C-terminal end of the long arm of laminin is composed of 5 globular domains named laminin globular (LG) domains G1–G5 [32, 35]. TEM revealed that gold-labeled recombinant PE bound to the C-terminal globular domain of laminin (Figure 6*B*). Because the LG domains G4 and G5 contain an active heparin-binding site [35], we performed inhibition experiments with heparin. Increasing concentrations of heparin inhibited the binding of laminin to PE^{22-160} in a dose-dependent manner (Figure 6*C*). Thus, the binding site of PE^{22-160} on the laminin molecule was located within LG domains G4 or G5.

DISCUSSION

An initial step in the pathogenesis of *H. influenzae* is adherence to the mucosa followed by attachment to epithelial cells and the ECM in the respiratory tract. NTHi expresses a number of adhesins that are involved in the success of NTHi colonization in patients with, for example, COPD [36]. Here we demonstrate a specific binding of the ECM protein laminin to NTHi. Interestingly, the adhesin PE appeared to have a dominant role in *Haemophilus*-dependent laminin binding to the well-known laminin-binding protein Hap [15].

H. influenzae PE is a 16 kDa lipoprotein with adhesive properties [19]. NTHi without PE showed a significantly decreased laminin-binding compared with that of the isogenic wild-type strain. In addition, Hap from *H. influenzae* also binds laminin [15]. Hap is an adhesin that mediates adherence to epithelial cells, ECM, bacterial aggregates, and microcolony formation [37]. When PE or Hap was deleted in our model strain NTHi3655, we observed a significantly decreased binding to both soluble and immobilized laminin, suggesting that these 2 proteins are the major laminin-binding proteins. However, the double mutant that lacked PE and Hap weakly bound to both soluble and immobilized laminin, suggesting additional laminin-binding proteins expressed by NTHi. The expression of multiple laminin-binding proteins has also been shown for several other pathogens, eg, *L. interrogans*, *Streptococcus pyogenes*, *Borrelia burgdorferi*, and *M. catarrhalis* [14, 38–44].

NTHi is among the most common pathogens found in exacerbations as well as in stable disease in patients suffering from COPD [22]. Among the major causal factors of COPD is smoking, and in smokers there are pathological changes such as loss of epithelial integrity, which results in exposure of the basement membrane where the laminin layer is thickened [23, 45]. The human lung contains several different forms of laminin, including the cruciform laminin-10 and laminin-11 [33]. Laminin-1 used in this study and laminin-10 both contain β1 and γ1 chains, suggesting an importance of the NTHi/laminin interaction during infections in the lung. Little is known about the distribution and alteration of laminin during pathological conditions such as COPD. In addition, whether bacterial infections can alter the laminin expression or composition remains to be studied. However, these patients also have an increased synthesis and deposition of ECM proteins, including laminin [46]. Smoking thus indirectly may promote a more efficient laminin-dependent NTHi colonization. Other pathogens that cause respiratory infections, such as *M. catarrhalis*, also possess laminin-binding proteins [14], suggesting adhesive mechanisms that are shared by several respiratory pathogens.

The major laminin-binding domain was found within PE^{41-68}, and inhibition experiments with peptides confirmed that PE^{41-68} was responsible for the interaction. In a recent paper, we showed that PE^{84-108} binds to epithelial cells in addition to

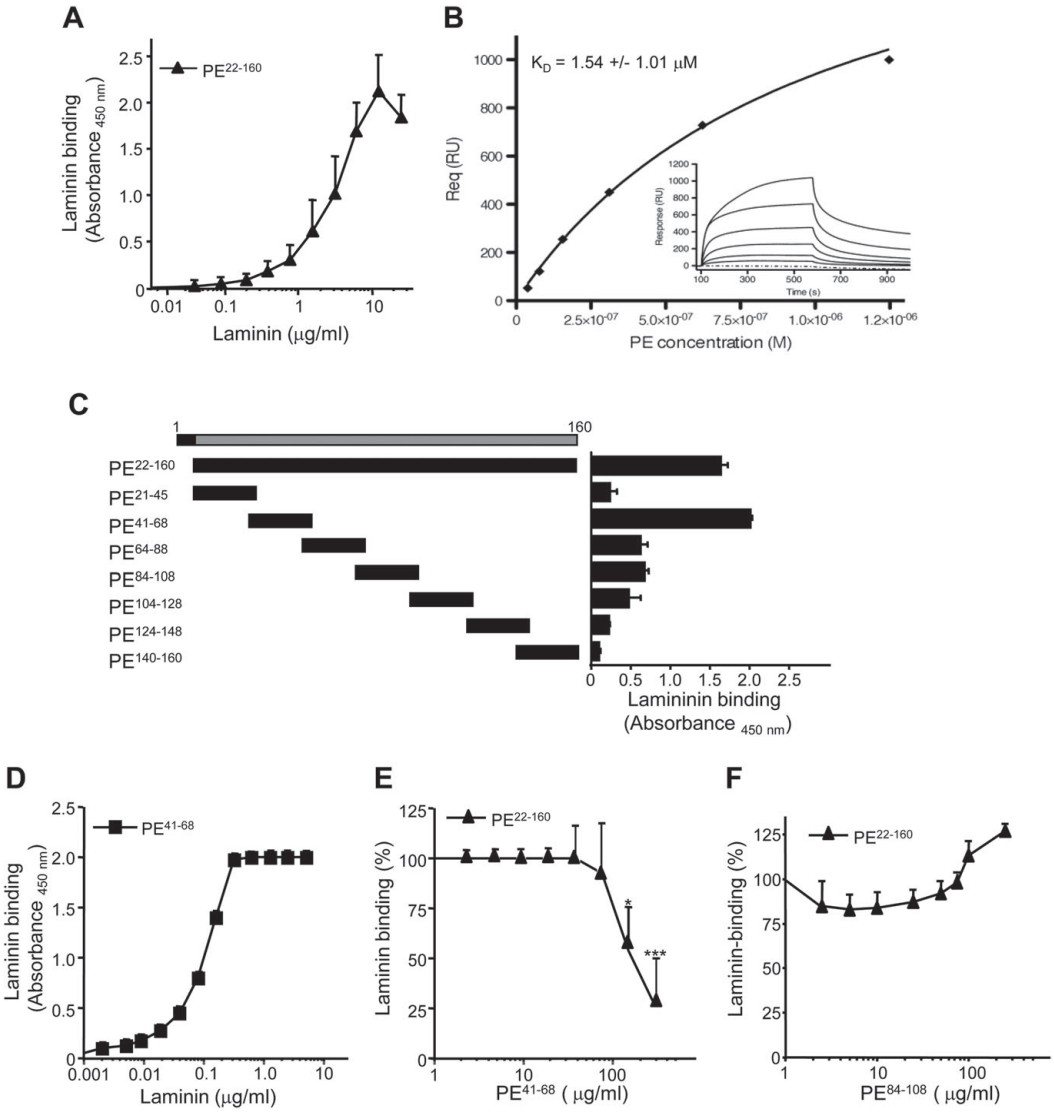

Figure 4. The laminin binding region is located within protein E (PE)$^{41-68}$. *A*, Immobilized PE$^{22-160}$ (5 μg/mL) binds laminin in a dose-dependent manner. The background binding was subtracted from all samples. *B*, PE $^{22-160}$ bound laminin as shown by surface plasmon resonance. *C*, The active laminin-binding region is located within PE$^{41-68}$. *D* and *E*, The binding of PE$^{41-68}$ to laminin is dose-dependent and specific. *F*, PE$^{84-108}$ does not inhibit laminin binding. PE$^{22-160}$ (5 μg/mL) (*A*), peptides (40 μM) spanning the entire PE molecule (with an overlap of 4 amino acids) (*C*), or PE$^{41-68}$ (*D*) was incubated with laminin (5 μg/mL) (*A, C*) or increasing concentrations of laminin (0–5 μg/mL) (*D*), and binding was analyzed in enzyme-linked immunosorbent assay (ELISA). In *E* and *F* laminin (5 μg/mL) was added with increasing concentrations of PE$^{41-68}$ (2-300 μg/mL) or PE $^{84-108}$ (2–300 μg/mL) to microtiter plates coated with PE$^{22-160}$. In *A* and *C–F*, bound laminin was detected with a rabbit antilaminin pAb followed by horseradish peroxidase (HRP)–conjugated goat antirabbit pAb. The mean values of 3 independent experiments are shown with error bars indicating standard deviation (SD). Statistical significance of differences was estimated using Student's *t* test. ***, $P \leq .001$, *, $P \leq .05$. *B*, Binding of PE to laminin was studied using surface plasmon resonance (Biacore 2000). Laminin was immobilized on a CM5 chip, and increasing concentrations of PE (40–1250 nM) were injected and sensorgrams recorded (inset). The sensorgram obtained for 1250 nM of Moraxella IgD binding protein (MID), a negative control, is shown as dotted line. Responses at equilibrium were plotted vs concentration of injected PE and the dissociation constant (K_D) = 1.54 ± 1.01 μM was calculated using a steady-state affinity equation in Biaevaluation 3.0.

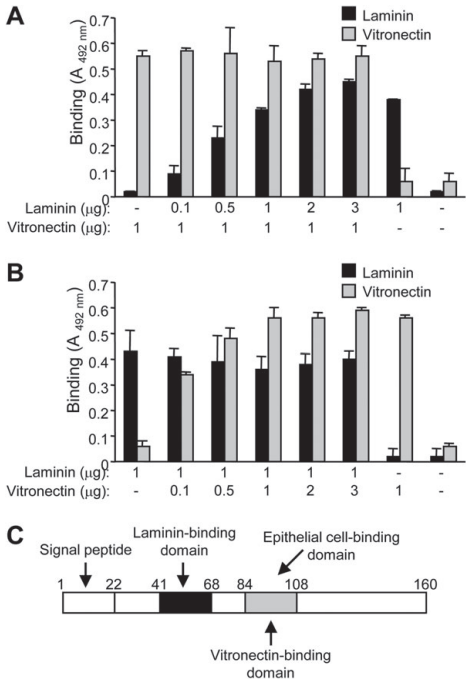

Figure 5. Protein E (PE) is a multifunctional protein that binds both laminin and vitronectin via different regions. *A, B,* Laminin and vitronectin bind concurrently to PE. *A,* Increasing laminin concentrations did not influence vitronectin binding to PE. *B,* Increased concentrations of vitronectin did not influence laminin binding to PE. *A, B,* PE^{22-160} was immobilized and concurrent binding of laminin and vitronectin (micrograms added are shown) were detected by specific antibodies in enzyme-linked immunosorbent assay (ELISA). *C,* Illustration showing the multifunctional PE. The laminin-binding region is located within PE^{41-68}, and the vitronectin and epithelial cell–binding regions are within PE^{84-108} [18, 20].

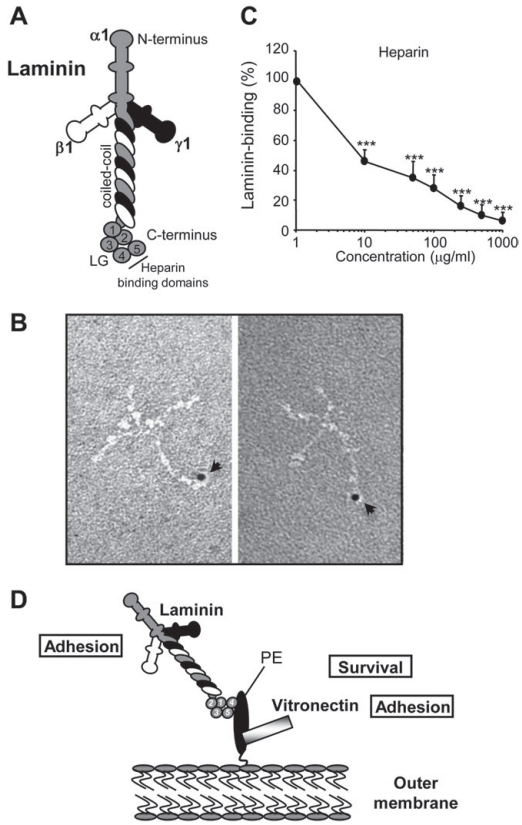

Figure 6. Protein E^{22-160} binds the C-terminal globular domains G4 and G5 of laminin. *A,* Schematic cartoon of laminin showing the composition of an α- (gray), β- (white), and γ-polypeptide chain (black) joined together through a coiled-coil with 1 long and 3 short arms [31]. The illustration is modified from McKee et al and Durbeej et al [31, 32]. The C-terminal end of the long arm is composed of 5 α-chain laminin globular (LG) domains G1–G5 and LG domains G4 and G5 contain a heparin-binding site. *B,* Protein E (PE) binds the C-terminal globular head of laminin. Gold-labeled PE^{22-160} was mixed with laminin and examined by electron microscopy. *C,* Increasing amounts of heparin inhibited the binding of laminin to PE^{22-160}. In *C,* immobilized PE^{22-160} was incubated with laminin and increasing concentrations of heparin, and bound laminin was detected with a rabbit antilaminin pAb followed by horseradish peroxidase (HRP)-conjugated antirabbit pAb. The mean values of 3 independent experiments are shown with error bars indicating standard deviation (SD). Statistical significance of differences was estimated using the Student *t* test. ***, $P \leq .001$. *D,* Schematic picture of simultaneous binding of PE to laminin and vitronectin. PE-dependent binding of laminin may contribute to nontypeable *Haemophilus influenzae* (NTHi) adhesion and colonization of the host. The PE-dependent binding of the complement regulator vitronectin to the surface of NTHi protects against complement-mediated attacks and significantly contributes to the survival of NTHi in human serum.

human vitronectin [18, 20]. Competition assays with laminin and vitronectin confirmed that both proteins are able to bind PE simultaneously, showing different binding sites on the PE molecule. These results reveal that various regions of PE have different, specific functions. Despite the small size (16 kDa), PE binds epithelial cells, vitronectin and laminin, suggesting that it is multifunctional (Figure 6*D*). Similar binding profiles have been shown for other bacterial proteins, eg, the *M. catarrhalis* UspA1/2 family that bind laminin, vitronectin, fibronectin, and C3 [14, 47–49]. Laminin is a glycoprotein that consists of an α-, β-, and γ-polypeptide chain joined together through a coiled coil with 1 long and 3 short arms [31]. There are 15 different forms of laminins, designated laminin-1 to laminin-15, belonging to 1 of 3 general types of laminin heterotrimer structures [33]. Most laminins, including laminin-1, belong to the cruciform structure (Figure 6*A*). Interestingly, full-length

PE bound the C-terminal globular domains of laminin as revealed by TEM. These domains also bind heparin [32, 35], and heparin inhibits the interaction between PE and laminin, confirming the involvement of the C-terminal globular domains G4 and G5. The ability to bind laminin, which is a major constituent of the ECM and basal membrane, suggests that bacteria may use this interaction for adherence to the lung parenchyma followed by an efficient colonization of the host. In fact, *M. tuberculosis* probably uses laminin as a target protein to facilitate adhesion to host epithelial cells [12]. Several pathogen-derived surface proteins that bind laminin are recently identified, eg, Scl1 from *S. pyogenes*, LipL53, Lsa21 and Lsa63 from *L. interrogans*, and CRASP-1 from *B. burgdorferi* [38, 40–42, 44].

In conclusion, we have shown that the adhesin and vitronectin-binding PE of NTHi has the basement-membrane glycoprotein laminin as a major target. The specific interaction with laminin may contribute to adhesion, bacterial colonization and spread. Laminin-binding proteins most likely play a larger role than previously anticipated both in the upper respiratory tract in children and in the airways of COPD patients. However, more investigations are required to fully delineate the importance of pathogen-dependent interactions with laminin.

