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THE ARCTIC UNIVERSITY OF NORWAY

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Non-beta-lactamase-mediated beta-lactam resistance in *Haemophilus influenzae*

Mechanisms, epidemiology and susceptibility testing

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In the precious memories of my father, mother and brother

The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.

Alexander Fleming in his Nobel lecture, December 1945 [133]

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Front page picture: word cloud based on the content of this thesis (www.wordle.net).

SUMMARY

Haemophilus influenzae is a major pathogen, with the ability to cause a wide spectrum of invasive and non-invasive infections. Beta-lactams are first-line drugs but beta-lactam resistant strains are common. Beta-lactamase (*bla*) producing isolates emerged in the 1970s, and non-*bla*-mediated resistance due to mutations in the *ftsI* gene encoding penicillin-binding protein 3, denoted 'rPBP3' in this project, has increased in recent years. Low-rPBP3 *H. influenzae* are defined by the absence of the S385T substitution and the presence of R517H (group I) or N526K (group II); these genotypes predominate in Europe, North America and Australia, whereas high-rPBP3 isolates (defined by the additional S385T substitution) are common in Japan and Korea. Data from the Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM) suggest that rPBP3 *H. influenzae* emerged in Norway in the early 2000s. In this project, two cross-sectional (I and II) and one longitudinal study (III) were performed to explore the resistance mechanisms, epidemiology and clinical characteristics of *H. influenzae* with non-*bla*-mediated beta-lactam resistance. The project was the first to characterize the resistance mechanism in Nordic *H. influenzae* with this phenotype.

Study I encompassed 46 respiratory *H. influenzae* from NORM 2004, including 23 isolates with phenotypes suggesting the presence of non-*bla*-mediated beta-lactam resistance mechanisms and 23 susceptible control isolates. Study II encompassed 196 respiratory isolates from NORM 2007, including 177 with non-wild type susceptibility to beta-lactams not explained by *bla*, and 19 susceptible controls. Characterization included pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *ftsI* sequencing with deduction of PBP3 substitution patterns (PBP3 typing), and susceptibility testing by determination of beta-lactam MICs.

The prevalence of rPBP3 isolates in 2007 was 14.6%. The exact prevalence in 2004 could not be calculated (≥4.8%), but was estimated to 5.7% based on NORM data and the rPBP3 prevalence / amoxicillin-clavulanic acid resistance rate ratio in 2007. These results indicate that the rPBP3 prevalence increased significantly in Norway from

2004 to 2007. Estimated rPBP3 prevalences in more recent NORM populations suggest a further increase to 16.6% in 2014. Enhanced selection pressure due to a 76% increase in amoxicillin usage between 2000 and 2012 may have contributed to the increased frequency of rPBP3 *H. influenzae* in Norway.

Isolates with group II low-rPBP3 genotypes accounted for most (96%) of rPBP3 *H. influenzae* in NORM 2007, and four clones with unique combinations of MLST allelic profiles and *ftsI* alleles accounted for 61% of all rPBP3 isolates. Analyses of clonality and comparison with other investigations showed that rPBP3 clones might persist over several years. The ST14/PBP3 type A clone appears to be particularly persistent, widespread and virulent. A few (n=13) *bla*-negative isolates with non-wild type beta-lactam susceptibility lacked rPBP3-defining substitutions in Study II, suggesting the existence of additional resistance mechanisms.

Study III encompassed 30 high-rPBP3 *H. influenzae* from Norway (2006-2013). Characterization included MLST, PFGE, *ftsI* sequencing, PBP3 typing and determination of broth microdilution (BMD) MIC for a wide range of agents. The strain collection is unique outside Japan. Of particular notice is the large number (n=23) of group III isolates (N526K + S385T), including 12 isolates with the additional L389F substitution associated with increased resistance. We suggest adding the suffix '(+)' for L389F positive isolates. The resistance rates for extended-spectrum cephalosporins were high in Study III, varying from 47% (ceftriaxone) to 97% (cefixime). Among the isolates were the first reported invasive group III(+) *H. influenzae* from Europe, and an extensively multi-drug-resistant (MDR) group III(+) high-rPBP3 ST159 strain, resistant to all extended-spectrum cephalosporins tested, and four classes of non-beta-lactams. This remarkable resistotype is previously unreported. The MDR strain was isolated from three patients at the same hospital within a period of four days, illustrating the potential for nosocomial spread.

Study III documented the emergence and spread of high-rPBP3 *H. influenzae* in Norway during the 2000s. A contribution of selective antimicrobial pressure is suggested by a 158% increase in extended-spectrum cephalosporin usage from 2000 to 2012, further underlining the importance of rational use of antibiotics.

This project was the first to report identical *ftsI* alleles in rPBP3 strains unrelated by MLST, suggesting that horizontal transfer of rPBP3-encoding *ftsI* gene sequences contributes to the evolution of new rPBP3 strains *in vivo*. The situation calls for improved surveillance. The MLST-*ftsI* typing approach, developed and validated in Study II, is a powerful tool for global molecular surveillance of rPBP3 *H. influenzae*. MLST-PBP3 typing offers lower resolution but may be used as a surrogate approach.

In Study IV, 154 *bla*-negative *H. influenzae* from Study II were used to evaluate nine disks as screening for isolates with rPBP3 genotypes, and Etest and EUCAST disk diffusion were evaluated for categorization of susceptibility to beta-lactams with BMD MICs as the gold standard. The benzylpenicillin 1 unit disk, recommended for screening by EUCAST and first evaluated in this project, detected rPBP3 *H. influenzae* with high sensitivity (96.2%) and specificity (94.0%) but is unsuitable for screening of *bla*-positive isolates. The cefuroxime 5 μg disk demonstrated high sensitivity (94.2%) and acceptable specificity (88.0%) and was superior to previously evaluated disks with *bla*-stable agents, including cefaclor 30 μg and cefuroxime 30 μg. Cefuroxime 5 μg appears to be the best current option for screening of *bla*-positive *H. influenzae* but the disk is not available from all manufacturers.

False susceptible rates were high with ampicillin Etest (88%) and disk diffusion with ampicillin 2 μg (EUCAST zone breakpoints, 77%; adjusted breakpoints, 28%). The poor performance may in part be explained by poor calibration of Etest and methodology-dependent test variation, but also reflects that current clinical breakpoints for aminopenicillins divide the low-rPBP3 population, making susceptibility categorization vulnerable to day-to-day variation. Breakpoint changes may improve agreement with reference methodology, but clinical data to support breakpoints for *H. influenzae* and beta-lactams are insufficient.

To minimize the clinical consequences of very major errors, a warning comment should be added for rPBP3 screening positive isolates susceptible to aminopenicillins by disk diffusion and gradient tests. *H. influenzae* positive by rPBP3 screening should be reported ampicillin resistant in cases of meningitis, irrespective of results by agent-directed testing.

ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AMR	Antimicrobial resistance
AOM	Acute otitis media
BAPS	Bayesian analysis of population structure
bla	Beta-lactamase
BLNAR	Beta-lactamase negative ampicillin resistant
BLNAS	Beta-lactamase negative ampicillin susceptible
BLPACR	Beta-lactamase positive amoxicillin-clavulanate resistant
BLPAR	Beta-lactamase positive ampicillin resistant
BMD	Broth microdilution
BPF	Brazilian Purpuric Fever
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CG	Clonal group
CLSI	Clinical and Laboratory Standards Institute
COPD	Chronic obstructive pulmonary disease
CSF	Cerebrospinal fluid
DAPC	Discriminant analysis of principal components
DDD	Defined daily dose
DNA	Deoxyribonucleic acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
ECM	Extracellular matrix
ECOFF	Epidemiological cut-off
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EU/EEA	European Union/European Economic Area

FSR	False susceptible rate
GLASS	Global Antimicrobial Resistance Surveillance System
HGT	Horizontal gene transfer
Hia, Hib etc.	*Haemophilus influenzae* serotypes a, b etc. (a - f)
HMW	High-molecular-weight
HTM	*Haemophilus* Test Medium
ICE	Integrative conjugative element
IRT	Inhibitor-resistant TEM-beta-lactamase
ISA	IsoSensitest Agar
KTG	Lys-Thr-Gly
LHB	Lysed horse blood
LMW	Low-molecular-weight
LOS	Lipooligosaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MDR	Multi-drug resistance
ME/mE	Major error / Minor error
MHA/MHB	Muller Hinton Agar / Muller Hinton Broth
MH-F	Muller Hinton Fastidious
MIC	Minimal inhibitory concentration
M.I.C.E.	MIC Evaluator
MLEE	Multilocus enzyme electrophoresis
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
MTS	MIC Test Strip
NAD	Nicotinamide adenine dinucleotide
NAG	N-acetylglucosamine (GlcNAc)
NAM	N-acetylmuramic acid (MurNAc)
NordicAST	Nordic Committee on Antimicrobial Susceptibility Testing
NORM	Norwegian Surveillance System for Antimicrobial Drug Resistance
NPV	Negative predictive value

NTHi	Nontypeable *Haemophilus influenzae*
NWGA	Norwegian Working Group on Antibiotics
ODC	Ornithine decarboxylase
OMP	Outer membrane protein
OMV	Outer membrane vesicle
ORF	Open reading frame
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PK/PD	Pharmacokinetic/Pharmacodynamic
PPV	Positive predictive value
QC	Quality control
QRDR	Quinolone resistance-determining region
ROC	Receiver operating characteristic
rPBP3	PBP3-mediated resistance present
SAST	Slide agglutination serotyping
S/I/R	Susceptible / Intermediate / Resistant
SLV/DLV/TLV	Single / Double / Triple locus variant
SNP	Single-nucleotide polymorphism
sPBP3	PBP3-mediated resistance absent (‘susceptible’ PBP3)
SSN	Ser-Ser-Asn
ST	Sequence type
STVK	Ser-Thr-Val-Lys
UPGMA	Unweighted pair group method with arithmetic mean
USS/pUSS	Uptake signal sequences / Partial uptake signal sequences
VME	Very major error
WGS	Whole-genome sequencing
WHB	Whole horse blood
WHO	World Health Organization

PREFACE

Just like a snowman starts with a snowflake, this project started with a single isolate (chapter 9). The project idea was born when we isolated a cefotaxime-resistant *Haemophilus influenzae* in a nasopharynx sample from a two-year old child with leukemia and otitis in May 2006.

We had just implemented a screening method for detection of non-beta-lactamase-mediated beta-lactam resistance in *H. influenzae*. Such isolates, often denoted 'beta-lactamase-negative ampicillin-resistant' ('BLNAR'), were considered rare and of little clinical relevance at the time, and resistance to extended-spectrum cephalosporins was almost unthinkable – as indicated by the term 'BLNAR'. The almost immediate detection of a cefotaxime-resistant isolate – the first of its kind in Norway – inspired us to initiate a project on non-beta-lactamase-mediated resistance in *H. influenzae*, based on isolates from the Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM) (Study I, II and IV).

The project was met with interest. As soon as a method for characterization of the most important resistance mechanism (altered penicillin-binding protein 3) was established, we started receiving *H. influenzae* isolates with unusual resistance profiles from Norwegian and Swedish routine and reference laboratories. In 2013, the number of cefotaxime-resistant isolates reached 30, and we decided to expand the project with a study on *H. influenzae* with high-level non-beta-lactamase-mediated beta-lactam resistance (Study III).

The complete project is presented in Part II of this thesis (chapters 9-15).

Part II rests heavily on Part I (chapters 1-8), in which previous research relevant for the project is critically reviewed, and current knowledge is summarized and put in a historical context. In addition, topics needing further elucidation are identified, and research ideas and hypotheses for future projects are presented.

Part I describes the universe of knowledge to which the project relates – or *the disciplinary matrix*, as Thomas S. Kuhn (1922-1996) might have expressed it. The voluminicity of Part I allows a considerably briefer and more to-the-point approach in Part II, and was found necessary due to the width and complexity of the issues elucidated in this thesis.

Studies on beta-lactam resistance require detailed understanding of the effect mechanism of beta-lactams, which in turn requires insight in the role of penicillin-binding proteins for the biosynthesis of peptidoglycan and cell division, and of course knowledge on beta-lactam resistance mechanisms in general. These topics are addressed in chapters 5-6, with particular emphasis on resistance caused by alterations in penicillin-binding protein 3 (chapter 6.3). As an introduction to multi-drug resistance, resistance to non-beta-lactams is briefly presented in chapter 7.

An adequate susceptibility test report starts with correct species identification and assessment of clinical relevance. As most *H. influenzae* isolates are sampled from non-sterile sites, reliable discrimination between *H. influenzae* and commensals, including closely related species and taxa within the *Haemophilus influenzae* group, is crucial. Current knowledge on phylogeny and pathogenicity, as well as approaches to species identification and epidemiological typing, are summarized in chapters 2-4. The principles, strengths and limitations of methods for phenotypic susceptibility testing of *H. influenzae* are presented in chapter 8.

But let us start with the beginning – in Berlin, 1891 (chapter 1).

PART I. BACKGROUND

Figure 1 Research pioneers on *H. influenzae* in the 19th and 20th centuries. Upper row: Richard Friedrich Johannes Pfeiffer, approximately 1894 (left) and Shibasaburo Kitasato in his forties (right); lower row: Theodor Thjøtta (left) and Margaret Pittman (right) in their laboratories. The respective pictures are used with permission from the copyright holders: Robert Koch Institute; the Kitasato Memorial Museum, Kitasato University; the University of Bergen; and the U.S. Food and Drug Administration

1 HISTORY

The bacterium we know as *Haemophilus influenzae* was described by the German physician and bacteriologist Richard Friedrich Johannes Pfeiffer (1858-1945) in 1892 [373]. Pfeiffer was head of the Scientific Section at the Berlin Institute for Infectious Diseases. The institute (today the Robert Koch Institute, www.rki.de) was founded in 1891 by the German physician Robert Koch (1843-1910), famous for his postulates on causal relationship between microorganisms and disease [235] and by many considered the founder of modern bacteriology [82].

In the spring of 1889, Europe was hit by the last influenza pandemic of the 19th century, denoted the 'Russian flu' due to the original peak in St. Petersburg in 1889 and probably caused by an H3N8 virus [535]. Inspired by the great achievements of Koch and other bacteriologists in the 1880s, with isolation of the bacilli of diphtheria, anthrax, tuberculosis and cholera [82], several researchers had tried to identify the etiological agent of influenza but with little success [190]. In 1891, at a late stage in the pandemic, Pfeiffer examined bronchial secretions from patients with influenza, pneumonia and 'ordinary bronchial catarrh' and observed large quantities of Gram-negative 'very tiny rodlets' in all 31 patients with influenza but none of the 'very numerous' control patients. Pfeiffer reported that inoculation experiments gave positive results with monkeys and rabbits but not with guinea-pigs, rats, pigeons or mice and drew the famous conclusion: 'In view of these results I consider myself justified in pronouncing the bacilli just described to be the exciting causes of influenza' [373].

The inoculation experiments were crucial in the chain of evidence, as they were needed to meet Koch's third postulate of ability to reproduce disease. Pfeiffer only referred to the experiments as 'positive' in the preliminary report but described the monkeys' reactions to inoculation in detail in the final report [374]. Retrospectively, his conclusion had obvious weaknesses: he managed to produce infectious processes in monkeys but the disease was not ambiguously influenza. However, Pfeiffer was

considered an authority in the field and the Koch Institute was one of the world's leading research institutions at the time, and the misinterpretation that the novel bacterium was the etiological agent of influenza was widely accepted [81,118,207]. In 1896, Pfeiffer was officially credited for the discovery when the bacterium was denoted *Bacillus influenzae* [257].

Pfeiffer had not yet succeeded in cultivating the bacterium beyond the 2nd generation when he published his preliminary results in The British Medical Journal in 1892 [373]. In the very same issue, the Japanese physician and researcher Shibasaburo Kitasato (1852-1931), since 1885 a visiting researcher at the Koch Institute, claimed to have cultivated the influenza bacillus to the 10th generation using glycerine agar [233]. Kitasato described the colonies as 'extremely small points like droplets of water, recognisable during the first twenty-four hours only with the aid of a lens', suggesting they might have been overseen by previous investigators (e.g. Pfeiffer). Convinced neither by Kitasato's observations nor by his method, Pfeiffer continued his efforts to culture the bacteria and soon discovered its requirement for blood. In his final report, Pfeiffer concluded that haemoglobin was the single crucial component and showed no mercy when he characterized the previously published observations of his visiting colleague: 'Die Angabe Kitasato's, dass es ihm gelungen sei, die Influenzabasillen auf einfachem Glycerinagar bis zur 10. Generation fortzupflanzen, muss ich jetzt nach diesen Resultaten definitive als irrig zurückweisen' [374].

During his time at the Koch Institute, Kitasato had become a skilled and respected bacteriologist. Not only was he the first to grow the tetanus bacillus in pure culture: together with the German physiologist Emil von Behring (1854-1917) he discovered and characterized the tetanus exotoxin and demonstrated neutralization of toxins *in vivo* in 1890 [33]. Kitasato ended his visit at the Koch Institute and returned to Japan in 1892. Probably inspired by his stay, he founded the Kitasato Institute for Infectious Diseases in Tokyo in 1914 [232]; today a leading research institution on antimicrobial resistance in *H. influenzae* (chapter 6.3.3).

The recognition of blood as an essential factor for bacterial growth gave rise to the term hemophilic (or hemoglobinophilic) bacilli, or simply hemophili (*hemo-*, from

Greek: *haima*, blood; *-philos*, Greek: dear, beloved). In 1917, the American Committee on Classification and Nomenclature introduced the generic name *Hemophilus* (sic) for small, non-motile Gram-negative rods that required blood or other body fluids for growth, with *Hemophilus* (sic) *influenzae*, 'the influenza bacillus', as the type species [560]. The genus also encompassed the 'Bordet-Gengou bacillus' (now *Bordetella pertussis*, the etiological agent of whooping cough), and the 'Koch-Weeks bacillus', today denoted *H. aegyptius or* '*H. influenzae* Biogroup aegyptius' (chapter 2.2.2). Despite the assignment to genus *Hemophilus* (the official spelling was later changed to *Haemophilus*), designations based on Pfeiffer's name, e.g. 'Pfeiffer's bacillus' and '*Bacillus Pfeifferi*', and hybrid designations such as '*Haemophilus influenzae* Pfeiffer' [265] were commonly used for decades.

Notably, *H. aegyptius* was described by Koch [236] nine years before Pfeiffer announced his discovery of the influenza bacillus and validly named *Bacillus aegyptius* by the Italian botanist Count Vittore Benedetto Antonio Trevisan di Saint-Léon (1818-1897) as early as 1889, seven years ahead of *Bacillus influenzae*. Thus, as the first validly published species, *H. aegyptius* should have been designated type species of genus *Haemophilus* instead of *H. influenzae* [581]. A reproduction of Trevisan's original treatise was printed in 1952 [195].

The requirement for growth factors was further explored by the Norwegian physician and microbiologist Theodor Thjøtta (1885-1955) [183], also known for his studies on dysentery and tularemia, and the Canadian bacteriologist and physician Oswald Theodore Avery (1877-1955) [332], who discovered that DNA serves as genetic material, at the Rockefeller Institute for Medical Research in New York. Through a series of experiments, Thjøtta and Avery identified two substances in red blood cells that were essential for growth. These were denoted the X factor, 'intimately associated with or a derivate of hemoglobin', and the V factor, which also could be extracted from bacterial and yeast cells and from fresh vegetables [503]. The letter V was chosen because the substance resembled vitamins, whereas the letter X reflected that the substance was 'less easily defined'. Later studies showed that factor X is identical to hemin [265] whereas factor V is nicotinamide adenine dinucleotide (NAD or NADP) [266]. The terms factor X and factor V are still used today (chapter 2.4).

The novel species designation *H. influenzae* reflects the organism's standing as the commonly accepted etiological agent of influenza in 1917. Although an increasing amount of research suggested that the organism was merely a secondary invader, the notion survived beyond the Spanish flu (1918-1919) [7,118,207]. A 'pseudo-influenza' bacillus was hypothesized to explain the presence of organisms morphologically identical to Pfeiffer's bacillus in patients without influenza. Typing studies did not confirm this hypothesis but significantly improved the understanding of the role of *H. influenzae* in disease other than influenza, such as meningitis [7]. In 1931, the American bacteriologist Margaret Pittman (1901-1995) at the Rockefeller Institute observed that strains with a smooth colony appearance ('S strains') were encapsulated and more virulent compared to strains with rough colony appearance ('R strains') [380]. By precipitation reactions with antisera, establishing the principle of serotyping (chapter 2.8), Pittman divided S strains into types a and b and found that all meningitis isolates in her study were type b. Pittman concluded that *H. influenzae* strains differ in pathogenicity and immunological specificity, similar to pneumococci [380]. Later studies revealed four additional serotypes (c-f) [238].

Despite increasing evidence for a small 'filter-passing micro-organism' as the etiological agent [259], Pfeiffer defended 'his' bacteria as the likely cause of influenza as late as 1931 [207]. Two years later, when influenza was established as a viral disease [472], *H. influenzae* was finally dethroned as the cause of influenza and the species designation instantly turned into an anachronism. Once characterized as 'a pathogen in search of a disease' [81], the organism is currently considered a major pathogen and a significant contributor to the global burden of disease by causing a variety of infections, many far more serious than influenza [208,537].

2 TAXONOMY, IDENTIFICATION AND CHARACTERIZATION

2.1 GENUS *HAEMOPHILUS*

Bergey's Manual of Systematic Bacteriology places genus *Haemophilus* [560] in the family *Pasteurellaceae*; order *Pasteurellales*; class *Gammaproteobacteria*; phylum Proteobacteria [357]. The genus consists of fastidious, facultatively anaerobic, Gram-negative, pleomorphic rod-shaped bacteria. With the exception of *H. ducreyi*, which requires special media and grows better at 33ºC, *Haemophilus* species are easily cultivable on sufficiently rich media, with optimal growth in air supplemented with 5-10% carbon dioxide at 35-37ºC [229,256]. *Haemophilus* species may be distinguished from other bacteria with similar morphology, such as *Bordetella* species and *Cardiobacterium hominis*, by their ability to reduce nitrate [228].

The systematics of the genus is a continuing process. According to the 'List of prokaryotic names with standing in nomenclature' (LPSN, www.bacterio.net) [115], 23 *Haemophilus* species have been validly published, but several species have later been reclassified. The genus currently includes 13 formally validated species [263], of which nine have specificity for humans [351]: *H. influenzae*, *H. aegyptius*, *H. ducreyi*, *H. haemolyticus*, *H. parahaemolyticus*, *H. parainfluenzae*, *H. paraphrohaemolyticus*, *H. pittmaniae* and *H. sputorum* (**Figure 2**). In addition, the genus includes four species with host specificity for animals: *H. felis* (cats) [196], *H. haemoglobinophilus* (dogs) [417], *H. paracuniculus* (rabbits) [501] and *H. parasuis* (pigs) [34].

The most recent changes include the addition of the novel species *H. sputorum* [348] and *H. pittmaniae* 2005 [352], and the transfer of *H. aphrophilus*, *H. paraphrophilus* and *H. segnis* to the novel genus *Aggregatibacter*, with the former species *H. aphrophilus* and *H. paraphrophilus* merged into the novel species *A. aphrophilus* [353]. The International Committee on Systematics of Prokaryotes, Subcommittee on the taxonomy of *Pasteurellaceae* has decided to conduct a taxonomic investigation with the aim of proposing a new genus that would include *H. ducreyi*, as this taxon is only distantly related to the type species of genus *Haemophilus* [67,227].

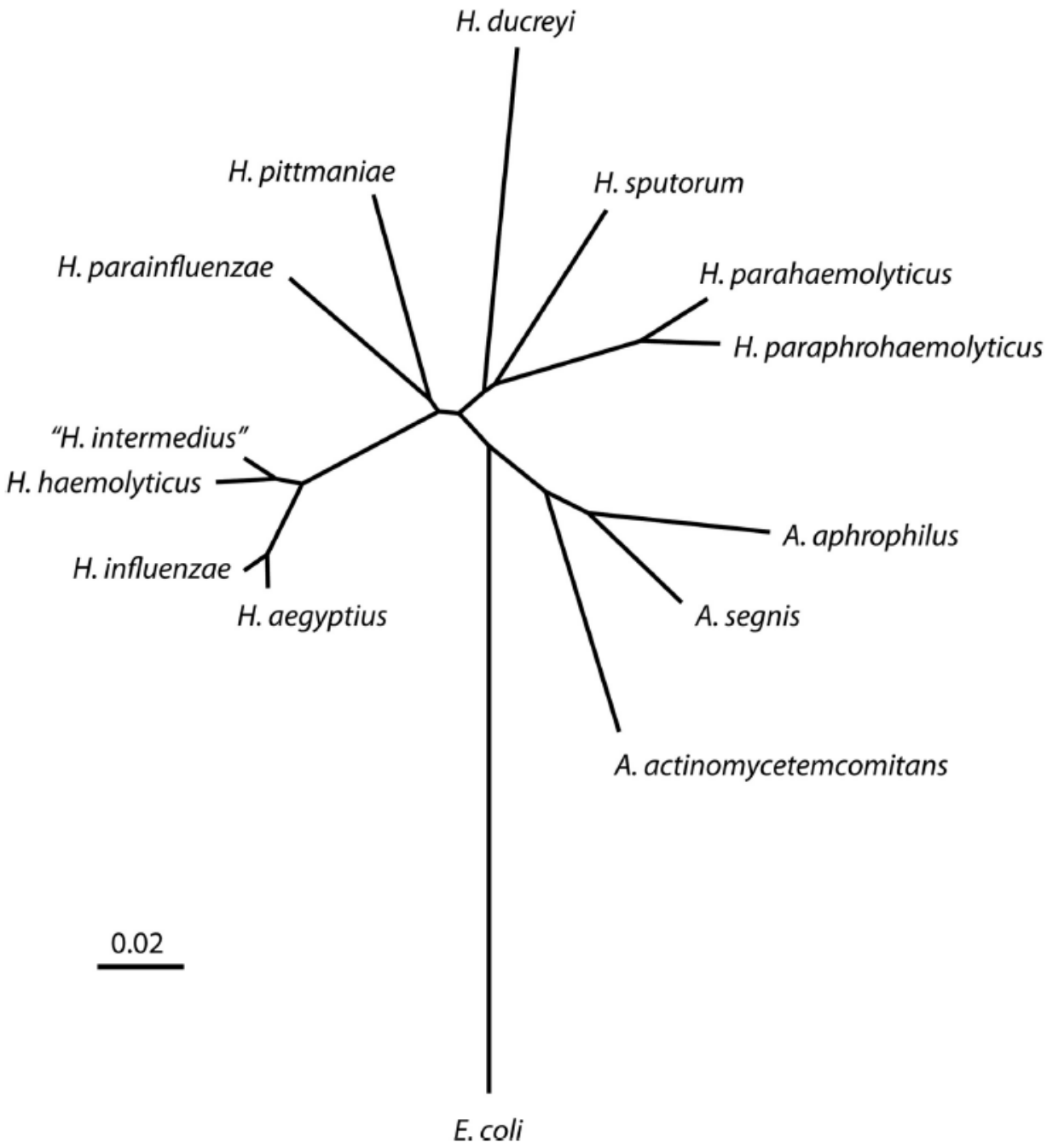


Figure 2 Phylogenetic relationship between *Haemophilus* and *Aggregatibacter* species with host specificity for humans, with *Escherichia coli* as outgroup. The dendrogram is based on concatenated sequences of near-full-length 16S rRNA genes and fragments of the housekeeping genes *infB*, *pgi* and *recA* of type strains. *H. influenzae*, *H. aegyptius*, *H. haemolyticus* and '*H. intermedius*' form a distinct phylogenetic group: the *Haemophilus influenzae* group. Reproduced from [351], with permission

2.2 THE *HAEMOPHILUS INFLUENZAE* GROUP

The three species *H. influenzae*, *H. aegyptius* and *H. haemolyticus* constitute a distinct phylogenetic clade of closely related organisms, denoted the *Haemophilus influenzae* group or '*Haemophilus* sensu stricto' [351] (**Figure 2**). According to Kilian in *Bergey's Manual of Systematic Bacteriology*, *Haemophilus* species not part of the *H. influenzae* group may from a phylogenetic view be considered as misclassified [227].

Recent investigations have significantly improved our understanding of the population structure within the *H. influenzae* group; this insight has revealed limitations and inconsistencies of current species designations [351,388]. A genomic analysis of 246 global isolates belonging to the *H. influenzae* group showed a population structure with four major branches: one consisting of *H. influenzae* phylogenetic division I, which includes *H. aegyptius*; one consisting of *H. influenzae* phylogenetic division II; one consisting of *H. haemolyticus* and related taxa (see below); and one consisting of 'fuzzy' isolates with genetic characteristics (*fucK* and *fucP* negative) separating them from typical *H. influenzae* [388] (**Figure 3**). Notably, the *H. haemolyticus* branch shares a node with *H. influenzae* phylogenetic division I, and the branch with the 'fuzzy' isolates shares a node with *H. influenzae* phylogenetic division II.

A similar population structure was suggested by a phylogenetic analysis based on concatenated partial sequences of six housekeeping genes (*adk*¸ *atpG*, *frdB*, *mdh*, *pgi*, and *recA*) from three type strains and 935 clinical isolates [351]. In that investigation, the *H. haemolyticus* branch, also denoted the 'variant cluster' [349], encompassed strains belonging to the non-validated taxa 'non-haemolytic *H. haemolyticus*', '*H. quentini*', and '*H. intermedius*'.

The close phylogenetic relationship between the species and taxa in the *H. influenzae* group complicates exact delineation of species borders within this group, and reliable species identification may be extremely difficult. Important clinical and epidemiological characteristics that may be helpful in recognizing and separating the various taxa are presented below.

2.2.1 *Haemophilus influenzae*

H. influenzae [560] is the type species and the primary pathogen in genus *Haemophilus* [560], and the primary focus of this thesis. The clinical and epidemiological characteristics of *H. influenzae* are presented in detail in chapter 4.

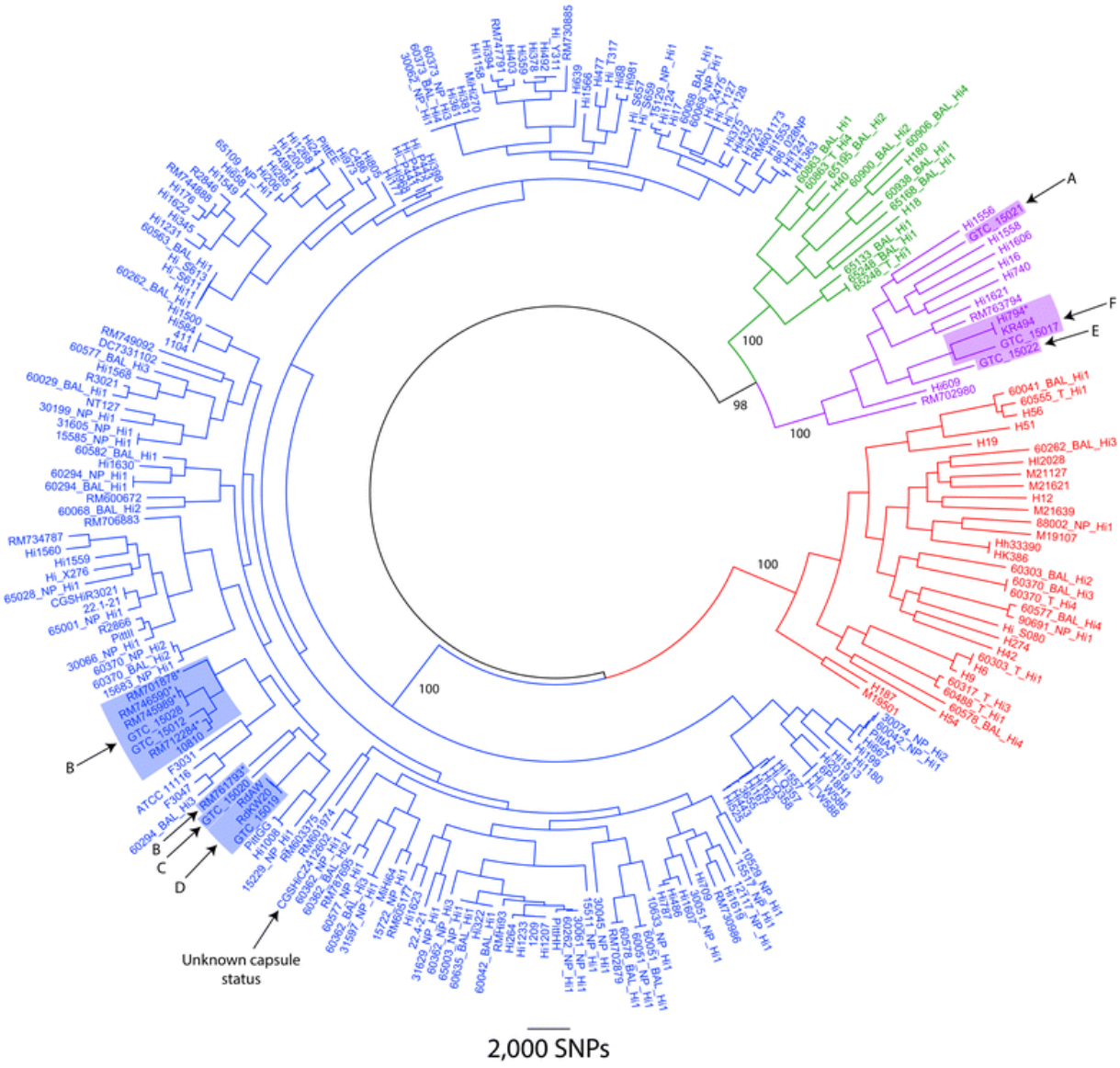


Figure 3 Population structure within the *H. influenzae* group. Phylogenetic analysis based on single-nucleotide polymorphisms in orthologous core genes in the genomes of 246 global isolates. Blue, *H. influenzae* (phylogenetic division I); purple, *H. influenzae* (phylogenetic division II); green; 'fuzzy' isolates (*fucK* and *fucP* negative); red, *H. haemolyticus*. Shading indicates isolates possessing capsular loci (irrespective of expression). ATCC 11116 *H. aegyptius* is located within phylogenetic division I (between the branches containing encapsulated isolates). Reproduced from [388] according to the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/)

2.2.2 *Haemophilus aegyptius*

H. aegyptius was first described by Koch in 1883 as a cause of purulent conjunctivitis in Egypt [236] and by Weeks in the US three years later [553]. Hence, the organism was first known as 'Koch-Weeks bacillus', a designation initially used for all haemophilic bacteria isolated from conjunctiva [337,382]. The name *Bacillus aegyptius* was introduced by Trevisan in 1889 [195].

It has been debated whether the organism should be classified as a species in genus *Haemophilus* or as a biogroup of *H. influenzae* [176,256,337,351,382]. Strains within this taxon are indistinguishable from *H. influenzae* by phylogenetic analyses based on housekeeping genes [351], 16S rRNA [351,357], and complete genomes (**Figure 3**) [362,388]. A formal obstacle to merging the two taxa into one species *H. influenzae* is that the epithet *H. aegyptius* has priority [581]. A pragmatic solution suggested by Nørskov-Lauritsen may be 'to accept *H. aegyptius* as a validly named species that designates a group of strains related to *H. influenzae* isolated during a short period from a single geographic region and to refrain from wider use of the name' [351].

According to Kilian in *Bergey's Manual of Systematic Bacteriology* [227], *H. aegyptius* is a frequent cause of conjunctivitis in hot climates, but due to the difficulties in separating these isolates from (other) *H. influenzae*, the natural history of such infections is not completely understood. In Brazil in 1984, a clone of *H. aegyptius* caused a novel syndrome denoted Brazilian Purpuric Fever (BPF) [175]. The illness was characterized by purulent conjunctivitis preceding bacteremia with septic shock and purpura, clinically resembling acute meningococcal disease. Sporadic cases of BPF have been reported in Australia, the US and Europe [176].The latest outbreak of BPF was reported in Brazil in 2007 [441].

Hemagglutinating factor has been suggested to be a major virulence determinant of the BPF clone [23]. Both conjunctival and BPF strains of *H. aegyptius* possess genes encoding IgA1 protease and homologs of high-molecular-weight (HMW) and Hia adhesins in *H. influenzae*, and several genes encoding novel adhesins and invasins [489]. BPF strains also possess the HP2 bacteriophage, associated with increased

virulence in nonencapsulated *H. influenzae* [557], and the HadA adhesin, which promotes adhesion and invasion of endothelial cells *in vitro* [452].

The phylogenetic relationship between the BPF clone and other strains of *H. aegyptius* is a matter of debate. The BPF clone is recognizable by multilocus enzyme electrophoresis (MLEE) pattern and rRNA gene restriction patterns (ribotyping) [176]. The multilocus sequence typing (MLST) allelic profile of the BPF clone (ST65) differs from the profiles of conjunctivitis strains (ST70 to ST77) and analysis of MLST sequences suggested only remote phylogenetic relationship [102], whereas genomic analysis has indicated close relationship [489].

2.2.3 *Haemophilus haemolyticus*

H. haemolyticus is the original representative and the only validated species of the variant cluster of the *H. influenzae* group [349]. The taxon was originally denoted 'Bacillus X' [389]. The species designation *H. haemolyticus* was introduced in the first edition of *Bergey's manual of Determinative Bacteriology* in 1923 [31]. When Pittman proposed the factor X-independent species *H. parahaemolyticus* in 1953, *H. haemolyticus* was redefined as haemolytic, XV-dependent *Haemophilus* [381].

Haemolysis is still part of the official species definition of *H. haemolyticus*. When present, this trait reliably separates the species from *H. influenzae* [229,288,321,349] but the ability to cause haemolysis may be lost during subculture [256] and non-haemolytic *H. haemolyticus* are frequent [288,321,347]. Such strains do not fit the original species description and may be considered an unnamed taxon [349,351]. In one study, 40% of sputum isolates and 27% of nasopharyngeal isolates of presumably *H. influenzae* were *H. haemolyticus* [321]. In two later investigations, 0.5-1.5% of phenotypically identified *H. influenzae* isolates were identified as *H. haemolyticus* by molecular methods (chapter 2.10) [126,579]. In a recent investigation using MALDI-TOF (with updated reference spectra; chapter 2.9) for identification, 81% and 4% of respiratory *Haemophilus* were *H. influenzae* and *H. haemolyticus*, respectively [280]. Notably, the two species were equally frequent in genital samples.

H. haemolyticus belongs to the human oral microbiota and is generally considered commensal. As opposed to *H. influenzae*, the acquisition of a new strain of *H. haemolyticus* is not associated with exacerbations of chronic obstructive pulmonary disease (COPD) [321]. Both *H. haemolyticus* and 'non-haemolytic *H. haemolyticus*' have been reported as causes of invasive disease [8].

Another recently discovered important feature of this species is the ability to exchange sequences of the *ftsI* gene, encoding penicillin-binding protein 3 (PBP3), in an inter-species manner with *H. influenzae* by horizontal gene transfer (HGT) and recombination [499,565]. *H. haemolyticus* plays an important role in the development and spread of PBP3-mediated resistance to beta-lactams (chapter 6.3.8).

2.2.4 *'Haemophilus quentini'*

'*H. quentini*' [227], also referred to as '*Haemophilus* cryptic genospecies biotype IV' [351], denotes a distinct group of XV-dependent *Haemophilus* strains associated with genito-urinary and neonatal infections [161,545]. As part of the variant cluster of the *H. influenzae* group, '*H. quentini*' are closely related to *H. haemolyticus* but the taxa are distinguishable by 16S rRNA PCR [402]. '*H. quentini*' strains have unique multilocus enzyme electrophoresis patterns, outer membrane protein profiles and fimbrial protein gene sequences [273], and a characteristic adhesin (Cha) [285].

During the 1980s there was an increase in serious mother and infant infections (including bacteremia and meningitis) caused by *H. influenzae* with biotype IV [545]. Quentin *et al.* characterized genital, obstetric and neonatal non-encapsulated isolates from France and the U.S. and identified several genetically distinct biotype IV isolates with <70% similarity with *H. influenzae* by DNA-DNA hybridization and proposed a previously unrecognized (cryptic) *Haemophilus* species [401]. The taxon was later assigned the (non-validated) species name '*H. quentini*' by Kilian in *Bergey's manual of systematic bacteriology* [227].

2.2.5 *'Haemophilus intermedius'*

The variant cluster also includes the non-validated taxon '*H. intermedius*' [227,351]. The clinical relevance is unknown. The species designation was suggested by Burbach in 1987, based on DNA-DNA hybridization studies and phenotypic characteristics [47]. Two subspecies were described: the X-independent and sucrose-fermenting subsp. *intermedius*; and the subsp. *gazogenes*, capable of producing gas from glucose. The latter may be separated from 'Non-haemolytic *H. haemolyticus*' by the ability to ferment mannose [351].

Early taxonomic studies based on analysis of quinone composition and polyamine patterns [54] indicated that *H. intermedius* was closely related to *H. parainfluenzae*. However, multilocus sequence phylogeny places both subspecies in the variant cluster, closely related to *H. haemolyticus*. By near-full length 16S rRNA phylogeny, '*H. intermedius* subspecies *gazogenes*' are located on a branch separated from the main variant cluster, adjacent to phylogenetic group II of *H. influenzae* [351].

2.3 OTHER *HAEMOPHILUS* SPECIES IN HUMANS

The remaining *Haemophilus* species with host specificity for humans include *H. ducreyi*, *H. parainfluenzae*, and the haemolytic species *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. pittmaniae* and *H. sputorum* (**Figure 2**). The sexually transmitted chancroid-causing *H. ducreyi* [514] forms a separate phylogenetic entity; the other species belong to the *Haemophilus parainfluenzae* group [351].

The species in the *H. parainfluenzae* group are generally commensal, but may occasionally have clinical relevance: *H. parainfluenzae* is the most frequent *Haemophilus* species causing infective endocarditis [44,86]. *H. sputorum* is the only species except *H. influenzae* for which a complete polysaccharide capsule biosynthesis locus has been observed [351]. Finally, *H. parahaemolyticus* express the virulence determinant IgA1 protease [227] and an association with acute pharyngitis has been suggested [381]. The clinical significance of the *H. parainfluenzae* group and *H. ducreyi* is outside the scope of this thesis and will not be further discussed.

2.4 FACTOR X AND FACTOR V DEPENDENCY

The variable requirements of *Haemophilus* species for factors X and V is a cornerstone in identification of *H. influenzae* and related species [503] (**Table 1**). The three valid species *H. influenzae*, *H. aegyptius* and *H. haemolyticus*, and most of the non-validated taxa in the *H. influenzae* group, require both factors X and V for growth.

Table 1 Requirement for factors X and V and haemolytic activity of various groups of *Haemophilus* species and former *Haemophilus* species transferred to genus *Aggregatibacter*. The dashed line separates the *H. influenzae* cluster and the variant cluster of the *H. influenzae* group. +/-, factor dependent/independent or haemolysis present/absent; d, delayed. Compiled from [47,196,228,256,321,348,351,353,353,402,501]

Groups, species and taxa	Factor X	Factor V	Haemolysis
***Haemophilus influenzae* group**			
H. influenzae	+	+	-
H. aegyptius	+	+	-
H. haemolyticus	+	+	+
'*H. haemolyticus*, non-haemolytic'	+	+	-
'*H. intermedius* subsp. *gazogenes*'	+	+	-
'*H. intermedius* subsp. *intermedius*'	-	+	-
'*H. quentini*'	+	+	-
***Haemophilus parainfluenzae* group**			
H. parainfluenzae	-	+	-
H. parahaemolyticus	-	+	+
H. paraphrohaemolyticus	-	+	+
H. pittmaniae	-	+	+
H. sputorum	-	+	+
H. ducreyi	+	-	-
Animal-*associated Haemophilus* species			
H. felis	-	+	d
H. haemoglobinophilus	+	-	-
H. paracuniculus	-	+	-
H. parasuis	-	+	-
***Aggregatibacter* species**			
A. actinomycetemcomitans	-	-	-
A. aphrophilus	-	-	-
'*A. aphrophilus*, factor V-dependent'	-	+	-
A. segnis	-	+	-

Hemin (factor X) is a crucial intermediate in the biosynthesis of respiratory cytochromes in bacteria [506]. Factor X-independent *Haemophilus* species form hemin by the biosynthetic pathway δ-aminolevulinic acid (ALA) → porphobilinogen → porphyrins → protoporphyrin IX → hemin [228]. Factor X-dependent organisms lack the enzymes for conversion of ALA into protoporphyrin and grow only on media containing sufficient concentrations of hemin (factor X), e.g. blood and chocolate agar. Growth may be obtained on hemin-deficient media if a hemin source is provided, e.g. via a paper disk [116]. Factor X requirement may also be demonstrated by the porphyrin test [225]. A positive result shows the ability to synthesize porphyrins from ALA, i.e. factor X is not required. *H. influenzae* is capable of anaerobic metabolism. Under anaerobic conditions, respiratory cytochromes are not formed and the hemin requirement is significantly reduced [228].

Factor V (nicotinamide adenine dinucleotide, NAD or NADP) is a coenzyme for a group of oxidation-reduction enzymes and is present in blood and yeast cells. *Staphylococcus* species and some other bacteria produce and excrete NAD, allowing factor V-dependent species to grow near staphylococcal colonies on NAD-deficient media (e.g. blood agars with intact erythrocytes). This 'satellite phenomenon' also provides an opportunity to assess the haemolyc abilities of V-dependent bacteria. Most species have similar haemolytic abilities on sheep, horse and bovine blood agars [229]. As satellite growth around a bacterial colony may be due to excretion of other substances than factor V, use of paper disks impregnated with NAD is more specific [116,228]. It has been claimed that *H. haemolyticus*, in contrast to *H. influenzae*, do not form satellites around *Staphylococcus* colonies [256]. The authors did not provide a reference for this observation, which is incompatible with the growth requirements of *H. haemolyticus* and therefore should be considered uncertain.

2.5 PHENOTYPIC CHARACTERISTICS

In addition to growth requirements and haemolysis (**Table 1**), *Haemophilus* species differ by a variety of phenotypic properties. Key biochemical reactions are fermentation of sucrose, lactose, mannose and xylose; presence of catalase and β-

galactosidase, H_2S production, and production of gas from fermentation of glucose. Additional phenotypic traits traditionally used for species identification include IgA cleavage and hemagglutination [87,501]. The typical profiles of *H. influenzae*, *H. aegyptius* and *H. haemolyticus* are presented in **Table 2**.

