# *Haemophilus influenzae* – typing, epidemiology and beta-lactam resistance

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Abstract

*Haemophilus influenzae* is a common cause of respiratory tract infections such as acute otitis media (AOM), exacerbations of chronic obstructive pulmonary disease (COPD) and pneumonia. The species is subdivided into encapsulated and non-encapsulated strains, designated type a-f and nontypeable *H. influenzae* (NTHi), respectively. Prior to introduction of polysaccharide-protein conjugate vaccines against *H. influenzae* type b (Hib) in childhood vaccination programmes this serotype frequently caused severe invasive infections in small children. Nowadays invasive disease by Hib is rare, but cases still occur. At present, NTHi is the dominating type to cause invasive disease and invasive NTHi disease appears to be increasing. Nontypeable *H. influenzae* disease severity traditionally has been considered largely host dependant. In parallel, non-beta-lactamase mediated beta-lactam resistance among NTHi is also increasing.

In the first two studies of this thesis, we investigated capsule typing of *H. influenzae* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a technology routinely used for bacterial species identification. Mass spectra of the different types of encapsulated *H. influenzae* were highly similar within each type and separable from each other. The differences in mass spectra relied on the clonal population structure of encapsulated *H. influenzae*, with conserved type specific genetic lineages. Mass spectra of NTHi were diverse, due to their genetic heterogeneity. Following construction of a comprehensive reference database, MALDI-TOF MS showed high accuracy for capsule typing of *H. influenzae*.

In the third study of the thesis, a clonal group of NTHi with resistance against beta-lactam antibiotics was investigated. The clonal group accounted for one quarter of clinical respiratory *H. influenzae* isolates with non-beta-lactamase mediated beta-lactam resistance in the study region. Furthermore, patients infected by isolates of the clonal group had an increased risk of hospitalization compared to patients infected by other NTHi, indicating enhanced virulence traits. The clonal group was also found among invasive isolates.

In the final study of the thesis we retrospectively compared benzylpenicillin, whose effect on *H. influenzae* is debated, to wide spectrum beta-lactams (WSBLs) as empirical treatment of *H. influenzae* lower respiratory tract infections in patients requiring hospitalization. Empirical treatment with benzylpenicllin was not associated with higher mortality or increased risk of hospital readmission compared to treatment with WSBLs. The early clinical response rate was, however, lower for patients receiving benzylpenicillin, which was attributed mainly to a lower response rate in patients infected with beta-lactamase producing isolates.

In conclusion this thesis shows that MALDI-TOF MS can be efficiently used for rapid capsule typing of *H. influenzae*. The newly developed method can be valuable for typing of invasive *H. influenzae* isolates and for surveillance of Hib vaccination efficacy. The studied clonal group appears to harbour enhanced virulence traits. This indicates that bacterial factors may affect NTHi disease severity more than previously considered, and possibly contribute to the increased incidence of invasive NTHi disease. Finally, although the effect of benzylpenicillin on *H. influenzae* is debated, empirical treatment of lower respiratory tract infections of mild to moderate severity caused by *H. influenzae* with the agent appears safe.



Key words: Benzylpenicillin, beta-lactam resistance, beta-lactams, BLNAR, capsule typing, clonal, *Haemophilus influenzae*, Hib, MALDI-TOF MS, MLST, NTHi, serotyping, virulence

Classification system and/or index terms: Bacterial capsules, bacterial typing, beta-lactam resistance, epidemiology, *Haemophilus influenzae*, MALDI

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Cover photo: Scanning electron microscopy picture of *Haemophilus influenzae* infecting lung tissue in an experimental mouse model. By Matthias Mörgelin, Colzyx AB, Lund, Sweden.



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# Contents

# List of papers

I. **Viktor Månsson***, Fredrik Resman*, Markus Kostrzewa, Bo Nilson, Kristian Riesbeck. Identification of *Haemophilus influenzae* Type b Isolates by Use of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry.
*Journal of Clinical Microbiology* 2015; 53(7):2215-24.
*These authors contributed equally to the article.

II. **Viktor Månsson**, Janet R Gilsdorf, Gunnar Kahlmeter, Mogens Kilian, J Simon Kroll, Kristian Riesbeck*, Fredrik Resman*. Capsule Typing of *Haemophilus influenzae* by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.
*Emerging Infectious Diseases* 2018; 24(3):443-52.
*These senior authors contributed equally to the article.

III. **Viktor Månsson**, Dagfinn Skaare, Kristian Riesbeck, Fredrik Resman. The spread and clinical impact of ST14CC-PBP3 type IIb/A, a clonal group of non-typeable *Haemophilus influenzae* with chromosomally mediated beta-lactam resistance – a prospective observational study.
*Clinical Microbiology and Infection* 2017; 23(3):209 e1- e7.

IV. John Thegerström, **Viktor Månsson**, Kristian Riesbeck, Fredrik Resman. Benzylpenicillin versus wide-spectrum beta-lactam antibiotics as empirical treatment of *Haemophilus influenzae*-associated lower respiratory tract infections in adults; a retrospective propensity score-matched study.
*European Journal of Clinical Microbiology Infectious Diseases* 2018; 37(9):1761-75.

# Abbreviations

| | |
|---|---|
| AOM | Acute otitis media |
| ATP | Adenosine triphosphate |
| BLNAR | Beta-lactamase negative ampicillin resistant |
| BLNAS | Beta-lactamase negative ampicillin susceptible |
| BLPAR | Beta-lactamase positive ampicillin resistant |
| BLPACR | Beta-lactamase positive amoxicillin-clavulanate resistant |
| CAP | Community-acquired pneumonia |
| CC | Clonal cluster |
| CCI | Charlson comorbidity index |
| CI | Confidence interval |
| CLSI | Clinical and Laboratory Standards Institute |
| COPD | Chronic obstructive pulmonary disease |
| CRP | C-reactive protein |
| ECM | Extracellular matrix |
| ECOFF | Epidemiological cut-off |
| ESBL | Extended spectrum beta-lactamase |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| hia | *Haemophilus influenzae* adhesin |
| Hia | *Haemophilus influenzae* type a |
| Hib | *Haemophilus influenzae* type b |
| Hic | *Haemophilus influenzae* type c |
| Hid | *Haemophilus influenzae* type d |
| Hie | *Haemophilus influenzae* type e |
| Hif | *Haemophilus influenzae* type f |
| HMW | High molecular weight protein |
| ICAM-1 | Intercellular adhesion molecule-1 |

| | |
|---|---|
| ICU | Intensive care unit |
| IgA1 | Immunoglobulin A1 |
| IgD | Immunoglobulin D |
| LOS | Lipooligosaccharide |
| LPS | Lipopolysaccharide |
| MALDI-TOF MS | Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry |
| MIC | Minimum inhibitory concentration |
| MLEE | Multilocus enzyme electrophoresis |
| MLST | Multilocus sequence typing |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MRSE | Methicillin-resistant *Staphylococcus epidermidis* |
| MSP | Main spectrum |
| NAD | Nicotinamide adenine dinucleotide |
| NordicAST | Nordic Committee on Antimicrobial Susceptibility Testing |
| NTHi | Nontypeable *Haemophilus influenzae* |
| OMV | Outer membrane vesicle |
| OR | Odds ratio |
| PBP | Penicillin-binding protein |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PcG | Penicillin G |
| PcV | Penicillin V |
| PCV | Pneumococcal conjugate vaccine |
| PRP | Polyribosylribitol phosphate |
| rPBP3 | Resistance mediated by amino acid substitutions in PBP3 |
| SAST | Slide agglutination serotyping |
| ST | Sequence type |
| WSBL | Wide spectrum beta-lactam |

# Populärvetenskaplig sammanfattning

*Haemophilus influenzae* är en bakterie som lever i människans luftvägar. Ofta orsakar bakterien ingen skada hos personen som bär den, men ibland orsakar *H. influenzae* luftvägsinfektioner såsom öroninflammation hos barn, akut försämring hos patienter med kroniskt obstruktiv luftvägssjukdom (KOL) samt lunginflammation. Bakterien kan också orsaka allvarliga infektioner såsom blodförgiftning och hjärnhinneinflammation. Dessa infektioner kallas invasiva, vilket innebär att bakterier kan odlas fram från en normalt sett bakteriefri (steril) vävnad, som till exempel blod. *H. influenzae* delas in i olika typer. Dessa benämns typ a till f beroende på vilken sorts skyddande kapselmolekyl de producerar och omger sig med. Det finns även bakteriestammar som inte producerar någon kapsel. Dessa kallas icke-kapslade *H. influenzae*. För att särskilja de olika typerna finns olika metoder för kapseltypning.

Allvarliga infektioner har historiskt nästan uteslutande orsakats av *H. influenzae* typ b (Hib) och främst drabbat barn i tidig förskoleålder. Sedan barnvaccination mot Hib införts i stora delar av världen under 1990- och 2000-talet har sjukdom orsakad av Hib minskat drastiskt. Idag orsakas både luftvägssjukdom och invasiv sjukdom i huvudsak av de icke-kapslade stammarna. Invasiv sjukdom med icke-kapslade *H. influenzae* verkar öka i förekomst, men anledningen till detta är inte klarlagd. Sjukdom framkallad av dessa stammar har till stor del ansetts vara beroende av en ökad infektionskänslighet hos personen som drabbas. Enstaka fall av svår sjukdom orsakad av Hib förekommer dock fortfarande även i befolkningar med hög vaccinationstäckning. Det är därför viktigt att undersöka vilken kapseltyp invasiva bakteriestammar tillhör och på så sätt säkerställa att vaccinationsprogrammen mot Hib fungerar.

Infektioner orsakade av *H. influenzae* behandlas ofta med så kallade betalaktamantibiotika, men resistens mot denna typ av antibiotika ökar. I många fall behandlas patienter med infektioner innan det är säkerställt vilken bakterie som orsakar infektionen. Vid lunginflammation som kräver sjukhusvård rekommenderas ofta läkemedlet benzylpenicillin för sådan initial behandling. Benzylpenicillin har god effekt på pneumokockbakterier, som är den vanligaste orsaken till lunginflammation, men osäker effekt på *H. influenzae*.

I de första två studierna i denna avhandling undersöktes möjligheten att använda *matrix-assisted laser desorption/ionization time-of-flight mass spectrometry* (MALDI-TOF MS) för kapseltypning av *H. influenzae*. Vid denna typ av masspektrometri analyseras ett bakterieprovs proteininnehåll och ett så kallat massfingeravtryck genereras. Analysen går snabbt och tekniken används idag i klinisk diagnostik för artbestämning av bakterier från patientprover. Studierna visade att de kapslade kapseltyperna (a till f) hos *H. influenzae* hade olika typspecifika massfingeravtryck och gick att skilja åt med hjälp av dessa. En ny

automatiserad kapseltypningsmetod med MALDI-TOF MS skapades och visade goda resultat jämfört med den nuvarande metoden för kapseltypning.

I den tredje studien i avhandlingen undersöktes en särskild antibiotikaresistent ”klon” av icke-kapslad *H. influenzae* som tidigare har kopplats till invasiv sjukdom och ett sjukdomsutbrott på ett äldreboende. Studien visar att klonen var vanligt förekommande i Skåne åren 2010 till 2012. Bland patienter med luftvägssjukdom orsakad av klonen var risken för sjukhusvård större än hos patienter som drabbats av infektioner med andra bakteriestammar. Detta tyder på att klonen orsakade mer allvarlig sjukdom än andra bakteriestammar. Under den studerade tidsperioden orsakade klonen även två fall av blodförgiftning (även kallat sepsis).

I avhandlingens sista studie undersöktes initial behandling av lunginflammation som kräver sjukhusvård och som i ett senare skede visar sig vara orsakad av *H. influenzae*. Vi kunde inte se någon ökad dödlighet eller ökad förekomst av sjukhusåterinläggningar hos patienter som fått initial behandling med benzylpenicillin jämfört med de som fått behandling med andra antibiotika.

Sammanfattningsvis visar denna avhandling att kapseltypning av *H. influenzae* med MALDI TOF MS har god träffsäkerhet. Metoden är snabb, enkel och kan underlätta vaccinationsövervakning och snabb kapseltypning av invasiva bakteriestammar. Avhandlingen visar också att det finns icke-kapslade stammar av *H. influenzae* som verkar vara särskilt aggressiva och medför en högre risk att patienten drabbas av allvarlig sjukdom. Förekomsten av sådana kloner skulle delvis kunna förklara den ökning av allvarlig sjukdom orsakad av icke-kapslade *H. influenzae* som noterats de senaste åren. Resultaten indikerar också att svårighetsgraden av sjukdom orsakad av icke-kapslade stammar inte endast beror på patientens infektionskänslighet, utan att även bakteriens egenskaper har betydelse. Den sista studien visar att initial behandling med benzylpenicillin av luftvägsinfektioner av mild till måttlig grad som senare visar sig vara orsakade av *H. influenzae* inte verkar öka risken för allvarliga komplikationer. Detta tyder på att nuvarande behandlingsrekommendationer med benzylpenicillin inte behöver ändras av den anledningen.

# Introduction

## Brief history of the bacterium *Haemophilus influenzae*

*Haemophilus influenzae* is a bacterium commonly residing in the human respiratory tract. Its relationship to its host is complicated and multifaceted. The bacterium was first discovered in the late 19th century by the German bacteriologist Richard Pfeiffer. Pfeiffer isolated the bacterium from patients with influenza. He mistakenly believed that the bacterium was the cause of influenza as he found that the bacteria produced disease when inoculated in apes and rabbits, and named it *Bacillus influenzae* (1). During the 1918 to 1920 influenza pandemic, research on the influenza disease intensified. It was shown that specimens from patients with influenza which had been cleared from bacteria could transmit the disease, and that the bacterial infection observed by Pfeiffer was only secondary to another infective agent (2). Later the influenza virus was discovered, but *H. influenzae*, which the bacterium was renamed to in 1917, remains named after the disease (3, 4).

Although not the cause of influenza, the bacterium was still the cause of other severe diseases. Infections such as meningitis, epiglottitis and bacteraemia were common, especially in children (5). In the 1930s, bacteriologist Margaret Pittman classified the species into smooth and rough strains and concluded that these types of strains were encapsulated and non-encapsulated, respectively. She further divided smooth strains into types a and b, based on precipitation reactions with antisera (6). Since then, another four capsule types (designated c-f) have been discovered. Pittman also concluded that type b strains where the most common in specimens from meningitis patients (6). Correctly, *H. influenzae* type b (Hib) was the serotype which, almost exclusively, caused invasive disease in children during the 20th century. Because of this, *H. influenzae* research focused on Hib and the development of an effective vaccine. In the 1980s Hib became the first pathogen against which glycoconjugate vaccines were developed and licensed. Since then, Hib disease has diminished greatly in countries implementing childhood vaccination (5).

Besides this, the species has been involved in other major scientific breakthroughs, such as the discovery of penicillin by Alexander Fleming. By adding the newly discovered agent penicillin to culture medium, Fleming could separate *H. influenzae* from more penicillin susceptible bacterial species, such as *Streptococcus pneumoniae* (7). The difference in penicillin susceptibility between *H. influenzae* and *S. pneumoniae* is still of great importance regarding the treatment of respiratory

tract infections. Intriguingly, *H. influenzae* strain Rd was the first free-living organism to have its entire genome sequenced in 1995, which is another example of a significant role of the species in scientific breakthroughs (8). The achievement was a major advancement in the field of genomic research. The genome data of strain Rd obtained in 1995, and laboratory transformants of the strain, have been used in the studies of this thesis.

Following implementation of childhood Hib vaccination programmes, the spectrum of *H. influenzae* disease has shifted. Although the burden of Hib disease has decreased, *H. influenzae* is still one of the major bacterial pathogens causing disease in humans. Most disease is now caused by nontypeable *H. influenzae* (NTHi), the strains Pittman called rough, which lack a polysaccharide capsule (6). Furthermore, the patients currently most affected by severe disease caused by *H. influenzae* are newborns, the elderly and patients with immune system impairments.

# Bacteriology of *Haemophilus influenzae*

## Basic characteristics

*H. influenzae* is a small, facultatively anaerobic, Gram-negative bacterium of the *Pasteurellaceae* family. The bacteria are generally rod-shaped coccobacilli but are often pleomorphic (9).

The species requires nicotinamide adenine dinucleotide (NAD, V factor) and haemin (X factor) for growth (10). Both factors can be found in erythrocytes, hence the species' generic name *Haemophilus* (blood loving). For the bacteria to utilize NAD the erythrocyte cell membrane must be disrupted, and NAD released. Special growth media, such as heat-treated red blood media (chocolate agar), is therefore needed for cultivation and standard blood agar is not sufficient.

The requirement of NAD and haemin for growth can be used to identify the species in the laboratory. More specifically it can be used to separate it from some other species of the *Haemophilus* genus. *H. parainfluenzae* is commonly found in the human airway, and requires only NAD for growth (11). To differentiate *H. influenzae* from the commensal *H. haemolyticus* the haemolytic ability of the latter can be used, although misclassifications are common (12). Matrix-assisted laser/desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been shown to reliably separate the three species excluding other more laborious tests (13-16).

*H. influenzae* is subdivided into encapsulated and non-encapsulated strains. Encapsulated strains possess one of six different types of capsule polysaccharides and are designated *H. influenzae* type a-f. Traditionally, typing was based on precipitation with specific antisera (serotyping). Strains which do not produce any

polysaccharide capsule cannot be serotyped and are therefore designated nontypeable *H. influenzae* (NTHi) (6).

Based on three biochemical tests (indole production, urease activity and ornithine decarboxylase activity), *H. influenzae* can be divided into different biotypes (17). This classification is useful for epidemiological typing, but its use has diminished since the development of other typing methods.

## Pathogenesis and virulence

*H. influenzae* is both a colonizer and pathogen, specialized mainly on the respiratory tract. In order to colonize the respiratory tract, bacteria need to access and attach to the mucosa of the upper airway. There it must survive and, to cause disease, migrate to other parts of the body such as the middle ear, the lungs or the bloodstream. Importantly, most colonization with *H. influenzae* does not result in infection and clinical disease, similarly to other common bacterial species found in the respiratory tract such as *S. pneumoniae* and *Moraxella catarrhalis* (18, 19).

In most cases, disease occurs when the balance between the commensal bacteria and host defence mechanisms is disrupted. Immature or defect immune systems, defect structural barriers, chronic inflammation and prior viral infections, separate or in combination, allow bacteria to migrate and grow, which can lead to clinical disease (18, 20-23). For *H. influenzae*, the capacity to cause disease, and thus the impact of the factors mentioned above on disease development, varies between different strains. One major determinant of virulence is the presence of a polysaccharide capsule. Besides capsule production, *H. influenzae* has developed several other strategies to colonize and cause disease in humans. The molecular complexity of the disease process is increasingly understood. The subject has been reviewed several times recently and is only briefly reported on here (24-26).

### *Adherence to the respiratory epithelium*

To attach to host epithelial cells and extracellular matrix (ECM), which is exposed if the epithelial cell layer is damaged, *H. influenzae* possesses several molecules with different effects and host targets (Figure 1).

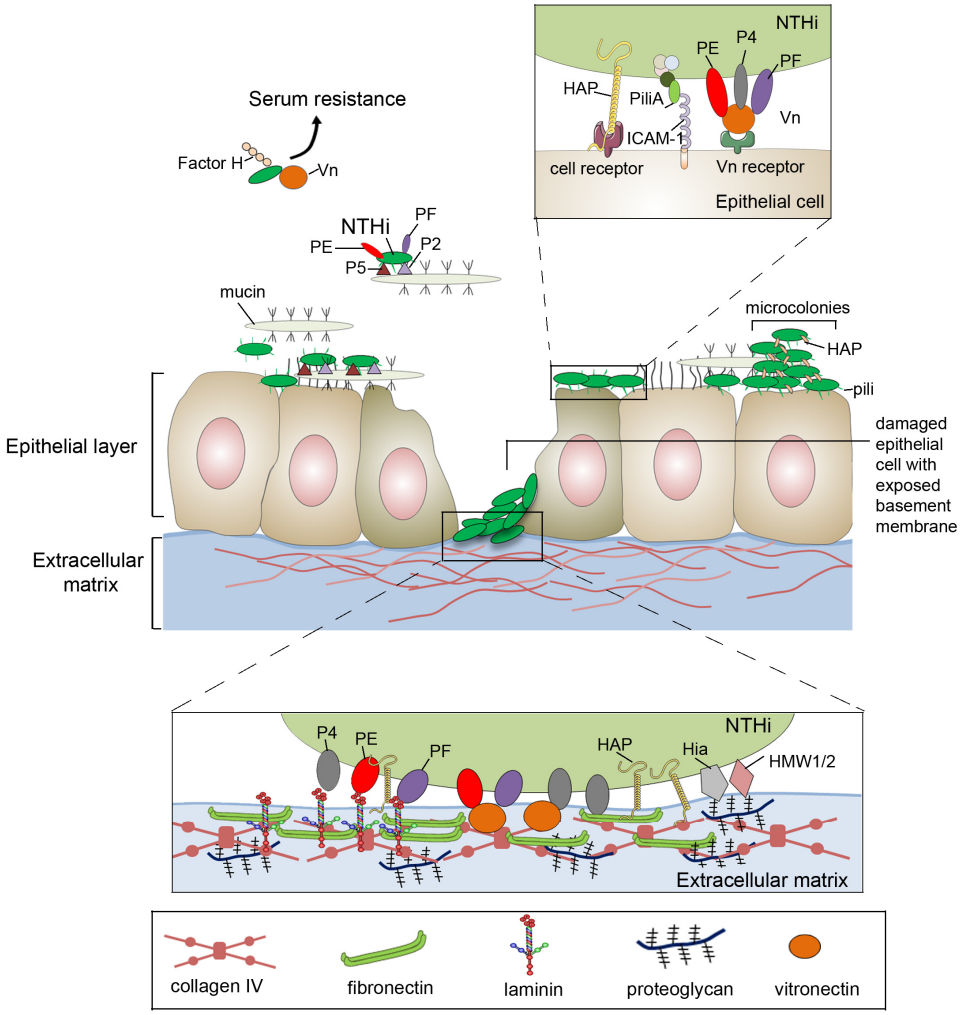



**Figure 1. Pathogen-host interactions of *H. influenzae* in the human respiratory tract.**
*H. influenzae* utilizes several adhesins to bind different host proteins which mediates attachment to the human epithelium and ECM of the respiratory tract. Adherence to the host tissue is essential for colonization and subsequent infection of the host. Vn, vitronectin. Image used with permission from the publisher (24).

Bacterial proteins which facilitate attachment to host tissue are most often incorporated in the outer membrane of the bacteria and are often multifunctional.

High molecular weight protein (HMW) 1 and 2 are adhesins expressed by approximately 75% of all NTHi strains (27, 28). These proteins bind to proteoglycans at the epithelial cell surface (29). *Haemophilus influenzae* adhesin (hia) is a similar adhesion protein, although with a different ligand, which most often is found in strains not expressing HMW 1 or 2 (28, 30). Proteins E and F are located

in the *H. influenzae* outer membrane, and confer adherence to epithelial cells and to the extracellular matrix of the respiratory tract by binding the major ECM components laminin and vitronectin (31-34). A similar effect is mediated by the protein P4 (also called lipoprotein e), which binds mainly fibronectin (also a component of the ECM), and to a lesser extent laminin (35). The *Haemophilus* adhesion and penetration protein (Hap) exhibits a comparable function by binding ECM components fibronectin, laminin and collagen IV (36). Several outer membrane proteins, including proteins P2 and P5, bind mucins, which are glycoproteins of the human nasopharyngeal mucus. If this is to advantage of the bacteria or the host is at present unclear (37). *H. influenzae* also expresses type IV pili, a protein structure important for bacterial motility (38). In addition, type IV pili confer attachment to the human mucosa by binding intercellular adhesion molecule-1 (ICAM-1) on epithelial cells (39, 40).

*Immune system evasion and modulation*

In the mucosa of the human respiratory epithelium, immunoglobulin A1 (IgA1) is continuously secreted and an important part of the local immune system. To counteract this most strains of *H. influenzae* produce an IgA protease, which cleaves human IgA1 (41). Higher IgA protease activity has been associated with disease causing strains, as opposed to commensal counterparts. The variation in activity was, however, large within the groups and the study sample relatively small (42). Different subtypes of IgA protease have been defined in *H. influenzae*, encoded by the genes *igaA* and *igaB*. Virtually all strains carry *igaA* while only some carry *igaB*. Strains carrying *igaB* are found in certain genetic groups of NTHi (43). Interestingly, carriage of *igaB* has been negatively associated with invasive disease and is instead more commonly found in strains isolated from samples derived from the respiratory tract (44). If this finding is attributed to *igaB* encoded IgA protease or other common characteristics of these strains is not currently known.

Furthermore, *H. influenzae* can disrupt the mucociliary clearance apparatus, which functions to remove bacteria and debris from the respiratory tract (45). This function is partly mediated via protein D, a surface protein ubiquitous for *H. influenzae* (46, 47). Protein D also functions as a hydrolase of glycerophosphodiesters and can thus aid the bacteria in the utilization of nutrients from the environment, such as choline from epithelial cells (48).

To protect itself from complement mediated killing, *H. influenzae* can bind C4b binding protein, which inhibits the classical pathway of the complement system (49). Furthermore, the proteins P4, P5, protein E and protein F have been shown to potentially protect bacterial cells from complement mediated clearance *in vitro*. This protection is mediated by the binding of host proteins, such as factor H and vitronectin, which inhibit complement activation (Figure 1) (33, 35, 50-52). Another ubiquitous protein of the outer membrane of *H. influenzae* is P6 (53). P6 is associated to the peptidoglycan layer of the bacterium and appears to protect against

complement mediated killing (54). Finally, a pro-inflammatory response is also mediated by P6 (55).

Lipooligosaccharide (LOS) is a glycolipid expressed by all *H. influenzae*. It is similar to lipopolysaccharide (LPS), which is typical for most other Gram-negative bacteria, but lacks the O-antigen. Lipooligosaccharide is an important part of the outer cell membrane and induces a pro-inflammatory response in host cells (56, 57). *H. influenzae* strains isolated from patients with chronic tonsillitis have the capacity to bind the B lymphocyte receptor immunoglobulin D (IgD). This results in a superantigen-dependent B lymphocyte activation with production of polyclonal antibodies not recognizing *H. influenzae*, and hence the immune system is potentially misled (58).

In addition to several other Gram-negative species, *H. influenzae* produces outer membrane vesicles (OMVs) which are budded off the outer membrane into the surroundings. The different functions of OMVs are not entirely clear, but similarly to LOS they induce a pro-inflammatory host response (59). It is possible that OMVs, containing LOS and other molecules of the periplasm and the outer membrane, act as decoys directing the host immune response away from the bacterial cell. Indeed, *H. influenzae* OMVs generate a similar unspecific response via the IgD receptor in B lymphocytes as do whole bacteria (60). Outer membrane vesicles from beta-lactamase producing *H. influenzae* have also been shown to protect *Streptococcus pyogenes* from amoxicillin *in vitro* (61). This OMV function may explain cases of penicillin treatment failure in respiratory tract infections primarily caused by susceptible Gram-positive species but where *H. influenzae* is also present. In other species OMVs have been shown to be highly multifunctional, and it is probable that they are for *H. influenzae* as well (62).

Biofilms are multicellular microbial communities, which also contain matrix material such as extracellular DNA. Biofilms protect the bacteria from the host immune system and antimicrobial agents. The type IV pili of *H. influenzae* appears to be involved in biofilm formation of the species (39). Another bacterial mechanism for avoiding the immune system is to invade and persist in host cells. Nontypeable *H. influenzae* has in fact been shown to remain viable for limited periods of time in both human epithelial and white blood cells (58, 63, 64). Immunoglobulin A proteases encoded by *igaB* appear to have a role in this persistence by inhibiting lysosomal activity of the human cells (65). The importance of biofilm and intracellular persistence in *H. influenzae* disease are not entirely clear, but these phenomena likely play an important role in persistent colonization and infections, such as recurring acute otitis media (AOM) and chronic obstructive pulmonary disease (COPD) (66).

## The polysaccharide capsule

The polysaccharide capsule of *H. influenzae* is a major virulence factor. The function of the capsule is primarily to help the bacteria avoid phagocytosis by immune cells and opsonization by the complement system. This results in a higher capability to survive in human blood (67). In animal experiments of isogenic capsule transformants, certain encapsulated isolates showed markedly higher virulence. The type b transformant was shown to be the most virulent, followed by type a and type f (68). The increased experimental virulence of Hib is in agreement with the epidemiology of invasive *H. influenzae* disease.

The production of capsular polysaccharide is regulated by the *cap* gene locus. The locus consists of three distinct regions, of which regions I and III are common to all capsule types and region II is type specific (69) (Figure 2).

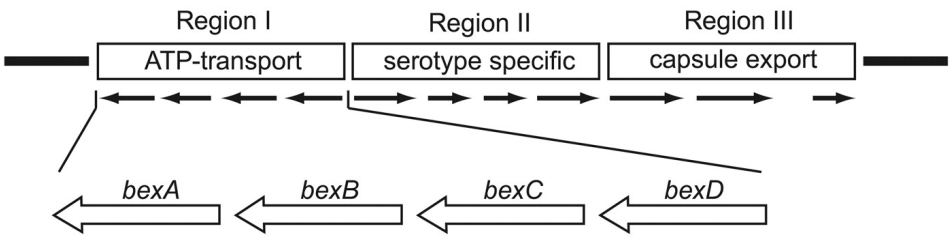



**Figure 2. The *H. influenzae cap* locus.**
The *H. influenzae cap* locus consists of regions I to III, of which region II is serotype specific. Image adapted and used with permission from the publisher (70).

Region II contains genes involved in biosynthesis of the type specific capsular polysaccharide (71). Region I contains the *bexABCD* genes, which encode proteins involved in adenosine triphosphate (ATP)-driven transport of synthesized polysaccharide (72). In region III the genes *hcsA* and *hcsB* encode products which are essential for export of the capsule polysaccharide from the periplasm across the outer membrane (73). The Hib capsule consists of polyribosylribitol phosphate (PRP) and originally the increased virulence of Hib strains was attributed to the unique structure of this polysaccharide (74). It has, however, been shown that the *cap* locus in Hib strains often is duplicated and flanked on both sides by an insertion element, IS*1016*, effectively making the locus a composite transposon (75). In addition, one of the repeats contains an IS*1016-bexA* partial deletion rendering a defect *bexA* gene (75, 76). This configuration of the *cap* locus makes amplification of the locus by recombination and subsequent increased capsule polysaccharide production possible, which generates a thicker capsule (76, 77). However, the configuration also makes capsule deficient mutants, which are unable to export the capsule material to the cell surface, occur at high frequencies due to genetic recombination events eliminating the intact *bexA* gene (78). All Hib of genetic

division I, which was the clearly dominating genetic lineage to cause invasive disease during the 20th century, share this *cap* locus configuration (75, 76, 79). The high pathogenicity of Hib strains might in part be due to this configuration and the following capability to produce large amounts of capsule polysaccharide. A similar *cap* locus duplication with an IS*1016*-*bexA* partial deletion has been reported in virulent *H. influenzae* type a (Hia) strains of genetic division I, but not in types c, d, e or f (69, 80-83).

Typing of *H. influenzae* has traditionally been performed by slide agglutination serotyping (SAST) with serotype specific antisera (6). Using this technique, misidentifications are, however, common. Especially non-encapsulated strains are often misclassified as encapsulated, due to false positive reactions with antisera. The method is also user dependent (84-86). The genetic capsulation status of an isolate can also be determined by polymerase chain reaction (PCR), which is a highly accurate method. However, the method does not demonstrate the existence of an actual capsule phenotype. Presence of the *cap* locus is investigated with primers complementary to the *bexA* gene in region I of the *cap* locus (87). In *bexA* positive isolates, this is followed by PCR with type specific primers complementary to region II of the *cap* locus to determine the serotype (88, 89). Primers complementary to *bexB* have also been developed to facilitate the differentiation of previously encapsulated capsule deficient strains with the IS*1016*-*bexA* partial deletion from strains which lack the entire *cap* locus (often deemed true NTHi) (70).

## Genetic variation and population structure

*H. influenzae* was at an early stage observed to be a heterogenous species, with variations in colony morphology, growth and metabolic properties (6).

According to the distributed genome hypothesis the total set of genes found in a bacterial species constitutes the supragenome. The supragenome consists of core genes, which are present in all strains of the species, and non-core genes, which are not present in all strains and can be laterally transferred between strains. Modern genomic studies have shown that the supragenome of *H. influenzae* may contain up to 6,000 genes, but that the core-genome only encompass about 1,500 genes (90, 91). Any single strain often carries between 1,700 and 2,100 genes (92). It is hypothesized that different *H. influenzae* strains exchange genetic material to adapt to environmental changes (90). The species is naturally competent with high DNA uptake *in vitro* (93, 94). DNA uptake and transformation varies greatly between different strains but is generally higher in resource scarce environments (95). Horizontal gene transfer has also been shown to occur between *H. influenzae* and *H. haemolyticus* (96).

Encapsulated *H. influenzae* can be divided into two major genetic divisions (division I and II) which contain separate genetic lineages representing strains of

specific capsule serotypes. This was first demonstrated by multilocus enzyme electrophoresis (MLEE) and has subsequently been shown with methods based on genetic sequencing, most notably multilocus sequence typing (MLST) which categorizes isolates into sequence types (STs) (97, 98). There are three major genetic groups of Hia and Hib isolates, divided between genetic division I and II. There is one group each for *H. influenzae* type c (Hic) and type d (Hid) (genetic division I) and *H. influenzae* type e (Hie) and type f (Hif) (genetic division II) (98). Hib of the ST6-related genetic lineage of genetic division I, which in general contain a duplicated *cap* locus with an IS*1016*-*bexA* partial deletion, was the dominating genetic group to cause invasive disease in the pre-vaccination era (75, 76, 79, 97). Within each serotype specific genetic lineage, strains are generally genetically conserved, although some genetic variations exist (98) (Figure 3).

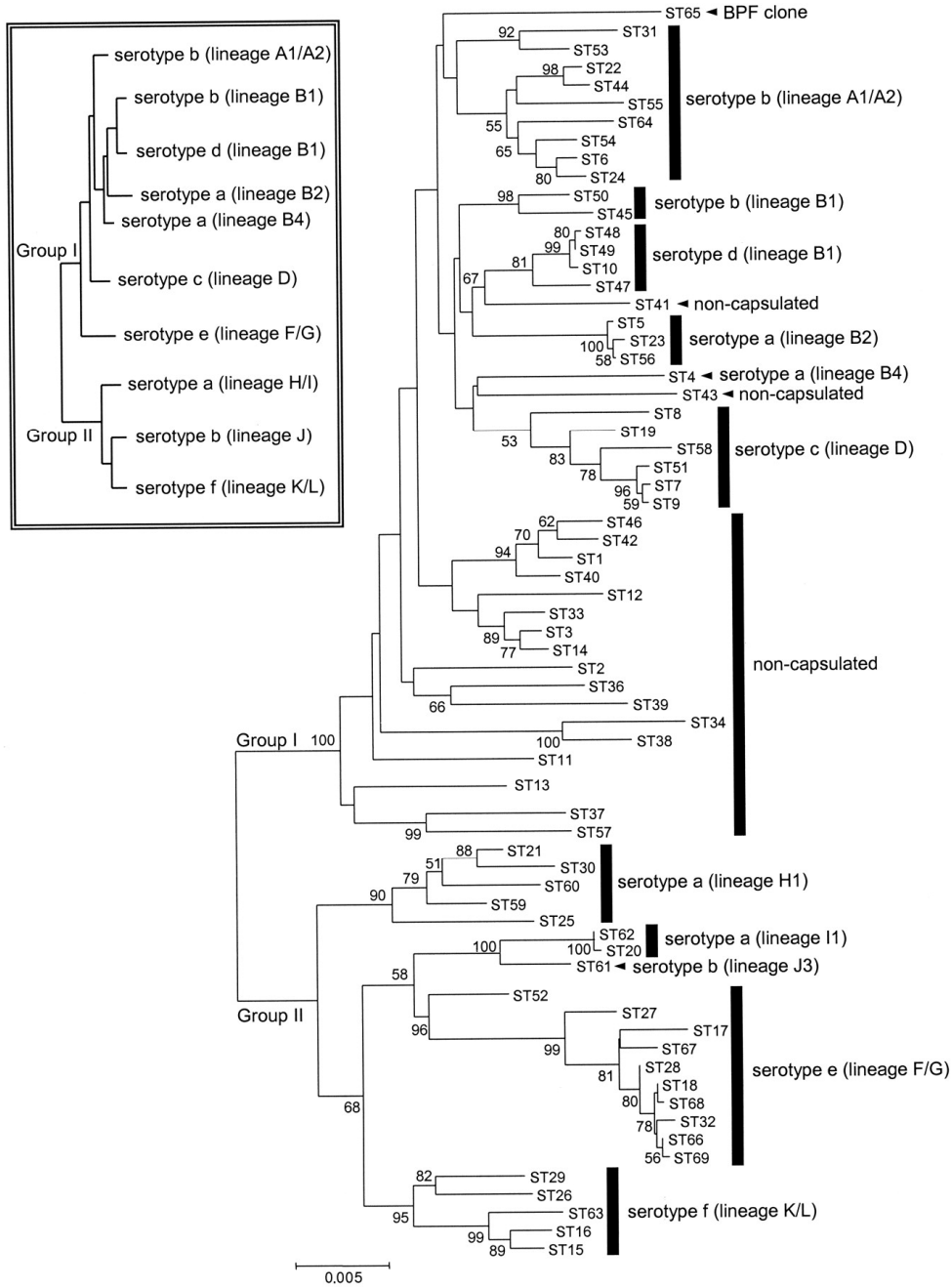



**Figure 3. Phylogenetic relationship of encapsulated *H. influenzae*.**
A minimum evolution tree based on concatenated MLST sequences. Different serotypes are genetically conserved and divided between major genetic divisions I and II. Isolates of Hia and Hib are found in both divisions. Tree in inset shows similar results based on MLEE analysis. Image used with permission from the publishers (98, 99).

NTHi make up a much more heterogenous entity than encapsulated *H. influenzae* and are found in both major genetic divisions of the species (100). Compared to encapsulated isolates, NTHi also appear more prone to genetic recombination events (101). However, studies using MLST and whole genome sequencing have separated NTHi into genetic groups which are designated clades. The clades, however, show a greater internal genetic variability compared to the genetic lineages of encapsulated *H. influenzae* (43, 100). Certain genetic groups of NTHi have been associated with expression of different virulence factors, but not to specific clinical infections (43, 100, 102). Several genes, although many with unknown function, have been shown to be more common in groups of commensal and pathogenic strains, respectively (91).

# Disease caused by *Haemophilus influenzae*

## Colonization and transmission

*H. influenzae* is exclusively found in humans, both as a commensal and pathogen, and no known animal reservoir exists. The bacterium is effectively transmitted between human hosts through infected respiratory droplets (103).

Asymptomatic carriage of NTHi in the respiratory tract is common, especially in children attending day-care facilities who on average are colonized in 30-40% of cases in different investigations (104-106). Colonization also occurs in adults (107). Carriage is dynamic, and strains are often exchanged between hosts (108). Colonization with a specific strain is often transient and the strain is in most cases cleared, and possibly replaced by another, within weeks or months (104, 105). Carriage of Hib strains is effectively reduced by vaccination and carriage of other encapsulated strains is relatively uncommon (104-106, 109).