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Paper II

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Binding of Complement Regulators to Invasive Nontypeable *Haemophilus influenzae* Isolates Is Not Increased Compared to Nasopharyngeal Isolates, but Serum Resistance Is Linked to Disease Severity▽

Teresia Hallström,[1] Fredrik Resman,[1,2] Mikael Ristovski,[1] and Kristian Riesbeck[1]*

Medical Microbiology, Department of Laboratory Medicine,[1] and Infectious Diseases, Department of Clinical Sciences,[2] University Hospital Malmö, Lund University, SE-205 02 Malmö, Sweden

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The aim of the present study was to analyze the importance of nontypeable *Haemophilus influenzae* (NTHi) isolated from patients with sepsis (invasive isolates) compared to nasopharyngeal isolates from patients with upper respiratory tract infection for resistance to complement-mediated attack in human serum and to correlate this result with disease severity. We studied and characterized cases of invasive NTHi disease in detail. All patients with invasive NTHi isolates were adults, and 35% had a clinical presentation of severe sepsis according to the ACCP/SCCM classification of sepsis grading. Moreover, 41% of the patients had evidence of immune deficiency. The different isolates were analyzed for survival in human serum and for binding of ^{125}I-labeled, purified human complement inhibitors C4b-binding protein (C4BP), factor H, and vitronectin, in addition to binding of regulators directly from serum. No significant differences were found when blood-derived and nasopharyngeal isolates were compared, suggesting that interactions with the complement system are equally important for NTHi strains, irrespective of isolation site. Interestingly, a correlation between serum resistance and invasive disease severity was found. The ability to resist the attack of the complement system seems to be important for NTHi strains infecting the respiratory tract as well as the bloodstream.

Haemophilus influenzae is an important human-specific pathogen that can be classified according to the presence of a polysaccharide capsule (20). The encapsulated strains cause invasive diseases, whereas the unencapsulated and hence nontypeable *H. influenzae* (NTHi) strains are mainly found in local upper and lower respiratory tract infections (2, 35). However, NTHi is, after *Streptococcus pneumoniae*, the most common microbe found in children with acute otitis media and is the main cause of exacerbations in patients suffering from chronic obstructive pulmonary disease and bronchiectasis (3, 4, 13, 28, 32, 34). NTHi can also cause sinusitis, conjunctivitis, and pneumonia in children (25). Thus, NTHi is a heterogenous species, capable of great variation in virulence, and is found in the airway either as a commensal or as a pathogen with the capacity to invade the airway epithelium.

Invasive disease caused by *H. influenzae* type b (Hib) mainly affects infants and children, causing potentially life-threatening conditions, such as meningitis, epiglottitis, and severe sepsis. After introduction of the conjugate vaccine against Hib in the early 1990s, the incidence of invasive disease caused by Hib has decreased substantially in the Western hemisphere (5). In contrast, it has been suggested that the incidence of NTHi septicemia is increasing (36). Most clinical studies of invasive *Haemophilus* infections have been about Hib, and less is known about the clinical characteristics of invasive disease caused by NTHi. In relation to its extensive presence in nasopharyngeal and sputum cultures, NTHi is infrequently found in the bloodstream and it seems likely that host factors are equally important as specific bacterial virulence in patients with NTHi sepsis.

The complement system is the first line of defense against pathogenic microorganisms (7). Activation of the complement system leads to a cascade of protein activation and deposition on the surface of the pathogen, resulting in formation of the membrane attack complex (MAC) and opsonization of the pathogen, followed by phagocytosis (38). The classical pathway of the complement system is activated by target-bound antibodies and C-reactive protein (37), whereas the alternative pathway is spontaneously activated through direct contact with foreign particles or cells (38). Both pathways lead to the formation of the C3 convertases, with subsequent cleavage of C3 to C3a and C3b. Thereafter, the C5 convertases are formed and the terminal pathway is activated, which results in the formation of the MAC and lysis of the cell. To prevent nonspecific damage from excess complement activation, the complement cascade is tightly regulated. Important regulators of the complement system are C4b-binding protein (C4BP) (governing the classical/lectin pathway) (6), factor H and factor H-like protein 1 (alternative pathway) (41), and vitronectin and complement factor H-related protein 1 (terminal pathway) (18, 33).

The complement system is classified as a part of serum, but there are several studies demonstrating the presence of complement in various sites of the body. Reports of the presence of complement components in the respiratory tracts of healthy individuals are scarce. There are several studies, however, indicating the importance of complement in the respiratory tract

* Corresponding author. Mailing address: Medical Microbiology, Dept. of Laboratory Medicine, University Hospital Malmo, Lund University, SE-205 02 Malmo, Sweden. Phone: 46-40-338494. Fax: 46-40-336234. E-mail: kristian.riesbeck@med.lu.se.

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during infections. The permeability of the mucosa increases during inflammation, and plasma, including complement proteins and immunoglobulins, enters the airway lumen (11, 12, 29). This process, designated plasma exudation, has been suggested to be the first line of the mucosal defense.

The pathogenesis of many microorganisms relies on their capacity to avoid, resist, or neutralize the host defense, including the complement system. Therefore, many pathogens have evolved different mechanisms to avoid complement-mediated killing. A frequent strategy used by some pathogens is binding of complement inhibitors such as C4BP, factor H, and vitronectin, which all protect from complement-mediated attacks (7, 22, 31). These inhibitors are captured on the bacterial surface in such a way that they are still functionally active. In previous studies, we have shown that NTHi binds C4BP and factor H and that these interactions significantly contribute to bacterial serum resistance (15, 17). In addition, *Haemophilus* surface fibrils that can be found solely in Hib, and protein E, which exists in both encapsulated and nontypeable strains (30), interact with vitronectin and thereby prevent complement-induced lysis, resulting in increased bacterial survival in normal human serum (NHS) (14, 16).

In the present study, the characteristics of invasive NTHi infections, including evidence of immune deficiency in the individual patient and the clinical presentation of the septic event, were studied. We correlated these findings with the capacity to bind specific complement regulators and the *in vitro* serum resistance of the individual isolates. The invasive NTHi isolates obtained from patients with sepsis were compared to nasopharyngeal strains from patients with upper respiratory tract infection. There was no clear difference in serum resistance or binding to complement inhibitors between the two groups of NTHi. Our findings also demonstrate that binding of complement regulators and resistance to human serum are important for NTHi isolates from the upper respiratory tract as well as those from blood samples. Furthermore, a significant correlation between disease severity and *in vitro* serum resistance was identified in cases of NTHi invasive disease.

MATERIALS AND METHODS

Patient data. Complete medical records, including clinical presentation, patient history, and laboratory results, from 17 out of 21 identified patients with invasive NTHi disease (bacteremia or meningitis) were studied and registered. The severity of the sepsis was graded according to the ACCP/SCCM classification of sepsis grading (8). Randomly selected nasopharyngeal cultures positive for NTHi ($n = 21$) were used as controls. All of these cultures had a referral history of airway infection.

Bacterial strains and culture conditions. Blood-derived NTHi isolates ($n = 21$) were obtained from patients with invasive NTHi disease (bacteremia or meningitis) in the county of Southwest Skane, Sweden, from 2001 to 2007. In addition, NTHi strains isolated from the nasopharynxes ($n = 21$) of patients (Southwest Skane in 2007) suffering from upper respiratory tract infection were included for comparison. Bacteria were routinely cultured in brain heart infusion (BHI) liquid broth supplemented with NAD and hemin (both at 10 μg/ml) or on chocolate agar plates at 37°C in a humid atmosphere containing 5% CO_2. All strains were characterized by standard bacteriological techniques, including oxidase, fermentation, satellite, and XV tests. Thereafter, the *H. influenzae* strains were typed by PCR. To exclude that *H. haemolyticus* was among the isolates, PCR and 16S rRNA sequencing was done (24).

Proteins and antibodies. C4BP and factor H from human plasma were purchased from Complement technology (Tyler, TX), and vitronectin from human plasma was from Sigma (Sigma-Aldrich, St. Louis, MO). The polyclonal rabbit anti-C4BP and goat anti-human factor H antisera were purchased from Complement technology or Calbiochem (La Jolla, CA). The horseradish peroxidase (HRP)-conjugated donkey anti-goat polyclonal antibody (pAb) was obtained from Serotech (Oxford, United Kingdom). The goat anti-human vitronectin and fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat pAbs were from Serotec (Oxford, United Kingdom). An antiserum obtained from a rabbit immunized with the clinical isolate NTHi 772 for 3 times at 2-week intervals was also included, as a control (1).

Serum bactericidal assay. Pooled NHS was obtained from healthy blood donors ($n = 5$) with informed consent. The NTHi strains were diluted in $DGVB^{++}$ (2.5 mM Veronal buffer, pH 7.3, containing 70 mM NaCl, 140 mM glucose, 0.1% [wt/vol] gelatin, 1 mM $MgCl_2$, and 0.15 mM $CaCl_2$). Bacteria (10^4 CFU) were incubated in NHS or heat-inactivated NHS (hiNHS) in a final volume of 100 μl at 37°C. After 20 min, 10-μl aliquots were removed and spread onto chocolate agar plates. After 18 h of incubation at 37°C, numbers of CFU were determined.

Flow cytometry. To analyze whether the NHS contained IgG directed against NTHi and if there were any differences in IgG deposition on NTHi isolates from blood and nasopharynx samples, NHS was tested for reactivity by flow cytometry using a FITC-conjugated mouse anti-human IgG pAb (Dakopatts). A rabbit anti-NTHi pAb was included as a positive control, and in these experiments, a FITC-conjugated goat anti-rabbit pAb (Dakopatts) was used as a secondary layer. Briefly, bacteria (10^7) were incubated with 50% hiNHS for 1 h on ice. Thereafter, bacteria were washed, followed by addition of the FITC-conjugated detection antibodies, additional washes, and finally flow cytometry analysis. All incubations were done in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA), and washings were done with the same buffer. Secondary antibodies were added separately as negative controls for each strain analyzed.

Protein labeling and direct binding assay. Purified C4BP, factor H, and vitronectin were labeled with 0.05 mol iodine (GE Healthcare, Buckinghamshire, United Kingdom) per mol protein, using the chloramine T method (10). The different *H. influenzae* strains were grown in BHI liquid broth overnight and washed in PBS containing 1% BSA (Saveen Werner, Malmo, Sweden) (PBS-BSA). Bacteria (2×10^7) were incubated with ^{125}I-labeled complement regulators at 37°C for 1 h. After incubation, bacteria were centrifuged ($10{,}000 \times g$) through a 20% sucrose column. The tubes were frozen and cut, and levels of radioactivity in the pellet and supernatant were measured using a gamma counter. Binding was calculated as amount of bound radioactivity (pellet) versus total radioactivity (pellet plus supernatant).

Serum binding assay. To analyze whether NTHi from different isolation sites bound C4BP or factor H directly from NHS, bacteria were grown overnight in BHI broth. NTHi bacteria (10^9) were incubated with hiNHS and buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4) for 1 h at 37°C. To remove unbound proteins, bacteria were washed 5 times with the same buffer. Thereafter, the bacterial pellet was resuspended in 150 μl of 0.1 M glycine-HCl, pH 2.0, in order to elute bound proteins (15, 17, 21). Bacteria without NHS were used as a negative control. After 15 min of incubation at 37°C with shaking, bacteria were centrifuged and the supernatants were subjected to SDS-PAGE (10%).

To analyze whether NTHi bound vitronectin from NHS, bacteria were grown overnight and incubated with hiNHS and PBS. To remove unbound proteins, NTHi bacteria were washed 5 times with the same buffer. Thereafter, the bacterial pellet was resuspended in 50 μl of 0.1% Triton X-100 (Darmstadt, Germany) and protease inhibitors (Complete; Roche, Mannheim, Germany). After 30 min of incubation at 4°C, bacteria were centrifuged and the supernatants were subjected to SDS-PAGE (10%). Electrophoretic transfer of protein bands from the gel to an Immobilon-P membrane (Millipore, Bedford, MA) was done at 35 V for 2 h. After transfer, the Immobilon-P membrane was blocked in PBS with 0.1% Tween 20 (PBS-Tween) containing 5% milk powder. After several washings, the membrane was incubated with rabbit anti-human C4BP, goat anti-human factor H, or goat anti-human vitronectin pAb, followed by incubation with HRP-conjugated swine anti-rabbit or donkey-anti-goat pAb. After incubation and additional washings in PBS-Tween, development was performed with enhanced chemiluminescence (ECL) Western blotting detection reagents (Pierce, Rockford, IL). No cross-reactivity of the anti-C4BP, anti-factor H, or anti-vitronectin pAbs was seen with bacteria incubated in the absence of NHS.

Statistical analysis. To test for differences in survival in serum and levels of complement inhibitor binding between the groups, a two-sided Mann-Whitney U test was used. This was done in consideration of small sample sizes and nonparametric data.

RESULTS

Clinical characteristics of invasive NTHi disease. Twenty-one cases of invasive NTHi were identified during the period

TABLE 1. Clinical and epidemiological data from 17 cases of invasive NTHi infection and survival of isolates in serum *in vitro*

Patient	Age (yr)	Gender	Immune status at time of sepsis	Sepsis grading[a]	% Survival of NTHi strain in serum *in vitro*[b]
1	52	Male	Agammaglobulinemia	Sepsis	5 ± 3
2	69	Male	No sign of immune deficiency	Sepsis	2 ± 3
3	60	Female	Metastatic breast cancer	Sepsis	18 ± 14
4	75	Male	Hypogammaglobulinemia	Sepsis	66 ± 31
5	75	Male	No sign of immune deficiency	Sepsis	8 ± 7
6	37	Female	Chemotherapy	Sepsis	23 ± 7
7	58	Male	Leukemia, neutropenia	Sepsis	63 ± 15
8	74	Female	No sign of immune deficiency	Sepsis	62 ± 0
9	36	Male	No sign of immune deficiency	Sepsis/meningitis	17 ± 8
10	73	Male	NA[c]	Sepsis/meningitis	44 ± 10
11	60	Male	No sign of immune deficiency	Sepsis/meningitis	79 ± 24
12	42	Female	No sign of immune deficiency	Severe sepsis	89 ± 2
13	39	Male	No sign of immune deficiency	Severe sepsis	81 ± 25
14	64	Female	No sign of immune deficiency	Severe sepsis	52 ± 4
15	89	Female	Chronic leukemia	Severe sepsis	86 ± 19
16	83	Male	No sign of immune deficiency	Severe sepsis	64 ± 40
17	74	Female	No sign of immune deficiency	Severe sepsis	58 ± 12

[a] Graded according to the ACCP/SCCM classification of sepsis grading.
[b] Values shown represent levels observed after 20 min of incubation with 5% NHS and are means ± SD of results from three experiments.
[c] NA, not available.

from 2001 to 2007, and complete medical records were obtained from 17 patients, as shown in Table 1. In contrast to invasive Hib disease, which in unvaccinated populations is mainly associated with younger patients, all patients with invasive NTHi disease were adults. The cases were divided into separate groups by severity of clinical disease according to the ACCP/SCCM classification (Table 1). Eight of the patients (47%) (patients 1 to 8) had a clinical presentation of sepsis, while nine (53%) (patients 9 to 17) had a presentation of sepsis with meningitis or severe sepsis. Interestingly, seven of the patients (41%) had evidence of immune deficiency. Out of these 7 patients, 6 presented secondary immune deficiencies such as leukemia and treatment-induced neutropenia. However, severe clinical presentations were not linked to immune deficiency. One patient died from the septic episode (28-day mortality; 6%), while 5 patients died within a year (1-year mortality; 29%). All but one of the patients that died within a year from their septic episode presented a mild clinical picture, suggesting the importance of underlying disease.

Serum resistance correlates with sepsis severity. Eight strains isolated from blood samples were studied with regard to survival at increasing concentrations of pooled NHS from 5 donors to determine the appropriate serum concentration for further serum resistance experiments (Fig. 1 and data not shown). When a titration of the serum concentration was performed, all strains except the isolate from patient 13 (Fig. 1C) were killed by 15% NHS after 20 min of incubation. All strains survived in 5% NHS, and therefore, this concentration was used for further studies.

When the cases of invasive disease were sorted according to disease severity, we considered the cases of sepsis with meningitis clinically severe and pooled these with the cases of severe sepsis according to the ACCP/SCCM grading. All NTHi strains were tested for serum resistance in 5% NHS for 20 min. Interestingly, NTHi isolates from patients with severe sepsis and meningitis ($n = 9$) had a significantly ($P = 0.04$) higher degree of serum resistance (mean, 63%) than NTHi isolates from patients with sepsis ($n = 8$) (mean, 31%) (Table 1). When we excluded the cases of sepsis with meningitis from the analysis, the level of serum resistance among the patients with severe sepsis ($n = 6$) (mean, 72%) was still significantly higher than that among the patients with sepsis ($P = 0.04$).