It should be noted that traditional approaches to separate *H. aegyptius* from *H. influenzae*, such as distinct morphology, growth characteristics in semifluid media, inability to grow on tryptic soy agar, inability to ferment xylose and the ability to hemagglutinate [176,284,382] have been demonstrated to be of limited value [58,351]. In addition, several of the characteristics typical for *H. haemolyticus* vary within the variant cluster, with lack of haemolysis [321] and the ability to cleave IgA in some strains [349] as the most notable examples.

Table 2 Typical phenotypic profiles of the validated species in the *H. influenzae* group. Compiled from [227-229,288,349,351]

Characteristic	*H. influenzae*	*H. aegyptius*	*H. haemolyticus*
Haemolysis	-	-	+
Sucrose fermentation	-	-	-
Mannose fermentation	-	-	-
Lactose fermentation	-	-	-
Xylose fermentation	+	-	+
Catalase activity	+	+	+
β-galactosidase activity (ONPG)	-	-	-
H_2S emission	-	-	+
Gas from glucose	-	-	+
IgA cleavage	+	+	-
Hemagglutination	-	+	-

2.6 BIOCHEMICAL IDENTIFICATION SYSTEMS

Identification systems based on biochemical profiles may be useful for species identification of *Haemophilus* isolates but should always be combined with determination of factors XV requirement to avoid misidentification.

Frequently used systems are API NH (manual) and Vitek 2 NH (automated) from bioMérieux (www.biomerieux.com). Vitek 2 NH is based on colorimetric technology

and compares the results from 30 biochemical reactions with reference profiles. The list of *Haemophilus* species (current and former) identified by Vitek 2 NH are *H. influenzae*, *H. haemolyticus*, *H. parahaemolyticus*, *H. parainfluenzae*, *A. aphrophilus* and *A. segnis* [38]. The API NH kit includes eight enzymatic reactions, tests for fermentation of glucose, fructose, maltose and sucrose, and a test for penicillinase. The organisms identified by API NH include *H. influenzae*, *H. parainfluenzae* and *A. aphrophilus*. In addition to species identification, API NH may also be used for biotyping of *H. influenzae* and *H. parainfluenzae* (see below) [317].

API NH showed good performance for the species included in the API NH database but misidentified *H. haemolyticus* as *H. influenzae* in an early study [21]. *H. aegyptius* was identified as *H. influenzae*. In a more recent investigation using 16S rRNA sequencing as the gold standard, the test correctly identified *H. influenzae* but misidentified '*H. quentini*' (belonging to the variant cluster, **Table 1**) as *H. influenzae* and failed to identify a *H. haemolyticus*-like isolate beyond genus level [271]. In another study, API NH identified a clinical '*H. quentini*' isolate from China as 99.5% *H. influenzae* biotype IV [273].

The performance of Vitek 2 NH for identification of *Haemophilus* species has been evaluated with 16S rRNA sequencing as gold standard [408,479]. In one study, the test misidentified *H. haemolyticus* as *H. influenzae*; *H. influenzae* was misidentified as *H. parainfluenzae* and vice versa; and *H. aphrophilus* (*A. aphrophilus*) and *H. parahaemolyticus* were misidentified as *H. segnis* (*A.segnis*) and *Actinobacillus ureae*, respectively [408]. In another investigation, the test misidentified *H. haemolyticus* (as *H. parainfluenzae*) and the haemolytic species *H. parahaemolyticus* and *H. pittmaniae*, and *A. ureae* was misidentified as *H. influenzae* [479].

2.7 BIOTYPING

H. influenzae may be divided into eight biotypes based on the three simple biochemical tests indole, urease and ornithine decarboxylase (ODC) [226]. There is a distinct relationship between biotypes, capsular serotypes, and population structure

(chapter 3.9). Encapsulated strains with serotypes a, b and f are biotype I; serotype c strains are usually biotype II; serotype d and e strains are biotype IV; and most nontypeable isolates associated with respiratory, ear and eye infections are biotype II or III. Biotyping is a useful supplementary tool for species identification within the *H. influenzae* group (**Table 3**). *H. aegyptius*, '*H. quentini*' and *H. haemolyticus* have the reaction patterns of biotypes III [21,229], IV [401], and II/III [228], respectively.

Table 3 Biotypes of species and taxa in the *H. influenzae* group. The dashed line separates the *H. influenzae* cluster and the variant cluster. Compiled from [226,228,229,256,349]

Species and taxa	Biotypes	Indole	Urease	ODC
H. influenzae	I	+	+	+
	II	+	+	-
	III	-	+	-
	IV	-	+	+
	V	+	-	+
	VI	-	-	+
	VII	+	-	-
	VIII	-	-	-
H. aegyptius	III	-	+	-
H. haemolyticus	II/III	+/-	+	-
'*H. quentini*'	IV	-	+	+
'*H. intermedius* subsp. *gazogenes*'	VIII	-	-	-
'*H. intermedius* subsp. *intermedius*'	I/II/III/IV	+/-	+	+/-

Biotyping may be misleading due to false positive or negative results, in particular for ODC. By two *H. influenzae* external quality assurance schemes distributed to European national reference laboratories by the European Centre for Disease Prevention and Control (ECDC), discrepant ODC results were reported by one or more laboratories for five of six strains in 2011 [104] and for all five strains in 2012 [105]. Similar observations have been made in studies on clinical isolates [273].

ODC, an enzyme involved in the synthesis of DNA-stabilizing polyamines, is regulated by a number of mechanisms [258]. It is not clear whether diverging test results are solely due to suboptimal test properties or if biological variation may contribute. Therefore, biotyping results should be interpreted with caution and other methods are necessary for strain discrimination.

2.8 CAPSULAR SEROTYPING

H. influenzae may possess polysaccharide capsule of one of six types (a through f) or be nonencapsulated [380]. Encapsulation is an important virulence determinant (chapter 4.2.1). The capsular serotype is traditionally determined phenotypically by slide agglutination serotyping (SAST) with serotype-specific antisera [380].

The *cap* locus contains genes needed for capsule production and expression in *H. influenzae* [238,443]. Except for *H. sputorum*, which carries a complete capsule biosynthetic locus, a polysaccharide capsule has not been reported in other *Haemophilus* species [351]. The *cap* locus consists of duplicated (Hib) or single-copy (Hia, Hic and Hid) ~17 kb DNA segments with three regions. Region II contains serotype-specific genes, whereas regions I and III are common in all encapsulated strains. Region I contains the *bexA* gene needed for capsule expression. In Hia, Hib, Hic and Hid strains in phylogenetic division I, the segments are flanked by the insertion sequence IS*1016*. Due to a 1.2 kb IS*1016–bexA* partial deletion at the 5' end of the *cap* locus, only one complete copy of *bexA* is present in Hib strains [238,443]. Curiously, the deletion is associated with increased pathogenicity [239].

Hib mutants with single copies of the DNA segment produce capsular material but do not export it to the surface of the bacteria due to the lack of *bexA*. Such strains, denoted Hib$^-$ ('Hib minus'), are nontypeable by phenotypic methods. Thus, molecular methods detecting both *bex* and *cap* genes are necessary for correct serotype assignment. Single-copy Hib variants possessing the *bexA* gene have been observed; such strains produce lower amounts of capsular material and may also be false negative by phenotypic tests [442,443].

A widely used molecular methodology for capsular serotyping, with amplification of *bexA* and serotype-specific *cap* genes by PCR and gel-based confirmation of the PCR product was developed by Falla *et al.* [119]. Due to mismatches between the original *bexA*, *cap* e and *cap* f primers and more recently added sequences in the EMBL database that may affect sensitivity; primer modifications may be necessary for optimal test performance. A real-time multiplex PCR methodology for capsular serotyping designed by Maaroufi *et al.* [268] showed 98.5% concordance with

conventional PCR as described by Falla *et al.* [119]; among 14 tested strains, two were typeable (Hib and Hie) by real-time PCR but could not be typed by conventional PCR. Both methods were superior to SAST, consistent with other reports [243,442].

Phenotypic tests may produce false negative/positive results due to cross-reaction, auto-agglutination and observer error, but such errors may be reduced with systems for standardization and quality control [243]. Among European reference laboratories participating in an external quality assurance scheme distributed by ECDC, the proportions of laboratories reporting the intended result by phenotypic serotyping ranged from 67% to 96% in 2011 [104] and from 73% to 96% in 2012 [105].

The term 'nontypeable *H. influenzae*' (abbreviated NTHi) is a commonly used designation for isolates lacking a capsule biosynthetic locus, although 'nonencapsulated' would be a more precise designation when the absence of a *cap* locus has been confirmed by molecular methods. Both terms are used in this thesis.

2.9 MALDI-TOF

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) offers significantly reduced analysis time for identification compared to conventional phenotypic methods and has become widely used in routine laboratories in recent years [450]. Identification of whole-cell bacteria is based on generation of a spectral profile of abundant bacterial proteins from the test strain, which is compared to profiles of bacteria of known identity in a reference database ('library') [253].

In a proof-of-concept study, Haag *et al.* demonstrated the differences between mass spectra of *H. influenzae*, *H. parainfluenzae*, *H. aphrophilus* (now *A. aphrophilus*) and *H. ducrey*i [168], but Seng *et al.* failed to identify two of seven *Haemophilus* species beyond genus level in a later study [450]. Recent investigations have indicated that MALDI-TOF may be used to identify *H. influenzae*, *H. parainfluenzae*, *H. haemolyticus* and *H. parahaemolyticus* [138], and to distinguish between *H. influenzae* and *H. haemolyticus* [46,580].

The ability of the approach to distinguish between test strains and type strains of *H. influenzae* and related species and taxa was investigated by Nørskov-Lauritsen in a recent review [351]. In contrast to previous studies [46,138,580], mass spectrometry analysis with calculation of log score similarities (Biotyper 3 software, Bruker Daltronic, www.bruker.com) gave insufficient resolution within the *H. influenzae* group, although comparison of strains of *H. influenzae* and *H. aegyptius* with reference spectra of *H. haemolyticus*, '*H. quentini*' and '*H. intermedius*' gave slightly reduced similarity log scores (1.63 – 1.90). There was also low resolution for separating *H. parahaemolyticus* from *H. paraphrohaemolyticus*, and *H. parainfluenzae* from *H. pittmaniae*. The author suggested that the discrepancy might be due to methodological bias, as the reference databases in previous investigations [138,580] partly were constructed using profiles of test strains [351].

In conclusion, MALDI-TOF may to some extent discriminate between the *H. influenzae* cluster and the variant cluster in the *H. influenzae* group, provided that the reference database contains a representative *H. haemolyticus* reference spectrum [46,138,351,580]. No study has thus far shown that the approach may be used to distinguish *H. influenzae* from *H. aegyptius*, or to separate *H. haemolyticus*, '*H. quentini*' and '*H. intermedius*' [351].

2.10 MOLECULAR METHODS FOR SPECIES IDENTIFICATION

DNA analysis is considered the gold standard for definitive species identification. Species identification may be performed by analysis of DNA sequences for single genes, multiple genes or the complete genome. According to the current species definition, a bacterial isolate can be assigned to a species if ≥70% DNA similarity with the species type strain can be demonstrated by DNA-DNA hybridization [485,551].

Whole genome sequencing (WGS) represents a modern approach to DNA analysis and has the advantage of offering identification, typing (chapter 3) and detection of virulence (chapter 4.3) and resistance genes (chapters 6-7) in one operation [367]. WGS technology has developed dramatically since *H. influenzae* became the first

bacterial species undergoing whole genome sequencing, through a 13-month analytic process performed by Fleischmann *et al.* in 1995 [131]. The development of commercial platforms for WGS has substantially reduced time and costs and bench-top instruments are available in an increasing number of routine laboratories [513].

Nucleic acid amplification tests (NAAT) such as polymerase chain reaction (PCR) are suitable for species identification in pure culture and for qualitative or quantitative detection of *H. influenzae* in respiratory tract secretions [1,2,142,216,403] and cerebrospinal fluids (CSF) [306,539,548]. Several genes have been suggested as targets for molecular tests but evaluations have shown that designing sensitive and specific NAAT for *H. influenzae* is challenging [186,351,378,388]. Discrimination between nontypeable *H. influenzae* (including *H. aegyptius*) and the variant cluster is particularly difficult.

Frequently used targets include *cap* genes (encapsulated isolates), the 16S rRNA gene; *omp*P2 and *omp*P6 (outer membrane proteins P2 and P6); *hpd* (Protein D); the lipooligosaccharide (LOS) genes *licA*, *lic2A* and *lgtC*, *igaA* (IgA1 protease); *hap* (adherence and penetration protein); the housekeeping genes *frdB*, *recA* and *fucK*; and *sodC* (Cu,Zn-superoxide dismutase). The *fucK* gene (fuculokinase) is absent in *H. haemolyticus* but also in some strains of *H. influenzae* [89,347,415]. Similarly, *hpd* is lacking in some *H. influenzae* strains [473]. The *sodC* gene is associated with *H. haemolyticus* [141], '*H. quentini*' [250] and both subspecies of '*H. intermedius*' [349] but may also be present in *H. influenzae* [126]. In addition, characteristic adhesins are present in the BPF clone of *H. aegyptius* (*hadA*) [452] and *'H. quentini'* (*cha*) [285].

The performance of molecular methods for species identification, with emphasis on differentiation between *H. influenzae* and *H. haemolyticus*, were summarized in three recent reviews [186,351,378]; the reader is referred to the papers for detailed description of performance and references. Of particular note is the poor performance of single PCR assays based on the 16S rRNA gene [1,37], *omp*P6 [1,37,388,539], *omp*P2 [37,388] and *hpd* [377,388]. In short, the authors of all three reviews concluded that no single target or methodology published so far accurately identify or discriminate between *H. influenzae* and *H. haemolyticus*, and multiple targets are

required for reliable identification. Accordingly, an algorithm based on real-time PCR assays for *hpd*, *fucK* and *sodC* identified *H. influenzae* and *H. haemolyticus* with 100% sensitivity and specificity [251].

Two most recent investigations evaluated novel genes as targets for molecular identification of *H. influenzae*. Reddington *et al.* found 100% concordance between a real-time PCR assay targeting the *smpB* gene and the gold standard (MALDI-TOF and *fucK*) for a collection of 44 clinical *Haemophilus* isolates [403]. Price *et al.* used comparative genomics comprising 246 *Haemophilus* isolates from various geographical locations to identify genetic loci specific for *H. influenzae* and to design a PCR assay targeting the *fucP* gene [388]. The test had 100% specificity but failed to detect isolates within a distinct phylogenetic group sharing a node with phylogenetic division II of *H. influenzae* (**Figure 3**). The cluster also also lacks *fucK* but groups with phylogenetic division II by housekeeping phylogeny with six alleles [89]. Previously assigned 'fuzzy species', such strains represent a particular challenge in species identification [37,89]. Although controversial from a phylogenetic point of view, Price *et al.* chose to define 'fuzzy' isolates as not *H. influenzae* [388].

Selected marker genes and conserved genes useful for discrimination between species, groups and taxa within the *H. influenzae* group are summarized in **Table 4**.

Table 4 Selected marker genes within the *H. influenzae* group and typical patterns in various species, groups and taxa. Compiled from [238,285,351,388,452]

Species and taxa	*cap*	*fucK*	*fucP*	*lgtC*	*igaA*	*sodC*	*hadA*	*cha*
Encapsulated *H. influenzae*	+	+	+	+	+	-	-	-
NTHi	-	+	+	+	+	-	-	-
H. aegyptius	-	+	+	+	+	-	+	-
'Fuzzy' *H. influenzae*	-	-	-	+	+	-	-	-
H. haemolyticus	-	-	-	-	-	+	-	-
'H. quentini'	-	-	-	-	-	+	-	+

3 GENETIC VARIATION AND EPIDEMIOLOGICAL TYPING

3.1 TO HIT A MOVING TARGET

A method for epidemiological typing should be able to determine if and at which level two isolates of the same species are related. Ideally, the results should also be unambiguous and easily comparable between study groups.

The most important methodologies for assessment of genetic relationship between *H. influenzae* isolates (reviewed by Harrison *et al.* [177]) are described in chapters 3.4 – 3.9. The various methods differ greatly in terms of resolution, and the reliability is variably affected by recombination and mutation rates and the degree of clonality in the population. Thus, which approach is the most suitable in each case depends on the purpose of typing (e.g. outbreak investigation in a hospital department or studying the dissemination of a global clone). In addition, the genetic characteristics of the population must be taken into account when selecting a typing method and interpretation of the results. The role of transformation and recombination for genetic variation in *H. influenzae* is described in chapters 3.2 – 3.3.

Irrespective of the typing method used, assessment of relatedness should always be supported by epidemiological data. Importantly, proximity in time and space is not included in the WHO outbreak definition, and an outbreak may extend over several countries and last for several years (www.who.int/topics/disease_outbreaks/en/, accessed 2016-01-11).

3.2 HORIZONTAL GENE TRANSFER

Acquisition of foreign genes by horizontal gene transfer (HGT) is an important mechanism contributing to adaptability and diversity in bacteria, including development and spread of antimicrobial resistance [184,491]. HGT may occur by transduction (transfer of DNA by bacteriophages), conjugation (transfer of DNA by cell-to-cell contact) or transformation (uptake of extracellular DNA followed by

homologous recombination). Mobile genetic elements such as plasmids, integrative conjugative elements (ICE), transposons, integrons, insertion sequence elements, gene cassettes and genomic islands are important vehicles in transduction and conjugation but are not involved in transformation. Genetic variation caused by transformation is of particular relevance for this thesis and is further elucidated below.

According to the distributed genome hypothesis, all genes available to a bacterial species constitute the 'supragenome', consisting of core genes present in all strains, and a pool of non-core genes that may be shared through HGT between naturally competent strains [189]. The *H. influenzae* supragenome has been estimated to consist of between 4425 and 6052 genes, whereas the core genome encompasses 1461 to 1485 genes [114,189]. The 81 *H. influenzae* genomes currently available at the National Center for Biotechnology Information (NCBI) possess between 1749 and 2352 genes (www.ncbi.nlm.nih.gov/genome/genomes/165, accessed 2015-10-01).

3.3 COMPETENCE AND RECOMBINATION

Natural competence is the genetically encoded ability of some bacteria of taking up extracellular DNA. This ability varies extensively between *H. influenzae* strains [283]. In competent strains, environmental double-stranded DNA fragments are bound and transported through outer membrane type II secretin pores by type IV pseudopili; the Rec2/ComF system then translocates single-stranded DNA across the inner membrane into the cytoplasm [294]. Recognition and efficient uptake of DNA depends on the presence of specific uptake signal sequences (USS) in the donor molecule [471].

Analyses of the *H. influenzae* genome identified the nine basepair sequence 5'-AAGTGCGGT as the probable USS in this species [165,471]. This was confirmed by deep sequencing experiments to investigate DNA uptake specificity in *H. influenzae*, in which the same uptake motif was identified in degenerate DNA fragments recovered from competent cells [294]. The four bases GCGG were critical for uptake, suggesting that these make strong specific contact with the uptake machinery. A USS-binding protein in *H. influenzae* has not yet been identified.

A number of genes, playing different roles in regulation and in binding and transporting DNA, are required for DNA uptake in *H. influenzae* [406]. Specific point mutations in the essential competence regulator gene *sxy* (also denoted *tfoX*) enhance spontaneous competence [405].

Mutations in the *murE* gene involved in the biosynthesis of peptidoglycan [474,515,528] have also been shown to significantly increase competence in *H. influenzae* [267]. This observation is of particular relevance for this thesis, as it suggests that transformation and peptidoglycan synthesis, both involved in the development of PBP-mediated beta-lactam resistance in *H. influenzae*, are not mutually independent processes.

Most DNA taken up from the environment undergoes degradation with recycling of the components in DNA synthesis and repair. In transformation, the uptake of DNA is followed by incorporation of fragments in the chromosome by homologous recombination, creating mosaic gene patterns. Homologous recombination requires a functional recA protein, encoded by the r*ecA* gene [419], a housekeeping gene included in the MLST scheme [289]. The *recA* gene is located adjacent to the *sxy* (*tfoX*) gene in *H. influenzae* [131]. RecA also plays an important role in the SOS response [461] and is thus involved in the control of cell division (chapter 5.7). Increased DNA uptake and higher transformation frequencies are induced by nutrient limitation, consistent with the theory that DNA is used as a nutrient source as well as a tool for DNA repair [283,406].

In contrast to point mutations, homologous recombinational events (HRE) may blur the phylogenetic signals obtained by phylogenetic analysis. The standard method for detection of HRE is based on comparison of phylogenetic trees; several algorithms and software packages are available [185]. Perez-Losada *et al.* described a statistical model for estimation of recombination and mutation rates based on MLST data in *H. influenzae* and other species [368]. In a more recent publication, Wang *et al.* were able to detect 31% to 61% of HRE using an algorithm based on single-nucleotide polymorphisms (SNPs) in bacterial genomes [547]; the software is freely available at http://sourceforge.net/projects/hrefinder/.

Investigations exploring the extent and significance of HRE in *H. influenzae* by analysis of housekeeping gene sequences have provided somewhat conflicting results. Feil *et al.* used five loci (*adk, pgi, recA, fucK*, and *mdh)* from a diverse selection of NTHi and encapsulated strains and found low recombination rates in *H. influenzae* compared to meningococci, pneumococci, *S. pyogenes* and *S. aureus* [124]. A comparative analysis based on all seven MLST loci by Meats *et al.* indicated that the impact of recombination is greater in NTHi compared to encapsulated strains [289]. This was supported by a later study by Connor *et al.* applying Bayesian analysis of population structure (BAPS) to MLST sequences [79]. By the BAPS approach, isolates are clustered based on shared polymorphisms. The polymorphism-population association may be used to identify sequences containing polymorphisms characteristic for more than one population; such sequences are likely the product of recombination [79]. The authors found evidence of recombination in a larger proportion of NTHi compared to encapsulated strains, with significant heterogeneity between different lineages.

In contrast, Pérez-Losada *et al.* [368] did not observe notable variation in average recombination and mutation rates between NTHi and encapsulated strains. Average recombination rate in *H. influenzae* was moderate (similar to *S. pyogenes*; higher than *E. coli* and *S. aureus*; lower than gonococci, meningococci and pneumococci). There was considerable variation between loci, with the highest recombination rates in *mdh* and *pgi*. Average recombination to mutation ratio was high, consistent with the observations in a more recent study by LaCross *et al.* [244]. The authors used MLST sequences to estimate the ratio of the recombination rate to the mutation rate in a collection of NTHi strains and concluded that recombination introduces five times more nucleotide substitutions than do point mutations.

WGS studies have revealed that transformation in competent strains of *H. influenzae* is more extensive than previously recognized and may cause allelic variation involving complete genes [387]. In one study, the mean length of 16 donor segments was 8.1±4.5 kb and the longest segment was 16.6 kb, suggesting that recombination of very short segments is rare [293].

Importantly, recombinational exchange of DNA occurs in an inter-species manner within the *H. influenzae* group, with recombinational exchange of *ftsI* gene sequences between *H. influenzae* and *H. haemolyticus* [288,499,565]. These observations are relevant for the development and spread of beta-lactam resistance in *H. influenzae* (chapter 6.3.8).

3.4 PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

By PFGE, total genomic DNA is processed by sequence-specific restriction endonucleases (most commonly SmaI) into a variable number of fragments, depending on the occurrence of polymorphisms within potential cleaving sites. The fragments are then separated by pulsed-field electrophoresis, creating individual patterns (pulsotypes). Several methods for pulsotyping of *H. influenzae* have been described [84,262,358,504]. PFGE is highly discriminative and generally considered suited for assessment of relatedness between epidemiologically connected isolates, particularly in populations with high recombination rates such as NTHi [502,504].

Tenover *et al.* proposed criteria for assessment of genetic relationship based on comparison of restriction patterns and the number of band differences [502]. Isolates with identical patterns are categorized as indistinguishable, isolates differing by 2–3 bands and 4–6 bands are classified as closely related and possibly related, respectively; isolates differing by seven bands or more are considered unrelated.

A commonly used approach to analysis of restriction patterns of multiple isolates is calculation of band similarity coefficients. Carrico *et al.* defined *S. pneumoniae* isolates with from one to six band differences (i.e. closely or possibly related according to Tenover criteria) as a ‘type’ and isolates with identical patterns as a ‘subtype’ and showed that with the Dice similarity index, 97% and 81% may be used as cut-off values for subtype and type assignment respectively [59].

3.5 MULTILOCUS ENZYME ELECTROPHORESIS (MLEE)

Assessment of genetic variation in conserved genes that are involved in central metabolic processes, denoted housekeeping genes, is a much used approach to studies of population structure and determination of phylogenetic relationship between isolates of *H. influenzae*. MLEE detects allelic variation within a number of housekeeping genes on the basis of the differing electrophoretic mobilities of their gene products with subsequent assignment of strains to electrophoretic types.

Musser *et al.* characterized a collection of 65 NTHi and 177 Hib isolates by MLEE with 15 loci and found the nontypeable isolates genetically heterogeneous and distinct from Hib strains [325]. Another collection of 2209 encapsulated *H. influenzae*, with all six serotypes represented, was characterized by MLEE with 17 loci and restriction fragment length polymorphism (RFLP) of the cap locus; two major phylogenetic groups (I and II) with seven (A-G) and five (H-L) major lineages, respectively, were identified [327]. Group I comprised strains of serotypes a, b, c, d and e; Group II encompassed serotypes a, b and f. The authors concluded that the population structure of encapsulated *H. influenzae* is clonal and suggested that the presence of lineages with serotype a and b strains in both major groups is due to horizontal transfer of serotype-specific *cap* sequences [326,328].

3.6 MULTILOCUS SEQUENCE TYPING (MLST)

By MLST, sequences of internal fragments of housekeeping genes are used to unambiguously characterize bacterial isolates of a species. For each gene, different sequences are assigned specific allelic numbers, and the allelic profile defines the sequence type (ST) [272]. Sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. A single genetic event may result in a new allele differing by one nucleotide (point mutations) or by several nucleotides (recombinational events). Thus, assessment of relatedness based on the number of nucleotide differences between alleles may be misleading. One advantage of MLST is that sequence data and allelic profiles are easily compared between study groups via

online databases [482]. Another advantage is that allelic profiles can be obtained by direct PCR and sequencing of normally sterile material.

The MLST scheme for *H. influenzae* was established in 2003 and assigns STs based on allelic profiles of the seven housekeeping genes *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi* and *recA* [289]. Notably, some strains lack the *fucK* gene [89,347,415]. Software for phylogenetic analysis and an updated database with STs, serotypes and clinical data are available at the PubMLST website http://pubmlst.org/hinfluenzae (previously http://haemophilus.mlst.net).

MLST allelic profiles may be analysed by the eBURST approach [125,480,527]. A clonal complex is defined as a group of strains sharing at least six alleles with at least one other member of the group. The term 'eBURST group' denotes a cluster of strains defined by the less stringent criterion of five shared alleles and must not be confused with clonal complexes [125]. For each clonal complex, eBURST identifies the ST that is most likely to represent the founding genotype (the primary founder) on the basis of parsimony as the ST that has the largest number of single locus variants (SLV). A ST with at least two descendent SLVs is defined as a subgroup founder.

By eBURST analysis, the allelic profiles currently (2014-10-28) included in the MLST database constitute 165 clonal complexes (of which 51 encompass ≥5 STs) and 371 singletons. The largest complex consists of Hib strains, with one ST (ST6) accounting for 41% (217/526) of the isolates, and encompasses 10.7% (149/1392) of all STs. The other serotypes and NTHi isolates cluster in separate complexes. An overview of the ten largest clonal complexes with predicted founders and associated capsular serotypes is shown in **Table 5**. The population structure of *H. influenzae* based on MLST allelic profiles, visualized using the eBURST software and setting the criterion for group formation to zero, is shown in **Figure 4**.

Turner *et al.* evaluated the performance of eBURST for assessment of phylogenetic relationship by using strains with known ancestry and showed that populations with high recombination rates relative to mutation can be recognized by their eBURST patterns [527]. While a typical clonal population (low recombination rates and genetic differences mostly due to point mutations) shows multiple radial groups and the largest

group contain a relatively low proportion of the STs, a population with high recombination rate typically consists of a large group with multiple linked radial groups and long chains of linked STs, where groups of different ancestry may be inappropriately linked together. Based on the eBURST population structure pattern for *H. influenzae*, with one large clonal complex and several smaller radial groups, the authors concluded that the species has moderate recombination to mutation rate and that eBURST performs very well.

The clonal complexes encompassing NTHi (CC 2, 3 and 6-9) generally tend to form chains, whereas the Hib (CC1 and CC10), Hie (CC5) and Hif (CC4) complexes have a strict radial appearance (**Figure 4**). According to Turner *et al.* [527], this may be suggestive of relatively higher recombination rates in NTHi subpopulations, and eBURST analysis may be less reliable. Consequently, eBURST analysis of NTHi strains should probably be restricted to assignment to clonal complexes, using the most stringent (default) criterion of six shared alleles [125]. Results obtained by phylogenetic analyses on 'eBURST groups' with five shared alleles [102,221] should be interpreted with caution.

Table 5 The ten largest clonal complexes (with predicted founders and associated capsular serotypes) by eBURST analysis of all isolates (n=2289) and STs (n=1392) in the MLST database (accessed 2014-10-28) using eBURSTv3 (http://haemophilus.mlst.net/eburst)

Clonal complex	Predicted founder	No. of sequence types	Serotype
1	ST6	149	b
2	ST1	40	non
3	ST3	31	non
4	ST124	31	f
5	ST18	29	e
6	ST584	23	non
7	ST103	22	non
8	ST503	20	non
9	ST57	19	non
10	ST222	19	b

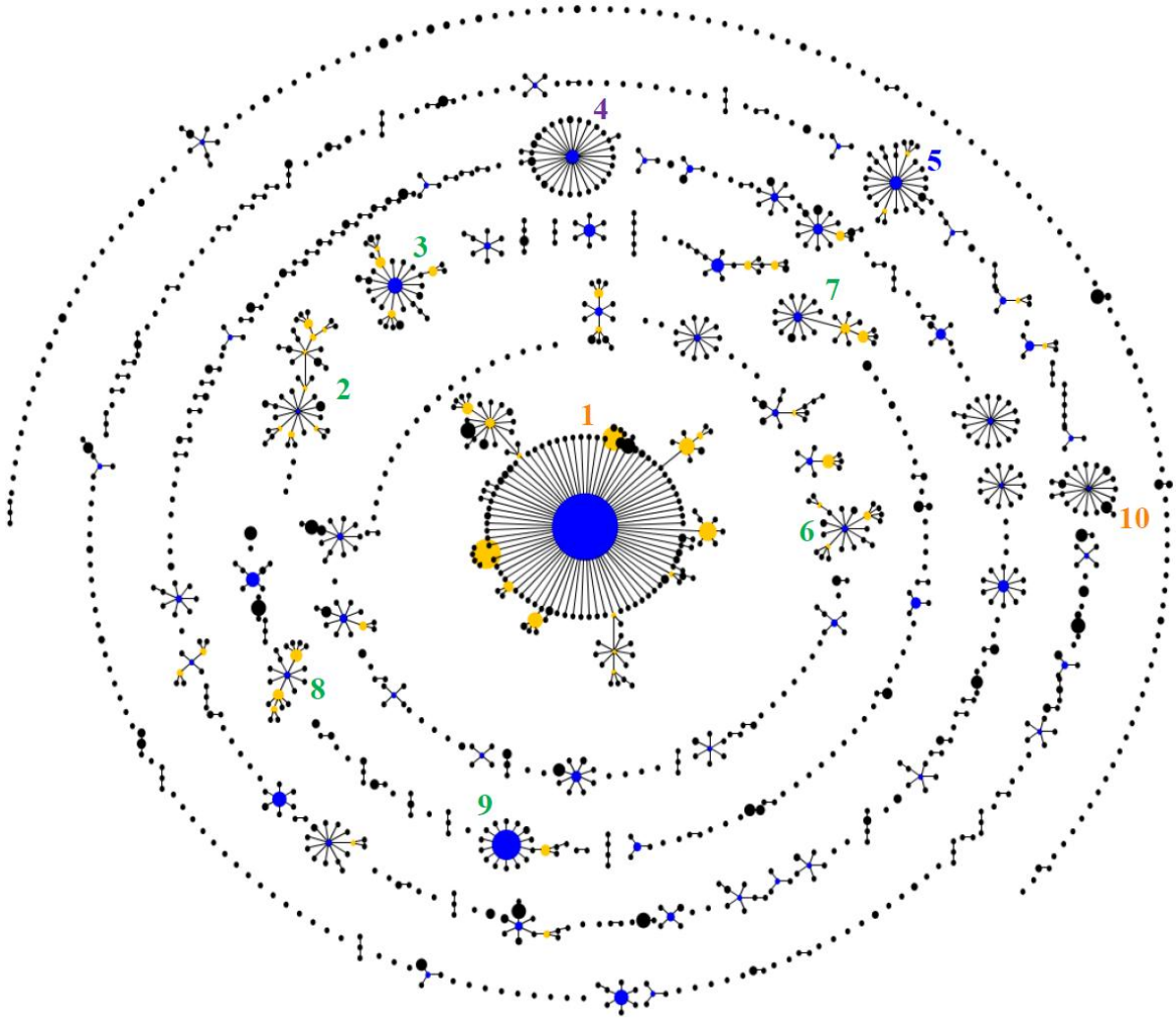


Figure 4 The population structure of *H. influenzae* by eBURST analysis of MLST allelic profiles using eBURSTv3 (http://haemophilus.mlst.net/eburst). The diagram is based on all isolates (n=2289) and STs (n=1392) in the MLST database (accessed 2014-10-28). Dot sizes indicate number of isolates. Connected STs indicate clonal complexes. Blue, predicted founders; yellow, predicted subgroup founders. The ten largest clonal complexes are indicated by numbers; colours indicate capsular serotype (**Table 5**)

3.7 MULTILOCUS SEQUENCE ANALYSIS (MLSA)

While eBURST analyses MLST data by comparing allelic profiles, MLSA denotes phylogenetic analysis of housekeeping genes by comparing concatenated DNA sequences [153,172]. MLSA is suitable for determination of the phylogenetic position of new species and the relationships between closely related taxa, species and genera as well as typing of isolates belonging to the same species.

The most commonly used MLSA scheme for typing of *H. influenzae* is based on MLST sequences. Phylogenetic analysis of the sequences of the 68 STs in the original MLST database by Meats *et al.* largely confirmed the population structure achieved by MLEE, with two major groups (phylogenetic division I and II) [289]. The most important difference between the two methods was the assignment of lineage F/G (Hie) to group/division I by MLEE and to group/division II by MLSA. NTHi strains formed several clusters within division I. Software for concatenation of MLST sequences and comparison with a reference set of STs (and MLEE lineages) is freely available at http://pubmlst.org/hinfluenzae.

Nørskov-Lauritsen performed MLSA based on six housekeeping genes (*fucK* excluded as the scheme was designed for all species within the *H. influenzae* group) for 900 STs from the MLST database (and 36 strains of *H. haemolyticus* and related species) and confirmed the existence of two major *H. influenzae* branches, one large branch (phylogenetic division I) encompassing the type strain, most NTHi, Hia and Hib strains, and all Hic and Hid strains; and a smaller branch (division II) with all Hie and Hif strains and some NTHi, Hia and Hib strains [351].

By maximum-parsimony analysis of concatenated MLST sequences from all isolates in the database per 2006 (n=655) and eBURST analysis (with five shared alleles), Erwin *et al.* identified 14 phylogenetic groups (Clades 1-13 and eBURST group 2) with different characteristics, including serotypes and virulence determinants [102]. Almost all (98%) encapsulated strains and 86% of NTHi strains could be assigned to one of the groups. Nine of 14 groups were predominated by NTHi (including one group with *H. aegyptius*) three were serotype-specific (two Hib, one Hid), and two groups contained NTHi-specific and serotype-specific clusters (one group with Hia, Hie, Hif and NTHi; one with Hic and NTHi). For comparison, Connor *et al.* analyzed concatenated sequences for all STs in the database per 2011 (n=819) using the BAPS approach with clustering of isolates based on shared polymorphisms [79]; the authors identified 12 clusters but found limited concordance with the groups described by Erwin *et al*. [102].

3.8 WHOLE-GENOME SEQUENCING (WGS)

WGS offers species identification and typing in one operation. Different statistical methods may be used for sequence analysis of complete genomic DNA. Phylogenetic analysis based on evolutionary stable SNPs is a robust measure for strain relatedness even in species with high recombination rates, and is considered the standard method for WGS-based phylogenetic analysis in *H. influenzae* [88,367,388]. The novel 'discriminant analysis of principal components' (DAPC) method correlates well to SNP-based phylogeny [88].

Discrepancies between WGS-based phylogenetic analysis and clustering based on MLST sequences have been reported. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on differences in non-core genes data for nine NTHi strains differed significantly from a dendrogram based on MLST sequences [189]. The authors suggested that the differences were due to recombinational exchange of non-core genes between strains with identical MLST sequence types. In another study, Strouts *et al.* analyzed protein-coding sequences for one Hib, three NTHi, two *H. aegyptius* (one conjunctivitis and one BPF isolate) and KW20 and found that BPF and conjunctivitis isolates of *H. aegyptius* were closely related [489]; the same strains were only remotely related according to previous MLSA [102].

Finally, De Chiara *et al.* identified six statistically supported clades (I-VI) using the DAPC method [88]. The collection comprised 90 NTHi, five Hib, one Hif and the KW20 strain. The population was bipartite, with Clade I being phylogenetically different from clades II-VI. Encapsulated strains were associated with DAPC clades I (Hif) and VI (Hib). Consistent with previous observations [102], the clades had different characteristics in terms of virulence determinants; for instance, *hmw* adhesins were associated with clades II-V and *igaB* was restricted to clades II and IV. According to the authors, classification of isolates into clades could not be predicted using MLST data: clades II-V correlated to some degree with an MLST-based minimum spanning tree, whereas no correlation was observed for clades I and VI.

3.9 CONSISTENCY BETWEEN TYPING METHODS

The *H. influenzae* population structures obtained by different typing approaches share important characteristics. First, the population is bipartite, with lineages of NTHi and encapsulated strains present in both major branches [88,289,325,351,388]. Second, there is a strong correlation between lineages and capsular serotypes, with a high degree of clonality among encapsulated strains [41,79,88,102,289,327,328,358]. NTHi are generally heterogeneous, but genetically distinct subpopulations exist; the exact delineation of these groups differs between methodologies [79,88,102,244,427].

Figure 5 shows the correlation between serotypes, MLEE lineages [327], phylogenetic divisions [289], MLST (ST and CC) [289], MLSA clades [102], and WGS (DAPC) clades [88]. DAPC clades and MLST CCs have different resolution levels, but there are no conflicts between the approaches for 55 STs assigned to DAPC clades [88]; the methods may thus be considered complimentary. The resolution levels of MLEE lineages and MLSA clades lie between DAPC clades and MLST CC, with MLSA clades being more discriminative than MLEE lineages. DAPC Clade I and MLSA Clade 2 correspond to phylogenetic division II, whereas the remaining DAPC and MLSA clades correspond to phylogenetic division I. Thus, in terms of resolution levels, the approaches may be ranked as follows: phylogenetic division < DAPC clade < MLEE lineage < MLSA clade < MLST clonal complex.

There are notable discrepancies (marked with blue) between population structures based on WGS and housekeeping phylogeny. Two NTHi-predominated MLSA clades suggest phylogenetic links between separate DAPC clades: MLSA Clade 10 connects DAPC clades IV and VI, and MLSA Clade 13 connects DAPC clades II and VI.

Figure 5 (next page). Consistency between different approaches to phylogenetic analysis and typing in *H. influenzae*. Compiled from [88,102,289,327]. Assignment to MLST clonal complexes (CC, named by predicted founder) by eBURST analysis (2014-10-28). The ten largest CCs are in bold (**Table 5**). Information about capsular serotypes was obtained from http://haemophilus.mlst.net. Colours and boxes indicate grouping by the respective approaches. Colour codes for the WGS-based DAPC clades are according to the original publication [88]. Blue marking of MLSA clades indicates conflicts with DAPC clades

Capsular serotypes	MLST sequence types and phylogenetic divisions			MLEE lineages	MLST CC (by founder)	MLSA clades	DAPC clades
	Division I	No data	Division II				
non	65				None	Indeterminate	
non	38				None	9	
non		156			ST472	9	III
non	34				ST34	9	III
non		396			ST396		III
non		697			None		V
non	11	103,134,145			**ST103**	eBURST gr 2	V
non	3,40				**ST3**	eBURST gr 2	V
non		267			ST267	eBURST gr 2	V
non	14				ST14	eBURST gr 2	V
non	33				ST33	eBURST gr 2	V
non	1				**ST1**	eBURST gr 2	V
non	46				**ST1**	1	
non	42				ST836	1	
non		245			ST836	1	V
non		142			ST142	Indeterminate	V
non		160			None	Indeterminate	V
non	12				ST12	Indeterminate	V
non	2				ST2	Indeterminate	IV
non		84			ST84	8	IV
non		321			ST349	8	IV
non	57				**ST57**	8	IV
non		411,422			ST422		IV
non	37				ST422	10	
non		334			ST1369	10	VI
non		70			None	4	VI
a	4			B	None	4	
a	5			B	None	Indeterminate	
a	23,56			B	ST23	Indeterminate	
d	47			B	ST47	7	
d	10,48,49			B	ST10	7	
b	45,50			B	**ST222**	5	
b		93			**ST222**	5	VI
b	22			A	None	3	
b	31			A	None	3	
b	24,44,53,54,55,64			A	**ST6**	3	
b	6	157,282		A	**ST6**	3	VI
b	44			A	**ST6**		
c	7,9,19,51			D	ST7	6	
c	8			D	None	6	
c	58			D	None	6	
non	43				None	6	VI
non		99,187			**ST584**	11	VI
non		238			ST896	11	VI
non		824			ST746		VI
non		879			None		VI
non		1012			None		VI
non		1063			None		VI
non		1223			ST706		VI
non		270			ST270	Indeterminate	VI
non	13				ST13	12	VI
non		262			ST262	13	VI
non	41				ST41	13	VI
non	39				None	13	
non		107			**ST503**	13	II
non		244			ST390	13	II
non		165			ST165	13	II
non		155			ST155	13	II
non		196			None	13	II
non	36				ST1020	Indeterminate	II
non		395			ST395		II
non		531			ST531		I
non		949			ST949		I
non		1131			None		I
non		111			None	2	I
non		35			None	2	
f		124			**ST124**	2	I
f			26,29	K/L	**ST124**	2	
f			16	K/L	ST123	2	
f			63	K/L	ST748	2	
f			15	K/L	None	2	
b			61	J	None	2	
e			27	F/G	None	2	
e			52	F/G	None	2	
e			18,66,67,68,69	F/G	**ST18**	2	
e			28	F/G	None	2	
e			17,32	F/G	ST121	2	
a			20,62	I	ST62	2	
a			21,30,59	H	ST21	2	
a			60	H	None	2	
a		25			None	2	

4 CLINICAL ASPECTS

4.1 COLONIZATION AND TRANSMISSION

Humans are the only natural host of the three XV-dependent species *H. influenzae*, *H. aegyptius* and *H. haemolyticus*. Nontypeable *H. influenzae* and *H. haemolyticus* colonize the upper respiratory tract in humans and are particularly frequent in children. In contrast to many other *Haemophilus* species, these organisms are exclusively found behind the palatine arches and not part of the mouth flora [227,228,321].

NTHi colonization is dynamic, and most children were occasional carriers in a longitudinal study of nasal isolates in healthy children in Korea [16]. Kindergarten children were significantly more often long-term carriers than school children. Molecular characterization (PFGE and MLST) of strains from long-term carriers showed that each child was colonized with different clones over time. Sequential colonization with different strains at high turnover rates was also reported in PFGE-based studies from Japan [181] and Portugal [426], and in a Swedish study using MLEE for characterization [524]. In the latter study, several strains were epidemic (present in >1 person on a single occasion) or endemic (present on >1 occasion in >1 person). Transmission is frequent between siblings [262] and between parent and child [549]. Extensive sharing of clones in a day-care setting was observed in Japan [181] and Portugal [426]. In contrast, the same strain was rarely found in different children in Korea [16]. Colonization varied from zero to 95% in a study comparing colonization rates and sharing of strains in day-care centers [24]. Colonization rates and strain sharing were significantly associated with suboptimal hygiene; colonization was also significantly associated with exposure for tobacco smoking.

Outbreaks of NTHi in respiratory wards and nursing homes (including transmission to staff members) have been reported, underlining the importance of hygienic measures [9,182,573]. Clones with increased capacity for transmission and/or prolonged colonization have been reported [426], consistent with the observations in a population study suggesting association between population structure and disease [244].

4.2 DISEASE AND EPIDEMIOLOGY

4.2.1 *Encapsulated H. influenzae*

The pathogenicity of *H. influenzae* depends largely on capsulation status (chapter 2.8). The polysaccharide capsule protects against the humoral immune system, including opsonophagocytosis [336] and complement-mediated killing [583]. Nasal inoculation of rats with transformed strains (identical except for capsular serotype) resulted in invasive disease in all animals inoculated with Hib strains and in a proportion of animals infected with Hia and Hif strains; no animals infected with Hic, Hid and Hie strains developed invasive disease [582].

Hib is strongly associated with severe infections such as meningitis and epiglottitis and was the leading cause of meningitis and other invasive disease in young children before effective vaccines were developed in the late 1980s [208,245,380]. Before the introduction of vaccines, Hib colonized nasopharynx in 0.5-3% of healthy children but was infrequently isolated from adults [61]. Invasive Hib disease occurred in approximately 0.5% of all children below the age of 5; two out of three cases occurred in children younger than 18 months [3,61,208]. The highest incidence rates were seen in Indigenous children in North America, the Arctic and Australia [525]. The pre-vaccine incidence of invasive Hib disease in Scandinavia was 30-60 cases per 100.000 children <5 yrs [70]. Age-specific annual incidence of epiglottitis in children 0-15 yrs in Sweden between 1971 and 1980 was 14/100.000 [69]. The estimated numbers of deaths from Hib disease among children <5 yrs in 2008 were 94.500 in Africa and 199.000 worldwide.