The introduction of pneumococcal conjugate vaccines (PCVs), of which the 10-valent (PCV10) includes *H. influenzae* protein D as a carrier protein, may well have substantial effects on the prevalence of NTHi colonization in the population. It has been hypothesized that the vaccine including protein D might have an additional effect in eliciting an immune response against *H. influenzae* and decrease colonization. However, the implementation of pneumococcal vaccinations might also give NTHi a greater ecological niche by reducing pneumococcal carriage. Randomized controlled trials of PCV10 have shown no consistent impact on *H. influenzae* colonization compared to other PCVs and control vaccines (110-113). Cross-sectional studies comparing pre- and post-vaccination carriage have shown varied results with both decreased and increased *H. influenzae* colonization after PCV10 vaccination in different studies (114, 115). For the PCVs not including protein D (7-valent and 13-valent), cross sectional studies have shown higher *H.*

*influenzae* colonization rates in vaccinated compared to unvaccinated children (116, 117). In a longitudinal study, *H. influenzae* carriage in children increased in the years following inclusion of PCV7 in the childhood vaccination programme (118). A recent randomized controlled trial comparing PCV10 and PCV13 showed no significant difference in NTHi colonization up to 6 months post vaccination (119). Regarding the genetic population structure of *H. influenzae*, a British study indicated limited changes among strains isolated from children before and after change from PCV7 to PCV13 (120). To summarize, the findings are divergent regarding PCV10 but indicate that PCVs not including *H. influenzae* protein D might increase *H. influenzae* carriage.

## Respiratory tract infections

*H. influenzae* is a colonizer of the respiratory tract, but also a cause of respiratory diseases, such as AOM, sinusitis, bronchitis, pneumonia and exacerbations and chronic infections in patients with COPD. Since both colonization and infection by *H. influenzae* is common, isolation of the species must be carefully evaluated regarding the clinical importance. The mucosal infections of *H. influenzae* are nowadays almost exclusively caused by NTHi and encapsulated isolates appear to be uncommon (121-123).

### *Otitis media*

Acute otitis media is characterized by otalgia, irritability and fever. Diagnosis is made by otoscopy and the bacterial aetiology is preferably determined by analysis of middle ear fluid, sampled by tympanocentesis or by collection of otorrhea if spontaneous perforation of the tympanic membrane has occurred. Acute otitis media is one of the most common infections of childhood. By 1 year of age 23% of children has had at least one episode of AOM and by 3 years of age this number has increased to 60% (124). Otitis media is most often a bacterial infection or a viral-bacterial co-infection, but approximately 10-20% of episodes are considered to be of entirely viral aetiology (125). *S. pneumoniae*, NTHi and *M. catarrhalis* are the most common bacterial pathogens in AOM (124). Nontypeable *H. influenzae* account for almost all episodes of *H. influenzae* dependent AOM (122, 123). The initial efficacy study of PCV10 showed a significant reduction of AOM caused by NTHi (126). This was not the case in a more recent study that, however, reported low AOM and low NTHi colonization rates in both the PCV10 and the control group (112).

In a recent American study, 30% of middle ear fluid cultures of patients with AOM were positive for *H. influenzae* (124). The proportion of *H. influenzae* AOM cases have been similar in other studies, as shown in a recent meta-analysis (122). Interestingly, a surge of *H. influenzae* cases (60% of culture-positive cases) was noted in the last year (2016) of the American study. Simultaneously, cases caused

by *S. pneumoniae* decreased to about 20% (124). Although likely a result of natural variation, it is possible that this is a consequence of PCVs, which might give NTHi a greater ecological niche, as discussed above. Importantly, the children in the study were vaccinated with PCV7 and PCV13, neither of which includes *H. influenzae* antigens. This trend needs to be followed and verified in the future.

*H. influenzae* has been associated with recurrent AOM and is more commonly found in otitis prone children (122, 124). These cases likely depend on both host and bacterial factors, such as immunological deficiencies and biofilm formation capability, respectively.

The detection of bacterial pathogens may increase when molecular methods such as PCR are included for pathogen detection. In one study of recurring AOM genetic material from *H. influenzae* was found in more than 50% of culture-negative specimens (123). However, if the presence of genetic material should be taken into clinical concern in all cases, e.g. by *H. influenzae* targeted antibiotic therapy, is not certain considering the high frequency of asymptomatic NTHi carriage in children.

*Community-acquired pneumonia*

Community-acquired pneumonia (CAP) is a common disease worldwide, with an annual incidence of about 1% (127, 128). The disease is common early and late in life, with the highest incidence in the elderly population (127, 129). Many patients require hospital care and the mortality in Sweden is about 4% for patients treated at an infectious diseases unit (130). In a recent German study on CAP, a lower 30-day mortality in patients with *H. influenzae* caused CAP of 2% for hospitalized patients and 0.8% in total (including out-patients) was reported (131).

In clinical practice the aetiology of CAP is often not determined, as it is difficult to retrieve reliable specimens representing the microbial flora of the lower airways. In observational reports where only culture-based methods are used, aetiology is often only determined in approximately 30-40% of cases (129, 132-135). *H. influenzae* has been reported the causative pathogen of CAP in 6-14% of cases. After *S. pneumoniae*, identified in 17-20% of cases, it is often considered the second most common CAP pathogen although there is some variation between different studies and settings (132-135). An increase in pathogen detection to almost 90% has been reported in two studies using molecular identification methods (quantitative PCR) for bacterial and viral pathogens on airway samples, besides regular culture-based methods (136, 137). In these studies, *H. influenzae* was identified in about 10 and 40% of cases, respectively. It was often found in combination with other potential bacterial or viral pathogens. The large difference in *H. influenzae* associated cases between the studies might, besides an actual difference, in part be explained by different target genes and cut-offs for the quantitative PCR.

In Sweden and many other countries CRB-65, a score of clinical parameters including confusion or altered mental state, respiratory rate, blood pressure and age, is often used to assess CAP disease severity (130). Higher CRB-65 scores are

correlated to higher mortality (138, 139). The score is often used to guide empirical antibiotic treatment of patients with CAP. In Sweden, benzylpenicillin 3 g t.i.d. is recommended as standard empirical treatment of hospitalized patients with mild to moderate disease, i.e. CRB-65 scores ≤2/4. The recommended definitive treatment if *H. influenzae* is identified as the causative agent is amoxicillin 750 mg t.i.d. for susceptible isolates, amoxicillin-clavulanate 500 mg/125 mg t.i.d. or doxycycline 200 mg q.d. for isolates with beta-lactamase mediated beta-lactam resistance and doxycycline 200 mg q.d. for isolates with non-beta-lactamase mediated beta-lactam resistance (130).

*Chronic obstructive pulmonary disease*

Chronic obstructive pulmonary disease is characterized by pulmonary emphysema and chronic bronchitis. The most common cause of the disease is smoking, but it can also be caused by air pollution, occupational exposure to irritative agents or alpha-1 antitrypsin deficiency (140).

Patients with COPD are often colonized with bacteria in the lower respiratory tract, both during the stable phases of the disease and exacerbations. Acute exacerbations are often caused by bacterial infections and the acquisition of new bacterial strains (141). *H. influenzae* is the most common pathogen associated with exacerbations and is estimated to account for 20-30% of exacerbations (142). It has also been shown that *H. influenzae* colonizes up to 60% of COPD patients and appears to have a major role in disease progression and severity, also during the stable phase of the disease (143, 144). Colonizing NTHi strains can persist in patients with COPD for long periods of time. In persistent strains, phase variation by slipped-strand mispairing affecting genes involved in e.g. adhesion, iron acquisition and LOS biosynthesis occurs. Gene gain and loss, however, appears limited (145). Presence of the species in the lower airways in the stable phase of COPD has been associated with increased inflammation, reduced pulmonary function, structural pulmonary abnormalities and a lower quality of life (146-148). Importantly, these studies were observational and causality between *H. influenzae* colonization and the reported changes was not further investigated.

## Invasive infections

*Before introduction of Hib vaccines in childhood vaccination programmes*

Severe disease caused by *H. influenzae* used to be clearly dominated by type b strains and affected mainly children below 5 years of age (5). Serum bactericidal activity against Hib in children was early shown to vary with age (149). Antibody levels against the Hib capsule were high in new born children due to maternal antibodies passing across the placenta. Levels subsequently decreased between 3 and 18 months of age, when levels successively started to rise again (21, 150). The

peak incidence of invasive disease coincided with the low antibody levels early in childhood (150-155). The most commonly reported invasive manifestations of Hib was meningitis, which accounted for approximately 50% of cases, followed by (in descending order) pneumonia, epiglottitis, septicaemia with no reported local infection, cellulitis and osteoarticular infections (5).

The incidence of Hib disease varied among different geographical regions. In Sweden the annual incidence of Hib meningitis was about 30/100,000 among children 0-4 years old and the incidence of all cases of invasive *H. influenzae* disease in the same age span 55/100,000 (156). In other Nordic countries, Hib disease rates were similar to those in Sweden (152, 157). Comparable incidences of invasive disease in small children (20-60/100,000) were also reported in other European countries (155, 158, 159). In North America invasive Hib disease was more common, sometimes with twice the incidences compared to European countries (151, 160, 161). Similar rates to those observed in Europe were reported from South America, Oceania, Japan and the Middle East (162-165). In Gambia the incidence of Hib meningitis was high, about 60/100,000 children under 5 years old (154). In Kenya and South Africa, the incidence of all invasive Hib disease cases in the same age group was 66/100,000 and 47/100,000, respectively (166, 167).

Several studies observed differences in susceptibility to Hib disease between different ethnic groups. In indigenous populations of North America, Hib disease incidences were much higher than the population average (150, 153, 160). Among Alaskan Inuit young children, the observed incidence was 705/100,000 (153). The indigenous people of Oceania were similarly more affected compared to people of European decent in the same countries (164). In the USA and South Africa, higher incidences of invasive disease were reported among African-American children and children of African descent, respectively (151, 161, 166).

Several environmental risk factors for invasive Hib disease exist. In pre-vaccination studies, attendance to day-care facilities, the presence of siblings and household crowding has been strongly associated with the disease (168, 169). Previous AOM and hospitalization were also associated with an increased risk, while breastfeeding after 6 months of age was protective (169). It is not clear if the observed differences in invasive Hib disease incidence between ethnic groups were due to genetic or environmental factors, or a combination of both.

Mortality from invasive Hib disease varied greatly between different countries in the pre-vaccination era. The mortality in Hib meningitis ranges from 4-37% in different reports from different countries (150, 154, 161-163, 166). In patients who recovered from Hib meningitis, neurological sequelae such as paresis, cognitive problems and impaired hearing were common (5, 150, 159, 163, 166).

*Development and epidemiological effect of Hib vaccines*

In 1939 it was shown that the serotype specific substance of Hib was a polysaccharide, which generated a highly specific immune response in immunized rabbits (170). The substance was later further characterized and identified as polyribosylribitol phosphate (PRP) (74). When injected in adult humans PRP generated an antibody response within two weeks. The bactericidal effect on Hib isolates of immunized serum increased due to antibodies directed to PRP, similarly to the sera of patients post Hib disease (21). In a large clinical trial, polysaccharide vaccines containing purified PRP showed antibody responses and protection against disease in children older than 18 months. No similar effects were observed among younger children, who have the greatest need of protection (171).

In the early 1980s, polysaccharide-protein conjugates were shown to induce bactericidal antibodies in mice in a T-cell dependent manner (172). It was subsequently shown that a PRP conjugate induce an antibody response in children below 18 months of age (173). In following clinical trials, different polysaccharide-protein conjugates were shown to be protective against invasive Hib disease in the same age group (174-177).

Hib conjugate vaccines to infants were first included in the national immunization programme of Finland, resulting in a dramatic decrease of severe Hib disease (178). Similar changes have been observed in other countries implementing vaccination in Europe (157, 179-181), North America (182-184), South America (185), Africa (167, 186), Asia (187), and Oceania (188). In Sweden, Hib conjugate vaccination has been included in the national vaccination programme for children since 1993 and Hib disease has declined dramatically (156). Contributing to the rapid decrease of invasive Hib disease, beside protection of the vaccines against disease, might be the potential of conjugate vaccines to decrease pharyngeal colonization, which consequently limits bacterial transmission (109).

Most countries now implement childhood Hib vaccination, and although coverage on a global basis is successively improving it is estimated that only 72% of eligible children receive full vaccination (189). In a report published in 2018, Hib was still estimated to cause 30,000 deaths in children under 5 years of age annually (190).

Hib vaccination failures have been described and more often occur in children with pre-existing medical conditions and immunoglobin deficiencies (191). In the United Kingdom a rise in invasive Hib disease was seen almost 10 years after vaccination was introduced which warranted a catch-up vaccination campaign. Contributing factors to the surge might have been omittance of a booster dose at age 12 months and the use of vaccinations containing PRP conjugate combined with specific acellular pertussis components, which may result in lower PRP antibody titres (192).

*Current epidemiology*

The epidemiology of invasive *H. influenzae* infections in countries implementing childhood vaccination is being increasingly studied. The current serotype distribution is considerably more diverse compared to the time before Hib vaccination. Several investigations in European and North American countries with Hib childhood vaccination programmes, concerning invasive *H. influenzae* disease in the late 1990s and first decade of the 21st century, show or indicate an increasing trend of invasive disease driven mainly by nontypeable strains (193-200). A few studies of the same time period, however, do not show this increase (201-204). The most recent and comprehensive studies support the notion that invasive NTHi disease is increasing compared to prior to the Hib vaccination era and is mainly affecting children below 1 year of age (especially neonates) and older adults (>65 years of age) (205-208). As these studies are observational and span over several years, many factors may contribute to the increased incidence. Besides an actual increased incidence, improved diagnostic procedures could be an important factor behind the increase. If the results from these studies reflect an actual increased incidence, the reason for this increase is not yet clear. Bacterial factors may contribute but also other factors, e.g. demographical changes and the increasing possibilities to treat chronic diseases. Current reports estimate the incidences of invasive NTHi disease in children less than 1 year old and adults more than 65 years old to 3 and 6/100,000 and 1 and 5/100,000, respectively (206, 207).

Increased incidences of invasive Hia disease in North and South America have also been reported (209-211). Children belonging to the indigenous population of North America are more affected by Hia disease than other groups (207, 210, 212-214). No similar trend has been observed among Australian indigenous children for Hia specifically, but for invasive *H. influenzae* in general (215, 216).

In a few post-Hib vaccination studies increased incidences and proportions of invasive Hie and Hif disease has been reported (193, 197, 217). Disease caused by Hic and Hid is only sporadically reported (206, 207).

Besides changes in serotype and age distribution, the post-vaccination era has also seen a notable shift in the clinical presentation of invasive *H. influenzae* disease. Septicaemia with no reported local infection and bacteraemic pneumonia are now the main clinical presentations, while the relative frequency of meningitis has decreased (206, 207). NTHi dominate all disease presentations, except for epiglottitis, which is still caused by Hib in a majority of cases (206). In contrast to disease caused by NTHi, Hia disease to a large extent resembles Hib in a clinical and epidemiological perspective, and childhood meningitis is a common presentation (209, 210, 214).

### *Nontypeable* H. influenzae *vaccine development*

As NTHi now is the dominating cause of both respiratory and invasive *H. influenzae* disease research efforts are increasingly directed towards development of an effective NTHi vaccine. Opposite to Hib and the capsule polysaccharide PRP, no singular highly surface exposed and immunogenic antigen is present in NTHi. To be suitable for inclusion in a vaccine, an antigen needs to be ubiquitous, highly conserved, exposed on the bacterial cell surface and immunogenic. Due to the genetic heterogeneity of NTHi, finding antigens with these properties is challenging. Several of the bacterial molecules involved in NTHi pathogenesis have, however, been suggested as vaccine candidates (26, 218).

As NTHi in many cases only colonizes the host and is part of the normal flora of the upper respiratory tract, a presumptive vaccine should reasonably be designed and used for protection of certain populations, such as patients with COPD.

Currently, there is one ongoing clinical NTHi vaccine trial. The trial is randomized, placebo controlled and observer blind. It is targeted at patients with COPD and the primary endpoint is the frequency of moderate and severe acute exacerbations. The trial vaccine includes protein D (which is also included in PCV10), a fusion protein of protein E and type IV pilus subunit A, and UspA2 which is a ubiquitous surface protein of *M. catarrhalis* (26, 219).

## Antimicrobial susceptibility and resistance of *Haemophilus influenzae*

### Epidemiological cut-offs and clinical breakpoints

All bacterial species have a wild-type population, with a specific range of intrinsic activity of any antimicrobial agent. The most reliable method to determine the level of susceptibility or resistance against a specific antimicrobial agent of a bacterial isolate, is to determine the corresponding minimum inhibitory concentration (MIC). The MIC is the lowest concentration of an antimicrobial agent which inhibits growth of the bacteria. In clinical settings the faster and less laborious method of disc diffusion antimicrobial susceptibility testing is often used instead of precise MIC determination (220).

Based on the distribution of MICs to a specific antimicrobial agent in the bacterial population, an epidemiological cut-off (ECOFF) for the agent can be established. The ECOFF aims to distinguish isolates with and without any acquired resistance mechanisms (221).

For clinical breakpoints, which seek to advice on whether treatment with a specific agent will have an expected therapeutic effect, more variables need to be

considered, such as pharmacodynamic and pharmacokinetic properties, site of infection and agent dosing. Generally, for breakpoints to be set, results from clinical studies are also required (222). The clinical breakpoint for resistance thus is not necessarily the same as the ECOFF. In Sweden, clinical breakpoints set by the Nordic Committee on Antimicrobial Susceptibility Testing (NordicAST), which are based on recommendations from the European Committee on Antimicrobial Susceptibility Testing (EUCAST), are commonly used (223). In many countries, clinical breakpoints set by the Clinical and Laboratory Standards Institute (CLSI) are used. Importantly, clinical breakpoints for several antimicrobial agents differ between EUCAST and CLSI recommendations (224, 225).

## Beta-lactam susceptibility and resistance

*Structure and classes of beta-lactams*

Beta-lactams are the most used antimicrobial agents globally today (226). The common structure essential for antibacterial activity is the beta-lactam ring. By binding to and halting the enzymatic activity of proteins involved in the synthesis of the peptidoglycan layer, an integral component of the bacterial cell wall, the beta-lactam agents exhibit their effect (227). The bacterial proteins bound by beta-lactams are designated penicillin-binding proteins (PBPs) and differ in number, size and function between different bacterial species (228, 229). The major beta-lactam classes used clinically today are penicillins, cephalosporins, carbapenems and monobactams. Differences in side structures confer different pharmacodynamic and pharmacokinetic properties, such as antibacterial spectrum and serum half-life, between different agents of the groups (Figure 4).

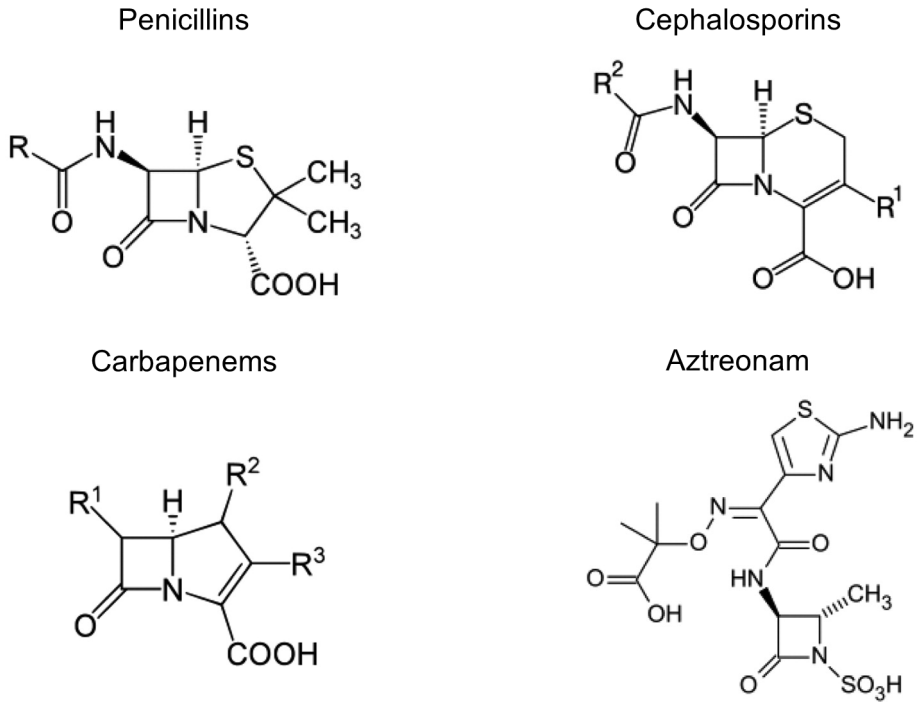



**Figure 4. General chemical structure of different classes of beta-lactam antibiotics.**
The major classes of beta-lactam antibiotics are penicillins, cephalosporins, carbapenems and monobactams. Aztreonam is the only commercially available monobactam. The antibacterial effect of beta-lactams is mediated via the beta-lactam ring, a structure common to all beta-lactams. Side structures determine pharmacodynamic and pharmacokinetic properties. Image adapted and used with permission from the publisher (230).

*Beta-lactam susceptibility of* H. influenzae

Penicillins with extended spectrum, such as the aminopenicillins ampicillin and amoxicillin, have for long been among the first choices for treatment of infections caused by *H. influenzae*.

Phenoxymethylpenicillin (penicillin V, PcV), an oral narrow spectrum penicillin used to treat infections caused by *S. pneumoniae*, is not effective against *H. influenzae* (231). This difference in penicillin susceptibility was noted by Alexander Fleming already during the initial discovery of penicillin (7). For benzylpenicillin (penicillin G, PcG), a parenteral agent with similar antimicrobial spectrum as phenoxymethylpenicillin, the effect on wild-type *H. influenzae* is debated. The European Committee on Antimicrobial Susceptibility Testing have not set any clinical breakpoints, referring to a lack of evidence for clinical effect. Monte Carlo simulations of pharmacokinetic and pharmacodynamic data, however, suggest a possible effect on wild-type *H. influenzae* if adequate dosage is used (232). In a recent retrospective study, definite treatment of *Haemophilus* spp. bacteraemia with benzylpenicillin was associated with a higher mortality compared to treatment with

the cephalosporin cefuroxime or an aminopenicillin. Treatment of *H. influenzae* bacteraemia specifically (other *Haemophilus* spp. excluded) with benzylpenicillin was also associated to a higher mortality (233). Wild-type *H. influenzae* are susceptible to most cephalosporins and to carbapenems (234).

*Beta-lactamase mediated beta-lactam resistance*

For *H. influenzae* beta-lactam resistant isolates are divided based on the production of beta-lactamase, which degrades the antibiotic agent. Two different classes of beta-lactamases are known to cause resistance in *H. influenzae*, called TEM and ROB. Both beta-lactamases confer high levels of resistance to beta-lactams of the penicillin group (235, 236). Cefuroxime (second-generation cephalosporin), third-generation cephalosporins and carbapenems are not affected by the enzymes. The beta-lactamases of *H. influenzae* are inhibited by beta-lactamase inhibitors such as clavulanate (237).

The TEM, but not ROB, beta-lactamase gene is most often found on a large, conjugative plasmid which can be integrated to the chromosome or reside circular in the cytoplasm (234). The plasmid often carries resistance genes to other, non-beta-lactam antibiotics (238). Furthermore, both TEM and ROB beta-lactamase genes can reside on smaller, non-conjugative, circular plasmids (234). Globally, TEM beta-lactamases account for about 95% of all beta-lactamase positive *H. influenzae* (237). Notably, beta-lactamases resistant to beta-lactamase inhibitors have been found in *H. parainfluenzae*, a human commensal and close relative of *H. influenzae* (239). The risk of a similar development in, or transfer of genes encoding these types of beta-lactamases to, *H. influenzae* is worrying.

*Non-beta-lactamase mediated beta-lactam resistance*

In beta-lactam resistant isolates lacking beta-lactamase production, the only fully established mechanism of resistance is decreased affinity of beta-lactams to penicillin-binding protein 3 (PBP3) (240). Although decreased affinity of beta-lactams to other PBPs has also been observed in resistant isolates, the significance of this is unclear (241).

Decreased affinity of beta-lactams to PBP3 is mediated via mutations in the PBP3 encoding *ftsI* gene, resulting in amino acid substitutions in the transpeptidase region of the protein. The amino acid substitutions Arg517His (R517H) or Asn526Lys (N526K) are strongly associated with resistance (242). The acquisition of one of these substitutions is considered necessary for resistance development, even though a study employing site-directed mutagenesis indicates the need for complementary factors (243). The amino acid substitutions R517H and N526K may confer increased MICs against penicillins, cephalosporins and carbapenems, but mainly ampicillin and amoxicillin MICs become elevated. Still, a large proportion of isolates with these alterations remain susceptible to ampicillin and amoxicillin according to clinical breakpoints (121, 242-245). Besides these amino acid

alterations, an additional substitution, Ser385Thr (S385T), is associated with higher level beta-lactam resistance, which includes resistance to third-generation cephalosporins. The MICs of cephalosporins are further elevated if the Leu389Phe (L389F) amino acid substitution is present. Besides these key substitutions, many more have been described but their implications for resistance level and spectrum are however not entirely clear (242-244, 246).

The different PBP3 have been divided in groups, based on which resistance-mediating amino acid substitution(s) are present, and subgroups, based on additional associated amino acids substitutions (121, 242, 244, 245, 247, 248). Importantly, this is a genotypic characterization, and the phenotype, that is level of beta-lactam susceptibility, can vary within the groups (Table 1).

**Table 1. Groups and subgroups of PBP3 with resistance mediating amino acid substitutions.**

| Main PBP3 group (242, 244, 248) | Key amino acid substitution(s) | Subgroups according to Dabernat (245) | PBP3 types according to Skaare (121, 247) |
|---|---|---|---|
| I | R517H | | |
| II | N526K | a-d | A-Q |
| III | S385T, N526K | | |
| III-like | S385T, R517H | | |
| III+ | S385T, L389F, N526K | | |
| ÍII-like+ | S385T, L389F, R517H | | |

In addition to the already mentioned PBP3 amino acid substitutions, new resistance mediating PBP3 alterations have recently been reported. The amino acid substitution Tyr528His (Y528H) was recently described to independently confer increased MICs for ampicillin and amoxicillin (249). In Japan, isolates with the Y528H substitution and an additional amino acid insertion in PBP3 resistant against certain carbapenems have recently been reported (250, 251).

All isolates with key amino acid substitutions in PBP3 do not have MICs elevated above the level of resistance according to clinical breakpoints. Ampicillin resistance, and resistance to other beta-lactams, can also vary between isolates with the same PBP3 amino acid substitutions. Furthermore, amoxicillin resistant isolates with wild-type PBP3 and no evidence of beta-lactamase production have been reported (234, 252). This indicates that other factors than PBP3 amino acid substitutions have a role in non-beta-lactamase mediated resistance. One possible mechanism is frame shift insertions in the *acrR* gene. The *acrR* gene product represses expression of the AcrAB efflux pump in the outer membrane. This has been investigated in a study of isolates with PBP3 amino acid substitutions and very high ampicillin MICs. Transformation of strain Rd with the *acrR* gene from isolates with high-level resistance increased the MIC significantly (253). However, in another study of isolates with both PBP3 alterations and *acrR* mutations, *acrR* frame shifts and the level of ampicillin resistance was not clearly associated (244).

Another possible mechanism conferring or regulating levels of beta-lactam resistance is decreased beta-lactam affinity in other PBPs than PBP3. Studies of other PBPs have however not shown any distinct association between amino acid substitutions and levels of beta-lactam resistance (242, 253-255). Variations in PBP synthesis levels and decreased permeability of beta-lactams in the outer membrane are other possible mechanisms which may contribute to resistance.

*Classification and terminology of beta-lactam resistant* H. influenzae

Strains phenotypically resistant to penicillins by beta-lactamase production are often termed beta-lactamase positive ampicillin resistant (BLPAR) as ampicillin is the traditional first choice treatment of *H. influenzae* infections. Similarly, strains with non-beta-lactamase mediated resistance to beta-lactams are often termed beta-lactamase negative ampicillin resistant (BLNAR). Strains with both resistance mechanisms are designated beta-lactamase positive amoxicillin-clavulanate resistant (BLPACR) and susceptible strains beta-lactamase negative ampicillin susceptible (BLNAS) (234).

One problem with this terminology is that all strains with resistance defining PBP3 amino acid substitutions do not have ampicillin MICs elevated above the clinical breakpoints and are thus classified as BLNAS. Furthermore, clinical breakpoints for ampicillin vary between countries. Because of this, the term genetically (g) has been introduced as a prefix (e.g., gBLNAR) to separate strains genotypically categorized (based on PBP3 amino acid substitutions and presence of a beta-lactamase gene) from those phenotypically categorized (256). Another problem with this terminology is that it does not imply resistance against other beta-lactams than ampicillin, although it is common among isolates with non-beta-lactamase mediated resistance.

The term rPBP3 (resistance mediated by alterations in PBP3) has been suggested to denote isolates with confirmed resistance-mediating PBP3 amino acid substitutions, regardless of phenotype and presence of concurrent beta-lactamase production (121).

*Beta-lactam susceptibility testing of* H. influenzae

According to the current NordicAST protocol, primary screening for beta-lactam resistance in *H. influenzae* is made by disk diffusion using a benzylpenicillin 1 unit disk. Isolates with indication of resistance (zone diameter <12 mm) are tested for beta-lactamase production (223, 257). A common method for this is the use of nitrocefin, a chromogenic cephalosporin, which changes colour from yellow to red when hydrolysed by beta-lactamases. Beta-lactamase positive isolates are further evaluated by disk diffusion test with cefaclor or cefuroxime. If the test result indicates resistance the isolate likely harbours non-beta-lactamase mechanisms of resistance, besides the production of beta-lactamase. Beta-lactamase negative isolates with a benzylpenicillin zone diameter <12 mm are suspected to harbour

non-beta-lactamase mediated beta-lactam resistance, and evaluation with cefaclor or cefuroxime disk diffusion is not needed. Importantly, the different mechanisms of beta-lactam resistance occur independently (223).

Beta-lactamase positive isolates are reported resistant to ampicillin and amoxicillin, but susceptible to aminopenicillins in combination with beta-lactamase inhibitors (e.g. amoxicillin combined with clavulanate), cephalosporins and carbapenems. If non-beta-lactamase mediated resistance is present, all beta-lactam agents (penicillins, cephalosporins and carbapenems) which could be used for treatment in the current case, need to be tested, as the level of resistance in these isolates varies.

In NordicAST and EUCAST recommendations, the MIC for ampicillin resistance is set to >1 mg/L and for amoxicillin >2 mg/L (223, 224). In CLSI recommendations the breakpoint for ampicillin resistance is an MIC of ≥4 mg/L. No breakpoints are provided for amoxicillin, but for the combination amoxicillin-clavulanate the breakpoint for resistance is ≥8/4 mg/L (225). Beta-lactamase positive isolates often have MICs clearly above the ampicillin and amoxicillin breakpoints. Isolates with PBP3 amino acid substitutions, however, often have MICs near the breakpoints and the population is divided by them (121, 242, 244, 245). Moreover, MIC determination by gradient tests compared to broth microdilution in these isolates may underestimate MICs, which makes misclassification of resistant isolates as susceptible possible (257).

*Epidemiology of beta-lactam resistance*

Ampicillin resistance in *H. influenzae* was first described in 1974 (258). In a recent global survey encompassing the years 2004-2012, the prevalence of ampicillin resistance in *H. influenzae* was 21.7% according to CLSI breakpoints. Beta-lactamase production was present in 20.2 % of isolates and was thus the headmost resistance mechanism. Notably, beta-lactamase mediated resistance declined somewhat over the study period. Resistance levels vary between different regions, with the highest levels (about 25-28% of isolates beta-lactamase positive) in North America and Asia (259). In Sweden, levels of beta-lactamase producing clinical isolates have been stable around 10-20% for several years (260-262).

Non-beta-lactamase mediated beta-lactam resistance constitutes an increasing problem, although globally it is still in minority compared to beta-lactamase mediated resistance (259). In Japan where beta-lactam resistance levels are highest, non-beta-lactamase mediated resistance is present in about 60% of isolates and resistance to cephalosporins is common. The reason for this might be a comparatively high use of oral cephalosporins, which may increase selective pressure for this type of resistance (263, 264). In Europe, non-beta-lactamase mediated resistance levels are lower but rising, which has been shown in studies from Sweden, Spain, France and Norway (121, 260, 265, 266).

Clonal dissemination of isolates with non-beta-lactamase mediated resistance has been noted in several studies (121, 244, 247, 248, 256, 260, 267-270). To facilitate comparison of this type of studies, MLST in combination with *ftsI* gene sequencing has been suggested as a surveillance tool (121).

## Non-beta-lactam susceptibility and resistance

Beta-lactam agents are often the first choice for treatment of *H. influenzae* infections. However, in some situations, such as patient allergy to beta-lactams or the occurrence of beta-lactam resistance, other antimicrobial agents may be used instead. Non-beta-lactam agents commonly used are folic acid metabolism inhibitors, tetracyclines and fluoroquinolones (234). The clinical effect of macrolide and ketolide agents on *H. influenzae* is disputed albeit the bacteria are susceptible *in vitro*. One study of AOM caused by *H. influenza*e shows a lower bacteriological cure rate for the macrolide azithromycin compared to amoxicillin-clavulanate (271). Another study showed similar, although not statistically significant, results (272). Importantly, many of the infections in these studies would likely heal spontaneously. While CLSI have set clinical breakpoints for some macrolide and ketolide agents EUCAST has not, referring to a lack of evidence for clinical effect (224, 225).

The folic acid inhibitors trimethoprim and sulfamethoxazole are used in combination to treat *H. influenzae* infections. The agents interfere with different enzymes involved in the production of tetrahydrofolate, which is important for bacterial metabolism and cell division. Resistance to the agents occurs by bacterial expression of modified enzymes, to which the antimicrobial agents have decreased affinity (234). In Sweden levels of resistance to trimethoprim-sulfamethoxazole between 2 and 20% have been reported (260).

Tetracyclines exert their antimicrobial effect by binding to the 30S subunit of the bacterial ribosome, which inhibits protein synthesis. Resistance against tetracyclines in *H. influenzae* is mediated via efflux pumps encoded by the *tet*(B) gene. Fluoroquinolones inhibit bacterial DNA replication and consequently bacterial reproduction. Resistance occurs by mutations in the genes encoding DNA gyrase and topoisomerase IV (234). Resistance to tetracyclines and fluoroquinolones is, however, relatively rare in Sweden (260).

# MALDI-TOF MS in clinical microbiology

## Principle and function

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a method for mass spectrometry combining the ionization technique MALDI with TOF mass analysis. MALDI is a soft ionization method, which refers to the limited fragmentation of the molecules this technique induces compared to other ionization techniques. The TOF mass analyser has a wide mass range, suitable for analysis of compounds including molecules of various sizes. These characteristics make the combination of these techniques suitable for mass analysis of biological specimens, such as microbiological samples (273).

Before MALDI-TOF MS analysis, the analyte is covered or mixed with a matrix consisting of a crystalline solid in a saturated organic solvent. The matrix aids in ionizing the analyte when it is irradiated by the laser of the MALDI instrument. In clinical microbiology the analyte is often whole cell bacteria taken directly from a culture plate. To improve the quality of results, different methods to extract the protein content from the bacterial cells can be applied. A commonly used method is ethanol and formic acid extraction, in which formic acid is used to disrupt the cell membrane to release the ribosomal proteins (274).

When the molecules of the analyte have been ionized by the instrument the ions are accelerated in an electrostatic field. Ions then drift in the TOF tube and, at the end of the tube, hit a detector. During drift, ions are separated according to mass, as their velocity is proportional to their mass to charge ratio (*m/z*). By measuring the ions time of flight, a spectrum of the *m/z* ratios is generated. As MALDI generally generates ions with a +1 electrical charge, a signal *m/z* ratio is often equivalent to the mass of a specific molecule. The spectrum generated represents the contents of the analyte within a specified mass range. In the case of microbial identification, the range of 2,000-20,000 Da is most commonly used. In clinical microbiology, the generated spectrum is often called the mass finger print of the analysed bacterial or fungal isolate. The workflow is presented in Figure 5 (273, 275).

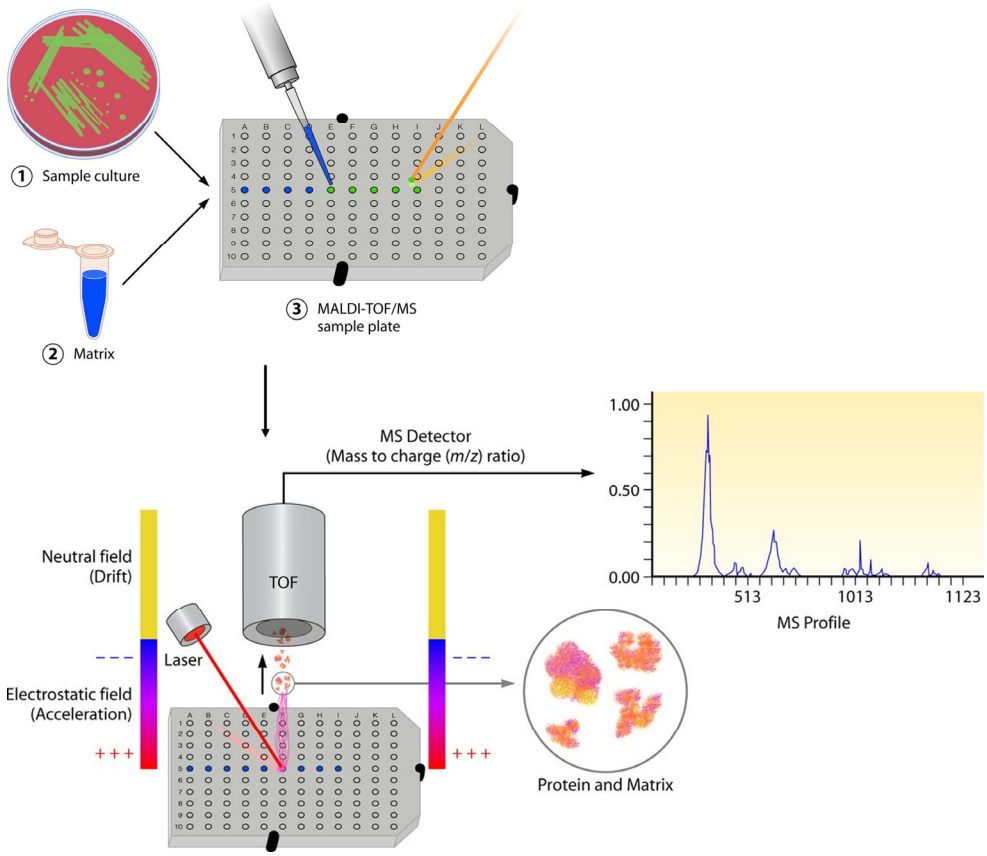



**Figure 5. Workflow and principle of MALDI-TOF MS in clinical microbiology.**
Bacterial material is spotted on the sample plate, covered with matrix and allowed to dry. In the MALDI-TOF mass spectrometer, the sample is desorped and ionized by a laser, a process aided by the matrix. The generated ions are accelerated in an electrostatic field and separated by size in the TOF mass analyser. The ions are registered by a detector and a spectrum representing the different ions in the sample is generated. Image used and adapted with permission from the publisher (273).