No difference in serum resistance was found between invasive and nasopharyngeal NTHi isolates. To test whether there was a difference regarding serum resistance between NTHi isolates from separate patients suffering from sepsis (blood-derived isolates) (Table 1) and upper respiratory tract infection (nasopharyngeal isolates) (Table 2), bacteria from the two

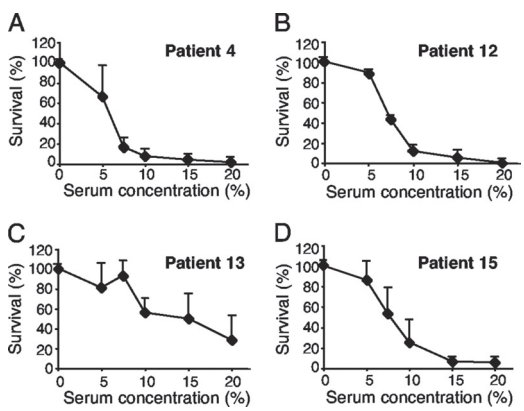

FIG. 1. Bactericidal activity against a series of randomly selected blood-derived isolates. NTHi bacteria were incubated in the presence of increasing concentrations of NHS (5 to 20%). Four typical strains are shown in panels A to D. After incubation for 20 min, bacteria were spread on chocolate agar plates to allow determination of the number of surviving bacteria. The number of bacteria (CFU) at the initiation of the experiment was defined as 100%. The mean values from three independent experiments are shown, with error bars indicating standard deviations (SD).

TABLE 2. Demographics of 21 cases of upper respiratory tract infection and serum resistance of NTHi nasopharyngeal isolates *in vitro*

Patient	Age (yr)	Gender	% Survival of NTHi strain in serum *in vitro*[a]
1	1	Male	51 ± 31
2	4	Female	82 ± 32
3	3	Female	4 ± 5
4	35	Male	69 ± 8
5	5	Male	45 ± 5
6	36	Male	60 ± 41
7	4	Male	22 ± 12
8	5	Female	58 ± 51
9	8	Male	28 ± 4
10	3	Female	70 ± 15
11	23	Female	60 ± 7
12	0	Female	54 ± 40
13	17	Male	52 ± 22
14	35	Male	42 ± 25
15	4	Male	15 ± 1
16	2	Male	67 ± 15
17	17	Male	27 ± 13
18	2	Female	5 ± 3
19	3	Female	94 ± 12
20	2	Male	4 ± 4
21	1	Male	24 ± 19

[a] Values shown represent levels observed after 20 min of incubation with 5% NHS and are means ± SD of results from three experiments.

sites of isolation were compared in a serum bactericidal assay. The nasopharyngeal strains were isolated from immunocompetent patients with suspected bacterial upper respiratory tract infection. Primary viral infection could not, however, be excluded. The age distribution of the patients from whom the nasopharyngeal control isolates were obtained slightly differed from that of the patients with invasive NTHi infection, reflecting the local clinical tradition of taking nasopharyngeal swabs primarily from children. After incubation in NHS (5%), the survival rates of the different NTHi isolates were determined (Fig. 2A and B). Survival rates varied between 2 and 89% for the blood-derived isolates and 4 and 94% for the nasopharyngeal isolates. No significant difference in survival rate was found between the two different sites of isolation, however. All strains included in the study were resistant to hiNHS (Fig. 2C).

To confirm that the NHS used in this study contained antibodies directed against NTHi isolates from both blood samples and nasopharynxes and whether there was a difference in binding of IgG between strains from blood samples and those from nasopharynxes, we analyzed IgG deposition on randomly selected blood-derived ($n = 3$) and nasopharyngeal ($n = 3$) isolates. Two typical strains are shown in Fig. 2D. Human IgG was deposited on the blood-derived isolates and the nasopharyngeal isolates to similar extents, suggesting that there were no significant difference in antibody binding to strains from the two anatomical sites of isolation. The anti-NTHi 772 rabbit antiserum was included as a positive control and bound all strains tested. Thus, invasive NTHi strains are not more serum resistant than strains isolated from the upper respiratory tract.

NTHi isolates from blood samples or nasopharynxes bind complement regulators. Several bacterial species have been shown to bind complement regulators and thereby counteract the different pathways of the complement system (7, 22, 31). To determine whether there was a difference in binding of various complement inhibitors between NTHi isolates from patients with invasive disease (blood-derived isolates) and those from patients with upper respiratory tract infection (nasopharyngeal isolates), a collection of NTHi strains was incubated with ^{125}I-labeled C4BP, factor H, or vitronectin, followed by separation of unbound ligand. Binding was calculated as the ratio of bound radioactivity versus total radioactivity added. Interestingly, there was no significant difference in binding between blood-derived and nasopharyngeal isolates (Fig. 3). Binding of the complement regulators varied between different strains, independently of isolation sites. The blood-derived and nasopharyngeal strains with the highest levels of C4BP binding bound 19.0% and 21.3%, respectively (Fig. 3A). When factor H was added, the blood-derived and nasopharyngeal strains with the highest levels of factor H binding bound 16% and 30.4% factor H, respectively (Fig. 3B). Finally, the blood-derived strain with the highest level of vitronectin binding bound 28.0% vitronectin, and the nasopharyngeal strain with the highest level of binding bound 24.1% vitronectin.

In order to analyze whether the strains isolated from blood samples or nasopharynxes also bound the three different complement inhibitors directly from NHS, bacteria were incubated with hiNHS for 1 h at 37°C. Thereafter, bound proteins were eluted, separated by SDS-PAGE, and analyzed by Western blot using specific antibodies directed against human C4BP, factor H, or vitronectin. In these experiments, binding of the three different regulators corresponded with the results from the direct binding assay. Moreover, no significant difference in binding of complement inhibitors could be detected when blood-derived and nasopharyngeal NTHi isolates were compared (data not shown). Taken together, there was no significant difference in binding of the three different complement regulators between blood-derived isolates and nasopharyngeal strains from patients with upper respiratory tract infection, suggesting the importance of the complement regulator binding capacity of NTHi in the upper respiratory tract as well.

DISCUSSION

The results in this study imply that invasive disease caused by NTHi is heterogenous in character. Although it has been reported that NTHi sepsis can have a severe clinical presentation mimicking that of encapsulated strains (40), the proportion of cases in the present study with a severe clinical picture was surprisingly high. The patients affected were all adults, and most cases could be sorted into one of two typical presentations, comprising patients with evidence of immune deficiency and mild clinical presentation and adults with no prior evidence of immune deficiency and severe clinical presentation. In the first category, host factors seem decisive for infection, but in the second category, the virulence of specific bacterial strains could be important, as supported by the higher degree of serum resistance *in vitro* in this group. Thus, invasive NTHi disease differs from invasive disease caused by Hib in epidemiology as well as general clinical presentation. NTHi can, however, still readily cause severe sepsis in previously healthy individuals. When the individual cases of sepsis were graded by severity according to the ACCP/SCCM grading system, a cor-

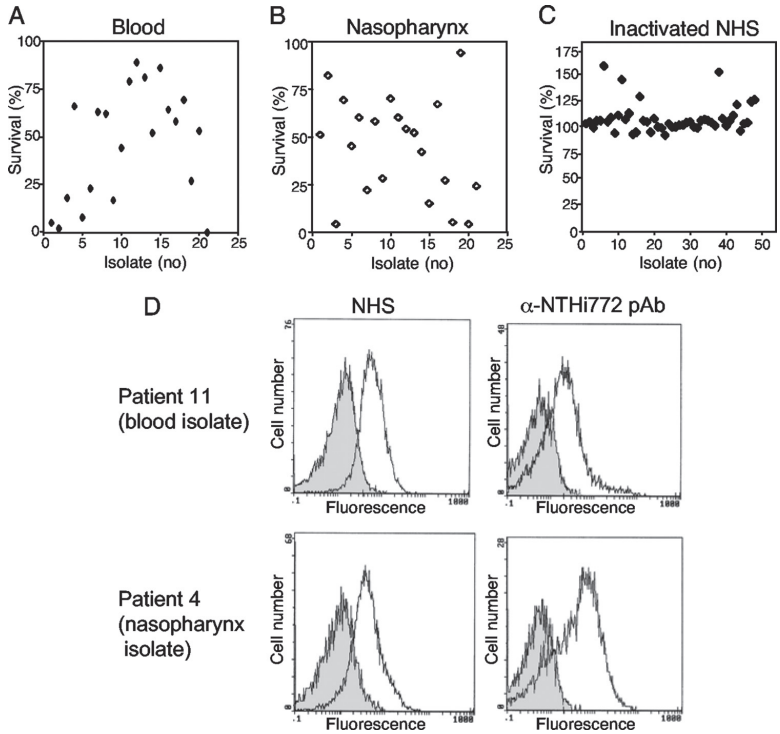

FIG. 2. NTHi isolates from blood samples (invasive disease) or nasopharynxes of patients with upper respiratory tract infection show variation in serum resistance independent of the presence of IgG in NHS. (A and B) No significant difference in survival rate in NHS was observed between NTHi isolates from blood samples and those from nasopharynxes. (C) All strains were resistant to hiNHS. NTHi isolates from blood samples (n = 21) (A) and nasopharynx (n = 21) (B) were analyzed for survival in human serum. The strains were incubated in the presence of 5% NHS or 5% hiNHS for 20 min. Thereafter, bacteria were spread on chocolate agar plates to allow determination of the number of surviving bacteria. The numbers of bacteria (CFU) at the initiation of the experiment was defined as 100%. (D) The NHS contained IgG directed against NTHi strains independently of whether they were isolated from blood samples or nasopharynxes. Flow cytometry profiles show the deposition of IgG and the binding of a rabbit anti-NTHi antiserum to the surfaces of the NTHi strains from patients 11 (blood) and 4 (nasopharynx). Bacteria were incubated with mouse anti-human IgG or rabbit anti-NTHi antiserum, followed by FITC-conjugated anti-mouse pAb or anti-rabbit pAb. Results for one representative experiment out of three independent ones performed are shown.

relation between severe clinical sepsis and bacterial *in vitro* serum resistance was found. This indicates that serum resistance is of great importance for the severity of the invasive disease. This finding is consistent with the theory that resistance to human serum facilitates spread of bacteria in the body but has not been demonstrated for NTHi sepsis prior to this study.

An analysis of the clinical data from the sepsis patients showed that many of the cases of NTHi sepsis occurred in immunocompromised hosts. The finding that the severity of the sepsis cases was significantly related to the survival rate of the NTHi strains in serum *in vitro* implies that while NTHi seems to act as an opportunistic agent in many cases of invasive disease, once it has caused invasive disease, higher complement resistance and survival rates in serum are related to a more clinically severe sepsis. Our data suggest that NTHi septic disease is heterogeneous, possibly reflecting the NTHi cluster in general. The number of cases of invasive disease in this study was limited, however, and this has to be taken into account in the interpretation of the results.

Studies using pulsed-field gel electrophoresis have shown that the dynamics of NTHi carriage in the airways is rapid and that one individual patient can carry more than a dozen different subtypes of NTHi at the same time (39). This raises the issue of selection bias in nasopharyngeal cultures. However, antibodies raised against one NTHi strain give considerable cross-protection against other strains (23). This could partly explain why the incidence of bloodstream infections caused by NTHi is low. In parallel, a decreased humoral immune competence caused by a disease such as leukemia or by immune-modulating pharmaceutical agents could explain some of the cases of NTHi sepsis seen and presented in this study.

In our initial experiments, we incubated invasive isolates with increasing concentrations of NHS and analyzed survival. All strains except one were killed by 15% NHS (Fig. 1 and data not shown). However, even if the majority of a population is

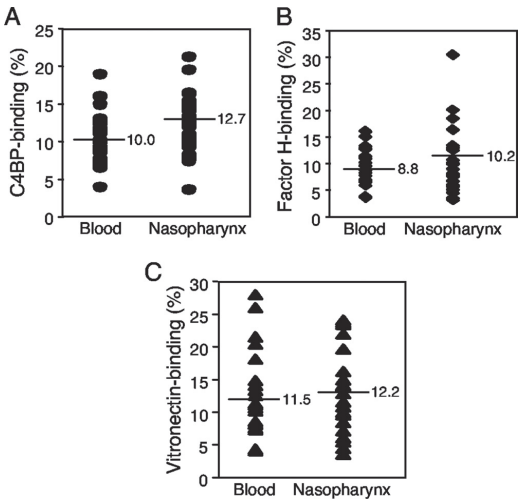

FIG. 3. Both blood-derived and nasopharyngeal NTHi isolates bind complement regulators. A series of clinical strains isolated from blood samples ($n = 21$; cases with invasive disease and an available patient history are listed in Table 1) or nasopharynxes ($n = 21$; patients with upper respiratory tract infection) was tested for C4BP (A), factor H (B), and vitronectin (C) binding. The different NTHi strains were grown overnight and incubated with ^{125}I-labeled C4BP, factor H, or vitronectin. The binding level was calculated as the percentage of bound radioactivity relative to the level of added radioactivity, measured after separation of free and bound ^{125}I-labeled protein over a sucrose column. The mean values of results from three independent experiments are shown. The mean value for all isolates in the same group is indicated by a line.

sensitive to NHS, it is likely that just a few bacteria may survive and that this particular subpopulation is able to multiply and initiate an infection. Importantly, no significant difference in survival between the various clinical strains from blood samples and those from nasopharynxes was found when serum resistance was determined, suggesting the need for NTHi, irrespective of isolation site, for resistance of bactericidal activity of human serum.

Previous studies showed that the NTHi strain R2866, which was isolated from a child with meningitis, had a high degree of serum resistance depending on the expression of the lipooligosaccharide biosynthesis gene *lgtC* (9, 19, 40). The phase-variable *lgtC* expression was demonstrated to inhibit C4b deposition and render the bacteria more resistant to human serum (19). Phase variation of outer membrane proteins and lipooligosaccharides also contributes to virulence of *H. influenzae* and is involved in evasion of the immune system (39). Intriguingly, phase variation can affect binding of regulatory proteins and has been shown to affect serum resistance (19). Since phase variation is a common phenomenon and depends on the current bacterial environment, it cannot be excluded that phase variation is a factor affecting the *in vitro* results of our study.

Another aim was to analyze the difference in binding of complement inhibitors and serum resistance between invasive and nasopharyngeal isolates. We have recently demonstrated that NTHi binds C4BP (15) and that both NTHi and Hib bind factor H (17) and vitronectin (14, 16). However, no major differences in binding of complement regulators were detected between strains from the two isolation sites. This indicates that binding of complement regulators is important and possibly facilitates survival and colonization of NTHi both in the airway and in the bloodstream. However, the variations of complement regulator binding capacity and ability to survive in human serum between the different invasive isolates suggest that additional factors are involved in the ability of NTHi to survive in the respiratory tract and in human serum.

The ability of nasopharyngeal isolates to bind factor H, C4BP, and vitronectin suggests that complement regulators may be present in the nasopharyngeal tract and thus would be utilized by NTHi. In fact, during inflammation, complement proteins as well as immunoglobulins and components of the coagulation system enter the airway lumen (11, 12, 29). In patients with chronic otitis media with effusion, local complement activation in the middle ear mucosa, including an intense deposition of C3, has been observed (27). In addition, factor H, factor H-like protein 1, and factor H-related proteins are complement components found in middle ear effusions of patients with otitis media (26).

In conclusion, NTHi isolates from patients with severe sepsis have a higher capacity to resist the bactericidal effect of human serum than NTHi isolates from patients with mild clinical sepsis. Our results also show that it is of importance for NTHi to bind specific complement regulators, irrespective of whether the bacteria are located in the bloodstream or in the nasopharynx, suggesting that this is an adaption to the innate immunity in the upper respiratory tract. In addition to a variation in host factors, virulence factors other than bacterial components binding complement inhibitors are required for determining the invasive capacity of a particular NTHi strain.