According to the World Health Organization (WHO), 189 countries had introduced the Hib vaccine by the end of 2013 [570]. Global coverage with three doses was 52% but only 18% in the Western Pacific Region and 27% in South-East Asia. In Norway, the Hib polysaccharide vaccine caused a rapid decline in invasive disease in children < 7 yrs when introduced in 1992 (**Figure 6**). A similar effect was seen in Iceland [32]. With the notable exception of Hia emerging as a major invasive disease in the Indigenous populations of North America [365,525], there is no convincing evidence of serotype replacement after introduction of Hib vaccines [3,32,106,245,537].

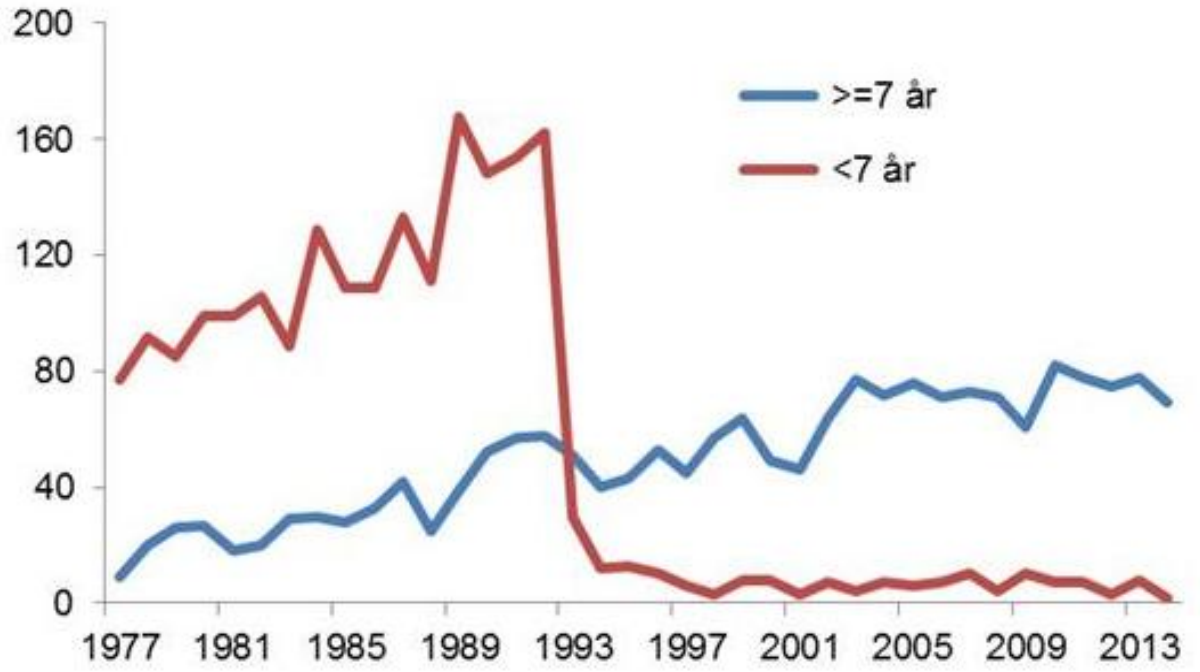


Figure 6 Annual incidence of invasive *H. influenzae* disease in Norway 1977-2014 by age groups ('år'). Blue, ≥7 years; red, <7 years. Number of cases on the x-axis. Reproduced from [354], with permission

Hif (72%) and Hie (21%) accounted for most cases of invasive non-Hib disease in a European surveillance study comprising 10.081 *H. influenzae* from 14 countries 1996-2006 [245]. Invasive Hia disease was rare but stronger correlated to meningitis in young children than other non-b serotypes. Hia and Hif strains have been reported as the etiological agent in septic arthritis [400], and Hif may cause necrotizing fasciitis and myositis [12,412].

4.2.2 *Nontypeable H. influenzae (NTHi)*

The underestimated clinical importance of NTHi was emphasized in recent reviews [154,248,537]. Most NTHi infections occur at sites contiguous with the upper respiratory tract, e.g. acute otitis media (AOM), sinusitis, conjunctivitis, and upper and lower respiratory tract infections [208,323]. There is a close correlation between nasopharyngeal colonization, conjunctivitis and AOM in children [222,262,490]. NTHi were the most frequent cause of all types of AOM in children and involved in 70% of clinically problematic AOM in a Spanish study [398]. NTHi was the main cause of bacteriological relapse following amoxicillin-clavulanate therapy for AOM in

an investigation from the U.S. [220]. In another study, 12 of 13 children with recurrent AOM had identical strains in nasopharynx and the middle ear during the first episode [262]. Of six children with a second episode, none had recurrent infection with the same strain but five had again identical strains in both locations. NTHi is the most common cause of COPD exacerbations and an important cause of exacerbations in cystic fibrosis [453]; there is a positive correlation between bacterial loads of *H. influenzae*, airway inflammation and COPD symptoms [25].

A total of 2152 cases of invasive *H. influenzae* disease were reported in EU/EEA countries in 2011 [106]. The highest number of isolates were reported from UK (n=746) and France (n=492), whereas Sweden and Norway had the highest notification rates (2.16 and 1.73 cases per 100.000/year, respectively) [106].

NTHi isolates account for the majority of invasive *H. influenzae* in countries where Hib vaccine is introduced [3,106,157,245,246,411,457,526,537]. Resman *et al.* reported a significant increase in invasive disease caused by NTHi and Hif in elderly (>60 yrs) in Sweden between 1997 and 2009 [411]. Similar observations have been made in Italy [158] and the U.S. [423].

Among invasive *H. influenzae* in Europe 1996-2006 (n=10.081), 44% were NTHi and 28% were Hib [245]. Case-fatality ratio (CFR) was higher for invasive NTHi (11.5%) than for Hib (4.4%) and particularly high for invasive NTHi in patients <1 yr (17.4%) and ≥65 yrs (14.9%). Most patients with invasive NTHi (n=3.172) had bacteremia (52.8%), pneumonia (12.6%), or meningitis (10.5%). The high meningitis proportion is consistent with later reports: NTHi accounted for 17 of 21 non-Hib CSF isolates from Portugal 2002-2010 [19] and 22 of 28 non-Hib CSF isolates from Italy 2007-2009 [158].

Among 71 invasive *H. influenzae* from Norway in 2014 (blood, n=67; CSF, n=4), 51 (71.8%) were NTHi, 12 (16.9%) were Hif, six (8.5%) were Hie, and Hia and Hib were represented with one isolate each [354]. All CSF isolates were NTHi. NTHi may also cause septic arthritis [400], and *H. influenzae* is the second most frequent *Haemophilus* species in endocarditis after *H. parainfluenzae* [44,86].

Perinatal invasive NTHi disease is strongly associated with premature birth and increased mortality and morbidity in mother and child [77,78,160,245,500]. In a study from Iceland, the risk of invasive *H. influenzae* was almost 26 times higher in pregnant women compared to nonpregnant women [32]. In a recent British study, the incidence of invasive NTHi was >17 times higher in pregnant compared to nonpregnant women, and infection was associated with poor pregnancy outcome [77]. In another study conducted by the same group, comprising 118 live-born neonates with invasive *H. influenzae* disease, 97% of strains were NTHi and 96% were early-onset (<48 h of birth) [78]. Only 15% of mothers had signs of infection.

An intriguing question is whether a proportion of presumably NTHi in perinatal disease may be misidentified '*H. quentini*' [401]. One of ten examined invasive presumably NTHi from infants in Italy was identified as '*H. quentini'* [159]. No strains were biotype IV in a Finnish study with eight NTHi (biotypes I-III) from early onset neonatal septicemia [500], suggesting that most cases are caused by 'true' NTHi.

In a Spanish investigation, Deza *et al.* highlighted *H. influenzae* as a cause of genital infections [92]. Among 413 males with acute urethritis, *H. influenzae* was the only detected pathogen in five; however, the method used for identification (API 20E) does not differentiate between *H. influenzae* and '*H. quentini*' (chapter 2.6).

4.3 VIRULENCE AND PATHOGENICITY

The ability of crossing the border between colonization and disease, i.e. to migrate from the upper respiratory tract to other parts of the body, such as the lungs, conjunctiva, paranasal sinuses and the middle ear, and then to survive and grow under various environmental conditions, differs between NTHi strains. Pathogenic strains are characterized by virulence determinants protecting the strain from the host's immune system, increasing the ability to adhere to and invade epithelial cells, and enabling the strain to deal with damaging physical or chemical stressors present within various environments [72]. Virulence determinants are variably present in NTHi strains [72,97,102,349,484].

Monitoring of transcriptional changes in NTHi and infected epithelial cells by dual RNA sequencing has provided new insights into the strategies exploited by NTHi to cause invasive disease [15] but the molecular aspects of NTHi pathogenesis are incompletely understood [202].

IgA1 is an important part of the local immune system in the respiratory tract, compromised by four variants of IgA1 proteases encoded by *igaA* and *igaB* genes; *igaA* is present in most NTHi whereas *igaB* is frequent in COPD strains and apparently important for intracellular survival [73,127,318,322]. Lipopolysaccharides (LPS) are involved in colonization and tissue injury and evasion of complement-mediated immune clearance [316] The mechanisms used by *H. influenzae* to evade attack by the host's complement system were reviewed by Hallström and Riesbeck [170].

The mechanisms by which lipooligosaccharides (LOS) act as virulence factors in NTHi are not fully understood but impaired local host defense due to ciliar damage has been suggested [413]. The surface lipoprotein Protein D (encoded by *hpd*) inhibits ciliary function [137] and promotes adherence to and internalization into epithelial cells [4]. Other major adhesins in NTHi are hemagglutinating pili [155], the low-molecular-weight lipoprotein Protein E [422], high-molecular-weight adhesins HMW-1 and HMW-2 [483], the 'adherence and penetration' (Hap) protein [128], the outer membrane lipoproteins P2 [404] and P5 [14], and *H. influenzae* adhesin (Hia) [484]. Heme acquisition genes are significantly more prevalent in AOM compared to throat strains and have been suggested to play a role in pathogenesis [174]. An investigation comparing the genomes of clinical NTHi strains identified 149 genes significantly associated with virulence or commensality; notably, none of the determinants above were among the 28 genes more likely to be present in pathogenic strains [114].

A notable trait of *H. influenzae* is the ability to adapt to the environment, evade immune attacks and regulate virulence behavior in an on/off manner through phase variation [28,316]. This phenomenon is caused by slipped-strand mispairment or other mechanisms of reversible genetic variation at 'contingency loci', consisting of hypermutable tandem repeats located within a promoter or coding sequence [28,197].

Colonization, migration and invasion is also regulated by complex interactions with competing microbes in the respiratory tract; these were reviewed by Bosch *et al.* [40]. In particular, pneumococci have developed mechanisms to kill and reduce the ability of *H. influenzae* to adhere to the surface of cells the respiratory tract.

4.4 BIOFILM

The ability to switch from a free-living, planktonic lifestyle with rapid cell division to an alternative life form characterized by cell-cell aggregation, surface attachment and low growth rate ('biofilm') is important for the survival of *H. influenzae* under variable and hostile conditions. The role of biofilm formation in chronic airway NTHi infections was reviewed by Swords [495]. The extracellular matrix (ECM) of NTHi biofilm contains significant amounts of extracellular, double-stranded DNA [213] and virulence determinants such as Hap and HMW adhesins, IgA1 protease and LOS [552]. The ECM of an AOM strain contained 265 different proteins [144].

Biofilm formation varies between strains [319] and is more frequent in NTHi than in Hib [399]. Biofilm producers are frequent among invasive [397], AOM [150,314,397] and adeno-tonsil isolates [145] but not in conjunctivitis isolates [309,355]. *In vitro* biofilm formation by COPD [319] and cystic fibrosis isolates [486] has been reported but is significantly less frequent compared to isolates associated with invasive disease and AOM [397]. Formation and maturation of biofilm *in vivo* is coordinated by quorum signaling and sensing, i.e. regulation of gene expression in bacterial communities by use of soluble signal substances [493,496]. It has been claimed that *H. influenzae* lack homologues of genes required for quorum sensing [316]; however, the genome of *H. influenzae* contains the *luxS* gene encoding the interspecies quorum signal dihydroxypentanedione (DPD) [493]. Current knowledge on quorum signaling and sensing in NTHi was reviewed by Swords [496].

Environmental factors, including beta-lactams and other antibiotics, may influence biofilm formation [198,249,495,572]. Restriction of heme-iron enhance biofilm architecture and the ability of NTHi to invade and form intracellular bacterial

communities in epithelial cells [498]. This is consistent with observations of NTHi within and between respiratory and adenoidal epithelial cells and macrophages, challenging the traditional notion of NTHi as an extracellular pathogen [72]. Intra- and paracellular communities of viable NTHi have been suggested to serve as seeds for recurrent and chronic infections, and may explain reports of antimicrobial therapy failure despite *in vitro* susceptibility [5,72,498,540]. NTHi isolates in biofilm are protected against antibiotics *in vitro* [469,486,572]. ECM destabilizers (e.g. EDTA and DNase) increase susceptibility to ampicillin and ciprofloxacin [60].

Garcia-Cobos *et al.* reported that 83% (40/48) of NTHi associated with recurrent infection, treatment failure or unresolved AOM produced biofilm [150]. There was no association between biofilm production and beta-lactam resistance mechanisms. In contrast, Mizrahi *et al.* characterized 216 NTHi and found no association between biofilm production and AOM treatment failure or recurrence [309]. There was a significant negative association between altered penicillin-binding protein 3 (PBP3) and biofilm production.

4.5 NTHI VACCINES

NTHi vaccines may reduce the global burden of disease substantially but effective vaccines are not available [154,208,320,323,537]. Due to genetic diversity, identification of cross-protective candidate antigens is difficult [316]. NTHi vaccines based on outer-membrane proteins have been unable to induce protective antibodies [39]. Outer membrane vesicles [421] and Protein F are new potential candidate antigens [201]. Other candidates are adhesins [561], LOS [65] and Protein D.

Protein D is the first NTHi antigen that has induced partial protective immune responses in humans [137]. A prototype 11-valent *S. pneumoniae* conjugate vaccine in which pneumococcal polysaccharides were conjugated to *H. influenzae* protein D (PHiD-CV11) reduced nasopharyngeal colonization with NTHi by 38.6% [391] and NTHi AOM by 35.3% [392]. However, a licensed 10-valent vaccine based on the same principles (PHiD-CV10) with protein D as a carrier for eight of ten

pneumococcal serotypes (Synflorix, GlaxoSmithKline Inc.) [393] does not seem to reduce NTHi carriage in healthy children [390,536]. On-going clinical trials are assessing the effect on AOM (NCT01735084, NCT01174849; http://clinicaltrials.gov).

Although the effectiveness of Protein D-based vaccines is compromised by *hpd* negative NTHi [388,473], they represent a proof of principle and development of broadly effective vaccines are expected within the next several years [320].

4.6 SURVEILLANCE

Vaccine coverage and the global burden of disease in terms of morbidity and mortality for Hib and other vaccine-preventable diseases is monitored by WHO [569].

In Europe, surveillance of invasive *H. influenzae* infections was initially (from 1996) performed as an EU-funded project, renamed European Union Invasive Bacterial Infections Surveillance Network (EU-IBIS) in 1998 (www.euibis.org) [245]. By 2006, 28 countries reported data to EU-IBIS. A final report with data on 10.081 invasive isolates (1996-2006) from the 14 countries where routine vaccination was implemented before 2000 and serotyping was performed for >50% of isolates was published in 2010 [245]. The responsibilities of EU-IBIS were transferred to ECDC (www.ecdc.eu) in 2007. According to the latest annual surveillance report, a total of 2152 cases of invasive *H. influenzae* disease were reported in EU/EEA countries in 2011, and 16 of 27 countries reported more than ten cases [106].

A recent review addressed the emergence and spread of strains with beta-lactam resistance due to altered penicillin-binding proteins and the implications for effective empirical therapy [537]. The authors emphasized the importance of standardized surveillance protocols and typing methodologies for global monitoring of antimicrobial resistance (AMR). WHO initiated the novel Global Antimicrobial Resistance Surveillance System (GLASS) in 2015 but whether the programme will encompass *H. influenzae* is unknown [571]. The organism is currently not included in the European Antimicrobial Resistance Surveillance Network (EARS-Net) for invasive isolates (http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance).

The Norwegian Institute of Public Health (www.fhi.no) monitors invasive *H. influenzae* disease in Norway, and the organism has been part of the Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM) (www.antibiotikaresistens.no) since 2000 for respiratory isolates and from 2012 for invasive isolates (chapter 6.6). Finally, the International Circumpolar Surveillance (ICS) network has monitored invasive *H. influenzae* disease in the Arctic Region since 2000; the surveillance program includes Northern Norway (from 2001) [365].

5 BETA-LACTAM ANTIBIOTICS AND THE CELL WALL

5.1 DEFINITIONS AND CHARACTERISTICS

Beta-lactams are hydrophilic drugs with a beta-lactam ring as the common structure and comprise penicillins, cephalosporins, carbapenems and monobactams [237,363,546]. A beta-lactam ring is a four-ring cyclic amide with the nitrogen atom adjacent to the carbon atom with the carbonyl group; this C-N-bond is denoted the beta-lactam bond and is the target for beta-lactamase enzymes (chapter 6.2). In penicillins (also denoted penams), the beta-lactam ring is fused with a saturated five-membered ring; in cephalosporins (cephems), the beta-lactam ring is fused with an unsaturated six-membered ring; in carbapenems, the beta-lactam ring is fused with an unsaturated five-membered ring; in monobactams, the beta-lactam ring is not fused to another ring (**Figure 7**). Antibacterial spectra and pharmacokinetic properties may be modified by adding different side chains and other chemical alterations.

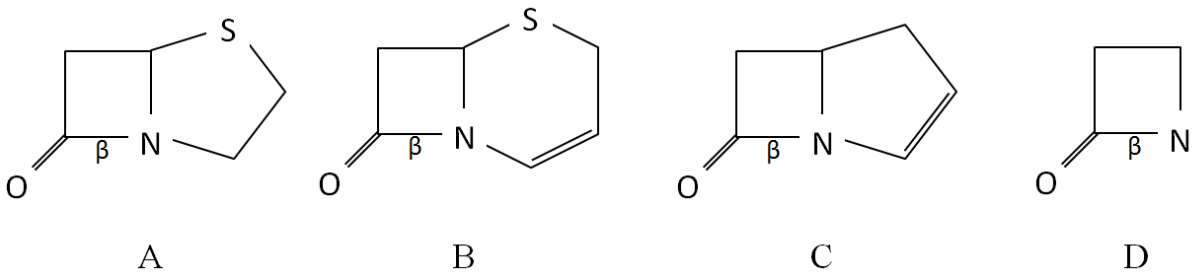


Figure 7 The core structures of penicillins (A), cephalosporins (B), carbapenems (C) and monobactams (D). The beta-lactam bond is indicated (β). Adapted from [237,363,546]

In addition to the narrow-spectrum benzylpenicillin (penicillin G, parenteral) and phenoxymethylpenicillin (penicillin V, oral), the penicillin group includes agents with extended spectrum, such as aminopenicillins (ampicillin and amoxicillin), ureido-penicillins (piperacillin), carboxypenicillins (e.g. ticarcillin) and amidinopenicillins (e.g. mecillinam); and penicillinase-stable penicillins (e.g. methicillin and cloxacillin). Beta-lactamase-sensitive penicillins are sometimes used in combinations with beta-lactamase inhibitors. The first beta-lactamase inhibitors developed for clinical use were clavulanic acid, sulbactam and tazobactam; these drugs are bicyclic beta-lactams

with structural similarities to penicillin and act as ‘suicide inhibitors’ by binding to and inactivating beta-lactamase enzymes through modifications of the active site [95].

Cephalosporins are (mostly) penicillinase-stable oral and parenteral agents. Antimicrobial spectra vary between generations; third-generation and upwards are referred to as extended-spectrum agents. Carbapenems are parenteral drugs with a wider antimicrobial spectrum than other beta-lactams [363]. Imipenem and meropenem are the clinically most important carbapenems. Monobactams constitute a small class of beta-lactams with aztreonam (parenteral) as the most important agent [416]. Importantly, extended-spectrum cephalosporins, carbapenems and monobactams exert beta-lactamase inhibitor effect in addition to antibacterial effect, through formation of sterically unfavorable acyl-enzyme complexes [95].

5.2 ACTIVITY AGAINST *H. INFLUENZAE*

Beta-lactam antibiotics are traditional first choice agents for treatment of *H. influenzae* infections, due to low toxicity and favorable pharmacokinetic and ecologic profiles. Ampicillin became available for general use in the early 1960s and revolutionized treatment of meningitis and other serious infections [334,455]. Aminopenicillins and other extended-spectrum penicillins are still preferred for treatment of infections caused by susceptible *H. influenzae* [516,537]. Aminopenicillins penetrate poorly into epithelial cells and are not active against intracellularly located *H. influenzae* [5,45]. Phenoxymethylpenicillin therapy is not recommended for *H. influenzae* infections, and benzylpenicillin therapy is associated with increased mortality compared to aminopenicillins and cefuroxime in *H. influenzae* bacteraemia [505].

Cephalosporins became widely used for treatment of infections caused by *H. influenzae* after the development of beta-lactamase-mediated resistance to penicillins in the 1970s (chapter 6.2). Most cephalosporins (except first-generation agents) are active against *H. influenzae*. The parenteral third-generation agents cefotaxime and ceftriaxone penetrate to the CSF [26] and are used for empirical therapy in invasive disease in many geographical regions [516]. The fifth-generation cephalosporin

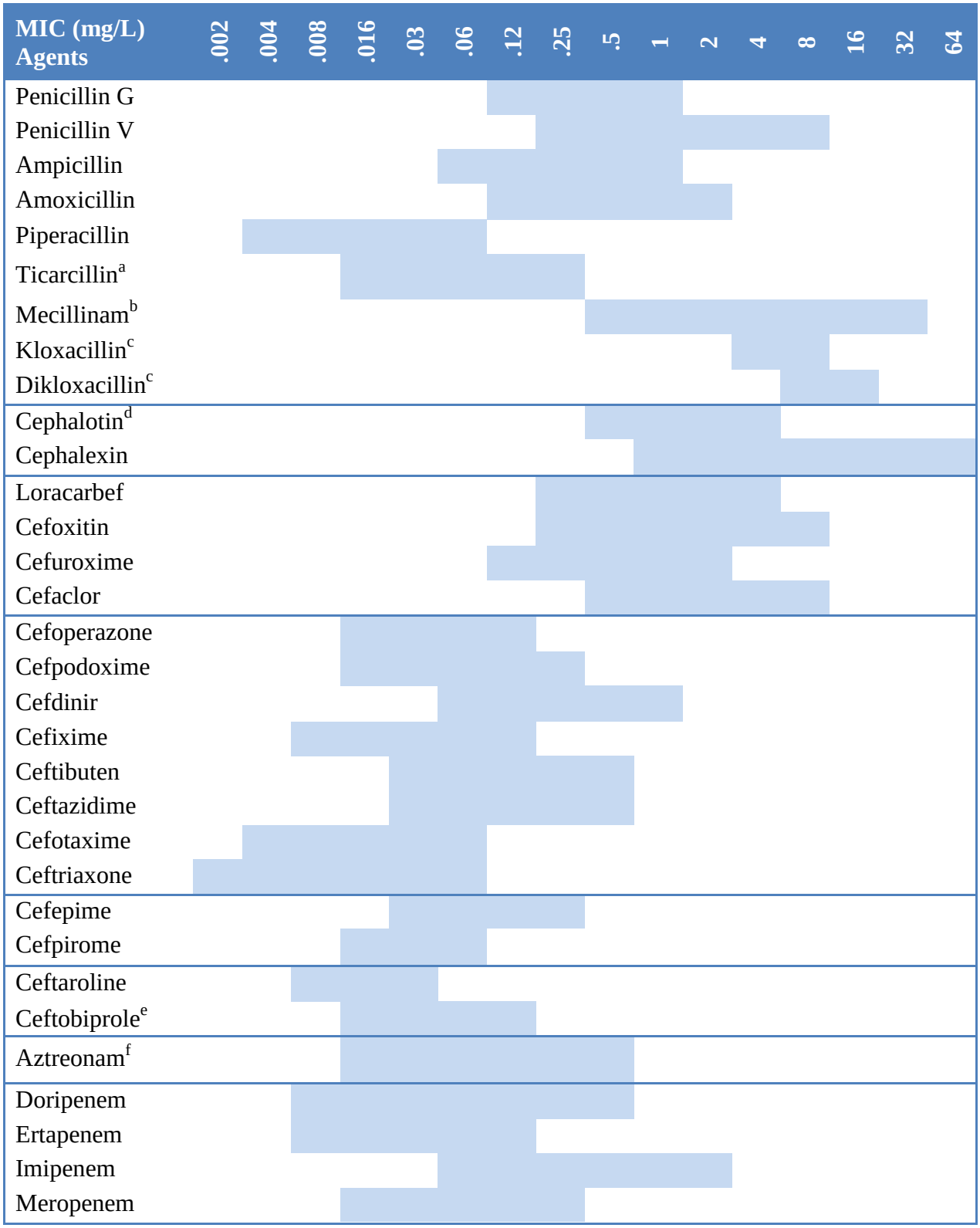
ceftaroline is approved by the European Medicines Agency (www.ema.europa.eu) for treatment of adults with community-acquired pneumonia. Other beta-lactam treatment options include extended-spectrum penicillins combined with beta-lactamase inhibitors (e.g. amoxicillin-clavulanic acid and piperacillin-tazobactam), second-generation cephalosporins (e.g. cefuroxime), and carbapenems, with meropenem so far representing the last stand for treatment of beta-lactam resistant *H. influenzae*.

Increased prevalence of resistance to extended-spectrum cephalosporins in Japan during the 1990s forced a second shift in empirical therapy in severe infections, from cephalosporins to meropenem [532]. Meropenem is as effective as cefotaxime in meningitis but therapeutic failures have been reported with both agents [346]. Due to neurotoxicity, imipenem is not suitable for treatment of meningitis.

Testing of susceptibility to ceftazidime and monobactams (i.e. aztreonam) is currently recommended by CLSI [75] but not by EUCAST [111]. According to EUCAST, *H. influenzae* is not a good target for therapy with ceftazidime, and the evidence for clinical efficacy of aztreonam is insufficient. Ceftazidime's *in vitro* activity against *H. influenzae* is inferior to cefotaxime but superior to that of second-generation cephalosporins [379]. Clinical data are lacking but the *in vivo* bactericidal activity of ceftazidime was equivalent to that of ceftriaxone in a rabbit model of experimental Hib meningitis [433]. In a rat model of Hib meningitis, ceftazidime penetrated better to the CSF and was significantly more active than ampicillin or chloramphenicol [286]. Aztreonam has high *in vitro* activity against *H. influenzae* [386,416] and has been used with success for treatment of Hib meningitis in children [156]. Aztreonam penetrated well to interstitial fluids and fibrin clots and killed *H. influenzae* more effectively than ampicillin and cefuroxime in an *in vivo* rabbit model [252]. The drug also penetrated well to the CSF and reduced bacterial load better than ampicillin or chloramphenicol in experimental meningitis in rabbits *in vivo* [287,446].

According to wild-type MIC distributions and epidemiological cut-off values (ECOFF) for beta-lactams and *H. influenzae*, piperacillin and the extended spectrum cephalosporins cefotaxime, ceftriaxone, cefpirome and ceftaroline are the most active beta-lactams *in vitro* against this organism (**Table 6**).

Table 6 Wild-type MIC distributions for beta-lactams against *H. influenzae*. Horizontal lines separate classes and cephalosporin generations. Loracarbef (carbacephem) and cefoxitin (cefamycin) are categorized with second-generation cephalosporins. Data from EUCAST (www.eucast.org/mic_distributions, accession date 2016-01-12) unless otherwise indicated



[a] [439]; [b] [507]; [c] [120] [d] [509]; [e] [122]; [f] [386]

5.3 PEPTIDOGLYCAN BIOSYNTHESIS

The peptidoglycan synthesis is the main target for beta-lactams. Peptidoglycan (also known as murein) is the stress-bearing component of the cell wall, and a functioning apparatus for peptidoglycan synthesis is vital for growth and morphogenesis in most bacteria [528]. In *H. influenzae* and other Gram-negative organisms, the peptidoglycan layer is located in the periplasm, defined as the space between the inner membrane (a classical phospholipid bilayer) and the outer membrane (composed of a mixture of lipopolysaccharides, lipooligosaccharides, phospholipids and lipoproteins) [261,546]. The biosynthesis of peptidoglycan is a complex process following a pathway which includes i) synthesis of nucleotide precursors in cytoplasma, ii) assembly of peptidoglycan monomers (see below) on the inner side of the cytoplasmic membrane, and iii) polymerization in the periplasmic space. A schematic overview of the pathway in *H. influenzae* was provided by Trepod and Mott [515].

Peptidoglycan biosynthesis has been little studied in *H. influenzae* but has been extensively investigated in *E. coli* [237,474,481,528,538,542]. The main principles are, however, believed to be common in most Gram-negative bacteria [474,528,538], and the *H. influenzae* genome has been shown to encode the same pathways characterized in *E. coli* [6,267,515,542]. Common ancestry and the large number of conserved genes in the two organisms [91] support the assumption that peptidoglycan synthesis is highly similar in *E. coli* and *H. influenzae*. Finally, *H. influenzae* peptidoglycan strongly resembles *E. coli* peptidoglycan with respect to monomer structure, chain lengths and degree of cross-linking [52].

In *H. influenzae* and other Gram-negative bacteria, a peptidoglycan monomer consists of a disaccharide; N-acetylglucosamine (GlcNAc or NAG) and N-acetylmuramic acid (MurNAc or NAM); and a pentapeptide chain linked to MurNAc. The monomers are synthesized in a series of enzymatic reactions catalyzed by four ligases encoded by *murC*, *murD*, *murE* and *murF*; these are responsible for the successive additions of the amino acids L-alanine, D-glutamate, diaminopimelic acid, and D-alanyl-D-alanine, respectively, to MurNAc [267]. The Mur ligases are required for cell viability [542]. The membrane steps include two enzymatic reactions catalyzed by the MraY and

MurG transferases, leading to the formation of the intermediates Lipid I and Lipid II; the latter reaction includes the addition of the second saccharide unit GlcNAc [538]. Lipid II, which includes the complete disaccharide-pentapeptide monomer unit, is then translocated (‘flipped’) across the inner membrane into the periplasmic space. The identity of the ‘flippase’ has been much debated; the cell division proteins FtsW, RodA and MurJ are the most likely candidates [456,474,528].

The modified monomers are polymerized and assembled into the cell wall structure by four membrane-associated reactions: disaccharides are chained into a glycan backbone in the transglycosylase (glycosyltransferase) reaction, and the peptide chains are cross-linked (the position 4 D-alanine of the donor peptide is linked with the position 3 diaminopimelic acid of the acceptor peptide) to form a mesh-like peptidoglycan layer in the transpeptidase reaction. Prior to the latter reaction, the terminal D-alanine residue is removed from some peptide side chains in the carboxypeptidase reaction, and existing peptide cross-links are broken in the endopeptidase reaction (autolysis) to allow insertion of new peptidoglycan.

The four reactions are catalyzed by enzymes collectively referred to as penicillin-binding proteins (PBPs) [223,237,474,481,538,544]. Several hydrolases in addition to the PBP endopeptidases contribute to autolysis [544]. The activity of PBPs is regulated by lipoproteins anchored in the outer membrane [474,528].

5.4 PENICILLIN-BINDING PROTEINS: BETA-LACTAM TARGETS

Penicillin-binding proteins differ in localization, size, structure, function and essentiality, and the number of PBPs varies between bacterial species. The proteins are numbered and categorized according to molecular weight and enzymatic activity. High-molecular-weight (HMW) PBPs are divided into two subclasses: class A PBPs are bifunctional transglycosylase and transpeptidase enzymes whereas class B PBPs have only transpeptidase activity. The low-molecular-weight (LMW) PBPs exert carboxypeptidase and/or endopeptidase activity [223,237,474,481,528,544,577]. The transpeptidase, carboxypeptidase and endopeptidase activity depends on three

conserved motifs Ser-X-X-Lys (SXXK), Ser-X-Asn (SXN) and Lys-Thr-Gly (KTG); amino acid sequences at these motifs are crucial for the tertiary structure of the active site pocket and have great impact on substrate specificity and enzymatic activity. The serine of the SXXK motif is essential for the catalytic mechanism and is the active residue. Serine is acylated and deacylated on each catalytic cycle [223,474,577].

Despite the term 'penicillin-binding' is the acyl-D-alanyl-D-alanine part of the peptidoglycan monomer the natural substrate for the transpeptidase moiety of PBPs. The exact binding location of the donor peptide in the active site pocket is unknown, and the transpeptidase reaction is incompletely understood on a molecular level [528]. It should also be noted that current understanding of the mechanisms and regulation of peptidoglycan synthesis is limited, partly because the different parts of this process have been traditionally studied independently rather than as an integrated macro-molecular machine [474]. PBPs interact with a large number of enzymes and proteins, and the activity of PBPs during bacterial growth and cell division is regulated in sophisticated ways (chapter 5.7). A schematic illustration of the peptidoglycan synthesis complexes and interactions between PBPs and other components in *E. coli* was provided in a recent review article by Typas *et al.* [528].

Beta-lactam antibiotics exert antibacterial effect by inhibiting the transpeptidase, carboxypeptidase and endopeptidase (but not the transglycosylase) activities of PBPs as substrate analogs of the acyl-D-alanyl-D-alanine component of the peptidoglycan monomer [237,481,544,546]. The disturbed balance between peptidoglycan synthesis and autolysis (caused by other hydrolases than the LMW PBPs) leads to weakening of the peptidoglycan layer, morphological changes and eventually lysis.

The antibacterial effect of beta-lactams depends on their binding affinity to the active site of specific PBPs (chapter 5.4), and the primary target of a beta-lactam may to some degree be deduced from the morphological effects. Early studies in *E. coli* identified seven PBPs and showed that simultaneous inhibition of PBP1A and 1B resulted in cell lysis, PBP2 inhibition resulted in spherical cells, and PBP3 inhibition induced filamentation; all effects were lethal. In contrast, inhibition of LMW PBPs (PBP 4-6) was not associated with morphological changes or death [481].

According to current knowledge [474,528], PBP1A and PBP2 interact and are central in cell elongation in Gram-negative bacteria, with PBP2 being particularly important for maintaining rod shape, whereas PBP1B and PBP3 cooperate in the synthesis of septal peptidoglycan during cell division. LMW PBPs are involved in control of morphology, autolysis and regulation of peptidoglycan synthesis but are generally not required for survival [223,237,474,528,544]. The role of LMW PBPs and other hydrolases was reviewed by Vollmer *et al.* [544].

Early observations suggested that the targets and effect mechanisms of beta-lactams in *H. influenzae* are similar to in *E. coli*. Klein and Luginbuhl reported that ampicillin induced filaments with 'periodic saccular outpouchings' in the ampicillin-susceptible *H. influenzae* strain ATCC 19418 (MIC = 0.1 mg/L) [234]. Makover *et al.* reported that exposure of *H. influenzae* to penicillin G, amoxicillin, cephalexin and amdinocillin resulted in morphological effects similar to those previously described in *E. coli*, including formation of spherical cells after exposure for amdinocillin [274].

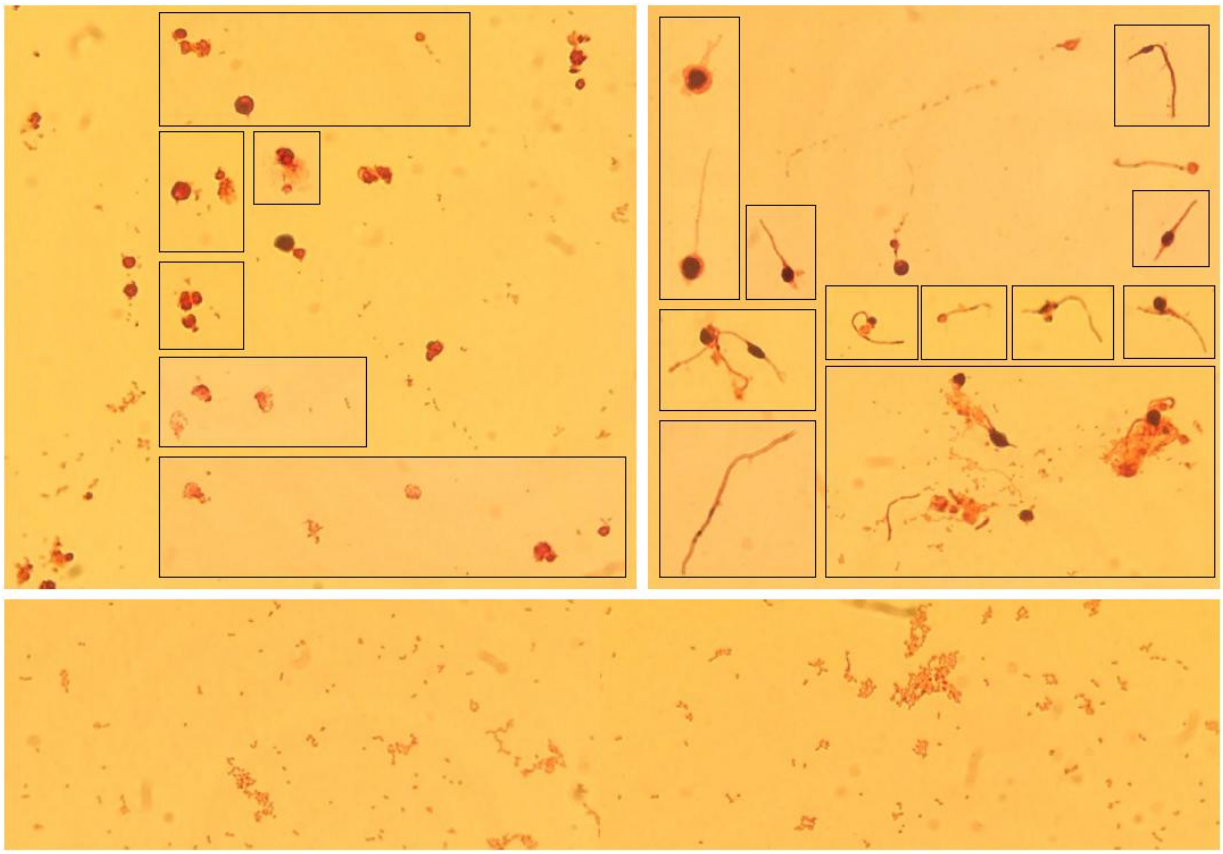


Figure 8 Collage of microphotographs (100x) showing the morphological effects of meropenem (top left) and ampicillin (top right) on *H. influenzae*. Bottom, cells with no exposure for antibiotics. All pictures are the same magnification. See text for details

The morphological effects of carbapenems on *H. influenzae* were investigated in a simple experiment for this thesis: three tubes with 5 ml BHI broth supplemented with haemin (10 μg/ml) and NAD (2 μg/ml) were inoculated with 2-3 colonies of a beta-lactamase negative isolate with increased ampicillin gradient MIC (2 mg/L). Paper disks with 10 μg ampicillin and 10 μg meropenem were added to separate tubes. Gram stained smears of sediment from each tube were examined by microscopy after 18 h incubation at 35 ±1ºC in air with 5% CO_2 (**Figure 8**). Filaments with saccular outpouchings were observed in the ampicillin tube, consistent with previous observations [234], whereas spherical cells were observed in the meropenem tube, indicating inhibition of a homologue to PBP2 in *E. coli*. Lysis occurred in both tubes.

5.5 PENICILLIN-BINDING PROTEINS IN *H. INFLUENZAE*

5.5.1 *Terminology*

The PBPs in *H. influenzae* were first studied by Makover *et al.* [274]. Radiolabeled penicillin G was bound to the membrane fraction of the beta-lactamase negative strain ATCC 19418 with subsequent gel electrophoresis. Electrophoretic patterns revealed eight major PBPs with molecular weights 90-27 kDa, designated PBP 1-8. Binding affinities for beta-lactams were determined by competition experiments. A factor adding some uncertainty to the results is the reported ampicillin MIC (3.3 mg/L), which is significantly higher than the value (0.1 mg/L) reported for this strain by others [234] and highly suggestive of acquired beta-lactam resistance mechanisms. In the following years, research groups in the U.S., Canada and Japan characterized PBPs in ampicillin susceptible isolates [71,276-278,296,298,301,303,304,366,451,530]. Variations in estimated molecular weights and numbers of detected PBPs make it difficult to compare results and observations from different studies, and the use of different numbering systems has caused some confusion. However, harmonization with current terminology is possible based on the reported binding affinities and morphological effects of PBP inhibition. An overview of reported PBPs in different studies and the correlation to current terminology is shown in **Figure 9**.

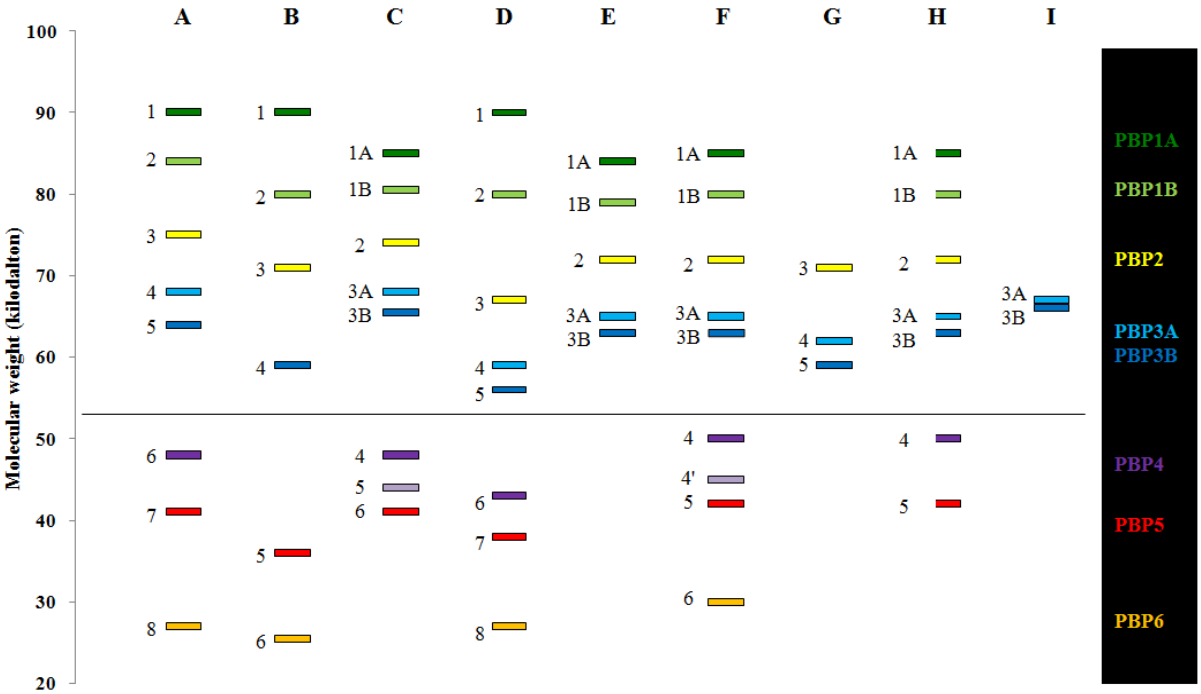


Figure 9 Penicillin-binding proteins (PBPs) in *H. influenzae*. Numbering and molecular weights (MW) is according to the respective authors. Colours indicate PBP identities according to current terminology, as shown to the right (not related to the MW scale). Only PBPs for which MW is presented in the original article are shown. The horizontal line separates high-molecular-weight and low-molecular-weight PBPs. Compiled from A [274], B [301], C [366], D [451], E [278], F [276,277], G [298], H [71], I [530]

Current terminology for HMW PBPs (1A, 1B, 2, 3A, 3B) and the LMW PBP with the highest molecular weight (PBP4) was proposed by Parr and Bryan in 1984 [366]. The two remaining LMW PBPs were assigned their current numbers (PBP5 and PBP6) by Malouin and Bryan in 1988 [276]; this terminology is used in most later publications (and in this thesis) [71,277,516,530]. In 1995, whole genome sequencing of *H. influenzae* Rd KW20 revealed seven PBP encoding genes: *ponA*, *ponB*, *pbp2*, *ftsI*, *dacA*, *dacB* and *pbp7* ('HI0364') [131]. In 2001, Ubukata *et al.* [530] showed by sequencing and transformational studies that the proteins denoted PBP 3A and 3B by Bryan and coworkers [276,277,366] were encoded by the *ftsI* gene and correspond to PBP3 in *E. coli*. In addition to PBP 3A and 3B, Ubukata *et al.* described six PBPs (1A, 1B, 2, 4 5 and 6); exact molecular weights were not presented but comparison of gel pictures indicates that these proteins correspond to the PBPs numbered similarly by Bryan and coworkers [276,277].

Accordingly, in a review on antimicrobial resistance, Tristram *et al.* stated that *H. influenzae* has eight PBPs, designated 1A, 1B, 2, 3A, 3B, 4, 5 and 6, and that the numbering is based on observations that the affinity of each PBP is similar to the corresponding PBPs in *E. coli* [516]. An overview of PBPs in *H. influenzae*, with the corresponding genes and enzymatic activity of the various proteins is presented in **Table 7**.

Table 7 Penicillin-binding proteins (PBPs) in *H. influenzae* with corresponding genes and functions (partly based on observations and terminology in *E. coli*). HMW, high-molecular-weight; LMW, low-molecular-weight; Tp, transpeptidase; Tg, transglycosylase; Cp, carboxypeptidase; Ep, endopeptidase. Compiled from [6,131,223,237,274,301,481,515,516,528,530]

PBP	1A	1B	2	3AB	4	5	6
Gene	*ponA*	*ponB*	*pbp2*	*ftsI*	*dacB*	*dacA*	*pbpG*
Class	HMW-A	HMW-A	HMW-B	HMW-B	LMW	LMW	LMW
Enzyme activity	Tp, Tg	Tp, Tg	Tp	Tp	Cp, Ep	Cp	Ep
Effects on morphology	Cell elongation	Cell division	Rod shape, Cell elongation	Cell division	None	Cell shape	None
Protein homologue in *E. coli* (synonymous gene names)	PBP1A (*mrcA*, *ponA*)	PBP1B (*mrcB*, *ponB*, *pbpF*)	PBP2 (*pbp2*, *mrdA*)	PBP3 (*ftsI*, *pbpB*)	PBP4 (*dacB*)	PBP5 (*dacA*, *pfv*)	PBP7 (*pbpG*, *yohB*)

The terminology implies at least two inconsistencies. First, only one gene (*ftsI*) encoding PBP3 is known in *H. influenzae* and *E. coli*, and the correspondence between this gene and the two proteins PBP 3A and 3B in *H. influenzae* is not clear (chapter 5.6). Secondly, whereas the genome of *H. influenzae* contains a homologue to the *pbpG* gene encoding PBP7 in *E. coli*, no homologue to the *dacC* gene encoding PBP6 in *E. coli* has been identified [131,223,515]. Thus, using the genes and PBPs of *E. coli* as reference, the correct designation for the PBP currently designated PBP6 in *H. influenzae* should probably be 'PBP7' [223]. Interestingly, PBP6 appears to consist of two proteins with slightly different molecular weights [299,530].