## Use in clinical microbiology

*Species identification*

The spectrum generated from MALDI-TOF MS analysis of a microbial isolate can be matched to a database and the species of the analysed isolate determined. The potential value of mass spectrometry in characterization of microorganisms was noted already in 1975 and MALDI-TOF MS was established as a valuable method for species identification in the 1990s (276-279). Commercial systems combining MALDI-TOF MS with software for microbial identification, have been shown highly precise in bacterial and fungal species determination compared to conventional phenotypic identification and 16S ribosomal RNA sequencing (280-

282). Due to its easy handling, swiftness and low per sample cost, MALDI-TOF MS is now a standard method for species identification in clinical microbiology laboratories (273, 281).

The technique was early shown to be potentially valuable in the difficult separation of species within the *Haemophilus* genus (283). More recent studies have demonstrated the instruments capability to differentiate *H. influenzae* from the commensals *H. parainfluenzae* and *H. haemolyticus* (13-16).

*Subtyping*

The possible use of MALDI-TOF MS for identification beyond the species level is intriguing, as it would allow the extraction of more diagnostic information without much increased use of time or resources. Several studies have been published suggesting the possibility to separate certain clones or groups of bacterial species. Typing by MALDI-TOF MS of the pathogen *Clostridium difficile* can be correlated to PCR ribotyping and used for surveillance of disease outbreaks. The method uses a different matrix and mass range (30,000-50,000 Da) than used for standard species identification to identify peaks correlating to S-layer proteins, which are usually analysed for ribotyping (284). Studies have also suggested the possible use of MALDI-TOF MS for the separation of different clonal groups of methicillin-resistant *Staphylococcus aureus* (MRSA) (285-289), extended spectrum beta-lactamase (ESBL)-producing and other subgroups of *Enterobacteriaceae* (290-292), and certain clones of *Pseudomonas aeruginosa* (293), among others.

Many studies, however, only compare a selected group of strains with known characteristics and lack testing and validation of the typing schemes on clinical isolates in a blinded fashion. There have also been concerns raised regarding the consistency and reproducibility of results from different studies (294, 295).

*Antimicrobial susceptibility testing*

MALDI-TOF MS has also been shown to be a possible tool for rapid detection of antimicrobial resistance.

The gene product of the *psm-mec* gene associated with the class A *mec* gene complex of MRSA and methicillin-resistant *Staphylococcus epidermidis* (MRSE) can be identified as a highly specific 2,415 *m/z* peak in MALDI-TOF MS analysis. This allows MALDI-TOF MS-based identification of MRSA and MRSE with a high positive predictive value (296, 297).

In another approach, the degradation products of carbapenems hydrolysed by carbapenemase-producing bacteria can be identified and the isolate classified as carbapenem susceptible or resistant. Variations of this method has been presented for *Bacteroides fragilis*, *Pseudomonas aeruginosa, Enterobacteriaceae* and *Acinetobacter baumanni* (298-303). These methods can, however, not identify non-enzymatic carbapenem resistance.

More general approaches to antimicrobial susceptibility testing using MALDI-TOF MS have also been investigated. In a recent study, droplets of blood culture specimens mixed with antibiotics were analysed by MALDI-TOF MS after a short incubation period. If the generated mass spectra could be used for successful species identification, the isolate of the culture was considered resistant to the added antibiotic. The method showed promising results compared to susceptibility testing by broth microdilution (304).

# The present investigation

## Aims

*The aims of the thesis were:*

- To investigate potential differences in MALDI-TOF mass spectra among different types of encapsulated *H. influenzae* and NTHi
- To investigate the capability of MALDI-TOF MS to separate Hib from non-type b *H. influenzae*.
- To develop a clinically applicable MALDI-TOF MS *H. influenzae* capsule typing method.
- To investigate the epidemiology and clinical characteristics of *H. influenzae* ST14CC-PBP3IIb/A, a clonal group of NTHi with non-beta-lactamase mediated beta-lactam resistance, in the county of Skåne.
- To compare benzylpenicillin to beta-lactams with established effect on *H. influenzae* as empirical treatment of lower respiratory tract infections caused by the species.

# Methods, Results and Discussion

## Paper I: Identification of *H. influenzae* type b by MALDI-TOF MS

Capsule typing of *H. influenzae* is traditionally made by conventional serotyping (SAST), but this method has limited specificity and misclassification of especially NTHi is common (6, 84-86). Typing with PCR is considered the gold standard and generates high quality results, but only addresses isolate genotype and not the phenotype (70, 87-89). Typing with PCR is also relatively time-consuming and laborious. Given the implementation of MALDI-TOF MS in many clinical laboratories for routine species identification and the genetically conserved capsule type specific lineages of *H. influenzae*, we sought to investigate the instruments capability to identify different capsule types of the species. In this study differences in mass spectra between Hib and the other capsule types of *H. influenzae* were investigated.

*Methods*

The culture collection of the study included Hib isolates ($n$=40) and *H. influenzae* isolates of all other capsule types ($n$=87). Most isolates were Swedish invasive isolates ($n$=114) but isolates from other countries, often representing rare capsule types, were also included. All isolates were capsule typed by PCR using *bexB* and type specific primers as previously described (70, 88, 89). Mass spectra were acquired for all isolates using a standard benchtop MALDI-TOF mass spectrometer. Mass spectra were manually analysed to investigate differences between Hib and non-Hib isolates. Selected isolates were further investigated by MLST.

Automatic Hib/non-Hib MALDI-TOF MS classification methods were constructed in ClinProTools and MALDI Biotyper using selected reference isolates. Classification methods relying on direct similarity with reference strains (MALDI Biotyper Main Spectra (MSPs) and subtyping MSPs) and methods classifying based on the absence or presence of specific peaks (ClinProTools) were constructed. Specially designed MALDI Biotyper MSPs, which included only two different mass peaks observed during manual analysis of mass spectra, were also constructed.

For validation of the methods isolates not included as reference isolates were used. Six spectra per isolate were acquired and classified by the methods. Isolates with ≥5/6 spectra classified as Hib were identified as Hib and isolates with ≤2/6 spectra classified as Hib were identified as non-Hib. If 3–4/6 spectra were classified as Hib, the isolate was identified as uncertain. To test the reproducibility of the results, mass spectra of 42 of the validation isolates (19 Hib and 23 non-Hib) were acquired on a different instrument and classified by the methods.

*Results*

Manual analysis of spectra showed that spectra within the Hib group were largely conserved. Several conserved peaks with apparent differentiating capability towards other capsule types were identified. Spectral differences appeared as either unique peaks lacking in other capsule types or, more commonly, as offsets in peaks (peak shifts) compared with other capsule types (Figure 6).

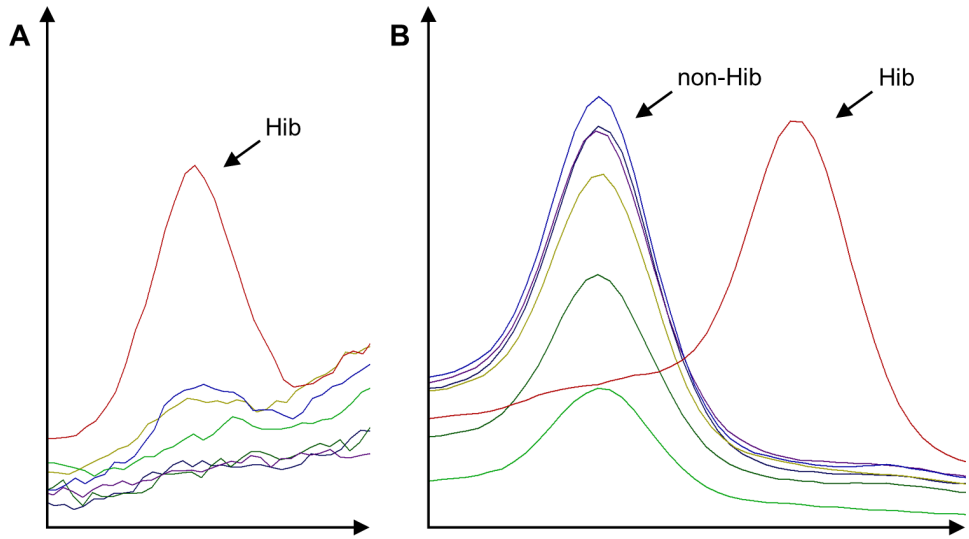



**Figure 6. Differences in mass spectra between Hib and non-Hib isolates.**
Differences in mass spectra between Hib and non-Hib isolates were observed as either unique peaks, such as peak 6,789 *m/z*, which was expressed by Hib but not non-Hib isolates (panel A), or peak shifts, such as peaks 8,332 *m/z* and 8,348 *m/z*, which were expressed by non-Hib and Hib isolates, respectively (panel B). Each capsule type is represented by a single representative isolate. x and y axes represent *m/z* ratio and relative intesity, respectively.

Similar type specific conservation of the spectral patterns was observed for isolates of other encapsulated types as well. Mass spectra of NTHi, however, were more heterogenous. One Hib isolate, KR194, had a markedly different mass spectrum compared to the other Hib isolates. By MLST, this isolate was shown to belong to one of the less common genetic lineages of Hib, whereas the other investigated Hib isolates all belonged to the more common ST6-related lineage (data not shown).

Based on MLST and differences in MALDI-TOF MS spectra, 7 Hib (6 variants from the ST6-related lineage and KR194) and 10 non-Hib isolates representing all capsule types including NTHi, were selected as reference isolates for the creation of automated MALDI-TOF MS classification methods.

All constructed MALDI-TOF MS Hib/non-Hib classification methods showed high sensitivity and specificity for identifying Hib when the remaining isolates ($n$=110) were used for validation. Reproducibility was highest for the ClinProTools method and lowest for the specially designed MALDI Biotyper MSPs (Table 2).

**Table 2. Evaluation of different Hib/non-Hib MALDI-TOF MS classfication methods.**

| Classification method | Hib (*n*=33) | Non-Hib (*n*=77) | Sensitivity (%) | Specificity (%) | Reproducibility (%)[1] |
|---|---|---|---|---|---|
| ClinProTools | | | 100 | 98.7 | 97.6 |
| Hib[2] | 33 | 1 | | | |
| Uncertain[3] | 0 | 0 | | | |
| Non-Hib[4] | 0 | 76 | | | |
| Biotyper MSPs | | | 100 | 98.7 | 90.5 |
| Hib | 33 | 0 | | | |
| Uncertain | 0 | 1 | | | |
| Non-Hib | 0 | 76 | | | |
| Biotyper subtyping MSPs | | | 97 | 100 | 92.9 |
| Hib | 32 | 0 | | | |
| Uncertain | 1 | 0 | | | |
| Non-Hib | 0 | 77 | | | |
| Biotyper specially designed MSPs | | | 81.8 | 100 | 76.2 |
| Hib | 27 | 0 | | | |
| Uncertain | 3 | 0 | | | |
| Non-Hib | 3 | 77 | | | |

1. Defined as the percentage of isolates with mass spectra acquired at two different instruments (*n*=42) classified to the same group in both classifications.
2. ≥5/6 spectra classified as Hib.
3. 3–4/6 spectra classified as Hib.
4. ≤2/6 spectra classified as Hib.

## *Discussion*

This study shows that Hib and non-Hib isolates of *H. influenzae* can be separated based on MALDI-TOF mass spectra. Furthermore, mass spectra were largely conserved within capsule types and other capsule types besides Hib appeared separable as well. Type specific differences observed in mass spectra were most often due to peak shifts, indicating amino acid variations in ribosomal proteins conserved within different capsule types. Unique peaks for Hib and other capsule types were also observed. Interestingly, one Hib isolate belonging to a different genetic lineage than the other Hib isolates had a markedly different mass spectrum. This indicates that the mass spectral differences between different capsule types are related to genetic differences between the isolates rather than differences in type specific capsule synthesis proteins, as expected.

Strengths of the study include the large and diverse culture collection and the careful selection and characterization of reference isolates used in the different Hib/non-Hib classification methods. The findings were robust, as indicated by the similar results obtained by the different Hib/non-Hib classification methods and the tests of reproducibility. One limitation in the study is that two genetic lineages of Hia and one of Hib were not represented. One Hib lineage included only one isolate, KR194, which was included as a reference isolate. The capability of MALDI-TOF MS to identify this lineage could hence not be tested.

In conclusion, this proof of concept study shows that MALDI-TOF MS can separate Hib from non-Hib isolates of *H. influenzae* with high accuracy.

## Paper II: Capsule typing of *H. influenzae* by MALDI-TOF MS

In Paper I it was noted that other capsule types besides Hib appeared separable in a similar way as Hib, with conserved unique peaks and peak shifts compared to other capsule types. Furthermore, Hib strain KR194, which belonged to a different genetic lineage than the other Hib strains in the study, had a deviant MALDI-TOF mass spectrum. This indicated that differences in mass spectra were a result of differences in ribosomal protein content, mirroring genetic relatedness, rather than differences in capsule synthesis proteins themselves. Nontypeable isolates, as expected due to their high genetic variability, showed a much greater inter-strain variability in mass spectra compared to encapsulated isolates.

This study is a continuation of the work from Paper I. The aim was to construct and validate a clinically applicable capsule typing method including all capsule types based on MALDI-TOF MS analysis. Our view was that a large and comprehensive reference database encompassing all genetic lineages of encapsulated isolates would be a key factor for success. Furthermore, we investigated whether the observed differences in mass spectra between different capsule types were due to differences in capsule biosynthesis proteins or associated to the clonal population structure of encapsulated *H. influenzae*.

*Methods*

A culture collection comprising 258 *H. influenzae* from various geographical regions representing all capsule types including NTHi were included in the study. This evaluation set of isolates was used to further investigate the type specific differences in mass spectra observed in Paper I. Furthermore, isogenic capsule type a, b, c and d transformants originating from strain Rd were included (68, 305). A separate, second culture collection, consisting of Swedish invasive isolates ($n$=126), was used to validate the new MALDI-TOF MS capsule typing method developed.

Concatenated MLST sequences of all Hia and Hib isolates (of which there are more than one established genetic lineage) and several type c to f and nontypeable isolates were used to construct a maximum-likelihood phylogenetic tree.

Mass spectra of all isolates were acquired using a benchtop MALDI-TOF mass spectrometer. Six spectra per isolate were acquired. Isolates in the evaluation set and capsule transformants were investigated by principal component analysis (PCA) and biomarker analysis. Principal component analysis is a method to reduce factors when there is redundancy in the data. The analysis converts observations and correlated variables to principal components, which are ranked by the proportion of variance from the original data set they contain. Principal components, often the first three as they contain the most variance, can be used to easily visualize the data in a three-dimensional space. In biomarker analysis, biomarkers (*m/z* values) separating different groups in the data set are discovered.

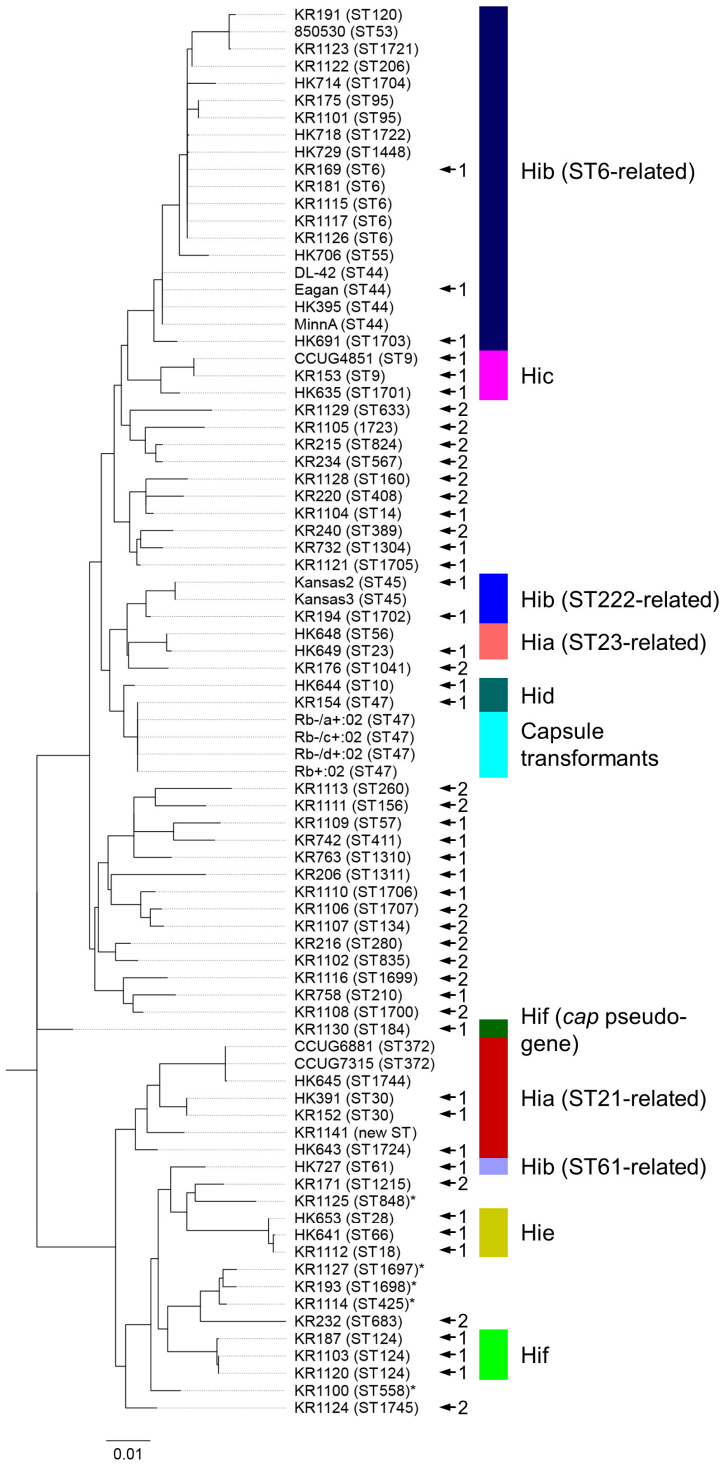
A new MALDI Biotyper reference database for capsule typing was constructed with isolates from the evaluation set. The database was initially validated and supplemented using the remaining isolates in the evaluation set. After supplementation, a final validation was performed by classification of the separate culture collection of Swedish invasive isolates.

As all isolates were *H. influenzae*, high score values were expected for all MSPs regardless of capsule type. Therefore, each spectrum was classified according to the best matching MSP. For isolate classification, ≥5/6 spectra classified to the same type (a to f or NTHi) were required. If ≤4/6 spectra were classified to the same type, the isolate was classified as inconclusive. All MALDI-TOF MS typing results were compared to PCR capsule typing by *bexB* and type specific primers (70, 88, 89).

*Results*

Phylogenetic analysis of MLST data clearly illustrated the clonal population structure of encapsulated *H. influenzae*, with isolates of different capsule types belonging to different capsule type specific genetic lineages (Figure 7). All three described genetic lineages of Hib and two of the three major genetic lineages of Hia were represented. Isolates of the other capsule types belonged to their respective established genetic lineage, as expected. One isolate, KR1130, was typed as Hif according to PCR but did not sort with the other Hif isolates. This isolate was subsequently shown to harbour a non-expressed pseudogene *cap* locus, rendering a false positive PCR result. All capsule transformants belonged to the serotype d specific genetic lineage, as their parental strain Rd (98).

**Figure 7. Phylogenetic analysis of *H. influenzae* isolates.**
The phylogentic relationship between isolates of the evaluation set in the study was investigated by MLST. All major genetic lineages of encapsulated *H. influenzae*, except the least common lineage of Hia, were represented in the collection. Isolate KR1130 did not belong to the established Hif lineage. Isolates included in the initial (1), and added to the supplemented (2), MALDI Biotyper typing database are indicated. A few NTHi which were misclassified in validation of the initial database could not be added to the database as they interfered with identification of Hie isolates. These isolates were included in the analysis and are indicated (*).


KR191 (ST120)
850530 (ST53)
KR1123 (ST1721)
KR1122 (ST206)
HK714 (ST1704)
KR175 (ST95)
KR1101 (ST95)
HK718 (ST1722)
HK729 (ST1448)
KR169 (ST6) ←1
KR181 (ST6)
KR1115 (ST6)
KR1117 (ST6)
KR1126 (ST6)
HK706 (ST55)
DL-42 (ST44)
Eagan (ST44) ←1
HK395 (ST44)
MinnA (ST44)
HK691 (ST1703) ←1
CCUG4851 (ST9) ←1
KR153 (ST9) ←1
HK635 (ST1701) ←1
KR1129 (ST633) ←2
KR1105 (1723) ←2
KR215 (ST824) ←2
KR234 (ST567) ←2
KR1128 (ST160) ←2
KR220 (ST408) ←2
KR1104 (ST14) ←1
KR240 (ST389) ←2
KR732 (ST1304) ←1
KR1121 (ST1705) ←1
Kansas2 (ST45) ←1
Kansas3 (ST45)
KR194 (ST1702) ←1
HK648 (ST56)
HK649 (ST23) ←1
KR176 (ST1041) ←2
HK644 (ST10) ←1
KR154 (ST47) ←1
Rb-/a+:02 (ST47)
Rb-/c+:02 (ST47)
Rb-/d+:02 (ST47)
Rb+:02 (ST47)
KR1113 (ST260) ←2
KR1111 (ST156) ←2
KR1109 (ST57) ←1
KR742 (ST411) ←1
KR763 (ST1310) ←1
KR206 (ST1311) ←1
KR1110 (ST1706) ←1
KR1106 (ST1707) ←2
KR1107 (ST134) ←2
KR216 (ST280) ←2
KR1102 (ST835) ←2
KR1116 (ST1699) ←2
KR758 (ST210) ←1
KR1108 (ST1700) ←2
KR1130 (ST184) ←1
CCUG6881 (ST372)
CCUG7315 (ST372)
HK645 (ST1744)
HK391 (ST30) ←1
KR152 (ST30) ←1
KR1141 (new ST)
HK643 (ST1724) ←1
HK727 (ST61) ←1
KR171 (ST1215) ←2
KR1125 (ST848)*
HK653 (ST28) ←1
HK641 (ST66) ←1
KR1112 (ST18) ←1
KR1127 (ST1697)*
KR193 (ST1698)*
KR1114 (ST425)*
KR232 (ST683) ←2
KR187 (ST124) ←1
KR1103 (ST124) ←1
KR1120 (ST124) ←1
KR1100 (ST558)*
KR1124 (ST1745) ←2
0.01
Hib (ST6-related)
Hic
Hib (ST222-related)
Hia (ST23-related)
Hid
Capsule transformants
Hif (cap pseudo-gene)
Hia (ST21-related)
Hib (ST61-related)
Hie
Hif

In the PCA NTHi formed a large heterogenous group, as expected (data not shown). Encapsulated isolates showed clustering according to capsule type and, for Hia and Hib isolates, genetic lineage. Isogenic capsule transformants clustered together, in proximity of Hid isolates, and were not distributed according to their respective capsule types (Figure 8).

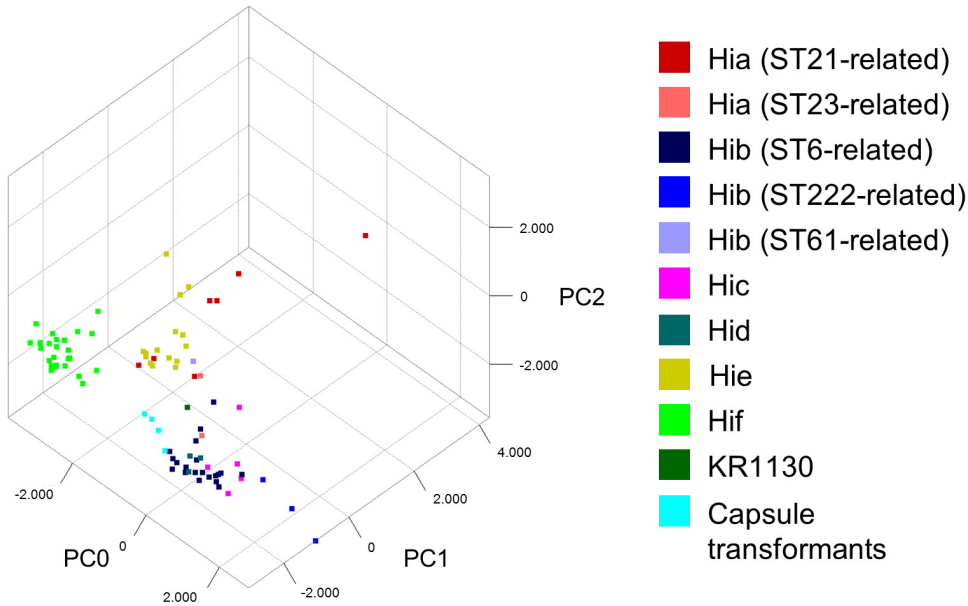



**Figure 8. Principal component analysis of mass spectra of encapsulated *H. influenzae* and isogenic capsule transformants.**
In the PCA, capsule type specific clustering was evident. Different genetic lineages of Hia and Hib isolates clustered separetaly. Isogenic capsule transformants clustered together and not with their respective capule type. Isolate KR1130 did not cluster with other Hif isolates.

The biomarker analysis revealed several mass peaks with separating capability between different capsule types and genetic lineages of Hia and Hib. The isogenic capsule transformants expressed similar peaks realtive to each other but differed in many peaks compared to wild-type isolates of the same capsule types (data not shown).

To construct a database for capsule typing in MALDI Biotyper, encapsulated isolates representing all capsule types and genetic lineages were selected (*n*=22). Nontypeable *H. influenzae* were included with the aim to include representatives of known genetic clades (*n*=9) (Figure 7) (100). Initial validation with the remaining isolates of the evaluation set (*n*=227) and capsule transformants (*n*=4) revealed perfect sensitivity for encapsulated isolates. Some NTHi, however, were misclassified as encapsulated which decreased specificity for encapsulated isolates.

As expected, all capsule transformants were classified as type d, the original capsule type of parental strain Rd (68, 305) (Table 3).

**Table 3. Validation of the initial MALDI-TOF MS capsule typing database.**

| Capsule type | n | Correct[1] | Inconclusive[2] | Incorrect[3] | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| Hia (ST21-related) | 4 | 4 | 0 | 0 | 100 | 99.1 |
| Hia (ST23-related) | 1 | 1 | 0 | 0 | 100 | 100 |
| Hib (ST6-related) | 17 | 17 | 0 | 0 | 100 | 100 |
| Hib (ST222-related) | 1 | 1 | 0 | 0 | 100 | 100 |
| Hic | 2 | 2 | 0 | 0 | 100 | 100 |
| Hid | 1 | 1 | 0 | 0 | 100 | 98.2 |
| Hie | 12 | 12 | 0 | 0 | 100 | 97.7 |
| Hif | 23 | 23[4] | 0 | 0 | 100 | 99.0 |
| All encapsulated isolates (a to f) | 61 | 61 | 0 | 0 | 100 | 92.2 |
| NTHi | 166 | 122 | 31 | 13[5] | 73.5 | 100 |
| Capsule transformants | 4 | 1[6] | 0 | 3[6] | N/A | N/A |

1. ≥5/6 spectra classified to the same, correct type.
2. ≤4/6 spectra classified to the same type.
3. ≥5/6 spectra classified to the same, incorrect type.
4. All classified to the established ST124-related Hif lineage.
5. Two isolates classified as Hia (ST21-related), 4 as Hid, 5 as Hie and 2 as Hif (ST124-related).
6. All classified as type d, i.e. the same type as the parental strain Rd, resulting in correct classification of the type d transformant and incorrect classification of the type a, b and c transformants.

Misclassified NTHi (*n*=19) were added to the typing database to improve its performance. A few NTHi could not be added to the database, as they interfered with the identification of Hie isolates (Figure 7). In a final, blinded validation of the supplemented database with clinical invasive strains (*n*=126), the sensitivity for encapsulated isolates (capsule types b, e and f) remained 100%. As none of the 5 nontypeable isolates which were not correctly identified were misclassified as encapsulated, specificity for typing of encapsulated isolates was 100% (Table 4).

**Table 4. Validation of the supplemented MALDI-TOF MS capsule typing database.**

| Capsule type | *n* | Correct[1] | Inconclusive[2] | Incorrect[3] | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| Hib | 8 | 8[4] | 0 | 0 | 100 | 100 |
| Hie | 5 | 5 | 0 | 0 | 100 | 100 |
| Hif | 15 | 15[5] | 0 | 0 | 100 | 100 |
| All encapsulated isolates (b, e and f) | 28 | 28 | 0 | 0 | 100 | 100 |
| NTHi | 98 | 93 | 5 | 0 | 94.9 | 100 |

1. ≥5/6 spectra classified to the same, correct type.
2. ≤4/6 spectra classified to the same type
3. ≥5/6 spectra classified to the same, incorrect type.
4. All classified to the ST6-related Hib lineage.
5. All classified to the established ST124-related Hif lineage.

*Discussion*

This study shows that following construction of a comprehensive database, MALDI-TOF MS can be utilized for capsule typing of *H. influenzae*.

The foremost strength of the study is the large collection of diverse strains collected at different times and from different regions, which ensure the robustness of the findings and the developed capsule typing method. By MLST, adequate coverage of different genetic lineages of encapsulated *H. influenzae* in the database was ensured. The database was furthermore carefully supplemented to reduce interference of the genetically heterogenous NTHi. Another major strength is the validation of the method using a collection of consecutive clinical isolates. One limitation is that no isolate in the culture collection belonged to the ST4-related genetic lineage of Hia and therefore could not be included in the typing database. For the least common genetic lineage of Hib (ST61-related) we only had access to one isolate which was included in the typing database.

Notably, isogenic capsule transformants clustered together in the PCA and showed similar peak patterns in biomarker analysis, confirming that differences in mass spectra is a result of general proteome differences related to genetic relationships rather than differences in capsule synthesis proteins. This notion is further supported by the separation of different genetic lineages of Hia and Hib isolates. The developed method relies on the clonal genetic population structure of encapsulated *H. influenzae* (97, 98). Each capsule type specific lineage is genetically relatively homogenous, which is reflected in the similarity of mass spectra of isolates within each lineage.

Compared to SAST and PCR typing, which identify the capsule polysaccharide and the *cap* locus, respectively, the indirect typing method of MALDI-TOF MS has both advantages and disadvantages. The diversity of NTHi constitutes a limitation of the typing method as complete coverage of the variations of NTHi of course cannot be guaranteed. Furthermore, a few NTHi were not added to the typing database as they interfered with identification of Hie isolates and a high sensitivity for encapsulated isolates was given priority to. These NTHi isolates were included in the phylogenetic analysis and shown to be closely related to the Hie genetic lineage (Figure 7). Due to the heterogeneity of NTHi, the specificity of the typing method for encapsulated isolates will not be perfect. This indicates that the method is mainly useful when the expected proportion of encapsulated isolates is substantial, such as in typing of isolates from sterile sites. Another potential drawback of the developed method is the possibility that new genetic lineages of encapsulated isolates, which the method would not identify, can occur. There is, however, little evidence that this has happened historically.

One advantage of MALDI-TOF MS typing is that identification of the genetic lineage of Hia and Hib isolates can be made simultaneously. Another advantage is that previously encapsulated capsule deficient strains, which might have lost their

capsule expression during infection or laboratory handling, are identified by the method (78, 305, 306).

A few studies have evaluated serotyping by MALDI-TOF MS of *S. pneumoniae*, for which there, similarly to *H. influenzae*, is a need of serotype surveillance. However, the results in these studies are less accurate compared to the accuracy for *H. influenzae* capsule typing in the current study (307, 308). The reason is likely the less clonal population structure of serotypes of *S. pneumoniae*, for which there exists more than 90 serotypes and serotype switching is not uncommon (309, 310). Subtyping by MALDI-TOF MS by separation of genetic lineages has also been investigated in other species, e.g. certain STs of *E. coli* and MRSA (285, 287, 288, 290, 292). For certain other species and subtyping the MALDI-TOF MS analysis is directed at certain proteins, such as the S-layer proteins of *C. difficile* (284).

In conclusion, Paper I and II show that MALDI-TOF MS can separate the different capsule types of *H. influenzae*. The capsule typing method developed in Paper II can become valuable as a rapid tool for typing of invasive *H. influenzae* and for surveillance of serotype distribution and Hib vaccination efficacy.

## Paper III: Investigation on the epidemiology and clinical impact of a clonal group of NTHi with non-beta-lactamase mediated beta-lactam resistance

While the level of beta-lactamase mediated beta-lactam resistance has been relatively stable, non-beta-lactamase mediated resistance has increased in Europe during the last decades (121, 247, 260, 265, 266). Furthermore, isolates with non-beta-lactamase mediated resistance are often genetically related (121, 244, 247, 248, 256, 260, 267-270). Incidences of invasive NTHi disease appear to be increasing (205-208). The reason for this is not fully known, and NTHi disease severity has been considered largely host dependant (204). In a recent Swedish study however, one distinct clonal cluster (CC) of NTHi (ST14CC) with specific alterations in PBP3 (group IIb, type A (242, 245, 247)) was responsible for several cases of invasive disease (260). The same clonal group was also associated with a high hospitalization rate in a Norwegian study and has caused a disease outbreak at a Swedish nursing home (121, 311). Sequence type 14 (PBP3 amino acid substitutions not reported) has also been observed in Canada, Spain and Italy (267, 268, 312, 313).

The aim of this study was to investigate the epidemiology and clinical significance of this specific clonal group, denoted ST14CC-PBP3IIb/A, in Skåne, a county in southern Sweden.

### *Methods*

For the years 2010-2012, all *H. influenzae* isolated from upper respiratory tract cultures in south Skåne (population approximately 500,000) and sterile sites from

all of Skåne (population approximately 1,200,000) were investigated. According to regional guidelines upper respiratory tract cultures are recommended in cases of recurrent AOM, treatment failure of AOM and CAP in adults. Cultures can also be obtained at the discretion of the referring physician.

The ST14CC-PBP3IIb/A clonal group was identified among isolates positive for screening of non-beta-lactamase mediated beta-lactam resistance by partial MLST. Initial screening of resistant isolates was made by sequencing of the *adk* gene, which appeared to have the highest discriminatory power for ST14 according to the *H. influenzae* MLST database (314). Isolates with *adk* 5 (specific *adk* allele of ST14) were further investigated by sequencing of *recA* and *ftsI*. In the *H. influenzae* MLST database all isolates with *adk* 5 and *recA* 5 are ST14 or single locus variants of ST14 (314). Deduced PBP3 amino acid sequences were compared with the amino acid sequence of *H. influenzae* Rd KW20 (GenBank accession number AAC22787). All isolates with *adk 5*, *recA* 5 and PBP3 group IIb, type A were considered part of the ST14CC-PBP3IIb/A clonal group. For confirmation, full MSLT was performed on a subgroup of randomly selected ST14CC-PBP3IIb/A isolates.

Minimum inhibitory concentrations for amoxicillin and cefotaxime were determined by gradient tests.

Cohorts of patients with disease caused by ST14CC-PBP3IIb/A, other NTHi with non-beta-lactamase mediated beta-lactam resistance and a control group of susceptible isolates, respectively, were identified. Data regarding age, sex, type of infection and hospitalization within five days of the culture date was retrieved from laboratory referrals and hospital records, and compared between the different cohorts. Significance in differences between groups were evaluated using the Pearson chi-squared test.

*Results*

Of respiratory tract isolates, 12.5% (400/3,192) were screening positive for non-beta-lactamase beta-lactam resistance and 360 were available for further typing. Of these isolates, 26.1% were identified as ST14CC-PBP3IIb/A. One isolate was excluded as it was encapsulated (capsule type e, PCR verified). Among invasive isolates, 13% (9/70) were screening positive for non-beta-lactamase mediated beta-lactam resistance. Of eight isolates available for typing two were ST14CC-PBP3IIb/A.

Although all ST14CC-PBP3IIb/A isolates had identical amino acid substitutions in PBP3, MICs determined by amoxicillin gradient test varied. Only 12% of non-beta-lactamase producing ST14CC/PBP3IIb/A isolates were classified as resistant according to EUCAST breakpoints. All but one ST14CC/PBP3IIb/A isolate were classified as susceptible to cefotaxime. Comparable proportions of other non-beta-lactamase producing beta-lactam resistant NTHi were classified as resistant to the tested agents (data not shown).

For patients infected by ST14CC-PBP3IIb/A and other isolates with non-beta-lactamase mediated beta-lactam resistance the proportion of patients residing in Malmö, the only large urban area in the region, increased during the study period. For patients with ST14CC-PBP3IIb/A the proportion increased from 15% in 2010 to 69% in 2012 (Figure 9).

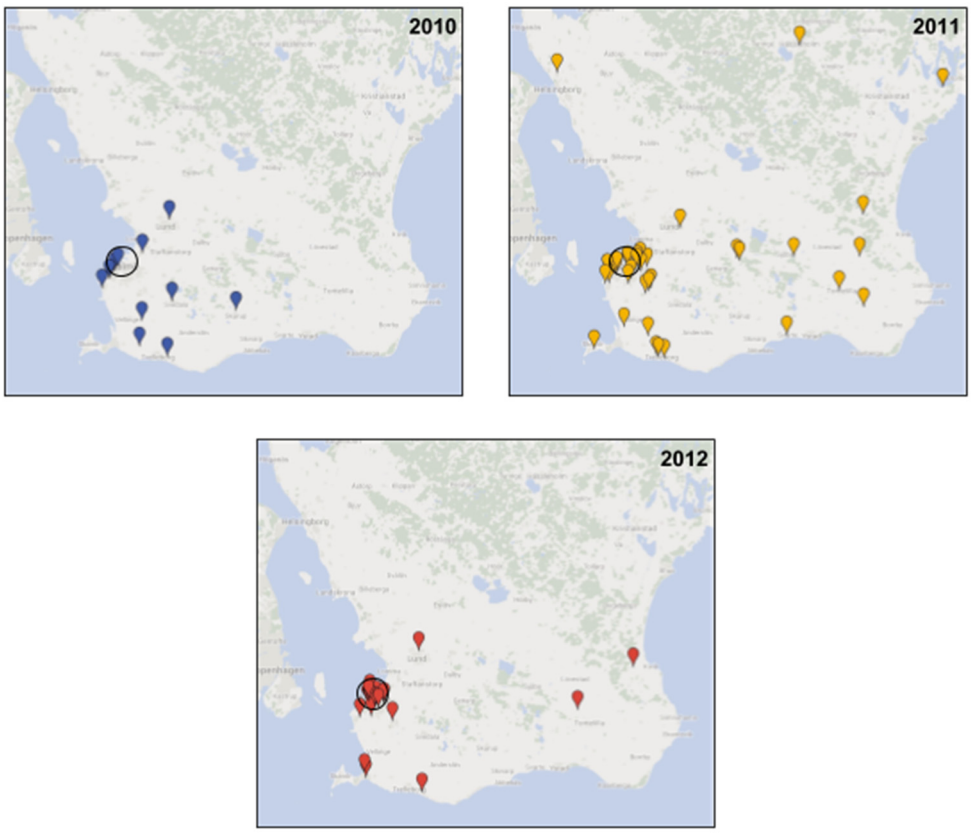



**Figure 9. ST14CC-PBP3IIb/A in southern Skåne 2010-2012.**
Postcodes of patients suffering from infection by ST14CC-PBP3IIb/A 2010-2012. During the study period, the proportion of patients residing in central Malmö increased from 15% (*n*=2/13) in 2010 to 69% (*n*=18/26) in 2012.