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Paper III

Invasive disease caused by *Haemophilus influenzae* in Sweden 1997–2009; evidence of increasing incidence and clinical burden of non-type b strains

F. Resman[1,2], M. Ristovski[1], J. Ahl[1,2], A. Forsgren[1], J. R. Gilsdorf[3], A. Jasir[4], B. Kaijser[5], G. Kronvall[6] and K. Riesbeck[1]

1) *Medical Microbiology, Department of Laboratory Medicine,* 2) *Infectious Diseases, Department of Clinical Sciences, Lund University, Skåne University Hospital, Malmö, Sweden,* 3) *Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, MI, USA,* 4) *Department of Clinical Microbiology and Immunology, Lund University, Skåne University Hospital, Lund,* 5) *Department of Clinical Bacteriology, Gothenburg University, Gothenburg and* 6) *Clinical Microbiology—MTC, Karolinska Institute, Karolinska Hospital, Stockholm, Sweden*

Abstract

Introduction of a conjugated vaccine against encapsulated *Haemophilus influenzae* type b (Hib) has led to a dramatic reduction of invasive Hib disease. However, an increasing incidence of invasive disease by *H. influenzae* non-type b has recently been reported. Non-type b strains have been suggested to be opportunists in an invasive context, but information on clinical consequences and related medical conditions is scarce. In this retrospective study, all *H. influenzae* isolates (n = 410) from blood and cerebrospinal fluid in three metropolitan Swedish regions between 1997 and 2009 from a population of approximately 3 million individuals were identified. All available isolates were serotyped by PCR (n = 250). We observed a statistically significant increase in the incidence of invasive *H. influenzae* disease, ascribed to non-typeable *H. influenzae* (NTHi) and encapsulated strains type f (Hif) in mainly individuals >60 years of age. The medical reports from a subset of 136 cases of invasive *Haemophilus* disease revealed that 48% of invasive NTHi cases and 59% of invasive Hif cases, respectively, met the criteria of severe sepsis or septic shock according to the ACCP/SCCM classification of sepsis grading. One-fifth of invasive NTHi cases and more than one-third of invasive Hif cases were admitted to intensive care units. Only 37% of patients with invasive non-type b disease had evidence of immunocompromise, of which conditions related to impaired humoral immunity was the most common. The clinical burden of invasive non-type b *H. influenzae* disease, measured as days of hospitalization/100 000 individuals at risk and year, increased significantly throughout the study period.

Keywords: *Haemophilus influenzae,* Hib, Hif, invasive disease, meningitis, sepsis
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Corresponding author: K. Riesbeck, Medical Microbiology, Department of Laboratory Medicine Malmö, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden
E-mail: kristian.riesbeck@med.lu.se

Introduction

Haemophilus influenzae is a frequent colonizer of the human respiratory tract. The species is subdivided into non-encapsulated and encapsulated strains that are typed based upon the presence of one of six antigenically distinct polysaccharide capsules designated a to f [1]. Strains that do not have a capsule are denoted non-typeable *H. influenzae* (NTHi). The carriage rates of NTHi in healthy pre-school children are as high as 65% [2], suggesting that NTHi is usually a commensal. However, NTHi frequently causes otitis media and pneumonia in children [3], and is the most common bacterial finding in exacerbations of chronic obstructive pulmonary disease [4]. Invasive NTHi cases are suggested to be opportunistic infections [3], even though information on correlated medical conditions is scarce.

Invasive disease by *H. influenzae* has historically been analogous with disease by encapsulated strain type b (Hib), a feared cause of sepsis, epiglottitis and meningitis in children and occasionally in adults [5]. In the early 1990s, the conjugated Hib vaccine was introduced in most countries in the Western World, and a dramatically reduced incidence of invasive Hib disease occurred [6]. The incidence of

bacteraemia caused by Hib in Sweden in the late 1980s was approximately 30/100 000 children, and these numbers had decreased 10-fold by 1994 [7]. The current Swedish Hib vaccine coverage rate is 99%.

Several reports on the epidemiology of invasive non-type b *Haemophilus* disease in the post-Hib-vaccine era have been published in recent years [8–13]. Indications of a rising incidence of invasive non-type b disease have been observed in North America [8,9,13,14]. The status in Europe is less clear, but a recent study encompassing surveillance data from 14 European countries between 1996 and 2006 showed a small but significant increase in incidence of invasive NTHi disease [15]. In contrast, a German study covering 1998–2005 did not reveal any increase in invasive non-type b disease [16]. Even though invasive Hib disease has been successfully repressed following the widespread introduction of the Hib vaccine, invasive disease by *H. influenzae* non-type b remains a clinical challenge.

Most epidemiological reports on invasive non-type b *H. influenzae* disease lack information on disease severity. Furthermore, although suggested as an opportunistic disease, there is little information on underlying conditions associated with invasive non-type b cases. In the present retrospective study we show that invasive disease caused by non-type b strains of *H. influenzae* has increased in incidence in Sweden in the period 1997–2009, that it readily affects individuals who are otherwise in good health, and is often clinically severe.

Materials and Methods

Bacterial strains and culture conditions

The *H. influenzae* collection comprised clinical isolates from three densely populated regions in Sweden: Stockholm, Gothenburg and Skåne county (see Supplementary material Fig. S1). All saved isolates from blood and cerebrospinal fluid (CSF) had been stored at −70°C. All available isolates (n = 250) were grown on chocolate blood agar and incubated at 35°C in a humid atmosphere containing 5% CO_2.

DNA preparation and molecular typing

To amplify the capsule transport gene, a *bexA* colony PCR was performed on all available strains (n = 250) [17]. To increase the sensitivity, all strains were also screened for *bexB* using primers 5′-TTGTGCCTGTGCTGGAAGGT TATG-3′ and 5′-GGTGATTAACGCGTTGCTTATGCG-3′. Strains positive for *bexA* and/or *bexB* were further tested using specific primers against types b, a, d and f, c, and e *cap* loci in sequential order. Whenever a strain had previously been typed by PCR, the result was included in the analysis in case the strain was not available (n = 21) for contemporary PCR testing. Results from serotyping by agglutination with antisera were not used because this method is considered inferior in specificity [18]. The caspsule gene was assumed to be expressed in all isolates carrying the *bex* and *cap* loci. The strict commensal *Haemophilus haemolyticus* can be indistinguishable from *H. influenzae* by standard bacteriological techniques so all isolates were tested by a slightly modified version of the PCR described by Murphy *et al.* [19]. Instead of a nested PCR, an initial PCR with primers denoted as 16S3′ and 16SNor [19] was performed. If a product of the correct size was not obtained, isolates (n = 6) were subjected to 16S rRNA sequencing.

Patient data

Basic epidemiological data such as culture date, age and gender were available for all strains. Medical reports from all patients in the county of Skåne (n = 136) were studied, and information on immunocompromise, sepsis severity, duration of stay in hospital, intensive care treatment and mortality was registered. Sepsis severity was defined according to the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) consensus document [20]. As a result of the retrospective nature of the study, not all medical reports contained complete information on all ACCP/SCCM criteria. Only objective and registered parameters were included in the analysis. Meningitis/epiglottitis with sepsis are life-threatening conditions, so these cases were considered severe regardless of other criteria. Immunocompromise was defined according to a definition adapted from the Merck Manual [21]; see Supplementary material Table S1.

Data sorting and estimates of population at risk

All blood and CSF samples taken in the included geographical areas were processed by four central laboratories that kept complete back records of invasive isolates by a database (three laboratories) or by a written list (one laboratory). All recorded *H. influenzae* isolates from blood and CSF were included in the study. Because of variations in storage routines, not all isolates had been saved, and only a few had been previously serotyped by PCR. Therefore, two separate analyses were performed. The primary analysis was based on all recorded strains regardless of serotype, and the secondary analysis was based only on years when at least half of the isolates from each laboratory could be or had been serotyped by PCR (n = 285). The laboratories were Malmö (all years 1997–2009), Lund (2004–2009), Gothenburg Östra Laboratory (1999–2009), Gothenburg Sahlgrenska Laboratory (1997–2000, 2003–2004 and

2007–2009) and Karolinska Laboratory Solna, Stockholm (1998, 2000 and 2004–2008). Any recorded strain from an included year in the secondary analysis that could not be retrieved was registered as 'not analysed (N/A)' (n = 17) and included in the subsequent analysis. Each geographical area had tertiary-care units, where patients referred from outside the area were treated. The addresses of patients (n = 11) with invasive *Haemophilus* disease at tertiary units were checked, and six of these could be confirmed as residents of the included geographical areas. For the remaining five patients, no information was available on living addresses at the time of sepsis. For each year in the study, two estimates of population at risk were defined (Table 1); one estimate for each of the described analyses. Population data were collected from the Swedish central statistics agency (http://www.ssd.scb.se). The estimates of population considered the population by geographical area served by each laboratory unit and year. In Lund and Malmö, the areas of Helsingborg and Kristianstad were added during the course of the study. In Stockholm, the population base was the northern part (with the addition of St Görans hospital in 1997 and 1998) for adults and the greater area of Stockholm for children. In Gothenburg, the inner city was the population base with the addition of North Bohuslän from 2001. The numbers for Stockholm and Gothenburg were double-checked by comparing the numbers of visits to the emergency-care units of the included hospitals with the total emergency-care visits in the greater counties (from which an exact population estimate was known each year).

Statistical analysis

Assuming a linear relation of data, trend tests using linear regression analysis were performed on all data using PAWS statistic 18.0.

Ethical approval

This study was approved by the regional ethical committee for medical research in Lund, Sweden (2009/536).

Results

Increasing incidence of invasive *H. influenzae* disease

Back records revealed 410 cases of invasive disease caused by *H. influenzae* in the defined geographical areas during the years 1997–2009 (Table 1). The incidence varied from 0.5 (in 1998) to 1.7 (in 2007) cases per 100 000 individuals (Fig. 1a). In the primary analysis, which included all recorded isolates regardless of serotype, there was a significantly increased incidence (constant = 0.082, 95% CI 0.040–0.123, $p \leq 0.001$). In the secondary analysis, only regions and years where >50% of isolates were typed by PCR (n = 285) were included (Table 1). As shown in Fig. 1(b), NTHi accounted for the majority (n = 191) of cases. The 77 encapsulated isolates were defined as Hib (n = 29), Hif (n = 44), Hie (n = 1), or 'encapsulated non-type b' (n = 3). A statistically significant increase in invasive disease by NTHi (constant = 0.079, 95% CI 0.046–0.111, $p \leq 0.001$) and Hif (constant = 0.023, 95% CI 0.003–0.043, p 0.025) was observed, whereas the incidence

TABLE 1. Total numbers of *Haemophilus influenzae* isolates and population base per study year

Year	All Hi (CSF)[a]	Secondary analysis					Total population base	Population base, secondary analysis[e]
		NTHi[b]	Hib[b]	Hif[b]	Non-b[c]	N/A[d]		
1997	25 (2)	4	2	1	1	1	2 407 000	1 102 000
1998	15 (3)	8	2	2	0	0	2 684 000	2 074 000
1999	16 (0)	3	0	1	0	1	2 433 000	1 222 000
2000	17 (2)	6	0	2	0	2	2 507 000	1 981 000
2001	20 (2)	3	0	0	0	1	2 720 000	801 000
2002	35 (5)	8	1	0	0	1	2 753 000	810 000
2003	31 (3)	6	1	0	0	1	2 774 000	1 352 000
2004	46 (4)	26	10	2	2	6	2 795 000	2 795 000
2005	35 (4)	20	2	3	0	1	2 815 000	2 274 000
2006	32 (4)	16	3	5	1	1	2 844 000	2 299 000
2007	49 (1)	35	4	8	0	2	2 874 000	2 874 000
2008	48 (5)	30	2	16	0	0	2 909 000	2 909 000
2009	41 (3)	26	2	4	0	0	2 935 000	2 164 000
Total	410 (38)	191	29	44	4	17		

[a]All invasive *H. influenzae* isolates during the study period and included in the primary analysis. Cerebrospinal fluid (CSF) isolates among the total number are given within parantheses.
[b]Numbers of isolates typed by PCR: NTHi, non-typeable *H. influenzae*; Hib, *H. influenzae* type b; Hif, *H. influenzae* type f.
[c]Comprising 'encapsulated non-type b' strains that were previously typed by a *capB* PCR using *bexA* as target. These strains had not been stored and were consequently not available for further analysis. The only *H. influenzae* type e isolate (from 2006) is also included here.
[d]N/A; not analysed. These isolates were not available for PCR serotyping, but were included in the secondary analysis as described in Materials and Methods.
[e]Population base including only years when >50% of strains were serotyped by PCR at each laboratory unit. This particular population out of the total population base was used in the secondary analysis.

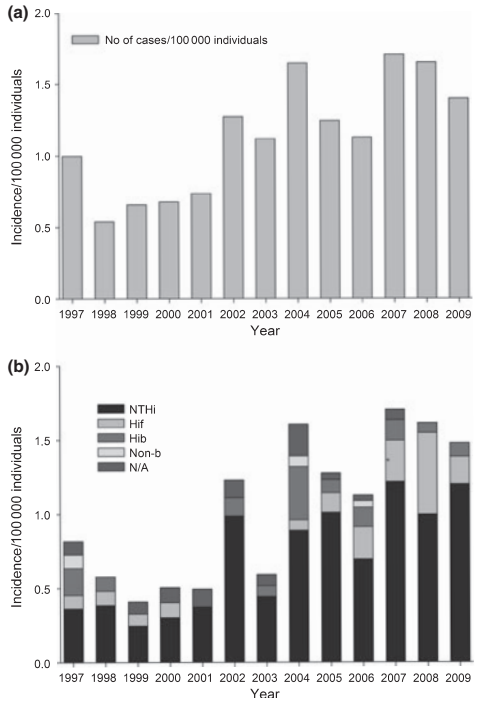

FIG. 1. The incidence of invasive *Haemophilus influenzae* disease increased significantly during the years 1997–2009. (a) Total incidence of invasive *H. influenzae* disease per 100 000 individuals and year from the primary analysis (regardless of serotype) (n = 410). The increase in incidence was statistically significant ($p \leq 0.001$). (b) Results from the secondary analysis, comprising 285 defined cases are shown. The increase was statistically significant (p 0.001 and p 0.025 for non-typeable *H. influenzae* (NTHi) and *H. influenzae* type f (Hif), respectively). Isolates (n = 17) that had not been saved or previously defined by PCR are indicated as not analysed (N/A). Regardless of the hypothetical outcome of the 17 non-analysed isolates, the increase of invasive NTHi disease remained statistically significant.

of Hib disease was unchanged during the study period. No *H. haemolyticus* isolate was identified in the material.

Increased incidence of invasive *H. influenzae* in individuals >60 years of age

The median ages of Hib, Hif and NTHi case patients were 38, 60 and 71 years, respectively. Only 11 of the 410 cases (3%) occurred in neonates (<28 days of age). NTHi was predominant in all age groups, including children <5 years of age. Based on an age-stratified population at risk (Fig. 2), it

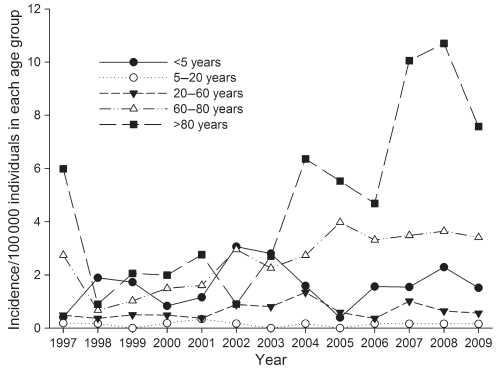

FIG. 2. The incidence per patient age group of invasive *Haemophilus influenzae* disease per 100 000 age-group-sorted individuals at risk is shown. The incidence increased significantly in the age groups 60–80 years and >80 years during the course of the study (p 0.002 and p 0.006, respectively). When the results from PCR serotyping were taken into account, the increase was mainly ascribed to non-typeable *H. influenzae* (NTHi), but in part also to *H. influenzae* type f (Hif).

was evident that the observed increase of invasive *H. influenzae* disease mainly occurred in individuals >60 years of age. The increase in both age groups 60–80 years (constant = 0.212, 95%CI 0.098–0.326, p 0.002) and >80 years (constant = 0.602, 95% CI 0.212–0.992, p 0.006) was statistically significant. No gender difference could be identified for any of the subspecies.