It should also be noted that other PBPs than the eight described above have been reported in *H. influenzae*. Malouin and Bryan observed a protein between PBP4 and PBP5 in the Rd strain (denoted PBP4´ in **Figure 9**), and Hie ATCC 8142 and Hib ATCC 9705 had a protein between PBP3B and PBP4 [276]. The significance of these observations is unknown.

Detection and characterization of PBPs may be affected by several factors. PBP3A and PBP4 are not always detectable when cells are in stationary phase [298]. Ubukata *et al.* reported that PBP4 could not be detected in strains with a 7-basepair deletion in the *dacB* gene resulting in a stop codon [530]. Malouin and Bryan reported that PBP 3A and 3B in strain Rd were temperature sensitive; at 42ºC the proteins did not bind penicillin G, and filamentous cells were formed [276]. These observations suggest that temperature may induce the SOS response in *H. influenzae* (chapter 5.8). The authors also showed that alterations in temperature affected the electrophoretic mobility of PBP3A and the binding affinity of PBP1A. Whether these observations have any clinical significance is not known.

5.5.2 *Essentiality*

Studies on essentiality of PBPs in *H. influenzae* have shown differing results. Akerley *et al.* assessed essentiality of by transposon mutagenesis and genetic footprinting [6]. As transposons normally cannot be inserted into essential genes, the number of insertions in the open reading frame of each gene (excluding 25% of the open reading frame in the 3' end) was used to assess essentiality. Genes with no insertions (e) or single insertions (e1) were considered putative essential for growth or viability, genes with two insertions (e2) were scored as equivocal and genes with multiple insertions were considered non-essential. Of the seven PBP genes, *ponB* (e), *dacB* (e), and *pbp2* (e1) were essential; *ftsI* (e2) was equivocal; and *dacA* and *pbpG* were non-essential. The essentiality of *ponA* could not be determined. These observations are consistent with observations in *E. coli*, where inactivation of PBP 1A and 1B, or PBP2, or PBP3, is lethal whereas inactivation of either PBP 1A or 1B is not [481].

Trepod *et al.* assessed essentiality of PBP genes in *H. influenzae* by insertional inactivation of genes using transposon mutagenesis and concluded differently [515]. Defining non-essentiality as cellular survival despite inactivation of the gene, the authors found that only *ftsI* was essential. The effect of simultaneous inactivation of two or more non-essential PBP genes was not investigated. Categorization of *pbp2* as non-essential is remarkable, considering the bactericidal effect of carbapenems [363] and that PBP2 is the primary target of carbapenems (see below). From these two studies it seems clear that *ftsI* is essential whereas *dacA* and *pbpG* are non-essential in *H. influenzae*. For the remaining genes, additional studies are needed. Clarification of the essentiality of the *pbp2* gene is of particularly importance.

5.5.3 *Beta-lactam affinity*

PBP binding affinities of beta-lactams may be determined by competition studies, in which whole cells or membrane preparations are allowed to react with beta-lactams at varying concentrations, followed by addition of radioactive penicillin G. The amount of radioactive penicillin G bound to each PBP (with and without the competing beta-lactam) is then measured. Binding affinity is expressed as the I_{50} value, which is the concentration of the drug required to reduce the binding of penicillin G by 50% [274]. A similar methodology using fluorescent penicillin may be used to calculate the IC_{50} value [313]. Low I_{50} or IC_{50} values indicate high affinity for the drug. Notably, I_{50} and IC_{50} values are not directly comparable, and inter-investigator variation must be taken into account when comparing results from different investigations.

Relative binding affinities indicate which PBP is the primary target of the agent but do not allow comparison of the activity of different drugs. Absolute binding affinities are comparable between agents, and may to some degree be used to predict antibacterial activity and vulnerability for resistance development due to target alterations (chapters 6.3 and 6.4.1). In *H. influenzae*, most beta-lactams (with carbapenems as a notable exception) have highest affinity for PBP1B and PBP3 (3A and/or 3B), but there are important differences between and within classes and subclasses (**Table 8**).

Table 8 Relative PBP affinities of beta-lactams in ampicillin-susceptible *H. influenzae*. The numbering of PBPs is harmonized according to current terminology (**Figure 9**). High-molecular-weight PBPs are in bold; PBP3A and 3B are highlighted (blue).

Agents	Relative affinities	References
Penicillin G	**1B** > 4 > **3A** = **3B** > **2** > **1A** = 5	[274]
	1B > **3B** > **3A** > 5 > **1A** > **2**	[298]
	6 > 4 > **1B** > **3B** > **3A** > 5 > **1A** > **2**	[299]
Ampicillin	**1B** = **3B** > **3A** > **2** = 4 > **1A** = 5	[274]
	3A > **1B** > 4 > **2** > **1A** > **3B**	[301]
	4 > **3A** > **1B** = **3B** > 6 > **2** > **1A** > 5	[298]
	4 > **1B** = **3A** = **3B** > **2** > **1A** > 5	[530]
Amoxicillin	**1B** = 4 > **2** > **3AB** > **1A** = 5	[274]
Piperacillin	**3AB** > **2** > **1AB**	[366]
	3B > **3A** > **2** > **1B** > **1A**	[313]
Amdinocillin	**2** > **1B** > 4 > **1A** > **3A** = **3B** = 5	[274]
Cephalexin	**1B** > 4 > **3AB** > **2** > **1A** > 5	[274]
Cephalotin	**1B** > **1A** > **3B** > **3A** > 4 > **2** = 5	[274]
Cefoxitin	**1AB** > **2** >=< **3AB**	[366]
Cefuroxime	**1B** > **3B** > **3A** > **1A** > **2**	[299]
Ceftibuten	**1B** > **3B** > **3A** > 6 > **1A** > 5	[303]
Cefdinir	**3A** = **3B** > **1B** > 4 > **1A** = **2** = 5	[530]
Cefditoren	**3A** = **3B** > **1B** > 4 > **1A** = **2** = 5	[530]
Cefixime	**1B** = **3A** > **3B** > **1A** > **2** > 5	[303]
Cefpodoxime	**1B** > **3A** > **3B** > 6 > **2** > **1A** > 5	[303]
	3A = **3B** > **1B** > 4 > **1A** = **2** = 5	[530]
Cefotaxime	**3A** > **3B** > 6 > **1B** > **1A** > **2** > 5	[303]
	3A = **3B** > **1B** > 4 > **1A** = **2** = 5	[530]
	3A > **3B** > **1B** > **1A** > **2**	[313]
Ceftriaxone	**3B** > **3A** > **1B** > **1A** > **2**	[313]
Imipenem	**2** > **1AB** > **3AB**	[366]
	2 > **1A** > **3B** > **3A** > **1B** > 5 >=< 6	[298]
	4 > **2** > **1B** > 5 > **1A** > **3B**	[217]
Meropenem	4 > **2** = **1B** > **1A** > **3A** = **3B** >= 5	[530]
	2 > 4 > **3B** > **1B** > 5 > **1A**	[217]

The strong affinity of cefotaxime and ceftriaxone for PBP3 is consistent with high *in vitro* activity; conversely, the low affinity of cephalexin is consistent with low activity of this drug against *H. influenzae* (**Figure 10**). Piperacillin has high affinity for both PBP3 and PBP2; the latter thus appears to be the secondary target for this drug.

Carbapenems have higher affinity for PBP2 than PBP3, consistent with the observed morphological effects of meropenem (**Figure 9**). The *in vitro* activity of these drugs is similar to or higher than the activity of ampicillin (**Table 6**) and they kill *H. influenzae* effectively *in vivo* [346]. Monobactam affinity studies in *H. influenzae* have not been identified but PBP3 is the primary target of monobactams in *E. coli* [416].

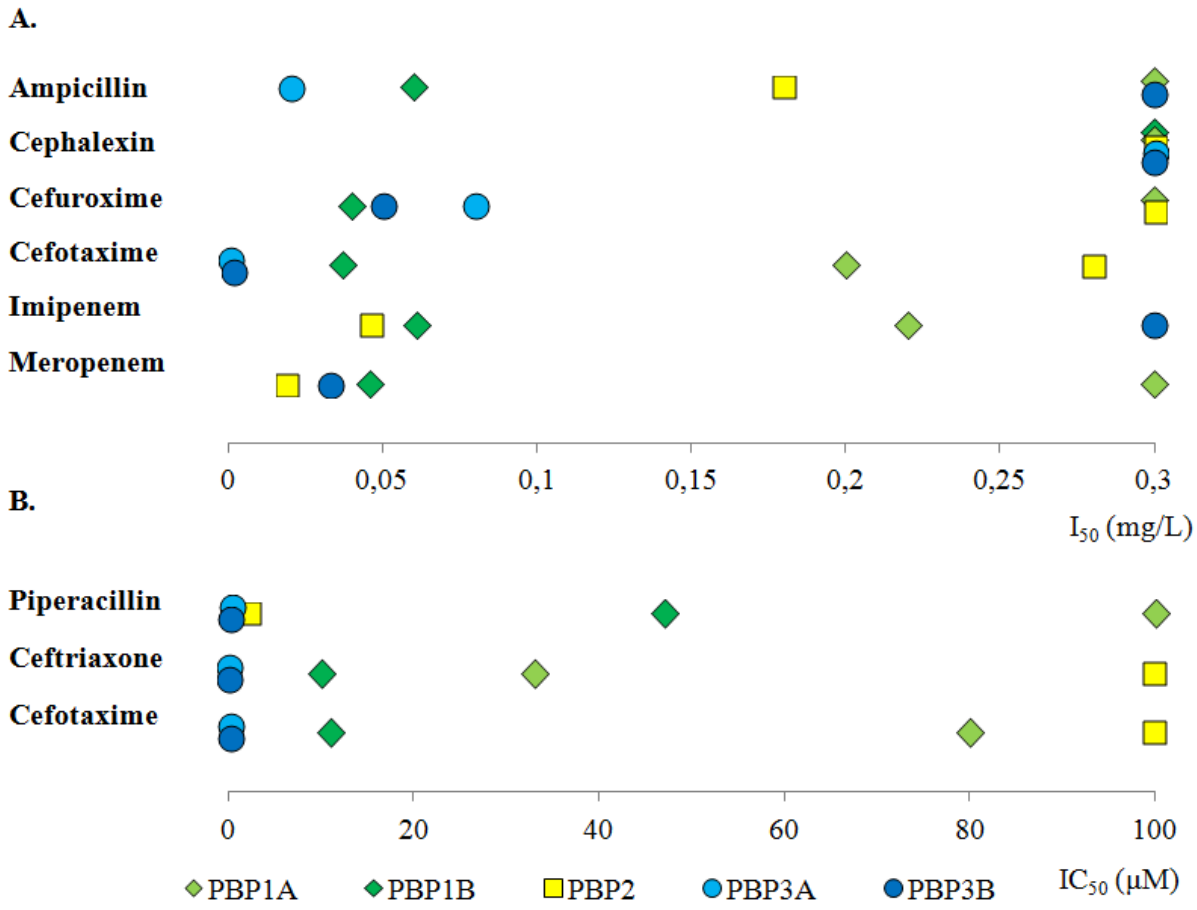


Figure 10 Absolute and relative binding affinities of beta-lactams for high-molecular-weight PBPs in ampicillin susceptible *H. influenzae*. A (I_{50}, mg/L): ampicillin [301], cephalexin [274], cefuroxime [299], cefotaxime [303], imipenem and meropenem [217]; B (IC_{50}, µM): piperacillin, ceftriaxone and cefotaxime [313]. I_{50} and IC_{50} values are truncated at 0.3 mg/L and 100 µM. Low values indicate high affinity. The affinities of cefotaxime are used to harmonize the scales. PBPs are numbered according to current terminology (**Figure 9**)

5.6 PBP3, CELL DIVISION AND THE *FTSI* GENE

Because PBP3 is the only PBP so far confirmed to be involved in clinically significant beta-lactam resistance in *H. influenzae* (chapters 6.3 and 6.4.1), this protein is given particular attention in this thesis.

The mature form of PBP3 is a class B HMW PBP with transpeptidase activity and essential for synthesis of septal peptidoglycans during cell division. The protein consists of a large periplasmic C-terminal transpeptidase domain, a membrane-spanning segment, and a small cytoplasmic N-terminal domain; the two latter constitute the membrane anchor and are necessary for normal cell division [554]. Beta-lactam affinity and transpeptidase activity depends on the three-dimensional structure of the active site pocket, for which the amino acid sequences near the conserved motifs S327TVK, S379SN and K512TG are particularly important [530].

The formation of a septum during cell division is mediated by a multi-protein complex of cell division proteins at the division site, denoted the *divisome* [528]. The first event is polymerization of the tubulin-like protein FtsZ into the Z-ring. This is followed by recruitment of other division proteins in a particular order, with the incorporation of PBP3 as a late event. Several cell division proteins such as FtsA, FtsQ, FtsL and FtsW are required for septal localization of PBP3 [305,554]. In the divisome, PBP3 interacts particularly close with PBP1B and the cell division protein FtsN [528]. Divisome PBP activity is in part controlled by outer membrane-anchored lipoprotein B [474,528]. It seems likely that the saccular outpouchings observed after exposure for ampicillin [234], depicted in **Figure 8**, represent dysfunctional divisomes with accumulation of cell division proteins.

The *ftsI* gene encoding PBP3 in *H. influenzae* consists of 1833 bp and has 51% homology to the *ftsI* gene (also denoted *pbpB*) encoding PBP3 in *E. coli*; for comparison, the homology identity between PBP3 in *H. influenzae* and PBP2X in pneumococci is 29% [530]. Comparison of the local context of the *ftsI* gene shows that the clusters of cell division genes (denoted *dcw*) are structurally similar in *H. influenzae* and *E. coli*, whereas clusters with the corresponding genes in other organisms are organized differently [542]. In addition to *ftsI* and other cell division

proteins, the *dcw* cluster comprises genes encoding murein ligases used for synthesis of peptidoglycan precursors (chapter 5.3), which are needed for biosynthesis of peptidoglycan during cell elongation as well as cell division. The genes of the *dcw* cluster in *H. influenzae* and the functions of the various gene products are presented in **Table 9**.

The relationship between the *ftsI* gene and the two proteins PBP 3A and 3B is incompletely understood. Ubukata *et al.* suggested that PBP 3A and 3B in *H. influenzae* are different forms transcribed from the same gene [530]. In *E. coli*, PBP3 is synthesized as a precursor which is processed into the mature form by a C-terminal protease, encoded by the *prc* gene (synonym *tsp*); the precursor and the mature protein have different electrophoretic mobilities [173]. The *prc* gene is present in *H. influenzae* [131] and a similar post-translational modification of PBP3 in this organism is likely.

Table 9 The *dcw* gene cluster (presented in order from 5' to 3') with the corresponding gene products and their functions in *E. coli* and *H. influenzae*. Compiled from [305,474,528,538,542,554]

Gene	Product	Function
mraZ	MraZ	Unknown
mraW	MraW	16S rRNA methyltransferase; methylates 16S rRNA
ftsL	FtsL	Cell division protein
ftsI	PBP3	Transpeptidase; catalyzes cross-linking of peptide sidechains
murE	MurE	Ligase; adds the third aa (diaminopimelic acid) to the peptide chain
murF	Mur F	Ligase; adds the last aa (D-alanyl-D-alanine) to the peptide chain
mraY	MraY	Transferase; converts the disaccharide-pentapeptide to lipid I
murD	MurD	Ligase; adds the second aa (D-glutamate) to the peptide chain
ftsW	FtsW	Cell division protein
murG	MurG	Transferase, converts lipid I to lipid II
murC	MurC	Ligase; adds the first aa (L-alanine) to MurNAc
ddlB	DdlB	Ligase; synthesizes D-alanyl-D-alanine from two D-alanine molecules
ftsQ	FtsQ	Cell division protein
ftsA	FtsA	Cell division protein
ftsZ	FtsZ	Cell division protein

5.7 REGULATION OF *FTSI* TRANSCRIPTION AND CELL DIVISION

Regulation of transcription of the *dcw* cluster is poorly investigated in *H. influenzae*. In *E. coli*, with identical organization of the *dcw* cluster [542], gene transcription is regulated in a sophisticated manner [162,542]. Considering their common ancestry [91], it seems likely that mechanisms similar to those regulating gene expression in *E. coli* may be present in *H. influenzae*; however, this assumption needs to be confirmed. Some regulatory mechanisms present in *E. coli* and their relevance for regulation of *ftsI* transcription and cell division in *H. influenzae* are described below.

In *E. coli*, five promoter sequences initiating transcription have been identified at the 5' end, and six promoters have been identified at the 3' end [542]. The closest promoter to the *ftsI* gene is localized ~400 bp upstream of the start codon, just upstream of the neighboring *ftsL* gene. Promoters of the *dcw* cluster have to my knowledge not been characterized in *H. influenzae*.

The pyruvate dehydrogenase complex regulator, PdhR, inhibits transcription of the *dcw* cluster in *E. coli* [162]. The pyruvate dehydrogenase complex consists of pyruvate dehydrogenase (*aceE*), dehydrolipoate acetyltransferase (*aceF*) and dehydrolipoamide dehydrogenase (*lpdA*), and catalyses the formation of acetyl-CoA from pyruvate. PdhR is inhibited by pyruvate and represents a link between cell division and nutritional status. The pyruvate dehydrogenase complex genes (*aceE*, *aceF* and *lpdA*) are present in *H. influenzae* [6] but the existence of a homologue to the gene encoding the complex regulator PdhR and the role of this putative gene product in the regulation of *dcw* transcription in this organism are to my knowledge not investigated.

Transcription of the *dcw* cluster at the 5' end is repressed by binding of LexA to at least three SOS boxes in *E. coli* [162,542]. The SOS response, reviewed by Simmons *et al.* [461], is a bacterial defense mechanism induced by DNA damage and other stressors. SOS induction leads to up-regulation of several genes, including *recA*, which is essential for DNA repair through recombination (chapter 3.3). RecA binds to single-stranded DNA (a signal of DNA damage) and forms a nucleoprotein which interacts with LexA to activate autocleavage, thereby increasing expression of the *dcw* cluster and other LexA repressed genes, such as *sulA* (also denoted *sfiA*, 'suppress

filamentation inhibitor'). The *sulA* gene encodes a cell division inhibitor (SulA) with the ability to prevent the formation of the Z-ring by blocking FtsZ (also known as SulB) [193]. This causes a rapid arrest in cell division. Combined with increased transcription of murein ligases, this results in filamentous growth, providing the cell with an opportunity to repair chromosomal damages before cell division [461]. The *H. influenzae* genome encodes both *sulA* and *lexA* [6], with 67% sequence identity between *lexA* in *H. influenzae* and *E. coli* [494]. The *sulA* gene is located adjacent to *recA* in *E. coli*, but not in *H. influenzae*.

In addition to DNA damage, a variety of environmental factors may induce the SOS response. Miller *et al.* showed that the response is initiated by inhibition of PBP3 by beta-lactams in *E. coli* [307]. The authors demonstrated that strains unable to generate an SOS response (mutations in *recA* or *dpiA*) or unable to inhibit FtsZ (mutations in *sulA*) were more effectively killed by ampicillin, and concluded that the SOS response represents a mechanism that protects against the lethal effects of beta-lactams. Observations by Malouin and Bryan suggest that temperature may activate the SOS response in *H. influenzae* [276]. At 42ºC, the authors observed filamentous growth and no binding of benzylpenicillin to PBP3, whereas binding to PBP1A was increased.

SOS boxes are located in the promoter regions of the genes they control [461,494,542]. All known SOS boxes in *E. coli* contain 5' CTGT alternating with $(AT)_4$ [461]. The consensus sequence TACTGTATATATATACAGTA, alternatively expressed as $TACTG(N)_{10}CAGTA$ or $TACTG(TA)_5CAGTA$, contains oligonucleotide repeats (TA) similar to the 'contingency loci' associated with phase variation in *H. influenzae* (chapter 4.3) [28]. Interestingly, induction of the SOS response destabilizes dinucleotide repeats in *E. coli*, with greatly increases replication slippage rates [312].

Based on these observations, it may be hypothesized that phase variation affecting SOS boxes in the *dcw* promoter region is involved in the regulation of cell division in *H. influenzae*. The correlation between the SOS response and phase variation in *H. influenzae* was explored by Sweetman *et al.* [494]. By searching for the sequence $CTG(N)_{10}CAG$, based on the consensus sequence for LexA-binding SOS boxes in *E. coli*, the authors identified 25 genes with SOS boxes within 200 basepairs of the start

codon in *H. influenzae* [494]. No SOS boxes were identified in the promoter regions of *sulA* or the *dcw* cluster; however, the structure of *dcw*-associated SOS boxes in *E. coli* (CTG$(N)_{11}$GAG) differs slightly from the consensus sequence (CTG$(N)_{10}$GAG) [199,542]. Some of the SOS boxes contained repeats, but RecA-dependent induction of the SOS response did not affect the frequency of phase variation at these loci. This does not, however, rule out the possibility of phase variation by RecA-independent mechanisms (e.g. slipped-strain mispairment) (chapter 4.3) [28].

Global stress responses other than the SOS response may also be involved in regulation of cell division. The stringent response is activated by starvation and other stress signals and allows the bacteria to adapt to changes in nutrient availability. The response is mediated by the stringent factors (also denoted 'alarmones') guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) synthesized by Rel/Spo homologues and small alarmone synthetase proteins [42]. These molecules downregulate transcription of genes involved in cell growth and division and thus interfere with the effect mechanism of beta-lactams. The *H. influenzae* genome encodes the genetic apparatus involved in the stringent response [131] and exposure to transcription inhibitors has been demonstrated to cause decreased concentrations of ppGpp in *H. influenzae* [117].

6 BETA-LACTAM RESISTANCE

6.1 AMPICILLIN THERAPY FAILURE: EARLY REPORTS

Beta-lactam resistance in *H. influenzae* has been reviewed repeatedly [164,333,383,410,470,516,537,558,559] since the first report of ampicillin therapy failure in *H. influenzae* meningitis in the New England Journal of Medicine in 1968 [64]. The authors concluded that the reason for failure was change from i.v. to oral therapy after eight days. Similar reports followed during 1969 [76,163,440] but therapy failure was in every case attributed to factors other than resistance to ampicillin [171,575]. In 1972, Shackelford *et al.* presented ten cases of *H. influenzae* meningitis initially treated with ampicillin, with slow bacteriological response and bacteriological relapse [455]. Two patients received high-dose (200-300 mg/kg/day) ampicillin i.v. for ten days but still experienced bacteriological relapse; notably, isolates from CSF in both patients had ampicillin MIC within the wild type range (0.37 mg/L and 0.97 mg/L). Although ampicillin resistance was not documented in that study, the authors concluded that therapy failure could be ascribed to dosage or route of administration in only three of the ten patients.

6.2 BETA-LACTAMASE-MEDIATED RESISTANCE

6.2.1 *Emergence and spread*

In 1974, resistance to ampicillin was confirmed by *in vitro* methods for the first time in *H. influenzae*. Nelson reported 17 ampicillin-resistant *H. influenzae* (mainly Hib) during 1972-1974; the first had ampicillin MIC = 20 mg/L and produced beta-lactamase [334]. In two separate publications, Thornsberry and Kirven reported increased ampicillin MIC (≥8 mg/L) in eleven isolates [509] and the presence of beta-lactamase (detected by an acidimetric test) in 18 of 20 isolates with ampicillin MIC ≥8 mg/L [508]. Beta-lactamase positive strains spread rapidly. Khan *et al.* reported 10% prevalence of ampicillin resistant Hib in Washington in 1974, confirmed beta-

lactamase production by a iodometric test in four isolates and showed that the enzyme had little or no effect on first-generation cephalosporins [224]. In a study with 1841 isolates (4.3% encapsulated) from 25 British laboratories in 1981, 106 (5.8%) beta-lactamase positive isolates were identified [375]. Four of 109 isolates (3.6%) produced beta-lactamase in a study from Northern Norway in 1982 [291]. The prevalence dramatically increased in many countries during the 1980s [164,559] but there were considerable geographical variations. In a study encompassing nine European countries in 1986 (n=1961), the mean proportion of beta-lactamase producers was 10%, with highest and lowest proportions in Spain (30.6%) and West Germany (1.6%) [269]. A study from the U.S. the same year showed that 31.7% of Hib (n=757) and 15.6% of non-b isolates (n=2054) were beta-lactamase positive [94].

The dissemination of beta-lactamase positive ampicillin resistant strains (often abbreviated BLPAR) prompted discussions on the safety of ampicillin for therapy in meningitis and other serious *H. influenzae* infections [334,455]. Therapeutic options in meningitis were largely restricted to penicillins and/or chloramphenicol until third-generation cephalosporins combining high antibacterial activity against *H. influenzae* [447] with penetration to CSF [26] were introduced in 1979 (cefotaxime) [334].

6.2.2 *TEM and ROB beta-lactamases*

Beta-lactamases (*bla*) are enzymes with the ability to inactivate beta-lactams [53]. Several schemes are used for classification; e.g. Ambler (based on amino acid sequence homology) and Bush-Jacoby-Medeiros (based on substrate inhibitor profile). Ambler class A, C and D are denoted serine beta-lactamases because of their active site serine; these enzymes attack and acylate beta-lactams (similar to PBPs) and use strategically positioned water molecules to hydrolyze the beta-lactam bond [95]. In 1975, Sykes *et al.* performed immunoisoelectric focusing and transferability studies for *bla* positive isolates of *H. influenzae* from the U.K. (n=6) and the U.S. (n=9) and showed that the enzyme in all cases was identical to the TEM enzyme (bla_{TEM}) in *E. coli* [497], which belongs to Ambler class A and Bush-Jacoby-Medeiros group 2b

[53]. Sykes *et al.* were also able to demonstrate inter-species transferability of $bla_{TEM,}$ suggesting that the gene was acquired from *E. coli* [497].

In a global prevalence study (PROTEKT) encompassing 14870 isolates (1999-2003), bla_{TEM} constituted approximately 95% of *bla* positive *H. influenzae* [123]. The bla_{TEM} gene is carried on transposons (Tn*A*), of which Tn*1*, Tn*2* or Tn*3* are most common [18]. In *H. influenzae*, Tn*3* is usually located at integrative conjugative elements (ICE) [212]. Less frequently, bla_{TEM} is carried by small (4.8-5.5 kB) non-conjugative plasmids associated with remnants of Tn*2* [523]; three distinct plasmids were identified and characterized in 98 Danish bla_{TEM} positive isolates [477]. In a Swedish study on respiratory (2009-2011; n=2845) and invasive (1997-2010; n=310) *H. influenzae*, small plasmids accounted for 15.8% of *bla* positive isolates [134]. Most bla_{TEM} positive *H. influenzae* possess bla_{TEM-1}, but a variant denoted bla_{TEM-2} characterized by the A4046C substitution accounted for 12.3% (8/65) of bla_{TEM} positive isolates in a Spanish study [311].

Another Ambler class A, Bush-Jacoby-Medeiros group 2b enzyme, ROB-1 (bla_{ROB}) is carried by most bla_{TEM}-negative *bla*-positive *H. influenzae* [519]. The first reported strain ('Hib-Rob') with bla_{ROB} was isolated from a U.S. child (blood and CSF) in 1981 [424]. Isolates with bla_{ROB} are considered rare outside North America [123], but a prevalence of 3.9% (with bla_{ROB} accounting for 9.7% of *bla*-positive *H. influenzae*) was reported in a Spanish study [311]. The enzyme is encoded by 4–5 kb plasmids related to plasmid pB1000 [434] and an animal source has been suggested [290]. The relatively lower frequency of bla_{ROB} compared to bla_{TEM} has been ascribed to higher fitness cost [434] and the easier transferability of ICEs [519]. Concomitant presence of bla_{TEM} and bla_{ROB} occur [519], and bla_{ROB} has been reported in strains with PBP3-mediated resistance [435].

Both bla_{TEM} and bla_{ROB} mediate high-level resistance to penicillins and are inhibited by clavulanic acid, sulbactam and tazobactam [53,95,143]. The ability of the enzymes to inactivate cefaclor (second-generation cephalosporin) and loracarbef (carbacephem) may vary: Molina *et al.* reported that 46% (30/65) of bla_{TEM} positive isolates from Spain had a 136 bp deletion upstream from the coding region (possibly affecting the

promoter region) and significantly higher MICs for cefaclor and loracarbef [311]. It should be noted that resistance mechanisms other than *bla* were not investigated. The deletion was also present in 18 of 29 invasive bla_{TEM} positive *H. influenzae* from Sweden [414].

Farrell *et al.* reported higher cefaclor MIC levels in bla_{ROB} isolates (n=102; MIC_{50}, 4 mg/L; MIC_{90}, 16 mg/L) compared to bla_{TEM} isolates (n=2885; MIC_{50}, 4 mg/L; MIC_{90}, 16 mg/L); none of the enzymes were active against cefuroxime or cefotaxime [123].

Increased prevalence of *bla* positive *H. influenzae* has been linked to antibiotic usage. Selection of bla_{ROB} isolates due to high consumption of cefaclor has been suggested to contribute to the high prevalence of this gene in the U.S. and Mexico [143,516]. Barbosa-Cesnik *et al.* studied predictors for NTHi among children in day-care centra in the U.S. and found that colonization with *bla* positive strains was significantly associated with previous antibiotic treatment (p=0.02) [24]. In a prospective cohort study, Chung *et al.* assessed the effect of beta-lactams on the presence of ICE*Hin1056* in *Haemophilus* isolates from children and found that amoxicillin increased carriage with ICE*Hin1056* positive strains from 35% to 83% [68].

It has been suggested that susceptible strains may be protected from penicillins by *bla* produced by other strains in mixed infections. Schaar *et al.* demonstrated that outer membrane vesicles (OMV) from NTHi contain functional *bla* and were able to protect group A-streptococci from amoxicillin *in vitro* [445]; similarly, amoxicillin susceptible NTHi were sheltered *in vitro* by *bla*-containing OMV from *Moraxella catarrhalis* [444]. However, Westman *et al.* previously found that a *bla* positive NTHi strain did not protect pneumococci from amoxicillin *in vivo* in an AOM rat model [555].

6.2.3 *Other beta-lactamases*

Extended-spectrum beta-lactamases (ESBL) and inhibitor-resistant TEM beta-lactamases (IRT) are derived from Bush-Jacoby-Medeiros group 2b enzymes and assigned to groups 2be and 2br, respectively [53]. Neither ESBL nor IRT have been confirmed in clinical isolates of *H. influenzae*, but *in vitro* studies have shown that the

resistance phenotypes of ESBL and IRT positive *H. influenzae* could easily be misinterpreted as concomitant bla_{TEM}/bla_{ROB} and PBP3-mediated resistance and such strains may have passed unnoticed.

Tristram and Burdach studied the effects of cloned IRTs on beta-lactam susceptibility in *H. influenzae* and observed increased amoxicillin-clavulanic acid MICs in Rd KW20 (up to 4 mg/L) and a strain with PBP3-mediated resistance (up to 8 mg/L), whereas cefotaxime MICs remained low (0.008-0.016 mg/L) [518]. In two investigations on the phenotypes of recombinant ESBL *H. influenzae* (TEM-3, TEM-4 and TEM-5), cefotaxime MIC was higher in recombinants with both ESBL and altered PBP3 (up to 8 mg/L) compared to recombinants with ESBL alone (up to 1 mg/L) [43,517]. Galan *et al.* explored the possibility of bla_{ROB} derived ESBL and obtained cefotaxime-resistant and *bla*-inhibitor-resistant mutants by transforming a hyper-mutagenic *E. coli* strain with a bla_{ROB} plasmid from *H. influenzae* and exposing the transformant to beta-lactams [143]. The resistance phenotypes were associated with distinct amino acid substitutions in bla_{ROB}.

Two *H. parainfluenzae* with TEM-15 ESBL and cefotaxime MICs of >16 mg/L were observed in South Africa in 2002; unrelated PFGE profiles suggested *in vivo* transfer [521]. Both isolates had altered PBP3. In a study on nasopharyngeal Hib (n=80) from children in Delhi, India (2005-2007), Saikia *et al.* reported high prevalence of resistance to extended spectrum cephalosporins (cefotaxime, 36%; cefepime, 45%), and that five (6.25%) bla_{TEM} isolates were ESBL-positive by double-disk diffusion according to CLSI guidelines [431]. As the agents used for double-disk diffusion were not specified, and the enzyme was not further characterized by molecular methods, this rather sensational report should be interpreted with caution.

6.2.4 *Beta-lactamase detection*

Several phenotypic tests may be used for detection of beta-lactamase activity in *H. influenzae*. These include colometric tests using the chromogenic cephalosporin nitrocefin (colour change from yellow to red on hydrolysis); iodometric tests based on iodine reduction by penicilloic acid (from hydrolysis of penicillin); and acidimetric

tests detecting acidification as a result of hydrolysis of the beta-lactam ring [260,264]. According to the latest review article on antimicrobial resistance in *H. influenzae* [516], conflicting information regarding the reliability of nitrocefin-based tests for detection of bla_{ROB} appears to be related to early works and difficulties related to the sensitivity of chromogenic cephalosporins has not been reported in more recent studies. Notably, Molina *et al.* reported that 24.6% (16/65) of bla_{TEM} PCR positive *H. influenzae* were negative by nitrocefin test [311]. The cloverleaf test, described by Ørstavik and Ødegaard in 1971 [359], is a reliable test for *bla* detection in *H. influenzae* [260,264]. The test principle is that *bla*, if produced by the test strain, allows a susceptible indicator strain to grow near a penicillin disk. The test is also denoted Hodge test, referring to a later publication by Hodge *et al.* [188].

A number of molecular tests have been designed for specific detection of bla_{TEM} and bla_{ROB} in *H. influenzae* [93,123,180,311,330,448,520,523]. Isolates positive by phenotypic tests and negative by bla_{TEM} and bla_{ROB} PCR have been reported [93,123,311,414,454,516]. Characterization of one such isolate [454] revealed the presence of a bla_{TEM} gene with a 27 bp deletion, interfering with PCR tests targeting regions outside the open reading frame (ORF); the authors concluded that PCR tests for detection of bla_{TEM} preferably should target regions within the ORF to avoid false negative results [523]. Reports of beta-lactamase-positive isolates negative by bla_{ROB} PCR and bla_{TEM} PCR with primers within the ORF [93,123] suggest the presence of hitherto uncharacterized beta-lactamases [523].

6.3 PENICILLIN-BINDING PROTEIN 3-MEDIATED RESISTANCE

6.3.1 *Emergence and spread*

Two *bla*-negative non-b *H. influenzae* with increased ampicillin MIC (8 mg/L) were reported by Thornsberry and Kirven in 1974 [508]. Beta-lactamase-negative ampicillin-resistant isolates (traditionally abbreviated BLNAR) attracted little attention compared to *bla*-positive isolates and were for decades considered rare, although they in fact accounted for a significant proportion of isolates with resistance to ampicillin.

Seven *bla* negative isolates (7%) with increased ampicillin MIC (1-8 mg/L) and six *bla* positive isolates were identified among 100 consecutive respiratory isolates from Australia in 1979 [29]. In 1981, 21 *bla* negative isolates (1.1%) with ampicillin MIC >1 mg/L and 106 *bla* positive isolates were identified among 1841 *H. influenzae* from the U.K. [375]. Of five ampicillin-resistant isolates in a collection of 109 *H. influenzae* from Northern Norway in 1981, one (0.9%) was *bla* negative [291]. In a study with *H. influenzae* (n=1961) from nine European countries in 1986, the authors reported that 'some isolates, especially from Spain, Belgium and the U.K.' were *bla* negative and resistant to ampicillin [269]. Finally, 71 of 2811 isolates (2.5%) in a U.S. surveillance study from 1986 were *bla* negative and had ampicillin MIC >1 mg/L [94]. In an early review on antimicrobial resistance in *H. influenzae*, Smith estimated that 5% of ampicillin resistant strains were *bla* negative [470].

It soon became evident that non-*bla*-mediated beta-lactam resistance was not restricted to aminopenicillins. *H. influenzae* with cefotaxime MIC up to 3.1 mg/L (six dilutions above ECOFF) were reported in 1979, before the drug was commercially available [335]. In 1983, Philpott and Williams investigated the activity of cephalosporins against *H. influenzae* from the U.K. and found higher MIC_{50} for cefuroxime (+3 dilutions) and cefotaxime (+2 dilutions) in *bla* negative isolates with amoxicillin MIC >0.5 mg/L (n=18) compared to isolates with lower MIC [376]. One isolate in the latter group and four *bla* positive isolates were cefotaxime-resistant by current EUCAST criteria [111]. In a 1983 review article, Smith reported four Hib with 1000-fold increased MICs to cefamandole and strains with resistance to third-generation cephalosporins [470]; the author hypothesized that the mechanism was altered PBPs and/or decreased permeability and predicted that resistance to third-generation cephalosporins would occur 'in a few years' as a result of increased use. As predicted, *H. influenzae* with non-*bla*-mediated beta-lactam resistance increased particularly rapidly in Japan, where the consumption of oral cephalosporins is high [180,532]. First reported in 1983, the proportion of respiratory isolates with non-*bla*-mediated beta-lactam resistance reached 10% in 1990 and exceeded 50% in 2002 [531].

6.3.2 *Clinical relevance*

The clinical relevance of non-*bla*-mediated beta-lactam resistance in *H. influenzae* has been a matter of debate for decades. In 1977, Markowitz isolated a *bla* negative ampicillin resistant Hib (MIC = 6.25 mg/L) from blood and CSF in a patient with ampicillin-treated endocarditis and meningitis [279]. Mendelman *et al.* later reported therapy failure with cefuroxime in a child with meningitis caused by a strain with PBP-mediated resistance (cefuroxime MIC = 4 mg/L) [299]. In 1986, Mendelman *et al.* stated that the correlation between *in vitro* and clinical resistance remained to be investigated [297]. Similarly, the authors of a 1995 review article concluded that 'the role in therapeutic failure of non-*bla*-mediated beta-lactam resistance has not been clearly established' [164]. The clinical relevance of non-*bla*-mediated beta-lactam resistance is acknowledged in more recent reviews [154,410,516,537] and is also supported by animal studies. In an AOM rat model, Melhus *et al.* observed delayed therapeutic effect of amoxicillin in animals infected with a *bla* negative NTHi transformant with increased amoxicillin MIC (2 mg/L) compared to the susceptible recipient strain (MIC = 0.5 mg/L) [292]. However, there is a lack of clinical data on the correlation between non-*bla*-mediated beta-lactam resistance and outcome [110,139,516,537]. The clinical relevance of current breakpoints for aminopenicillins is debated [537] and the criteria for clinical susceptibility categorization of *H. influenzae* against beta-lactams differ between authorities (chapter 8.1).

6.3.3 *Characterization of the resistance mechanism*

Several investigations during the 1980s and 1990s explored the role of target alteration in non-*bla*-mediated beta-lactam resistance in *H. influenzae*. Most early studies were performed by research groups from Canada [71,276-278,366] and the U.S. [298-300,300,301,303,451]. In 1984, the Canadian group used a previously described method [541] to transform broad-spectrum beta-lactam resistance from a *bla* negative isolate with increased ampicillin MIC (1.56 mg/L) into a susceptible strain [366]. Comparison of PBP profiles of the transformant and the parent strain showed that resistance correlated with the acquisition of low-affinity PBP 3A and 3B. Further

analyses using recombinant DNA techniques revealed that resistance was due to two distinct chromosomal mutations [275].

Similarly, the U.S. group used DNA from four *bla* negative ampicillin-resistant isolates (MIC 8-16 mg/L) from New Zealand and managed to transform ampicillin resistance into a susceptible strain in three cases and concluded that altered PBP was the primary resistance mechanism in these isolates [301]. The Canadian group later transformed the Rd strain and found a correlation between decreased affinity of PBP 3A and 3B, beta-lactam resistance levels and the degree of filamentation [71]. This observation indicated that PBP3 alterations resulted in septal dysfunction, consistent with the role of PBP3 in the synthesis of septal peptidoglycans in *E. coli* [481].

The major breakthrough came as a result of novel molecular technologies and the sequencing of the complete genome of *H. influenzae* Rd KW20 in 1995 [131]. A research group led by Kimiko Ubukata, connected to the Kitasato Institute for Life Sciences in Tokyo (chapter 1), showed that PBP3 is encoded by the *ftsI* gene in *H. influenzae* [530], as it is in *E. coli* (chapter 5.5.1).

Ubukata *et al.* sequenced the *ftsI* transpeptidase region in 22 *bla* negative *H. influenzae* with ampicillin MIC >0.5 mg/L and found PBP3 substitutions in all of them. 3D modeling indicated that substitutions near the 379-Ser-Ser-Asn (SSN) and 512-Lys-Thr-Gly (KTG) motifs surrounding the active site were associated with resistance, consistent with similar observations in penicillin-resistant pneumococci and *Neisseria* spp. [577]. The change from a neutral to a basic amino acid (Asn to Lys) in position 526 had particularly large impact on the structure of the active site pocket, and on the susceptibility to beta-lactams [530].

The association between altered PBP3 and resistance was supported by transformation of mutant PBP3 into an isogenic background. By transformation of the Rd strain with 2.2-kB fragments containing the *ftsI* gene (1.8 kB), Ubukata *et al.* obtained transformants with low-affinity PBP 3A and 3B and increased ampicillin, cefotaxime and meropenem MICs [530]. The transformants had MICs similar to or 1-2 dilutions lower than the donors; the discrepancy was most prominent in transformants with PBP3 from high-level resistant strains (group III, see next chapter).

6.3.4 *Categorization and terminology*

Ubukata *et al.* categorized strains and transformants as group I (Arg-517 to His; R517H), group II (Asn-526 to Lys, N526K) and group III (Met-377 to Ile, M377I; Ser-385 to Thr, S385T; Leu-389 to Phe, L389F; N526K) according to PBP3 substitution patterns and resistance levels [530]. Compared to the recipient strain, MICs increased by two (ampicillin), three (cefotaxime) and two (meropenem) dilutions in group I/II transformants, and by three (ampicillin), six (cefotaxime) and three dilutions (meropenem) in group III transformants.

The original 'Ubukata system' for genotypic categorization of isolates with PBP3-mediated resistance has later been modified and supplemented. Ubukata and coworkers introduced the designation 'low-BLNAR' for *bla*-negative group I/II isolates to separate them from group III isolates, denoted 'BLNAR' [180]. The term 'BLNAR' (i.e. group III) was subsequently redefined as *bla*-negative isolates with the SSN-near S385T substitution in addition to the KTG-near N526K substitution [178], and Garcia-Cobos *et al.* introduced the designation 'group III-like' for isolates with S385T + R517H [148].

This 'modified Ubukata system' (**Table 10**) is currently used by most groups for categorization of *H. influenzae* with PBP3-mediated resistance. However, some authors prefer to categorize R517H positive isolates as group I irrespective of S385T [17,83], and reserve group III for isolates possessing M377I and L389F in addition to S385T and N526K [83].

Table 10 The 'modified Ubukata system' for genotypic categorization of *H. influenzae* with penicillin-binding protein 3-mediated resistance (rPBP3). Compiled from [148,178,180,530]

Resistance level	Group	SSN motif	KTG motif	
		S385	R517	N526
Low	I		H	
	II			K
High	III-like	T	H	
	III	T		K

The traditional terminology distinguishes between *bla*-negative ('BLNAR') and *bla*-positive isolates with PBP3-mediated resistance (*bla*-positive amoxicillin-clavulanic acid-resistant; 'BLPACR') [180]. Ubukata and coworkers proposed the terms 'BLPACR I' and 'BLPACR II' for bla_{TEM} positive strains with the same substitutions as low-BLNAR and BLNAR strains, respectively [178]. Hotomi *et al.* added the prefix 'g' for strains with genetically confirmed PBP3-mediated resistance (e.g. 'gBLNAR') to separate them from strains categorized by phenotypic criteria [191].

This use of the originally phenotypic designations BLNAR, BLPACR, BLPAR (*bla* positive ampicillin resistant) and BLNAS (*bla*-negative ampicillin-susceptible) to denote resistance genotypes is problematic for several reasons. First, there is no consensus on phenotypic definitions of ampicillin and amoxicillin-clavulanic acid resistance. This has resulted in terms like 'BLNAI' [414,537] and 'almost-BLNAR' [180]. Second, discrepancy between phenotype and genotype is common and strains with gBLNAR/gBLPACR genotypes may present with BLNAS/BLPAR phenotypes. Third, 'BLPACR' would be a very precise description of the phenotype of inhibitor-resistant bla_{TEM}-positive (IRT) strains [518]. Fourth, as the two mechanisms (*bla* and altered PBP3) occur independently, otherwise genetically indistinguishable isolates may be categorized as gBLNAR and gBLPACR depending on individual acquisition or loss of *bla*. Finally, the terms give the impression that PBP3-mediated resistance affects aminopenicillins only, concealing that many strains are resistant to extended-spectrum cephalosporins. For clarity and simplicity, the designations 'rPBP3' and 'sPBP3' are used in this thesis to denote isolates with (gBLNAR/gBLPACR) and without (gBLNAS/gBLPAR) resistance-defining PBP3 substitutions.

Osaki *et al.* proposed a slightly different genotypic categorization system [360]. The authors explored the impact of PBP3 substitutions on beta-lactam susceptibility using recombinants and mutants obtained by transformation of Rd KW20 and site-directed mutagenesis. Consistent with previous reports [530], Osaki *et al.* observed that mutagenically introduced S385T and/or L389F substitutions in N526K positive strains led to a two- to four-fold additional increase in cephalosporin resistance. Importantly, L389F positive recombinants and mutants had higher cephalosporin MICs compared to strains lacking this substitution [360]. Accordingly, the authors proposed a three-

stage categorization system with six classes (I-VI), based on the four substitutions S385T, L389F, R517H and N526K. The principal difference compared to the modified Ubukata system is that the 'Osaki system' distinguishes between group III and group III-like isolates with (+) and without (-) the L389F substitution [360]. The two systems are combined to form the 'modified Ubukata-Osaki system' in **Figure 11**.