When patient cohorts with NTHi in respiratory tract cultures (disease caused by ST14CC-PBP3IIb/A, other resistant and susceptible NTHi, respectively) were compared, no statistically significant differences were observed in diagnosis according to culture referrals. The most common diagnoses were unspecified upper respiratory tract infection and otitis, according to culture referrals. Young children and adults dominated in all three cohorts. Patients with ST14CC-PBP3IIb/A caused disease had a significantly higher hospitalization rate compared to patients infected

by other non-beta-lactamase producing beta-lactam resistant NTHi and susceptible NTHi (Table 5). Furthermore, hospitalization appeared to more often be primarily related to the NTHi infection. The most common diagnosis for hospitalized ST14CC-PBP3IIb/A patients was pneumonia (data not shown).

All invasive disease caused by non-beta-lactamase producing beta-lactam resistant NTHi occurred in adults, of which 7 of 8 were above 65 years old. ST14CC-PBP3IIb/A accounted for two cases, one with pneumonia and one with cholangitis. All patients with disease caused by other non-beta-lactamase producing beta-lactam resistant NTHi suffered from pneumonia. One patient from each group died.

**Table 5. Clinical data of patients with ST14CC-PBP3IIb/A compared to patients with other non-beta-lactamase producing beta-lactam resistant and susceptible NTHi.**

| Patient cohort (*n*) | Suspected diagnosis/site of infection[1] (%) | | | | | | | Age in years (%) | | | | | Hospital-ized patients (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Unspecified URI[2] | Ear | Sinus | Eye | LRI[3] | Other | Un-known | <1 | 1-5 | 6-20 | 21-65 | ≥66 | |
| ST14CC-PBP3IIb/A (83) | 17 (20) | 20 (24) | 7 (8) | 12 (14) | 9 (11) | 0 | 18 (22) | 14 (17) | 35 (42) | 7 (8) | 23 (28) | 4 (5) | 14 (17)[4] |
| Other resistant NTHi (255) | 70 (28) | 44 (17) | 19 (7) | 36 (14) | 20 (8) | 4 (2) | 62 (24) | 36 (14) | 78 (31) | 21 (8) | 96 (38) | 24 (9) | 21 (8.2) |
| Suscepti-ble NTHi (192) | 70 (36) | 29 (15) | 6 (3) | 9 (5) | 14 (7) | 4 (2) | 60 (31) | 25 (13) | 74 (39) | 24 (13) | 56 (29) | 13 (7) | 13 (6.8) |

1. According to culture referral.
2. Upper respiratory tract infection
3. Lower respiratory tract infection
4. Statistically significant difference compared to patients with other non-beta-lactamase producing beta-lactam resistant NTHi (RR 2.0 (95% CI 1.1-3.8), p=0.025) and susceptible NTHi (RR 2.5 (95% CI 1.2-5.0), p=0.01.

*Discussion*

This study demonstrates that a single clonal group NTHi accounted for one quarter of isolates with non-beta-lactamase mediated beta-lactam resistance in the investigated geographical region and spread dynamically during the study period. Furthermore, the clonal group was associated with a higher risk of patient hospitalization compared to both other resistant and susceptible isolates, indicating enhanced virulence properties.

The major strength of the study is the large and near-complete collection of isolates and cases studied. A potential limitation is the partial MLST scheme used to identify clonal isolates, which may result in minor differences in the number of isolates identified as ST14CC-PBP3IIb/A compared to full MLST. Furthermore, the disk used for initial screening for beta-lactam resistance in the clinical laboratory was changed from phenoxymethylpenicillin 10 µg to benzylpenicillin 1 unit during the study period (early 2011), which may affect the number of isolates classified as resistant (257). Given the short time period of the study data regarding temporal variations should be interpreted with caution.

The findings in this study agree well with previous reports which have associated this clonal group to disease outbreaks, invasive disease and high rates of hospitalization (121, 260, 311). The current study however includes a larger sample of both ST14CC-PBP3IIb/A strains and controls, making comparison of clinical data more reliable. Since publication of this study, ST14 (PBP3 amino acid substitutions not reported) has also been found to be the most common ST among invasive NTHi in an Italian surveillance study (315).

In a study of NTHi isolated from COPD patients, a specific clonal group (ST159) was identified in approximately 10% of cases (316). The clonal group (ST159) was, however, not associated with increased risk of COPD exacerbations or prolonged colonization. The study was smaller compared to the present study, including a total of 134 strains. No data on antimicrobial susceptibility or PBP3 amino acid substitutions was reported . In a study regarding the genetic population structure of NTHI including otitis media and commensal isolates, two different genetic clusters contained only disease causing and no commensal strains. The study however included no further clinical data (317). Other studies have linked genetically related groups of NTHi to different potential virulence factors, but not specific clinical infections or disease severity (43, 100, 102). Thus, previous studies have indicated that despite the large heterogeneity of NTHi isolates, clonal groups with specific virulence traits exist. The current study, in contrast to the mentioned studies on COPD and otitis media patients, included all respiratory tract isolates, regardless of patient diagnosis. We found no specific association to the type of infection for ST14CC-PBP3IIb/A, but instead an increased patient hospitalization rate, indicating increased clinical virulence.

In laboratory studies, increased invasion rates into epithelial cells have been shown in isolates with altered PBP3 (318, 319). There was however a high variation

within the group, and the altered PBP3 in itself does not appear to mediate the effect (319). Genotypic characterization was not performed in these studies and it is possible that clonal isolates with common virulence factors generated the observed difference in invasiveness.

Most ST14CC-PBPIIb/A isolates in the current study were classified as susceptible to both amoxicillin and cefotaxime. The number of phenotypically amoxicillin resistant isolates might be underestimated, as gradient tests were used for MIC determination (257). The results highlight the difficulties in classification of non-beta-lactamase producing beta-lactam resistant isolates and the range of amoxicillin and cefotaxime MICs within this clonal group with identical PBP3 underlines that other mechanisms than PBP3 alterations affect beta-lactam susceptibility.

In summary, although NTHi are genetically heterogenous, the investigated clonal group ST14CC-PBP3IIb/A appears to be widespread and associated with increased clinical virulence. This notion is supported by the current, previous and subsequent studies of the clonal group (121, 260, 311, 315).

## Paper IV: Retrospective comparison of benzylpenicillin and wide-spectrum beta-lactams as empirical treatment of lower respiratory tract infections caused by *H. influenzae*

In Sweden, benzylpenicillin is recommended as empirical treatment for mild to moderate CAP in adult patients requiring hospitalization (130). Considering benzylpenicillin's debated effect on *H. influenzae* and the suggested increased proportion of cases of CAP caused by the species there is a need to assess the outcome of empirical treatment of benzylpenicillin on lower respiratory infections subsequently shown to be caused by *H. influenzae* (136, 232). This is further underlined by the increase in Europe in non-beta-lactamase mediated beta-lactam resistance, which might affect benzylpenicillin susceptibility (121, 247, 260, 265, 266). Importantly, in cases shown to be caused by *H. influenzae* definitive treatment with an agent with clinical breakpoints against the species should be given (130).

This retrospective study compared the outcomes of hospitalized patients with lower respiratory tract infections caused by *H. influenzae* who were empirically treated with either benzylpenicillin or a wide spectrum beta-lactam (WSBL) with established effect against *H. influenzae*.

### *Methods*

Cases were identified from the records of the clinical microbiological laboratory in Skåne and included only patients ≥18 years of age. Two case definitions were used: [1] bacteraemia with *H. influenzae* and a lower respiratory tract infection during 1997-2016 or [2] pure culture of *H. influenzae* in respiratory tract samples in patients

hospitalized for lower respiratory tract infections during 2015-2016. Cases according to definition [2] were excluded if any other potential respiratory pathogen was recorded. Data was also retrieved regarding beta-lactam resistance, which is routinely investigated for.

Descriptive and clinical data was collected from patient records. Descriptive data included age and sex. Comorbidities were registered according to the Charlson comorbidity index (CCI) (320). Immunosuppression was registered and defined as ongoing primary immune deficiency or immunosuppressive therapy not denoted in the CCI. Clinical data recorded included sepsis severity score, maximum C-reactive protein (CRP) levels, CRB-65 score and admittance to an intensive care unit (ICU) (321). All cause 30-day mortality, 30-day hospital readmission rates and early clinical response rates were used as outcomes. Early clinical response was defined as no signs of fever, tachycardia, hypotension, hypoxemia or tachypnoea on day 4 after hospital admission (322). If complete data on all parameters regarding early clinical response was not available in records, they were complemented by an assessment whether a substantial general improvement in the patient's condition had occurred.

Cases were sorted into patients receiving empirical treatment with benzyl-penicillin, any other beta-lactam antibiotic with established clinical breakpoints against *H. influenzae* and other empirical treatment regimens. Patients receiving more than one antimicrobial agent active against *H. influenzae* as empirical therapy were excluded. Outcomes were compared between the first two patient groups by logistic regression and comparison of propensity score matched cohorts. Multivariate logistic regression models were fitted using the purposeful selection algorithm (323).

Since the purposeful selection algorithm potentially risks to exclude covariates of intermediate value and since the study groups were assumed to be inherently different due to indication bias, a propensity-matched cohort was also created and analysed. Propensity scores were calculated in a logistic regression using treatment group (benzylpenicillin or a WSBL) as outcome and included categorized age, sex, ICU care, maximum levels of C-reactive protein (CRP), CCI, bacteraemia, immune suppression not denoted in the CCI and sepsis severity score as covariates. The CRB-65 scores were not included in propensity score calculation due to a high number of missing values. A 1-1 nearest neighbour matching without replacement with a caliper of 0.2 was performed and the balance in the covariates in the matched cohort was verified. Associations between treatment and outcomes in the matched cohort were assessed with full cohort logistic regression and conditional logistic regression on matched pairs.

*Results*

From blood and respiratory cultures 140 and 341 cases of lower respiratory tract infection caused by *H. influenzae* were identified, respectively. The median age was

75 years (interquartile range 66-84 years). The most common diagnosis was CAP (*n*=418, 87%). The all-cause 30-day mortality was 9%.

Empirical monotherapy treatment was given with benzylpenicillin in 199 cases and a WSBL in 213 cases. The most common WSBLs were cefotaxime (76%, *n*=162), piperacillin-tazobactam (8.5%, *n*=18) and cefuroxime (7.5%, *n*=16). Between the benzylpenicillin and WSBL treatment group there was significant differences ($p \leq 0.05$) regarding CCI, the proportions of patients given ICU care, the proportion of patients with bacteraemia and sepsis severity. The mortality was 5% and 13% in the benzylpenicillin and WSBL treatment group, respectively. In univariate logistic regression, WSBL treatment was associated with an increased mortality. The difference between treatment groups was however not significant in a multivariate model, adjusted for age, sepsis severity and CCI score (Table 6)

Similarly, when including only bacteraemic patients (*n*=120), a significantly increased mortality was noted for patients given empirical treatment with a WSBL in univariate regression (odds ratio (OR) 5.55, 95% confidence interval (CI) 1.20-25.7, *p*=0.028), but not in multivariate regression adjusted for age and CCI (OR 4.86, 95% CI 0.98-24, *p*=0.054).

No statistically significant differences regarding 30-day readmission rate in univariate or multivariate regression were observed. Although rates of early clinical response were high in both treatment groups, there was a significantly increased rate in the WSBL treatment group in multivariate regression (Table 6).

**Table 6. Univariate and multivariate logisitc regression comparing WSBL treatment and benzylpenicillin treatment of H. influenzae lower respiratory tract infections.**

| Outcome | WSBL treatment univariate OR[1] (95% CI) | *p* | WSBL treatment multivariate OR[1] (95% CI) | *p* |
|---|---|---|---|---|
| 30-day all cause mortality | 2.59 (1.25-5.35) | 0.010 | 2.03 (0.91-4.50)[2] | 0.082 |
| 30-day readmission | 1.23 (0.72-2.13) | 0.45 | 1.16 (0.66-2.05)[3] | 0.61 |
| Early clinical response | 1.28 (0.76-2.16) | 0.35 | 2.28 (1.21-4.31)[2] | 0.011 |

1. Benzylpenicillin treatment reference.
2. Adjusted for age, CCI category and sepsis severity.
3. Adjusted for age and CCI category.

The construction of a propensity score matched cohort resulted in the omission of many of the more severely ill patients (CRB-65≥3, ICU care, severe sepsis or septic shock). These patients were primarily found in the WSBL treatment group, which is reasonable as benzylpenicillin is not recommended as empirical therapy in these cases. The matched cohort consisted of 151 patients in each treatment group. Results regarding the different outcomes in the analyses of the propensity score matched cohort resembled those obtained from multivariate logistic regression. Treatment with a WSBL was associated with a higher, although not significantly higher, mortality (OR 2.14, 95% CI 0.93-4.92, *p*=0.075 and OR 1.89, 95% CI 0.84-4.23, *p*=0.12 for the full cohort and in conditional regression, respectively). Similarly, a

higher rate of early clinical response for patients treated with WSBLs was observed in this cohort both in full cohort logistic regression (OR 2.14, 95% CI 1.07-4.27, *p*=0.031) and conditional logistic regression (OR 2.5, 95% CI 1.20-5.21, *p*=0.014). There was no difference between treatment groups regarding hospital readmission.

Potential effect modification of treatment by isolates positive for beta-lactam resistance (beta-lactamase producing and non-producing) was investigated by comparison of odds ratios for the different outcomes stratified by treatment group and resistance mechanism between the propensity score matched cohorts. Significant effect modification was observed for beta-lactamase production regarding early clinical response and treatment group (interaction term for beta-lactamase × treatment group (benzylpenicillin as reference); β=3.12, 95% CI 0.82-5.43, *p*=0.008), with a lesser extent of early clinical response in the benzylpenicillin group compared to the WSBL group in patients infected by beta-lactamase producing isolates (Table 7).

**Table 7. Effect modification by isolate beta-lactamase production on early clinical response in the propensity score matched cohort.**

| Isolate beta-lactamase production | Benzylpenicillin treatment | | WSBL treatment | | OR within strata of beta-lactamase production, (95% CI), *p* |
|---|---|---|---|---|---|
| | *n* with early clinical response (%) | OR (95% CI), *p* | *n* with early clinical response (%) | OR (95% CI), *p* | |
| No beta-lactamase production | 109/125 (87.2%) | Reference | 107/120 (89.2%) | 1.21 (0.55-2.63), *p*=0.63 | 1.21 (0.55-2.63), *p*=0.63 |
| Beta-lactamase production | 10/21 (48%) | 0.13 (0.05-0.36), *p*<0.001 | 25/26 (96%) | 3.67 (0.46-29.0), *p*=0.22 | 27.5 (3.13-242), *p*=0.003 |

No similar effect modification was seen for non-beta-lactamase beta-lactam resistant isolates regarding early clinical response (data not shown). No significant effect modification was observed for any resistance mechanism regarding the other outcomes (data not shown).

*Discussion*

In this retrospective study of lower respiratory tract infections caused by *H. influenzae*, treatment with benzylpenicillin was not associated with a significantly increased risk of mortality or hospital readmission compared to treatment with a WSBL. Even though the rates of early clinical response were generally high, empirical treatment with benzylpenicillin was associated with a significantly lower rate of early clinical response compared to treatment with a WSBL. This difference was to a large extent explained by a lower response rate in patients infected with beta-lactamase producing isolates.

One major strength of this study is the relatively large size of the cohort, which was constructed in an un-biased way by identification of cases from culture findings.

The data has been thoroughly analysed and the results are robust, as shown by the similar results attained by both logistic regression and propensity score matching of the treatment groups.

One limitation of the study is the risk of indication bias due to its retrospective design. Possibly because of this, WSBL treatment was associated with worse outcomes in the crude models. The models were adjusted for risk factors of worse outcomes (such as comorbidities) using the purposeful selection algorithm to adjust for possible confounding, and this reduced the association. However, it still bordered significance, indicating possible residual confounding not adjusted for. Using another algorithm for variable selection more variables could have been included in the final models and possibly reduce this confounding. This would, however, have increased the risk of overfitting, especially as the numbers of the different outcomes were relatively low. Instead, the data was also investigated by creating a propensity score matched cohort based on regression analysis including several more variables. Furthermore, due to the limitations of diagnostic tools used for determination of CAP aetiology, it is not possible to know for sure that all cases included in the study had infections attributed to *H. influenzae* although the study method carefully tried to address this. Hopefully, implementation of modern molecular methods, such as quantitative PCR, can improve the diagnostic yield in the future (136, 137). Another limitation of the study is its limited power, due to the relatively low outcome rates. For mortality, which was 14% in the WSBL group, the study has power to detect an OR of approximately 2 (given $\alpha$=0.05, $\beta$=0.8 and a two-sided test of significance).

Benzylpenicillin has been compared to treatment with WSBLs in patients with *Haemophilus* spp. bacteraemia in a previous retrospective study (233). In the study, definite (as opposed to empirical) treatment with benzylpenicillin compared to treatment with an aminopenicillin or cefuroxime was associated with an increased 30-day mortality for patients with *H. influenzae* bacteraemia. There are notable differences between the previous and the current study. The previous study investigated bacteraemic patients regardless of infection focus, as opposed to only respiratory tract infections. In the benzylpenicillin treatment group of the previous study, more than 30% had an unknown site of infection. The overall mortality was also considerably higher, 22% compared to 9%. Furthermore, several patients in the benzylpenicillin group hade polymicrobial infections and many patients received combination treatment. Adjustment for potential confounders was made only in separate bivariate regressions for each covariate. These factors, separate or in combination, could reasonably explain the previous study's different results compared to the current.

In another recent retrospective study, treatment with phenoxymethylpenicillin or benzylpenicillin was compared to WSBL treatment in patients with CAP and a CRB-65 score $\leq$2 (134). The study found, similarly to the current study, no differences regarding 30-day or 90-day mortality between the treatment groups.

Treatment with WSBLs was associated with ICU care, possibly due to similar indication bias as in our study. The study included cases of pneumonia regardless of causative organism. *H. influenzae* accounted for 7.1% of cases in patients with CRB-65 score ≤1, and apparently differences in mortality among these patients were (at least) not large enough to significantly affect mortality in the different treatment groups in total.

Different dosing regimens might affect the effectiveness of benzylpenicillin. In this study, 1 g t.i.d. (40% of cases) and 3 g t.i.d. (49% of cases) were the most used regimens. Monte Carlo simulations calculated by EUCAST suggest markedly lower attainment rates of a time above a concentration of 1 mg/L (ECOFF for *H. influenzae*) for at least 30% or 40% of the dosing interval if the former dosing regimen is used (232). In patients receiving high dose (3 g t.i.d. or higher) and low dose (1 g t.i.d. or lower) treatment the mortality was 3% (*n*=4 of 116) and 8% (*n*=7 of 93), respectively. Statistical comparison between the groups was not made in the study due to a too small number of events.

The lower rate of early clinical response in patients infected by beta-lactamase producing isolates treated with benzylpenicillin is understandable as *H. influenzae* beta-lactamases are active against benzylpenicillin but not cefotaxime, piperacillin/tazobactam and cefuroxime, which were the most used agents in patients treated with WSBLs (234).

In conclusion, the results from this study suggest that empirical treatment with benzylpenicillin in patients with *H. influenzae* caused respiratory tract infections of mild to moderate severity is not associated with increased mortality or risk of hospital readmission. To fully determine optimal empirical treatment of these infections prospective randomized controlled trials are needed.

# Ethical considerations

Papers I and II did not involve human subjects and no ethical permit was needed. Papers III and IV involved data on human subjects and ethical permits were required. The studies were approved by the Regional Ethical Review Board at Lund University (registration number 2014/533 and 2016/743 with addendum 18-391, respectively). All patient data in the studies was collected in retrospect and no interventions in the treatment given to patients were made. All data was anonymized and presented at group level in the published papers.

# Concluding remarks and future perspectives

Despite the success of Hib childhood vaccination programmes, *H. influenzae* still is a major cause of disease in humans. With increasing incidences of invasive NTHi disease and remaining sporadic cases of invasive Hib disease, surveillance of invasive *H. influenzae* disease and Hib vaccination efficacy is still highly important. The capsule typing method developed in Paper II showed highly accurate results for encapsulated isolates. Future work will concern the further evaluation and implementation of the method in clinical laboratories, where the method can be valuable for rapid and cost-effective capsule type surveillance of invasive *H. influenzae*.

Typing of other bacterial species by MALDI-TOF MS is another research area which warrants further study. The possibility of typing by the instrument could be helpful in areas such as infection prevention and control. Although previous studies have investigated typing of bacterial strains with antimicrobial resistance, this form of MALDI-TOF MS typing is to a large extent not yet in clinical use. Development of clinically applicable typing methods in these cases could be highly useful.

As NTHi now are the most prevalent cause of both mucosal and invasive *H. influenzae* infections, future research will naturally focus on these isolates. The complex system of bacterial and host factors leading to NTHi disease is not fully understood. Paper III and previous studies have indicated ST14CC-PBP3IIb/A to be a clonal group of NTHi with enhanced virulence traits. The bacterial factors conferring this virulence have, however, not yet been investigated and poses an interesting area of further research. Given the rapid development of genetic sequencing methods and the possibilities to handle large amounts of genomic data, this could be a possible initial approach for investigation. Severe *H. influenzae* disease occurs only in a minority of patients colonized with the bacterium. Besides bacterial characteristics, host factors play a large role. It is, however, not entirely clear which factors contribute most to the risk of severe disease. Further investigations including both bacterial factors and patient risk factors are required to understand this interplay better. With increasing research focus on the development of an NTHi vaccine, studies in these areas could be of high value both for vaccine development and for information on which patients who would benefit most from such vaccination.

The increase in non-beta-lactam mediated beta-lactam resistance in *H. influenzae* is worrying. Although Paper IV provided no evidence for treatment failures with benzylpenicillin resulting in severe outcomes in patient with lower respiratory tract infections as a result of this kind of beta-lactam resistance, the issue should be taken seriously. Understanding this type of resistance is important to facilitate actions against further increase in resistance prevalence and could potentially be a source of new antimicrobial therapies.

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# Paper I

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# Identification of *Haemophilus influenzae* Type b Isolates by Use of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry

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***Haemophilus influenzae* type b (Hib) is, in contrast to non-type b *H. influenzae*, associated with severe invasive disease, such as meningitis and epiglottitis, in small children. To date, accurate *H. influenzae* capsule typing requires PCR, a time-consuming and cumbersome method. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) provides rapid bacterial diagnostics and is increasingly used in clinical microbiology laboratories. Here, MALDI-TOF MS was evaluated as a novel approach to separate Hib from other *H. influenzae*. PCR-verified Hib and non-Hib reference isolates were selected based on genetic and spectral characteristics. Mass spectra of reference isolates were acquired and used to generate different classification algorithms for Hib/non-Hib differentiation using both ClinProTools and the MALDI Biotyper software. A test series of mass spectra from 33 Hib and 77 non-Hib isolates, all characterized by PCR, was used to evaluate the algorithms. Several algorithms yielded good results, but the two best were a ClinProTools model based on 22 separating peaks and subtyping main spectra (MSPs) using MALDI Biotyper. The ClinProTools model had a sensitivity of 100% and a specificity of 99%, and the results were 98% reproducible using a different MALDI-TOF MS instrument. The Biotyper subtyping MSPs had a sensitivity of 97%, a specificity of 100%, and 93% reproducibility. Our results suggest that it is possible to use MALDI-TOF MS to differentiate Hib from other *H. influenzae*. This is a promising method for rapidly identifying Hib in unvaccinated populations and for the screening and surveillance of Hib carriage in vaccinated populations.**

*Haemophilus influenzae* type b (Hib) has been, and in some regions still is, the dominating cause of severe invasive disease associated with the species *H. influenzae*. More specifically, it is (or used to be) a feared cause of meningitis and epiglottitis in small children (1, 2). The conjugate vaccines against Hib that were introduced in the early 1990s have resulted in a steep decline in invasive Hib disease (1, 3–5). However, Hib still causes 5 to 10% of invasive *H. influenzae* disease in Sweden, and occasional cases of fully vaccinated children with invasive Hib disease have been reported in several countries (6, 7). Hib is estimated to cause a substantial number of infections and deaths among young children each year on a global basis (8). PCR is at present required for accurate Hib capsule typing (9–11) but is a relatively time-consuming and laborious method. Agglutination with antisera is an alternative method used for capsular typing. Even though this is a faster method than PCR, studies have shown that its accuracy is comparatively poor (12).

Early studies have suggested that the Hib population consists of two genetically distinct clusters (13, 14). This population structure was later confirmed using multilocus sequence typing (MLST), since practically all Hib isolates that had been submitted to the MLST database from all over the world could be sorted into one of the two clusters (15). The dominant cluster comprises the main portion of Hib isolates and is centered on sequence type 6 (ST6) in the MLST database. The second Hib cluster is comparatively uncommon, and the predominant sequence type in this cluster is ST93. The fact that there are two distinct Hib clusters is an important factor to take into account when investigating methods of identifying Hib that do not focus on capsule identification *per se*.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has revolutionized clinical microbiological diagnostics and is used nowadays to identify fungal and bacterial species, including *H. influenzae* (16, 17). The technique has also been suggested to be a useful tool for various subtyping of bacteria, testing of antimicrobial susceptibility, and identification of virulent bacterial clones (18–21). However, MALDI-TOF MS is currently used only to identify *H. influenzae* at the species level and has not yet been established for use in subtyping.

The aim of this study was to investigate whether MALDI-TOF MS can be used to differentiate Hib from other *H. influenzae* capsular types and nontypeable (unencapsulated) *H. influenzae* (NTHi). The results demonstrate that MALDI-TOF MS can successfully be used to discriminate between Hib and non-b *H. influenzae*, but the choice of testing algorithm is essential to achieve

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optimal results. MALDI-TOF MS can thus be used as a highly valuable cost- and time-effective methodology for typing of Hib.

## MATERIALS AND METHODS

**Bacterial isolates.** The culture collection in this study comprised 127 isolates. The majority of the isolates ($n = 114$) were invasive *H. influenzae* isolates collected in four Swedish metropolitan areas (Gothenburg, Lund, Malmö, and Stockholm) between 1997 and 2010. The remaining isolates were collected from other countries, such as the United States, or were obtained from culture collections (Culture Collection, University of Gothenburg [CCUG], Sweden, and the National Collection of Type Cultures [NCTC], Public Health England, London, United Kingdom). Isolates were identified as *H. influenzae* using routine clinical methods (hemin and NAD required for growth) and standard MALDI-TOF MS analysis using the MALDI Biotyper software. All isolates were stored in glycerol at −80°C and cultured on chocolate agar plates at 37°C and 5% $CO_2$ for 18 to 24 h before use.

**Preparation of bacterial DNA.** To prepare DNA, a few colonies were suspended in 100 μl of distilled water and heated at 98°C for 10 min. After centrifugation, the supernatant was collected and used as the template for PCR. To obtain high-quality sequencing results, extraction of genomic DNA was performed with the GenElute bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO).

**Capsular typing by PCR.** All isolates in this study were capsule typed by PCR. To determine the presence of a capsule, PCR to detect the common capsule transport complex (*bexB*) was performed as previously described (9). On isolates positive for *bexB*, capsule type-specific PCRs were performed as described previously (10, 11).

**MLST.** MLST was performed as described by Meats et al. (22) using primers available in the MLST database (www.mlst.net). The resulting PCR products were sequenced, analyzed using Geneious 7.1.3 (Biomatters, Auckland, New Zealand), and the STs of the isolates were determined using information in the MLST database.

**MALDI-TOF MS data acquisition.** Isolates were prepared for acquisition of spectra using a standard ethanol-formic acid extraction protocol developed by the mass spectrometer manufacturer (Bruker Daltonics, Bremen, Germany). The bacterial pellets were allowed to dry completely (up to 1 h) following ethanol washing. The volume of formic acid and acetonitrile used (10 to 40 μl) was based on pellet size. Reference and test isolates were applied on 8 and 2 spots, respectively. Three spectra per spot were acquired, giving 24 spectra per reference isolate and 6 spectra per test isolate.

Mass spectra of the reference and test isolates were acquired using a microflex MALDI-TOF mass spectrometer with flexControl software (Bruker Daltonics, Bremen, Germany) using default settings (mass range of spectra, *m/z* 2,000 to 20,000 in linear positive-ionization mode). Each spectrum consisted of 240 shots, divided into 40-shot increments. For reproducibility testing of the generated classification methods, 19 Hib and 23 non-Hib test isolates were randomly selected for repeated extraction and measurement on a different MALDI-TOF MS instrument (ultrafleXtreme; Bruker Daltonics) in another laboratory using default settings (a total of 800 shots divided into 100-shot increments). Before each run, the instruments were calibrated with Bacterial Test Standard (BTS) (Bruker Daltonics).

**Manual analysis of mass spectra and selection of reference isolates.** Manual analysis of mass spectra, using the ClinProTools 3.0 software (Bruker Daltonics), was performed to identify peak differences between Hib and non-Hib isolates. Reference Hib isolates were selected from a screen of 19 randomly chosen Hib isolates based on a manual analysis of their spectral profile and MLST. Reference isolates were chosen in order to cover the broadest possible range within the MLST Hib clusters. Isolates not chosen as reference isolates were included among the test isolates. The non-Hib reference isolates included all capsular types except Hib, as well as five NTHi.

**Hib/non-Hib classification methods.** Four different methods were each tested in two separate ways, resulting in a total of 8 different classification algorithms, and these were evaluated for differentiation of Hib and non-Hib. ClinProTools was used for the generation of classification models using raw spectra of the reference isolates. The default settings were used for spectrum preparation (resolution, 800; baseline subtraction, top-hat, 10% minimal baseline width; recalibration, 1,000 ppm maximal peak shift and 30% match to calibrant peaks), peak calculation (peak picking on the total average spectrum; signal-to-noise ratio, 5), and peak selection (use all peaks). The models in ClinProTools were generated using three different algorithms (genetic, supervised serial network, and quick classifier). The number of peaks to be included in the genetic algorithm varied between 5 and 25. This was considered a suitable range, based on manual analysis for capsule-specific peak differences of the spectra of reference isolates. The other two algorithms detect the optimal number of peaks to be included automatically. The model with the best Hib/non-Hib differentiation (when analyzing 6 spectra per test isolate) was chosen for further testing with only two spectra per test isolate and reproducibility testing (see below).

The MALDI Biotyper 3.1 software was used to classify spectra using three different methods; regular main spectra (MSPs), subtyping MSPs, and specially designed MSPs. Before MSPs were created, the quality of the reference isolate spectra was checked using FlexAnalysis 3.4 (Bruker Daltonics). Smoothing and baseline subtraction were performed, and outlier (lacking a peak or having an extra peak) or low-quality (peaks outside a 500-ppm range) spectra were removed. The remaining spectra, ≥20 of each isolate, were used to create the different types of MSPs in Biotyper.

Regular MSPs of Hib and non-Hib reference isolates were created using default settings (maximum, 70 peaks; mass range, *m/z* 3,000 to 15,000). For the creation and testing of subtyping MSPs, the preprocessing, MSP creation, and subtyping MSP creation methods in Biotyper were modified to use a wider mass range (*m/z* 2,500 to 15,500) and to allow more peaks in the MSPs (maximum, 150 peaks). MSPs were created for all reference isolates. The subtyping MSPs of each Hib reference isolate were then individually generated against the MSPs of the non-Hib isolates (not together with other Hib MSPs) to avoid Hib subtyping MSPs to differentiate between different Hib clones.

Specially designed MSPs of the Hib reference isolates were created using default mass range settings (*m/z* 3,000 to 15,000) but with an increased number of peaks allowed (maximum, 150 peaks). The MSP peak lists were modified to include only two peaks with masses of approximately *m/z* 6,789 and *m/z* 8,348 (Fig. 1). These peaks were selected based on a manual evaluation of spectra for their capacity to differentiate between Hib isolates of the ST6 cluster and non-Hib isolates. *H. influenzae* strain KR194 is not a part of the ST6 cluster and does not express these peaks.

**Testing of different classification algorithms.** Each method was tested in two different ways using 33 Hib and 77 non-Hib isolates (Table 1). First, the methods were tested by classification of all six spectra acquired for each test isolate. Isolates with ≥5 of 6 spectra classified as Hib were identified as Hib, and isolates with ≤2 of 6 spectra classified as Hib were identified as non-Hib. Isolates with 3 to 4 of 6 spectra classified as Hib were identified as uncertain. Second, to mimic the everyday clinical standard procedure, methods were tested using only the first spectrum acquired per spot, giving two spectra per test isolate. In this setting, isolates with ≥1 spectrum classified as Hib were identified as Hib, isolates with no spectra classified as Hib were identified as non-Hib. The identification category uncertain was not used.

ClinProTools models classify all spectra as either of the classes used to generate the models (e.g., Hib or non-Hib). In MALDI Biotyper, scores are calculated for each spectrum. When using regular MSPs, a spectrum was classified as Hib if it had a Hib MSP as the best match (since all MSPs were of the same species, scores were expected to be high on all MSPs, regardless of the capsular type of the test isolates). When using subtyping MSPs, spectra were classified as Hib if the score was >2.0 for any Hib

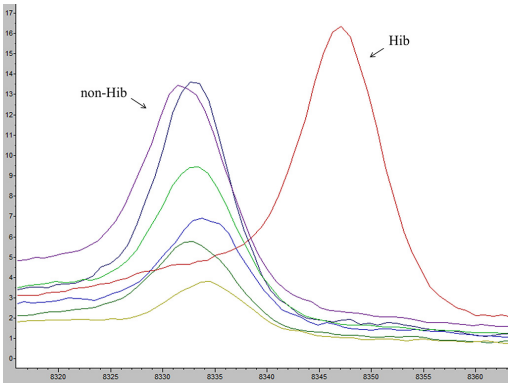


**FIG 1** Illustration of peak *m/z* 8,332 and peak *m/z* 8,348 as potential differentiators between Hib and non-b *H. influenzae*. Non-Hib (Hia, Hic, Hid, Hie, Hif, and NTHi) isolates expressed a peak of approximately *m/z* 8,332. In contrast, Hib isolates expressed an offset peak of approximately *m/z* 8,348. Each capsular type is represented by a single isolate. *x* and *y* axes show *m/z* values and intensity (in arbitrary units), respectively.

subtyping MSP. When using specially designed MSPs, spectra were classified as Hib if the score was >1.3 for any Hib MSP, as 1.301 was the score yielded by spectra expressing both peaks included in these MSPs.

A flowchart of the testing procedure of the different classification methods is presented in Fig. 2.

**Analysis of test results.** Sensitivity, specificity, and discriminatory accuracy (expressed as the area under the receiver operating characteristic [AU-ROC]) were calculated for each Hib/non-Hib classification algorithm based on the results generated on the microflex instrument. AU-ROC was calculated using SPSS Statistics 22.0 (IBM, Armonk, NY) based on correct or false identification compared to PCR (golden standard). All isolates identified as uncertain were considered misidentified in the analysis. The reproducibility of each classification method was calculated as the percentage of tested isolates that was sorted into the same category (Hib, uncertain, or non-Hib) in both measurements (microflex and ultrafleXtreme).

## RESULTS

**MLST analysis and selection of reference isolates.** MLST was performed on 19 Hib isolates. Seven different STs were represented in the group (Table 2). The isolates KR194, L2, KR211, and M17 belonged to new STs. All were single-allele variants from previously known STs. All isolates belonged to the worldwide dominant ST6 cluster, except KR194, which belonged to the established second cluster (single-allele variant from ST93). By choosing KR194 and 6 different variants from the ST6 cluster as reference isolates for MALDI-TOF MS classification, we aimed for optimal coverage of known Hib clusters.

For the non-Hib reference group, one isolate of each non-Hib capsular type (a, c, d, e, and f) and five NTHi were selected as reference isolates. Five NTHi isolates with different spectral and MLST patterns were chosen as references. Even though the reference NTHi were genetically and spectrally heterogenous, we did not cover the full and very wide range of NTHi. The focus of the selection was based on their spectral variation in key Hib peaks. The selected reference isolates are presented in Table 2.

**Identification of Hib using MALDI-TOF MS.** Four different classification methods were tested in two different ways (Fig. 2). Sensitivity, specificity, AU-ROC, and reproducibility were calculated for all classification algorithms. A full summary of the test results is presented in Table 3.

**Identification of Hib using ClinProTools.** The best model in ClinProTools was generated using the genetic algorithm and was based upon 22 peaks. Other models generated by the genetic algorithm achieved results close to those of the 22-peak model. The results from the 22-peak model (referred to as the ClinProTools model) are presented below.

When 6 spectra per isolate were used, all Hib ($n = 33$) and 98.7% of non-Hib (76 of 77) isolates were correctly identified. The non-Hib isolate KR183 was identified as Hib. All 6 spectra of all individual Hib isolates were correctly classified, and all KR183 spectra were incorrectly classified. Three non-Hib isolates each had one of six spectra that was incorrectly classified. The reproducibility was 97.6%, as all isolates except one Hib isolate (identified as uncertain by the ultrafleXtreme) were sorted into the same category by both instruments. When only two spectra per isolate were analyzed, the NTHi isolate M5 was incorrectly identified as Hib according to our definitions (since one of the two first spectra was incorrectly classified in this isolate) along with KR183. Here, all measured isolates were identified in the same category on the second instrument, giving a reproducibility of 100%.

**Identification of Hib using regular Biotyper MSPs.** When using 6 spectra, all Hib isolates were correctly classified on all spectra. A large portion (98.7%) of the non-Hib isolates were correctly identified. Several of the non-Hib isolates had one or two spectra incorrectly classified but only one isolate was incorrectly identified as uncertain, with four spectra classified as Hib. When repeated on a different instrument, 16 of 19 Hib and 22 of 23 non-Hib isolates were identified to the same category, resulting in a reproducibility of 90.5%.

When two spectra per isolate were analyzed, all Hib isolates were correctly identified, with both spectra classified as Hib. The majority (97.4% [75 of 77]) of the non-Hib isolates were correctly identified. When measured using the other instrument, all Hib ($n = 19$) and 21 of 23 non-Hib isolates were identified to the same category, resulting in a reproducibility of 95.2%.

**Identification of Hib using Biotyper subtyping MSPs.** When using subtyping MSPs, a spectrum was classified as Hib if it had any Hib subtyping MSP score of >2.0. When 6 spectra per isolate were analyzed, 97.0% (32 of 33) of the Hib isolates were correctly identified. One isolate was classified as uncertain. All non-Hib isolates were correctly identified, and all non-Hib spectra (6 per isolate) had Hib subtyping scores of ≤2.0. When the analysis was repeated with the ultrafleXtreme instrument, the absolute scores were lower in general. However, relative differences in scores between Hib and non-Hib isolates remained. The score cutoff on Hib subtyping MSPs for Hib classification was consequently adjusted for these spectra. The absolute reproducibility with this method was therefore low, but with the adjusted cutoff, the reproducibility was 92.9%, with 16 of 19 Hib and all non-Hib ($n = 23$) isolates were identified to the same category by the two instruments.

When using only two spectra per isolate, all Hib isolates and all non-Hib isolates were correctly identified. All isolates extracted and measured by the other instrument were also correctly identified, giving a reproducibility of 100% (implying adjusted cutoff).