High proportion of sepsis severity in non-type b *H. influenzae* cases

When medical records were analysed in detail we found that the most common clinical presentation of invasive non-type b disease (in this context presence of bacteria in blood or CSF) was pneumonia (70%) but a wide variety of other presentations such as meningitis, epiglottitis, soft tissue infections and cholangitis was seen. As many as 48% of NTHi cases (n = 101) met the criteria of severe sepsis or septic shock, and 20% were admitted to intensive care units (Table 2). Interestingly, 62% of invasive NTHi cases occurred in individuals without evidence of immunocompromise, although one-third of these patients were >80 years of age. The case mortality (within 28 days) was 8%, whereas the 1-year mortality of invasive NTHi cases was 29%. Hif generally caused severe disease; 59% of Hif cases (n = 22) met the criteria of severe sepsis or septic shock, and 36% of cases were admitted to intensive care units. The majority of invasive Hif cases (68%) occurred in individuals without evidence of immunocompromise, whereas <10% of patients were

TABLE 2. Disease severity, need for intensive care treatment, mortality and data on state of immunocompromise from a total of 123 cases of invasive *Haemophilus* non-type b disease in Skåne county 1997–2009

	NTHi (n = 101)			Hif (n = 22)		
ACCP/SCCM category	**Sepsis**	**Severe sepsis**	**Septic shock**	**Sepsis**	**Severe sepsis**	**Septic shock**
Patients (total n)	53	41	7	9	10	3
Immunocompromise per sepsis category (n)[a]	20	15	3	5	1	1
Age >80 years per sepsis category (n)[b]	12	10	1	1	1	0
Need for intensive care (n)	20			8		
28-day mortality (n)	8			3		
1-year mortality (n)	29			5		
Immunocompromise[c] (total n)	38			7		
Acquired immunodeficiency (n)	25 (6 solid tumours and 19 blood cancers/myelomas)			5 (2 solid tumours and 3 blood cancers)		
Chronic disease (n)	9 (3 severe COPD, 3 dialysis patients, 2 dysregulated diabetes mellitus, and 1 heart disease)			1 (severe COPD)		
Iatrogenic condition (n)	3 (1 organ transplant and 2 chemotherapy)			1 (chemotherapy)		

ACCP/SCCM, American College of Chest Physicians/Society of Critical Care Medicine; COPD, chronic obstructive pulmonary disease; Hif, *H. influenzae* type f; NTHi, non-typeable *H. influenzae*.
[a]All ages of patients including individuals >80 years of age with immunocompromise.
[b]Patients >80 years of age with no sign of immunocompromise.
[c]In addition to acquired immunodeficiency, chronic disease and iatrogenic conditions, one patient with a primary immunodeficiency was identified in the material. This particular patient with Good's syndrome presented with an NTHi sepsis.

>80 years of age. Case mortality for Hif was 14% and 1-year mortality was 27%. Patients with invasive Hib infections (n = 13) were included as a control group and 77% of these patients met the criteria of severe sepsis/septic shock and 54% were admitted to an intensive care unit.

Increased clinical burden of invasive non-type b *H. influenzae*

To monitor the clinical burden of invasive *H. influenzae* non-type b disease, the demand for hospitalization days per year and individual at risk was studied (Fig. 3; filled circles). A statistically significant increase of hospital days/100 000 individuals at risk was identified (Constant = 1.31, 95% CI = 0.03–2.60, p 0.046). During the same time period, the average duration of hospitalization per case of any bacterial pneumonia or sepsis decreased in the same geographical area (Fig. 3; black and grey bars, respectively). Hence, despite shorter hospitalization periods for severe infections in general, the required days of hospitalization for *H. influenzae* non-type b invasive disease/100 000 individuals increased, indicating that the nominal increase in incidence was followed by an increase in clinical burden.

Discussion

The present study shows an unambiguous increase in the incidence of invasive disease caused by *H. influenzae* in southern Sweden from 1997 to 2009. The observed increase is explained by an increase in invasive disease caused by NTHi and Hif, mainly in individuals >60 years of age, so this does not imply that the vaccination campaign against Hib has been unsuccessful. The incidence of invasive Hib disease in Sweden remains stable at a very low level. However, the Hib cases that were studied in detail were generally severe, with a high

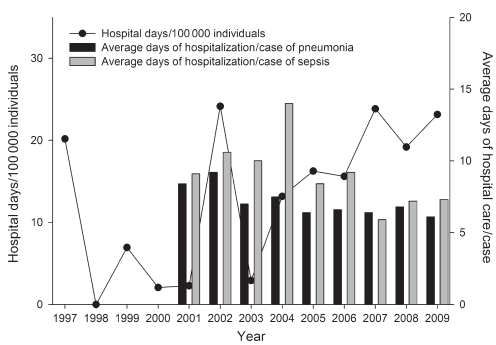

FIG. 3. The clinical burden from invasive *Haemophilus influenzae* non-type b disease is increasing. The line with filled circles shows total days of hospitalization of patients with invasive *H. influenzae* non-type b disease per 100 000 individuals at risk in the county of Skåne (left y-axis). The number from 2002 is high because of two cases with unusually long periods of hospitalization (>50 days). The increase in hospitalization days during the years 1997–2009 was significant (p 0.046). For comparison, the black and grey bars show the decreased average times of hospitalization/case due to pneumonia and sepsis in the city of Malmö, Skåne county 2001–2009 (right y-axis).

proportion of cases requiring intensive care treatment. The findings therefore support continued vaccination against and surveillance of Hib. Our study has a few limitations. We have no data from before 1997 because of the unavailability of isolates. However, there was still 13 years of observations, allowing for trends to be observed. Furthermore, not all recorded isolates could be retrieved and serotyped by PCR. This was addressed by the performance of a secondary analysis excluding years and laboratories where <50% of isolates could be retrieved.

In a recent European surveillance study [15], the total incidence of invasive *H. influenzae* disease was 0.4–0.5 cases per 100 000 individuals (2000–2006). The incidence varied between regions from 0.02 cases per 100 000 individuals in Italy to 1.0 case per 100 000 individuals in Norway. In our study, the incidence of invasive *H. influenzae* disease in Sweden almost reached 2.0 cases per 100 000 individuals, numbers supported by Swedish national surveillance data for 2007–2009 [7]. This suggests that Sweden has a current incidence of invasive *H. influenzae* disease that is three-fold higher than Europe in general, and 50- to 100-fold higher than in Italy. These conflicting data may be the result of actual, unexplained, differences in incidence, but could also merely reflect problems with regional variations in surveillance.

The present epidemiological observations are in line with studies from other parts of the world. The epidemiology of invasive *Haemophilus* disease in the post-Hib-vaccine era seems to be shifting from Hib in children towards non-type b strains in individuals >60 years of age [9,22]. Non-type b strains now dominate in all age groups, including children <5 years of age. We further identified that a major proportion of invasive non-type b cases met the criteria for severe disease. While Hib cases had the highest proportion of severe disease (77%), as many as 47% of invasive NTHi and 59% of invasive Hif cases met the criteria of severe sepsis or septic shock. Invasive disease by non-type b strains was not confined to immunocompromised patients or individuals of extreme age; findings that are in contrast to the widely held view that invasive disease by *H. influenzae* non-type b is mild and opportunistic in nature. Invasive non-type b cases with a severe clinical presentation often occurred in patients with no evidence of immunocompromise, suggesting a significant impact of bacterial virulence in these cases. However, fatal outcome was mainly observed in elderly or immunocompromised patients, and this small group of cases seemed truly opportunistic. Interestingly, the most common immuno compromising conditions that are associated with invasive *Haemophilus* disease were chronic lymphatic leukaemia and multiple myeloma, highlighting the role of humoral immunity in the control of *H. influenzae* disease [23,24]. Less efficient B-cell function is also a major part of immunosenescence [25], which is intriguing considering the high incidence of invasive *Haemophilus* disease in elderly individuals.

The increased incidence of invasive *H. influenzae* disease is probably explained by a combination of contributing factors. Though the Swedish incidence of multiple myeloma or chronic lymphatic leukaemia has not increased in recent years [26], prolonged survival may have increased disease prevalence. Another possible contributing factor is that the total number of blood cultures taken in Sweden increased during the study period. This may have led to the identification of more cases of pneumonia with bacteraemia. New guidelines in Swedish pneumonia care stressing blood culture as a quality indicator were introduced in 2007. However, the proportion of severe invasive cases did not decrease during the study period, indicating that modified clinical routines cannot fully explain the observed increase in incidence.

The introduction of the conjugated Hib vaccine has decreased Hib airway carriage in children [27], and arguably in adults. NTHi is a common colonizer of the human airway, with or without airway disease, but information on nasopharyngeal carriage rates of encapsulated *Haemophilus* other than type b in healthy children is scarce. Interestingly, *Streptococcus pneumoniae* and *H. influenzae* are niche competitors in the upper airway and are suggested to be negatively correlated [28]. Pneumococcal vaccines may, however, affect the future incidence of invasive *H. influenzae* disease because an increased burden of *H. influenzae* disease following pneumococcal vaccination has been suggested for other conditions, such as otitis media [29]. In fact, a heptavalent pneumococcal vaccine was introduced in the national vaccination programme for children in Sweden in 2008 or 2009 depending of geographical region, and most probably had no effect on the epidemiological results of the present study. Another issue is that a conjugated pneumococcal vaccine with *H. influenzae* protein D is now widely available, and the effect of this vaccine on the occurrence of invasive *H. influenzae* disease remains to be studied.

In conclusion, the present study demonstrates a statistically significant increase in the incidence of invasive *H. influenzae* disease in southern Sweden 1997–2009, explained by an increase in NTHi and Hif infections in individuals >60 years of age. Strikingly, many patients with invasive non-type-b disease presented no evidence of immunocompromise, and a surprisingly high proportion of cases were severe according to the ACCP/SCCM grading system. The results call for continued surveillance and active monitoring of invasive disease caused by *H. influenzae*.

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Transparency Declaration

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Figure S1. Map of Sweden including population density and geographical regions that are covered in the present study.

Table S1. Study definition of immunocompromise.

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Paper IV

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Increase of β-Lactam-Resistant Invasive *Haemophilus influenzae* in Sweden, 1997 to 2010

Fredrik Resman,[a,b] Mikael Ristovski,[a] Arne Forsgren,[a] Bertil Kaijser,[c] Göran Kronvall,[d] Patrik Medstrand,[a] Eva Melander,[e] Inga Odenholt,[b] and Kristian Riesbeck[a]

Medical Microbiology, Department of Laboratory Medicine Malmö, Lund University, Sweden[a]; Infectious Diseases Unit, Department of Clinical Sciences Malmö, Lund University, Sweden[b]; Institute of Laboratory Medicine, Deptartment of Clinical Bacteriology, Gothenburg University, Gothenburg, Sweden[c]; Clinical Microbiology, MTC, Karolinska Institute, Karolinska Hospital, Stockholm, Sweden[d]; and Infection Control, Laboratory Medicine, Skåne County, Skåne, Sweden[e]

The proportions of *Haemophilus influenzae* resistant to ampicillin and other β-lactam antibiotics have been low in Sweden compared to other countries in the Western world. However, a near-doubled proportion of nasopharyngeal Swedish *H. influenzae* isolates with resistance to β-lactams has been observed in the last decade. In the present study, the epidemiology and mechanisms of antimicrobial resistance of *H. influenzae* isolates from blood and cerebrospinal fluid in southern Sweden from 1997 to 2010 ($n = 465$) were studied. Antimicrobial susceptibility testing was performed using disk diffusion, and isolates with resistance to any tested β-lactam were further analyzed in detail. We identified a significantly increased ($P = 0.03$) proportion of β-lactam-resistant invasive *H. influenzae* during the study period, which was mainly attributed to a significant recent increase of β-lactamase-negative β-lactam-resistant isolates ($P = 0.04$). Furthermore, invasive β-lactamase-negative β-lactam-resistant *H. influenzae* isolates from 2007 and onwards were found in higher proportions than the corresponding proportions of nasopharyngeal isolates in a national survey. Multiple-locus sequence typing (MLST) of this group of isolates did not completely separate isolates with different resistance phenotypes. However, one cluster of β-lactamase-negative ampicillin-resistant (BLNAR) isolates was identified, and it included isolates from all geographical areas. A truncated variant of a β-lactamase gene with a promoter deletion, $bla_{\text{TEM-1}}$-PΔ dominated among the β-lactamase-positive *H. influenzae* isolates. Our results show that the proportions of β-lactam-resistant invasive *H. influenzae* have increased in Sweden in the last decade.

Invasive disease caused by the respiratory pathogen *Haemophilus influenzae* has in the past been synonymous with disease by encapsulated *H. influenzae* type b (Hib), a cause of meningitis and epiglottitis, mainly in children (6). Following the introduction of the conjugated Hib vaccine in the early 1990s (introduced in the National Swedish Childhood Immunization Schedule in 1992), a rapid decline in invasive Hib disease occurred (23). Invasive disease by non-type b isolates of *H. influenzae*, including nontypeable *Haemophilus influenzae* (NTHi) and encapsulated serotypes other than Hib, has mainly been considered an opportunistic infection. In the last decade, however, a number of reports indicated increasing incidence rates of invasive non-type b *Haemophilus* disease that were not merely related to infections in immunocompromised individuals (1, 3, 35). A similar increase of invasive disease by non-type b *H. influenzae* in Sweden during the years 1997 to 2009 was recently confirmed by us (26). Importantly, we found that both NTHi and *Haemophilus influenzae* type f (Hif) often cause severe sepsis in individuals with no evidence of immune suppression. More than 70% of bacteremic cases also had concurrent pneumonia (26). From our study and others, it is evident that the epidemiology of invasive *H. influenzae* disease in general has changed. Invasive *H. influenzae* disease mainly affected children in the pre-Hib vaccine era, but now it affects both the very young and the very old, and cases are most commonly seen in older adults.

Resistance to ampicillin in *H. influenzae* was first described in 1974 (17). In Sweden, as in many other countries, ampicillin is the main drug of choice in proven *H. influenzae* infections and the primary empirical treatment choice for respiratory tract infections, where *H. influenzae* can be suspected. Ampicillin resistance in *H. influenzae* is now globally widespread, with incidence rates varying from 8 to 30% in different European countries and North America to more than 50% in some East Asian countries (12, 13).

The nomenclature of resistant *H. influenzae* is complex, and since definitions vary between different studies and regions, the definitions used by us are outlined in Table 1. Isolates with resistance to ampicillin can be sorted into two main categories: those that carry a β-lactamase, and those that do not. The most common mechanism of β-lactam resistance in *H. influenzae* is by TEM-1 or ROB-1 β-lactamases (7), and such isolates are denoted β-lactamase positive, ampicillin resistant (BLPAR). The commonly used term β-lactamase negative, ampicillin resistant (BLNAR) is used for isolates with ampicillin resistance but no evidence of β-lactamase production. After this definition was established, it was concluded that ampicillin resistance in such isolates was due to key mutations in the *ftsI* gene (encoding penicillin-binding protein 3 [PBP-3]) that lowered the affinity for β-lactams (36). Subsequently, it became clear that some isolates had such mutations but were not ampicillin resistant according to phenotype testing. Isolates with key mutations in PBP-3 regardless of resistance phenotype are designated genomic BLNAR (gBLNAR), a group of isolates that overlaps with, but does not match, the BLNAR group (34, 36).

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Address correspondence to Kristian Riesbeck, kristian.riesbeck@med.lu.se.

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TABLE 1 Study definitions of the different types of β-lactam-resistant invasive *H. influenzae* isolates[a]

Abbreviation	Name	Study definition	*n*
BLPAR	β-Lactamase positive, ampicillin resistant[b]	Resistance to penicillin according to disk diffusion testing, using SRGA[c] breakpoints for the study period; nitrocefin positive	45
BLNAR	β-Lactamase negative, ampicillin resistant	MIC of ampicillin, ≥2 mg/liter; nitrocefin negative	11[d]
gBLNAR	Genomic β-lactamase negative, ampicillin resistant	Substitutions in PBP-3: genotype I, Arg517His; genotype II, Asn526Lys; genotype III, Met377Ile, Ser385Thr, Leu389Phe, and Asn 526Lys; nitrocefin negative	16[d]
BLNBR	β-Lactamase negative, β-lactam resistant	Resistance to one or more tested β-lactam antibiotics (penicillin V/G, ampicillin, a cephalosporin, or a carbapenem) according to SRGA breakpoints; nitrocefin negative	43
BLPACR	β-Lactamase positive, amoxicillin-clavulanate resistant	Resistance to ampicillin or penicillin and a tested cephalosporin according to SRGA breakpoints; nitrocefin positive	3

[a] The BLNAR and gBLNAR groups have substantial overlap and are both subsets of the BLNBR group.
[b] All studied isolates that were nitrocefin positive had an ampicillin MIC of ≥2 mg/liter.
[c] SRGA, Swedish Reference Group for Antibiotics.
[d] Group sizes (*n*) are from a total of 465 tested isolates, but since only a portion of isolates were available for Etest and sequencing, the numbers for BLNAR and gBLNAR were obtained from fewer isolates and are therefore not comparable to the other group sizes.