A large number of PBP3 substitutions occur in addition to S385T, L389F, R517H and N526K and might contribute to resistance [516]. Straker *et al.* used protein modeling to demonstrate that an S357N substitution alters the tertiary structure of PBP3 in a way that denies certain beta-lactams (e.g. cefuroxime) access to the active site [487]. Dabernat *et al.* suggested subgrouping of group II low-rPBP3 based on the presence of N526K (IIa), A502V (IIb), A502T (IIc) and I449V (IId) [84]. The system is widely used despite no convincing correlation with phenotypic resistance levels. Garcia-Cobos *et al.* reported minor differences between the subgroups, with lower MIC_{50} values in subgroup IId compared to IIa-c [148]. Bengtsson *et al.* observed lower MICs in subgroups IIc and IId compared to IIa and IIb [30], whereas no differences were found in two studies from Korea [17,364].

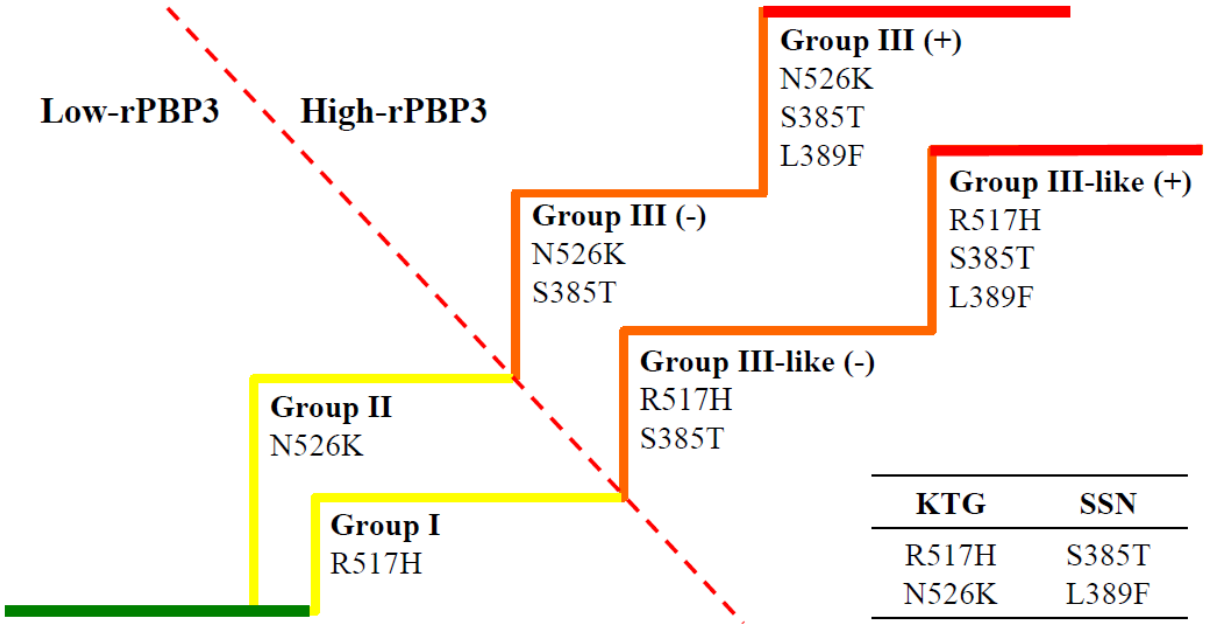


Figure 11 The 'modified Ubukata-Osaki system' for genotypic categorization of *H. influenzae* with penicillin-binding protein 3-mediated resistance (rPBP3) according to the presence of PBP3 amino acid substitutions in four positions. Compiled from [148,178,180,360,530]

6.3.5 *Resistance genotypes and phenotypic susceptibility profiles*

Tristram *et al.* presented an overview of the correlation between genotypes and phenotypic resistance (data from [179,436]) in the latest review on antimicrobial resistance in *H. influenzae* [516]. According to this, the difference between median MICs in low- and high-rPBP3 *H. influenzae* is less marked for ampicillin (1 mg/L versus 2 mg/L in *bla*-negative isolates) than for cefotaxime (0.063 mg/L versus 0.5 mg/L), whereas meropenem susceptibility is similarly affected in low-rPBP3 and high-rPBP3 isolates (median MICs of 0.125 mg/L in both groups) [516].

Table 11 shows beta-lactam susceptibility profiles in clinical isolates according to the six defined rPBP3 genotypes (**Figure 11**). For aminopenicillins and cephalosporins, resistance correlates well to genotypes, and group III(+) isolates have the highest resistance levels. Practically all low-rPBP3 are susceptible to cefotaxime while most high-rPBP3 are cefotaxime-resistant according to EUCAST breakpoints [111], supporting the relevance of the categorization system.

Notably, ceftriaxone activity is less affected by PBP3 alterations compared to cefotaxime [178,530], and the activity of piperacillin is less affected than the activity of other penicillins and cephalosporins [187,436]. Consistent with the latter observation, Morikawa *et al.* reported that piperacillin was more effective than third-generation cephalosporins against rPBP3 *H. influenzae* in a time-kill study; the authors suggested that the low impact of PBP3 alterations on piperacillin susceptibility was due to its high affinity for PBP2 [313].

Early studies by Powell *et al.* showed that non-*bla*-mediated mechanisms in *H. influenzae*, conferring resistance to ampicillin, cephalosporins and aztreonam, had little or no effect on carbapenem susceptibility, and there was poor correlation between increased MICs for imipenem and meropenem [384,385]. Meropenem MIC is only slightly increased in rPBP3 isolates, and varies little between genotypes [178,231]. While Ubukata *et al.* obtained 2-3 dilutions increase in meropenem MIC by transformation of the Rd strain with *ftsI* from rPBP3 strains [530], Osaki *et al.* did not manage to transfer reduced susceptibility to meropenem by *ftsI* recombination or site-directed mutagenesis [360].

Table 11 Beta-lactam MICs for clinical isolates of *H. influenzae* according to rPBP3 genotype (genotyping based on *ftsI* sequencing)

Agents	ECOFF[a]	Genotypes[b] and MICs (mg/L)[c]						References
		I	II	III-like		III		
				(-)	(+)	(-)	(+)	
Ampicillin[d]	1	0.063-0.5	0.5-4	1-2	1	1-4	0.5-16	[360]
		1-4	1-4	-	-	-	2-4	[530]
		0.5-2	0.5-8	0.5-2[e]		-	-	[148]
		-	2-4	2	-	-	4-8	[17]
		0.60	*1.29*	*1.23*	-	*2.57*	*5.62*	[436]
		-	-	*1*	*8*	*2*	*4*	[178]
		-	**1**	**4**[e]		**4**	**16**	[231]
Amoxicillin[d]	2	0.5-2	0.5-8	-	-	-	4	[84]
		0.25-2	0.25-4	0.5-2[e]		-	-	[148]
		1.12	*2.87*	*3.25*	-	*7.05*	*17.21*	[436]
		-	**4**	**4**[e]		**32**	**32**	[231]
Piperacillin[d]	0.064	*0.060*	*0.050*	*0.065*	-	*0.095*	*0.084*	[436]
Cefuroxime	2	1-2	0.25-8	-	-	-	-	[84]
		0.5-2	0.5-≥16	2-≥16[e]		-	-	[148]
		-	2-16	8-16	-	-	4-32	[17]
Cefixime	0.125	0.06-0.12	≤0.001-0.25	0.5-4[e]		-	-	[148]
Ceftriaxone	0.064	0.007	0.003-0.06	-	-	-	-	[84]
		0.008-0.031	0.008-0.063	-	-	-	0.25	[530]
		-	-	*0.063*	*0.25*	*0.063*	*0.125*	[178]

Table 11 (continued)

Agents	ECOFF[a]	Genotypes[b] and MICs (mg/L)[c]						References
		I	II	III-like		III		
				(-)	(+)	(-)	(+)	
Cefotaxime	0.064	0.03-0.12	0.007-0.12	-	-	-	0.5	[84]
		0.031	0.031-0.12	0.063-0.5	0.5	0.12-0.5	0.5-1	[360]
		0.063-0.25	0.063-0.125	-	-	-	1-2	[530]
		-	0.063-0.2	0.25	-	-	1-2	[17]
		≤0.001-0.12	≤0.001-0.5	0.25-4	-	-	-	[148]
		-	-	*0.25*	*1*	*0.25*	*0.5*	[178]
		-	**0.063**	**1**	**1**	**1**	**1**	[231]
Imipenem	2	0.063-0.5	0.25-8	0.12-0.5	0.12	2-8	1-32	[360]
		0.43	*1.33*	*0.76*	-	*3.76*	*2.37*	[436]
Meropenem	0.25	0.016-0.063	0.063-0.25	0.031-0.12	0.031	0.12-0.25	0.031-1	[360]
		0.031-0.25	0.063-0.5	-	-	-	0.125-0.5	[530]
		-	-	*0.125*	*0.125*	*0.25*	*0.25*	[178]
		-	**0.5**	**0.25**	**0.25**	**0.5**	**0.5**	[231]

[a] Epidemiological cut-off MIC values (mg/L) (www.eucast.org/mic_distributions/)

[b] Categorization according to the modified Ubukata-Osaki system (**Figure 11**)

[c] Plain text, MIC ranges; italic, geometric mean MIC or MIC_{50}; bold, MIC_{90}

[d] *Bla* negative isolates or tested in combination with a *bla* inhibitor

[e] Combined data for group III-like (-) and group III-like (+) isolates

The exact mechanism behind increased meropenem resistance needs to be elucidated. As meropenem and piperacillin bind strongly to both PBP2 and PBP3 (**Figure 10**), there is a possibility that resistance to these agents requires alterations in both proteins.

In contrast to meropenem, imipenem resistance does appear to correlate with rPBP3 genotypes [360,436]. The association was supported by an experiment performed by Cerquetti *et al.*, in which resistance to imipenem (MIC 4-8 mg/L) was transferred to the Rd strain by transformation with PCR-amplified full-length *ftsI* (including upstream and downstream regions) from a clinical isolate with imipenem MIC ≥32 mg/L [62]. These observations contradict previous observations by Powell *et al.* [384] and are difficult to reconcile with the low affinity of imipenem for PBP3 (**Figure 10**).

As PBP2 is the primary target for imipenem (**Table 8**), PBP2 alterations may theoretically contribute to reduced susceptibility to imipenem; it could also be hypothesized that PBP2-mediated resistance ('rPBP2') would affect imipenem more than meropenem because of the higher affinity of the latter for PBP3 [217]. This intriguing possibility of an additional PBP-mediated resistance mechanism in *H. influenzae* is barely investigated (chapter 6.4.1).

6.3.6 *Geographic distribution of rPBP3 genotypes*

Table 12 summarizes the distribution of PBP3 resistance genotypes in clinical isolates. Group II low-rPBP3 isolates predominate in Western Europe, North America and Australia, whereas high-rPBP3 isolates are prevalent in Japan and Korea. Data based on *ftsI* sequencing of isolates from other regions are not available.

Respiratory low-rPBP3 *H. influenzae* increased gradually in Japan during the 1980s and 1990s [531]. A genotype shift occurred when the prevalence of low-rPBP3 isolates approached 20%, and the prevalence of high-rPBP3 isolates increased from zero to 30% between 1996 and 2002. This shift was also evident in CSF isolates: the first high-rPBP3 meningitis case was reported in 2000 and the proportion of high-rPBP3 isolates in CSF exceeded 50% only seven years later [532]. A similar development was observed in respiratory isolates from Korea 2005-2010 [17,230,364].

Table 12 Geographic distribution of rPBP3 genotypes. Only publications with genotyping based on *ftsI* sequencing are included

Country	Period	Source[a]	*ftsI*[b] (n)	rPBP3[c] (n)	Genotypes[c] (n)								References
					NS	Mis	I	II	III-like (-)	III-like (+)	III (-)	III (+)	
Japan	2005-2008	MEF	114[d]	111	0	0	0	13	3	9	6	83	[231]
	2002-2003	Mixed	156	140[e]	49	0	0	0	0	0	0	91	[492]
	1995-2003	Respiratory	621	277	0	8	65	108	15	4	13	64	[436]
	1999-2002	CSF	62[d]	62	0	0	0	0	10	2	8	42	[178]
	1998-2000	No data	279	151	115	0	5	12	3	1	4	11	[360]
	1997-2000	Respiratory	9	9	0	0	0	4	0	0	1	4	[281]
	1995-2000	Respiratory	25	20	0	0	0	7	0	0	0	13	[240]
	1998	Respiratory	30	25	0	0	7	12	2	0	0	4	[530]
Korea	2010	Respiratory	123	78[e]	0	0	1	48	3	0	0	26	[364]
	2005-2006	Respiratory	61	59	0	0	0	48	5	0	2	4	[17]
	2000-2005	Respiratory	175	86	0	1	4	81	0	0	0	0	[230]
Australia	No data	No data	18[d]	7	0	0	1	1	0	0	1	4	[562]
	No data	No data	43	36[e]	0	0	3	32	1	0	0	0	[566]
Spain	2008-2013	Invasive	82	26	0	0	4	22	0	0	0	0	[395]
	2004-2009	Invasive	162	68[e]	0	0	2	64	2	0	0	0	[146]
	2000-2009	Respiratory	95	29	0	0	2	27	0	0	0	0	[394]
	2005-2007	No data	196	159	0	1	1	145	12	0	0	0	[454]
	2001-2006	Mixed	354	220	0	0	10	198	10	2	0	0	[148]
Portugal	2002-2010	Invasive	12	11	0	0	1	10	0	0	0	0	[19]
	2001-2008	Mixed	240	141[e]	0	0	3	136	2	0	0	0	[22]
Italy	2007-2009	Invasive	78	3	0	0	0	3	0	0	0	0	[158]
	2004-2009	Invasive	1	1	0	0	0	1	0	0	0	0	[435]
	2004-2006	Invasive	6	2	0	0	0	2	0	0	0	0	[62]
	1997-2006	CSF	9	1	0	0	0	1	0	0	0	0	[56]

Table 12 (continued)

Country	Period	Source[a]	*ftsI*[b] (n)	rPBP3[c] (n)	Genotypes[c] (n)								References
					NS	Mis	I	II	III-like (-)	III-like (+)	III (-)	III (+)	
France	2001-2008	Mixed	241	241	0	0	11	225	4	0	0	1	[83]
	1991-2000	Mixed	117	108	0	0	7	101	0	0	0	0	[84]
Germany	2009-2012	Invasive	157	36	0	0	0	34	0	2	0	0	[247]
Switzerland	2009-2014	Mixed	32	30	0	0	0	28	1	1	0	0	[63]
Sweden	2011	Respiratory	14	14	0	0	0	14	0	0	0	0	[9]
	2006-2011	Respiratory	74	61	0	0	0	61	0	0	0	0	[30]
	1997-2010	Invasive	36	16[e]	0	0	1	15	0	0	0	0	[414]
	1991-1996	Respiratory	103	98	0	0	0	97	1	0	0	0	[30]
Denmark	2007	Mixed	147	47	0	0	2	45	0	0	0	0	[350]
U.K.	Unknown	Respiratory	17	13	0	0	1	12	0	0	0	0	[487]
Europe[f]	2006-2007[g]	Respiratory	60	54[e]	0	0	1	52	1	0	0	0	[204]
	2004-2005	Respiratory	65	50	0	0	2	48	0	0	0	0	[203]
	1997-2003	Respiratory	30	26	0	0	1	25	0	0	0	0	[135]
Canada	2008-2009	Invasive	98	23[e]	3	0	1	18	0	0	0	1	[457]
	1990-2006	Invasive	21	21	0	0	0	21	0	0	0	0	[458]
U.S.	1996-2001	No data	12	12	0	0	1	11	0	0	0	0	[214]

[a] MEF, middle ear fluid; CSF, cerebrospinal fluid

[b] Number of isolates for which *ftsI* sequencing was performed

[c] Number of isolates with rPBP3-defining substitutions according to the modified Ubukata-Osaki system (**Figure 11**) and assignment to genotypes. Mis (miscellaneous), combinations of substitutions not fitting into any of the six genotypes; NS, not specified

[d] Strain inclusion based on prior PBP3 genotyping by PCR (chapter 6.3.9)

[e] Most frequent substitution patterns specified in **Table 13**

[f] Isolates from Austria, France, Germany, Ireland, Italy, the Netherlands, Poland, Portugal, Spain, Turkey and the U.K.

[g] Includes isolates from Canada

The majority of high-rPBP3 isolates in Japan and Korea have the group III(+) genotype, expressing the highest resistance levels [169,364,532]. In contrast, very few group III isolates have been reported in systematic surveys outside Asia; these include one invasive group III(+) isolate from Canada [457] and one group III(+) AOM isolate from France [83]. Group III-like high-rPBP3 isolates occur more frequently in Europe, in particular in Spain [454].

Subgrouping of group II low-rPBP3 according to Dabernat [84] provides some additional epidemiological information in regions with predominance of group II isolates. Bengtsson *et al.* reported that subgroups IIc and IId were the most common genotypes in Sweden during 1991-1996, whereas IIa and IIb were most frequent during 2006-2011 [30]. Comparison of complete PBP3 substitution patterns offers higher resolution than Dabernat subgroups but variable lengths of the sequenced *ftsI* fragments complicate inter-investigation analyses.

The most common PBP3 substitution patterns (hereafter denoted 'PBP3 types') in representative surveillance studies from different geographical regions are presented in **Table 13**. PBP3 sequences of different lengths, with identical substitution patterns as far as comparison is possible, are in the following denoted 'compatible'. Of particular notice is that the most frequent PBP3 type in respiratory high-rPBP3 isolates from Korea in 2010 [364] was compatible with the most frequent PBP3 type in an earlier Japanese surveillance study (2002-2003) [492].

Two PBP3 types are particularly common in low-rPBP3 isolates. PBP3 type A or a compatible pattern was present in 19-50% of rPBP3 isolates in surveillance studies from Europe [22,63,204,247,394,414], Canada [457], Australia [566] and Korea [364], whereas PBP3 type B accounted for >10% of rPBP3 isolates in surveillance studies from Sweden [414], Switzerland [63], Canada [457], Australia [566] and Korea [364]. Notably, the apparent absence of PBP3 types A and B in a French investigation [83] may be due to a typographic error: the two most frequent patterns (aa 350-532) in that study corresponded to PBP3 types A and B minus the M377I substitution, and figured twice in the results table.

Table 13 Most frequent PBP3 substitution patterns (present in >10% of rPBP3 isolates) in selected surveillance studies from different regions

Country	Period	Source	rPBP3 (n)	Genotype[a]	Substitutions[b]												PBP3 type[c]	n (%)[d]	References
					D350	S357	M377	S385	L389	I449	G490	A502	N526	A530	V547	V562	N569		
Sweden	2006-2011	Invasive	16	IIb	N		I					V	K		I		S	**A**	8 (50)	[414]
				IId						V			K		I		S	**B**	2 (11)	
				IIb	N		I				E	V	K		I		S	-	2 (11)	
Portugal	2001-2008	Mixed	141	IIb	N		I					V	K		I		S	**A**	44 (31)	[22]
				IIb	N		I				E	V	K		I		S	-	14 (10)	
Spain	2004-2009	Invasive	68	IIb	N		I					V	K					**(A)**	13 (19)	[146]
				IIc	N							T	K					-	12 (18)	
				IIc								T	K					(E)	11 (16)	
Europe[e]/ Canada	2008-2009	Respiratory	54	IIb	N		I					V	K					**(A)**	11 (20)	[204]
				IIb							E	V	K					(Q)	6 (11)	
Canada	2008-2009	Invasive	20	IIb	N		I					V	K					-	5 (25)	[457]
				IIb	N		I					V	K		I			**(A)**	4 (20)	
				IId						V			K		I			**(B)**	3 (15)	
Australia	No data	Respiratory	36	IIb	N		I					V	K		I			**(A)**	8 (22)	[566]
				IId						V			K		I			**(B)**	4 (11)	
				IIa	N						E		K	S				(D)	4 (11)	
				IIc	N							T	K		I			-	4 (11)	
Korea	2010	Respiratory	78	IIb	N		I					V	K		I		S	**A**	26 (33)	[364]
				III(+)	N	N	I	T	F				K		I	L	S	2	15 (19)	
				IId						V			K		I		S	**B**	9 (12)	
Japan	2002-2003	Respiratory	140	III(+)	N	N	I	T	F				K					(2)	91 (65)	[492]

[a] Categorization according to the modified Ubukata-Osaki system (**Figure 11**). Subgrouping of group II isolates according to Dabernat *et al.* [84]
[b] Shaded area, not sequenced
[c] Letters (low-rPBP3) and numbers (high-rPBP3) correspond to the PBP3 type assignments used in **Table 26**. Brackets, incomplete patterns compatible with a distinct PBP3 type. '-', not assigned to a PBP3 type. Colours, identical or compatible patterns. Bold, frequent at ≥3 continents
[d] Numbers and proportions (of all rPBP3 isolates) with each pattern
[e] Isolates from Austria, France, Germany, Ireland, Italy, the Netherlands, Poland, Portugal, Spain, Turkey and the U.K.

As a distinct PBP3 type may be encoded by different *ftsI* sequences, DNA sequence analysis is necessary to determine whether identical PBP3 types in separate isolates are encoded by identical or evolutionary related alleles. Phylograms based on *ftsI* sequences for *bla*-positive and *bla*-negative rPBP3 *H. influenzae* from Spain were presented by Sevillano *et al.* [454]. The two most frequent alleles accounted for 44% of *bla*-negative (n=118) and 71% of *bla*-positive (n=41) isolates, and the predominating *ftsI* allele in *bla*-positive isolates (present in 51%) encoded a PBP3 substitution pattern compatible with PBP3 type A (**Table 13**).

Andersson *et al.* recently reported that 15 isolates of on outbreak strain in a Swedish nursing home possessed the same PBP3 type A-encoding *ftsI* sequence [9]; identical *ftsI* alleles were present in a previously reported cluster of genetically related invasive NTHi from other parts of Sweden [414].

A notable correlation between PBP3 genotypes and geographical regions at DNA level is that the lysine residue in the N526K substitution is encoded by the AAA codon in a significant proportion of rPBP3 isolates in Europe and Australia, whereas the AAG codon predominates in Japan [532,562].

6.3.7 *Molecular epidemiology and clonal spread*

In contrast to encapsulated strains, NTHi are genetically diverse (chapter 3.9). Thus, the presence of identical PBP3 types in rPBP3 NTHi from separate geographical regions (**Table 13**) and identical *ftsI* alleles in separate isolates [9,454] suggest dissemination of resistant clones and/or horizontal spread of *ftsI* gene sequences encoding resistance.

Karlowsky *et al.* observed identical PFGE band patterns in all nine ampicillin-resistant *bla* negative *H. influenzae* in a U.S. surveillance study (2000-2001), collected at two different hospitals [218]. The resistance mechanism was not characterized. Limited clonal dissemination of genetically confirmed rPBP3 NTHi strains has later been observed in surveillance studies from several countries. Hotomi *et al.* assessed clonality among 61 respiratory rPBP3 *H. influenzae* from Japan (2003) [191]. Three

clusters, each consisting of 5-6 epidemiologically unrelated isolates with identical or highly similar PFGE band patterns were identified. PCR-based genotyping showed that one cluster consisted entirely of group III high-rPBP3 isolates. Garcia-Cobos *et al.* identified 13 PFGE clusters with 2-4 isolates among 72 rPBP3 *H. influenzae* from Spain (2001-2006); all isolates within each cluster had identical PBP3 substitution patterns (aa 350-532) [148]. Three clusters consisted of group III-like isolates. Similarly, Barbosa *et al.* observed 12 PFGE clusters with 2-5 isolates among 74 rPBP3 *H. influenzae* from Portugal (2001-2008); several clusters consisted of isolates with identical PBP3 patterns (aa 350-569) [22].

Resman *et al.* reported that seven of 16 invasive rPBP3 *H. influenzae* in Sweden belonged to the same MLSA cluster and had identical PBP3 patterns (PBP3 type A, **Table 13**); the seven clonal isolates were collected during 2008-2010 from three separate geographical regions [414]. The same group recently reported an outbreak in a Swedish nursing home in 2011, affecting 15 individuals including eight residents; the outbreak clone (mainly ST14) was genetically related to the previously reported invasive cluster and carrying identical *ftsI* alleles encoding PBP3 type A [9].

Exact knowledge on the global molecular epidemiology of rPBP3 strains is limited, as most rPBP3 isolates and clones have been characterized by PFGE or other methods for epidemiological typing complicating inter-investigator comparison. In contrast to PFGE, MLST provides unambiguous, easily comparable data. Several investigators [19,56,146,361,394,395,414,457,458,492] have performed both MLST and rPBP3 genotyping, but linked MLST data and PBP3 substitution patterns for individual isolates are presented in very few publications [9,56,146,394,492].

Cardines *et al.* reported the substitution pattern of a single group II low-rPBP3 ST368 NTHi meningitis isolate from Italy (1998) [56]. Sunakawa *et al.* reported MLST allelic profiles (but not STs) for 83 respiratory group III(+) high-rPBP3 isolates from Japan with identical substitution patterns (PBP3 type 2, **Table 13**) [492]. The largest cluster consisted of seven isolates with a novel allelic profile not assigned to any known ST (ST1-DLV); five clusters consisted of four isolates each (ST34; ST107; ST411;

ST156-SLV; ST395-DLV), and three clusters encompassed three isolates each (ST57; ST855; ST3-SLV). Thus, nine STs accounted for 43% (36/83) of the isolates [492].

In two more recent publications, Garcia-Cobos *et al.* [146] and Puig *et al.* [394] reported STs and PBP3 substitution patterns (aa 350-532) for 18 invasive (2004-2009) [146] and 29 respiratory (2000-2009) [394] rPBP3 *H. influenzae* from Spain. The most common PBP3 pattern in invasive isolates was compatible with PBP3 type A (**Table 13**) [146]. Three (of 13) isolates with this pattern were characterized by MLST; two were ST367 and one had a related profile (ST1114, DLV). Five of 12 invasive isolates with the second most frequent substitution pattern (**Table 13**) were characterized by MLST; four had related allelic profiles (ST949; ST1123, SLV; ST1122, TLV). In addition, two invasive group III-like high-rPBP3 isolates had related STs (ST155; ST1118, SLV).

Respiratory isolates were more genetically diverse [394]. No PBP3 substitution pattern was present in more than two isolates of identical STs, but one ST367 isolate and one related isolate (ST14, TLV) had a substitution pattern compatible with PBP3 type A, similar to two invasive isolates [146]. The two most frequent PBP3 substitution patterns in respiratory isolates were distributed to eight isolates/seven STs and seven isolates/five STs, respectively. Both patterns were among the three most frequent in invasive isolates [146] (**Table 13**).

6.3.8 *Evolution of rPBP3*

The traditional view is that rPBP3 *H. influenzae* develops through spontaneous point mutations [83,516,577]. Consistent with this notion, Takahata *et al.* showed that *ftsI* sequences encoding the four rPBP3-defining substitutions S385T, L389F, R517H and N526K are absent in the type strains of *Haemophilus* species other than *H. influenzae* (including species transferred to genus *Aggregatibacter*) [499]. As long as no donor (with naturally occurring resistance-encoding *ftsI* sequences) has been identified, point mutations remain the most likely primary cause of these substitutions.

However, there is increasing evidence that horizontal gene transfer (HGT) in terms of transformation and homologous recombination contributes to the evolution of rPBP3 strains through spread of mutant *ftsI* genes [478,499,564,565]. Successful transformation of susceptible *H. influenzae* with *ftsI* genes from resistant strains *in vitro* [71,301,366] suggest that new rPBP3 strains may evolve *in vivo* by transfer of resistance-conferring *ftsI* sequences from a resistant to a susceptible strain if both are present in the same patient [516].

Importantly, species barriers within the *H. influenzae* Group do not restrict HGT. Witherden *et al.* showed that rPBP3-defining substitutions are frequent in *H. haemolyticus* [564] and that *ftsI* gene sequences are exchanged between *H. influenzae* and *H. haemolyticus in vivo*, resulting in mosaic patterns [565]. Søndergaard *et al.* made similar observations and found no differences between inter- and intra-species transformation frequencies [478]. The authors also reported that the entire ORF of *ftsI* was replaced in eight of 40 transformants. It should be noted that DNA was introduced in the recipients by electroporation, and the results may not be fully representative of transformation *in vivo*. It seems likely that the commensal *H. haemolyticus* is more frequently exposed for antibiotics than *H. influenzae*, and the species may function as a birthplace and reservoir for resistance genes, available for its more pathogenic relative [321,565]. This is analogous to the development of PBP-mediated beta-lactam resistance in pneumococci, gonococci and meningococci through recombination with commensal *Streptococcus* and *Neisseria* species [577].

DNA uptake *in vivo* depends on the presence of specific uptake signal sequences (USS) in the donor molecule. The USS in *H. influenzae* has been identified as the nine-bp sequence 5'-AAGTGCGGT (chapter 3.3) [165,471]. *H. influenzae* Rd KW20 and *H. haemolyticus* ATCC 33390 both possess two USS at identical positions in the *ftsI* genes [499]. One copy is located in the middle of the *ftsI* gen, between the 327-Ser-Thr-Val-Lys (STVK) and SSN motifs, another is situated 22 bp downstream of the ORF [565]. In addition, the *ftsI* sequence in *H. influenzae* Rd KW20 [131] contains four partial USS (pUSS) with the critical four-bp sequence 5'-GCGG [294]: one at the beginning of the ORF, two between the SSN and KTG motifs, and one downstream of KTG (**Figure 12**).

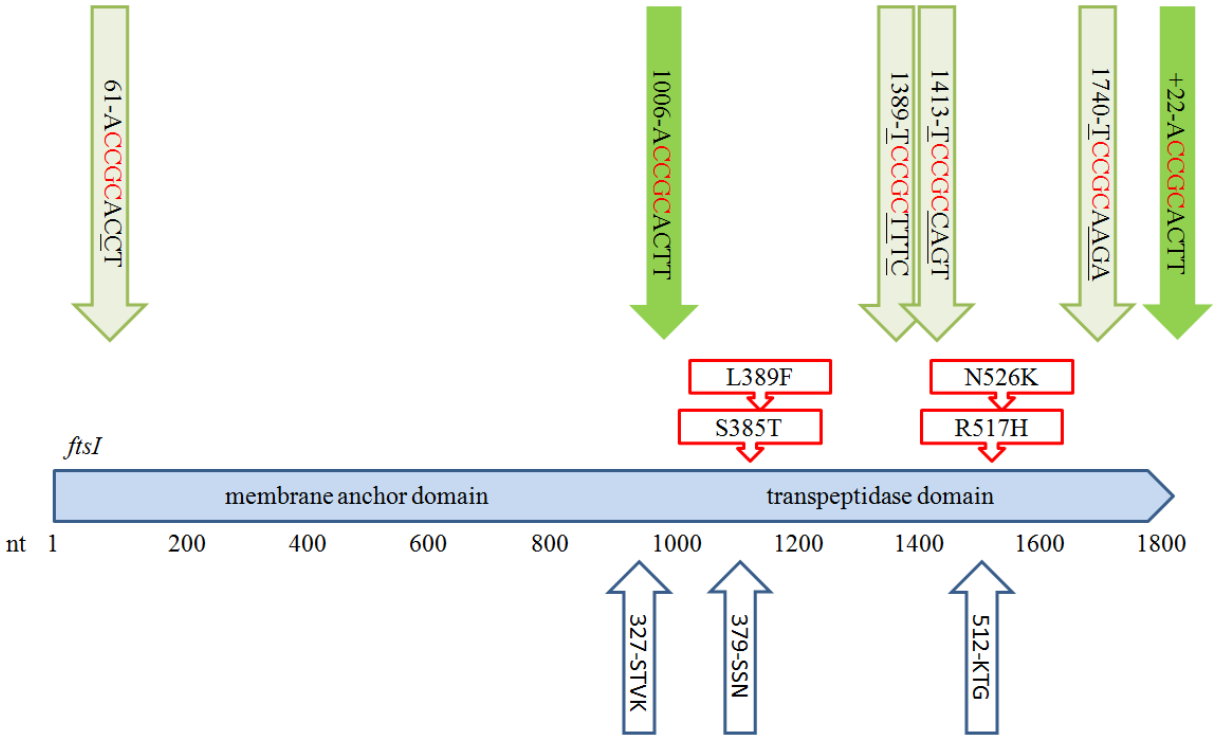


Figure 12 Complete and partial uptake signal sequences (USS and pUSS) in the *ftsI* gene of *H. influenzae* Rd KW20 [131]. Green arrows indicate locations and DNA sequences (reverse) of USS and pUSS (brighter green); the critical four-bp sequence [294] (red) and nucleotides differing from the complete USS (underscored) are shown. Red boxes, resistance-conferring substitutions; blue arrows, conserved motifs surrounding the active site pocket

The high density of HGT-facilitating USS/pUSS in the transpeptidase region, surrounding the SSN and KTG motifs, is consistent with observations that recombinational events are particularly frequent at the 3' end of the *ftsI* gene in clinical *H. influenzae* isolates [565].

As shown by Søndergaard *et al.* [478] and Witherden *et al.* [565], fragments encompassing the complete transpeptidase region, and even the entire ORF, may be transferred as the result of a single recombinational event. Considering the short distance between the S385T and L389F substitutions of the SSN motif, it seems likely that the two are transferred concomitantly if both are present in the donor molecule. The transferred sequence may include the KTG motif, with one of the rPBP3-defining substitutions R517H and N526K, or combine with the KTG motif of a R517H/N526K positive low-rPBP3 strain into a new high-rPBP3 strain.

In conclusion, rPBP3 strains of various genotypes may evolve stepwise through i) a series of point mutations, ii) a combination of recombinational events and point

mutations, or iii) sequential recombinational events, or *en bloc*, as the result of a single recombinational event.

Clinical isolates with combinations of the substitutions S385T, L389F, R517H and N526K other than the six encompassed by the rPBP3 categorization system (**Figure 11**) are extremely rare (**Table 12**), and were not obtained by in site-directed mutagenesis experiments (in which all four substitutions occurred) [360]. It seems likely that such combinations result in dysfunctional PBP3 proteins incompatible with bacterial survival. These observations would suggest that stepwise acquisition of the four substitutions follows strict rules:

1. The presence of N526K blocks the acquisition of R517H and *vice versa*
2. The acquisition of S385T requires the presence of N526K or R517H
3. The acquisition of L389F requires the presence of S385T

It could be hypothesized that first-stage substitutions (R517H and N526K) increase beta-lactam resistance, but represent a fitness cost, and that second-stage (N526K) and third-stage (L389F) substitutions are compensatory substitutions, primarily reducing fitness cost but also increasing resistance. Second-site compensatory substitutions may occur and resistance may be maintained in the absence of selective pressure caused by antibiotics [206,270,295].

Growth rate or ability to compete with other strains in laboratory media are frequently used as measures of fitness [295]. Acquisition of non-*bla*-mediated resistance did not affect the growth rate of a *H. influenzae* strain in a transformation experiment conducted by Melhus *et al.* but the PBP3 substitution pattern was not characterized [292]. To my knowledge, the impact of distinct PBP3 substitutions on fitness has not been investigated in *H. influenzae*.

6.3.9 *Molecular detection and genotyping*

A multiplex PCR methodology for detection of the rPBP3 genotype was designed by Hasegawa *et al.* [180]. Two primer sets were used: one to detect N526 (wild-type) isolates (amplification interpreted as sPBP3) and one to detect N526K + S385T

positive isolates (amplification interpreted as high-rPBP3). Non-amplification by both primer sets was interpreted as low-rPBP3. A different multiplex PCR entirely based on positive amplification was designed by Nakamura *et al.* [330]. The test included one primer set to detect N526K isolates (amplification interpreted as low-rPBP3) and one primer set to detect N526K + S385T isolates (amplification interpreted as high-rPBP3). Non-amplification by both primer sets was interpreted as sPBP3.

The tests [180,330] have important limitations. First, as they were not designed to detect the R517H substitution, both will categorize group I low-rPBP3 and group III-like high-rPBP3 as sPBP3. Second, Witherden *et al.* showed that both methods are specific for N526K substitutions encoded by the AAG codon and unable to detect N526K encoded by the AAA codon, which account for a significant proportion of rPBP3 isolates in Europe and Australia [562].

To avoid false negative results by rPBP3 PCR, Witherden *et al.* [562] suggested that the primer set designed by Hasegawa *et al.* [180] to detect wild-type isolates (N526) is used for screening and that non-amplification is followed by *ftsI* sequencing. The same group later designed new primer sets to amplify N526K in sPBP3 isolates and both variants of the N526K substitution (AAA and AAG) in rPBP3 isolates [563]. The tests demonstrated high sensitivity (100% and 84% respectively) and specificity (both tests 100%) by testing of *H. influenzae* and *H. haemolyticus*.

Notably, none of the approaches will prevent false negative results for rPBP3 strains with R517H as the first stage substitution. It should also be noted that the limitations described above significantly affect the results when rPBP3 genotyping is based on PCR alone [166,169,179,180,187,192,361,432,468] or if selection of isolates for *ftsI* sequencing is based on genotyping by PCR [178,231], in particular when older PCR assays [180,330] are used.

6.4 OTHER RESISTANCE MECHANISMS

Altered PBP3 is by far the most important non-*bla*-mediated beta-lactam resistance mechanism in *H. influenzae* but does not explain phenotypic resistance in all isolates

[516]. For instance, Fluit *et al.* reported two *bla* negative ampicillin resistant isolates (MIC = 8 mg/L) with no PBP3 substitutions [135]. In addition, Ubukata [530] and Kaczmarek [214] observed higher resistance levels in clinical rPBP3 isolates compared to transformants with identical *ftsI* genes, and Osaki *et al.* obtained higher resistance levels in transformants with *ftsI* genes from clinical sPBP3 isolates compared to mutants with similar PBP3 substitutions caused by site-directed mutagenesis [360].

These observations suggest one or more additional non-*bla*-mediated resistance mechanisms that may occur independently of PBP3 substitutions or modulate resistance in rPBP3 isolates. In this chapter, some possible non-*bla*-mediated resistance mechanisms other than rPBP3 in *H. influenzae* are presented.

6.4.1 *PBP-mediated resistance other than rPBP3*

PBP-mediated beta-lactam resistance was reviewed by Malouin and Bryan [275], Georgopapadakou [152], and more recently by Zapun *et al.* [577]. According to the latter, such resistance may be caused by i) alteration of PBPs by spontaneous point mutations or homologous recombination, ii) hyperproduction of an endogenous low-affinity PBP, iii) acquisition of an additional low-affinity PBP, or iv) a combination of these mechanisms [577]. Current knowledge regarding alternatives i-iii, with emphasis on alterations in PBPs 1A, 1B, 2 and 4-6 (characteristics presented in chapter 5.5), regulatory mechanisms (chapter 5.7), and acquisition of additional PBPs as potential contributors to beta-lactam resistance in *H. influenzae* is summarized below.

PBP1A (*ponA*) and PBP1B (*ponB*) are poorly studied in *H. influenzae* despite the close interaction between PBP1B and PBP3 during cell division [528]. Kaczmarek *et al.* sequenced *ponA* and *ponB* in four *bla* negative low-rPBP3 isolates with unusually high ampicillin MIC but found no amino acid substitutions in the transpeptidase region [214]. The Rd strain and two rPBP3 strains with low-level resistance were transformed with full-length *ponA* and *ponB* genes but no transformants with increased resistance were obtained by selection with ampicillin and cefaclor.

PBP2 (*pbp2*) interacts with PBP1A during cell elongation and is important for maintenance of rod shape [528]. PBP2 deserves special attention as an important target for carbapenems and piperacillin in *H. influenzae* (**Table 8** and **Figure 10**).

As described in chapter 6.3.5, there is some evidence that carbapenem resistance is associated with PBP3 alterations, but data are conflicting and the mechanism is incompletely understood. Theoretically, it would make sense to characterize PBP2 in isolates with resistance to piperacillin and/or carbapenems, in particular in the absence of resistance to ampicillin and cephalosporins. To my knowledge, such investigations have not been performed.

In an early study, Mendelman *et al.* reported that I_{50} for PBP2 could not be determined in *H. influenzae* transformants with DNA from isolates with non-*bla*-mediated resistance and hypothesized that this was due to protein loss or reduced affinity [301]. In a later investigation, the same group observed that two transformants with alterations in PBP2 and PBP3 had higher ampicillin MICs than to transformants with PBP3 alterations only, and concluded that PBP2 is associated with resistance [298].

Powell *et al.* described resistance to imipenem without cross-resistance to ampicillin and meropenem but did not perform sequencing of PBP genes [384-386]. Cardines *et al.* reported 17.7% imipenem resistance among 79 *H. influenzae* from Italian cystic fibrosis patients [57]. The collection included imipenem-resistant, ampicillin-susceptible isolates but the resistance mechanism was not characterized.

High-level resistance to imipenem [308] and meropenem [180] has also been reported in *bla*-negative ampicillin-resistant *H. influenzae* from Japan. In one study [180], sPBP3 isolates with remarkably high meropenem MICs (32 mg/L) were reported. As PBP3 genotyping was performed by PCR, these isolates may represent miscategorized group III-like high-rPBP3 isolates (chapter 6.3.9). PBP2 was not characterized.

Kaczmarek *et al.* sequenced *pbp2* in four *bla*-negative low-rPBP3 *H. influenzae* with unusually high ampicillin MIC but found no amino acid substitutions in the transpeptidase region of PBP2 [214]. The Rd strain and two rPBP3 strains were transformed with full-length *pbp2* but no transformants with increased resistance were

obtained by selection with ampicillin or cefaclor. The effect on susceptibility to carbapenems was not investigated.

In conclusion, carbapenem resistance in *H. influenzae* is incompletely understood and the hypothetic resistance mechanism altered PBP2 ('rPBP2') is barely investigated.

PBP4 (*dacB*), PBP5 (*dacA*) and PBP6 (*pbpG*) are LMW PBPs with carboxypeptidase and/or endopeptidase activity (chapter 5.5). Reduced affinity for LMW PBPs has been observed in *H. influenzae* with non-*bla*-mediated beta-lactam resistance [300,301,530]. In an early study, Mendelman *et al.* reported that PBP4 was detectable only during growth but found no uniform relationship between PBP4 affinity and resistance [298]. Similar conclusions have been drawn in later investigations on the correlation between PBP4 and PBP5 alterations and beta-lactam resistance in *H. influenzae*.

Ubukata *et al.* sequenced *dacB* in 25 rPBP3 and five sPBP3 isolates and identified substitutions in the transpeptidase region of PBP4 in two group II low-rPBP3 isolates; no substitutions were observed close to the conserved SDN and KTG motifs [530]. A seven bp deletion downstream of SDN caused a stop codon in two isolates with undetectable PBP4 by fluorography but did not correlate to beta-lactam resistance.

Straker *et al.* sequenced *dacB* and *dacA* in 14 cefuroxime-resistant and three susceptible *H. influenzae* [487]. Several PBP4 substitutions were present in susceptible and resistant isolates, whereas no PBP5 substitutions were detected. The authors concluded with no obvious correlation between PBP4 alterations and resistance. Similarly, Cerquetti *et al.* detected several PBP4 substitutions in the transpeptidase region of two low-rPBP3 isolates with heterogeneous imipenem resistance and four sPBP3 isolates, but found no correlation between substitutions and resistance [62].

Investigations on the effect of PBP6 alterations in *H. influenzae* have not been identified during the work with this thesis.

Acquisition of additional PBPs has not been described as a mechanism of PBP-mediated resistance in *H. influenzae*. However, PBP binding assays have suggested the presence of additional PBPs in strains with non-*bla*-mediated resistance in several

investigations. Mendelman *et al.* reported marked PBP profile heterogeneity with detection of 5-10 PBPs in individual resistant isolates [300]. Makover *et al.* reported an extra HMW protein between PBP2 and PBP3A, and that PBP5 appeared to consist of at least two proteins in the (normally) ampicillin susceptible strain *H. influenzae* ATCC 19418 [274]; however, ampicillin MIC (3.3 mg/L) suggests acquired resistance. Serfass *et al.* observed 1-2 additional LMW proteins between PBP5 and PBP6 in two *bla* negative isolates with decreased PBP3 affinity and ampicillin MIC 4-8 mg/L [451]; similar observations were made by Mendelman *et al.* in two cefuroxime-resistant CSF isolates [299].

Whether these observations represent known PBPs with altered electrophoretic mobilities and/or acquired additional PBPs, and the relevance for beta-lactam resistance, is unknown.

Hyperproduction of low-affinity PBPs may confer beta-lactam resistance [577]. The mechanism has not been described in *H. influenzae*, but increased *ftsI* transcription and PBP3 hyperproduction would theoretically enhance the impact of PBP3 alterations and increase beta-lactam MICs. Several promoter sequences controlling transcription of the *dcw* cluster (including the *ftsI* gene) are described in *E. coli* (chapter 5.7); the corresponding promoters in *H. influenzae* have to my knowledge not been characterized. Kaczmarek *et al.* sequenced a 225 bp region upstream of the *ftsI* start codon in four *bla* negative low-rPBP3 isolates with unusually high ampicillin MIC but found no alterations compared to the Rd strain [214]. Cerquetti *et al.* sequenced a 166 bp region upstream of *ftsI* and a 100 bp region downstream of the *ftsI* stop codon; except for one nucleotide (upstream) no differences were observed compared to the Rd strain [62]. Notably, the sequenced regions would not encompass promoters with locations similar to promoters in *E. coli* (approximately 400 bp upstream of *ftsI*) [542].

As described in chapter 5.7, activation of the SOS response in *E. coli* (and likely *H. influenzae*) leads to increased transcription of the *dcw* cluster and a concomitant arrest in cell division, resulting in filamentous growth [542]. As inhibition of PBP3 by beta-lactams may initiate the SOS response, it has been suggested that the response may protect bacteria against beta-lactams [307]. The SOS response is suppressed by the

binding of LexA to SOS boxes located in the promoter region of the *dcw* cluster in *E. coli* [162,542]. The SOS box consensus sequence in *E. coli* contains TA repeats, resembling the contingency loci associated with phase variation through slipped-strain misplacement in *H. influenzae* (chapter 4.3) [28]. If the SOS response in *H. influenzae* is regulated in similar manners, and SOS boxes in the *dcw* promoter region contain contingency loci, an intriguing hypothesis might be that phase variation is involved in the regulation of *ftsI* transcription and cell division in *H. influenzae*.