**TABLE 1** Clinical isolates and laboratory reference strains used to test Hib/non-Hib classification

| Isolate | Capsular type | Location | Yr | Site of isolation |
|---|---|---|---|---|
| Hib | | | | |
| Clinical isolates | | | | |
| M58 | b | Malmö | 1997 | Blood |
| M61[a] | b | Malmö | 1997 | Blood |
| KR181[a] | b | Stockholm | 1998 | CSF[b] |
| G4[a] | b | Gothenburg | 2002 | Blood |
| M41 | b | Malmö | 2004 | Blood |
| M35[a] | b | Malmö | 2004 | Blood |
| L40 | b | Lund | 2004 | Blood |
| L2[a] | b | Lund | 2004 | Blood |
| KR196 | b | Stockholm | 2004 | Blood |
| KR192[a] | b | Stockholm | 2004 | Blood |
| M31 | b | Malmö | 2005 | Blood |
| L35[a] | b | Lund | 2005 | Blood |
| M66 | b | Malmö | 2006 | Blood |
| KR211 | b | Stockholm | 2006 | Blood |
| KR209[a] | b | Stockholm | 2006 | Blood |
| L58 | b | Lund | 2007 | Blood |
| L63[a] | b | Lund | 2008 | Blood |
| G32[a] | b | Gothenburg | 2008 | CSF |
| M7[a] | b | Malmö | 2009 | Blood |
| G26 | b | Gothenburg | 2009 | Blood |
| S86[a] | b | Stockholm | 2010 | Blood |
| S79 | b | Stockholm | 2010 | Blood |
| M81[a] | b | Malmö | 2010 | CSF |
| M75 | b | Malmö | 2010 | Blood |
| G74[a] | b | Gothenburg | 2010 | CSF |
| G72 | b | Gothenburg | 2010 | Blood |
| G67[a] | b | Gothenburg | 2010 | Blood |
| Laboratory reference strains | | | | |
| CCUG 18095 (Eagan)[a] | b | Boston, MA | 1968 | CSF |
| MinnA[a] | b | Minneapolis, MN | 1979 | CSF |
| DL-42 | b | Dallas, TX | Before 1984 | Unknown |
| 850530[a] | b | Netherlands | 1985 | Unknown |
| HK 691[a] | b | Netherlands | Before 1985 | Unknown |
| HK 729 | b | United States | Before 1988 | CSF |
| Non-Hib | | | | |
| Clinical isolates | | | | |
| L59[a] | e | Lund | 2007 | Blood |
| KR228 | e | Stockholm | 2008 | Blood |
| KR236 | e | Stockholm | 2008 | CSF |
| KR138[a] | e | Unknown | Unknown | Unknown |
| KR147 | e | Unknown | Unknown | Unknown |
| KR569 | e | Malmö | 2009 | Tonsil |
| M63 | f | Malmö | 1997 | Blood |
| KR179[a] | f | Stockholm | 1998 | CSF |
| M54[a] | f | Malmö | 1999 | Blood |
| KR187 | f | Stockholm | 2000 | CSF |
| L45 | f | Lund | 2005 | Blood |
| L44 | f | Lund | 2005 | Blood |
| KR200 | f | Stockholm | 2005 | Blood |
| M65 | f | Malmö | 2006 | Blood |
| M29 | f | Malmö | 2006 | CSF |
| L11[a] | f | Lund | 2006 | Blood |
| KR210 | f | Stockholm | 2006 | Blood |
| L50[a] | f | Lund | 2007 | Blood |
| L19 | f | Lund | 2007 | Blood |
| L18 | f | Lund | 2007 | Blood |
| L16 | f | Lund | 2007 | Blood |

(Continued on following page)

**TABLE 1** (Continued)

| Isolate | Capsular type | Location | Yr | Site of isolation |
|---|---|---|---|---|
| M14 | f | Malmö | 2008 | Blood |
| M12 | f | Malmö | 2008 | Blood |
| M10[a] | f | Malmö | 2008 | Blood |
| L64 | f | Lund | 2008 | punctate |
| L22 | f | Lund | 2008 | Blood |
| L21 | f | Lund | 2008 | Blood |
| KR233[a] | f | Stockholm | 2008 | Blood |
| G34 | f | Gothenburg | 2008 | Blood |
| G22 | f | Gothenburg | 2008 | Blood |
| G20 | f | Gothenburg | 2008 | Blood |
| G19 | f | Gothenburg | 2008 | Blood |
| G18[a] | f | Gothenburg | 2008 | Blood |
| L29 | f | Lund | 2009 | Blood |
| L25 | f | Lund | 2009 | Blood |
| L24 | f | Lund | 2009 | Blood |
| S78 | f | Stockholm | 2010 | Blood |
| M60 | NT[c] | Malmö | 1997 | Blood |
| M59 | NT | Malmö | 1997 | Blood |
| KR170[a] | NT | Stockholm | 1998 | Blood |
| G1[a] | NT | Gothenburg | 1999 | Blood |
| KR183 | NT | Stockholm | 2000 | Blood |
| M51[a] | NT | Malmö | 2001 | CSF |
| G2[a] | NT | Gothenburg | 2001 | Blood |
| M45 | NT | Malmö | 2002 | Blood |
| M36 | NT | Malmö | 2004 | Blood |
| L3[a] | NT | Lund | 2004 | Blood |
| L17 | NT | Lund | 2004 | Blood |
| KR189 | NT | Stockholm | 2004 | Blood |
| KR201 | NT | Stockholm | 2005 | Blood |
| L9 | NT | Lund | 2006 | Blood |
| L8 | NT | Lund | 2006 | Blood |
| L48 | NT | Lund | 2006 | Blood |
| M23 | NT | Malmö | 2007 | Blood |
| M21 | NT | Malmö | 2007 | Blood |
| L61 | NT | Lund | 2007 | Blood |
| L57[a] | NT | Lund | 2007 | Blood |
| L56 | NT | Lund | 2007 | Blood |
| L55[a] | NT | Lund | 2007 | Blood |
| L20 | NT | Lund | 2007 | Blood |
| G14 | NT | Gothenburg | 2007 | Blood |
| M9 | NT | Malmö | 2008 | Blood |
| M13 | NT | Malmö | 2008 | Blood |
| KR223[a] | NT | Stockholm | 2008 | Blood |
| G17 | NT | Gothenburg | 2008 | Blood |
| M5 | NT | Malmö | 2009 | Blood |
| M4[a] | NT | Malmö | 2009 | Blood |
| M38 | NT | Malmö | 2009 | Blood |
| L26 | NT | Lund | 2009 | Blood |
| G40[a] | NT | Gothenburg | 2009 | Blood |
| Laboratory reference strains | | | | |
| CCUG 6881[a] | a | Unknown | Before 1973 | Unknown |
| CCUG 7315 | a | United States | 1941 | Respiratory tract |
| CCUG 4851[a] | c | United States | 1942 | Sputum |
| CCUG 4852 | c | New York, NY | Before 1950 | CSF |
| NCTC 8470[a] | d | Netherlands | 1937 | Throat |
| CCUG 15521[a] | e | Unknown | Before 1984 | Unknown |
| CCUG 6877 | NT | United States | 1941 | Unknown |

[a] To test reproducibility, extraction and MALDI-TOF MS measurements were also performed on a different instrument in a second laboratory.
[b] CSF, cerebrospinal fluid.
[c] NT, nontypeable.

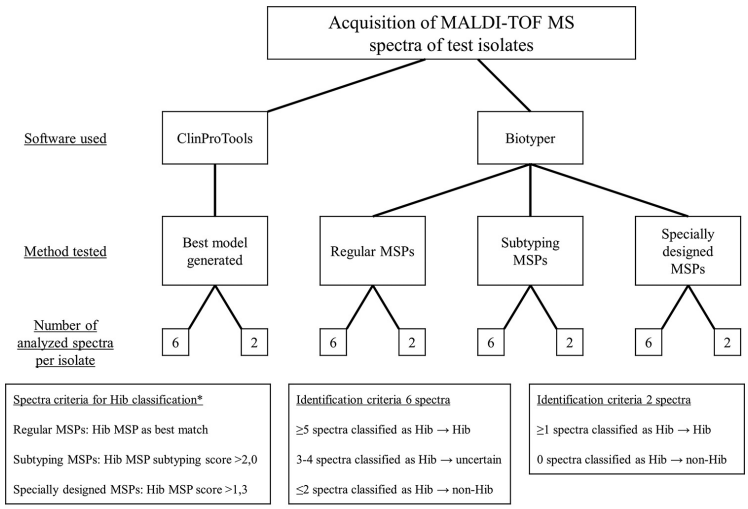


**FIG 2** Flowchart presenting the testing procedures of different Hib/non-Hib classification methods. The ClinProTools and MALDI Biotyper softwares were used to separate Hib and non-Hib test isolates. In ClinProTools, the best model was chosen for further testing. In Biotyper, regular MSPs, subtyping MSPs, and specially designed MSPs were used to classify test isolate spectra. Each method was tested with both 6 and 2 spectra per test isolate. Extraction and MALDI-TOF MS spectra acquisition were repeated on 19 Hib and 23 non-Hib isolates on a different MALDI-TOF MS instrument to test the reproducibility of the classification methods.

**Identification of Hib using specially designed Biotyper MSPs.** For all Hib reference isolates except KR194, which belongs to the less common of the two established genetic clusters of Hib, MSPs were generated. A score of >1.3 on any of the Hib MSPs was required for Hib classification.

When 6 spectra per isolate were used, 81.8% (27 of 33) of the Hib isolates were correctly identified. Three isolates were identified as uncertain, and three isolates were identified as non-Hib. All non-Hib isolates ($n = 77$) were identified as non-Hib. When analyzed using the ultrafleXtreme instrument, only 9 of 19 Hib isolates were assigned to the same category as by the microflex instrument. However, all non-Hib isolates were correctly identified, and the reproducibility was 76.2%.

When two spectra per isolate were used, 97.0% (32 of 33) of the Hib isolates and 98.7% (76 of 77) of the non-Hib isolates were correctly identified. When the measurements were repeated with the other instrument, 5 of 19 Hib isolates and 1 of 23 non-Hib isolates were assigned to a different category. Thus, 6 isolates changed categories, and the reproducibility was 85.7%.

## DISCUSSION

In the present study, we show that MALDI-TOF MS can be used to differentiate Hib from non-b *H. influenzae*. All classification algorithms that were generated and tested identified Hib among the *H. influenzae* isolates, with variations in sensitivity and specificity. The ClinProTools model had the best and most robust results, performing similarly to or better than models based on this software in previous studies (23–25).

A major strength in our work is the generation, testing, and comparison of several different classification algorithms to test our hypothesis. The careful selection of genetically different reference isolates is also important, especially since there have been reports on increased genetic diversity within the ST6 cluster since vaccination against Hib was introduced (26). Another strength of this study is the large selection of well-characterized isolates used to validate the classification algorithms. The isolates were collected from several geographical areas during a wide time range to ensure that the algorithms are applicable not just in a Scandinavian setting. The robustness of the algorithms was validated by repeated extraction and spectral acquisition on another MALDI-TOF MS instrument in a different laboratory.

One limitation of our study is that it has not been possible to fully evaluate the capacity of the different algorithms to identify Hib from the less common genetic cluster (represented by KR194 among our reference isolates). None of our test isolates (neither Hib nor non-Hib) were fully similar to KR194 in its capsule type-defining peaks during manual analysis, indicating that this genetic cluster most likely was not represented among our test isolates. This is not surprising, since isolates from this cluster are uncommon both in Sweden and worldwide (22). A search in the MLST database (www.mlst.net) reveals that isolates from this cluster are found mostly in Russia but also in a few other countries. In these countries, they still represent only a very small portion of Hib isolates. KR194 was, however, separable from the non-Hib isolates in manual analysis, suggesting that identification of this cluster with MALDI-TOF MS should be possible. It is likely that our methods do not identify the type b capsule itself but rather identify proteins common to the Hib population due to the clonal relationship of the isolates. It is therefore important to keep this in

**TABLE 2** MLST of Hib isolates and reference isolates used for creation of Hib/non-Hib classification methods

| Isolate | Capsular type | MLST results | | | | | | | | Location | Yr | Site of isolation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *adk* | *atpG* | *frdB* | *fucK* | *mdh* | *pgi* | *recA* | ST | | | |
| MLST of Hib isolates[a] | | | | | | | | | | | | |
| G4 | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Gothenburg | 2002 | Blood |
| G6[b] | b | 31 | 14 | 4 | 5 | 4 | 7 | 8 | 95 | Gothenburg | 2004 | Blood |
| G26 | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Gothenburg | 2009 | Blood |
| G32 | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Gothenburg | 2008 | CSF |
| KR169[b] | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Stockholm | 1998 | Blood |
| KR181 | b | 31 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Stockholm | 1998 | CSF |
| KR191[b] | b | 10 | 14 | 5 | 7 | 57 | 7 | 8 | 120 | Stockholm | 2004 | Blood |
| KR194[b] | b | 6 | 30 | 23 | 9 | 33 | 29 | 7 | New ST[c] | Stockholm | 2004 | Blood |
| KR209 | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Stockholm | 2009 | Blood |
| KR211 | b | 10 | 14 | 4 | 5 | 4 | New | 8 | New ST[d] | Stockholm | 2009 | Blood |
| L2 | b | 10 | New | 4 | 5 | 4 | 7 | 8 | New ST[e] | Lund | 2004 | Blood |
| L35 | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Lund | 2005 | Blood |
| L40 | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Lund | 2004 | Blood |
| L58 | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Lund | 2007 | Blood |
| L62[b] | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Lund | 2007 | Blood |
| L63 | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Lund | 2008 | Blood |
| M16[b] | b | 10 | 14 | 4 | 1 | 4 | 7 | 8 | 206 | Malmö | 2007 | Blood |
| M17[b] | b | 10 | 14 | 5 | 7 | 4 | New | 8 | New ST[d] | Malmö | 2007 | Blood |
| M41 | b | 10 | 14 | 4 | 5 | 4 | 7 | 8 | 6 | Malmö | 2004 | Blood |
| Non-Hib reference isolates | | | | | | | | | | | | |
| KR152[f] | a | 20 | 12 | 25 | 7 | 20 | 23 | 19 | 30 | Unknown | Unknown | Unknown |
| KR153[f] | c | 7 | 11 | 6 | 8 | 6 | 16 | 9 | 9 | Unknown | Unknown | Unknown |
| KR154[f] | d | 5 | 15 | 7 | 9 | 7 | 5 | 11 | 47 | Unknown | Unknown | Unknown |
| L13 | e | 18 | 6 | 3 | 7 | 10 | 28 | 12 | 18 | Lund | 2006 | Blood |
| M1 | f | 22 | 19 | 11 | 11 | 22 | 19 | 15 | 124 | Malmö | 2009 | Blood |
| G23 | NT[g] | 5 | 1 | 1 | 1 | 1 | 2 | 5 | 14 | Gothenburg | 2008 | Blood |
| G63 | NT | 14 | 7 | 13 | 7 | 17 | 13 | 17 | 57 | Gothenburg | 2010 | Blood |
| KR206 | NT | 11 | 2 | 15 | 8 | 28 | 26 | 3 | 34 | Stockholm | 2008 | Blood |
| L1 | NT | 1 | 1 | New | 14 | 9 | 14 | 3 | New ST[h] | Lund | 2004 | Blood |
| M15 | NT | 1 | 1 | 1 | 1 | 7 | 2 | 5 | New ST[i] | Malmö | 2008 | Blood |

[a] MLST was performed on 19 Hib isolates. The selection of Hib reference isolates was based on MLST and spectral characteristics. Hib not selected as reference isolates were used as test isolates for Hib/non-Hib classification methods. Non-Hib reference isolates and their characteristics are also presented.
[b] Selected as Hib reference isolate.
[c] Most similar to ST93 among previously known STs.
[d] Most similar to ST53 among previously known STs.
[e] Most similar to ST6 among previously known STs.
[f] Unfortunately, for the Hia, Hic, and Hid reference isolates, the location, year, and tissue from which they were isolated are unknown.
[g] NT, nontypeable.
[h] Most similar to ST103 among previously known STs.
[i] Most similar to ST3 among previously known STs.

mind and perhaps regularly evaluate the sensitivity of MALDI-TOF MS as a capsule typing method.

Two of the classification algorithms, the ClinProTools model and the Biotyper subtyping MSPs, showed better results. These algorithms reliably separated Hib from non-Hib isolates, and the separation could be fully or almost fully reproduced on another instrument in another laboratory. Reproducibility of a classification algorithm is important, since it increases the possibility of using MALDI-TOF MS in clinical routine diagnostics. The use of different instrument models (i.e., microflex and ultrafleXtreme) should not affect the test results. Each MALDI-TOF MS instrument should, however, be individually calibrated for optimal outcome. The Biotyper subtyping MSPs had lower sensitivity than the ClinProTools model when 6 spectra per isolates were used for testing. Our requirements for Hib identification (≥5 of 6 spectra classified as Hib) were high, considering that the Hib isolate that was not correctly identified in the original measurement had four spectra classified as Hib, while no isolate in the non-Hib group had any spectra classified as Hib using our method. It was therefore not surprising that the sensitivity for the Biotyper subtyping MSPs improved when using only two spectra per test isolate, while the specificity remained high.

Regular Biotyper MSPs performed somewhat less well in separating Hib and non-Hib isolates, even though it cannot be ruled out that the results could be improved with optimized instrument settings. The slightly poorer performance, however, was not unexpected, as regular MSPs take all peaks of an isolate into account when generating a score. Since this study investigated subtyping within one species, the spectral patterns as a whole were much alike, increasing the risk of misidentification using regular MSPs. The advantage of the ClinProTools model and the Biotyper subtyping MSPs is that spectral differences, rather than similarities,

**TABLE 3** Evaluation of different MALDI-TOF MS Hib/non-Hib classification methods

| Results by no. of spectra for: | Hib ($n = 33$) | Non-Hib ($n = 77$) | Sensitivity (%)[a] | Specificity (%)[a] | AU-ROC[b] | Reproducibility (%)[c] |
|---|---|---|---|---|---|---|
| ClinProTools | | | | | | |
| 6 spectra | | | 100 | 98.7 | 0.994 | 97.6 |
| Hib | 33 | 1 | | | | |
| Uncertain | 0 | 0 | | | | |
| Non-Hib | 0 | 76 | | | | |
| 2 spectra | | | 100 | 97.4 | 0.987 | 100 |
| Hib | 33 | 2 | | | | |
| Non-Hib | 0 | 75 | | | | |
| Biotyper MSPs: | | | | | | |
| Regular | | | | | | |
| 6 spectra | | | 100 | 98.7 | 0.994 | 90.5 |
| Hib | 33 | 0 | | | | |
| Uncertain | 0 | 1 | | | | |
| Non-Hib | 0 | 76 | | | | |
| 2 spectra | | | 100 | 97.4 | 0.987 | 95.2 |
| Hib | 33 | 2 | | | | |
| Non-Hib | 0 | 75 | | | | |
| Subtyping | | | | | | |
| 6 spectra | | | 97.0 | 100 | 0.985 | 92.9[d] |
| Hib | 32 | 0 | | | | |
| Uncertain | 1 | 0 | | | | |
| Non-Hib | 0 | 77 | | | | |
| 2 spectra | | | 100 | 100 | 1.000 | 100[d] |
| Hib | 33 | 0 | | | | |
| Non-Hib | 0 | 77 | | | | |
| Specially designed | | | | | | |
| 6 spectra | | | 81.8 | 100 | 0.909 | 76.2 |
| Hib | 27 | 0 | | | | |
| Uncertain | 3 | 0 | | | | |
| Non-Hib | 3 | 77 | | | | |
| 2 spectra | | | 97.0 | 98.7 | 0.978 | 85.7 |
| Hib | 32 | 1 | | | | |
| Non-Hib | 1 | 76 | | | | |

[a] Sensitivity and specificity were calculated based on the results from the original laboratory (Malmö).
[b] Area under the receiver operator characteristic curve calculated based on correct or false result from the measurements with the microflex instrument compared to those with PCR. All uncertain isolates were considered falsely identified. AU-ROC values were calculated using SPSS Statistics 22.0 (IBM, Armonk, NY).
[c] Reproducibility was calculated as the percentage of isolates MALDI-TOF MS measured on both the microflex and ultrafleXtreme instrument, which were identified to the same category (Hib, uncertain, or non-Hib) on both instruments.
[d] Reproducibility with adjusted cutoff.

are highlighted. This might also explain the slightly lower reproducibility of the regular Biotyper MSPs. The creation of specially designed MSPs with only two peaks was performed to test if these peaks were sufficient for Hib/non-Hib differentiation. Regarding reproducibility, this classification method was not, however, as robust as the ClinProTools model and subtyping MSPs, indicating that it is more sensitive to differences in spectral quality and between different MALDI-TOF MS instruments.

One isolate (NTHi KR183) was incorrectly classified as Hib using ClinProTools. The known genetic variation within the NTHi population makes the design of a set model with full specificity for NTHi isolates challenging. However, if these novel methods are used in a clinical setting, it would likely be used as a screening for Hib among *H. influenzae* isolates. In this case, sensitivity, which was very good in both of our best methods, is of higher priority than specificity. Hib isolates identified in a MALDI-TOF MS screening should be confirmed by capsule PCR.

Several studies have illustrated the value of MALDI-TOF MS in difficult microbiological diagnostics and bacterial subtyping. Bruin et al. (27) showed that MALDI-TOF MS effectively differentiated *H. influenzae* from the commensal *Haemophilus haemolyticus*, which has been difficult with traditional microbiological techniques. A few recent studies have also been published that use ClinProTools software for bacterial classification, for example, for the differentiation of *Shigella* species from *E. coli* and *Streptococcus pneumoniae* from *Streptococcus mitis* (23, 24). These studies also used ethanol-formic acid extraction. The direct transfer method and extended direct transfer method were used on a sample of test isolates in this study to test our classification methods. However, the results were less accurate than when ethanol-formic acid extraction was used. It is likely that small spectral differences and low-intensity peaks, although constant, are not as clear when using the simpler preparation methods before mass spectrometry measurement. This is not surprising, since it is well known that the quality and reproducibility of weaker peaks are better when the extraction method is used, and the method results in improved scores with the MALDI Biotyper classification software (28).

In conclusion, the present study shows that rapid identification

of Hib among *H. influenzae* is possible using a routine sample preparation and MALDI-TOF MS analysis of *H. influenzae* isolates. After methodological optimization, this novel finding may be implemented in clinical microbiology laboratories. *H. influenzae* isolates identified by standard MALDI-TOF MS analysis could then be directly reanalyzed using the ethanol-formic acid extraction procedure to identify Hib isolates. This would improve the efficiency of Hib identification and make rapid intervention possible in cases of vaccine failure or poor vaccination coverage. MALDI-TOF MS could also be used as a surveillance tool in vaccinated populations to monitor sustained vaccine efficacy and as an initial screening method of invasive *H. influenzae* isolates.

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# Paper II

# Capsule Typing of *Haemophilus influenzae* by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry[1]

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Encapsulated *Haemophilus influenzae* strains belong to type-specific genetic lineages. Reliable capsule typing requires PCR, but a more efficient method would be useful. We evaluated capsule typing by using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Isolates of all capsule types (a–f and nontypeable; n = 258) and isogenic capsule transformants (types a-d) were investigated. Principal component and biomarker analyses of mass spectra showed clustering, and mass peaks correlated with capsule type-specific genetic lineages. We used 31 selected isolates to construct a capsule typing database. Validation with the remaining isolates (n = 227) showed 100% sensitivity and 92.2% specificity for encapsulated strains (a–f; n = 61). Blinded validation of a supplemented database (n = 50) using clinical isolates (n = 126) showed 100% sensitivity and 100% specificity for encapsulated strains (b, e, and f; n = 28). MALDI-TOF mass spectrometry is an accurate method for capsule typing of *H. influenzae*.

*Haemophilus influenzae* is subdivided into encapsulated strains, which express different serotypes of capsular polysaccharide (designated types a–f), and nonencapsulated strains, which are designated nontypeable *H. influenzae* (NTHi) (*1*). Since the introduction of conjugate vaccines against *H. influenzae* type b (Hib), a common cause of meningitis, epiglottitis, and sepsis in small children, the epidemiology of invasive *H. influenzae* disease has changed dramatically, with an increase in the diversity of serotypes responsible for illness.

Although the incidence of Hib disease has decreased in countries implementing childhood vaccination (*2*), invasive disease caused by NTHi has become more prominent during the same period, especially among newborns and the elderly (*3*–*6*). In the postvaccination era, increasing incidences and outbreaks of invasive *H. influenzae* type a (Hia) infections have been reported in South and North America (*7*–*10*), particularly among the indigenous populations in Canada and the United States (*7,8,10*). Studies have also suggested an increase in cases of invasive *H. influenzae* type e (Hie) and type f (Hif) disease (*4,11,12*). Hib vaccine failures have been described (*13*), and omission of booster dose(s) appears to result in a rapidly increased incidence of invasive Hib disease (*14,15*), suggesting continued circulation of Hib isolates in the community. Globally, one third of eligible children still do not receive adequate vaccination (*16*).

Encapsulated *H. influenzae* strains are generally genetically clonal. This finding was first demonstrated by multilocus enzyme electrophoresis, by which encapsulated isolates could be separated into different genetic lineages that correspond to different capsule types (*17*). This clonal population structure has been confirmed by multilocus sequence typing (MLST), which assigns isolates to different sequence types (STs), although some differences have been observed in the organization of different lineages (*18*). There are 3 known major genetic groups of Hia and Hib (*18,19*). Two genetic groups of Hia (related to ST21 and ST23) account for most Hia isolates in the MLST database (*20*). For Hib, ST6-related isolates account for most cases (*17,18*), whereas the second most common genetic group is related to ST222 (*18,21*). There is 1 known lineage each for serotypes c through f (*18,19*). In contrast, NTHi are genetically heterogenous (*19*).

Capsule typing of *H. influenzae* has traditionally been performed by using slide agglutination with antisera (conventional serotyping), but incorrect results are common,

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[2]These senior authors contributed equally to this article.

and specificity for encapsulated isolates is low (*22,23*). Determination of presence of the capsule gene complex (*bexA* or *bexB*) by PCR, followed by type-specific *cap* a–f PCRs has excellent sensitivity and specificity but is laborious and time-consuming (*24–27*). Because of limitations of current typing methods, typing might be delayed or not performed in clinical practice. However, rapidly obtained information on capsule type is still of interest for the treating clinician (*5*) and, in particular, for monitoring of capsule type distribution and effectiveness of Hib vaccination programs, especially with respect to invasive disease.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is commonly used to identify bacterial and fungal species, including *H. influenza*e, by analyzing the composition of ribosomal proteins in a sample. It is a rapid and convenient method and has a low cost per sample (*28*). Recently, we have shown that MALDI-TOF mass spectrometry can separate Hib from non-b *H. influenzae* (*29*). In this study, we examined the capacity of MALDI-TOF mass spectrometry to perform full capsule typing of *H. influenzae*. This method would be valuable for first-line diagnostics of *H. influenzae* to identify patients at risk for immunodeficiency or anatomic cerebrospinal fluid space defect, and to detect rapidly outbreaks caused by specific capsule types. It would also increase time and cost effectiveness of surveillance of *H. influenzae* epidemiology and Hib vaccination efficacy.

## Materials and Methods

### Bacterial Isolates

We used 2 culture collections in this study (Figure 1). The first collection was an evaluation set of isolates used to construct a coherent reference database and was composed of 258 *H. influenzae* strains. It included isolates from 3 major clinical laboratories in Sweden (Malmö/Lund, Gothenburg, and Stockholm) obtained in 1997–2011 but also a wide range of international strains from different countries, continents, and time periods (n = 41; online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/3/17-0459-Techapp1.pdf). In addition, we included 4 isogenic capsule-transformed strains of types a ($Rb^-/a^+$:02), b ($Rb^+$:02), c ($Rb^-/c^+$:02), and d ($Rb^-/d^+$:02) (*30*) in the study. These strains originate from strain Rd, a capsule-deficient type d strain (*31*). For validation of the new MALDI-TOF mass spectrometry typing method, we used a second collection composed of 126 bloodstream and cerebrospinal fluid *H. influenzae* isolates obtained in Sweden during 2010 and 2013–2016. All isolates were identified as *H. influenzae* by using standard laboratory taxonomy techniques and were grown on chocolate agar plates overnight (18–24 h) in a humid atmosphere at 37°C containing 5% $CO_2$ before any experiments were conducted.

### PCR for Capsule Typing and MLST

We prepared DNA by adding a few colonies of bacteria to distilled water. After heating at 98°C for 10 min, we centrifuged each sample at 16,000 × *g* for 5–10 min and collected the supernatant. In a few instances, we extracted DNA by using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. We performed capsule typing by PCR using *bexB* and type-specific *cap* primers for all isolates as described (*24–26*).

We performed PCR for MLST genes as described (*18*) and sequenced the resulting PCR products by using the forward primer and, if necessary for adequate sequence quality, the reverse primer. We trimmed and

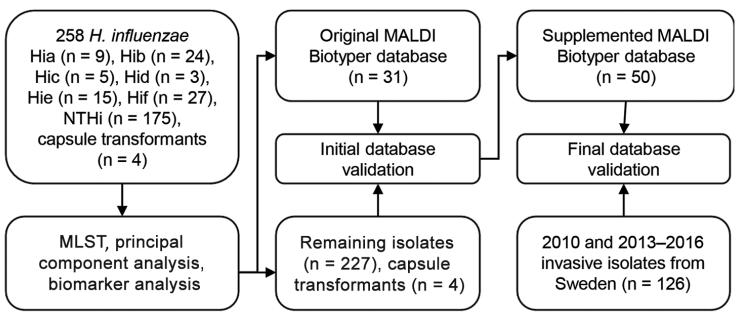


**Figure 1.** Culture collections and methods used to investigate capsule typing of *Haemophilus influenzae* by MALDI-TOF mass spectrometry. An evaluation set of *H. influenzae* isolates of all capsule types from diverse geographic origins and time periods and isogenic capsule transformants (*30*) were used to investigate capsule type-specific differences in MALDI-TOF mass spectra. MLST was used to ensure adequate coverage of different genetic lineages of encapsulated *H. influenzae*. Reference isolates from the evaluation set (encapsulated and nonencapsulated) were selected to construct a new typing database in MALDI Biotyper. This database was tested with the remaining isolates in the set, and misclassified isolates were added to the database. The final supplemented database was blindly validated with a second culture collection that consisted of clinical invasive isolates. Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MLST, multilocus sequence typing; NTHi, nontypeable *H. influenzae*.

edited sequences before concatenation (total length 3,057 bp). We deposited MLST nucleotide sequences in GenBank (accession nos. MG550316–MG550889). Some isolates had been previously typed by MLST. In these instances, we retrieved MLST data from the MLST database (*20*).

### Analysis of MLST Data

We determined sequence types by using the MLST database. We aligned concatenated sequences in Geneious 9.1.8 (Biomatters, Auckland, New Zealand) and used the PAUP* 4.0a158 plug-in (http://phylosolutions.com/paup-test/) to construct a maximum-likelihood phylogenetic tree. The best fitting model was estimated to be the generalized time-reversible model including invariant sites and gamma distribution by using the Akaike information criterion in jModelTest 2.1.10 (*32,33*). We visualized the resulting tree by using FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). All isolate and ST information has been submitted to the MLST database.

### Acquisition of MALDI-TOF Mass Spectrometry Data

We acquired mass spectra by using a Microflex LT MALDI-TOF mass spectrometry system (Bruker Daltonics, Bremen, Germany), with default settings as described (*29*). We prepared all isolates for acquisition of spectra by using the ethanol–formic acid procedure described by the instrument manufacturer. We spotted isolates on 2 spots and analyzed each spot 3 times, resulting in 6 spectra/isolate. Isolates in the reference database were spotted on 8 spots, resulting in 24 spectra, before being added to the database.

### Analysis of MALDI-TOF Mass Spectrometry Data

In Mass-Up 1.0.13 (*34*), we preprocessed and analyzed raw spectra of all isolates in the evaluation set (n = 258) and capsule transformants (n = 4) by using the integrated MALDIquant analysis package for R (http://strimmerlab.org/software/maldiquant/). We performed preprocessing with intensity transformation (square root), smoothing (Savitzky–Golay), baseline correction (Top-Hat), and intensity standardization (total ion current). We performed peak detection with a signal-to-noise ratio of 2, a half window size of 50, and no minimum peak intensity. We calculated a consensus spectrum for each isolate with a peak tolerance of 0.002 and percentage of presence of 60%. For principal component analysis (PCA) (*35*) and biomarker analysis, we performed intersample matching with a peak tolerance of 0.002. PCA was performed with default settings (maximum number of components = –1 and 0.95 of the total variance covered). In the biomarker analysis, we calculated a p value for each peak by using the randomization test of independence.

### Construction and Validation of a MALDI Biotyper Database for Capsule Typing

We used selected isolates from the evaluation set to create main spectra (MSPs) for a new MALDI Biotyper 4.1 database (Bruker Daltonics) (Figure 1). These reference isolates were selected to represent all capsule types and genetic lineages. NTHi strains were selected with the aim of including isolates of all known genetic clades (*19*). Spectra of reference isolates were controlled by using FlexAnalysis (Bruker Daltonics). We performed smoothing (Savitzky-Golay) and baseline correction (Top-Hat) and excluded spectra with outlier appearance (lacking or having an extra peak) and low quality (peaks outside a 500 ppm range). If <20 spectra remained after the control, new spectra for that specific isolate were obtained. We used default settings for spectra preprocessing, MSP creation, and identification as described (*29*).

We used isolates in the evaluation set not selected as reference isolates for initial validation of the database (Figure 1). Because all isolates were *H. influenzae*, high score values (≥2.0) were expected. Thus, we classified each spectrum according to the top matching MSP in the new database. For isolate classification, ≥5/6 spectra classified to the same type (a–f or NTHi) were required. If ≤4/6 spectra were classified to the same type, the isolate was classified as inconclusive. To improve the specificity of the typing method, considering the known heterogeneity of NTHi, we supplemented the capsule typing database with NTHi isolates not correctly classified in the initial validation until all isolates in the evaluation set not included in the database were correctly classified on every single spectrum. Finally, we blindly validated the supplemented database by using MALDI-TOF mass spectrometry classification of invasive isolates (n = 126) obtained during 2010 and 2013–2016 and calculated sensitivity and specificity by using PCR typing as the standard (Figure 1).

## Results

### Genetic Lineages of Encapsulated *H. influenzae* in the Evaluation Set

To construct a clinically useful reference database for capsule typing by MALDI-TOF mass spectrometry, we aimed to identify and collect isolates from all known lineages of encapsulated *H. influenzae* (Figure 2; online Technical Appendix Table). We performed MLST for all Hia (n = 9) and Hib isolates (n = 24) in the evaluation set, in addition to a subset of isolates of other capsule types (c–f), capsule transformants (n = 4), and NTHi. Phylogenetic analysis confirmed that the collection contained isolates from different genetic lineages of encapsulated *H. influenzae*, including the 2 major genetic groups of type a and all 3 lineages of type b (Figure 2). Capsule transformants belonged

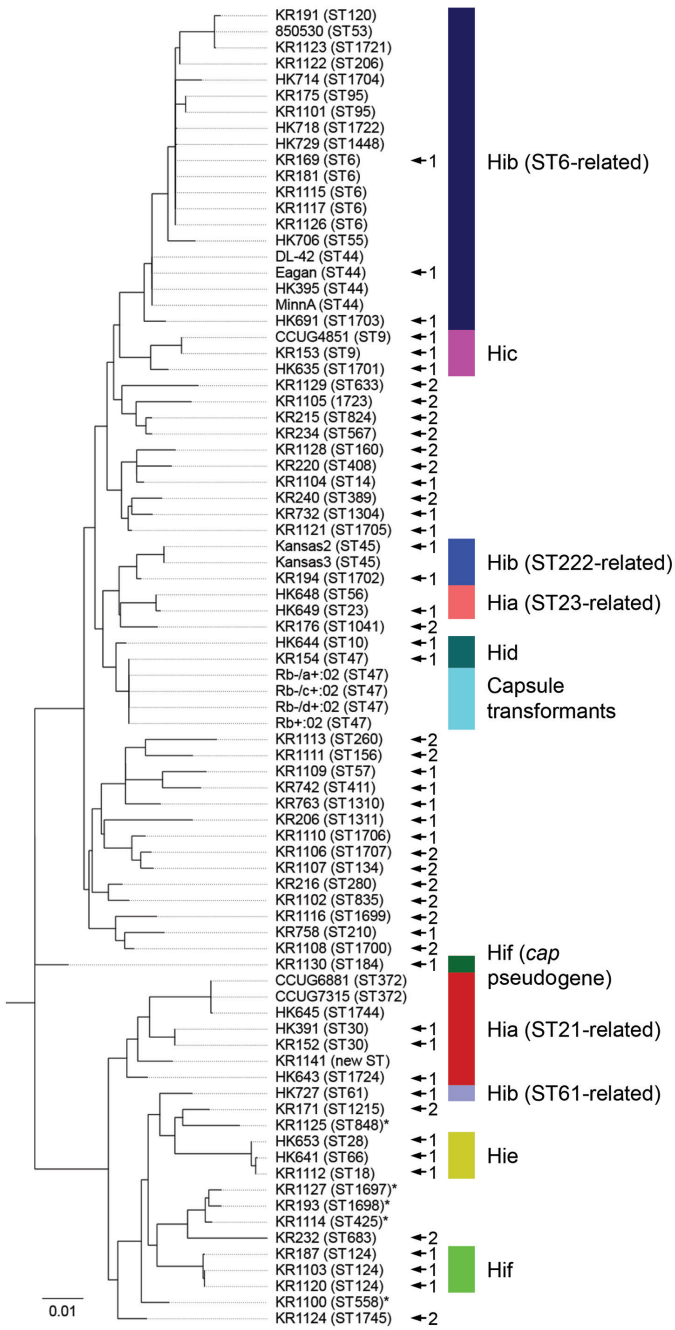


**Figure 2.** Multilocus sequencing typing (MLST) of encapsulated and nonencapsulated *Haemophilus influenzae* isolates. MLST was performed on a subset of encapsulated isolates (n = 44) from the evaluation set, including all type a and type b isolates (n = 33). All major genetic lineages (indicated by colors), except the least common lineage of Hia (ST4-related), of encapsulated *H. influenzae* were represented in the collection, including the 2 more common lineages of Hia and all 3 lineages of Hib. An isolate typed by PCR as Hif (KR1130) with a nonexpressed pseudogene *cap* locus was also included. This isolate was not part of the established Hif lineage, and was initially suspected to be an outlier on the basis of differences from other Hif in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra. All capsule transformants (n = 4) were ST47 (same as the parental strain Rd) (*30,31*) and were part of the Hid lineage. Nontypeable *H. influenzae* (NTHi; no color) included as reference isolates in the capsule typing databases (n = 28) were included in the analysis. NTHi in the evaluation set misclassified as type e (n = 5, indicated by asterisks) were also included. These isolates belonged to 3 separate genetic lineages, all related to the Hie lineage. Isolates included as references in MALDI-TOF mass spectrometry databases are indicated by arrows and numbers (1 for isolates in the original database and 2 for isolates added during supplementation of the database). Scale bar indicates nucleotide substitutions per site. Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; ST, sequence type.

to the known genetic lineage of Hid isolates (Figure 2) and were the same ST as the parental strain Rd (*18*). One isolate (KR1130) was typed by PCR as Hif (*bexB*- and *cap f*-positive) but phylogenetically belonged to a lineage separate from all other Hif isolates. Thus, this isolate was not part of the established, ST124-related Hif lineage (Figure 2). The *cap* locus of this isolate was sequenced and found to be a nonexpressed pseudogene (data not shown).