Clinical isolates that are susceptible to ampicillin but resistant to other β-lactams are consequently not included in the BLNAR definition. However, β-lactam antibiotics other than ampicillin are often used empirically in infections where *H. influenzae* can be the pathogen. Due to this, resistance of *H. influenzae* to β-lactam antibiotics other than ampicillin needs to be considered. For many years, the screening method for identification of β-lactam-resistant *H. influenzae* in Sweden has been disk diffusion testing for penicillin and cefaclor/loracarbef followed by a nitrocefin β-lactamase test. Even though penicillin rarely is an alternative for treatment of *H. influenzae* infections, experience suggests that this method is suitable for resistance surveillance, allowing for sensitive monitoring of β-lactam resistance. In this study, we refer to the β-lactamase-negative isolates with resistance (according to disk diffusion test screening) to any tested β-lactam antibiotic as β-lactamase negative, β-lactam resistant (BLNBR). This term includes the BLNAR isolates as a subset. Finally, isolates with both a β-lactamase and chromosomally derived resistance are defined as β-lactamase positive, amoxicillin-clavulanate resistant (BLPACR).

The epidemiological trends of antimicrobial resistance in *H. influenzae* vary in different areas of the world. The proportions of β-lactam-resistant isolates in general, and specifically BLNARs, are high in Japan and its neighboring countries, as described in several reports (10, 11, 28). In Europe, reports are less consistent, with some reports suggesting increasing proportions of isolates with ampicillin resistance (14, 32), albeit at a lower level than in Japan. In contrast, a recent Spanish report showed a decrease in proportions of ampicillin-resistant strains (24), demonstrating the local differences in resistance epidemiology. The proportion of β-lactam-resistant *H. influenzae* has been consistent, and comparatively low, in Sweden. However, in the last decade a 2-fold increase of β-lactam-resistant strains has been observed in the yearly national surveillance of Swedish nasopharyngeal *H. influenzae* isolates (http://www.smi.se/upload/stat/haemophilus-influenzae-99-09.gif). The aim of the current study was to investigate the epidemiology, mechanisms, and clonality of antimicrobial resistance in invasive *H. influenzae* in Sweden from 1997 to 2010.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The collection comprised clinical *H. influenzae* isolates from three densely populated regions in Sweden, i.e., Skåne County, Stockholm, and Gothenburg. All isolates from blood and cerebrospinal fluid collected between 1997 and 2010 ($n = 465$) were registered, and available isolates ($n = 301$) were stored at −70°C. Bacteria were cultured on chocolate blood agar plates and incubated for 18 h at 35°C in a humid atmosphere containing 5% CO_2.

DNA preparation and capsule typing by PCR. In order to release bacterial DNA, 5 bacterial colonies were heated in sterile distilled water at 96°C for 10 min. To amplify the capsule transport gene, a *bexA* PCR was performed on all available strains ($n = 301$) (5). To further increase the sensitivity, all available strains were screened for *bexB* by PCR using the primers 5′-TTGTGCCTGTGCTGGAAGGTTATG-3′ and 5′-GGTGATTAACGCGTTGCTTATGCG-3′ (annealing temperature, 54°C), resulting in a product size of 567 bp. Strains positive for *bexA* and/or *bexB* were further tested for capsule type by using specific primers against types b, a, d and f, c, and e *cap* loci in sequential order (5). Whenever a strain had previously been capsule typed by *bex/cap* PCR, the result was included in the analysis in case the strain was not available ($n = 21$). Results from serotyping by agglutination with antisera were not used, since this method is considered inferior in specificity compared with PCR (29). For all saved isolates from 1997 to 2009, a PCR to exclude the presence of *Haemophilus haemolyticus* isolates was performed (21). However, instead of a nested PCR, an initial PCR with primers 16S3′ and 16SNor was performed (26). If a product of correct size was not obtained, isolates were subjected to 16S rRNA sequencing. Since not a single isolate of *H. haemolyticus* was identified, the procedure was discontinued in 2010.

Antimicrobial susceptibility testing. The disk diffusion method was used for antimicrobial susceptibility testing (4). Although not all strains were available for further analysis, all the clinical isolates were or had been tested for resistance to penicillin V, ampicillin, and trimethoprim-sulfamethoxazole. The majority of strains had been tested for resistance to tetracycline (95%), a cephalosporin (cefaclor/loracarbef and cefuroxime-axetil or cefotaxime; 98%), and a fluoroquinolone (nalidixic acid/ciprofloxacin/moxifloxacin or levofloxacin; 86%). Only a few isolates had been tested for resistance to a carbapenem (imipenem/meropenem; 39%), chloramphenicol (6%), or an aminoglycoside (4%). Antimicrobial susceptibility was interpreted according to Swedish Reference Group for Antibiotics (SRGA) breakpoints of the study period (www.srga.org/ZONTAB/Zontab2a.htm and www.srga.org/ZONTAB/Zontab2b.htm). Isolates were defined as β-lactam resistant according to SRGA breakpoints for penicillin V (10 μg) or for another tested β-lactam. All isolates with β-lactam resistance according to these breakpoints were or had been tested for β-lactamase production by using a commercial disk test (Cefinase disks; bioMérieux, Marcy l'Etoile, France). The cefinase disks contain nitrocefin, which is a chromogenic cephalosporin. Since susceptibility testing for amoxicillin-clavulanate was not routinely performed, the iden-

tification of true BLPACR isolates (β-lactamase positive, amoxicillin-clavulanate resistant) was not possible. The definition refers to isolates with both β-lactamase production and chromosomal resistance, and since the TEM-1 or ROB-1 β-lactamases of *H. influenzae* do not confer resistance to cephalosporins, BLPACR isolates were defined as those β-lactamase-positive isolates with resistance to a tested cephalosporin. β-Lactam-resistant isolates were thereby defined as BLPAR, BLNBR, or BLPACR based on results from nitrocefin testing and cefaclor (30 μg)/loracarbef (10 μg) tests, respectively. E-tests for ampicillin (Biodisk, Solna, Sweden) were performed on all available β-lactam-resistant isolates.

PCR and sequencing for detection of bla_{TEM} and bla_{ROB}. All available β-lactam-resistant isolates that tested positive for β-lactamase production were subjected to PCR to detect the specific β-lactamase gene. First, a bla_{TEM-1} PCR was performed, and on TEM-1 PCR-negative isolates, a bla_{ROB-1} PCR was then performed (30). Since the bla_{TEM-1} PCR resulted in products of two distinct sizes, bla_{TEM} PCR products from representative isolates were sent for sequencing and compared to known bla_{TEM-1} variants (20, 34). The sequenced isolates were included as controls in the bla_{TEM-1} PCR.

PBP-3 sequencing. All available isolates that were either defined as BLNBR or BLPACR based on the method described above were subjected to an *ftsI* PCR, amplifying the transmembranous part of PBP-3 by using primers 5′-CCTTTCGTTGTTTTAACCGCA-3′ and 5′-AGCTGCTTCA GCATCTTG-3′ (annealing temperature, 52°C), resulting in a product size of 770 bp. All products were sent for sequencing, analyzed for amino acid substitutions, and compared to the wild-type *H. influenzae* RdKW20 PBP-3 by using the CLC-DNA workbench (CLC bio, Aarhus, Denmark).

Multilocus sequence Typing (MLST). All available BLNBR and BLPACR isolates were sequence typed using PCR primers and conditions according to the *H. influenzae* protocol described on the MLST.net web page (http://haemophilus.mlst.net/). Sequences were trimmed manually, concatenated, and aligned using ClustalX (19). A best-fitting nucleotide substitution model was estimated using the Akaike information criterion corrected for small sample sizes (AICc) as implemented in jModeltest 0.1.0 (25). A neighbor-joining (NJ) tree was constructed in PAUP* version 4.0b10 (33) using the AICc model (HKY+I+G). Support for internal branches was obtained with 1,000 bootstrap replicates in PAUP*. The resulting phylogenetic tree was visualized using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree).

Data sorting and estimates of population at risk. The laboratories in Stockholm, Gothenburg, and Malmö/Lund (Skåne County) kept complete records of all *H. influenzae* isolates from blood and cerebrospinal fluid ($n = 465$). Due to variations in storage routines, not all strains had survived during the years. Of the 465 isolates, 340 were or had been serotyped by PCR. If less than 50% of isolates from one laboratory were or had been serotyped by PCR in a year, all results from that laboratory were excluded from the serotype epidemiology analysis for that particular year, and the population data were adjusted accordingly. From the isolates defined as BLPAR or BLPACR, 69% (33/48) were available for detailed study. From the isolates defined as BLNBR or BLPACR, 80% (36/46) were available for further study. All population data by region and year were collected from the Swedish Central Statistics Agency (www.scb.se).

Statistical analyses. To test the significance of the increase in proportions of *H. influenzae* β-lactam resistance, trend tests using yearly proportions of each type of resistance as a dependent variable in linear regression analyses were initially performed. These analyses gave significance levels of the increase and confidence intervals (CIs). We had *a priori* knowledge that the data set was skewed toward the end of the study period, and considering the fact that the dependent variable was binomial, logistic regressions were also performed on the three data sets. After plotting the three data sets, the assumption of a linear relation of data used in both the linear and logistic regressions could not be assumed for the BLNBR data set nor the data set with all ampicillin-resistant isolates. The curve fit of these two data sets suggested that a quadratic polynomial regression should be used. For the BLNBR data set, a cubic equation (a third-degree polynomial equation) fit the data almost equally well. For these two data sets, quadratic logistic regressions were performed with centered squared years and centered years used as covariates. Years, and not exact dates, were used as time points, since we know that there is a seasonal variation in *H. influenzae* disease. The most conservative estimate of significance was used. The data were analyzed using PASW statistics version 20.0.

RESULTS

Increasing numbers and proportions of invasive β-lactam-resistant *H. influenzae* in Sweden from 1997 to 2010. We recently observed an increase of invasive *H. influenzae* disease in Sweden from 1997 to 2009 (26), which was in parallel with similar epidemiological findings in North America as well as in Europe (18, 35). In the present study, results from 2010 were also included. Since 2010 holds the highest incidence per 100,000 individuals during the study period, a continued increasing incidence trend is suggested (Fig. 1A). The increase was dominated by NTHi.

As revealed by disk diffusion, 91 out of 465 *H. influenzae* isolates were defined as β-lactam resistant, of which 43 isolates were β-lactamase negative. The total numbers of isolates for each group are shown in Table 1. The absolute numbers (ranging from 1 to 5 for 1997 to 2000 to 12 to 15 for 2007 to 2010) as well as the proportion of β-lactam-resistant invasive *H. influenzae* isolates increased (Fig. 1B). The increase in proportion of β-lactam-resistant isolates was significant in a linear regression ($P = 0.01$; 95% CI, 0.36 to 2.26), as was the increase of BLNBR isolates ($P = 0.04$; 95% CI, 0.08 to 1.94), whereas the increase in BLPAR isolates was not statistically significant ($P = 0.13$; 95% CI, 0.11 to 0.72). Since the plots of the data sets, except for BLPAR data, suggested a quadratic equation, a logistic regression of the data using a quadratic regression was performed. The observations were confirmed, and the increase of the BLNBR isolates ($P = 0.02$) as well as the increase of all β-lactam-resistant isolates ($P = 0.03$) remained significant. A logistic regression of the BLPAR data set further stressed that these isolates did not increase in incidence ($P = 0.67$). β-Lactam resistance in Swedish *H. influenzae* isolates appeared almost exclusively in NTHi isolates, since only eight encapsulated strains displayed this characteristic during the study period.

We also studied the susceptibility to other antimicrobial agents. The proportion of isolates resistant to trimethoprim-sulfamethoxazole varied from 6 to 20% per year, and no trend suggesting increasing incidence rates was seen throughout the study period. This contrasts to the national nasopharyngeal surveillance data, where an increasing trend of resistance to the folic acid antagonists has been observed (http://www.smi.se/upload/stat/haemophilus-influenzae-99-09.gif). Finally, resistance to fluoroquinolones and tetracycline remained low during the study period, at 2.1% and 1.9%, respectively.

The gene variant with a promoter deletion, bla_{TEM-1}-PΔ, dominates among BLPAR isolates. All identified β-lactamase-positive isolates (BLPAR or BLPACR) (Table 1) that were available for further analysis ($n = 33$) were resistant to ampicillin (the MIC for ampicillin ranged from 4 to 256 mg/liter). The corresponding β-lactamase gene was defined by PCR in 30 out of 33 isolates, and we found that bla_{TEM-1} dominated ($n = 29$). Only one isolate carrying the bla_{ROB-1} gene was found. The gene product encoding TEM-1 was detected in two variants, resulting in different DNA products when using the same primer pair

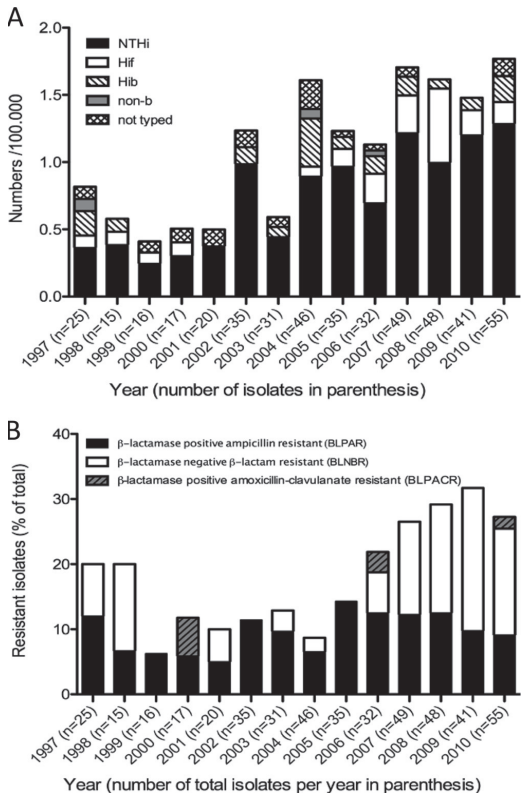

FIG 1 Incidence of invasive *Haemophilus influenzae* as well as β-lactam-resistant invasive *H. influenzae* increased from 1997 to 2010. (A) The incidence of all *H. influenzae* strains, as well of NTHi and Hif strains, increased during the observation period. For all years, laboratories in which less than 50% of strains had been or could be capsule typed by PCR were excluded. The denotation "non-b" reflects a small group of isolates that had been serotyped by PCR against only *bexA* and *capB* and were sorted as encapsulated but not Hib. The denotation "not typed" describes isolates that were included in the analysis but not available for capsule typing by PCR. (B) The proportion of β-lactam-resistant isolates as a percentage of all invasive isolates is shown per study year. BLPAR isolates, BLNBR isolates, and BLPACR isolates are shown separately. The total proportion increased significantly throughout the study period, as did the proportion of β-lactamase-negative β-lactam-resistant isolates.

(Fig. 2A). After sequencing, it was clear that the larger product (600 bp) represented the wild-type $bla_{\text{TEM-1}}$ gene, whereas the smaller product represented a $bla_{\text{TEM-1}}$ gene with a 135-bp deletion in the promoter region. This corresponded to the $bla_{\text{TEM-1}}$-PΔ gene previously described in Spain by Molina and colleagues (20). In our clinical collection, the variant $bla_{\text{TEM-1}}$-PΔ dominated during the study period (18 had the $bla_{\text{TEM-1}}$-PΔ gene, whereas 11 isolates carried the wild-type $bla_{\text{TEM-1}}$ gene). The median MIC for ampicillin, however, was the same for the two identified bla_{TEM} gene variants. Finally, we found three β-lactamase-positive (as revealed by nitrocefin testing), ampicillin-resistant *H. influenzae* isolates (BLPAR) from 2009 and 2010 that were negative for both bla_{TEM} and bla_{ROB} genes when we used the described primers.

Amino acid substitutions in PBP-3 are found mainly in BLNAR isolates and are less common in other BLNBR strains. A total of 46 isolates were defined as β-lactamase negative, β-lactam resistant (BLNBR), or BLPACR (Table 1). Of these isolates, 12 were penicillin resistant only, and 34 isolates were resistant to penicillin and another tested β-lactam. Of the total 46 isolates, 36 were available for further testing, and these were subjected to an ampicillin Etest followed by PBP-3 sequencing. Several of the isolates were true BLNAR (11/36; ampicillin MIC, ≥2 mg/liter) or gBLNAR (16/36) (amino acid substitutions Arg517His or Asn526Lys). In Table 2, we show all variants of PBP-3 that were identified among the BLNBR isolates and the correlating MIC ranges for ampicillin. Genotype II dominated among BLNAR isolates, and a correlation between the BLNAR genotypes and ampicillin resistance phenotype was confirmed. However, several isolates that were resistant to other β-lactams but susceptible to ampicillin did not have BLNAR-defining substitutions in PBP-3. Seven BLNBR isolates did not have any mutations at all in PBP-3. These findings imply that mechanisms other than β-lactamase production and substitutions in PBP-3 contribute to β-lactam resistance in *H. influenzae*.