Not only does this hypothesis suggest that the SOS response may be initiated through reversible mutations affecting LexA-binding SOS boxes; it also raises the possibility that *ftsI* transcription (and PBP3 production) may be switched on and off through reversible mutations (e.g. slipped-strain misplacement) affecting promoter sequences.

These hypothesized mechanisms of PBP3 hyperproduction are relevant for future investigations on some well-known, incompletely understood phenomenons:

Paradoxical effect, also referred to as 'the Eagle effect', was described by Eagle in 1948 [96]. Eagle observed that penicillin was optimally effective at a certain concentration and that killing rates were reduced at higher concentrations. Consistent with Eagle's observations in other organisms, Woolfrey *et al.* reported that killing of *H. influenzae* decreased rapidly at ampicillin concentrations 2-4 times MIC [567,568].

Bacterial persistence, first described in 1944 [35], denotes the ability of a subpopulation ('persisters') of susceptible bacteria to survive exposure to bactericidal antibiotics [20]. The persister phenotype is characterized by slow growth and acquired through a reversible switch [242]. Persistence has not been described in *H. influenzae*.

Heteroresistance denotes variable expression of a resistance mechanism within an isogenic population. The phenomenon has been known since 1947 but the mechanisms are incompletely understood and the clinical relevance uncertain [98]. Heteroresistance to imipenem in rPBP3 *H. influenzae* was reported by Cerquetti *et al.* [62].

Theoretically, bacteria with increased tolerance to beta-lactams due to PBP3 hyperproduction would be expected to have normal cell morphology, whereas filamentous forms would be expected in bacteria surviving beta-lactam exposure due

to activated SOS response and inhibited cell division. Woolfrey *et al.* described hazy growth of *H. influenzae* on agar dilution plates and within inhibition zones by disk diffusion [567]. The resistance mechanisms were not characterized, but the phenotypes of some of the study isolates (ampicillin MIC = 2 mg/L) strongly suggest altered PBP3. Gram staining revealed filamentous forms with outpouchings at concentrations immediately above the MIC, and variously sized and shaped cells at higher concentrations. In both cases, cellular morphology normalized in subcultures. Whether *H. influenzae* strains use phase variation to increase beta-lactam tolerance and/or to regulate resistance levels in rPBP3 strains remains to be investigated.

Interaction between PBP3 and other cell division proteins is crucial for cell division [528]. Gene products interacting with PBP3 in *E. coli* and likely in *H. influenzae* include the cell division protein FtsA, which plays a structural and regulatory role in peptidoglycan synthesis and is encoded by the *ftsA* gene, located at the 3' and of the *dcw* cluster (**Table 9**). Tormo *et al.* reported that *ftsA* mutations was associated with a significant decrease in the binding of ampicillin to PBP3 and with resistance to lysis by beta-lactams in *E. coli* [512]. The interaction between FtsA and PBP3 and the relevance for phenotypic resistance has not been investigated in *H. influenzae*.

6.4.2 *Impermeability*

The outer membrane of Gram-negative bacteria represents a barrier for beta-lactams and other hydrophilic drugs. For these targets, access to target depends on the presence of outer membrane proteins (OMP) forming permeable channels, denoted porins [534]. Porin loss or alterations might lead to reduced permeability and increased resistance to beta-lactams. Assays for measurement of permeability and OMP profiling were described by Mendelman *et al.* [301] and Sanchez *et al.* [438].

The lower beta-lactam MICs in *H. influenzae* compared to *Enterobacteriaceae* has been ascribed to a higher degree of penetration of the outer membrane [80,438]. It is generally accepted that OMP2, encoded by the *omp2* gene, is the only porin in *H. influenzae* [437,534]. The porin function of OMP2 was demonstrated by Burns *et al.*,

who showed that transformants lacking this protein had higher MICs to hydrophilic drugs such as chloramphenicol and beta-lactams [49].

There is considerable variation in *omp2* sequences and the size (35-42 kDa) of OMP2 in NTHi [136]. The correlation between composition and pore function of OMP2, PBP affinities and beta-lactam susceptibility in NTHi was investigated by Regelink *et al.* [407]. The authors found that ampicillin MIC differed significantly between isolates with different variants of OMP2 with different pore function; the observations could not be explained by differences in PBP affinity profiles.

Early investigations suggested that OMPs with sizes different from OMP2 may act as porins and be involved in beta-lactam resistance in *H. influenzae*. Mendelman *et al.* reported significantly reduced permeability in a ampicillin resistant transformant (MIC = 8 mg/L) with concomitantly reduced affinity for PBP3; notably, the transformant lacked a 27-kDa OMP which was present in the recipient strain [301]. In another study, Parr and Bryan observed significantly lower permeability in an ampicillin resistant isolate (MIC 1.56 mg/L) with low affinity for PBP3 compared to a susceptible isolate (ampicillin MIC = 0.185 mg/L) [366]. The authors also noted different OMP profiles, with a 45-kDa protein in the resistant strain, but not in the susceptible strain nor in the Rd strain. The low-affinity PBP3 and the resistance phenotype were transferred by transformation of the Rd strain, but not the 45-kDa protein nor the reduced permeability.

The observations of Mendelman *et al.* [301], Parr and Bryan [366], Burns *et al.* [49] and Regelink *et al.* [407] were not addressed by Tristram *et al.* in the latest review on beta-lactam resistance in *H. influenzae* [516]. Instead, the authors referred to negative observations made by Clairoux *et al.* [71] and Kaczmarek *et al.* [214], and concluded that a role of altered outer membrane proteins and impermeability has not been demonstrated. Further investigations are needed to clarify whether impermeability due to porin alterations may contribute to beta-lactam resistance in *H. influenzae*.

6.4.3 *Increased efflux*

The concentration gradient of beta-lactams across the outer membrane may be expressed as the balance between influx (through porins) and efflux. The genome of *H. influenzae* contains homologs of several multi-drug efflux pumps [131,437]. The *acrRAB* gene cluster contains genes encoding the AcrAB efflux pump and the *acrR* suppressor gene [131]. Sanchez *et al.* found that this efflux pump was important for baseline susceptibility to erytromycin and rifampicin in *H. influenzae*, but disruption of the pump in the Rd strain did not affect susceptibility to beta-lactams, quinolones, tetracycline and chloramphenicol (in contrast to in *E. coli*) [437]. The effect of AcrAB derepression on beta-lactam susceptibility was not investigated.

Kaczmarek *et al.* demonstrated that four low-rPBP3 isolates from North America (1996-2001) with unusually high ampicillin MICs (8-16 mg/L) had increased efflux due to frame shift insertions in *acrR* [214]. The authors obtained significantly higher ampicillin and erythromycin MICs by transformation of the Rd strain with *acrR* genes from clinical isolates, and also reduced mean ampicillin MIC from 10.3 mg/L to 3.67 mg/L (typical for low-rPBP3) through an *acrR* knock-out experiment. OMP characterization showed no differences between resistant isolates and control strains or transformants. The authors concluded that regulation of AcrAB might increase as well as decrease ampicillin MIC in rPBP3 *H. influenzae*, challenging the traditional view that efflux does not affect the activity of beta-lactams in this organism [437].

The conclusions of Kaczmarek *et al.* were questioned by Garcia-Cobos *et al.* in a later study [148]. Characterization of the *acrR* genes of 72 *H. influenzae* from Spain revealed *acrR* mutations predicting early termination of the ORF in eight isolates, but none of these had ampicillin MIC exceeding 2 mg/L. Transformation experiments were not conducted in that study. With conflicting observations in the two studies, the impact on *acrR* mutations on beta-lactam susceptibility in *H. influenzae* needs further elucidation. An attractive hypothesis might be that a third, unknown mechanism is required to increase beta-lactam MICs in rPBP3 isolates with increased efflux due to *acrR* mutations.

6.5 NON-*BLA*-MEDIATED RESISTANCE AND PATHOGENICITY

Several investigations have addressed the association between beta-lactam resistance and pathogenicity. Peptidoglycan from *H. influenzae* mediates inflammation in experimental meningitis, and peptidoglycan from strains with non-*bla*-mediated resistance mediates as stronger inflammatory response compared to peptidoglycan from susceptible strains [50]. Burroughs *et al*. found different peptidoglycan structures in susceptible and resistant strains [52] and suggested an association between peptidoglycan structure and pathogenicity [51].

Rubin *et al.* found that *H. influenzae* transformants with non-*bla*-mediated resistance were associated with lower mortality and reduced ability to cause bacteremia in a rat model [425]. The authors suggested that reduced pathogenicity was due to reduced growth rates, i.e. that acquisition of resistance was associated with a fitness cost [295]. However, in a transformation experiment by Melhus *et al.* [292], reduced susceptibility to amoxicillin was transferred but the growth rate remained unchanged after transformation of a susceptible recipient strain with DNA from a strain with non-*bla*-mediated resistance and lower growth rate. The authors also concluded that the transformant expressed no obvious alterations in virulence in a rat model of acute otitis media. The exact resistance mechanism was not characterized in the two studies.

Clinical studies have shown no significant association between non-*bla*-mediated resistance and disease severity in patients with *H. influenzae* pneumonia [331] and other respiratory disease [169]. Okabe *et al.* reported increased ability of an NTHi strain with PBP3-mediated resistance to invade bronchial epithelial cells *in vitro* and hypothesized that altered PBP3 may enhance virulence by acting as an adhesion molecule [157]. In a later investigation, Atkins *et al.* confirmed an increased capacity of some clinical rPBP3 strains to invade respiratory epithelial cells but transformation experiments showed that this was not related to the altered PBP3 [13]. Strain characterization by molecular methods for epidemiological typing was not performed in either of the studies referred above.

6.6 BETA-LACTAM RESISTANCE EPIDEMIOLOGY

Table 14 shows global beta-lactam susceptibility rates according to current EUCAST breakpoints [111], and **Table 15** summarizes beta-lactam susceptibility from the Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM) (2000-2014) [339-345].

Bla positive *H. influenzae* are less prevalent in Europe compared to other regions, and susceptibility rates for amoxicillin-clavulanic acid and ceftaroline suggest that non-*bla*-mediated resistance in general and high-rPBP3 isolates in particular are more frequent in the Asia-Pacific region and South Africa compared to Europe and the Americas. A global surveillance study comparing the periods 2004-2008 (n=8732) and 2009-2012 (n=6038) indicated increasing prevalence of *bla* negative, ampicillin-non-susceptible *H. influenzae* in Latin America (3.3%/4.0%), Asia/Pacific region (1.1%/2.9%) and North America (1.0/1.3%), and decreasing prevalence in Africa (2.5%/1.6%) and Europe (1.9%/1.3%) [511].

In Norway, both *bla* positive *H. influenzae* and isolates with non-*bla*-mediated resistance increased considerably in frequency between 2000 and 2014. With the notable exception of cefuroxime, suggesting that the prevalence of rPBP3 *H. influenzae* is higher in Norway compared to the rest of Europe, beta-lactam susceptibility rates for non-invasive isolates from Norway in 2011 [343] were largely comparable to the rates for respiratory isolates from Europe in the period 2009-2012 [121,428].

Table 14 Beta-lactam susceptibility of *H. influenzae* isolates in surveillance studies from different geographical regions. Proportions (%) of isolates categorized as susceptible by current EUCAST breakpoints (chapter 8.1, **Table 17**) [111]

Agents	Europe	U. S.	Asia-Pacific region and South Africa	Latin America
bla negative	87.2[a]	73.2[c]	77.9[e]	70.6[f]
Ampicillin	86.4[a]	73.1[c]	75.5[e]	No data
Amoxicillin-clavulanic acid	91.3[a]	>90.0[c]	84.1[e]	88.9[f]
Cefuroxime[g]	94.8[a]	No data	No data	No data
Ceftriaxone	99.6[b]	99.5[d]	99.3[e]	99.2[f]
Ceftaroline	97.7[a]	No data	93.4[e]	96.0[f]
Meropenem	100.0[b]	100.0[d]	No data	No data

[a] Community-acquired respiratory tract infections, 2010 (n=515) [121]
[b] Pneumonia, hospitalized patients, 2009-2012 (Europe and the Mediterranean region) (n=189) [428]
[c] Respiratory and bloodstream isolates, 2008-2010 (n=1545) [371]
[d] Pneumonia, hospitalized patients, 2009-2012 (n=251) [428]
[e] Community-acquired respiratory tract infections, 2010 (n=453) [429]
[f] Various infection types and locations, 2011 (n=126) [130]
[g] Isolates with cefuroxime MIC = 2 mg/L (intermediate) are included in the susceptible proportion

Table 15 Beta-lactam susceptibility of non-invasive (2000-2014) and invasive (2013-2014) *H. influenzae* from Norway (compiled from NORM [339-345]). Proportions (%) of isolates categorized as susceptible by current EUCAST breakpoints (chapter 8.1, **Table 17**) [111]

Agents[a]	Non-invasive[b]						Invasive[c]	
	2000 n=355	2001 n=704	2004 n=513	2007 n=808	2011 n=677	2014 n=463	2013 n=79	2014 n=69
bla negative	92.9	93.0	91.2	89.5	87.7	82.7	84.8	87.0
Ampicillin	93.6	91.8	88.9	86.6	81.7	80.3	82.3	79.7
Amoxicillin-ca[d]	100.0	98.7	96.9	92.0	93.8	90.9	94.9	94.2
Cefuroxime[e]	No data	No data	No data	83.7	83.0	86.6	81.0	87.0
Cefotaxime	No data	No data	No data	99.5	100.0	99.4	98.7	98.6
Ceftriaxone	No data	No data	No data	No data	No data	No data	100.0	100.0

[a] MIC determination by gradient tests (Etest, 2000-2011; non-specified, 2013-2014)
[b] Respiratory, eye and ear isolates
[c] Isolates from blood, CSF and other sterile materials
[d] Amoxicillin-clavulanic acid
[e] Isolates with cefuroxime MIC = 2 mg/L (intermediate) are included in the susceptible proportion

7 NON-BETA-LACTAM ANTIMICROBIAL AGENTS

7.1 AGENTS WITH ACTIVITY AGAINST *H. INFLUENZAE*

Non-beta-lactam resistance in *H. influenzae* has been reviewed repeatedly [164,269,333,383,410,470,516,558]. Although the topic is outside the primary scope of this thesis, a brief summary is presented in this chapter because any phylogenetic, epidemiologic or mechanistic links between beta-lactam and non-beta-lactam resistance in *H. influenzae* would be highly relevant.

H. influenzae without acquired resistance mechanisms are susceptible to a wide range of non-beta-lactam antimicrobial agents *in vitro*. Commonly used therapeutic agents with clinical breakpoints defined by EUCAST include quinolones, tetracyclines, chloramphenicol and trimethoprim-sulfamethoxazole; rifampicin may be used for prophylaxis [111].

In addition, CLSI have defined clinical breakpoints for the macrolides clarithromycin, azithromycin (azalide) and telithromycin (ketolide) [75]. EUCAST currently define the wild type population as intermediate susceptible to these agents due to intrinsic resistance [255] and weak correlation between MICs and outcome [111]. *In vitro* and *in vivo* studies have shown bactericidal effect of azithromycin [103], telithromycin [356] and clarithromycin [194,329] on NTHi in AOM and/or pulmonary infections. The effect of azithromycin correlates to MIC and clinical breakpoints [103]. Clarithromycin may have *in vivo* bactericidal effect despite increased MIC by *in vitro* susceptibility testing [329]. Clinical trials indicate that azithromycin is non-inferior to amoxicillin-clavulanic acid for treatment of AOM [11] and lower RTI [576].

The ability of azithromycin, telithromycin and clarithromycin to kill intercellular bacteria reflects the good intracellular penetration of these drugs [5,103,329]. Intracellular accumulation is also an important hallmark of fluoroquinolones, such as ciprofloxacin [5] and levofloxacin [449]. Other non-beta-lactams with demonstrated activity against intracellularly located NTHi include tetracycline [45,540], quinupristin/dalfopristin [5] and rifampicin [540].

Synergy between beta-lactams and non-beta-lactams (e.g. quinolones) against rPBP3 *H. influenzae* may be observed *in vitro* (**Figure 13**). Uemura *et al.* compared concurrent administration of imipenem-levofloxacin and imipenem-clarithromycin with single administration and observed significantly reduced biofilm formation [533]. These observations suggest that combined therapy may be a useful therapeutic approach in infections where biofilm and/or the formation of intracellular bacterial communities are important for pathogenesis (chapter 4.4).

Clinical breakpoints for *H. influenzae* and aminoglycosides have not been defined by EUCAST [111] and CLSI [75]. MIC-distributions suggest that the *in vitro* activity of gentamicin against *H. influenzae* is similar to the activity against other Gram-negative organisms, e.g. *Acinetobacter baumannii* (www.eucast.org/mic_distributions/). Gentamicin kills *H. influenzae* effectively *in vitro* but has no intracellular activity [540] and is generally considered unsuitable for treatment of pulmonary infections.

7.2 NON-BETA-LACTAM RESISTANCE IN *H. INFLUENZAE*

EUCAST define resistance to ciprofloxacin as an exceptional phenotype in *H. influenzae* [255] and recommend that such isolates are referred to a reference laboratory [111]. Resistance to quinolones is associated with hypermutability [369] and usually due to substitutions in the quinolone resistance-determining regions (QRDR) of subunit A of topoisomerase II (GyrA) and subunit A of topoisomerase IV (ParC); the resistance level depends largely on the number of substitutions in the positions 84 and 88 in both proteins [151]. Isolates with single substitutions are usually low-level resistant but additional mechanisms may increase resistance [369]. The plasmid-mediated acetyl transferase gene *aac(6')-lb-cr* may contribute to quinolone resistance [372]. Other plasmid-mediated quinolone resistance determinants such as *qnr* has been reported in *H. parasuis* [167] but not in *H. influenzae*.

Nalidixic acid (30 µg) is superior to ciprofloxacin (5 µg) for detection of low-level resistance and may be used for screening [370]. Global surveillance studies indicate that 10-30% of *H. influenzae* may possess first-step QRDR substitutions [85]. In Spain

(2000-2013), only 28/7267 isolates (0.39%) had ciprofloxacin MIC >1 mg/L and the proportion remained stable during the study period [396]. Notably, dissemination of levofloxacin resistant clones was recently reported from Taiwan, with an increase in isolates with MIC >2 mg/L from 2.0% in 2004 to 24.3% in 2010 [241].

Resistance to trimethoprim-sulfamethoxazole in *H. influenzae* is caused by reduced activity of trimethoprim due to alterations in the *dfrA* gene encoding the enzyme dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolate to tetrahydrofolate [90], and/or resistance to sulfamethoxazole due to acquisition of *sul* genes and mutations in the *folP* gene encoding dihydropteroate synthase (DHPS) [99]. Tetracycline resistance in *H. influenzae* is due to increased efflux mediated by the transferable *tet*(B) gene [66]. Resistance to chloramphenicol may be caused by the acquired enzyme chloramphenicol acetyltransferase, encoded by the *cat* gene [418], and occasionally by impermeability due to loss of an outer membrane porin [48].

Non-beta-lactam resistance rates for respiratory and invasive isolates from Norway based on surveillance data from NORM [343,344] are presented in **Table 16**.

Table 16 Susceptibility to non-beta-lactams in non-invasive and invasive *H. influenzae* from Norway according to NORM 2014 [345] and EUCAST breakpoints [111]. S, susceptible; I, intermediate; R, resistant

Agents	MIC breakpoints[a]	Non-invasive[b] (n=463)			Invasive[c] (n=69)		
		S	I	R	S	I	R
Trim-sulfa[d]	0.5/1	78.0	3.0	19.0	87.0	2.9	10.1
Tetracycline	1/2	98.5	0.2	1.3	98.6	0.0	1.4
Chloramphenicol	2/2	99.1	-	0.9	100.0	-	0.0
Ciprofloxacin	0.5/0.5	99.6	-	0.4	8.6	-	1.4

[a] S≤/R> (mg/L) [111]
[b] Respiratory, eye and ear isolates
[c] Isolates from blood, CSF and other sterile materials
[d] Trimethoprim-sulfamethoxazole

7.3 MULTI-DRUG RESISTANCE (MDR)

In 1984, Mendelman *et al.* reported a case of meningitis caused by MDR *H. influenzae* [302]. The resistotype (*bla* positive and co-resistance to chloramphenicol and tetracycline) was mediated by a 43-megadalton R-plasmid. The first Nordic case of *H. influenzae* septicemia with a similar strain was reported by Vik *et al.* in 1986 [543]. Later investigations showed that the resistance determinants in such strains are located at an ICE [254], now referred to as ICE*Hin1056*, with bla_{TEM} residing in one transposon (Tn*3*) and *tet* and *cat* genes in another (Tn*10*) [212,310]. ICE*Hin1056* may be detected by molecular methods [430]. Transfer occurs with a frequency of between 10^{-1} and 10^{-2} in *H. influenzae* [212]. The association between *bla*, *tet* and *cat* genes is reflected by surveillance data: resistance to tetracycline and chloramphenicol was more frequent in *bla* positive versus *bla* negative isolates in NORM 2011 [343].

MDR *H. influenzae* with combinations of chromosomally mediated resistance mechanisms have also been reported. Campos *et al.* reported two isolates with resistance to ciprofloxacin and concomitant *bla* and chloramphenicol acetyltransferase activity, and two ciprofloxacin-resistant isolates with concomitant non-*bla*-mediated ampicillin resistance [55]. A strain with non-*bla*-mediated beta-lactam resistance (resistant to amoxicillin-clavulanic acid and imipenem) co-resistant to quinolones and trimethoprim-sulfamethoxazole caused a nosocomial outbreak in Taiwan in 2008 [573]. More recently, a group II low-rPBP3 strain with co-resistance to quinolones was reported from Germany [372]. Surveillance data support a link between various chromosomally mediated resistance mechanisms (beta-lactams, quinolones and trimethoprim-sulfamethoxazole). Resistance to trimethoprim-sulfamethoxazole is more frequent in *H. influenzae* with with non-*bla*-mediated beta-lactam resistance in Norway [342], and resistance to trimethoprim-sulfamethoxazole is prevalent among *H. influenzae* with QRDR substitutions in Spain [396].

Finally, extensively MDR *H. parainfluenzae* with resistance to ciprofloxacin, tetracycline, chloramphenicol, azithromycin and extended-spectrum cephalosporins were reported from Switzerland [510]; a similar accumulation of resistance mechanism has to my knowledge not been reported in *H. influenzae*.

8 TESTING OF SUSCEPTIBILITY TO BETA-LACTAMS

8.1 CLINICAL BREAKPOINTS

The minimum inhibitory concentration (MIC) value is an objective measure of drug tolerance and denotes the lowest concentration (expressed as mg/L or μg/mL) of a drug that prevents visible growth of a microorganism. Clinical MIC breakpoints are used to categorize the organism as susceptible (S), intermediate (I) or resistant (R) based on MIC [315]. Breakpoint setting requires information on dosages, pharmacokinetics and pharmacodynamics (PK/PD), resistance mechanisms, and MIC distributions with epidemiological cutoff (ECOFF) values, defined as the highest MIC of isolates without acquired resistance mechanisms (wild-type). Clinical breakpoints should be supported by clinical data and not divide the wild-type population [315].

Current breakpoints for *H. influenzae* and beta-lactams from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org) [111] and Clinical and Laboratory Standards Institute (CLSI, http://clsi.org) [75] differ significantly and are not identical for any of the 17 agents listed in **Table 17**.

EUCAST and CLSI both use ECOFF to separate between ampicillin susceptible (MIC ≤1 mg/L) and non-susceptible (MIC >1 mg/L) isolates. In contrast to CLSI, EUCAST have not defined an intermediate category for ampicillin. Clinical data for *H. influenzae* and aminopenicillins are insufficient and the relevance of current breakpoints is debated [110,139,516,537]. Ampicillin breakpoints from CLSI were originally set to separate between *bla* positive and *bla* negative strains [139].

Non-susceptibility to extended-spectrum cephalosporins is characterized as an 'exceptional phenotype' [255]. Both EUCAST and NordicAST recommend that such isolates are sent to a reference laboratory [111,338]. EUCAST breakpoints for extended-spectrum cephalosporins are largely based on ECOFFs (**Table 17**), whereas CLSI breakpoints mostly correlate to non-species-related (PK/PD) breakpoints [516]. As different breakpoints lead to differences in susceptibility rates, surveillance data are sometimes reported according to both guidelines [121,130,428,429].

Table 17 Clinical breakpoints for *H. influenzae* and beta-lactams from EUCAST [111] and CLSI [75] compared to epidemiological cut-off (ECOFF) values and non-species related (PK/PD) breakpoints [111] (S≤/R>, mg/L)

Agents	Class[c]	Adm[d]	ECOFF[e]	CLSI [75]	EUCAST [111]	PK/PD [111]
Ampicillin	Ap	p.o.	1	1/2	-	-
Ampicillin	Ap	i.v.	1	1/2	1/1	2/8
Ampicillin-sulbactam[a,b]	Ap-i	i.v.	1	-	1/1	2/8
Amoxicillin[a,b]	Ap	p.o.	2	-	-	-
Amoxicillin[a,b]	Ap	i.v.	2	-	2/2	2/8
Amoxicillin-clavulanic acid[a]	Ap-i	p.o.	2	4/4	-	-
Amoxicillin-clavulanic acid[a]	Ap-i	i.v.	2	-	2/2	2/8
Piperacillin-tazobactam[a,b]	Ur-i	i.v.	0.06	1/1	-	4/16
Cefuroxime[a]	Cs II	p.o.	2	4/8	0.125/1	-
Cefuroxime[a]	Cs II	i.v.	2	4/8	1/2	4/8
Ceftibuten	Cs III	p.o.	0.5	2/-	1/1	-
Cefixime	Cs III	p.o.	0.125	1/-	0.125/0.125	-
Cefpodoxime	Cs III	p.o.	0.25	2/-	0.25/0.5	-
Cefotaxime	Cs III	i.v.	0.06	2/-	0.125/0.125	1/2
Ceftriaxone	Cs III	i.v.	0.06	2/-	0.125/0.125	1/2
Ceftazidime	Cs III	i.v.	0.5	2/-	-	4/8
Cefepime	Cs IV	i.v.	0.25	2/-	0.25/0.25	4/8
Ceftaroline	Cs V	i.v.	0.03	0.5/-	0.03/0.03	0.5/0.5
Aztreonam	Mb	i.v.	0.5	2/-	-	4/8
Doripenem	Cp	i.v.	0.5	1/-	1/1	1/2
Ertapenem	Cp	i.v.	0.125	0.5/-	0.5/0.5	0.5/1
Imipenem	Cp	i.v.	2	4/-	2/2	2/8
Meropenem (non-CSF)	Cp	i.v.	0.25	0.5/-	2/2	2/8
Meropenem (CSF)	Cp	i.v.	0.25	0.5/-	0.25/1	-

[a] CLSI: infer susceptibility to amoxicillin from ampicillin; consider ampicillin-resistant beta-lactamase negative isolates resistant to ampicillin-sulbactam, amoxicillin-clavulanate, piperacillin-tazobactam and cefuroxime despite apparent *in vitro* susceptibility [75]

[b] EUCAST: infer susceptibility to amoxicillin from ampicillin; infer susceptibility to ampicillin-sulbactam and piperacillin-tazobactam from amoxicillin-clavulanate [111]

[c] Ap, aminopenicillin; i, beta-lactamase inhibitor; Up, ureidopenicillin; Cs, cephalosporin; Mb, monobactam; Cp, carbapenem; I-V, cephalosporin generations

[d] Administration route; p.o., per os; i.v., intravenous

[e] Epidemiological cut-off values (EUCAST; www.eucast.org/mic_distributions_ecoffs)

As illustrated by the differences between EUCAST and CLSI breakpoints, setting of clinical breakpoints may be difficult. Notably, there is also a lack of consensus on non-species-related (PK/PD) breakpoints between the two breakpoint committees. For instance, PK/PD breakpoints for ampicillin and cefotaxime calculated by EUCAST suggest that isolates with ampicillin MIC 4-8 mg/L and cefotaxime MIC 2-8 mg/L are resistant to standard doses of the drugs, but susceptible to high-dose therapy [111]. In contrast, the PK/PD breakpoints for ampicillin and cefotaxime (S≤2/R>2 mg/L, both agents) presented in the latest review article on beta-lactam resistance in *H. influenzae* [516] suggest that increased doses of these drugs do not affect clinical efficacy.

The MIC distributions for *H. influenzae* with and without resistance mechanisms further complicate setting of clinical breakpoints for this organism. The rPBP3 population overlaps with the wild-type population for several agents, and no breakpoint will reliable discriminate between the two populations. For instance, rPBP3 isolates have ampicillin MIC range 0.5-16 mg/L (*bla* negative) [139,516,537] and cefotaxime MIC range 0.03-2 mg/L [180,516,531]; both ranges include isolates with MIC below ECOFF (**Table 17**).

Antimicrobial susceptibility testing is associated with uncertainty due to biological and technical variation. A precision of ±1 dilution is generally accepted for MIC methods [209]. Consequently, when the breakpoints divide the resistant population, MIC-based susceptibility categorization will in some cases lead to categorization errors. For ampicillin, the S/R-breakpoint from EUCAST divides the low-rPBP3 population (MIC_{50} = 1 mg/L), and the I/R-breakpoint from CLSI divides the high-rPBP3 population (MIC_{50} = 2 mg/L) [516]. For cefotaxime, the EUCAST breakpoints divide the high-rPBP3 population (MIC range 0.125-2 mg/L, MIC_{50} = 0.5 mg/L) [516].

8.2 BROTH DILUTION

MIC determination by dilution methodology using serial dilutions of the drug (twofold dilutions including the value 1 mg/L) is considered the gold standard for susceptibility testing of bacteria [107]. Methodologies for broth microdilution (BMD) are described

by EUCAST [107,109] and CLSI [75]; both methods are based on the early works of Ericsson & Sherris [101]. Serial dilutions may be produced in-house from antibacterial powders and broth from approved manufacturers, but commercial dried trays for reconstitution with broth are more convenient for use in routine laboratories [209].

EUCAST and CLSI methodologies are both based on Mueller-Hinton broth (MHB) but differ with respect to the supplements recommended for testing of *H. influenzae*. EUCAST recommend Mueller-Hinton-Fastidious (MH-F) broth, consisting of MHB supplemented with 5% defibrinated horse blood and 20 mg/L beta-NAD [109,282]. CLSI recommend *Haemophilus* Test Medium (HTM), composed by MHB supplemented with 5 g/L yeast extract, 15 μg/mL beta-NAD, and 15 μg/mL hematin [74,211]. Different media for susceptibility testing of *H. influenzae* were compared in a multicenter study by Jacobs *et al.* [200]. MICs obtained with HTM (from different manufacturers), another MHB-based medium (with 2% lysed horse blood and 15 mg/L beta-NAD), and two media based on IsoSensitest broth were within ±1 dilution for 15 of 21 agents tested, and inter-laboratory differences were more marked than the differences between media. Ampicillin, amoxicillin-clavulanic acid and ceftriaxone were among the most reproducible agents tested.

Fuchs and co-workers evaluated media for determination of ampicillin broth dilution MIC in *H. influenzae*. In two studies with 206 isolates (including 61 *bla* positive) [140] and 143 *bla* negative isolates [27], respectively, HTM was compared with the MH-F-like LHB-3 medium (MHB supplemented with 3% lysed horse blood and 20 mg/L beta-NAD). Geometric mean MIC was essentially the same with HTM (2.49 mg/L) and LHB-3 (2.55 mg/L) and 96.1% of the results were within ±1 dilution step [140]. Susceptibility categorization (CLSI breakpoints) was essentially the same with both media [27]. The authors also investigated the influence of variations in colony age, inoculum, incubation time and atmosphere and observed that a 10-fold increase in inoculum increased ampicillin MIC from 1.50 to 2.45 mg/L [140].

EUCAST and CLSI guidelines also differ with respect to susceptibility testing of *bla* positive isolates to ampicillin-sulbactam and amoxicillin-clavulanic acid. EUCAST recommend fixed concentrations of the *bla* inhibitor (sulbactam, 4 mg/L; clavulanic

acid, 2 mg/L) [111], whereas CLSI recommend a 2:1 ratio [74]. Clavulanic acid has low antibacterial activity against *H. influenzae* (MIC range 25-125 mg/L) [129].

Although BMD is considered reference methodology for MIC determination, validation of data requires adequate quality control (QC). QC MIC ranges for *H. influenzae* reference strains are defined by EUCAST [113] and CLSI [75]. Notably, as QC MICs within ±1 dilutions of target are accepted, median MICs for a particular agent-organism combination determined in separate investigations using the same test population may differ by two dilutions if MIC in the two investigations systematically deviates from target by +1 and -1 dilution, respectively. To avoid systematic errors, all QC MICs should be within range, and median MIC should ideally be on target. Also, as systematic errors outside the MIC range of the strain will not be detected if only one reference strain is used, QC should ideally be performed with strains covering different parts of the MIC scale. MIC data may also be compared to MIC distributions for clinical isolates as an additional control of validity.

Other methods for susceptibility testing than MIC determination by broth dilution should be calibrated and evaluated against reference methodology. Commonly used acceptance criteria are >90% overall essential agreement (±1 dilution) and <1.5% very major errors (VME) and <3% major errors (ME) for individual species-drug combinations [209]. Notably, VME, ME, minor errors (mE) and categorical agreement are calculated with the complete test population as the denominator and thus strongly influenced by the proportion of resistant isolates in the test population (prevalence). Categorical errors and categorical agreement rates may be used for comparison of methods for susceptibility testing using identical populations, but the usefulness of acceptance criteria based on defined categorical error rates without taking into account the representativeness of the test population is debatable. In contrast to VME, the false susceptible rate (FSR; the number of VME divided by the number of isolates resistant by reference method) is independent of prevalence and may be used for comparison of results obtained with different methods and test populations.

It should be noted that all the parameters described above are dependent on MIC breakpoints, and that rates achieved with different guidelines may not be compared.

8.3 GRADIENT DIFFUSION

Gradient diffusion tests are commonly used for MIC determination in routine laboratories [209]. A strip coated with an antimicrobial agent concentration gradient on one side and an MIC scale printed on the other side is applied on an agar plate inoculated with a suspension of the test strain, and MIC is read directly where bacterial growth intersects with the strip. Three tests are commonly used: Etest (bioMérieux; www.biomerieux.com), MIC test strip (MTS) (Liofilchem; www.liofilchem.net) and M.I.C.Evaluator (M.I.C.E.) (Thermo Fisher Scientific; www.thermoscientific.com). EUCAST provides a list of compliance of manufacturers with EUCAST recommendations [108]. Etest, MTS and M.I.C.E. may all be used with MH-F agar (and with HTM, according to the manufacturers). Notably, as per September 2015, only one manufacturer (Liofilchem) offers amoxicillin-clavulanic acid and ampicillin-sulbactam strips with fixed inhibitor concentrations [108].

Several studies have indicated that Etest and M.I.C.E. may be unreliable for susceptibility testing of *H. influenzae* to beta-lactams (in particular aminopenicillins), but the results are conflicting [27,36,149,324,409,522]. No publications evaluating the performance of MTS and *H. influenzae* have been identified during the work with this thesis; there is also a general lack of evaluations of gradient tests with MH-F agar.

Mushtaq *et al.* compared Etest and M.I.C.E. with agar dilution (IsoSensitest Agar, ISA) for several agents and species [324]. Overall essential agreement was poorest for the 56 *Haemophilus* isolates (*H. influenzae*, n=46; *H. parainfluenzae*, n=10) (Etest, 74.4%; M.I.C.E., 76.9%), lower for beta-lactams than for non-beta-lactams, and particularly low for ampicillin and amoxicillin. Essential agreement rates for ampicillin, amoxicillin, amoxicillin-clavulanic acid (2:1), cefotaxime low (32 mg/L) and cefotaxime high (256 mg/L) were 60.5%, 42.4%, 65%, 55.8% and 85.7% for Etest, and 73.8%, 59.4%, 73.8, 83.7% and 68.8%, for M.I.C.E., respectively. Correlation coefficients (r) between gradient and reference MICs were 0.6-0.9 for Etest and 0.7-0.9 for M.I.C.E.; poor correlation ($r \leq 0.7$) was seen for amoxicillin (Etest) and amoxicillin-clavulanic acid (both tests).

Billal *et al.* reported even lower essential agreement in a study comparing Etest to BMD (HTM) or categorization of 87 *bla* negative rPBP3 strains [36]. The authors found 48%, 49% and 60% essential agreement for ampicillin, amoxicillin-clavulanic acid (2:1) and ceftriaxone, respectively. For all agents, a large number of Etest MICs were ≥6 dilutions higher than the corresponding BMD MIC.

Overestimation of ampicillin resistance was also observed by Rennie *et al.* [409]. The authors compared the performance of Etest and M.I.C.E. for susceptibility testing of fastidious species with BMD (HTM) as the gold standard. The test population included 39 *H. influenzae* and ten isolates of other *Haemophilus* species, and the agents tested included ampicillin, amoxicillin, amoxicillin-clavulanic acid (2:1) and cefotaxime. Detailed results for *H. influenzae* were not presented but the authors stated that major errors were mainly observed for ampicillin and *Haemophilus* spp.; for this agent-species combination, major errors were seen for 4/49 (8.2%) isolates with M.I.C.E. and 3/49 (6.1%) isolates with Etest.

In contrast, Fuchs and co-workers tested 143 *bla* negative *H. influenzae* and found a higher ampicillin susceptibility rate with Etest (HTM) (68%) compared to BMD (HTM) (59%) [27]. The authors also evaluated Etest with the MH-F-like media WHB (MHB with 5% whole horse blood and 20 mg/L beta-NAD) and LHB-3 (MHB with 3% lysed horse blood and 20 mg/L beta-NAD) and obtained susceptibility rates slightly closer to the gold standard (WHB, 66%; LHB-3, 64%). The same group compared BMD (HTM) and ampicillin Etest with six different media, including HTM, WHB and LHB-5 (MHB with 5% lysed horse blood and 20 mg/L beta-NAD), for a collection of 145 *bla* negative (including 59 isolates with ampicillin MIC >1 mg/L) and 61 *bla* positive *H. influenzae*. The authors obtained lower ampicillin MICs with Etest (HTM, 1.23 mg/L; WHB, 1.53 mg/L; LHB-5, 1.62 mg/L) compared to BMD (2.49 mg/L) [140]. Both reports suggest that Etest underestimates MIC for ampicillin at levels near the clinical breakpoint, irrespective of media.

Similarly, Garcia-Cobos *et al.* obtained a higher ampicillin susceptibility rate with Etest compared to BMD (HTM) in 34 *bla* negative rPBP3 isolates (88.2% versus 76.5%); Etest MIC was 1-2 dilutions lower than BMD MIC for 41% (14/34) of the

isolates [149]. Overall essential agreement for ampicillin (all resistance genotypes, n=94) was 80.9% and varied between genotypes (*bla* negative sPBP3, 100%; *bla* negative rPBP3, 97%; *bla* positive sPBP3, 25%; *bla* positive rPBP3, 86.7%). The corresponding results for amoxicillin and amoxicillin-clavulanic acid (2:1) were 80.9% (100%; 73.5%; 15%; 87.7%) and 84% (96%; 73.5%; 100%; 66.7%), respectively.

Tristram compared Etest and M.I.C.E. with BMD (HTM) and reported excellent correlation between the gradient tests, and between the gradient tests and BMD [522]. Overall essential agreement rates for ampicillin, amoxicillin-clavulanic acid (2:1) and cefotaxime were 81%, 91% and 93%, respectively. Essential agreement for ampicillin was higher for *bla* negative (27/30, 90%) compared to *bla* positive isolates (28/40, 70%). Notably, for *bla* negative rPBP3 isolates, 47% (14/30) of ampicillin gradient MICs were one (n=12) or two (n=2) dilutions lower than reference MIC.

Poor agreement between gradient tests and BMD for testing of susceptibility of *bla* positive isolates to ampicillin [149,522] is consistent with previous observations. Jorgensen *et al.* compared Etest with BMD (HTM) for susceptibility testing of *H. influenzae* (n=100) and found essential agreement for ampicillin, cefuroxime and cefotaxime of 76.5%, 95% and 96%, respectively [210]. The poor agreement for ampicillin reflects that the test was difficult to interpret for *bla* positive strains due to growth of small colonies within the inhibition ellipse. EUCAST recommend that *bla* positive isolates of *H. influenzae* are reported resistant to aminopenicillins without *bla* inhibitor, irrespective of MIC [111]. Notably, 6.3% (5/80) of *bla* positive *H. influenzae* had ampicillin MIC <1 mg/L in NORM 2014 [345]. Methods for detection of *bla* in *H. influenzae* are described in chapter 6.2.4.

8.4 DISK DIFFUSION

In the very first report of laboratory-confirmed ampicillin resistance (*bla*) in *H. influenzae*, Nelson stated that susceptibility testing of this organism is ‘a tricky business’ and characterized disk testing as ‘notoriously unreliable’ [334].

Modern disk diffusion is a simple and cost-effective method suitable for large-scale testing of clinical isolates in routine laboratories [209]. In short, a paper disk with a standardized content of an antimicrobial agent is applied on an agar plate inoculated with the test strain according to a standardized procedure. A concentration gradient is established by drug diffusion. Inhibited growth at concentrations exceeding the MIC of the isolate results in an inhibition zone around the disk; the diameter corresponds to the MIC value. A linear relationship between the zone diameter and antimicrobial log concentration of various drugs was demonstrated by Ericsson *et al.* [100].

The principle of agar diffusion (and broth dilution) was demonstrated by Fleming as early as 1929 [132]. Inspired by his famous observation that a substance produced by a *Penicillium* colony inhibited the growth of staphylococci, Fleming embedded a standardized amount of mould broth filtrate (denoted 'penicillin') in growth agars inoculated with various organisms, including '*Bacillus influenzae* (Pfeiffer)', and obtained inhibition zones of different sizes.

As a consequence of the emergence of ampicillin resistant *H. influenzae*, a disk diffusion method for susceptibility testing of this organism was approved by the National Committee for Clinical Laboratory Standards (NCCLS) (now CLSI) in 1986 [139]. The originally used LHB medium was replaced by HTM in 1990 [211]. In Europe, several disk diffusion methodologies were developed by different European national breakpoint committees; most were based on MHA or ISA [10]. The EUCAST methodology, with zone diameter breakpoints calibrated against EUCAST clinical MIC breakpoints, was launched in 2009 and is widely used, primarily in Europe [282]. EUCAST developed and recommend MH-F agar for susceptibility testing of *H. influenzae* [111,215].

Zone diameters are not comparable between various disk diffusion methodologies. In addition to being calibrated against different sets of breakpoints, major differences include different agar bases, different supplements for fastidious organisms (e.g. *H. influenzae*) and different inoculums (confluent or semi-confluent). The consistency of susceptibility categorization of *H. influenzae* to various antimicrobial agents by different test methodologies and interpretative guidelines was investigated in an

international collaborative quality assessment study [574]. Testing of 150 isolates at 15 laboratories in six European countries and the U.S. revealed significant discrepancies, especially for *bla* negative ampicillin resistant isolates.

The impact of different media for susceptibility testing of *H. influenzae* (*bla* negative isolates, n=143) to ampicillin by disk diffusion (10 µg) was investigated by Barry *et al.* [27]. Compared to HTM, larger inhibition zones were obtained with LHB-3, WHB, ISA (supplemented with 5% whole horse blood and 20 mg/L beta-NAD), and chocolate agar, resulting in underestimation of resistance. In contrast, Fuchs *et al.* obtained smaller zones with LHB-5, WHB and ISA compared to HTM when 206 isolates (including 61 *bla* positive) were tested with the same disk [140].

The performance of disk diffusion for susceptibility categorization depends on the correlation between inhibition zone and reference MIC, which may be expressed as the Pearson correlation coefficient (r) (perfect correlation, r=1). MIC-zone correlation varies between agents and with disk potency. Zerva *et al.* evaluated beta-lactam disks (HTM) for susceptibility testing of *H. influenzae* (n=300) with BMD (HTM) as the gold standard and observed superior correlation and interpretative accuracy with ampicillin 2 µg (r=0.94) compared to ampicillin 10 µg (r=0.90) [578]. Others have reported similar results [149,219,297]. Superiority of low-potency disks compared to disks with higher drug content for categorization of *H. influenzae* is also reported for amoxicillin-clavulanic acid 2-1 µg versus 20-10 µg [149,219]. Consistent with previous observations [386], Zerva *et al.* also reported poor MIC-zone correlation with imipenem 10 µg (r=0.41) and meropenem 10 µg (r=0.28), and the disks did not separate between wild-type isolates and isolates with increased MIC [578].

EUCAST has defined clinical zone breakpoints for most beta-lactams with MIC breakpoints [111]. In contrast to CLSI [75], EUCAST recommend low-potency disks for testing of ampicillin, amoxicillin-clavulanic acid and cefotaxime. MIC-zone correlations produced according to EUCAST recommendations are available at www.eucast.org/zone_diameter_distributions. Categorical agreement, error rates and FSR based on correlations for beta-lactams are shown in **Table 18**. The data indicate high FSR (>10%) for ampicillin, extended-spectrum cephalosporins and carbapenems.