### MALDI-TOF Mass Spectrometry of Genetic Lineages of Encapsulated *H. influenzae*

We performed PCA for all isolates in the evaluation set (n = 258) and the capsule transformants (n = 4). As expected, NTHi formed a large heterogeneous group, but clustering of encapsulated isolates of the same capsule types was found (Figure 3, panel A). When PCA was performed on encapsulated isolates (n = 83) and capsule transformants (n = 4) only, the clustering became clearer and was particularly evident for Hib, Hie, and Hif isolates (Figure 3, panel B). Encapsulated isolates segregated in groups according to capsule type and, for Hia and Hib isolates, by genetic lineage according to MLST (Figure 2; 3, panel B). Capsule transformants were found as a separate group in close proximity of Hid isolates and not distributed according to their respective capsule type (Figure 3, panel B). Isolate KR1130 did not cluster with Hif isolates of the ST124-related lineage (Figure 3, panel B).

Biomarker analysis of encapsulated isolates and capsule transformants identified several peaks conserved within the different genetic lineages of capsule types, indicating the possibility of separating them on the basis of MALDI-TOF mass spectra (Figure 4). Capsule transformants expressed similar peak patterns relative to each other but differed in many peaks when compared with wild-type strains of the same capsule types.

### Sensitivity and Specificity of Automated Capsule Typing by MALDI-TOF Mass Spectrometry

An initial capsule typing reference database was constructed in MALDI Biotyper. Encapsulated isolates (n = 22) representing all major genetic lineages of encapsulated *H. influenzae* were included (Figure 2). To ensure adequate coverage of potential variation within each lineage, multiple reference isolates were chosen for each lineage (when possible) on the basis of geographic origin and variations in mass spectra. In addition, NTHi (n = 9) representing 8 of 10 known genetic clades of NTHi (*19*) were included in the database (Figure 2).

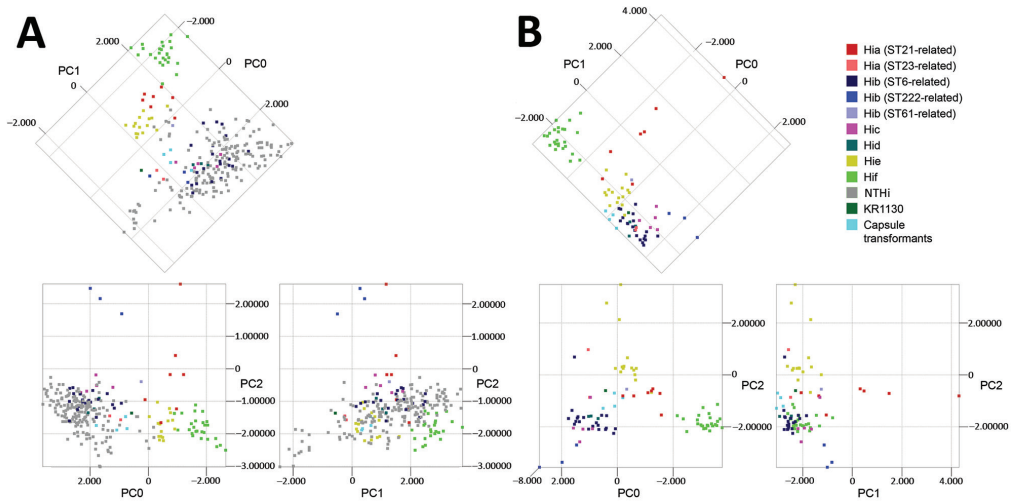


**Figure 3.** Principal component analysis (PCA) of matrix-assisted laser desorption/ionization time-of-flight mass spectra of encapsulated and nonencapsulated *Haemophilus influenzae*. A) PCA of all isolates (n = 258) of *H. influenzae* in the evaluation set representing all capsule types, which are color-coded according to capsule type and for Hia and Hib isolates by genetic lineage as shown by multilocus sequence typing (MLST) and capsule transformants (n = 4). The first 3 principal components (PC0, PC1, and PC2) are shown in 2-dimensional plots. Analysis showed the diversity of nontypeable *H. influenzae* (NTHi). Encapsulated isolates showed discrete clustering, which was further evaluated by PCA of encapsulated isolates separately. B) PCA of encapsulated isolates in the evaluation set (n = 83) and capsule transformants (n = 4) presented and color-coded as in panel A. Clustering of isolates on the basis of capsule type was evident, particularly for Hib, Hie, and Hif isolates. Different genetic lineages of the same capsule type (Hia and Hib) clustered separately. KR1130 (with a pseudogene type f *cap* locus) did not cluster with the other Hif isolates. Capsule transformants clustered together in proximity of Hid isolates, and not with their respective capsule type. Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; ST, sequence type.

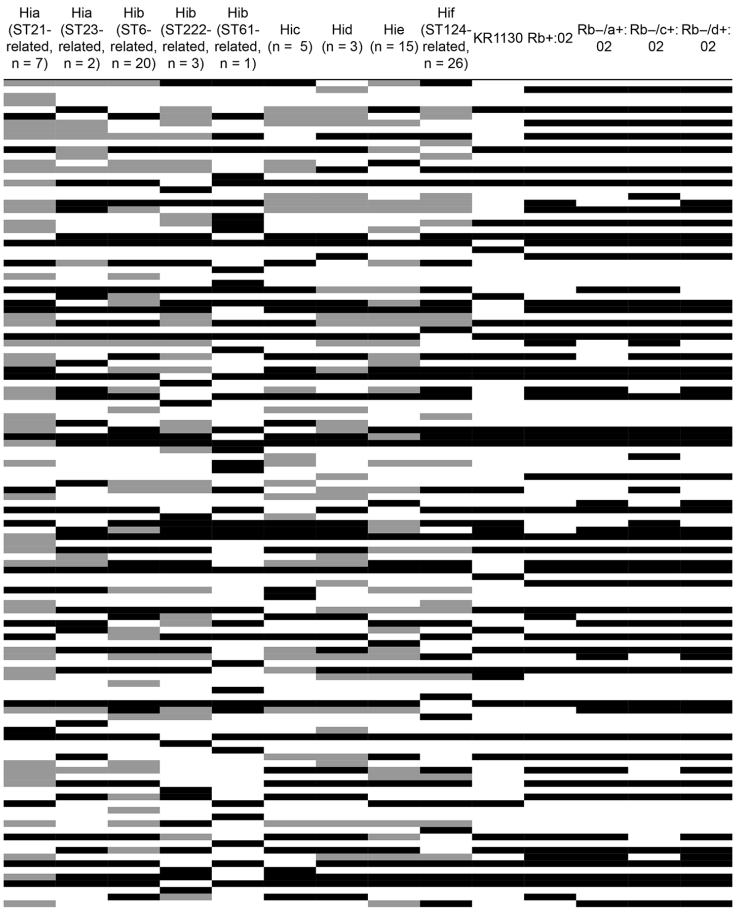


**Figure 4.** Biomarker analysis of matrix-assisted laser desorption/ionization time-of-flight mass spectra of encapsulated *Haemophilus influenzae*. Analysis was performed on all encapsulated isolates in the evaluation set (n = 83) and capsule transformants (n = 4). Rows represent peaks (2,000–20,000 m/z in descending order), and columns represent groups of encapsulated *H. influenzae*. A total of 124 peaks with discriminatory power ($p<0.05$) between different capsule types and genetic lineages were identified. Peak expression is indicated by shades of black (black >75%, gray ≥25% but ≤75%, and white <25% of isolates in the group express the peak). Several peaks conserved within capsule types and genetic lineages with the possibility for separation were observed, as indicated by the mosaic of peak patterns. Capsule transformants showed similar peak patterns, and lacked many of the capsule type-specific peaks for their respective phenotypic capsule types. KR1130 expressed different peaks than Hif of the ST124-related lineage. Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; ST, sequence type.

Validation of the original database (n = 31) using the remaining isolates in the evaluation set (n = 227) showed 100% sensitivity for encapsulated isolates (Table 1), and every isolate was correctly classified on every spectrum. All capsule transformants were classified as type d, the original serotype of the parental strain Rd (*31*). No isolate matched KR1130, the isolate typed by PCR as Hif with a pseudogene *cap* locus.

A few NTHi were either inconclusively typed or misclassified as encapsulated, resulting in reduced specificity for encapsulated isolates (Table 1). For this reason, we supplemented the capsule typing database with misclassified NTHi from the evaluation set until the database correctly classified all the remaining isolates in the evaluation set on every single spectrum. This modification resulted in an additional 19 NTHi being added to the MALDI Biotyper database (Figure 2). When PCA was performed separately for NTHi in the evaluation set, it was evident that the supplemented database covered the heterogeneity of NTHi better than the original database (Figure 5). The same finding was evident from phylogenetic analysis (Figure 2). Five NTHi were misclassified as Hie and could not be added to the database because they interfered with classification of true Hie isolates and would decrease sensitivity for Hie (Figure 2).

As a final performance test, we blindly validated the supplemented database (n = 50) by using a separate culture collection consisting of clinical invasive isolates from Sweden (n = 126) obtained during 2010 and 2013–2016. When we compared MALDI-TOF mass spectrometry capsule typing results with PCR capsule typing results, all encapsulated isolates (types b, e, and f; n = 28)

**Table 1.** Validation of the original MALDI-TOF mass spectrometry capsule typing database (n = 31) by classification of the remaining 227 isolates in the evaluation set and 4 capsule transformants of *Haemophilus influenzae**

| Capsule type | No. | No. correct† | No. inconclusive‡ | No. incorrect§ | Sensitivity, % | Specificity, % |
|---|---|---|---|---|---|---|
| Hia, ST21-related | 4 | 4 | 0 | 0 | 100 | 99.1 |
| Hia, ST23-related | 1 | 1 | 0 | 0 | 100 | 100 |
| Hib, ST6-related | 17 | 17 | 0 | 0 | 100 | 100 |
| Hib, ST222-related | 1 | 1 | 0 | 0 | 100 | 100 |
| Hic | 2 | 2 | 0 | 0 | 100 | 100 |
| Hid | 1 | 1 | 0 | 0 | 100 | 98.2 |
| Hie | 12 | 12 | 0 | 0 | 100 | 97.7 |
| Hif | 23 | 23¶ | 0 | 0 | 100 | 99.0 |
| All encapsulated isolates, a–f | 61 | 61 | 0 | 0 | 100 | 92.2 |
| NTHi | 166 | 122 | 31 | 13# | 73.5 | 100 |
| Rb-negative capsule transformants | 4 | 1** | 0 | 3** | NA | NA |

*Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NA, not applicable; NTHi, nontypeable *H. Influenzae*; ST, sequence type.
†≥5/6 spectra classified to the same correct type.
‡≤4/6 spectra classified to the same type.
§≥5/6 spectra classified to the same incorrect type.
¶All classified to the established ST124-related Hif lineage.
#Two isolates classified as Hia (ST21-related), 4 as Hid, 5 as Hie, and 2 as Hif (ST124-related).
**All classified as type d (i.e., the same type as the parental strain Rd) (*30,31*), resulting in correct classification of isolate Rb–/d+:02 and incorrect classification of isolates Rb+:02, Rb–/a+:02, and Rb–/c+:02.

were correctly classified on every single spectrum (Table 2). Of 98 NTHi, only 5 were not correctly classified. These isolates were all classified as inconclusive. Thus, no NTHi was incorrectly classified as encapsulated, and the resulting sensitivity and specificity of capsule typing was 100% in the final validation (Table 2).

## Discussion

In this study, we have shown that encapsulated *H. influenzae* have different MALDI-TOF mass spectra that correlate with genetic lineages representing different capsule types. We have demonstrated that, after construction of a comprehensive reference database, routine MALDI-TOF mass

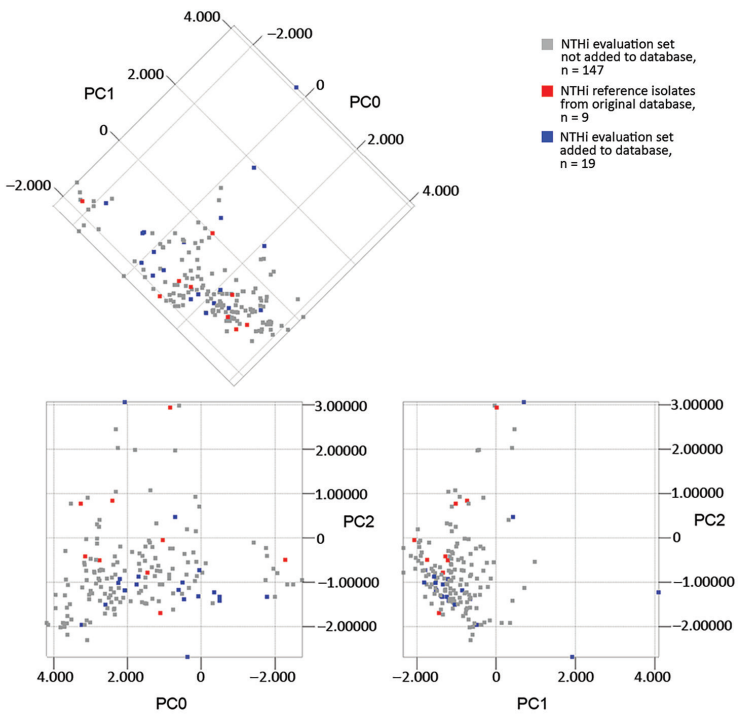


**Figure 5.** Principal component analysis of matrix-assisted laser desorption/ionization time-of-flight mass spectra of NTHi in the original and supplemented databases. Isolates are color-coded according to database affiliation, and the first 3 principal components (PC0, PC1, and PC2) are shown in 2-dimensional plots. No clustering similar to that for encapsulated isolates was observed. NTHi reference isolates in the original capsule typing database (n = 9), representing different genetic clades, were evenly distributed in the group. Supplementing the reference database with another 19 isolates improved coverage of the heterogeneity of NTHi. NTHi, nontypeable *H. influenzae*; PCA, principal component analysis.

**Table 2.** Validation of the supplemented MALDI-TOF mass spectrometry capsule typing database (n = 50) by classification of 126 invasive isolates of *Haemophilus influenzae* from Sweden*

| Capsule type | No. tested | No. correct† | No inconclusive‡ | No. incorrect§ | Sensitivity, % | Specificity, % |
|---|---|---|---|---|---|---|
| Hib | 8 | 8¶ | 0 | 0 | 100 | 100 |
| Hie | 5 | 5 | 0 | 0 | 100 | 100 |
| Hif | 15 | 15# | 0 | 0 | 100 | 100 |
| All encapsulated isolates (b, e, and f) | 28 | 28 | 0 | 0 | 100 | 100 |
| NTHi | 98 | 93 | 5 | 0 | 94.9 | 100 |

*Hib, *H. influenzae* type b; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NTHi, nontypeable *H. influenzae*; ST, sequence type.
†≥5/6 spectra classified to the same correct type.
‡≤4/6 spectra classified to the same type.
§≥5/6 spectra classified to the same incorrect type.
¶All classified to the ST6-related Hib lineage.
#All classified to the established ST124-related Hif lineage.

spectrometry analysis has excellent capacity to identify type-specific genetic lineages associated with encapsulated *H. influenzae* and thereby can be used for capsule typing of *H. influenzae*.

Our study had several strengths. We analyzed a large collection of well-characterized strains collected at different times from various geographic regions to ensure the robustness of our findings. Using MLST, we ensured adequate coverage of the major genetic lineages of encapsulated *H. influenzae* in the MALDI-TOF mass spectrometry reference database. Moreover, the database was carefully evaluated and supplemented to ensure adequate coverage of the heterogeneity of NTHi. We blindly validated the supplemented database to mimic an authentic clinical or epidemiologic situation and demonstrated excellent sensitivity and specificity compared with conventional PCR-based typing. During construction of the capsule typing database, we identified several isolates previously typed by PCR or agglutination (by us or others) in which the MALDI-TOF mass spectrometry results did not match the suggested capsule type. When we retyped these isolates by PCR, the capsule type suggested by MALDI-TOF mass spectrometry proved to be correct in all instances (except for the NTHi typed as Hie) (Figure 2), and isolates were reassigned to a new capsule type, further supporting the capacity of MALDI-TOF mass spectrometry for capsule typing.

Our study had some limitations. The first limitation reflects the limited availability of some rare variants. Our collection contained no Hia isolates belonging to the uncommon ST4-related genetic group. For the ST61-related lineage of Hib, we had access to only 1 isolate, which was included in the reference database and thus not represented in the test collection. However, our ST61 isolate was separable when mass spectra were analyzed by PCA and biomarker analysis, and no isolate was misclassified to this lineage in the initial or final validation of the typing databases. Furthermore, we have demonstrated that identification of the ST222-related Hib lineage by MALDI-TOF mass spectrometry is possible (Table 1), which was not the case previously (*29*). The second potential limitation arises through the genetic heterogeneity of NTHi, making adequate representation in the reference database a challenge (*19*). This limitation was apparent during the initial evaluation of the typing method, when some NTHi were misclassified. To address this issue, we supplemented the database with 19 additional reference NTHi strains. The final validation of our typing method demonstrated excellent specificity for NTHi, but the sensitivity for identifying encapsulated isolates remained unchanged. Because most invasive infections in countries implementing Hib vaccination are caused by NTHi, a high specificity is desirable (*3*–*5*).

MALDI-TOF mass spectrometry has proved valuable in subtyping several clinically relevant bacteria, including *Clostridium difficile* (*36*), methicillin-resistant *Staphylococcus aureus* (*37,38*), and enterohemorrhagic *Escherichia coli* (*39*). Subtyping generally relies on common genetic differences between isolates, reflected in the composition of the proteins measured. In our study, wild-type isolates of different capsule types could be separated, but isogenic capsule transformants could not. These isolates were classified as type d, the original capsule type of the parental strain Rd. This finding confirms that capsule type identification is based on a proxy identification of genetic lineage, rather than identification of capsule biosynthesis-associated proteins. Thus, our method is an indirect typing method, as opposed to serotyping, which identifies the capsule polysaccharide, and PCR, which identifies the capsule gene complex directly.

Although there is little evidence that new lineages of encapsulated *H. influenzae* have appeared historically, novel lineages of encapsulated strains might appear and be missed by the method. Isolate KR1130 used in this study was initially suspected to represent such a lineage. However, its *cap* locus was shown to be on a nonexpressed pseudogene. Only 1 other isolate of the same ST (ST184) is currently registered in the MLST database, and it is a nontypeable isolate. No other Hif strain in this study or the MLST database belongs to this genetic lineage (*20*).

One advantage of indirect capsule type identification by MALDI-TOF mass spectrometry is that determination of genetic lineage of encapsulated isolates can be made without further analysis. The method can also identify

previously encapsulated capsule-deficient strains, which have lost parts or all of the *cap* locus, either during infection or laboratory handling (*40*–*42*).

A concern regarding subtyping by MALDI-TOF mass spectrometry (*43*) is the potential need for special sample preparations, such as growth conditions and type of matrix. In several studies, differences in mass spectra between subtypes of various species were observed but no automated classification methods were was reported (*43*), which might limit general applicability. In this study, we used standard growth conditions, as well as routine ethanol–formic acid extraction and mass spectra acquisition protocols. The software used (MALDI Biotyper) also has the advantage of being a standard software used in clinical settings. These factors greatly increased the chance of clinical implementation of our findings.

In conclusion, our study demonstrated that rapid capsule typing of *H. influenzae* by identification of capsule type-specific genetic lineages using routine MALDI-TOF mass spectrometry is possible and highly accurate. After further large-scale validation, this method has the potential for clinical and research use. With the increasing heterogeneity in capsule types of disease-causing *H. influenzae* observed since Hib conjugate vaccines were introduced, the method can become a valuable tool in clinical diagnostic laboratories.

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# Capsule Typing of *Haemophilus influenzae* by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

## Technical Appendix

**Technical Appendix Table.** Isolates of *Haemophilus influenzae* used to develop a MALDI-TOF mass spectrometry typing database*

| Isolate | Other designation | Capsule type | Location | Year | Tissue/infection |
|---|---|---|---|---|---|
| International | | | | | |
| CCUG6881 | Smith | a | Unknown | Before 1973 | Unknown |
| CCUG7315 | NCTC8465 | a | USA | 1941 | Respiratory tract |
| HK391 | ATCC9327, NCTC8466 | a | USA | 1942 | CSF/nasal secretion |
| HK643 | Fin31 | a | Finland | 1974–1976 | Respiratory tract |
| HK645 | Harding | a | Boston, MA, USA | 1981 | CSF |
| HK648 | CDC78, E202 | a | Unknown | Unknown | Unknown |
| HK649 | CDC22, CDC D5361 | a | USA | 1976 | Blood |
| KR152 | | a | Sweden | Unknown | Unknown |
| KR1141 | | a | Angola | 2016 | Ear swab |
| DL-42 | | b | Dallas, TX, USA | Before 1984 | Unknown |
| Eagan | CCUG18095 | b | Boston, MA, USA | 1968 | CSF |
| HK395 | | b | USA | Before 1954 | Unknown |
| HK691 | A1132st | b | USA | Before 1985 | Unknown |
| HK706 | M1053 | b | USA | 1979 | Unknown |
| HK714 | M1062 | b | USA | 1980 | Unknown |
| HK718 | M1071 | b | USA | 1941 | Unknown |
| HK727 | M1084 | b | USA | 1947 | CSF |
| HK729 | M1077 | b | USA | 1980 | CSF |
| Kansas2 | | b | Missouri, USA | 2014 | Epiglottitis |
| Kansas3 | | b | Missouri, USA | 2014 | Osteomyelitis |
| MinnA | | b | Minneapolis, MN, USA | 1979 | CSF |
| 850530 | | b | The Netherlands | 1985 | Unknown |
| CCUG4851 | NCTC8469, ATCC9007 | c | USA | 1942 | Sputum |
| CCUG4852 | Ruggerio | c | New York, NY, USA | Before 1950 | CSF |
| HK635 | 43/LA | c | Papua New Guinea | 1980 | Lung aspirate |
| HK688 | | c | Copenhagen, Denmark | 1984 | CSF |
| KR153 | | c | Sweden | Unknown | Unknown |
| HK644 | 51/LA | d | Papua New Guinea | 1979 | Unknown |
| KR154 | | d | Sweden | Unknown | Unknown |
| NCTC8470 | | d | The Netherlands | 1937 | Respiratory tract |
| CCUG15521 | | e | Probably Sweden | Before 1984 | Unknown |
| KR1142 | | e | Unknown | Unknown | Unknown |
| HK636 | CDC54 | e | Unknown | Unknown | Unknown |
| HK641 | Hepke | e | Denver, CO, USA | 1980 | Unknown |
| HK653 | 45/LA | e | Papua New Guinea | 1979 | Unknown |
| KR138 | A76/01 | e | Unknown | Unknown | Unknown |
| KR147 | A77/99 | e | Unknown | Unknown | Unknown |
| CCUG15435 | Shawn | f | Unknown | Unknown | Unknown |
| CCUG6877 | | NT | USA | 1941 | Unknown |
| CCUG15519 | | NT | Unknown | Before 2001 | Unknown |
| HK224 | | NT | Denmark | Before 1976 | Otitis media |
| Sweden | | | | | |
| 11 | | b | Gothenburg, Lund/Malmö, Stockholm | 1998–2007 | Blood, CSF |
| 8 | | e | Lund/Malmö, Stockholm | 2006–2009 | Blood, CSF, respiratory tract |

| Isolate | Other designation | Capsule type | Location | Year | Tissue/infection |
|---|---|---|---|---|---|
| 26 | | f | Gothenburg, Lund/Malmö, Stockholm | 1997–2009, 2011 | Blood, CSF |
| 172 | | NT | Gothenburg, Lund/Malmö, Stockholm | 1997–2011 | Blood, CSF, respiratory tract |

*ATCC, American Type Culture Collection; CCUG, Culture Collection of the University of Göteborg; CDC; US Centers for Disease Control and Prevention; CSF, cerebrospinal fluid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NCTC, National Collection of Type Cultures; NT, nontypeable.

# Paper III

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Original article

# The spread and clinical impact of ST14CC-PBP3 type IIb/A, a clonal group of non-typeable *Haemophilus influenzae* with chromosomally mediated β-lactam resistance—a prospective observational study

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ABSTRACT

*Objectives:* Increasing incidences of non-typeable *Haemophilus influenzae* (NTHi) with β-lactam resistance mediated through mutations in penicillin-binding protein 3 (BLNAR or rPBP3) have been observed in the past decades. Recently, an rPBP3 NTHi sequence type (ST) 14 with PBP3 type IIb/A caused a disease outbreak in a nursing home in Sweden with severe outcomes, indicating increased bacterial virulence. In this prospective observational study, the epidemiology and clinical significance of the corresponding clonal group (ST14CC-PBP3IIb/A) in Skåne, Sweden was investigated.

*Methods:* ST14CC-PBP3IIb/A isolates were identified through partial multilocus sequence typing and *ftsI*(PBP3 gene)-sequencing of prospectively collected *H. influenzae* from patients with respiratory tract or invasive infections ($n = 3262$) in 2010–2012. Data on type of infection, hospitalization and outcomes were analysed, and the geographical distribution of isolates from different groups was investigated.

*Results:* Isolates belonging to ST14CC-PBP3IIb/A constituted 26% ($n = 94/360$) of respiratory tract rPBP3 NTHi. From mapping of patient addresses, a dynamic geographical spread was apparent during the study period. Furthermore, patients with infections by ST14CC-PBP3IIb/A isolates had higher hospitalization rates compared with patients infected with other rPBP3 NTHi (14/83 versus 21/255, p 0.025) and to a group of patients infected with susceptible NTHi (14/83 versus 13/191, p 0.010). ST14CC-PBP3IIb/A isolates constituted 25% ($n = 2/8$) of invasive rPBP3 NTHi during the study period.

*Conclusions:* Our investigation suggests that the ST14CC-PBP3IIb/A clonal group is associated with increased clinical virulence, resistance to several antimicrobial agents, and is persistent over time. We conclude that virulence varies between different NTHi, and that NTHi disease may be more dependent on bacterial factors than previously recognized. **V. Månsson, CMI 2017;23:209.e1–209.e7**



## Introduction

*Haemophilus influenzae* is a common colonizer of the human respiratory tract. The species is subdivided into encapsulated and non-encapsulated (non-typeable) strains (NTHi) [1]. NTHi is often commensal but also a frequent cause of infections such as acute otitis media, pneumonia and exacerbations of chronic obstructive pulmonary disease (COPD) [2,3]. Since the introduction of conjugate vaccines against *H. influenzae* type b, studies suggest increasing incidences of invasive NTHi disease, especially in the elderly [4,5].

Multilocus sequence typing (MLST) is a well-established method for typing of *H. influenzae* [6]. Classification of the *H. influenzae* population into different genetic clades by phylogenetic analysis through MLST predicts carriage of virulence factor genes [7,8]. Although specific sequence types (STs) have been linked to mucosal infections or to certain anatomical sites [9,10], no report has linked NTHi disease severity to specific genetic clades or clonal complexes (CCs). Rather, it has been suggested that disease severity may be largely host dependent [11,12].

β-lactam resistance in *H. influenzae* is mediated via β-lactamase production (termed β-lactamase-positive ampicillin-resistant isolates; BLPAR) or by key alterations (Arg517→His or Asn526→Lys) in penicillin-binding protein 3 (PBP3) (termed β-lactamase-negative ampicillin-resistant isolates; BLNAR) [1]. Current European Committee on Antimicrobial Susceptibility Testing (EUCAST)

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breakpoints for ampicillin effectively split the population with key PBP3 alterations in half, so the terms genetic BLNAR (gBLNAR) or rPBP3 (PBP3-mediated resistance) have been introduced to denote the full population with key substitutions, and we will hence use the term rPBP3 in this work [13,14]. PBP3 alterations have been classified into functional groups and subgroups by Ubukata, Dabernat, Skaare and their co-workers [15–17]. Although the proportion of BLPAR isolates has been relatively stable in the past decades, rPBP3 is an increasing problem in Europe, as incidences have risen, with group II rPBP3 isolates being particularly common [14,17–21]. In addition, rPBP3 isolates are often genetically related [14,17,19,21,22]. As a consequence, sequencing of the *ftsI* gene, encoding PBP3, has been suggested as an addition to the MLST scheme [14].

A distinct clonal group of rPBP3 isolates, consisting of sequence type (ST) 14 and related STs with PBP3 type IIb/A (amino acid substitutions Asp350→Asn, Met377→Ile, Ala502→Val, Asn526→Lys, Val547→Ile and Asn569→Ser) [14,16,17] has been highlighted in recent studies in Scandinavia. This clonal group (ST14CC-PBP3IIb/A) caused an outbreak of respiratory tract infections in a nursing home in southern Sweden, resulting in several hospitalizations and one fatality [23]. In addition, a previous study on invasive rPBP3 isolates in Sweden revealed a cluster of invasive cases later confirmed to be caused by ST14CC-PBP3IIb/A isolates [19]. Furthermore, a Norwegian study indicated that ST14CC-PBP3IIb/A had the highest hospitalization rate of all STs in a 2007 surveillance population [14].

Although ST14CC-PBP3IIb/A has been described in general studies on *H. influenzae* epidemiology, the recent outbreak prompted us to investigate the epidemiology and clinical significance of this clonal group. In this study we examined the prevalence of ST14CC-PBP3IIb/A among respiratory and invasive *H. influenzae* isolates in our geographical area. The epidemiology and clinical presentation of isolates from this clonal group were compared with those of other rPBP3 NTHi as well as those of β-lactam susceptible NTHi, with the hypothesis that this is a lineage of NTHi with increased virulence.

## Materials and methods

### *Study setting*

This study was carried out in Skåne, Sweden. During the years 2010–2012, all *H. influenzae* isolated from upper respiratory tract cultures (nasopharyngeal swabs and secretions from the middle ear, sinuses and conjunctivae) from patients with symptoms of respiratory tract infections referred to the Clinical Microbiology Laboratory at Skåne University Hospital, Malmö, were prospectively stored at −80°C. This laboratory receives clinical bacterial cultures from all health centres and hospitals in southern Skåne (population approximately 500 000). Furthermore, all hospitals in the region share the same electronic medical records system, allowing thorough epidemiological investigations. According to regional guidelines, upper respiratory tract cultures are recommended in cases of recurrent acute otitis media, treatment failure of acute otitis media and community-acquired pneumonia in adults. Cultures can also be obtained at the discretion of the referring physician.

Invasive NTHi isolates (cultures from blood and cerebrospinal fluid) from 2010 to 2012, from Skåne county (population of approximately 1 200 000), were also prospectively collected and studied.

### *Antimicrobial susceptibility testing and interpretation*

All isolates were investigated for susceptibility to β-lactam antibiotics by EUCAST disc diffusion testing [24], a method demonstrated to accurately identify rPBP3 isolates [25]. Results were interpreted according to the recommendations for the time of isolation (www.eucast.org). Isolates were grown on Müller–Hinton-fastidious agar and screened for β-lactam resistance using discs with phenoxymethylpenicillin (10 μg) or benzylpenicillin (1 U). Resistant isolates were examined by a nitrocefin β-lactamase test and β-lactamase non-producing isolates were presumed to be rPBP3. β-lactamase-producing, cefaclor-resistant (30 μg disc) isolates were considered both β-lactamase-producing and rPBP3. All isolates with a phenotype suggesting PBP3-mediated β-lactam resistance, both β-lactamase-producing and non-producing, were selected for further study (see Supplementary material, Fig. S1). Not all recorded isolates could be retrieved, but apart from non-retrieval, no exclusion criteria were applied. A group of randomly selected, β-lactam-susceptible isolates ($n = 193$) were included as references in the study (Fig. 1).

Minimum inhibitory concentrations (MICs) for amoxicillin and cefotaxime were determined for respiratory isolates identified as ST14CC-PBP3IIb/A, as well as for a randomly selected subset of non-ST14CC-PBP3IIb/A rPBP3 isolates ($n = 95$), by gradient tests (Etest, bioMérieux, Marcy-l'Étoile, France).

### *Culture conditions, preparation of bacterial DNA and PCR*

Bacterial culturing and DNA preparation were performed as previously described [26]. PCR for MLST genes and the transpeptidase region of the *ftsI* gene (encoding PBP3) was performed as described previously [6,17]. Sequenced PCR products were analysed using Geneious R9 (Biomatters, Auckland, New Zealand).

### *Identification of ST14CC-PBP3IIb/A isolates*

Following determination of the discriminatory power of each MLST gene to identify ST14 (www.pubmlst.org), suspected rPBP3 isolates were screened by sequencing of the *adk* gene. Isolates with *adk* 5 (specific *adk* allele of ST14) were further investigated by sequencing of *recA* and *ftsI*. In the MLST database (www.pubmlst.org), all isolates with *adk* 5 and *recA* 5 are ST14 or single locus variants of ST14. Deduced PBP3 amino acid sequences were compared with the amino acid sequence of *H. influenzae* Rd KW20 (GenBank accession number AAC22787). Isolates with *adk* 5, *recA* 5 and PBP3IIb/A [14] were considered part of the ST14CC-PBP3IIb/A clonal group. To validate the method, full MLST was performed on a subset of ST14CC-PBP3IIb/A isolates ($n = 9$). Furthermore, resistance patterns of non-β-lactam antibiotics were compared, and the *ftsI* DNA sequences (nucleotides 1010–1719) were compared with the previously reported PBP3IIb/A-encoding *ftsI* allele *lambda-1* (EMBL: HG818630) associated with ST14 [14]. The typing process is outlined in Fig. 1.

### *Patient cohorts and patient data*

*Respiratory isolates.* Since β-lactam resistance in itself could be a risk factor for treatment failure and more severe infections, clinical data were compared between patient cohorts with three groups of isolates: (a) ST14CC-PBP3IIb/A, (b) other rPBP3 screening positive isolates and (c) β-lactam-susceptible isolates. If a patient had sequential cultures of NTHi within 6 months belonging to the same group, only the first was included in the patient cohort analysis. Laboratory referrals were examined for patient age and sex, and the working diagnosis of the referring physician. This working diagnosis could not, however, be validated and the information was treated accordingly. Medical records were investigated for information on hospital admission in any of the regional hospitals within 5 days of the culture date. For hospitalized patients,

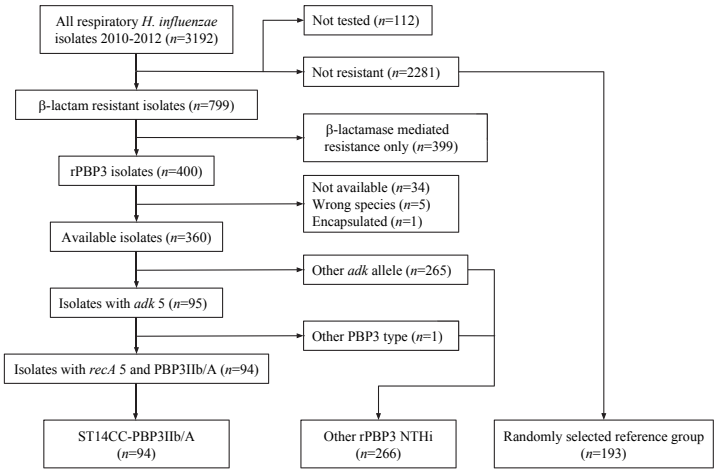


**Fig. 1.** Identification of ST14CC-PBP3IIb/A isolates. To identify ST14CC-PBP3IIb/A isolates, a simplified multilocus sequence typing (MLST) scheme (using only the *adk* and *recA* gene) was adopted in combination with *ftsI* gene sequencing and translation. All rPBP3 non-typeable *Haemophilus influenzae* (NTHi) isolates with *adk* 5 and *recA* 5 (the *adk* and *recA* alleles of ST14) and PBP3IIb/A were considered ST14CC-PBP3IIb/A. One isolate had *adk* 5 and *recA* 5, but a completely different PBP3 substitution pattern (Gly490→Glu, Asn526→Lys and Ala530→Ser). All isolates with *adk* 5 and PBP3IIb/A also had *recA* 5. A randomly selected subset of ST14CC-PBP3IIb/A isolates ($n = 9$) were confirmed ST14 by full MLST, to validate the typing scheme. Clonality of ST14CC-PBP3IIb/A isolates was further confirmed by coherent antibiotic susceptibility by disc diffusion testing for trimethoprim-sulfamethoxazole (R), nalidixic acid (S) and tetracycline (S) and analysis of *ftsI* sequences. Of β-lactam susceptible isolates ($n = 193$), about half ($n = 91$) were evaluated with the MLST-PBP3 typing scheme. Only three isolates had *adk* 5 and of these, all carried wild-type PBP3 and none had *recA* 5. Non-ST14CC-PBP3IIb/A rPBP3 isolates and the randomly selected β-lactam susceptible isolates were used to identify control patient cohorts to evaluate the clinical impact of ST14CC-PBP3IIb/A.

**Table 1**
Respiratory rPBP3 NTHi isolates and patients in the study region

| Year (total respiratory *H. influenzae* isolates ($n$)) | Incidence[1] | | | | | rPBP3 isolates[2, 3] | | | Patient cohorts[4] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0–5 | 6–20 | 21–65 | 66+ | Total | ST14CC-PBP3IIb/A (%) | Other rPBP3 (%) | Total | ST14CC-PBP3IIb/A (%[5]) | Other rPBP3 (%[5]) | Susceptible[6] | |
| 2010 (1185) | 2010 | 138 | 101 | 74 | 243 | 13 (21.3) | 48 (78.7) | 61 | 13 (22.4) | 45 (77.6) | 41 | |
| 2011 (1062) | 1453 | 140 | 118 | 83 | 216 | 48 (28.2) | 122 (71.8) | 170 | 40 (25.2) | 119 (74.8) | 75 | |
| 2012 (945) | 1319 | 117 | 94 | 89 | 190 | 33 (25.6) | 96 (74.4) | 129 | 30 (24.8) | 91 (75.2) | 66 | |
| 2010-2012 (3,192) | 1587 | 131 | 104 | 82 | 216 | 94 (26.1) | 266 (73.9) | 360 | 83 (24.6) | 255 (75.4) | 192 | |

[1] Yearly incidence of respiratory NTHi per 100 000 age group-sorted individuals.
[2] Of 400 suspected rPBP3 isolates, 34 were not available. Six isolates were excluded, five were of other species and one was encapsulated (type e, PCR-verified).
[3] Isolates obtained before and after 20 January 2011 were initially screened for β-lactam resistance with 10 μg phenoxymethylpenicillin and 1 U benzylpenicillin discs, respectively.
[4] If the same patient had more than one respiratory culture of NTHi within 6 months, only the disease episode related to the first culture was included in the patient data analyses, unless the separate isolates belonged to different groups (i.e. ST14CC-PBP3IIb/A, other rPBP3 or susceptible isolate).
[5] Per cent of patients with rPBP3 NTHi cultures.
[6] Identified from a randomly selected subset of susceptible isolates ($n = 193$).