A marked increase of β-lactamase-negative β-lactam-resistant isolates was found from 2007 and onwards (Fig. 2B), with consistent yearly proportions above 10%. In the years 1997 and 1998, the proportion of BLNBR isolates was relatively high, but this was based on a very limited number of isolates. This makes the data from these years less reliable and more difficult to interpret. For comparison, the definitions were also adjusted to the definition used in the national surveillance program described earlier, which only includes isolates resistant to both penicillin and cefaclor/loracarbef in the BLNBR group. From 2007 and onwards, we observed consistently higher proportions of β-lactamase-negative β-lactam-resistant invasive isolates than the proportions seen in the national surveillance data for nasopharyngeal isolates, for which numbers never reached 5%.

Identification of a cluster of BLNAR genotype IIb isolates with limited genetic variation. To identify putative clusters, MLST based upon 7 different genes was performed on the invasive BLNBR isolates. Even though alleles were shared, all analyzed isolates had different ST profiles, as revealed by the MLST. The clonal relation of the BLNBR isolates was analyzed using concatenated MLST sequences. In the resulting neighbor-joining analysis, clusters supported by bootstrap values of >70% were considered well supported (Fig. 2C). The phylogenetic analysis identified several clusters with bootstrap support of 70% or more, of which one cluster contained 7 BLNAR isolates (Fig. 2C). Interestingly, this BLNAR cluster comprised isolates from all three distinct geographical areas in the study, all from the period 2008 to 2010. Furthermore, all of the isolates in the cluster had identical PBP-3 sequences, belonging to genotype IIb according to the classification of Dabernat and colleagues (2). Even though the numbers are small, together these findings suggest a clonal spread of this particular cluster.

DISCUSSION

This study identifies an increase in proportions of β-lactam resistance among invasive *H. influenzae* isolates in Sweden during the years 1997 to 2010. The proportions of β-lactam-resistant isolates reached 30% in the final years of the study period. The observed increase was not mainly due to an increase of β-lactamase-pro-

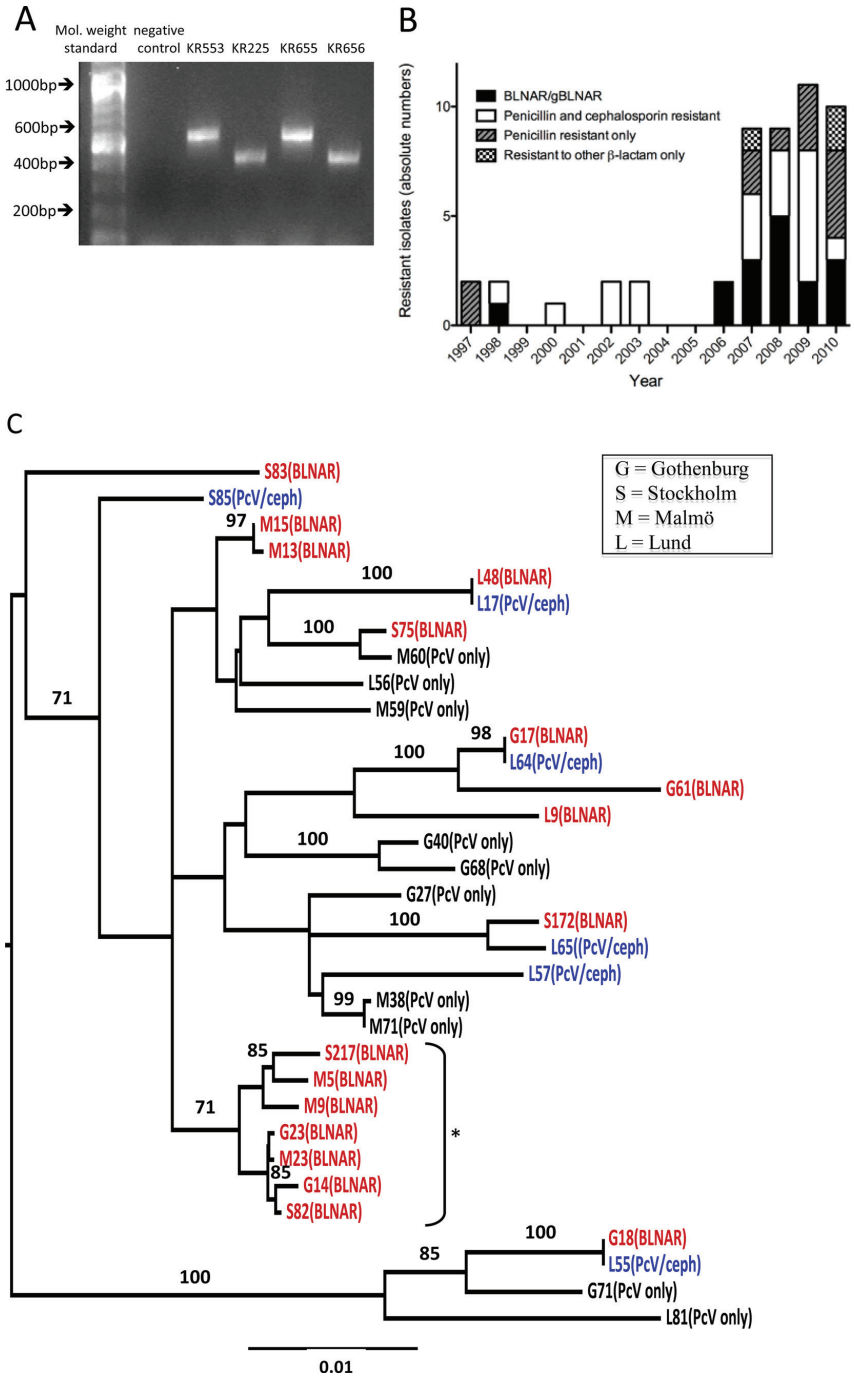
A
Mol. weight standard
negative control
KR553
KR225
KR655
KR656
1000bp
600bp
400bp
200bp
B
BLNAR/gBLNAR
Penicillin and cephalosporin resistant
Penicillin resistant only
Resistant to other β-lactam only
Resistant isolates (absolute numbers)
Year
C
G = Gothenburg
S = Stockholm
M = Malmö
L = Lund
S83(BLNAR)
S85(PcV/ceph)
M15(BLNAR)
M13(BLNAR)
L48(BLNAR)
L17(PcV/ceph)
S75(BLNAR)
M60(PcV only)
L56(PcV only)
M59(PcV only)
G17(BLNAR)
L64(PcV/ceph)
G61(BLNAR)
L9(BLNAR)
G40(PcV only)
G68(PcV only)
G27(PcV only)
S172(BLNAR)
L65((PcV/ceph)
L57(PcV/ceph)
M38(PcV only)
M71(PcV only)
S217(BLNAR)
M5(BLNAR)
M9(BLNAR)
G23(BLNAR)
M23(BLNAR)
G14(BLNAR)
S82(BLNAR)
G18(BLNAR)
L55(PcV/ceph)
G71(PcV only)
L81(PcV only)
0.01

TABLE 2 Amino acid substitutions in PBP-3 of 36 invasive β-lactamase-negative β-lactam-resistant *H. influenzae* isolates[a]

BLNAR genotype	*n*	Amino acid substitution															Ampicillin MIC[b] (range; mg/liter)
		Asp 350	Ala 368	Asp 373	Met 377	Ala 395	Ala 437	Ile 449	Ile 475	Gly 490	Ala 502	Arg 517	Asn 526	Ala 530	Val 547	Asn 569	
I	**1**											**His**					**1**
IIb	**8**	**Asn**			**Ile**						**Val**		**Lys**		**Ile**	**Ser**	**0.5–4**
IIb	**2**	**Asn**			**Ile**					**Glu**	**Val**		**Lys**		**Ile**	**Ser**	**2**
IIb	**1**			**Asn**		**Gly**					**Val**		**Lys**		**Ile**	**Ser**	**2**
IIb	**1**				**Ile**						**Val**		**Lys**		**Ile**		**8**
IId	**2**							**Val**					**Lys**		**Ile**	**Ser**	**2**
II	**1**	**Asn**								**Glu**			**Lys**	**Ser**			**0.5**
—[c]	3	Asn													Ile	Ser	0.25–0.5
—	2	Asn					Ser								Ile	Ser	0.5
—	2			Asn													0.5
—	1								Leu								1
—	2		Thr														0.25–0.5
—	3														Ile		0.25
—	7	(No substitution[d])															0.25–256

[a] All gBLNAR variants are highlighted in boldface.
[b] MICs were determined by Etest.
[c] —, the isolate was not gBLNAR.
[d] Two strains produced β-lactamases (BLPACR) and therefore there was a broad MIC range.

ducing isolates, but among these a $bla_{\text{TEM-1}}$ variant with a promoter deletion dominated (i.e., $bla_{\text{TEM-1}}$-PΔ). The increase was mainly due to a recent rise in BLNBR isolates. Since such isolates have a potential for resistance to multiple antibiotics (34), the observation is of concern. Not all of the BLNBR isolates displayed true BLNAR phenotypes, but most isolates were resistant to multiple β-lactam antibiotics. Our study also confirms a strong, but not perfect, correlation between BLNAR-defining amino acid substitutions and the ampicillin resistance phenotype established in earlier studies (2, 9, 31, 36). However, it is evident that other mechanisms than PBP-3 mutations or β-lactamase production contribute to β-lactam resistance in *H. influenzae*. A few such mechanisms, including disrupted repression of the *acrR* efflux pump, have been suggested (15).

Since the study design was retrospective, our study has limitations. Not all isolates were available for detailed study, and since the absolute numbers of *H. influenzae* isolates were limited, the statistical calculations as well as the indications from the MLST analysis should be interpreted with caution. Furthermore, the reliability of the disk diffusion method for defining precise levels of β-lactam resistance in *H. influenzae* has been questioned. However, as a primary screening method for resistance in clinical isolates, when followed by a detailed examination, the disk diffusion method was considered suitable. Previous reports that have studied clonal relations of resistant *H. influenzae* have used pulsed-field gel electrophoresis (PFGE) (9, 32), and PFGE is a common method for studying clonal relations in local outbreaks with a limited geographical distribution. Even though all methods have limitations, we believe that MLST is advantageous, with its benefits of a high resolution power and the possibility of international comparisons.

Acquisition of antimicrobial resistance is often thought to imply a fitness cost and thereby theoretically reduced bacterial fitness and virulence. However, evidence points to antimicrobial resistance in Gram-negative bacteria that can be linked to a higher degree of virulence (27), possibly due to cocarriage of resistance and virulence genes. The explanation for the increase of the proportion of resistant invasive *H. influenzae* isolates is likely to be multifactorial. Selection pressure from liberal use of antibiotics for upper airway infections may be a contributing factor, and there is support for this mechanism from earlier reports (8). Moreover, a contribution of the spread of dominant clones of *H. influenzae* with antimicrobial resistance should be considered. Such patterns were suggested in earlier studies (12, 16). The MLST results from the present study of invasive isolates suggest a spread of one BLNAR clone with close genetic relation, but the absolute number of isolates was too small to fully conclude this as a fact. Two observations strengthening this indication is that the cluster was comprised of isolates from all three geographical areas of the study, and all of the isolates of this cluster had identical PBP-3

FIG 2 Two variants of $bla_{\text{TEM-1}}$ and a steep increase of β-lactamase-negative invasive isolates with a cluster of BLNAR isolates were identified. (A) The agarose gel shows an example of a $bla_{\text{TEM-1}}$ PCR result from four different invasive NTHi strains with β-lactamase production. The lanes are, from left to right, molecular weight standard, negative control, and the clinical NTHi isolates KR553, KR225, KR655, and KR656. Sequencing revealed that the products of KR553 and KR655 are $bla_{\text{TEM-1}}$ wild type, whereas the products of KR225 and KR656 are representative of the $bla_{\text{TEM-1}}$-PΔ. (B) The recent increase of H. *influenzae* isolates with a β-lactamase-negative, β-lactam-resistant phenotype. The absolute numbers of invasive BLNBR isolates during 1997 to 2010, sorted by resistance phenotype, are shown. The black bars show BLNAR isolates, and the white bars show isolates resistant to penicillin and a cephalosporin. The striped gray bars show isolates resistant to penicillin only, while the checked bars show isolates resistant to only a cephalosporin or a carbapenem. (C) A neighbor-joining phylogenetic tree was constructed based on concatenated MLST data from all available invasive BLNBR isolates. The BLNAR isolates are indicated in red. Isolates with penicillin and cephalosporin (PcV/ceph) resistance are indicated in blue, while isolates with only penicillin (PcV only) resistance are shown in black. The prefix letter of the isolate name indicates the laboratory where the isolate was isolated: G, Gothenburg; S, Stockholm; M, Malmö; L, Lund. Clusters with >70% bootstrap support are indicated with their bootstrap values, and one cluster of seven gBLNAR/BLNAR isolates, including isolates from all three geographical areas of the study, is indicated by an asterisk.

sequences. Among the BLPAR isolates, the reason for the spread and domination of the bla_{TEM-1}-PΔ variant needs further investigation.

The finding of higher proportions of β-lactamase-negative β-lactam-resistant *H. influenzae* invasive isolates, including BLNAR, than that found in surveillance of nasopharyngeal disease carriage strains is intriguing. Since not all isolates were tested for cephalosporins or carbapenems, and since not all isolates were available for PBP-3 sequencing, the numbers in this group may be an actual underestimate. The possibility of a higher invasive capacity of resistant strains cannot be excluded, and such suggestions have been made for BLNAR isolates in earlier work (22). Since the study is skewed toward metropolitan areas of Sweden, however, the risk of the results reflecting local Swedish differences in resistance epidemiology also has to be considered. Interestingly, when the BLNBR data set was statistically examined, the curve could be fitted almost equally well with a cubic equation as the quadratic one used in this analysis. One may argue that a cubic equation, with a reduction in the rate of increase at the end of the study period, may be a more plausible estimate. The coming years will show which model best predicts future incidence rates.

To assess the relevance of studying *H. influenzae* resistance to all β-lactams, and not only to ampicillin, in a clinical setting, we registered the initial antibiotic given to the patients in 106 cases of *H. influenzae* sepsis in the county of Skåne (data not shown). The majority (53%) were primarily given an expanded- or broad-spectrum cephalosporin. Interestingly, 28% were given benzylpenicillin, 15% were given a carbapenem, and only one single patient was administered ampicillin as a starting antibiotic. This observed empirical treatment strategy reflects the clinical need to consider resistance of *H. influenzae* to also β-lactams other than ampicillin, most notably cephalosporins and benzylpenicillin.

To harmonize resistance testing, a novel disk diffusion method to detect β-lactam resistance in *H. influenzae* was issued by the European Committee on Antimcrobial Susceptibility Testing (EUCAST; www.eucast.org) in 2011. The new method sorts β-lactam-resistant isolates by using benzylpenicillin disks (1 U) in Mueller-Hinton agar. Preliminary results from our laboratory suggest a higher incidence of β-lactamase-negative β-lactam-resistant nasopharyngeal *H. influenzae* isolates in 2011. Whether this reflects a true increase of β-lactam resistance in *H. influenzae* or merely improved diagnostics is unclear for the time being. Since the two methods are not entirely interchangeable, only results from the one used during the study period (1997 to 2010) were included in the present study. Regardless of the specific method utilized, it is clear that the proportion of β-lactam-resistant *H. influenzae* in Sweden is no longer low, as roughly 30% of invasive isolates displayed β-lactam resistance in the final years of this study. These results call for continued surveillance and active measures to restrain the use of unnecessary antibiotics in upper airway infections.

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Paper V

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CASE REPORT

Necrotizing myositis and septic shock caused by Haemophilus influenzae type f in a previously healthy man diagnosed with an IgG3 and a mannose-binding lectin deficiency

FREDRIK RESMAN[1,2], TOR SVENSJÖ[3], CAN ÜNAL[1], JONAS CRONQVIST[2], HÅKAN BRORSON[3], INGA ODENHOLT[2] & KRISTIAN RIESBECK[1]

From the [1]Medical Microbiology, Department of Laborative Medicine, [2]Infectious Diseases Unit, and [3]Plastic and Reconstructive Surgery, Department of Clinical Sciences, Lund University, Skåne University Hospital, Malmö, Sweden

Abstract
An increased incidence of infections by Haemophilus influenzae type f (Hif) has recently been suggested, but such infections have mainly been regarded as opportunistic. We present here a dramatic case of Hif necrotizing myositis and septic shock. A subsequently diagnosed IgG3 and mannose-binding lectin deficiency possibly contributed to the severe outcome.