Table 18 Performance of EUCAST disk diffusion based on MIC-zone correlations and interpretation according to EUCAST MIC and zone breakpoints [111]. Data from www.eucast.org/zone_diameter_distributions (accessed 2015-09-25)

Agents	Disk (µg)	n[a]	R or I/R[b] (n)	CA[c] (%)	mE[d] n (%)	ME[e] n (%)	VME[f] n (%)	FSR[g] (%)
Ampicillin	2	265	81	87.5	-	22 (8.3)	11 (4.2)	13.6
Amoxicillin-ca[h]	2-1	60	3	50.0	-	30 (50.0)	0 (0.0)	NA
Cefuroxime	30	217	32/85	81.1	38 (17.5)	2 (0.9)	1 (0.5)	1.2
Ceftibuten	30	165	15	95.8	-	5 (3.0)	2 (1.2)	13.3
Cefixime	5	148	15	97.3	-	3 (2.0)	1 (0.7)	6.7
Cefpodoxime	10	146	9/17	91.8	9 (6.2)	0 (0.0)	3 (2.1)	17.6
Cefotaxime	5	417	32	97.1	-	6 (1.4)	6 (1.4)	18.8
Ceftriaxone	30	60	6	81.7	-	9 (15.0)	2 (3.3)	33.3
Cefepime	30	148	22	93.2	-	1 (0.7)	9 (6.1)	40.1
Doripenem	10	133	14	90.2	-	1 (0.8)	12 (9.0)	85.7
Ertapenem	10	148	1	99.3	-	0 (0.0)	1 (0.7)	100.0
Imipenem	10	148	21	79.7	-	24 (16.2)	6 (4.1)	28.6
Meropenem[i]	10	154	0	100.0	-	0 (0.0)	0 (0.0)	NA

[a] Number of observations
[b] Intermediate or resistant by MIC
[c] Categorical agreement
[d] Minor error (resistant or susceptible by MIC and intermediate by disk diffusion, or intermediated by MIC and resistant or susceptible by disk diffusion)
[e] Major error (susceptible by MIC and resistant by disk diffusion)
[f] Very major error (resistant by MIC and susceptible by disk diffusion)
[g] False susceptible rate (VME divided by the number of resistant isolates)
[h] Amoxicillin-clavulanic acid (fixed)
[i] General breakpoints are used for interpretation. A total of 15 isolates were intermediately susceptible by MIC with meningitis breakpoints (zone breakpoint are not defined)

I am aware of only one previously published evaluation of EUCAST disk diffusion for susceptibility categorization of *H. influenzae* to beta-lactams, using reference MIC as the gold standard. Søndergaard *et al*. performed susceptibility testing to ampicillin, cefuroxime and cefpodoxime by disk diffusion and BMD (HTM) for 135 *bla* negative isolates, including 44 (33%) with the rPBP3 genotype [475]. Categorical agreement and error rates were not presented, but the authors reported that six isolates were ampicillin resistant by both disk diffusion and BMD, whereas 10% (i.e. 13 or 14

isolates) were resistant by BMD only. Thus, FSR by disk diffusion (ampicillin 2 µg; AMP2) was >50% (7/13 or 8/14).

For comparison, Zerva *et al.* tested 183 *bla* negative *H. influenzae* (including 22 ampicillin non-susceptible isolates) with AMP2 and HTM agar and found 96% (176/183) categorical agreement with BMD [578]. No isolates with MIC >1 mg/L were susceptible by disk diffusion, i.e. FSR was 0%. In a more recent multi-centre study, Kärpänoja *et al.* tested two ampicillin susceptible and three ampicillin resistant (MIC > 1 mg/L) *bla* negative control strains with AMP2 (HTM) [219]. Using the same interpretative criteria as Zerva *et al.* [578], the authors reported an overall FSR at 8% (sensitivity 92%), and the individual FSR for one ampicillin resistant strain (MIC = 8 mg/L) was 24%.

To my knowledge, no previously published study has compared EUCAST disk diffusion and gradient tests for susceptibility categorization of *H. influenzae*, with reference methodology as the gold standard.

Garcia-Cobos *et al.* performed susceptibility testing of 89 *H. influenzae* isolates to ampicillin and amoxicillin-clavulanic acid by EUCAST disk diffusion and Etest (HTM) [147]. The authors focused on genotype assignment and did not assess agreement with reference methodology for susceptibility categorization, although BMD MICs (HTM) were determined in a previous study [149].

In another study, Cherkaoui *et al.* compared results obtained by EUCAST disk diffusion and ampicillin Etest for 78 *bla* negative *H. influenzae* (nine rPBP3 isolates with Etest MIC = 1 mg/L, and 69 isolates of unknown resistance genotype with Etest MIC ≤0.5 mg/L) [63]. All isolates were negative by screening with the benzylpenicillin 1 unit disk (chapter 8.5). The authors reported discrepancy between Etest (susceptible) and disk diffusion (resistant) for seven rPBP3 isolates and 26 isolates of unknown genotype. Whether the discrepancy was due to false resistance by disk diffusion or false susceptibility by Etest is unknown, as MIC determination by reference methodology was not performed.

8.5 SCREENING FOR RESISTANCE MECHANISMS

Disk diffusion with interpretative criteria based on zone ECOFFs provides a useful tool for discrimination between wild-type isolates and isolates with acquired beta-lactam resistance. With a sufficiently sensitive screening method, agent-directed testing may be restricted to screening positive isolates.

Shortly after their discovery that most isolates with PBP3-mediated resistance have the N526K substitution (chapter 6.3.3), Ubukata and co-workers developed a screening algorithm based on four disks for categorization according to resistance genotype [529]. The authors tested 228 *H. influenzae* isolates (including 53 low-rPBP3, 29 high-rPBP3 and 32 bla_{TEM} positive; five isolates had both mechanisms) with six disks (HTM). Isolates were categorized as sPBP3 ('BLNAS') if susceptible by ampicillin 10 µg and cefaclor 30 µg, and as high-rPBP3 ('BLNAR') if resistant by cefpodoxime 10 µg and cefdinir 5 µg. Remaining isolates were categorized as low-rPBP3 ('low-BLNAR'). Using PCR-based genotyping as the gold standard, sPBP3 and high-rPBP3 isolates were identified with ≥95% accuracy. Interpretative criteria for *bla* positive isolates were not presented, and the fact that the proposed algorithm would categorize *bla* positive sPBP3 isolates as low-rPBP3 was not discussed.

Nørskov-Lauritsen *et al.* evaluated screening disks using ISA (with NAD and horse blood) and chocolate agar [350]. The authors identified 47 low-rPBP3 isolates by disk diffusion testing of 470 *H. influenzae* and *ftsI* sequencing of 147 isolates with non-wild-type zone for cefaclor 30 µg (CEC30), corresponding to an rPBP3 prevalence of 10%. CEC30 was superior to ampicillin 10 µg and cefuroxime 30 µg (CXM30) and identified rPBP3 isolates with sensitivities/specificities of 98%/99% (chocolate) and 96%/96% (ISA). The reported sensitivities and specificities may be biased as calculations were based on the assumption that the inclusion criteria for *ftsI* sequencing identified all rPBP3 isolates in the original population.

In the Nordic countries, the Swedish Reference Group for Antibiotics (SRGA) and the Norwegian Working Group on Antibiotics (NWGA) previously recommended phenoxymethylpenicillin 10 µg (PV10) and CEC30 as screening for beta-lactam resistance in *H. influenzae* (www.antibiotikaresistens.no, AFA/Brytningspunkter).

Following the launching of the EUCAST disk diffusion methodology in 2009 [282], the benzylpenicillin 1 unit (PG1) disk was introduced for screening in the first version of the NordicAST breakpoint table in 2010 (www.nordicast.org/aldre-versioner). Screening with PG1 was included in the EUCAST breakpoint table from 2012 (www.eucast.org/ast_of_bacteria/previous_versions_of_documents/). In a EUCAST validation of the screening method (breakpoint S≥12 mm), the disk correctly categorized 98% (102/104) of *bla* negative isolates according to the presence of 'PBP mutations', with a sensitivity of 98% (53/54) and specificity of 98% (49/50) [112].

For comparison, Søndergaard *et al.* evaluated the ability of seven disks (EUCAST methodology) to categorize *H. influenzae* isolates according to the presence of the N526K substitution [475]. The authors tested 135 *bla* negative isolates (including 44 rPBP3) and found 91% sensitivity and 99% specificity with PG1 and EUCAST screening breakpoints. With 33 *bla* positive isolates (including one rPBP3) added to the collection, three disks with *bla* stable agents had practically equal sensitivities/specificities (CEC30, 89%/90%; CXM30, 91%/90%; cefoxitin 30 µg (FOX30), 89%/92%).

In another investigation, Garcia-Cobos *et al.* tested two collections of *H. influenzae* isolates (54 *bla* negative, including 30 rPBP3; 35 *bla* positive, including 15 rPBP3) with 12 disks (not PG1) [147]. CXM30 was superior to CEC30 and FOX30 (sensitivity/specificity; *bla* negative, 83%/92%; *bla* positive, 93%/100%). Based on their results, the authors suggested a screening algorithm, based on the CXM30 disk.

Because PG1 does not separate between *bla* positive isolates with and without additional resistance mechanisms [111,112], NordicAST currently recommend CEC30 or CXM30 for screening of *bla* positive isolates [338]. Notably, cefaclor is less stable to *bla* compared to other cephalosporins (chapter 6.2.2), suggesting that separate screening breakpoints for *bla* positive and *bla* negative isolates may be warranted for this agent [338].

8.6 INTERPRETING DIFFICULTIES

Some characteristic phenomenons may complicate interpreting of agar-based methods for susceptibility testing of *H. influenzae*. These include synergistic effects between neighboring disks, double zones [350] and hazy growth within the inhibition zone [219,567] by disk diffusion, and regrowth at concentrations above MIC by agar dilution [567,568] and gradient tests (**Figure 13**). Mechanisms possibly contributing to these poorly understood observations are described in chapter 6.4.1.

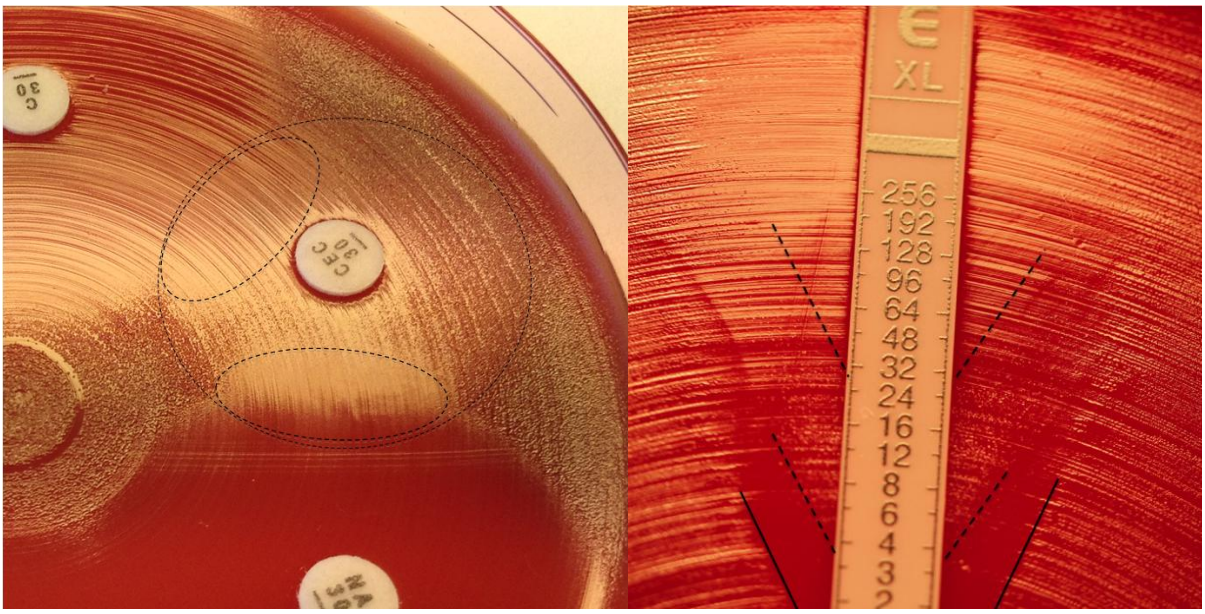


Figure 13 Phenomenons complicating interpreting of susceptibility testing of *H. influenzae* by disk diffusion (EUCAST) and Etest. Left, hazy growth within the zone (cefaclor, CEC30) and synergistic effects between CEC30 and chloramphenicol (C30), and between CEC30 and nalidixic acid (NA30). Right, inhibited growth at 2 mg/L (not shown) and regrowth at higher concentrations (4-32 mg/L; dashed lines) by amoxicillin-clavulanic acid (2:1) Etest (MH-F). Photography by Lene Haakensen, Norwegian Institute of Public Health, with permission

PART II. THE PROJECT

Idea

Study IV

Study III

Study II

Study I

www.cliparthut.com

9 THESIS AT A GLANCE

The idea (2006)

A cefotaxime^{R} *H. influenzae* isolated from nasopharynx in a child with ALL and AOM

Paper I (2010)

H. influenzae (n=46) from NORM 2004 (n=484) were characterized by *ftsI* sequencing, PBP3-typing, PFGE, and Etest. All isolates with non-*bla*-mediated resistance (n=23) were group II low-rPBP3 (prevalence ≥5%). Most rPBP3 isolates (83%) belonged to two clones. Clonality analysis suggested HGT of mutant *ftsI in vivo*.

Paper II (2014)

H. influenzae (n=196) from NORM 2007 (n=808) were characterized by *ftsI*/PBP3-typing, PFGE, MLST, and BMD MIC. Novel terminology (rPBP3/sPBP3) was introduced. MLST-*ftsI* typing was established. The prevalence of PBP3-mediated ampicillin^{R} was 9%. The rPBP3 prevalence was 15%. Group II predominated (96%). Four MLST-*ftsI* types accounted for 61% of rPBP3 isolates. The first group III rPBP3 isolate from Northern Europe was reported. Clonality analysis supported a contribution of HGT to the evolution of rPBP3 strains.

Paper III (2014)

High-rPBP3 *H. influenzae* (group III, n=23; group III-like, n=7) from Norwegian routine laboratories (2006-2013) were characterized by *ftsI*/PBP3-typing, MLST, and BMD MIC. Extended-spectrum cephalosporin^{R} was frequent (47%-97%). Ceftriaxone^{R} was restricted to L389F positive isolates (n=16). The suffix '(+)' was introduced for this genotype. Clonal spread was evident. An outbreak (n=3) with a group III(+) MDR strain (bla_{TEM} positive and resistant to four non-beta-lactams) was described. The first invasive group III(+) isolate from Europe was reported.

Paper IV (2015)

H. influenzae (n=154) (*bla* negative, 68% low-rPBP3) from Study II were used to evaluate screening disks for detection of rPBP3 isolates, and to evaluate Etest and EUCAST disk diffusion for testing of beta-lactam susceptibility. The PG1 and CXM5 disks identified rPBP3 with the highest accuracies. False susceptibility rates were high with ampicillin Etest (88%) and the AMP2 disk (EUCAST breakpoints, 77%; adjusted breakpoints, 28%). A comment recommending high-dose aminopenicillin therapy in severe infections with screening-positive isolates susceptible to aminopenicillins by gradient or disk diffusion was suggested.

10 PAPERS

I. Skaare D, Allum AG, Anthonisen IL, Jenkins A, Lia A, Strand L, Tveten Y, Kristiansen BE

Mutant *ftsI* genes in the emergence of penicillin-binding protein-mediated beta-lactam resistance in *Haemophilus influenzae* in Norway

Clin Microbiol Infect. 2010 Aug;16(8):1117-24. doi: 10.1111/j.1469-0691.2009.03052.x. Epub 2009 Sep 8. PMID: 19737286 [464]

II. Skaare D, Anthonisen IL, Caugant DA, Jenkins A, Steinbakk M, Strand L, Sundsfjord A, Tveten Y, Kristiansen BE

Multilocus sequence typing and *ftsI* sequencing: a powerful tool for surveillance of penicillin-binding protein 3-mediated beta-lactam resistance in nontypeable *Haemophilus influenzae*

BMC Microbiol. 2014 May;14:131. doi: 10.1186/1471-2180-14-131. PMID: 24884375 [465]

III. Skaare D, Anthonisen IL, Kahlmeter G, Matuschek E, Natås OB, Steinbakk M, Sundsfjord A, Kristiansen BE

Emergence of clonally related multi-drug resistant *Haemophilus influenzae* with penicillin-binding protein 3-mediated resistance to extended-spectrum cephalosporins in Norway 2006 – 2013

Euro Surveill. 2014 Dec;19(49). pii: 20986. PMID: 25523969 [466]

IV. Skaare D, Lia A, Hannisdal A, Tveten Y, Matuschek E, Kahlmeter G, Kristiansen BE

***Haemophilus influenzae* with non-beta-lactamase-mediated beta-lactam resistance: easy to find but hard to categorize**

J Clin Microbiol. 2015 Nov;53(11):3589-95. doi: 10.1128/JCM.01630-15. Epub 2015 Sep 9. PMID: 26354813 [467]

11 OBJECTIVES AND RESEARCH QUESTIONS

The overall objectives of this project were to:

i) Elucidate the resistance mechanisms and the molecular epidemiology of Norwegian *H. influenzae* with non-*bla*-mediated beta-lactam resistance

ii) Evaluate and improve methods and algorithms for *in vitro* susceptibility testing of *H. influenzae* to beta-lactam antibiotics

The major research questions were (studies/papers in brackets):

- How important is PBP3-mediated resistance (rPBP3) for the increased prevalence of *H. influenzae* with non-*bla*-mediated beta-lactam resistance in Norway? (I-II)
- Which rPBP3 genotypes occur in Norwegian *H. influenzae*? (I-III)
- What is the correlation between rPBP3 genotypes and phenotypic susceptibility profiles? (II-III)
- How clonal are rPBP3 *H. influenzae*? (I-III)
- Does horizontal gene transfer with recombinational exchange of mutant *ftsI* genes contribute to the development of rPBP3 strains? (I-III)
- Are there any associations between phylogeny, rPBP3 genotypes and pathogenicity? (II-III)
- Which screening disk is the most reliable for detection of rPBP3 isolates? (IV)
- How reliable are routine methods (disk diffusion and gradient tests) for susceptibility categorization of *H. influenzae* to beta-lactams? (IV)

12 STUDY DESIGN

Study I Cross-sectional epidemiologic and laboratory study

Study II Cross-sectional epidemiologic and laboratory study

Study III Longitudinal epidemiologic and laboratory study with case reports

Study IV Methodology evaluation (diagnostic) study

13 MATERIALS AND METHODS

13.1 BACTERIAL ISOLATES

13.1.1 *Study I*

A total of 46 *H. influenzae* isolates were included in Study I [464]. The isolates were selected from a nationwide surveillance population of consecutive respiratory, eye and ear isolates (n=484), collected as part of standard health care in January – March 2004 (NORM 2004) [341]. Detailed information on primary sampling, characterization and susceptibility testing is available in the surveillance report [341]. Selection of isolates was based on susceptibility data reported by the primary laboratories. Inclusion criteria were constructed to encompass all isolates resistant to benzylpenicillin and non-susceptible to aminopenicillins (according to 2004 breakpoints), and an equal number of susceptible control isolates (**Figure 14**).

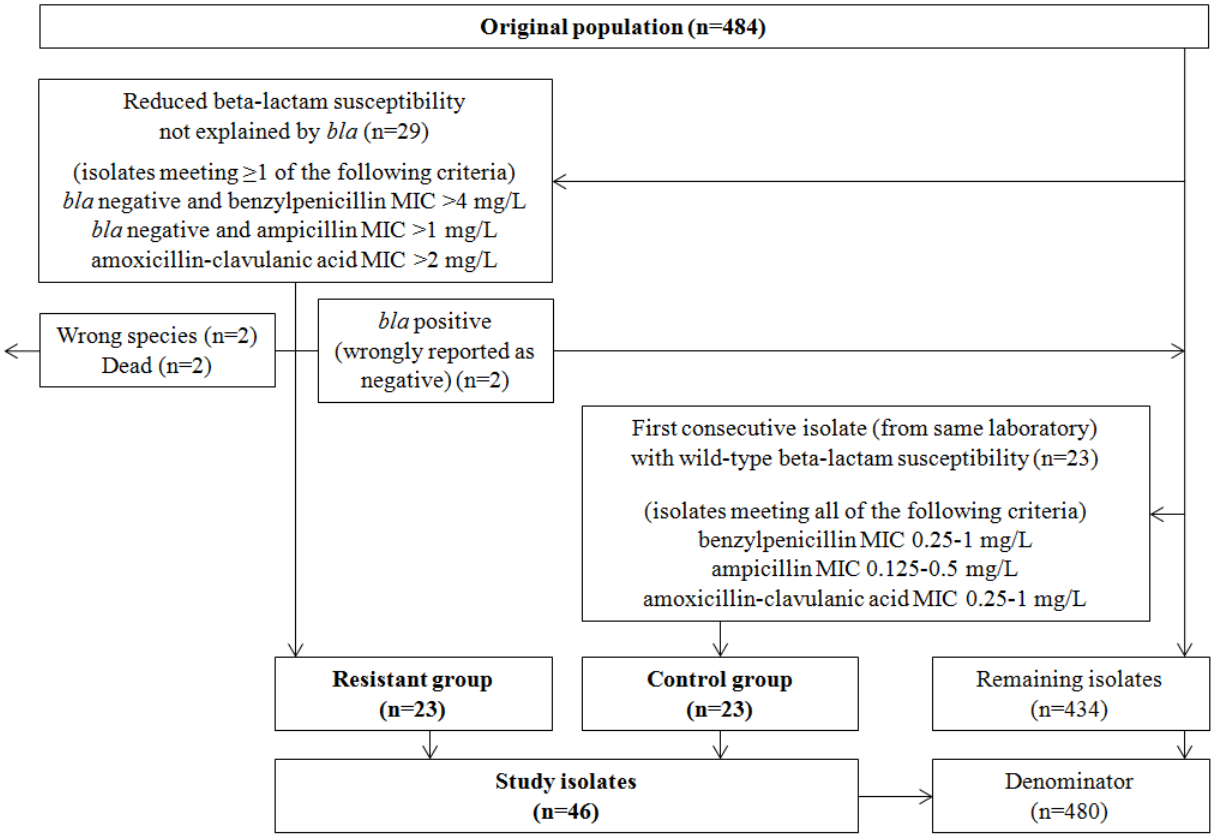


Figure 14 Flowchart showing selection and inclusion of isolates for Study I [464]

13.1.2 *Study II*

A total of 196 *H. influenzae* isolates were included in Study II [465]. The isolates were selected from a nationwide surveillance population of consecutive respiratory, eye and ear isolates (n=808) collected as part of standard health care in January and February 2007 (NORM 2007) [341]. Detailed information on primary sampling, characterization and susceptibility testing is available in the surveillance report [341]. An overview of the inclusion procedure for Study II is presented in **Figure 15**.

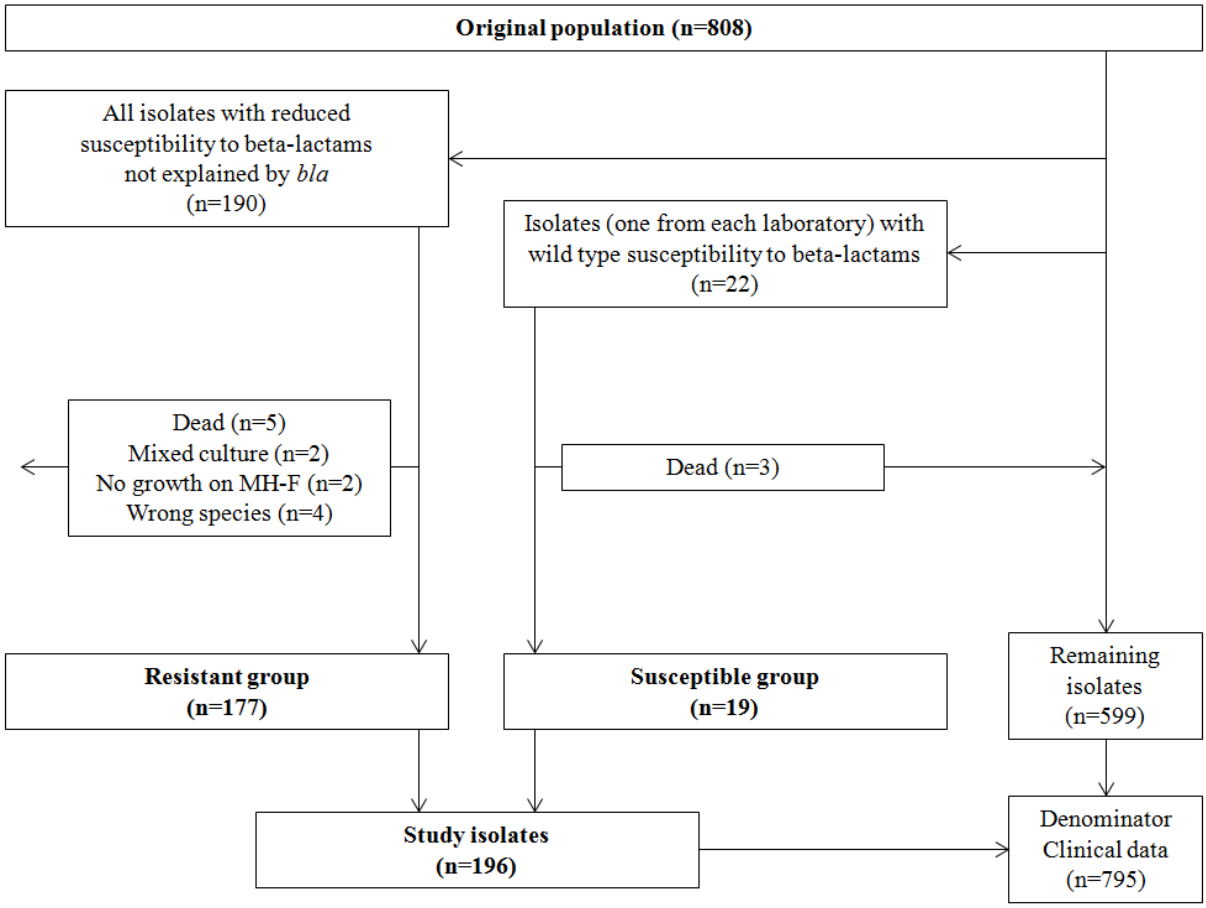


Figure 15 Flowchart showing selection and inclusion of isolates for Study II [465]

Selection of isolates was based on the susceptibility data reported by the primary laboratories. Inclusion criteria were constructed to identify all isolates with reduced beta-lactam susceptibility not explained by *bla*. The exact criteria for the R-group were cefaclor (30 µg) zone <17 mm, amoxicillin-clavulanic acid (2:1) MIC >1 mg/L, cefuroxime MIC >2 mg/L, *and/or* cefotaxime MIC >0.06 mg/L (all isolates); phenoxymethylpenicillin (10 µg) zone <13 mm, ampicillin (2 µg) zone <16 mm,

and/or ampicillin MIC >0.5 mg/L (*bla* negative isolates). One isolate with wild-type susceptibility from each laboratory (randomly selected from the remaining isolates) was included in the S-group (control isolates).

Four selected isolates were identified as other species than *H. influenzae (H. parainfluenzae*, n=3; *H. haemolyticus*, n=1) and excluded from the study. Clinical data for the complete population (except 13 patients and isolates excluded from the R-group) was included in the statistical analyses (n=795).

13.1.3 *Study III*

A total of 30 high-rPBP3 *H. influenzae* from Norway, isolated between May 2006 and July 2013, were included in Study III [466] **(Figure 16**).

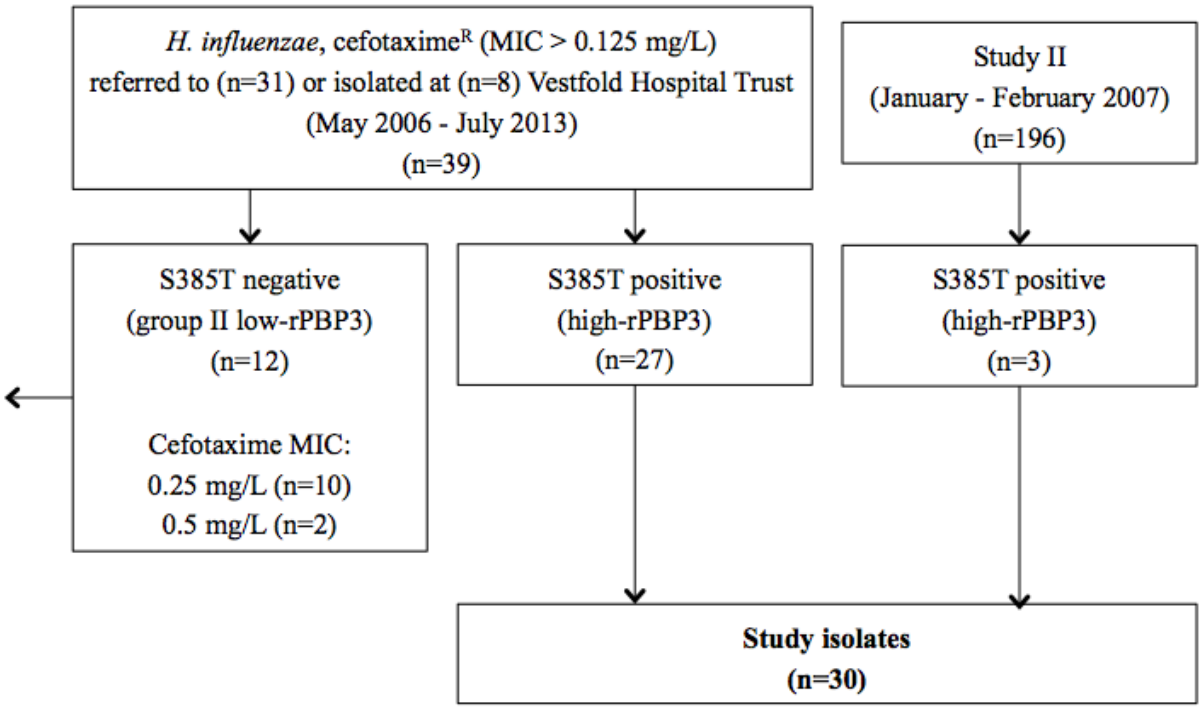


Figure 16 Flowchart showing selection and inclusion of isolates for Study III [466]

The only inclusion criterion was presence of the S385T substitution. Three isolates were recruited from Study II [465]. The remaining 27 isolates were included based on results from *ftsI* sequencing of *H. influenzae* isolated at Vestfold Hospital Trust or referred from Norwegian routine laboratories, according to recommendations from NWGA (from 2007) and NordicAST (from 2010), stating that *H. influenzae* resistant

to extended-spectrum cephalosporins be sent to reference laboratory [338]. This service was established at Vestfold Hospital Trust in 2007 and is offered to Norwegian routine laboratories in agreement with the *Haemophilus* Reference Laboratory at the Norwegian Institute of Public Health and the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-Res).

13.1.4 *Study IV*

A total of 154 isolates (sPBP3, n=50; rPBP3, n=104) from Study II were included in Study IV [467]. Inclusion criteria were constructed to encompass i) *bla* negative group II low-rPBP3 isolates, and ii) sPBP3 isolates with wild-type BMD MICs to all beta-lactams tested. Study II isolates with the following characteristics (n=42) were excluded from Study IV:

- *bla* positive (n=16)
- rPBP3 other than group II (n=5)
- sPBP3 and cefuroxime MIC = 2 mg/L (wild-type MIC but intermediately susceptible according to EUCAST breakpoints [111]) (n=9)
- sPBP3 and MIC above ECOFF for ≥1 beta-lactam (n=13) (see **Table 28**)

13.1.5 *Reference strains*

H. influenzae reference strains used in the project are presented in **Table 19**.

Table 19 *H. influenzae* reference strains

Strain	Characteristics	Purpose	References
Rd KW20[a]	Species type strain	DNA reference sequence	[131,556]
ATCC 49766	sPBP3	Quality control (MIC)	[75,113]
ATCC 49247	Group II low-RPBP3	Quality control (MIC)	[75,113]
ATCC 35056	bla_{TEM} positive	Quality control (*bla* inhibition)	
NCTC 8468	sPBP3	Quality control (zone)	[113]

[a] ATCC 51907; GenBank:U32793

13.2 CLINICAL DATA

Demographic data and sampling information are registered as part of the NORM surveillance system and were readily available for patients and isolates included in Study I [464] and Study II [465].

For patients and isolates included in Study III [466], demographics, sampling information and brief clinical information (as provided by the clinician) were acquired from the primary laboratories. Three patients recently hospitalized with X-ray confirmed pneumonia most likely caused by study isolates were selected for clinical case reports. For these patients, relevant supplementary information about diagnosis, antimicrobial therapy and clinical outcome was extracted from the medical records. Additional information was achieved through phone interviews with the patients.

Basic clinical data for the patients and isolates included in this project is summarized in **Table 20**.

Table 20 Demographics and clinical data

Parameter	Study I [464]	Study II [465]	Study III [466]
Patients[a]	46	196	30
Denominator[b]	480	795	Not applicable
Laboratories[c]	15	22	10
Isolate from			
Respiratory tract	42 (91%)	110 (56%)	24 (80%)
Ear	4 (9%)	24 (12%)	3 (10%)
Eye	-	53 (27%)	2 (7%)
Blood	-	-	1 (3%)
No data	-	9 (5%)	-
Patient characteristics			
Median age (range)	30 (0-86)	5 (0-86)	29 (0-91)
Male/female ratio	0.7	1.0	0.8
Hospitalized	19 (41%)	60 (31%)	10 (33%)

[a] Corresponds to the number of study isolates (one isolate per patient)
[b] Size of the original surveillance population after exclusion (**Figures 15-16**)
[c] Number of primary laboratories contributing with study isolates

13.3 ETHICS

The bacterial isolates and patient information used in Study I, Study II and Study IV were collected as part of the Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM) (www.antibiotikaresistens.no). The use of NORM data in this project is warranted in Norwegian law (http://lovdata.no, FOR-2003-11-14-1353) and no further ethical approval was required.

The use of isolates and data in Study III was approved by the Regional Committees for Medical and Health Research Ethics in Norway (reference 2014/411). Written consent was provided for the patients selected for clinical case reports.

13.4 LABORATORY METHODS

13.4.1 *Overview*

An overview of laboratory methods used in this project, including information on manufacturers, is presented in **Table 21**.

13.4.2 *Susceptibility testing*

BMD MICs were determined by in-house prepared serial dilutions in Study II [465] and with commercial dried trays for reconstitution in Study III [466]. BMD MICs were imported to the EUCAST database (www.eucast.org/MIC_distributions) in June 2010 (Study II) and March 2014 (Study III).

Etest was used for MIC determination in Study I [464] and evaluated with previously determined BMD MICs as the gold standard in Study IV [467]. Study IV also evaluated susceptibility categorization by disk diffusion according to EUCAST methodology [282], and the proficiency of nine disks to screen for isolates with the rPBP3 genotype.

Details regarding guidelines, media and manufacturers are specified in Table 21.

Table 21 Laboratory methods

Procedure	Study I [464]	Study II [465]	Study III [466]	Study IV [467]
Species identification	XV (Oxoid, UK)	XV (Oxoid, UK); *ompP6* and 16S rRNA PCR [465,488]; 16S rRNA sequencing[a] [420]	XV (Oxoid, UK); API NH (bioMérieux, France); MALDI-TOF (Bruker, Germany)	Performed previously [465]
Biotyping	No	No	API NH (bioMérieux, France) [226]	No
Serotyping	SAST (Remel, UK)	*bexA* and *cap*[a] PCR [119,465]	*cap* PCR [119,466]	Performed previously [465]
Molecular typing	PFGE [84,464]	PFGE[a] [84,464]; MLST [289]	PFGE[a] [84,464]; MLST [289]	Performed previously [465]
Gene sequencing	*ftsI* (1010-1719) [464]	*ftsI* (1010-1719) [464]	*ftsI* (1010-1719) [464]; *gyrA* and *parC* (QRDR)[a] [370]	Performed previously [465]
Beta-lactamase detection	Nitrocefin (Oxoid, UK)	Acidimetric agar[a] [260]; bla_{TEM} and bla_{ROB} PCR[a] [84,464]	Acidimetric agar [260]; bla_{TEM} and bla_{ROB} PCR [84,464]	Performed previously [465]
BMD MIC	No	HTM (Oxoid, UK)[b] [75][c]	HTM (TREK, UK)[d] [75] or MH-F (BD, U.S.)[d] [107,109]	Performed previously [465]
Gradient MIC	Etest (AB Biodisk, Sweden) w/ supplemented[e] MHA (BD, U.S.)	No	No	Etest (bioMérieux, France) w/ HTM (Oxoid, UK)
Disk diffusion	No	No	No	EUCAST methodology [111] w/ disk and media from Oxoid, UK
Breakpoints	EUCAST [111]	EUCAST [111]	EUCAST [111]	EUCAST [111]; CLSI [75]

[a] Selected isolates; [b] In-house prepared serial dilutions; [c] With modifications (fixed concentrations of *bla* inhibitors); [d] Commercial dried trays; [e] 1% haemoglobin and 1% IsoVitaleX (BD, U.S.)

13.4.3 *New and modified molecular tests*

Several molecular tests were designed particularly for the project by dr. scient. Inger Lill Anthonisen (Vestfold Hospital Trust). In addition, some previously described tests were used with modifications. Details are specified in **Table 22**.

Table 22 New and modified primers and primes used in this project

Name	Function	Target	Sequences (5′ to 3′)[a]	Original[b]	Reference
SSNF2	F-primer	*ftsI*	CCTTTCGTTGTTTTAA CCGCA	-	[464]
KTGR2	R-primer	*ftsI*	AGCTGCTTCAGCATC TTG	-	[464]
*bexA*Fb	F-primer	*bexA*	CGTTTATRTGATGTTG ATCCTGA	HI-1	[119]/[465]
*bexA*Rb	R-primer	*bexA*	TGTCCATATCTTCAAA ATGGTG	HI-2	[119]/[465]
*bexA*P	Probe	*bexA*	FAMATGCAAGYCGRG CTTTCATCCCTG-BHQ	-	[465]
Hinf_fR	R-primer	*cap* (f)	GGTACTATCAAGTCC AAATC	f1	[119]/[465]
Hinf_eR2	R-primer	*cap* (e)	CTAATTGTTCTTTCTG TCTA	-	[465]
*ompP6*P	Probe	*ompP6*	ACGTGGTACACCAGA ATACAACATCGA	-	[465]
H16SP	Probe	16S rRNA	TCGCTCCACCTCGCA GCTTCGCT	-	[465]
TEMP	Probe	bla_{TEM}	CAGCTCCGGTTCCCA ACGATCAAG	-	[465]
ROBP	Probe	bla_{ROB}	TAGCGACAACAGCGC GACCAATTTG	-	[465]
e1.1[c]	F-primer	*cap* (e)	TTTGGTAACGAATGT AGTGGTAG[c]	e1	[119]/[466]
e2.1[c]	R-primer	*cap* (e)	ATAGCTTTACTGTATA AGTCTTAG[c]	e2	[119]/[466]

[a] Underscored, sites of modification

[b] Original designation (in cases of modification)

[c] Modified primers designed at the *Haemophilus* Reference Laboratory at the Norwegian Institute of Public Health

13.4.4 *DNA sequence analyses*

Sequencing of gene fragments was performed at GATC Biotech AG (www.gatc-biotech.com), with the exception of MLST (performed at the Norwegian Institute of Public Health). Gene sequences were deposited in the EMBL-EBI Nucleotide Sequence Database (www.ebi.ac.uk) (**Table 23**).

Table 23 Accession numbers[a] for gene sequences produced in the respective studies

Gene	Study I [464]	Study II [465]	Study III [466]
ftsI	FM161990, FM163633 - FM163679	HG818627 - HG818822	HG983286 - HG983315
gyrA	-	-	HG983316 - HG983320
parC	-	-	HG983321 - HG983325

[a] EMBL-EBI Nucleotide Sequence Database (www.ebi.ac.uk)

Sequences were analyzed using Lasergene software (DNASTAR, www.dnastar.com). Amino acid substitutions were deduced from comparison with the sequences of *H. influenzae* Rd KW20 [131] (**Table 19**).

Phylogenetic analysis of *ftsI* gene sequences (nucleotides 1010–1719) was performed by construction of UPGMA (unweighted pair group method with arithmetic mean) phylograms by distance methods using ClustalW2 (www.ebi.ac.uk) and displayed using Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) and TreeDyn (www.phylogeny.fr) softwares. *H. influenzae* Rd KW20 was used as reference and *H. parainfluenzae* (EMBL:AB267856) as outgroup.

13.4.5 *Epidemiological typing*

Dendrograms of PFGE band patterns were constructed using GelCompar II software (Applied Maths, www.applied-maths.com), Dice coefficients and the UPGMA algorithm. Clusters of related or possibly related isolates were identified according to degree of similarity and comparison of band patterns according to the Tenover criteria [502] (chapter 3.4).

MLST was performed according to the scheme constructed by Meats *et al.* [289]. Allelic profiles were analyzed using eBURSTv3 software, with six shared alleles for definition of clonal complexes (chapter 3.6). Based on data from this project and previous investigations, STs were assigned to phylogenetic division I or II [289], WGS clades [88], and MLSA-based 'phylogroups' (Clades 1-13 and eBURST group 2) [102] (chapters 3.6-3.8),

Assessment of clonality was carried out by combined analyses of PBP3 types, *ftsI* alleles, PFGE band patterns and/or MLST allelic profiles. The novel concepts of MLST-PBP3 typing and MLST-*ftsI* typing as tools for molecular surveillance of rPBP3 strains of *H. influenzae* were established and validated (see chapter 14.3.2).

13.5 STATISTICS

Descriptive statistics were used in the two cross-sectional epidemiological studies (Study I [464] and Study II [465]), and in the longitudinal study (Study III [466]). Analysis and presentation of data focused on beta-lactam susceptibility rates, rPBP3 prevalence and distribution of rPBP3 genotypes, the correlation between rPBP3 genotypes and phenotypic resistance, and the dynamics of rPBP3 clones.

Comparison of rates, frequencies and proportions in Study II [465] was performed with calculation of significance levels by Fisher's exact probability test. Multivariate logistic regression analysis was performed to search for associations between resistance genotypes and various clinical characteristics. Predictive Analytics Software (PASW) Statistics v17.0 (IBM Corp., www.ibm.com) was used for statistical analyses.

A qualitative approach was used for analysis of clinical data for the case reports in Study III [466], with emphasis on infection type, co-morbidity, antimicrobial therapy (agents and doses) and clinical outcome.

In the diagnostic study (Study IV) [467], the performance of Etest was evaluated using continuous (MIC) and categorical data (S-I-R). Essential and categorical agreement with reference MIC (BMD) at different MIC levels was visualized by plotting BMD MIC at the x-axis and Etest MIC (log difference) at the y-axis. This approach is a

modification of the Bland-Altman plot for comparison of two tests (mean at the x-axis and log difference at the y-axis).

Agreement rates, categorical errors and false susceptible rates by disk diffusion and Etest were calculated with BMD MIC interpretead according to current clinical breakpoints as the gold standard. Test performances by the two methods were compared and significance levels were calculated using Chi-square test and MedCalc software (www.medcalc.net).

Receiver operating characteristic (ROC) analysis and calculation of test performance parameters (sensitivity, specificity, predictive values) with optimized screening breakpoints was used to evaluate and compare the ability of screening disks to identify isolates with the rPBP3 genotype. Positive and negative predictive values (PPV and NPV, respectively) at a prevalence representative for a Norwegian population were calculated using Bayes' theorem [205] and the following formulas:

$$PPV = \frac{\text{sensitivity x prevalence}}{\text{sensitivity x prevalence} + (1 - \text{specificity}) \text{ x } (1 - \text{prevalence})}$$

$$NPV = \frac{\text{specificity x } (1 - \text{prevalence})}{(1 - \text{sensitivity}) \text{ x prevalence} + \text{specificity x } (1 - \text{prevalence})}$$

Finally, descriptive statistics were used for presentation of supplemental disk diffusion data. Histograms were used to show MIC-zone and genotype-zone correlations; a scatter plot was used to show zone-zone correlations [467].

14 RESULTS AND DISCUSSION

14.1 CHARACTERIZATION OF STUDY ISOLATES

14.1.1 *Species identification*

Correct species identification of *H. influenzae* may be challenging (chapter 2). By confirmatory molecular species identification, ten of 196 isolates in Study II were positive by *ompP6* PCR and negative by 16S PCR [465,488]; the isolates were confirmed to be *H. influenzae* by MLST and 16S rRNA sequencing [420]. Conversely, conventional methods revealed that two of 52 isolates selected for Study I (**Figure 14**) [464], and four of 212 isolates selected for Study II (**Figure 15**) [465], belonged to other species. In other words, 2.3% (6/264) of isolates selected from the NORM database were not *H. influenzae*.

Four *H. parainfluenzae* were distinguishable from *H. influenzae* by being dependent on factor V but not factor X. In addition, one XV-dependent isolate selected for Study II (from the ear of a three-year old) expressed beta-haemolysis by satellite test and was identified as presumptive *H. haemolyticus*. Species identification of this isolate has later been confirmed by MALDI-TOF (Bruker) (A++, score >2.1, repeated analysis).

Notably, characterization by MLST (with detection of the *fucK* gene) revealed no non-haemolytic *H. haemolyticus* among the 196 isolates included in Study II, neither among the 30 isolates (identified by conventional methods and MALDI-TOF) included in Study III (**Figure 16**) [466]. Thus, the frequency of *H. haemolyticus* among XV-dependent *Haemophilus* isolates in this project was considerably lower than reported by Murphy *et al.* [321] but in accordance with more recent reports [126,280,579].

These observations indicate that misidentification of *H. haemolyticus* as *H. influenzae* by conventional methods is infrequent in clinical samples from eye, ear and the respiratory tract, and illustrate the usefulness and high reliability of such methods and MALDI-TOF (with updated database) for identification of *H. influenzae* in routine laboratories. The 16S PCR assay [488] used for identification in Study II is unreliable.

14.1.2 *Capsular serotypes and biotypes*

All isolates in this project were sampled from non-invasive locations, except one blood isolate in Study III (**Table 20**). As expected for collections of non-invasive *H. influenzae*, almost all were NTHi (98.9%, 369/372). Two Hif isolates (from ear and respiratory tract) were included in Study II [465], and one Hif isolate (from ear) was included in Study III [466]. It should be noted that the method used for capsular serotyping in Study I (SAST) may produce false negative results, and *bexA*-deficient Hib isolates (Hib$^-$) (chapter 2.8) would not be detected by the approach used in Study II (screening with *bexA* PCR).

The 30 isolates in Study III were assigned to biotypes I (n=3), II (n=4), III (n=14) and IV (n=9) [466]. Within a clonal group of ten ST1197 isolates, six had biotype III reaction patterns and four were assigned to biotype IV. Similarly, among a clonal group of four ST836 isolates, biotypes III and IV accounted for two isolates each. The two biotypes differ by the ornithine decarboxylase (ODC) reaction (**Table 3**). As described in chapter 2.7, false positive and negative ODC results are frequent [104,105]. However, ODC reactions for the isolates in the two clones described above were reproducible and easy to interpret, suggesting that different test results reflect true differences in ODC enzyme activity. Variations in phenotypic expression of ODC activity may be related to several regulatory mechanisms [258]. The observations suggest that biotyping has poor epidemiological value.