**Table 2**
MICs of respiratory ST14CC-PBP3IIb/A and other rPBP3 NTHi for amoxicillin and cefotaxime

| Type of NTHi | Isolates ($n$) | β-lactamase ($n$) | Amoxicillin | | | | Cefotaxime | |
|---|---|---|---|---|---|---|---|---|
| | | | Including β-lactamase producing isolates[2] | | Excluding β-lactamase producing isolates | | | |
| | | | $MIC_{50}/MIC_{90}$ (mg/L) | S/R[3] (%) | $MIC_{50}/MIC_{90}$ (mg/L) | S/R[3] (%) | $MIC_{50}/MIC_{90}$ (mg/L) | S/R[3] (%) |
| ST14CC-PBP3IIb/A | 94 | 5 | 2/4 | 84/16 | 1/4 | 88/12 | 0.064/0.125 | 99/1 |
| Other rPBP3 | 95[1] | 12 | 2/64 | 71/29 | 2/4 | 81/19 | 0.064/0.125 | 100/0 |

[1] For cefotaxime, gradient tests were performed on 29 randomly selected isolates.
[2] As expected β-lactamase producing isolates had higher amoxicillin MICs than non-producing isolates (range 4–256 and 64–256 mg/L for ST14CC-PBP3IIb/A and other rPBP3, respectively).
[3] Susceptibility according to EUCAST clinical breakpoint (S≤/R>) for amoxicillin (2/2) and cefotaxime (0.125/0.125). All β-lactamase-producing isolates were classified as resistant for amoxicillin.

**Table 3**
Descriptive data and geographical distribution of patients with respiratory NTHi infections

| Patient cohort | Patients (*n*) | Suspected diagnosis/site of infection[1] (%) | | | | | | Age (years) (%) | | | | | Sex (%) | | Postcode available (%) | Postcode in central Malmö (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Unspecified URI[2] | Ear | Sinus | Eye | LRI[3] | Other | Unknown | <1 | 1-5 | 6-20 | 21-65 | ≥66 | Female | Male | | 2010 | 2011 | 2012 |
| ST14CC-PBP3IIb/A | 83 | 17 (20) | 20 (24) | 7 (8) | 12 (14) | 9 (11) | 0 | 18 (22) | 14 (17) | 35 (42) | 7 (8) | 23 (28) | 4 (5) | 42 (51) | 41 (49) | 76 (92) | 2 (15) | 10 (27) | 18 (69)[4] |
| Other rPBP3 | 255 | 70 (28) | 44 (17) | 19 (7) | 36 (14) | 20 (8) | 4 (2) | 62 (24) | 36 (14) | 78 (31) | 21 (8) | 96 (38) | 24 (9) | 140 (55) | 115 (45) | 249 (98) | 14 (31) | 49 (43) | 46 (51)[5] |
| Susceptible | 192 | 70 (36) | 29 (15) | 6 (3) | 9 (5) | 14 (7) | 4 (2) | 60 (31) | 25 (13) | 74 (39) | 24 (13) | 56 (29) | 13 (7) | 107 (56) | 85 (44) | 183 (95) | 11 (29) | 31 (42) | 20 (28) |

[1] According to culture referral.
[2] Upper respiratory tract infection.
[3] Lower respiratory tract infection.
[4] Significantly increased, compared to 2010 (p 0.002). When *lambda-2* carrying ST14CC-PBP3IIb/A isolates were excluded, the significant increase remained (p 0.003).
[5] Significantly increased, compared to 2010 (p 0.029).

information on the cause of hospitalization, final diagnosis (International Classification of Diseases 10th revision), recent antibiotic treatment, causal treatment and outcomes were collected.

Postcodes of patients in the three cohorts were registered and changes in geographical distribution of cases during the study period were analysed. Postcodes for patients with ST14CC-PBP3IIb/A infections were plotted on maps to illustrate the spread of the clonal group.

*Invasive isolates*. Invasive isolate patient data were analysed separately for patient age, diagnosis and outcome.

### *Statistical analyses*

Comparisons between the different patient cohorts and changes in geographical distribution of disease cases (categorical data) were performed using chi-square test. In the latter instance by comparing the proportions of patients living in and outside central Malmö (the only large urban area in the region), comparing the first (2010) and last (2012) year of the study.

### *Ethical considerations*

This study was approved by the Regional Ethics Committee at Lund University (2014/533).

## Results

### *ST14-PBP3A isolates in the study region*

*Respiratory isolates*. During the study period, the yearly incidence of cultured *H. influenzae* from patients with respiratory tract symptoms was 216/100 000 individuals, and was highest among young children (Table 1). A total of 3192 respiratory tract *H. influenzae* were registered. Susceptibility testing by disc diffusion screening (Fig. 1) identified 400 (12.5%) likely rPBP3 isolates, of which 360 were available for further typing (Table 1). Among β-lactam-susceptible isolates, a randomly selected group ($n = 193$) was included as reference.

When subjected to the MLST-PBP3 typing scheme (Fig. 1), 26.1% ($n = 94$) of all rPBP3 isolates belonged to ST14CC-PBP3IIb/A according to the typing scheme. In full MLST performed on a subset of these isolates ($n = 9$), all were confirmed ST14. All ST14CC-PBP3IIb/A isolates were resistant to trimethoprim-sulfamethoxazole, compared with 37.1% of other rPBP3 isolates ($n = 266$). Of ST14CC-PBP3IIb/A isolates, 98% ($n = 92$) carried the *ftsI* allele *lambda-1*. In the two remaining isolates, PBP3A was encoded by *ftsI* allele *lambda-2* [14], with four single nucleotide polymorphisms compared with *lambda-1*. These isolates were also confirmed ST14 by full MLST. As these isolates deviated slightly genetically, two separate analyses of patient data were made based upon inclusion and exclusion of the patients infected by these isolates. Of susceptible isolates evaluated according to the typing scheme ($n = 91$), none were ST14 (Fig. 1).

The 94 ST14CC-PBP3IIb/A isolates were isolated from 83 (81 when excluding the *lambda-2*-carrying isolates) individual patients, while rPBP3 non-ST14CC-PBP3IIb/A isolates ($n = 266$) were isolated from 255 patients (Table 1). Hence, ST14CC-PBP3IIb/A constituted 24.6% (alternatively 24.1% when excluding the *lambda-2*-carrying isolates) of non-redundant infections caused by rPBP3 NTHi during the study period. The β-lactam-susceptible isolates were isolated from 192 individual patients (Table 1).

*Invasive isolates*. All invasive *H. influenzae* ($n = 70$) from 2010–2012 were cultured from blood, no case of meningitis was reported. Of 13% ($n = 9$) with phenotypes suggesting rPBP3, eight isolates were available for typing. Two (25%) were ST14CC-PBP3IIb/

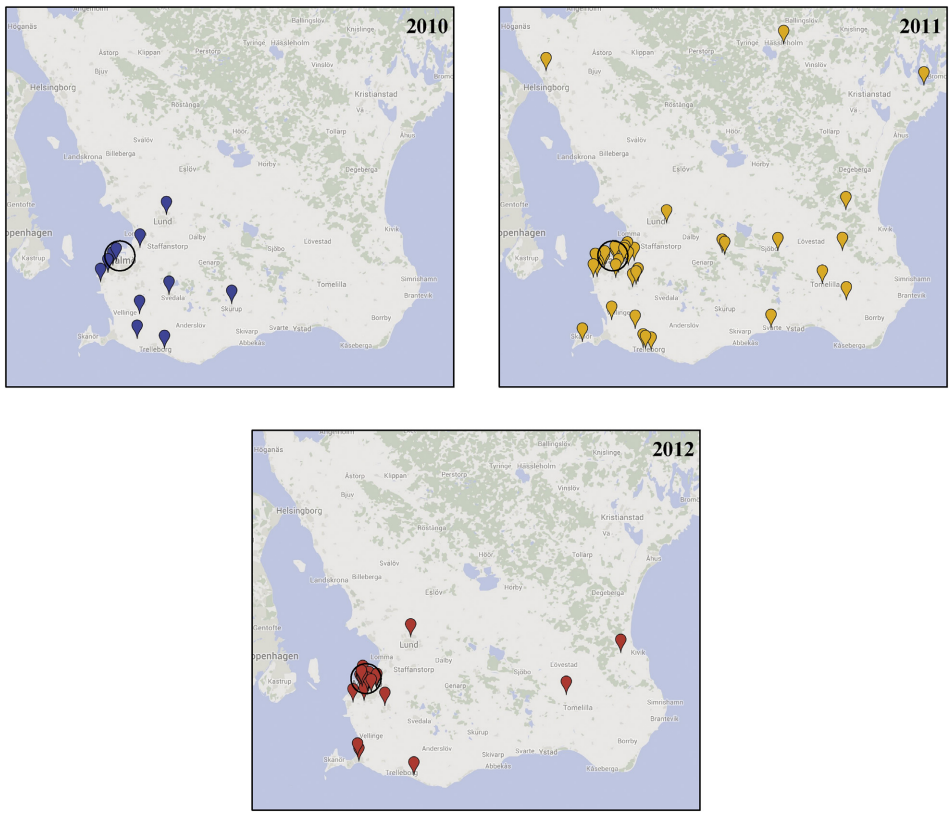


**Fig. 2.** The spread of ST14CC-PBP3IIb/A in southern Skåne. Available postcodes of patients suffering from infections by ST14CC-PBP3IIb/A 2010 ($n = 13$), 2011 ($n = 37$) and 2012 ($n = 26$), respectively, were plotted on maps. The proportion of patients with addresses in central Malmö (the only large urban area in the region, indicated) increased during the study years. Patients residing in inner-city Malmö constituted 15% ($n = 2$), 27% ($n = 10$) and 69% ($n = 18$) of cases in 2010, 2011 and 2012, respectively.

A, both with *ftsI* allele *lambda-1*, according to the MLST-PBP3 typing scheme. These isolates were confirmed as ST14 by full MLST.

### *β-lactam-resistance among respiratory rPBP3 NTHi isolates*

ST14CC-PBP3IIb/A isolates and control rPBP3 NTHi isolates had comparable MICs for amoxicillin and cefotaxime (Table 2). Even though the amoxicillin MIC ranges of β-lactamase non-producing isolates were narrow for both populations, both were divided by the amoxicillin breakpoints. As expected, β-lactamase-producing isolates had higher MICs for amoxicillin (Table 2).

### *Patient data and clinical impact of ST14CC-PBP3IIb/A infections*

*Respiratory isolates–referral data* No statistically significant differences were observed between patients isolated with ST14CC-PBP3IIb/A, other rPBP3 or susceptible NTHi regarding suspected clinical diagnosis, age or sex (Table 3). Young children (age 0–5 years) and adults dominated all three cohorts. Nine cases of neonatal infections, evenly spread between the groups, were noted (data not shown).

*Geographical distribution.* For ST14CC-PBP3IIb/A as well as for other rPBP3, the proportions of infected patients residing in the urban Malmö region increased significantly over the study period (Table 3). The centralization of patient cases to Malmö was, however, distinctly greater for ST14CC-PBP3IIb/A with an increase of >400% (from 2/13 in 2010 to 18/26 cases in 2012), as visualized in Fig. 2, compared with 65% for other rPBP3. No significant changes in geographical distribution were seen for the patient cohort with susceptible NTHi.

*Hospital data.* A significantly larger proportion of patients infected with ST14CC-PBP3IIb/A were hospitalized compared with patients infected with other rPBP3 (14/83 versus 21/255, p 0.025) and susceptible strains (14/83 versus 13/192, p 0.010) (Table 4). When excluding cases caused by *lambda-2*-carrying ST14CC-PBP3IIb/A isolates, a significant difference was still observed (Table 4). Four individual patients (one repeat isolate) had infections caused by β-lactamase-producing ST14CC-PBP3IIb/A isolates, one of which was hospitalized. When a subgroup analysis was performed (including only β-lactamase-negative ST14CC-PBP3IIb/A isolates), the difference compared with the non-ST14CC-PBPIIb/A rPBP3 cohort remained statistically significant (p 0.035). Patients with infections caused by ST14CC-PBP3IIb/A were more often hospitalized primarily because of their respiratory tract infection,

**Table 4**
Hospitalized patients with respiratory NTHi infections

| Patient cohort | Patients (*n*) | Hospitalization data | | | | | Prior disease | | | Final diagnosis | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Hospitalized patients[1] (%) | Medical records available | Hospitalization primarily related to NTHi infection[3] (%) | Other reason for hospitalization (%) | Median hospital time (days) | Antibiotics prior to hospitalization (%) | Immuno-compromised[4] (%) | COPD (%) | Pneumonia[5] (%) | AOM (%) | Other infection (%) |
| ST14CC-PBP3IIb/A | 83 | 14 (17)[2] | 14 | 13 (93) | 1 (7) | 5 | 5 (36) | 0 | 3 (21) | 11 (79) | 2 (14) | 0 |
| Other rPBP3 | 255 | 21 (8.2) | 19 | 12 (63) | 7 (37) | 4 | 3 (16) | 2 (11) | 6 (32) | 8 (42) | 0 | 4[6] (21) |
| Susceptible | 192 | 13 (6.8) | 13 | 9 (69) | 4 (31) | 3,5 | 5 (38) | 0 | 1 (8) | 5 (38) | 1 (8) | 3[7] (23) |

[1] Admitted to hospital within 5 days of positive culture.
[2] Statistically significant, compared with patients infected by other rPBP3 and susceptible NTHi (p 0.025, RR 2.0 (95% CI 1.1–3.8) and (p 0.010, RR 2.5 (95% CI 1.2–5.0), respectively). Significant differences remained when cases caused by *lambda-2* carrying isolates were excluded (p 0.042 and p 0.017 for other rPBP3 and susceptible isolates, respectively).
[3] Hospitalized due to distinct bacterial infection related to the culture that responded to adequate antibiotic treatment.
[4] Defined as neutropenia, congenital immunodeficiency or current chemotherapy.
[5] Radiographically confirmed.
[6] Including three chronic obstructive pulmonary disease (COPD) exacerbations with no signs of pneumonia on chest X-ray.
[7] Including one COPD exacerbation with no signs of pneumonia on chest X-ray.

and they suffered less frequently from COPD compared with patients infected by other rPBP3 NTHi. However, these differences were not statistically significant (Table 4). No hospitalized patient died, but one patient each from the ST14CC-PBP3IIb/A and the other rPBP3 NTHi cohort received intensive care. It is difficult to draw firm conclusions on treatment effects of specific antibiotics in this limited-size cohort, but five patients with rPBP3-related pneumonia were treated with penicillins only (high-dose intravenous benzylpenicillin and/or high-dose oral amoxicillin) with no record of relapse. The corresponding isolates all had MICs for amoxicillin of 1–2 mg/L.

*Invasive isolates.* All cases of invasive rPBP3 NTHi disease occurred in adults, of which seven were >65 years of age. The patients with ST14-PBP3IIb/A disease ($n = 2$) suffered from cholangitis and pneumonia. The patients with other rPBP3 NTHi isolated from blood ($n = 6$) had pneumonia. One patient from each group died. Due to the limited number of cases, no statistical analyses were performed.

## Discussion

In this study we show that ST14CC-PBP3IIb/A accounted for approximately 25% of respiratory rPBP3 NTHi in Skåne, Sweden 2010–2012. We further demonstrate that ST14CC-PBP3IIb/A carries a significantly higher patient hospitalization rate compared with other respiratory rPBP3 and susceptible NTHi; the clonal group is also present among invasive isolates and has established itself in a densely populated urban area.

ST14CC-PBP3IIb/A was first recognized among Norwegian respiratory isolates and was the most prevalent rPBP3 clone in a 2004 surveillance study [17], and the fourth most prevalent in 2007 [14]. In addition, the clonal group has been observed among Swedish invasive isolates [19] and associated with pneumonia in Spain [27]. Recently, it was reported to have caused a severe disease outbreak in a Swedish nursing home [23]. ST14 (PBP3 alterations not reported) has also been observed among invasive isolates in Canada and Italy [28,29]. The number of previous studies performing MLST-PBP3 typing is limited, making it difficult to draw definitive conclusions about the spread and clinical impact of ST14CC-PBP3IIb/A. The present study confirms that the clonal group is widely established and persistent. Interestingly, ST14CC-PBP3IIb/A cases clustered in the urban Malmö region during the study period. It is possible that this particular clonal group, in addition to increased virulence, has an increased capacity of inter-individual spread that would be enhanced in a densely populated area. This would be in line with the recently reported outbreak [23]. Isolates in the clonal group are resistant to commonly used antimicrobials, and consistent with previous indications [14] a significantly higher hospitalization rate of ST14CC-PBP3IIb/A patients was observed in the present study. The clonal group appears to be endemic in Scandinavia [14,17,19], and possibly far beyond [27–29].

Two isolates in our ST14CC-PBP3IIb/A collection expressed PBP3 encoded by the slightly different *ftsI* allele *lambda-2*, an allele previously reported to appear in other groups of rPBP3 NTHi [14]. Horizontal gene transfer of the *ftsI* gene is a possible cause of this distribution, and has been shown *in vitro* [30]. Although these isolates met the criteria of being part of the clonal group (ST14CC and PBP3IIb/A), we chose to reperform statistical analyses to confirm significant differences between ST14CC-PBP3IIb/A and control isolates, with these isolates excluded.

Notably, five ST14CC-PBP3IIb/A isolates had acquired β-lactamase genes, consistent with previous reports that the two major β-lactam-resistance mechanisms occur independently [21].

This study has several strengths. We have analysed a large and near-complete collection of prospectively collected clinical isolates

from patients with respiratory tract and invasive infections, allowing a comprehensive epidemiological analysis. By investigating medical records, we have demonstrated an increased clinical virulence of ST14CC-PBP3IIb/A. The study also used the novel approach of combining MLST and *ftsI*-typing to compare rPBP3 strains between study groups [14]. A potential limitation of the study is the algorithm used to identify ST14CC-PBP3IIb/A isolates. However, in the MLST database (www.pubmlst.org), all isolates with *adk* 5 and *recA* 5 are ST14, or single locus variants of ST14. Furthermore, in a study of 196 Norwegian clinical respiratory tract *H. influenzae*, all isolates expressing *adk* 5 and PBP3IIb/A were ST14 ($n = 11$) or single locus variants of ST14 ($n = 1$) [14].

In conclusion, this study demonstrates the presence of a prevalent, widespread and persistent clonal group of rPBP3 NTHi with increased clinical virulence. The results call for continued epidemiological surveillance of NTHi, further studies regarding NTHi virulence mechanisms, and supports recent shifts in attitude towards NTHi as a pathogen capable of causing severe disease not only in a host-dependent manner.

## Transparency declaration

None of the authors have any conflict of interest to disclose.

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## Appendix A. Supporting information

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2016.11.006.

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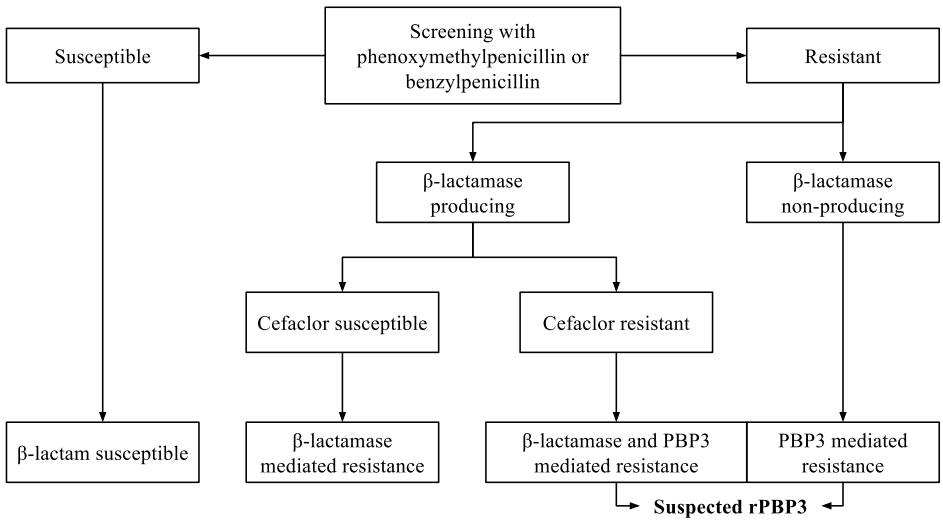
The spread and clinical impact of ST14CC-PBP3 type IIb/A, a clonal group of non-typeable *Haemophilus influenzae* with chromosomally mediated β-lactam resistance—a prospective observational study

Supplementary material.

Fig. S1. Antibiotic susceptibility testing by disc diffusion.



# Paper IV

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ORIGINAL ARTICLE

# Benzylpenicillin versus wide-spectrum beta-lactam antibiotics as empirical treatment of *Haemophilus influenzae*-associated lower respiratory tract infections in adults; a retrospective propensity score-matched study

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**Abstract**
There is consensus that definitive therapy for infections with *H. influenzae* should include antimicrobial agents with clinical breakpoints against the bacterium. In Scandinavia, benzylpenicillin is the recommended empirical treatment for community-acquired pneumonia (CAP) except in very severe cases. However, the effect of benzylpenicillin on *H. influenzae* infections has been debated. The aim of this study was to compare the outcomes of patients given benzylpenicillin with patients given wide-spectrum beta-lactams (WSBL) as empirical treatment of lower respiratory tract *H. influenzae* infections requiring hospital care. We identified 481 adults hospitalized with lower respiratory tract infection by *H. influenzae*, bacteremic and non-bacteremic. Overall, 30-day mortality was 9% (42/481). Thirty-day mortality, 30-day readmission rates, and early clinical response rates were compared in patients receiving benzylpenicillin ($n = 199$) and a WSBL ($n = 213$) as empirical monotherapy. After adjusting for potential confounders, empirical benzylpenicillin treatment was not associated with higher 30-day mortality neither in a multivariate logistic regression (aOR 2.03 for WSBL compared to benzylpenicillin, 95% CI 0.91–4.50, $p = 0.082$), nor in a propensity score-matched analysis (aOR 2.14, 95% CI 0.93–4.92, $p = 0.075$). Readmission rates did not significantly differ between the study groups, but early clinical response rates were significantly higher in the WSBL group (aOR 2.28, 95% CI 1.21–4.31, $p = 0.011$), albeit still high in both groups (84 vs 81%). In conclusion, despite early clinical response rates being slightly lower for benzylpenicillin compared to WSBL, we found no support for increased mortality or readmission rates in patients empirically treated with benzylpenicillin for lower respiratory tract infections by *H. influenzae*.

**Keywords** *Haemophilus influenzae* · Benzylpenicillin · Beta-lactam antibiotics · Community-acquired pneumonia · Empirical antibiotic treatment · Propensity score

## Introduction

*Haemophilus influenzae* is considered the second most common bacterial cause of community-acquired pneumonia (CAP) after *Streptococcus pneumoniae* [1]. Surveillance data have suggested an increased incidence of invasive infections with *H. influenzae* in recent years, and since the introduction of capsule type b polysaccharide conjugate vaccines, non-encapsulated strains (NTHi) dominate, followed by capsule type f [2–4]. This has led to a shift in the clinical epidemiology of severe *H. influenzae* infections, as most cases now present as pneumonia in older adults or patients with comorbidities, most notably chronic obstructive pulmonary disease (COPD) [2, 3, 5]. With the widespread introduction of conjugated pneumococcal vaccines, there is also concern that the proportion of CAP caused by *H. influenzae* may increase [6]. Although this has yet to be confirmed, one recent study from Great Britain using molecular diagnostics showed it to be the most common agent in CAP, contributing to 40% of cases [7].

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In Scandinavian countries, high-dose benzylpenicillin (PcG) is still recommended as the empirical treatment for patients with CAP requiring hospitalization, with the exception of patients with immunosuppression or very severe presentation (CRB-65 > 2 or concomitant severe sepsis [8]). There is a long tradition of benzylpenicillin treatment in Scandinavia, which remains effective against the majority of pneumococci and has limited collateral ecological effects compared to other empirical alternatives [9].

The activity of benzylpenicillin against *H. influenzae* has been debated. Recommendations are based mainly on clinical experience combined with theoretical assumptions from PK/PD simulations and time-killing experiments [10]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has not defined clinical breakpoints for benzylpenicillin, referring to insufficient evidence from clinical studies [11]. Moreover, in recent years, the proportion of *H. influenzae* isolates with reduced susceptibility to aminopenicillins due to alterations in penicillin-binding protein 3 (termed rPBP3) has increased, reaching 10–20% [12, 13]. In addition, a stable proportion (an additional 10–20%) of isolates expresses beta-lactamases [14, 15]. Both of these resistance mechanisms are likely to increase the risk of treatment failure with use of empirical benzylpenicillin. The increase of these resistance mechanisms may make benzylpenicillin a less viable option in the case of *H. influenzae* CAP. One recent retrospective study from Denmark showed a significantly higher 30-day mortality for patients receiving benzylpenicillin as definitive treatment for *H. influenzae* bacteremia, compared to those who received cephalosporins or aminopenicillins [16].

Considering the proposed increase in the proportion of *H. influenzae* as a cause of CAP and the current empirical treatment recommendation of benzylpenicillin, there is a need to assess the outcome of empirical benzylpenicillin treatment in cases of lower respiratory tract infection by *H. influenzae*. The primary objective of this study was to compare the 30-day mortality between adults who had received benzylpenicillin as empirical treatment for *H. influenzae* lower respiratory tract infections (with or without bacteremia) requiring hospital care with individuals who had received empirical treatment with wide-spectrum beta-lactams (WSBL). As secondary objectives, 30-day readmission rates and estimations of early clinical response were compared between the two groups.

## Materials and methods

### Study population and setting

Patients with positive cultures of *H. influenzae* were identified at the clinical microbiology laboratories in Malmö and Lund, Sweden. The catchment area of these laboratories corresponds to Skåne county in southern Sweden (adult population of 1,045,792 in 2016 [17]), and healthcare was provided by Skåne University Hospital and surrounding regional hospitals. All blood and respiratory tract cultures sampled in the catchment area were analyzed in these two laboratories.

### Case definitions and exclusion criteria

Two case definitions were applied: (1) bacteremia with *H. influenzae* in an individual ≥ 18 years of age 1997–2016 with a lower respiratory tract infection or (2) pure culture of *H. influenzae* from a respiratory tract sample in an individual hospitalized due to a lower respiratory tract infection 2015–2016.

Patients aged ≥ 18 years with positive blood cultures of *H. influenzae* between 1997 and 2016 were identified through the laboratories' records. All individuals with positive blood cultures were included in the study if they had a concurrent respiratory tract infection, except for those with epiglottitis (in which the recommended treatment in the area is cefotaxim). Recurrent episodes of bacteremia were only recorded once.

Individuals above 18 years of age with positive cultures from sputum or nasopharynx in Skåne county in 2015–2016 were also identified. All individuals with positive cultures were included in the study if (1) they were admitted more than 24 h to a hospital ward due to a lower respiratory tract infection, (2) no other respiratory tract pathogen (see below) was present in any microbiological sample, (3) they had been diagnosed with lower respiratory tract infection (pneumonia or COPD exacerbation) at discharge from hospital, and (4) no alternative foci of infection were identified except the lower respiratory tract.

In cultures from sputum and nasopharyngeal swabs, concurrent growth of *Streptococcus pneumoniae*, beta-haemolytic streptococci and *Moraxella catharrhalis* were regularly sought for, and if any of these pathogens were identified in a culture, the patient was excluded. If a respiratory tract sample indicated the presence of influenza virus, respiratory syncytial virus, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Chlamydia psittaci*, *Legionella pneumophila*, or *Pneumocystis jirovecii* through polymerase chain reaction (PCR), the patient was excluded. Finally, patients were excluded if the presence of *Streptococcus pneumoniae* or *Legionella pneumophila* was demonstrated by urine antigen detection tests.

### Microbiological methods and antimicrobial susceptibility testing

All blood samples were cultured using the automated BacTAlert system (bioMérieux, Marcy l'Etoile, France) (1997–December 2014) or the BACTEC system (BD diagnostic systems, Sparks, MD) (December 2014–2016). Respiratory tract specimens from sputum and nasopharyngeal swabs were cultured using standard microbiological techniques. Isolates were identified by typical colony morphology on agar plates, through standard biochemical tests and by

MALDI Biotyper analysis (Bruker Daltonics, Bremen, Germany). Capsule typing was performed by PCR for *H. influenzae* isolated from blood. Isolates from respiratory tract samples were not routinely capsule typed and were not saved (and thus not available for capsule typing at the time of the study).

Antimicrobial susceptibility testing was performed according to local laboratory guidelines until 2009, and thereafter according to the EUCAST algorithm [18], which is based upon a disk diffusion screen of 1 U benzylpenicillin on fastidious Mueller Hinton solid medium (MH-F) for identification of beta-lactam resistance. Beta-lactamase production was confirmed by a standard nitrocefin assay in screening-positive cases. Gradient tests (Etest, bioMérieux, Marcy l'Etoile, France) were used to determine the actual MICs of beta-lactam agents if the first disk diffusion screen was positive. Data on antimicrobial susceptibility, including beta-lactam resistance by rPBP3 and beta-lactamase production, were obtained from laboratory records.

## Clinical definitions

The following clinical descriptive patient data were recorded from hospital medical records: age, sex, immunosuppression, and comorbidities according to Charlson/Deyo comorbidity index [19]. The study definition of immunosuppression was ongoing primary immune deficiency or immunosuppressive therapy not denoted in the Charlson/Deyo comorbidity index, in order to avoid overfitting in the statistical analysis. Regarding the presenting infection, the following was recorded in addition to the antibiotic treatment strategy: sepsis severity score (SCCM/ESICM/ACCP/ATS/SIS criteria [20]), the maximal concentration of C-reactive protein during hospitalization, the CRB-65 score, and admittance to an intensive care unit. The following was recorded regarding the outcome of the infection: all-cause 30-day mortality, readmission to hospital within 30 days from discharge, and early clinical response. Early clinical response was defined as no signs of fever, tachycardia, hypotension, hypoxemia, or tachypnea on day 4 following admission, according to FDA criteria [21]. Since complete data on all parameters by day 4 were not always available, they were complemented by an evaluation of whether a substantial general improvement of the patient's condition had occurred by day 4.

Patients were sorted into three groups according to type of empirical antibiotic therapy: (1) patients receiving intravenous (i.v.) benzylpenicillin as empirical therapy, (2) patients receiving any other i.v. beta-lactam agent with clinical breakpoints against *H. influenzae*, and (3) patients receiving other empirical treatment regimens. Empirical treatment was defined as the initial antibiotic agent the patient received upon admittance, prior to culture results. Clinical outcomes were compared between groups 1 and 2. In the multivariate regressions and propensity score-matched analyses, all patients who received concomitant empirical therapy with another antibiotic active against *H. influenzae* (fluoroquinolone, tetracycline, aminoglycoside) were excluded.

## Statistical analysis

Data were analyzed using Stata 14 (StataCorp, College Station, TX) and SPSS statistics version 24 (IBM, Armonk, NY). The results were expressed as counts and percentages for categorical variables and as medians and interquartile ranges for continuous variables. Comparisons of baseline statistics between the empirical treatment groups were assessed using $Chi^2$-test for categorical variables, the Mann-Whitney *U* test or the Kruskal-Wallis test for continuous variables. *P* values $\leq 0.05$ were considered statistically significant. Univariate logistic regressions were performed to establish associations between outcomes (30-day all-cause mortality, 30-day readmission, and early clinical response) and collected predictors as well as covariates.

Multivariate logistic regression models were fitted for the defined outcomes. The main predictor was empirical antibiotic treatment (benzylpenicillin monotherapy versus i.v. WSBL monotherapy). Considering the relatively low number of outcomes, to avoid over-fitting, all multivariate models were fitted using the purposeful selection algorithm [22]. Briefly, the main predictor and all covariates with a $p$ value of $< 0.2$ in the univariate analysis were included in a crude model. The least significant covariate was step-wise removed from the model, unless the removal changed the adjusted odds ratios by more than 20%, until only significant and strongly influential covariates remained. A separate analysis was performed for 30-day mortality on only bacteremic patients.

A propensity score-matched analysis was performed to assess the effect of the two empirical treatment groups on 30-day mortality, 30-day readmission, and early clinical response. Propensity scores were calculated in a logistic regression using the treatment group as outcome. The following variables were used as covariates in this regression: age (categorized), sex, ICU-care, maximum CRP, CCI, bacteremia, immune suppression, and sepsis severity score. A 1–1 nearest neighbor matching without replacement was performed using the psmatch2 module [23], with a caliper of 0.2. The propensity scores were plotted graphically to verify spread and overlap, and balance in the covariates in the matched cohort was verified (Table 5). Finally, the associations between treatment and outcomes in the matched cohort were assessed in two ways, with full-cohort logistic regression as well as conditional logistic regression on matched pairs. In all analyses, assessments were made only on individuals with complete outcome data for the respective outcome.

The potential effect modification of beta-lactamase production and rPBP3 on the associations between treatment group and outcomes was investigated by stratifying the outcomes per treatment group and resistance mechanism in the propensity-matched cohort and comparing the odds ratios

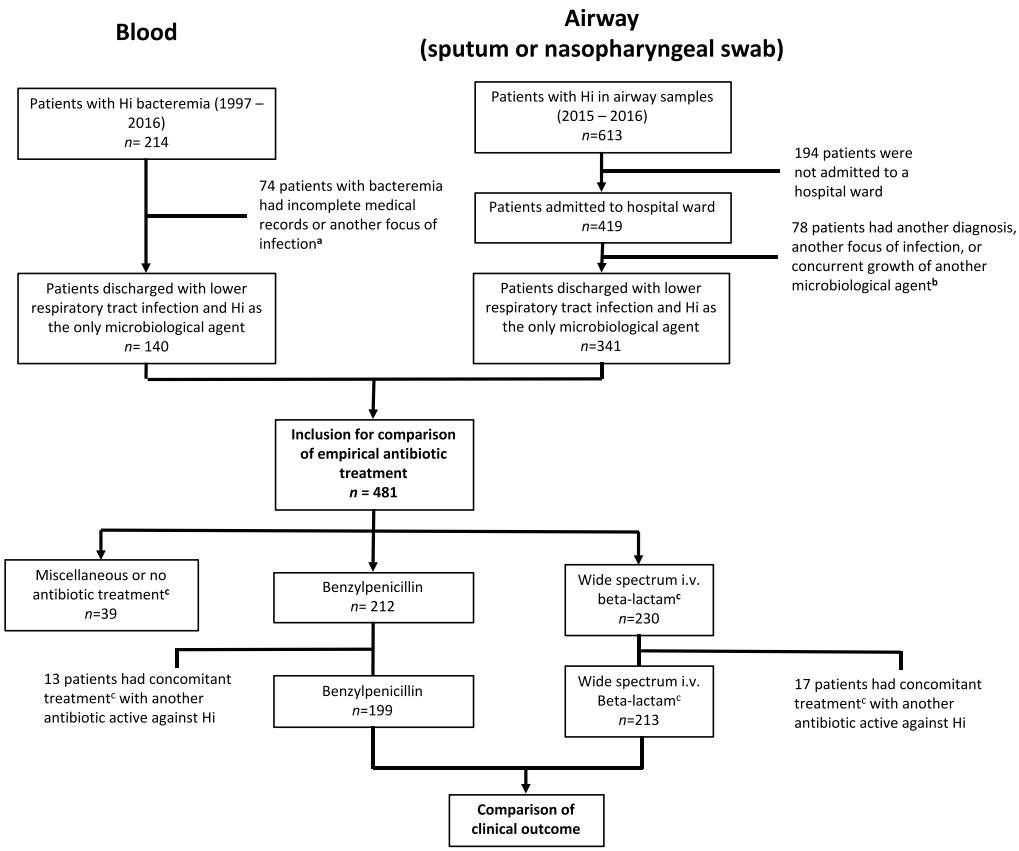


**Fig. 1** Cases included in the study. Flowchart summarizing the number of included and excluded patients in the study as well as the reason for exclusion. Hi = *Haemophilus influenzae*. [a, b]See appendix Table 6 for diagnosis of the excluded patients. [c]See appendix Table 7 for the specific antibiotic agents administered in the different groups

within each strata, including estimation of the beta-coefficient, 95% confidence interval, and *p* value for the respective interaction term in logistic regressions.

## Results

A total of 214 unique episodes of bacteremia with *H. influenzae* were identified in the catchment area between 1997 and 2016. Of these, 140 individuals had a lower respiratory tract infection (the vast majority had CAP, $n = 135$, 96%). In addition, a total of 613 unique adult patients had growth of *H. influenzae* in sputum or nasopharyngeal swabs taken at an emergency department in the catchment area between 2015 and 2016. Of these, 419 were admitted to a hospital ward for more than 24 h. A total of 341 of these patients had been diagnosed with a lower respiratory tract infection (the vast majority were CAP), while at the same time not meeting any of the defined microbiological exclusion criteria. Thus, in total 481 individual cases were included for further analysis (Fig. 1; Appendix Table 6).

### Descriptive and demographic characteristics of the crude study population

Out of the 481 included patients, 281 (58%) were women. The median age was 75 years (interquartile range (IQR), 66–84). A total of 25 patients (5.2%) met the study criteria of immunosuppression, while the median Charlson comorbidity index (CCI), unadjusted for age, was 2 (IQR 1–3). One out of four patients in the study ($n = 120$) had COPD. The most commonly confirmed diagnosis was pneumonia ($n = 418$, 87%)

followed by COPD exacerbation ($n = 39$, 8%). A total of 55 patients (12%) had severe sepsis or septic shock upon admission, whereas 30 (6%) had a CRB-65 score of > 2. The median CRP level was 237 mg/L (IQR 157–318 mg/L), and 21 individuals (4%) were admitted to an ICU. In total, 108 isolates (23%) were screening positive for beta-lactam resistance through rPBP3 in screening, and 65 (14%) were beta-lactamase positive.

Forty-two individuals died within 30 days after cultures were being taken, resulting in an all-cause 30-day mortality of 9%. The 30-day mortality in patients with concurrent bacteremia was 12% ($n = 17$) and among patients with growth of *H. influenzae* exclusively in respiratory tract samples 7% ($n = 25$). The readmission rate 30 days after discharge was 15% ($n = 72$) for the whole study cohort, whereas 77 patients (16%) failed to meet the criteria of early clinical response on day 4.

Patients were sorted into three groups depending on which empiric antibiotic regimen they had been given. The first group comprised all patients treated with benzylpenicillin ($n = 212$, 44%), the second group all patients treated with a WSBL antibiotic (total $n = 230$, 48%; cefotaxim ($n = 175$), piperacillin-tazobactam ($n = 20$), cefuroxime ($n = 16$), imipenem-cilastatin ($n = 8$), meropenem ($n = 8$), ampicillin ($n = 2$), ceftazidime ($n = 1$)), and the third group those who received alternative treatment options ($n = 39$, 8%). A comparison of clinical characteristics between the three groups is presented in Appendix Table 8. Concomitant empirical therapy with another agent active against *H. influenzae* was administered to 13 patients (6%) in the benzylpenicillin group (fluoroquinolone ($n = 3$), doxycycline ($n = 1$), aminoglycoside ($n = 9$)) and to 17 patients (7%) in the wide spectrum beta-lactam group (fluoroquinolone ($n = 8$), doxycycline ($n = 3$), aminoglycoside ($n = 6$)). Thus, 199 patients received benzylpenicillin monotherapy and 213 patients received WSBL monotherapy and were included in the multivariate analysis and formed the basis for propensity score matching (Fig. 1).