Keywords: *Haemophilus influenzae, myositis, IgG3 deficiency, MBL deficiency*

Introduction

Necrotizing soft tissue infections are amongst the most dramatic conditions in the field of infectious diseases, often requiring radical and repeated surgery combined with intensive care treatment. The mortality is approximately 25% even if treatment is prompt and correct, and a cured patient often suffers substantial consequential morbidity [1]. In most clinical cases etiological factors, such as trauma, peripheral vascular disease, or immunosuppression, are evident. The majority of cases of necrotizing soft tissue infections are considered polymicrobial. Monomicrobial cases are usually caused by exotoxin-producing strains of streptococci or staphylococci. Many other species can occasionally cause necrotizing soft tissue infections, especially in elderly or immunocompromised patients, warranting the use of broad-spectrum antibiotics prior to identification of a causative microorganism [1]. We could not identify any prior description of a monomicrobial necrotizing myositis caused by Haemophilus influenzae type f (Hif) in a previously healthy patient. However, a single case of Hif necrotizing fasciitis has been described [2]. This previously described case occurred in a patient with a number of evident predisposing factors.

Severe invasive disease caused by H. influenzae has historically been analogous with disease by encapsulated type b (Hib). Since the introduction of a conjugated Hib vaccine in the early 1990s, the incidence of invasive Hib disease has decreased dramatically [3]. Recent reports have shown increasing incidences of invasive disease by non-type b strains of H. influenzae in regions where vaccination has been implemented [4,5]. In Europe, Hif is now identified as the most common encapsulated non-type b H. influenzae in invasive disease [5,6]. Fortunately, unlike Hib, invasive Hif infections have been suggested to be opportunistic in nature, occurring mainly in immunocompromised individuals or individuals of extreme age [7].

Case report

A 70-y-old man presented to the emergency room due to a sudden sharp pain in the left hip region. The patient had no prior medical conditions, apart from a left-side coxarthrosis, and was on no medication but glucosamine and paracetamol. The initial medical report included the patient's self-reported airway symptoms, including a sore throat and a persistent

Correspondence: F. Resman, Medical Microbiology, Department of Laboratory Medicine Malmö, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden. Tel: +46 40331000. Fax: +46 40336234. E-mail: fredrik.resman@med.lu.se

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cough in the weeks prior to admission. The patient did not have an increased body temperature on admission, but C-reactive protein (27 mg/l) and the white blood cell count (12.9×10^9/l) were slightly elevated.

After initial examination, the patient was admitted to an orthopaedic ward. An X-ray of the left hip verified coxarthrosis, but offered no explanation to the sudden pain. In the following 2 days the patient gradually developed fever, while the pain in the hip persisted despite opiate analgesics. On the 3rd day following admission, symptoms of shock emerged, including hypotension and tachypnoea, and the patient was transferred to an intensive care unit (ICU). At this time, blood cultures were drawn, and renewed laboratory testing revealed multiple organ failure, including thrombocytopenia (16×10^9/l), leukopenia (1.4×10^9/l), and elevated serum creatinine (455 μmol/l) suggesting tubular necrosis. Treatment with meropenem was initiated, as well as intensive fluid therapy (12 l of crystalloid fluid was administered in the first 24 h in the ICU) and inotropic therapy. Despite these efforts, the clinical picture was aggravated with signs of metabolic encephalopathy, and mechanical ventilation was started. Meanwhile, symptoms from the patient's right upper arm had become evident, with sharp pain, a rash, and swelling. Due to these symptoms, together with the aggravated hip pain, septic shock, and elevated myoglobin levels (3093 μg/l), necrotizing myositis was suspected. The patient was taken to surgery 12 h after initiation of antibiotic therapy.

Exploration of the oedematous areas of the body was performed by physicians from the departments of hand, orthopaedic, and plastic surgery, and included the right lower and upper arm, left hip region, and left elbow. Necrotic parts of the affected muscles were removed and only muscles that displayed spontaneous contraction upon pinching and apparent bleeding were retained. Muscles that were partially or totally resected included the flexors of the right arm (Figure 1A), musculus (m.) gluteus maximus, m. biceps femoris, and m. quadriceps femoris (Figure 1B). Tissue samples from the right arm and the left hip and thigh were collected for routine pathology and culture. Histology from all regions showed an intense inflammatory response, small abscesses, and muscle necrosis (Figure 1C). A computed tomography (CT) scan performed after the first surgery was helpful to identify perfusion disturbances of the quadriceps and hamstring muscles, which led to revisions in a second procedure. An olecranon abscess/necrosis of the left arm was also revised. Unfortunately, all toes of the left and right foot displayed ischemia and had to be amputated. Wounds were managed with moist dressings or negative pressure therapy before delayed primary closure was performed after 3 weeks. The patient was treated in the ICU for a total period of 12 days. All blood cultures ($n = 4$) and 1 peroperative tissue culture from the oedematous muscle in the left hip area showed growth of H. influenzae, typed by polymerase chain reaction (PCR) as an encapsulated strain type f (Hif).

Following recovery, the patient was tested for predisposing immune deficiencies and was interviewed. The patient had led a healthy life, and had not suffered from repeated infections. Even though analyses of total immunoglobulin (Ig) and total haemolytic complement were normal, laboratory tests revealed low levels of IgG3 (0.18 g/l, reference 0.24–1.25 g/l) combined with high levels of IgG4 (1.98 g/l, reference 0.052–1.25 g/l) and a mannose-binding lectin (MBL) deficiency, genotype LYPB/LYPB [8], with less than 10% function of the lectin pathway. Repeated laboratory testing after 3 months confirmed these results. No pre-immune serum prior to the septic episode was available for analysis.

Three years after the myositis episode, the patient still suffers from the consequences of the severe infection, with chronic pain in the hip and shoulder region and impaired mobility. The wounds on both feet have been subject to further revision and are not yet fully healed.

Further experiments

Was the clinical Hif myositis isolate (strain KR494) more virulent as compared to other Hif and nontypeable Haemophilus influenzae (NTHi) carrier strains? To answer this question the strain was tested for virulence properties suggested to affect disease severity. Well-characterized Hib and NTHi strains (MinnA and RM3655, respectively) and a bacteremic Hif strain causing a milder sepsis, were used as controls. No difference in epithelial cell adhesive or invasive capacity of KR494 to A549 cells [9] was found compared with controls. The transmigratory capacity of KR494 through NCI-H292 cells [10] was intermediate, whereas the biofilm-forming capacity, as assessed by crystal violet staining, was lower than the reference strain RM3655. The serum resistance of KR494 [11] was also comparable to that of controls. The multiplication of the capsule locus by an IS1016-*bexA* deletion is considered as a major Hib virulence determinant, and a PCR to detect this deletion was performed [12]. MinnA, but not KR494 or any other control strain, had evidence of multiple capsulation. Thus, in these well-documented in vitro assays, the myositis Hif strain did not appear more virulent than our selected Haemophilus control strains.

Since an MBL deficiency and low levels of IgG3 were identified in our patient, an attempt to study the

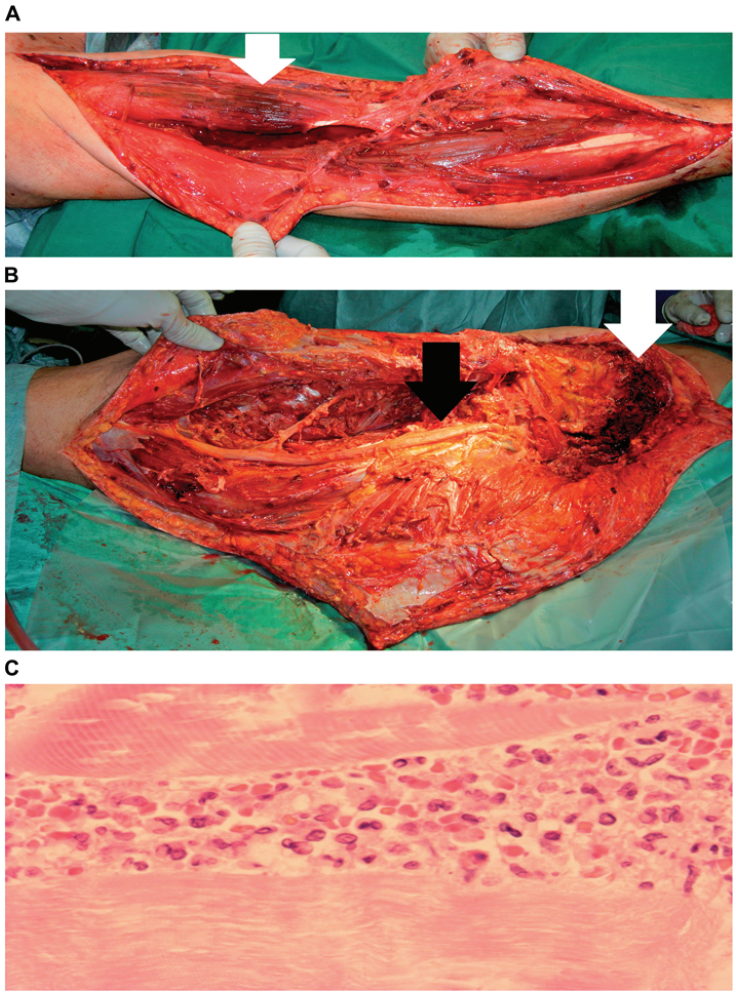

Figure 1. Myositis caused by Haemophilus influenzae type f. (A) Right arm during the first revision; white arrow indicates area of partial necrosis of the biceps muscle. (B) Left hip and thigh region after the first revision of gluteal muscles (white arrow indicates the proximal remains of gluteal muscles) and biceps femoris; black arrow indicates the sciatic nerve that was left intact. (C) Haematoxylin and eosin staining of removed muscle tissue displaying intense acute and chronic inflammatory infiltrate amid the partially necrotic muscle fibres. All photographs were used with the informed consent of the patient.

relevance of these findings was made. MBL deficiencies are common in the general population (>10%), and although study results are conflicting, MBL deficiencies alone are not convincingly associated with an increased risk of invasive infections, but with an increased risk of severe outcome [8]. MBL has been shown to bind to a number of bacterial species, including H. influenzae [13]. We studied the binding of recombinant MBL (R&D Systems) to the clinical Hif isolate KR494 with an FITC-conjugated anti-MBL monoclonal antibody (mAb) (Hycult Biotech) by flow cytometry. Recombinant MBL bound to KR494 as well as to NTHi and Hib controls, but the binding was low and inconsistent compared to the positive control Staphylococcus aureus Cowan I (data not shown).

Although IgG3 deficiency is the most frequently diagnosed Ig subclass disorder, mainly IgG2 deficiency has been associated with increased susceptibility to encapsulated bacteria [14]. The IgG2, IgG3, and IgG4 binding to Hif KR494, as well as to NTHi and Hib control isolates, was assessed after

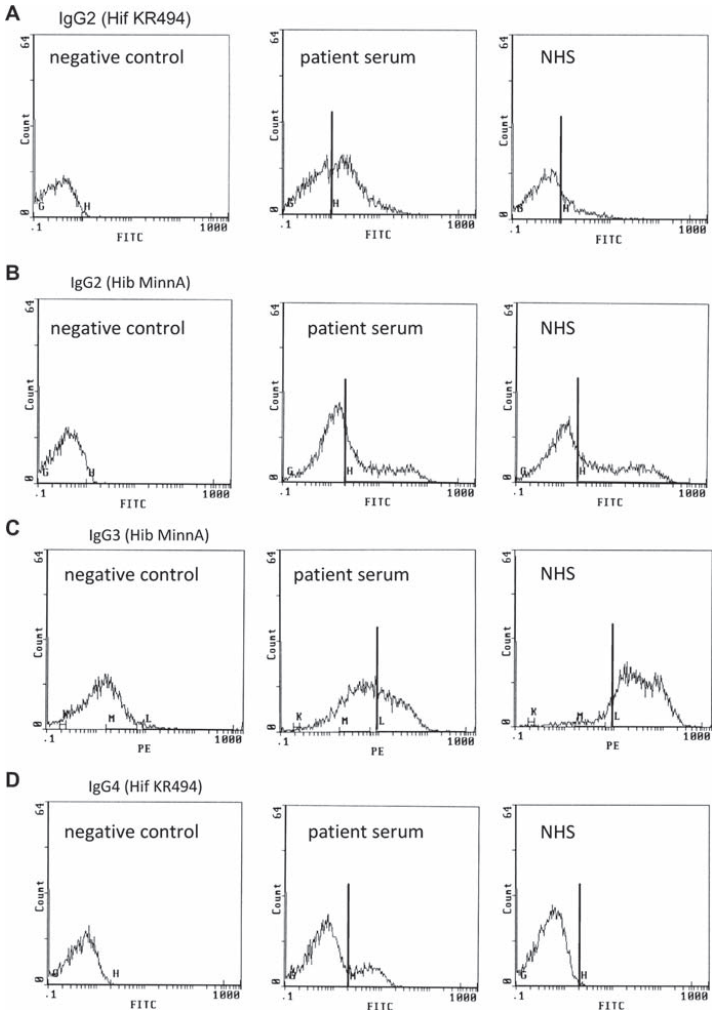

Figure 2. Flow cytometry profiles of Haemophilus influenzae incubated with patient sera and normal human serum (NHS). (A) Binding of patient IgG2 to the myositis strain Hif KR494 was slightly higher as compared to IgG2 in pooled NHS. FITC-conjugated mouse anti-human IgG2 was incubated with Hif KR494 in the absence of serum (negative control), with patient serum or pooled NHS. (B) IgG2 binding to Hib MinnA did not differ between the patient's serum and the pooled NHS. FITC-conjugated mouse anti-human IgG2 was incubated with Hib MinnA without serum (negative control), with patient serum or pooled NHS. Similar results were obtained with the control H. influenzae strains RM3655 and Hif KR462. (C) A lower binding of IgG3 to all studied strains, here exemplified by Hib MinnA, was found with the patient's serum as compared to pooled NHS. Mouse biotin-conjugated anti human-IgG3 mAb using streptavidin–RPE as detection system was used. (D) An increased binding of IgG4 to all strains, here exemplified by Hif KR494, was detected with the patient's serum compared to pooled NHS. A FITC-conjugated mouse anti-human IgG4 mAb was used. All panels show representative examples from experiments performed on 3 separate occasions.

incubation with sera from our patient or pooled normal human serum (NHS) from healthy donors ($n = 5$). Labelling with FITC-conjugated anti-IgG2, anti-IgG4, and biotin-conjugated anti-IgG3 mAb (all Sigma-Aldrich) was performed [15]. In flow cytometry, NHS IgG3 bound to 50–80% of the bacterial population, IgG2 bound to 25–40%, while IgG4 detected $< 10\%$ of the studied Haemophilus strains. There was a higher binding of patient IgG2 to KR494 as compared to IgG2 derived from NHS (Figure 2A), but no such difference was found with the control strains including Hib MinnA (Figure 2B). The

binding of patient IgG3 to all studied strains was lower compared to binding of NHS IgG3 as exemplified with Hib MinnA in Figure 2C. While there was no binding of NHS IgG4 to any studied isolate, 5–15% of bacteria was bound by patient IgG4 (Figure 2D).

Conclusions

Based upon the experimental findings we suggest: (1) that IgG3 plays an significant role in immune reactions against Hif and other H. influenzae strains, (2) that the patient in this study had an altered humoral immunity against Haemophilus compared with healthy controls, with a low activity of IgG3 that may in part have been compensated for by IgG4, and finally (3) that the MBL deficiency alone is less likely to explain the severe outcome in the presented case.

We have described a unique case of necrotizing myositis and septic shock by the emerging pathogen H. influenzae type f in a previously healthy patient. Experiments with the clinical isolate did not identify an explanatory virulence trait. We suggest a correlation between the IgG3 deficiency, possibly in conjunction with the MBL deficiency, and the severe clinical presentation. Results from in vitro experiments indicate that the patient has an altered serum immune response to H. influenzae compared with healthy subjects. This case is of general interest since the incidence of invasive infections by non-type b strains of H. influenzae is increasing in the Western world [4,5].

This case report was written with the patient's informed consent.

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