**Table 1** Descriptive characteristics of the non-adjusted final cohort on which the logistic regressions are performed, including all individuals receiving empirical monotherapy with benzylpenicillin (PcG) or a wide-spectrum beta-lactam (WSBL). Significant *p* values are in italics

| Covariate | | PcG, $n = 199$ | WSBL[a], $n = 213$ | *P* | Missing values (*n*) |
|---|---|---|---|---|---|
| Age, *n* (%) | | | | 0.50 | – |
| | 0–40 years | 12 (6.0) | 11 (5.2) | | |
| | 40–60 years | 11 (5.5) | 20 (9.4) | | |
| | 60–80 years | 103 (51.8) | 104 (48.8) | | |
| | > 80 years | 73 (36.7) | 78 (36.6) | | |
| Sex, *n* (%) | Female | 112 (56.3) | 128 (60.1) | 0.43 | – |
| Maximum CRP, median (IQR) | | 250 (173–322) | 241 (172–311) | 0.53 | 4 |
| ICU care, *n* (%) | | 3 (1.5) | 12 (5.7) | *0.024* | 2 |
| CCI category (age not included), *n* (%) | | | | *0.001* | 4 |
| | 0–1 | 82 (41.6) | 69 (32.7) | | |
| | 2–3 | 87 (44.2) | 84 (39.8) | | |
| | 4–5 | 24 (12.2) | 35 (16.6) | | |
| | > 5 | 4 (2.0) | 23 (10.9) | | |
| Bacteremia, *n* (%) | | 48 (24.1) | 72 (33.8) | *0.031* | – |
| Immune suppression, *n* (%) | | 10 (5.0) | 13 (6.1) | 0.63 | 4 |
| Sepsis severity, *n* (%) | | | | *0.042* | 8 |
| | no SIRS | 21 (10.6) | 18 (8.7) | | |
| | sepsis | 162 (81.8) | 158 (76.7) | | |
| | severe sepsis | 15 (7.6) | 24 (11.7) | | |
| | septic shock | – | 6 (2.9) | | |
| CRB-65, *n* (%) | | | | 0.12 | 108 |
| | 0–1 | 112 (72.2) | 92 (61.7) | | |
| | 2 | 35 (22.6) | 43 (28.9) | | |
| | 3–4 | 8 (5.2) | 14 (9.4) | | |
| **Potential effect modifiers** | | | | | |
| Beta-lactamase, *n* (%) | | 29 (14.6) | 30 (14.2) | 0.92 | 2 |
| rPBP3, *n* (%) | | 53 (26.6) | 40 (19.0) | 0.064 | 2 |

[a] i.v. WSBL antibiotics (monotherapy only): cefotaxim ($n = 162$), piperacillin-tazobactam ($n = 18$), cefuroxime ($n = 16$), imipenem-cilastatin ($n = 7$), meropenem ($n = 7$), ampicillin ($n = 1$), ceftazidime ($n = 1$)

## Clinical outcome in the two treatment groups according to uni- and multivariate logistic regression analysis

In the group receiving benzylpenicillin as empirical monotherapy, 11 patients (5%) died within 30 days after cultures were taken. In the group receiving WSBL monotherapy, 28 patients (13%) died. There was a significant difference between the two groups in terms of CCI, proportion of patients receiving ICU care, proportion of patients with bacteremia, and sepsis severity. Age, sex, maximal level of CRP, the proportion of immunosuppression, antimicrobial susceptibility to beta-lactam antibiotics, and CRB-65 score did not significantly differ between the two groups (Table 1).

To compare all-cause 30-day mortality between the group treated with benzylpenicillin and the group treated with WSBL, uni- and multivariate logistic regression was performed (Table 2). In the univariate analyses, a significantly increased 30-day mortality was seen in the group treated with WSBL compared to the benzylpenicillin group. Increasing age, CCI score, sepsis severity, and CRB-65 score were also associated with higher 30-day mortality. In the fitted multivariate model, adjusted for age, sepsis severity, and CCI-score, antibiotic treatment with WSBL was no longer significantly associated with increased mortality (OR 2.03, 95%CI 0.91–4.50, $p = 0.082$).

We also compared mortality in patients with only *H. influenzae* bacteremia treated with either benzylpenicillin or other intravenous beta-lactams in a multivariate logistic regression model (Appendix Table 9). Thirty-day mortality was higher in the WSBL group, bordering on statistical significance (age- and CCI-adjusted OR 4.86, 95% CI of 0.98–24, $p = 0.054$), compared to the benzylpenicillin group.

There was no significant association between empirical antibiotic treatment and 30-day readmission rates. Increasing age and CCI were both significantly associated with the risk of readmission in the multivariate regression model (Table 3).

In contrast to 30-day mortality and 30-day readmission rate, the proportion of cases with early clinical response (in

**Table 2** Univariate and multivariate logistic regressions with 30-day mortality as outcome. Significant *p* values in the univariate regressions are in italics

| Thirty-day mortality ($n = 410$) Events = 39 | | Univariate OR (95% CI) | *p* | Multivariate adjusted OR (95% CI), $n = 399$ | *p* | Missing values (*n*) |
|---|---|---|---|---|---|---|
| WSBL vs PcG (ref) empirical monotherapy | | 2.59 (1.25–5.35) | *0.010* | 2.03 (0.91–4.50) | 0.082 | – |
| Age, continuous[a] | | 1.05 (1.02–1.08) | *0.003* | 1.06 (1.03–1.10) | 0.001 | – |
| Sex, female vs male (ref) | | 0.73 (0.38–1.42) | 0.35 | | | – |
| Maximum CRP | | 1.00 (1.00–1.01) | 0.21 | | | 4 |
| ICU care | | 1.48 (0.32–6.81) | 0.62 | | | 2 |
| CCI category (age not included) | | | | | | 4 |
| | 0–1 | ref. cat | – | ref. cat | – | |
| | 2–3 | 1.53 (0.65–3.60) | 0.33 | 0.94 (0.37–2.39) | 0.89 | |
| | 4–5 | 2.17 (0.77–6.12) | 0.15 | 1.06 (0.34–3.36) | 0.92 | |
| | > 5 | 6.64 (2.29–19.3) | *< 0.001* | 5.44 (1.69–17.5) | 0.004 | |
| Bacteremia | | 1.79 (0.91–3.51) | 0.093 | | | – |
| Immune suppression | | 1.46 (0.41–5.16) | 0.56 | | | 4 |
| Sepsis severity | | | | | | 8 |
| | no | ref. cat | – | ref. cat | – | |
| | sepsis | 1.53 (0.35–6.73) | 0.57 | 1.40 (0.30–6.42) | 0.67 | |
| | severe sepsis | 5.4 (1.08–26.9) | *0.04* | 5.21 (0.96–28.3) | 0.056 | |
| | septic shock | 18 (2.1–153) | *0.008* | 28.1 (2.49–316) | 0.007 | |
| CRB-65[b] | | | | | | 108 |
| | 0-1 | ref. cat | – | | | |
| | 2 | 3.5 (1.54–7.96) | *0.003* | | | |
| | 3–4 | 6 (1.99–18.1) | *0.001* | | | |

[a] Used as a continuous variable due to no events in the youngest age group

[b] Not used in the multivariate analysis due to the number of missing values

which data could be obtained) was higher in the WSBL group ($n = 170$, 84%) compared with the benzylpenicillin group ($n = 153$, 81%). This difference was statistically significant in a multivariate regression model after adjustment for age, bacteremia, CCI, and sepsis severity (OR 2.28, 95% CI 1.21–4.31, $p = 0.011$) (Table 4).

## Clinical outcomes in the two treatment groups in the propensity score-matched cohort

To further adjust for potential confounders between the two treatment groups, a propensity score-matched cohort was constructed. The matched cohort consisted of 151 individuals treated with empirical benzylpenicillin monotherapy and 151 individuals receiving empirical WSBL treatment. In this matched cohort, 9 patients (6%) died within 30 days in the benzylpenicillin group and 18 patients (12%) died in the WSBL group (Appendix Table 10). Table 5 shows the balance in covariates between the two groups. The propensity score matching resulted in the omission of the most severely ill patients in the WSBL group, which is reasonable considering that these patients are not recommended empirical benzylpenicillin treatment.

Thirty-day mortality was still higher, but not significantly higher in the WSBL group when comparing the matched groups both in a full cohort logistic regression model (OR 2.14, 95% CI 0.93–4.92, $p = 0.075$) and in a conditional regression model (OR 1.89, 95% CI 0.84–4.23, $p = 0.12$). There were still no significant differences in 30-day readmission rates (Appendix Table 10). Early clinical response rates remained significantly higher in the WSBL group both by full cohort logistic regression (OR 2.14, 95% CI 1.07–4.27, $p = 0.031$) and by conditional logistic regression (OR 2.5, 95% CI 1.20–5.21, $p = 0.014$).

## Effect modification

In order to evaluate any potential effect modification by beta-lactamase production and rPBP3, the odds ratios of the stratified outcomes per treatment group and resistance mechanism in the propensity-matched cohort were compared (Appendix Tables 11and 12). The presence of a beta-lactamase was a significant effect modifier of the association between treatment group and early clinical response (the interaction term for beta-lactamase × treatment group (benzylpenicillin as reference): $\beta = 3.12$, 95% CI 0.82–5.43, $p = 0.008$), explaining a substantial portion of the difference in early clinical response. This was not the case for

**Table 3** Univariate and multivariate logistic regressions with 30-day readmission as outcome. Significant $p$ values in the univariate regressions are in italics

| Thirty-day readmission ($n = 369$) Events = 63 | | Univariate OR (95% CI) | $p$ | Multivariate adjusted OR (95%CI), $n = 369$ | $p$ | Missing values ($n$) |
|---|---|---|---|---|---|---|
| WSBL vs PcG (ref) empirical monotherapy | | 1.23 (0.72–2.13) | 0.45 | 1.16 (0.66–2.05) | 0.61 | – |
| Age, continuous[a] | | 1.04 (1.01–1.06) | *0.003* | 1.03 (1.01–1.06) | 0.013 | – |
| Sex, female vs male (ref) | | 0.95 (0.55–1.64) | 0.84 | | | – |
| Maximum CRP | | 1.00 (0.19–4.05) | 0.87 | | | 2 |
| ICU care | | 0.88 (0.32–6.81) | 0.62 | | | 1 |
| CCI category (age not included) | | | | | | – |
| | 0–1 | ref. cat | – | ref. cat | – | |
| | 2–3 | 2.27 (1.14–4.55) | *0.020* | 1.80 (0.88–3.68) | 0.11 | |
| | 4–5 | 2.84 (1.20–6.72) | *0.018* | 2.23 (0.92–5.39) | 0.075 | |
| | > 5 | 4.73 (1.62–13.8) | *0.005* | 3.98 (1.32–12.0) | 0.014 | |
| Bacteremia | | 1.44 (0.81–2.56) | 0.21 | | | – |
| Immune suppression | | 1.15 (0.37–3.55) | 0.81 | | | – |
| Sepsis severity | | | | | | 12 |
| | no SIRS | ref. cat | – | | | |
| | sepsis | 1.26 (0.47–3.40) | 0.65 | | | |
| | severe sepsis | 2.56 (0.73–8.93) | 0.14 | | | |
| | septic shock | no events | – | | | |
| CRB-65[b] | | | | | | 93 |
| | 0-1 | ref. cat | – | | | |
| | 2 | 1.38 (0.67–2.84) | 0.39 | | | |
| | 3–4 | 3.18 (1.09–9.28) | *0.035* | | | |

[a] Used as a continuous variable due to no events in the youngest age group

[b] Not used in the multivariate analysis due to the number of missing values

**Table 4** Univariate and multivariate logistic regressions with early clinical response as outcome. Significant *p* values in the univariate regressions are in italics

| Early clinical response ($n = 392$) (events = 323) | | Univariate OR (95%CI) | *p* | Multivariate, adjusted OR (95%CI), $n = 385$ | *p* | Missing values (*n*) |
|---|---|---|---|---|---|---|
| WSBL vs PcG (ref) empirical monotherapy | | 1.28 (0.76–2.16) | 0.35 | 2.28 (1.21–4.31) | 0.011 | – |
| Age, continuous[a] | | 0.98 (0.96–1.00) | *0.031* | 0.97 (0.95–0.99) | 0.012 | – |
| Sex, female vs male (ref) | | 1.02 (0.60–1.73) | 0.93 | | | – |
| Maximum CRP | | 0.99 (0.99–1.00) | *< 0.001* | | | 2 |
| ICU care | | 0.17 (0.05–0.52) | *0.002* | | | – |
| CCI category (age not included) | | | | | | – |
| | 0–1 | ref. cat | – | ref cat | – | |
| | 2–3 | 1.33 (0.73–2.45) | 0.35 | 2.72 (1.30–5.70) | 0.008 | |
| | 4–5 | 0.97 (0.44–2.11) | 0.93 | 1.33 (0.54–3.27) | 0.532 | |
| | > 5 | 0.54 (0.21–1.36) | 0.19 | 0.47 (0.16–1.37) | 0.17 | |
| Bacteremia | | 0.28 (0.16–0.49) | *< 0.001* | 0.37 (0.20–0.69) | 0.002 | – |
| Immune suppression | | 2.09 (0.48–9.20) | 0.33 | | | – |
| Sepsis severity | | | | | | 7 |
| | no SIRS | ref. cat | – | ref. cat | | |
| | sepsis | 0.31 (0.072–1.34) | 0.12 | 0.27 (0.06–1.21) | 0.087 | |
| | severe sepsis | 0.06 (0.013–0.30) | *0.001* | 0.05 (0.010–0.27) | < 0.001 | |
| | septic shock | 0.01 (0.001–0.18) | *0.001* | 0.009 (0.001–0.15) | 0.001 | |
| CRB-65[b] | | | | | | 98 |
| | 0-1 | ref. cat | – | | | |
| | 2 | 0.75 (0.37–1.51) | 0.41 | | | |
| | 3–4 | 0.17 (0.06–0.43) | *< 0.001* | | | |

[a] Used as a continuous variable due to no non-events in the youngest age group

[b] Not used in the multivariate analysis due to the number of missing values

isolates with rPBP3 (the interaction term for rPBP3 × treatment group (benzylpenicillin as reference): $\beta = 0.74$, 95% CI − 1.05–2.54, $p = 0.42$). Neither the associations between all cause 30-day mortality nor between 30-day readmission rates and treatment group were significantly modified by the presence of either resistance mechanism.

## Discussion

In the present study, the all-cause 30-day mortality of severe lower respiratory tract infections caused by *H. influenzae* was 9%. In an analysis adjusted for potential confounders, empirical monotherapy with benzylpenicillin was not significantly associated with increased risk of mortality or readmission. Early clinical response rates were high in both treatment groups, but significantly higher in the group receiving empirical monotherapy with a WSBL in an adjusted analysis, a difference largely explained by effect modification of benzylpenicillin treatment by beta-lactamase-producing strains.

Previous studies have reported mortality rates between 8 and 22% for *Haemophilus* bacteremia, results that are in good agreement with our findings of 12% in the bacteremia cohort [4, 5, 16]. Data on fatality rates of in-patients with pneumonia is scarce, with one recent study reporting a 30-day case fatality ratio of 2% [24]. This is lower than what we found in the non-bacteremia group (7%). The lower overall mortality in our study (9%) supports the notion that lower respiratory tract infections with *H. influenzae* are generally associated with less severe presentation compared with *S. pneumoniae* [1].

To the best of our knowledge, only one previous report has compared treatment outcome of benzylpenicillin in severe infections by *H. influenzae* [16]. That study was done in a retrospective cohort of bacteremia cases with various foci of infection, in Copenhagen, Denmark. The authors found a significantly increased 30-day mortality when using benzylpenicillin as a definitive treatment for bacteremia. As for empirical treatment, the results were not significant, but there was a trend towards higher mortality in the benzylpenicillin group ($p = 0.06$). This study also had a higher overall case fatality rate, reaching 22%. These results contrast our findings, where no such difference in outcome could be shown between treatment groups, neither in the bacteremia cohort nor in the overall study cohort. Since our study population only comprised respiratory tract infections, and empirical, as opposed to definite, treatment with benzylpenicillin, the results are not fully comparable.

**Table 5** Descriptive characteristics for the 8 covariates matched for in the propensity-matched cohort, based on individuals receiving empirical monotherapy with benzylpenicillin (PcG) or a wide-spectrum beta-lactam (WSBL), using a caliper of 0.2 ($n = 302$)

| Covariate | | PcG, $n = 151$ | WSBL, $n = 151$ | $p$ | Missing values ($n$) |
|---|---|---|---|---|---|
| Age, $n$ (%) | | | | 0.97 | – |
| | 0–40 years | 8 (5.3) | 7 (4.6) | | |
| | 40–60 years | 11 (7.3) | 13 (8.6) | | |
| | 60–80 years | 74 (49.0) | 74 (49.0) | | |
| | > 80 years | 58 (38.4) | 57 (37.8) | | |
| Sex, $n$ (%) | Female | 95 (62.9) | 91 (60.3) | 0.64 | – |
| Maximum CRP, median (IQR) | | 223 (142–306) | 226 (167–304) | 0.74 | – |
| ICU care, $n$ (%) | | 3 (2.0) | 2 (1.3) | 0.65 | – |
| CCI category (age not included), $n$ (%) | | | | 0.93 | – |
| | 0–1 | 53 (35.1) | 58 (38.4) | | |
| | 2–3 | 71 (47.0) | 68 (45.0) | | |
| | 4–5 | 23 (15.2) | 22 (14.6) | | |
| | > 5 | 4 (2.7) | 3 (2.0) | | |
| Bacteremia, $n$ (%) | | 38 (25.1) | 41 (27.2) | 0.69 | – |
| Immune suppression, $n$ (%) | | 8 (5.3) | 9 (6.0) | 0.80 | – |
| Sepsis severity, $n$ (%) | | | | 0.63 | – |
| | no SIRS | 13 (8.6) | 17 (11.3) | | |
| | sepsis | 126 (83.4) | 123 (81.5) | | |
| | severe sepsis | 12 (8.0) | 10 (6.6) | | |
| | septic shock | – | 1 (0.7) | | |
| CRB-65[a], $n$ (%) | | | | 0.96 | 79 |
| | 0–1 | 82 (70.0) | 73 (68.9) | | |
| | 2 | 28 (23.9) | 27 (25.5) | | |
| | 3–4 | 7 (6.0) | 6 (5.7) | | |

[a] Not used for matching due to the number of missing values

In another recent, propensity score-matched study, benzylpenicillin and phenoxymethylpenicillin were compared with WSBL as empirical treatment of pneumonia with CRB-65 score ≤ 2 regardless of etiological agent [25]. No significant difference in mortality was seen between the two groups.

In contrast to 30-day mortality, there was a significantly lower chance of early clinical response among patients treated with benzylpenicillin in our study. However, the early clinical response rate in the benzylpenicillin group was still above 80%, and beta-lactamase production was found to be a significant effect modifier. When comparing patients infected with non-beta-lactamase-producing isolates in the propensity-matched cohort, early clinical response rates were 87% in the benzylpenicillin group compared to 89% in the WSBL group (Appendix Table 11).

The strength of this study includes its substantial size of an unselected, population-based homogenous cohort of cases with *H. influenzae* respiratory tract infection and a thorough analysis of data. The risk of an indication bias in a retrospective analysis of antibiotic treatment is always substantial. To counter this, the treatment groups were adjusted for known covariates that could confound the outcome association, including age, comorbidities, infection severity, and maximal level of CRP. However, the retrospective design of the study still entails a risk for residual confounding, supported by the counter-intuitive result that the mortality was higher in the wide-spectrum treatment group, and that this difference in outcome was bordering significance in multivariate analysis even after adjusting for confounders. On the other hand, this type of study would be very challenging to perform prospectively, as the causative agent in CAP is rarely known at the start of treatment. Another limitation of this study is its limited power due to the relatively low mortality rate. Given the number of patients in the two treatment groups ($n = 199$ for the benzylpenicillin group and $n = 213$ for the WSBL group), a defined $\alpha = 0.05$ and $\beta = 0.8$, and a mortality of 14% in the WSBL group, this study has power to significantly detect an odds ratio of approximately 2, assuming a two-sided test of equality.

We have not performed a statistical comparison between high-dose (3 g t.i.d or higher) and low-dose (1 g t.i.d. or less) benzylpenicillin treatment. Monte Carlo simulations calculated by EUCAST suggest significantly lower target attainment rates for the latter regimen, which might lead to treatment failure [11]. Therefore, current Swedish guidelines advocate the use of high-dose benzylpenicillin in CAP treatment [9]. In our cohort, the fraction of patients receiving high-dose versus low-dose

treatment were quite similar (55% (116/212) versus 44% (93/212), appendix Table 8). There was a trend towards fewer case fatalities in the high-dose group ($n = 4$ versus $n = 7$), but the number of events was too small to allow further statistical comparison.

Almost all rPBP3 isolates in Europe are still considered susceptible to third generation cephalosporins, whereas the susceptibility to aminopenicillins, and thus also to regular benzylpenicillin remains a matter of controversy [26]. In our study, the presence of rPBP3 isolates does not seem to have any detrimental effect on the clinical outcome in the benzylpenicillin group compared to patients treated with WSBL. Beta-lactamase production, in contrast to rPBP3, was a significant effect modifier, associated with a risk of reduced early clinical response in the benzylpenicillin group. This is intuitive, since third generation cephalosporins are generally stable to beta-lactamases expressed by *H. influenzae*, whereas benzylpenicillin is not [14]. Thus, beta-lactamase expression may well account for cases of treatment failure in this group. The proportion of beta-lactamase producing strains, however, has been stable over the past decades, whereas the increase in rPBP3 isolates has been worrisome [12, 13].

There has been debate on the optimal empirical treatment of respiratory tract infections and CAP caused by *H. influenzae*. Although definitive therapy with an agent with clinical breakpoints against *H. influenzae* always should be used following a positive culture, the present study suggests that empirical benzylpenicillin treatment is not associated with higher mortality in patients with mild to moderate lower respiratory tract infection caused by *H. influenzae.*

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** The study design was reviewed and approved by the Regional Ethical Review Board in Lund, Sweden (Dnr 2016/743, addendum 18-391).

**Research involving human participants and/or animals** The study design was reviewed and approved by the Regional Ethical Review Board in Lund, Sweden (Dnr 2016/743, addendum 18-391). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

**Informed consent** Individual informed consent was waived in this retrospective observational study, but information about the study and its purpose was announced in local and regional newspapers. The study design was approved by the regional ethical review board.

## Appendix

**Table 6** Patients excluded from the initial cohort due to other foci of infection or other diagnosis

| Patients excluded from bacteremia group due to other foci of infection | |
|---|---|
| Epiglottitis | $n = 14$ |
| Meningitis | $n = 11$ |
| Abdominal infection | $n = 8$ |
| Urinary Tract Infection | $n = 3$ |
| Arthritis | $n = 3$ |
| Peritoneal infection | $n = 2$ |
| Gynecological infection | $n = 2$ |
| Soft tissue infection | $n = 2$ |
| Aortitis | $n = 1$ |
| Unknown focus of infection | $n = 7$ |
| Incomplete medical records | $n = 21$ |
| Total | $n = 74$ |
| **Patients excluded from the sputum/nasopharyngeal sample group due to other diagnosis** | |
| Other respiratory tract pathogens | |
| Influenza virus | $n = 11$ |
| *Streptococcus pneumoniae* | $n = 6$ |
| Respiratory syncytial virus | $n = 4$ |
| *Mycoplasma pneumoniae* | $n = 3$ |
| β-hemolytic Streptococci | $n = 3$ |
| *Legionella pneumonia* | $n = 1$ |
| *Pneumocystis jirovecii* | $n = 1$ |
| *Chlamydophila pneumoniae* | $n = 1$ |
| Total | $n = 30$ |
| Other focus of infection than the respiratory tract (w or w/o other pathogens) | |
| Urinary tract infection | $n = 7$ |
| *Escherichia coli* bacteremia | $n = 4$ |
| *Staphylococcus aureus* bacteremia | $n = 3$ |
| *Streptococcus mitis* bacteremia | $n = 2$ |
| *Pseudomonas aeruginosa* bacteremia | $n = 1$ |
| Erysipelas | $n = 1$ |
| *Clostridium difficile* enteritis | $n = 1$ |
| Unknown focus of infection | $n = 9$ |
| Total | $n = 28$ |
| No signs of infection | $n = 20$ |
| Total | $n = 78$ |

**Table 7** Detailed description of the empirical antibiotic therapy given

| Description of the empirical antibiotic treatment given: | | | |
|---|---|---|---|
| Empiric antibiotic treatment (*n*, %) | Group 1) | Benzylpenicillin | 212 (44%) |
| | | Total | 212 (44%) |
| | Group 2) | Cefotaxime | 175 (36%) |
| | | Cefuroxime | 16 (3%) |
| | | Piperacillin-tazobactam | 20 (4%) |
| | | Imipenem-cilastatin | 8 (2%) |
| | | Meropenem | 8 (2%) |
| | | Ampicillin | 2 (< 1%) |
| | | Ceftazidime | 1 (< 1%) |
| | | Total | 230 (48%) |
| | Group 3) | Doxycycline | 12 (2%) |
| | | Clindamycine | 6 (1%) |
| | | Amoxicillin | 5 (1%) |
| | | Erythromycine | 3 (< 1%) |
| | | Ciprofloxacine | 2 (< 1%) |
| | | Phenoximethyl penicillin | 2 (< 1%) |
| | | Trimetoprim / sulfamethoxazole | 2 (< 1%) |
| | | Amoxicillin / clavulanic acid | 1 (< 1%) |
| | | Cloxacillin | 1 (< 1%) |
| | | Levofloxacine | 1 (< 1%) |
| | | Roxithromycine | 1 (< 1%) |
| | | No antibiotic treatment | 2 (< 1%) |
| | | Missing data | 1 (< 1%) |
| | | Total | 39 (8%) |
| Allergy to penicillin (*n*, %) | | No | 461 (96%) |
| | | Yes | 15 (3%) |
| | | Missing data | 5 (1%) |
| Concomitant antibiotic treatment active against H. influenzae in Benzylpenicillin group (*n*, % within group) | | None | 199 (94%) |
| | | Fluoroquinolone | 3 (1%) |
| | | Doxycycline | 1 (< 1%) |
| | | Aminoglycoside (1 dose) | 9 (4%) |
| | | Total | 212 (100%) |
| Concomitant antibiotic treatment active against H. influenzae in wide-spectrum beta-lactam group (*n*, % within group) | | None | 213 (93%) |
| | | Fluoroquinolone | 8 (4%) |
| | | Doxycycline | 3 (1%) |
| | | Aminoglycoside (1 dose) | 6 (3%) |
| | | Total | 230 (100%) |
| Concomitant antibiotic treatment active against H. influenzae in the miscellaneous group (*n*, % within group) | | None | 36 (92%) |
| | | Fluoroquinolone | 1 (2%) |
| | | Doxycycline | 1 (2%) |
| | | Aminoglycoside (1 dose) | 1 (2%) |

**Table 7** (continued)

| Description of the empirical antibiotic treatment given: | | |
|---|---|---|
| | Total | 39 (100%) |
| Dosage of benzylpenicillin | 1 g t.i.d. | 84 (40%) |
| (*n*, % within total group) | 3 g t.i.d. | 104 (49%) |
| | 1 g b.i.d. | 9 (4%) |
| | 2 g t.i.d. | 11 (6%) |
| | 1 g q.i.d. | 1 (< 1%) |
| | Missing data | 3 (1%) |
| | Total | 212 (100%) |
| 30-day mortality (no of casualties, % within dosage group) in the benzylpenicillin group sorted by dosage | | |
| | 1 g t.i.d. | 5 (6%) |
| | 3 g t.i.d. | 4 (4%) |
| | 1 g b.i.d. | 2 (22%) |
| | Other dosages | 0 |

**Table 8** Descriptive characteristics of the crude cohort, including all individuals in the study, stratified on treatment group. Significant differences are italics

| Potential confounders | | PcG, *n* = 212 | WSBL[a], *n* = 230 | Other/no antibiotic *n* = 39 | *p* | Missing values (*n*) |
|---|---|---|---|---|---|---|
| Age *n* (%) | | | | | 0.13 | – |
| | 0–40 years | 12 (5.7) | 11 (4.8) | 6 (15.4) | | |
| | 40–60 years | 13 (6.1) | 20 (8,7) | 5 (12.8) | | |
| | 60–80 years | 110 (51.9) | 115 (50.0) | 18 (46.2) | | |
| | > 80 years | 77 (36.3) | 84 (36.5) | 10 (25.6) | | |
| Sex *n* (%) | Female | 118 (55.7) | 137 (59.6) | 26 (66.7) | 0.39 | – |
| MaxCRP (median) | | 251 | 242 | 127 | *< 0.001* | 4 |
| ICU care *n* (%) | | 5 (2.4) | 15 (5.7) | 1 | 0.082 | 2 |
| CCIcat (unadjusted for age) *n* (%) | | | | | *< 0.001* | 4 |
| | 0–1 | 89 (42.4) | 75 (32.9) | 21 (53.9) | | |
| | 2–3 | 90 (42.9) | 90 (39.5) | 11 (28.2) | | |
| | 4–5 | 27 (12.9) | 38 (16.7) | 7 (18.0) | | |
| | > 5 | 4 (1.9) | 25 (11.0) | – | | |
| Bacteremia *n* (%) | | 53 (25.0) | 78 (33.9) | 9 (23.1) | 0.082 | – |
| Immune suppression *n* (%) | | 11 (5.2) | 14 (6.1) | – | 0.28 | 4 |
| Sepsis severity *n* (%) | | | | | 0.12 | 11 |
| | no SIRS | 22 (10.4) | 18 (8.1) | 5 (13.9) | | |
| | sepsis | 170 (80.6) | 172 (77.1) | 28 (77.8) | | |
| | severe sepsis | 19 (9.0) | 25 (11.2) | 2 (5.6) | | |
| | septic shock | – | 8 (3.6) | 1 (2.8) | | |
| CRB65 *n* (%) | | | | | 0.26 | 123 |
| | 0–1 | 119 (71.7) | 100 (60.6) | 12 (70.6) | | |
| | 2 | 36 (21.7) | 48 (29.1) | 3 (17.7) | | |
| | 3–4 | 11 (6.6) | 17 (10.3) | 2 (11.8) | | |
| Potential effect modifiers | | | | | | |
| Beta-lactamase *n* (%) | | 32 (15.1) | 31 (13.6) | 2 (5.1) | 0.25 | 2 |
| rPBP3 *n* (%) | | 56 (26.4) | 44 (19.3) | 8 (20.5) | 0.19 | 2 |
| Low dose Benzyl-penicillin[b] *n* (%) | | 93 (45.6) | – | | | 3 |

[a] Wide spectrum i.v. beta lactam antibiotics: cefotaxim (*n* = 175), piperacillin-tazobactam (*n* = 20), cefuroxime (*n* = 16), imipenem-cilastatin (*n* = 8), meropenem (*n* = 8), ampicillin (*n* = 2), ceftazidime (*n* = 1)

[b] 1 g t.i.d. or lower

**Table 9** Univariate and multivariate logistic regressions with outcome 30-day mortality in only bacteremic isolates receiving empirical monotherapy with an i.v. beta-lactam. Significant *p* values in the univariate regressions are in italics

| Thirty-day mortality, ($n = 120$) Events = 16 | | Univariate odds ratio (95%CI) | *P* | Multivariate adjusted OR (95%CI), $n = 116$ | *p* | Missing values (*n*) |
|---|---|---|---|---|---|---|
| WSBL vs PcG (ref) empirical monotherapy | | 5.55 (1.20–25.7) | *0.028* | 4.86 (0.98–24) | 0.054 | – |
| Age, continuous[a] | | 1.05 (1.00–1.10) | *0.004* | 1.06 (1.01–1.12) | 0.025 | – |
| Sex, female vs male (ref) | | 0.68 (0.24–1.95) | 0.47 | | | – |
| Maximum CRP | | 1.00 (1.00–1.01) | 0.35 | | | 4 |
| ICU care | | no events | – | | | 2 |
| CCI category (age not included) | | | | | | 4 |
| | 0–1 | ref. cat | – | ref cat | – | |
| | 2–3 | 1.09 (0.27–4.33) | 0.91 | 0.65 (0.15–2.83) | 0.57 | |
| | 4–5 | 3.33 (0.63–17.6) | 0.16 | 1.65 (0.26–10.3) | 0.59 | |
| | > 5 | 8.0 (1.51–42.4) | *0.015* | 4.59 (0.74–28.6) | 0.10 | |
| Immune suppression | | no events | – | | | 2 |
| Sepsis severity | | | | | | 6 |
| | No | ref. cat | – | | | |
| | sepsis | 0.72 (0.08–6.86) | 0.78 | | | |
| | severe sepsis | 0.71 (0.06–8.40) | 0.79 | | | |
| | septic shock | 3.33 (0.20–54) | 0.40 | | | |
| CRB65[b] | | | | | | 47 |
| | 0-1 | ref. cat | – | | | |
| | 2 | 1.75 (0.39–7.8) | 0.46 | | | |
| | 3–4 | 3.75 (0.68–21) | 0.13 | | | |

[a] Used as a continuous variable due to no events in the youngest age group

[b] Not used in the multivariate analysis due to the number of missing values

**Table 10** Outcomes stratified by treatment group in the propensity-matched cohort

| No | *n* (%) | | logistic regression, OR (95%CI) | | | conditional logistic regression, OR (95%CI) | | |
|---|---|---|---|---|---|---|---|---|
| | Benzyl-penicillin | WSBL | Benzyl-penicillin | WSBL | *p* value | Benzyl-penicillin | WSBL | *p* value |
| 30-day all cause mortality, ($n = 300$) | 9/150 (6.0%) | 18/150 (12.0%) | 1 (ref) | 2.14 (0.93–4.92) | 0.075 | 1 (ref) | 1.89 (0.84–4.23) | 0.12 |
| 30-day all cause hospital readmission, ($n = 275$) | 24/143 (16.8%) | 21/132 (15.9%) | 1 (ref) | 0.94 (0.49–1.78) | 0.85 | 1 (ref) | 0.89 (0.46–1.72 | 0.74 |
| Early clinical response at day 4 ($n = 292$) | 119/146 (81.5%) | 132/146 (90.4%) | 1 (ref) | 2.14 (1.07–4.27) | 0.031 | 1 (ref) | 2.5 (1.20–5.21) | 0.014 |

**Table 11** Potential effect modification of the treatment group by betalactamase production on the respective outcomes in the propensity-matched cohort, balanced by covariates earlier described

| Thirty-day all-cause mortality ($n = 300$) | Benzylpenicillin treatment | | Wide-spectrum betalactamase treatment | | OR within STRATA of beta-lactamase presence |
|---|---|---|---|---|---|
| | *n* with outcome (%) | OR (95% CI), *p* | | OR (95% CI), *p* | |
| Isolates without betalactamase | 8/129 (6.2%) | 1 (ref) | 16/125 (12.8%) | 2.22 (0.91–5.39), $p = 0.078$ | 2.22 (0.91–5.39), p = 0.078 |
| Isolates with beta-lactamase | 1/21 (4.7%) | 0.76 (0.090–6.38), $p = 0.80$ | 2/25 (8.0%) | 1.32 (0.26–6.60), $p = 0.74$ | 1.74 (0.15–20.6), $p = 0.66$ |
| 30-day all-cause readmission ($n = 275$) | | | | | |
| Isolates without betalactamase | 21/124 (16.9%) | 1 (ref) | 19/108 (17.6%) | 1.05 (0.53–2.07), $p = 0.90$ | 1.05 (0.53–2.07), p = 0.90 |
| Isolates with beta-lactamase | 3/19 (15.8%) | 0.92 (0.25–3.44), p = 0.90 | 2/24 (8.3%) | 0.45 (0.097–2.04), $p = 0.30$ | 0.48 (0.072–3.25), $p = 0.46$ |
| Early clinical response on day 4 ($n = 292$) | | | | | |
| Isolates without betalactamase | 109/125 (87.2%) | 1 (ref) | 107/120 (89.2%) | 1.21 (0.55–2.63), $p = 0.63$ | 1.21 (0.55–2.63), p = 0.63 |
| Isolates with beta-lactamase | 10/21 (47.6%) | 0.13 (0.05–0.36), $p < 0.001$ | 25/26 (96.1%) | 3.67 (0.46–29.0), $p = 0.22$ | 27.5 (3.13–242), $p = 0.003$ |

Interaction term for betalactamase × treatment group (Benzyl-pc as ref) for 30-day mortality: β = − 0.24 (− 2.97–2.38), $p = 0.86$

Interaction term for betalactamase × treatment group (Benzyl-pc as ref) for 30-day readmission: β = − 0.77 (− 2.79–1.25), $p = 0.46$

Interaction term for betalactamase × treatment group (Benzyl-pc as ref) for early clinical response: β = 3.12 (0.82–5.43), $p = 0.008$

**Table 12** Potential effect modification of the treatment group by rPBP3 on the respective outcomes in the propensity-matched cohort, balanced by covariates earlier described

| Thirty-day all-cause mortality ($n = 300$) | Benzylpenicillin treatment | | Wide-spectrum betalactamase treatment | | OR within STRATA of rPBP3 |
|---|---|---|---|---|---|
| | *n* with outcome (%) | OR (95% CI), *p* | | OR (95% CI), *p* | |
| Isolates without rPBP3 | 4/110 (3.6%) | 1 (ref) | 13/121 (10.7%) | 3.19 (1.01–10.1), $p = 0.048$ | 3.19 (1.01–10.1), $p = 0.048$ |
| Isolates with rPBP3 | 5/40 (12.5%) | 3.79 (0.96–14.9), $p = 0.06$ | 5/29 (17.2%) | 5.52 (1.38–22.1), $p = 0.016$ | 1.46 (0.38–5.6), $p = 0.58$ |
| 30-day all-cause readmission ($n = 275$) | | | | | |
| Isolates without rPBP3 | 17/106 (16.0%) | 1 (ref) | 19/106 (17.9%) | 1.14 (0.56–2.34), $p = 0.72$ | 1.14 (0.56–2.34), $p = 0.72$ |
| Isolates with rPBP3 | 7/37 (18.9%) | 1.22 (0.46–3.23), $p = 0.69$ | 2/26 (7.7%) | 0.44 (0.094–2.02), $p = 0.29$ | 0.35 (0.068–1.88), $p = 0.22$ |
| Early clinical response on day 4 ($n = 292$) | | | | | |
| Isolates without rPBP3 | 87/105 (82.9%) | 1 (ref) | 105/117 (89.7%) | 1.81 (0.83–3.96), $p = 0.14$ | 1.21 (0.55–2.63), $p = 0.63$ |
| Isolates with rPBP3 | 32/41 (78.0%) | 0.74 (0.30–1.80), $p = 0.50$ | 27/29 (93.1%) | 2.79 (0.61–12.8), $p = 0.19$ | 3.80 (0.75–19.1), $p = 0.11$ |

Interaction term for rPBP3 × treatment group (Benzyl-pc as ref) for 30-day mortality: β = − 0.78 (− 2.55–0.99), $p = 0.39$

Interaction term for rPBP3 × treatment group (Benzyl-pc as ref) for 30-day readmission: β = − 1.16 (− 2.97–0.65), $p = 0.21$

Interaction term for rPBP3 × treatment group (Benzyl-pc as ref) for early clinical response: β = 0.74 (− 1.05–2.54), $p = 0.42$

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