14.1.3 *Phylogenetic analyses*

Phylogenetic analysis of the isolates belonging to the ten most frequent STs in Study II [465] is summarized in **Table 24**, with assignment to MLSA clade [102], WGS clade [88] and phylogenetic division [289] deduced from MLST or PFGE (**Figure 17**). Seventy STs were represented; 15 were novel (ST1190-ST1204) [465]. Ten STs had >3 representatives and accounted for 58% (114/196) of the isolates. Seven of the ten isolates negative by 16S rRNA PCR (chapter 14.1.1) were ST425 (n=5) and ST124 (n=2). By eBURST analysis [125], the 70 STs were grouped into 39 clonal complexes (CC) and three singletons. Ten of 14 previously described MLSA clades [102] were

represented; 69% of the isolates belonged to Clade 13 (n=59), eBURST group 2 (n=50) and Clade 9 (n=26), whereas no isolates belonged to Clade 3 (Hib), Clade 4 (*H. aegyptius*), Clade 5 (Hib) and Clade 7 (Hid). Nine of the ten most frequent STs belonged to phylogenetic division I and to WGS clades II, III, IV and V. Phylogenetic division II was represented by two ST124 Hif isolates, belonging to WGS Clade I.

PFGE analysis of the 46 isolates in Study I [464] and the 177 isolates in the R-group in Study II identified 24 clusters and 21 singletons (**Figure 17**). Eight clusters counted >5 isolates. In Study I, 28 isolates (61%) were assigned to clusters 1 (n=12), 2 (n=7), 3A (n=3), 6C (n=3) and 4 (n=3). PFGE clusters 6A and 3B, both among the four most frequent in Study II (2007), were not represented in Study I (2004).

As expected, pulsotypes varied within STs. Conversely, most isolates with the same pulsotypes shared the same ST, but several examples of isolates with identical pulsotypes and different STs were observed. PFGE merged CC-ST201 and CC-ST503; the clusters otherwise corresponded well to MLST CCs. Notably, PFGE Cluster 6B, largely corresponding to CC-ST12, also comprised one ST180 (CC-ST3) and one ST14 (CC-ST14) isolate. The STs belong to WGS Clade V (**Table 24**), indicating that PFGE is able to trace relationship not detected by housekeeping phylogeny.

Table 24 Phylogenetic characterization of isolates in Study II [465] (ten most prevalent STs)

ST [289]	n	PFGE[a]	CC [125]	MLSA [102]	WGS [88]	Division [289]
ST367	29	6A	CC-ST3	eBURST gr 2	V[b]	I[b]
ST396	16	2	CC-ST396	Clade 9[b]	III	I[c]
ST201	15	3A	CC-ST201	Clade 13	II[d]	I[d]
ST159	12	3A	CC-ST503	Clade 13	II[b]	I[c]
ST14	11	1	CC-ST14	eBURST gr 2	V	I
ST12	8	6B	CC-ST12	None	V	I
ST395	8	3B	CC-ST395	Clade 13[b]	II	I[c]
ST57	6	7,8,9	CC-ST57	Clade 8	IV	I
ST425	5	20	CC-ST425	No data	No data	No data
ST2	4	No data	CC-ST2	None	IV	I

[a] Associated cluster. Some isolates of the respective STs were assigned to other PFGE clusters
[b] Indirect assignment via another ST assigned to the same CC
[c] Indirect assignment via assignment to WGS clade
[d] Indirect assignment via assignment to PFGE cluster

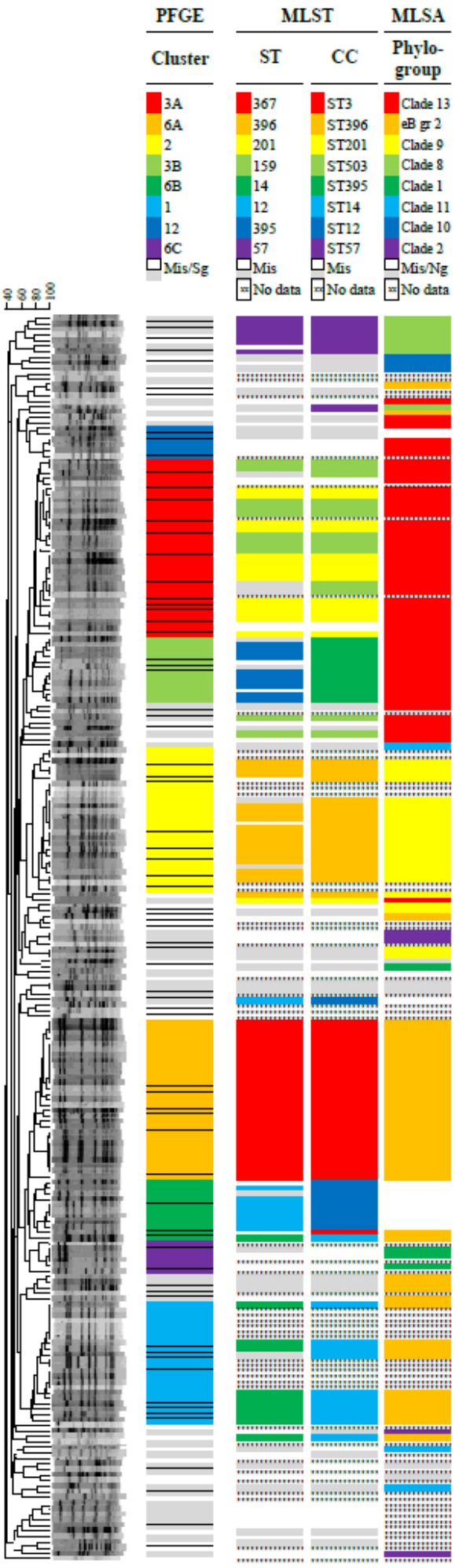


Figure 17 UPGMA dendrogram of band patterns for the 46 isolates in Study I [464] and the 177 isolates in the R-group in Study II [465] showing the correlation between PFGE (pulsotype and cluster) and housekeeping phylogeny (not performed for Study I isolates; indicated as 'No data'). MLSA phylogroups are based on phylogenetic analysis of concatenated MLST sequences by Erwin *et al.* [102]. PFGE clusters of related or possibly related isolates based on analysis of band patterns and Dice coefficient of similarity are indicated by colours (similarity coefficients 71%-76%). Horizontal lines separate different pulsotypes within each cluster. The colour scales indicate relative frequencies of the eight most prevalent PFGE clusters, STs, CCs and phylogroups in Study II. eB gr2, eBURST group 2; Mis, miscellaneous; Sg, singletons; Ng, no phylogroup. An error in CC-ST57 occurring in the previously published figure [465] has been corrected

Table 25 shows the phylogenetic characteristics for the 30 isolates included in Study III [466]. Four WGS clades, five MLSA clades, eight CCs and 12 STs were represented, including two novel STs (ST1282 and ST1287).

Although only one of the ten most frequent STs in Study II (ST159) was represented in Study III, there were obvious phylogenetic similarities on a superior level: two of the three most prevalent STs in Study III belonged to CCs occurring frequently in Study II (CC-ST395 and CC-ST503); except for WGS Clade III being absent, all Study III isolates that could be assigned to a WGS clade belonged to the same clades as the ten most frequent STs in Study II; and phylogenetic division I predominated in both studies. Finally, phylogenetic division II and WGS Clade I were represented through ST124 Hif isolates in both studies.

The phylogenetic characteristics of the two strain collections illustrate that NTHi are genetically diverse, but that most isolates belong to a limited number of lineages (chapter 3.9). The observations in this project also show that the fundamentally different typing methods PFGE, MLST and WGS are complimentary and may be combined to determine phylogenetic relationship at different resolution levels.

Table 25 Phylogenetic characterization of isolates (n=30) in Study III [466]

ST [289]	n	CC [125]	MLSA [102]	WGS [88]	Division [289]
ST1197	10	CC-ST395	Clade 13[a]	II[a]	I[b]
ST159	4	CC-ST503	Clade 13	II[a]	I[b]
ST836	4	CC-ST245	Clade 1[a]	V	I[a]
ST155	3	CC-ST155	Clade 13	II	I[b]
ST422	2	CC-ST422	Clade 10[a,c]	IV[c]	I[a]
ST124	1	CC-ST124	Clade 2	I	II[a]
ST142	1	CC-ST142	None	V	I[b]
ST148	1	CC-ST245	Clade 1	V[a]	I[a]
ST160	1	CC-ST160	None	V	I[b]
ST408	1	CC-ST3	eBURST gr 2[a]	V[a]	I[a]
ST1282	1	CC-ST503	Clade 13[a]	II[a]	I[b]
ST1287	1	None	No data	No data	No data

[a] Indirect assignment via another ST assigned to the same CC
[b] Indirect assignment via assignment to WGS clade
[c] Discrepancy between MLSA and WGS phylogeny (see **Figure 5**)

14.2 RESISTANCE MECHANISMS AND EPIDEMIOLOGY

14.2.1 *Terminology and categorization*

A novel terminology for *H. influenzae* with/without genetically confirmed PBP3-mediated resistance to beta-lactams (rPBP3/sPBP3) was introduced in Paper II [464] and later used in Paper III [466], Paper IV [467], and in this thesis. The rationale is presented in detail in chapter 6.3.4. In short, the designations rPBP3 and sPBP3 are equivalent to the traditionally used designations gBLNAR/gBLPACR and gBLNAS/gBLPAR, respectively, but differ in that they i) do not include information on beta-lactamase status, ii) may be used independently of guidelines for interpretation of MICs to ampicillin and amoxicillin-clavulanic acid, and iii) do not give the false impression that PBP-mediated resistance is restricted to aminopenicillins.

The rPBP3 (i.e. gBLNAR/gBLPACR) genotype is defined by the presence of the R517H or N526K substitution, whereas sPBP3 (i.e. gBLNAS/gBLPAR) isolates lack both substitutions. There is, however, no international consensus on subcategorization of rPBP3 isolates (chapter 6.3.4). The 'modified Ubukata system' (my designation), based on publications by Ubukata and coworkers [178,180,530] and Garcia-Cobos *et al.* [148], is summarized in **Table 10**.

In Paper III, we proposed a further modification by adding the L389F substitution to the categorization system [466]. High-rPBP3 isolates (S385T positive) with and without the additional L389F substitution were designated 'third stage' and 'second stage', respectively, and the suffix '(+)' or '(-)' was added to the group designation. This modification was based the classes suggested by Osaki *et al.* [360], and phenotypic susceptibility profiles observed in in Study III and in previous studies (**Table 11**). The 'modified Ubukata-Osaki system' is presented in **Figure 11**.

As demonstrated in this thesis, the introduction of the novel terminology, and the modification to the categorization system, have clinical and epidemiological relevance and simplify precise and unambiguous communication on the molecular basis of non-*bla*-mediated beta-lactam resistance in *H. influenzae*.

14.2.2 *Resistance genotypes in rPBP3 H. influenzae from Norway*

Prior to this project, the resistance mechanism in Nordic (Norway, Sweden, Denmark, Finland and Iceland) isolates of *H. influenzae* with non-*bla*-mediated beta-lactam resistance had to my knowledge not been characterized. **Table 26** summarizes the resistance genotypes (chapter 6.3.4) of the rPBP3 isolates in this project (n=169).

In the two cross-sectional studies, encompassing *H. influenzae* isolates from 2004 and 2007, group II low-rPBP3 predominated, consistent with previous European surveillance studies (**Table 12**). In Study I [464], all isolates in the R-group (n=23) were group II low-rPBP3. Data were first presented at the 24th Annual Meeting of the Scandinavian Society for Antimicrobial Chemotherapy (SSAC) in 2007 [463]. Of the 177 R-group isolates in Study II [465], 116 (66%) were rPBP3; predominantly group II (n=111, 96%). Two group III-like(-) and one group III(-) isolate were identified; the latter represents the second group III high-rPBP3 *H. influenzae* reported from Europe, and the first with this genotype from Northern Europe (**Table 12**).

The three high-rPBP3 isolates from Study II were included in the longitudinal Study III, adding up to 30 high-rPBP3 *H. influenzae* (2006-2013) [466]. This highly remarkable collection is unique outside Japan (**Table 12**). The high proportion of group III isolates (23/30), with more than half (12/23) being group III(+) (i.e. third stage high-rPBP3) is of particular notice. Only two isolates with similar genotypes have been reported previously in Europe and the Americas: one group III(+) invasive isolate from Canada [457] and one group III(+) AOM isolate from France [83]. Accordingly, the single group III(+) blood isolate in Study III is the first reported invasive isolate from Europe with this genotype.

Table 26 also presents PBP3 types with designations used in the respective studies. There are striking similarities between the PBP3 types in Norwegian rPBP3 isolates and previous surveillance studies from other geographical regions (chapter 6.3.6). First, PBP3 type 1 (n=10) and PBP3 type 2 (n=10) accounted for 67% of the high-rPBP3 isolates in Study III. PBP3 type 2 is identical to the most frequent PBP3 type in respiratory high-rPBP3 isolates from Korea in 2010 [364], and compatible with the most frequent PBP3 type in an earlier Japanese study (2002-2003) [492] (**Table 13**).

Table 26 Overview of resistance genotypes in rPBP3 *H. influenzae* from Norway (n=169)

Genotypes[a]	PBP3 types[b]	Study[c]			PBP3 substitutions[d]																	
		I [464]	II [465]	III [466]	D350	S357	A368	M377	S385	L389	A437	I449	G490	A502	R517	N526	A530	T532	V547	A554	Y557	V562	N569
III(+)	2			10 (7)	N	N		I	**T**	**F**						**K**			I			L	S
	5			1	N	N		I	**T**	**F**				T		**K**							
	6			1	N	N		I	**T**	**F**				T		**K**						L	
III-like(+)	3			4 (3)	N	N		I	**T**	**F**					**H**			S	I		H		S
III	1		1	10 (1)	N	N			**T**					T		**K**			I				S
	7			1					**T**					T		**K**			I				S
III-like	4		2	3	N	N		I	**T**						**H**			S	I				
II	A	11	48 (5)		N			I						V		**K**			I				S
	B	7	19 (5)									V				**K**			I				S
	C	2	5		N			I					E			**K**			I				S
	D	1	17		N								E			**K**	S						
	E	1												T		**K**							
	F	1	1													**K**							
	G															**K**							
	H		6											V		**K**							
	I		4		N						S			V		**K**			I				S
	J		3		N											**K**			I				S
	K		2				T									**K**							
	L		1		N								E			**K**			I	D			S
	M		1		N									V		**K**			I				S
	N		1 (1)		N						S	V				**K**			I				S
	O		1													**K**			I				S
	P		1													**K**			I				
	Q		1										E	V		**K**			I				S
I			2												**H**				I	T			

[a] According to the modified Ubukata-Osaki system for categorization of rPBP strains (**Figure 11**)
[b] According to the designations used in the respective studies
[c] Number of isolates with the respective PBP3 types (number of *bla* positive isolates in brackets)
[d] Substitutions included in the categorization system in bold

Second, PBP3 types A (n=59), B (n=26) and D (n=18) accounted for 77% (67%, 19% and 13%, respectively) of the 134 group II low-rPBP3 isolates in Study I and Study II. PBP3 type A is frequent in Europe [22,63,204,247,394,414], Canada [457], Australia [566] and Korea [364], whereas PBP3 type B is frequent in Sweden [414], Switzerland [63], Canada [457], Australia [566] and Korea [364] (**Table 13**).

The significance of these observations with respect to the molecular epidemiology of rPBP3 strains is discussed in chapter 14.3.2.

14.2.3 *Correlation between rPBP3 genotypes and phenotypic resistance*

Correlations between resistance genotypes and phenotypic susceptibility profiles, based on data from Study II [465] and Study III [466], are presented in **Table 27** (median MICs) and **Table 28** (proportions of isolates categorized as susceptible).

Table 27 Correlation between resistance genotypes (**Figure 11**) and phenotypic beta-lactam susceptibility (MIC_{50}, mg/L)

Agents	sPBP3 (n=80) [465]	I (n=2) [465]	II (n=111) [465]	III-like(-) (n=3) [466]	III(-) (n=11) [466]	III-like(+) (n=4) [466]	III(+) (n=12) [466]
Ampicillin	0.25	0.5	2[a]	2	2[b]	8[b]	4[b]
Amoxicillin	0.5	0.25	4[a]	4	8[b]	4[b]	8[b]
Piperacillin	0.004	0.03	0.03[c]	-	-	-	-
Cefuroxime	0.5	0.5	8	>8	8	8	8
Cefotaxime	0.004	0.06	0.03	≤0.125	0.25	0.5	1
Ceftriaxone	-	-	-	0.06	0.06	0.25	0.25
Cefixime	-	-	-	>1	0.25	>1	0.5
Cefepime	-	-	-	0.5	0.5	1	2
Ceftaroline	-	-	-	0.03	0.06	0.125	0.25
Meropenem	0.03	0.016	0.125	0.125	0.25	0.25	0.5
Imipenem	-	-	-	1	1	≤0.5	1

[a] Susceptibility testing of *bla* positive isolates to ampicillin and amoxicillin performed in the presence of sulbactam (4 mg/L) and clavulanic acid (2 mg/L), respectively
[b] Susceptibility testing of *bla* positive isolates to ampicillin and amoxicillin performed in the presence of sulbactam (2:1) and clavulanic acid (2:1), respectively
[c] Susceptibility testing of *bla* positive isolates to piperacillin performed in the presence of tazobactam (4 mg/L)

With the notable exception of imipenem, genotype-MIC correlations were largely consistent with previous data (**Table 11**). Median MICs were generally ≥2 dilutions higher for group II rPBP3 isolates compared to sPBP3 [465], and median cefotaxime MICs for second-stage and third-stage high-rPBP3 isolates exceeded low-rPBP3 by three and five dilutions, respectively. Interestingly, 11 isolates in Study II has piperacillin MICs above ECOFF (range 0.125-0.25 mg/L), including four sPBP3 isolates. The significance of this observation is discussed in chapter 14.2.4.

The majority of group II isolates were categorized as resistant to ampicillin, amoxicillin and cefuroxime (**Table 28**). Significant proportions were resistant to cefotaxime (7/111, 6%) and (in the case of meningitis) non-susceptible to meropenem (22/111, 20%). Notably, 12% (13/111) of group II isolates were categorized as susceptible to all tested agents, whereas 11% (9/80) of sPBP3 isolates were non-susceptible to ≥1 beta-lactam (not including isolates intermediately susceptible to cefuroxime, n=10). The latter observation is further discussed in chapter 14.2.4.

All 30 high-rPBP3 isolates [466] were categorized as resistant to ≥1 of the extended-spectrum cephalosporins cefotaxime, ceftriaxone, cefixime, cefepime and ceftaroline; the proportions of resistant isolates varied from 47% (ceftriaxone) to 97% (cefixime). Cefixime differed from the other cephalosporins by being less active in isolates with R517H-based genotypes compared to genotypes with N526K as the first stage substitution. Genotype-MIC correlations for cefepime and ceftaroline (or other fourth- and fifth-generation cephalosporins) are not reported previously (**Table 11**).

Of particular notice are the higher MICs to ampicillin, cefotaxime and ceftriaxone in third-stage high-rPBP3 (group III-like(+) and group III(+)) compared to second-stage (group III-like and group III). This is in accordance with previous reports (**Table 11**). With current EUCAST breakpoints [111], the differences in MIC levels greatly affected categorization of susceptibility to ceftriaxone (**Table 28**): no isolates with second-stage genotypes had ceftriaxone MIC above the S-breakpoint, whereas 88% of isolates with third-stage genotypes were categorized as resistant [466].

Table 28 Correlation between resistance genotypes (**Figure 11**) and phenotypic beta-lactam susceptibility (% susceptible by EUCAST breakpoints [111]). Green, >90%; yellow, 50-90%; orange, 25-50%; red, <25%, grey, no data

Agents	sPBP3 (n=80) [465]	1st stage rPBP3 (n=113) [465]	2nd stage rPBP3 (n=14) [466]	3rd stage rPBP3 (n=16) [466]
Ampicillin	98.8	40.7[a]	7.1[b]	0.0[b]
Amoxicillin	97.5	46.0[a]	0.0[b]	0.0[b]
Cefuroxime[c]	87.5	44.2	0.0	0.0
Cefotaxime	98.8	93.8	28.6	0.0
Ceftriaxone			100.0	12.5
Cefixime			7.1	0.0
Cefepime			14.3	0.0
Ceftaroline			28.6	0.0
Meropenem[d]	100.0	80.5	85.7	50.0
Meropenem[e]	100.0	100.0	100.0	100.0
Imipenem			100.0	100.0

[a] Susceptibility testing of *bla* positive isolates to ampicillin and amoxicillin performed in the presence of sulbactam (4 mg/L) and clavulanic acid (2 mg/L), respectively
[b] Susceptibility testing of *bla* positive isolates to ampicillin and amoxicillin performed in the presence of sulbactam (2:1) and clavulanic acid (2:1), respectively
[c] Proportion of isolates categorized as S or I
[d] Meningitis breakpoints
[e] General breakpoints

Most group III(+) (8/12, 67%), some group III(-) (2/11, 18%), and no group III-like isolates were non-susceptible to meropenem (meningitis breakpoints). In contrast, all isolates had imipenem MICs below ECOFF. This is consistent with the low affinity of this agent for PBP3 (chapter 6.3.5) but differ from previously reported genotype-MIC correlations (**Table 11**). However, imipenem-resistant *H. influenzae* have with few exceptions [308,436] been identified using agar-based MIC methods [57,62,360,384-386]. Further studies are needed to determine the correlation between rPBP3 genotypes, increased imipenem MIC and clinical resistance.

The genotype-MIC correlations and susceptibility profiles observed in this project underline the importance of confirming susceptibility to extended-spectrum cephalosporins and meropenem in severe *H. influenzae* infections such as meningitis and septicaemia, even in geographical regions where low-rPBP3 genotypes

predominate. Finally, the observed differences in resistance phenotypes between isolates with second-stage and third-stage high-rPBP3 genotypes data support the suggested modification to the rPBP3 categorization system (**Figure 11**).

14.2.4 *Evidence of non-bla-mediated resistance other than rPBP3*

Mechanisms regulating the resistance phenotypes in rPBP3 strains and the possible contribution of other suggested and hypothesized mechanisms to non-*bla*-mediated resistance in *H. influenzae* are described in chapter 6.4. This project confirmed that *H. influenzae* with non-*bla*-mediated beta-lactam resistance other than rPBP3 (as defined in **Table 10**) are rare in Norway. However, analysis of resistance phenotypes suggested the existence of additional, uncharacterized resistance mechanisms.

First, in Study III [466], the single invasive group III(-) high-rPBP3 isolate was susceptible to cefotaxime and had generally lower beta-lactam MICs than other group III(-) isolates. Conversely, the single group III(+) Hif isolate was more resistant than NTHi isolates with comparable genotypes. There were no indications of increased efflux-mediated beta-lactam resistance due to derepression of the AcrAB efflux pump [214], as all isolates expressed wild-type susceptibility to macrolides [466]. These observations suggest that strain-associated mechanisms other than increased efflux may modify beta-lactam resistance levels in rPBP3 strains, consistent with previous observations [214,530]. These issues will be further explored in future studies.

Second, in Study II [465], ampicillin, cefuroxime and cefotaxime MIC_{50} values for sPBP3 isolates in the R-group were one dilution higher compared to sPBP3 isolates in the S-group (**Figure 18**). The two sPBP3 populations were phylogenetically different: a total of 39 STs were represented, but only five were present in both study groups.

Furthermore, 13 of 71 (18%) *bla* negative sPBP3 isolates (including two isolates in the S-group) had MIC above ECOFF for at least one agent; reduced beta-lactam susceptibility was supported by positive PG1 screening in three (**Table 29**). Four isolates had ≥3 PBP3 substitutions; most were also present in fully susceptible isolates. One screening-positive ST368 isolate had the A554T substitution present in *H. influenzae* ATCC 49247, which expresses higher beta-lactam resistance levels than

most other group II low-rPBP3 [464]. A554T was also present in two additional ST368 sPBP3 isolates with identical pulsotypes (not in the table); both were screening positive (PG1 zones 6 and 9 mm, respectively). Two isolates had single, unique substitutions: the novel SSN-near P392S and the KTG-near V511A. Whether either of the P392S, V511A and A554T substitutions may contribute to reduced susceptibility will be investigated through PBP3 modeling and transformational studies.

Seven of the thirteen isolates had no PBP3 substitutions. Among these were four screening-negative ampicillin-susceptible isolates with increased piperacillin MIC. An intriguing hypothesis may be that increased piperacillin MICs in these isolates is due to altered PBP2 (chapter 6.4.1). Interestingly, the three isolates belonged to a clonal group of nine ST395 isolates with related pulsotypes; the remaining six isolates were fully susceptible to piperacillin (MIC ≤0.016 mg/L). Characterization of this clone (including PBP2 typing) will be carried out in a future study.

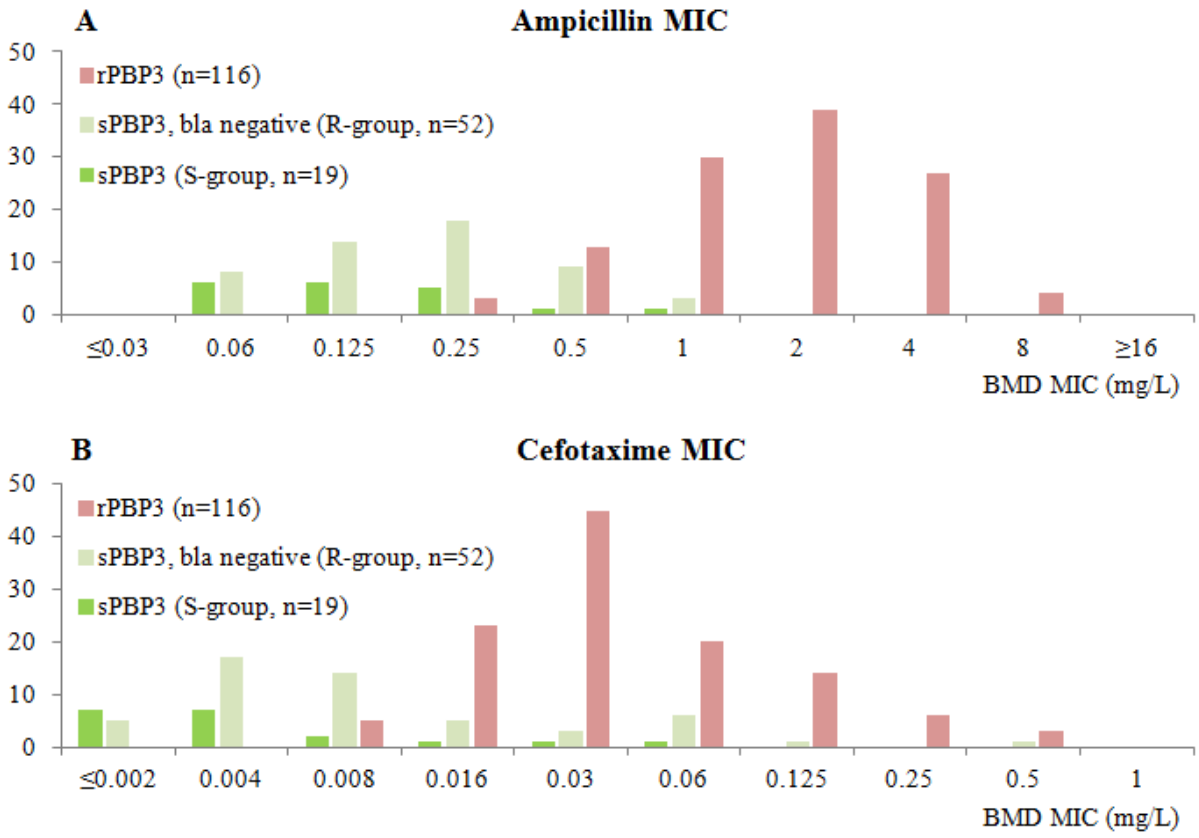


Figure 18 MIC distributions for ampicillin (A) and cefotaxime (B) by resistance genotype and study group in Study II [465]. Median MICs for isolates in the S-group, *bla*-negative sPBP3 isolates in the R-group, and rPBP3 isolates, respectively, were 0.125 mg/L, 0.25 mg/L, and 2 mg/L (ampicillin); and 0.004 mg/L, 0.008 mg/L, and 0.03 mg/L (cefotaxime)

Table 29 Non-*bla*-mediated beta-lactam resistance in isolates categorized as sPBP3 according to the rPBP3 categorization system (**Table 10**). Isolates and data are from Study II [465]. PG1 zone data were obtained as part of Study IV [467] before the isolates were excluded. Study group, phylogenetic assignment (MLST and PFGE), phenotypic susceptibility profiles and PBP3 substitutions are shown. All isolates were *bla* negative by acidimetric test and bla_{TEM}/bla_{ROB} PCR. MICs above ECOFF (**Table 17,** www.eucast.org/MIC_distributions) and PG1 zones below the screening breakpoint recommended by EUCAST (<12 mm) [111] are highlighted. Bold, PBP3 substitutions possibly contributing to reduced susceptibility (see text)

Study group[a]	MLST[b]		PFGE cluster	PG1[c] zone (mm)	MIC (mg/L)						PBP3 substitutions
	ST	CC			AMP[c]	AMX[c]	PIP[c]	CXM[c]	CTX[c]	MEM[c]	
S	ST34	CC-ST34	No data	15	0.25	0.25	0.25	8	0.03	0.125	None
R	ST155	CC-ST155	3C	14	0.5	0.5	<0.002	16	0.016	0.06	**V511A**
R	ST159	CC-ST503	3A	17	0.25	1	0.004	8	0.008	0.03	None
R	ST210	CC-ST176	Single	17	0.25	1	0.008	4	0.004	0.016	None
R	ST368	CC-ST368	12	6	0.5	1	0.016	4	0.008	0.03	V547I, **A554T**, N569S
R	ST395	CC-ST395	3B	16	0.25	2	0.125	2	0.06	0.03	None
R	ST395	CC-ST395	3B	14	0.125	1	0.25	0.5	0.016	0.06	None
R	ST395	CC-ST395	3B	15	1	1	0.125	>16	0.03	0.125	None
R	ST425	CC-ST425	20	14	0.25	1	0.016	>16	0.008	0.06	D350N, V547I, N569S
R	ST430	CC-ST425	19	6	0.25	0.5	0.03	>32	0.03	0.25	None
S	ST907	CC-ST425	No data	18	0.25	4	0.016	16	0.008	0.03	D350N, V547I, N569S
R	ST558	CC-ST906	21	10	0.5	2	0.016	8	0.03	0.25	D350N, A502T, V547I, N569S
R	ST1198	Single	Single	16	0.25	4	0.004	1	0.008	0.06	**P392S**

[a] See **Figure 15**
[b] ST, sequence type; CC, clonal complex (named by founder)
[c] PG1, benzylpenicillin 1 unit; AMP, ampicillin; AMX, amoxicillin; PIP, piperacillin; CXM, cefuroxime; CTX, cefotaxime; MEM, meropenem

Further investigations are needed to clarify whether the unexplained phenotypes may be mediated by novel resistance mechanisms or simply are due to measurement errors. As correct assignment to susceptibility categories was considered unclear, the 13 isolates were excluded from (the methodology) Study IV [467] (chapter 13.1.4).

14.2.5 *Prevalence of phenotypic resistance and resistance mechanisms*

The prevalence of phenotypic non-*bla*-mediated beta-lactam resistance to Norwegian *H. influenzae* is monitored by NORM (**Table 15**) [339-345]. Among the agents tested are amoxicillin-clavulanic acid, cefuroxime, cefotaxime (from 2007) and ceftriaxone (invasive isolates, from 2013). Reduced susceptibility to these drugs is not explained by commonly occurring beta-lactamases (bla_{TEM} and bla_{ROB}) and indicates the presence of other acquired resistance mechanisms. Testing in NORM is strictly phenotypic and resistance mechanisms are not characterized by molecular methods.

When this project was initiated, *H. influenzae* categorized as resistant to beta-lactams due to mechanisms other than *bla* were still rare in Norway. According to the most recent Norwegian surveillance data at the time (NORM 2004) [341], 3.1% of respiratory isolates were resistant to amoxicillin-clavulanic acid, an increase from 1.3% three years earlier [340]. Beta-lactam resistance mechanisms other than *bla* had not yet been characterized in Nordic isolates and the prevalence of *H. influenzae* possessing such resistance mechanisms was unknown.

Study I aimed to assess the contribution of rPBP3 to phenotypic beta-lactam resistance in the NORM 2004 surveillance population [464]. The study confirmed that altered PBP3 was the predominating mechanism in Norwegian isolates of *H. influenzae* with non-*bla*-mediated resistance. Consistent with previous studies from Europe and North America (**Table 12**), group II low-rPBP3 was the predominating genotype (**Table 26**).

All 23 isolates included in the R-group were categorized as rPBP3, indicating a prevalence of ≥4.8% (23/480). As the inclusion criteria (**Figure 14**) failed to identify all rPBP3 in the original population, the exact prevalence could not be calculated.

The considerably wider inclusion criteria for Study II (**Figure 15**) appeared to be well under the cut-off for rPBP3 isolates in the NORM 2007 surveillance population, as only 66% (116/177) of the isolates in the R-group had the rPBP3 genotype [465]. The calculated rPBP3 prevalence in the original population was 14.6% (116/795). Similar to three years earlier, group II low-rPBP3 was the predominating genotype (**Table 26**).

As shown in chapter 14.2.4, an additional 13 isolates (1.6%) in Study II expressed non-*bla*-mediated beta-lactam resistance other than rPBP3. If the phenotypes of these isolates reflect the presence of novel resistance mechanisms, the prevalence of isolates with non-*bla*-mediated beta-lactam resistance would reach 16.2% (129/795).

For most beta-lactams, the prevalence of non-*bla*-mediated non-susceptibility by *in vitro* susceptibility testing and interpretation according to clinical breakpoints is considerably lower than the prevalence of isolates with rPBP3 genotypes. As indicated in **Table 15**, the proportions of non-susceptibility vary considerably between beta-lactams. In Study II, the calculated prevalences of rPBP3-mediated non-susceptibility to ampicillin (or ampicillin-sulbactam), amoxicillin (or amoxicillin-clavulanic acid), cefotaxime and meropenem with EUCAST breakpoints [111] were 8.8%, 8.1%, 1.3%, and 2.9%, respectively [465].

The datasets from Study I and Study II do not allow direct comparison of rPBP3 prevalences. However, assuming that the correlation between the proportion of isolates with rPBP3 genotypes and phenotypic resistance rates is constant in comparable populations, this correlation may be used to estimate rPBP3 prevalences if the phenotypic resistance rate is known. According to the NORM 2007 surveillance report, the amoxicillin-clavulanic acid resistance rate was 8.0% [342], consistent with the calculated rPBP3-mediated resistance rate of 8.1% for this agent in Study II. Consequently, the rPBP3 prevalence / amoxicillin-clavulanic acid resistance rate ratio in NORM 2007 was 14.6% / 8.0% = 1.825. Based on this ratio, the rPBP3 prevalence in the NORM 2004 surveillance population may be estimated to 5.7% (1.825 x 3.1% [341]), suggesting a significant increase in the prevalence of rPBP3 isolates from 2004 to 2007 ($p<0.0001$). Using the most recent surveillance data, the rPBP3 prevalence in 2014 may be estimated to 16.6% (1.825 x 9.1% [345]).

Figure 19 shows estimated rPBP3 prevalences for respiratory *H. influenzae* based on amoxicillin-clavulanic acid resistance rates in NORM 2000-2014 [339-345] (recalculated according to current EUCAST breakpoints [111]). Beta-lactamase prevalences and human use of amoxicillin (oral) in Norway during the same period are also depicted. Notably, the increased frequency of both resistance mechanisms occurred concomitantly with a 76% increased usage of amoxicillin from 2000 to 2012 (from 0.83 to 1.45 DDD/1000 inhabitants/day), while phenoxymethylpenicillin usage decreased by 7.3% during the same period (from 4.45 to 4.07 DDD) [342,345]. Norwegian antibiotic prescriptions do currently not include information on diagnoses, but it may be assumed that amoxicillin and phenoxymethylpenicillin are mainly used for respiratory tract infections (including otitis media and sinusitis). Consequently, the observed alterations in use of oral penicillins in Norway suggest that outpatients with respiratory tract infections are increasingly frequently treated with amoxicillin.

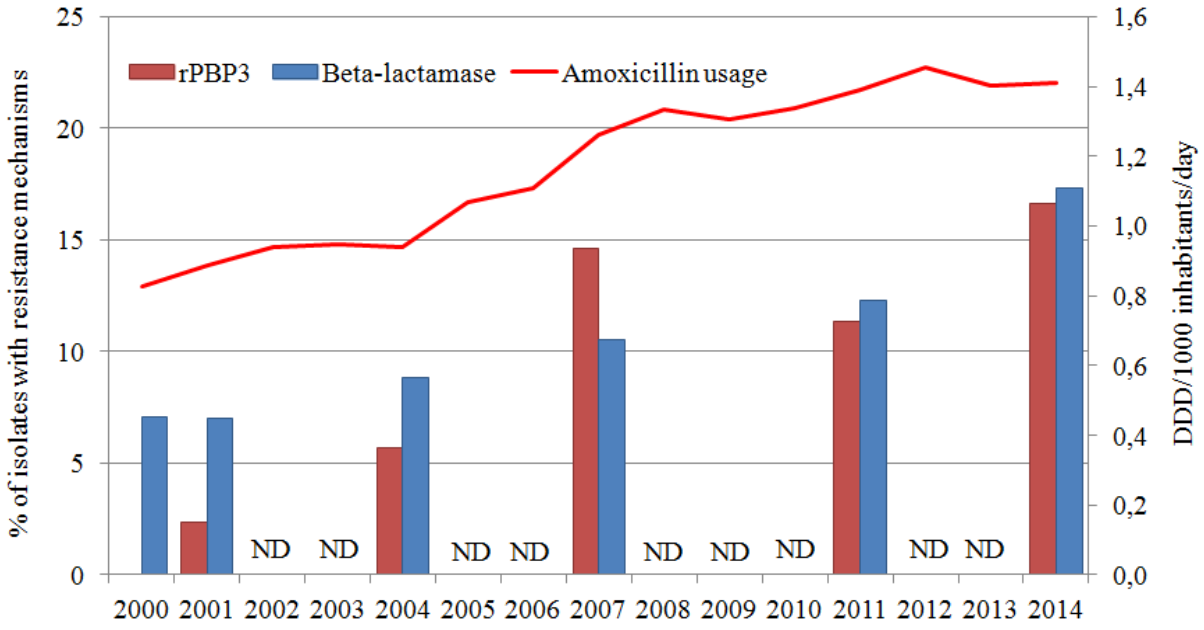


Figure 19 Usage of amoxicillin (oral) and prevalences of respiratory *H. influenzae* with beta-lactam resistance mechanisms (rPBP3 and beta-lactamase) in Norway 2000-2014. Amoxicillin usage data (total human use) and beta-lactamase prevalence data are from NORM surveillance reports [342,345]. The rPBP3 prevalence for 2007 is from Study II [465]; prevalences for 2000, 2001, 2004, 2011 and 2014 are estimates based on recalculated amoxicillin-clavulanic acid resistance rates in NORM [339-343,345] (**Table 15**) and a conversion factor of 1.825 (see text). ND, no data

An association between amoxicillin usage and *bla* positive *H. influenzae* was demonstrated in a previous study [68], and the rapid increase in rPBP3 *H. influenzae* in Japan during the 1990s was linked to high consumption of oral extended-spectrum cephalosporins [180,532]. To my knowledge, an association between amoxicillin usage and rPBP3 *H. influenzae* is not previously reported. It seems likely that amoxicillin may increase the selective pressure for ampicillin-resistant rPBP3 strains as well as for *bla* positive strains. Thus, it may be hypothesized that the increased usage of this drug in Norway during the 2000s contributed to the increasing frequency of rPBP3 and *bla* positive *H. influenzae* in this period.

14.2.6 *Emergence of high-rPBP3 strains in Norway*

The gradual increase in the prevalence of rPBP3 *H. influenzae* in Norway (**Figure 19**) is comparable to the development in Japan during the 1980s and 1990s [531] (chapter 6.3.6). A rapid shift in genotypes from low-PBP3 to high-rPBP3 occurred in Japan when the prevalence of low-rPBP3 isolates approached 20%. A similar development took place in Korea during the 2000s [17,230,364]. A genotype shift from low-rPBP3 to high-rPBP3 in Norway would compromise current empirical therapy (usually cefotaxime) in severe infections with *H. influenzae*.

Study III was a longitudinal study focusing on the epidemiology and characteristics of high-rPBP3 *H. influenzae* from Norway [466]. An overview of the 30 study isolates according to year of isolation, resistance genotypes and susceptibility to cefotaxime is presented in **Figure 20**.

Notably, the study design does not allow conclusions with respect to changes in incidence. The material is not complete and the true annual incidences are likely considerably higher. Nevertheless, although the study was closed for inclusion in July 2013, this year had the highest annual incidence of high-rPBP3 (n=9). In addition, four high-rPBP3 isolates with cefotaxime MIC 1-2 mg/L (gradient test) identified between August and December were not included in the study, adding up to 13 high-rPBP3 isolates in 2013. The data also suggest a shift in high-rPBP3 genotypes from second-stage to third-stage during the study period.

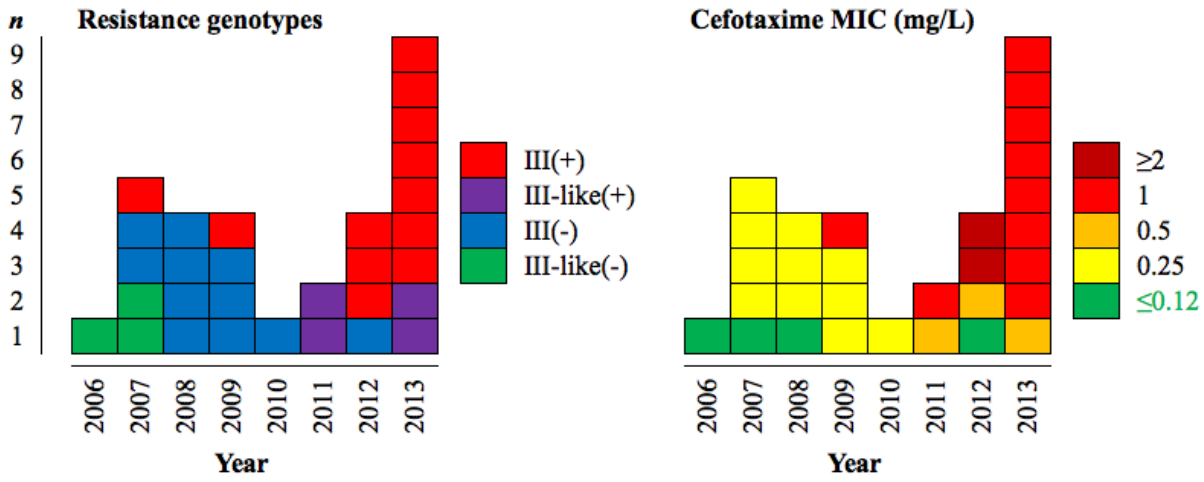


Figure 20 High-rPBP3 *H. influenzae* from Norway (May 2006 – July 2013). Left, genotypes according to the modified Ubukata-Osaki system (**Figure 11**); right, susceptibility to cefotaxime (BMD MIC interpreted with EUCAST breakpoints [111]). Data from [466]

The emergence of high-rPBP3 strains in Norway and the shift from second-stage to third-stage genotypes occurred concomitantly with 158% increased usage of extended-spectrum cephalosporins between 2000 and 2012 (from 0.06 to 0.16 DDD/1000 inhabitants/day) [342,345].

The results from Study I indicate that high-rPBP3 *H. influenzae*.were absent in NORM 2004 [464]. Study II showed a high-rPBP3 prevalence of 0.4% (3/795) in NORM 2007, and high-rPBP3 strains accounted for 2.6% (3/116) of rPBP3 isolates [465]. Recent surveillance data indicate that low-rPBP3 still is the predominating genotype in Norway. Based on gradient MICs and EUCAST breakpoints [111], the prevalence of cefotaxime-resistant isolates in NORM 2014 was low (respiratory, 0.6%; invasive, 1.4%) and stable (**Table 15**).

Selection of isolates for Study III was based on cefotaxime resistance by gradient MIC and EUCAST breakpoints **(Figure 16**). PBP3 typing showed that 69% (27/39) of the selected isolates were high-rPBP3, suggesting that this criterion is a sensitive indicator of the high-rPBP3 genotype.

In conclusion, there is so far no evidence of a shift from low-PBP3 to high-rPBP3 in Norwegian *H. influenzae*. However, the emergence of high-rPBP3 strains is a cause for concern, and the epidemiological situation may be dramatically altered in few

years (chapter 6.3.6). The temporal association between increased usage of extended-spectrum cephalosporins and the emergence of high-rPBP3 strains in recent years suggests a role of increased selective pressure and underlines the importance of rational antibiotic usage.

14.2.7 *Molecular epidemiology of rPBP3 strains*

As described in chapter 6.3.7, limited clonal dissemination of rPBP3 *H. influenzae* within geographically restricted areas has been reported in several previous investigations. However, there is a general lack of knowledge on the global molecular epidemiology of rPBP3 strains, as only a few studies [56,146,394,492] have linked information about resistance genotypes to epidemiological typing by unambiguous methods (i.e. MLST), which is required for inter-investigator comparison.

Clonal expansion of rPBP3 strains in Norway was confirmed in Study I [464]. Most rPBP3 isolates (19/23, 83%) belonged to PFGE clusters 1 and 2, consisting of 12 isolates carrying PBP3 type A, and seven with PBP3 type B, respectively. Sequence analysis of the *ftsI* gene fragment used for deduction of PBP3 substitutions revealed that all isolates within each of the two PFGE clusters had identical *ftsI* alleles. The two clones were widespread, with distances of up to 970 and 410 km, respectively, between domiciles of the corresponding patients.

Based on these observations, and the striking similarities between the PBP3 substitution patterns in rPBP3 *H. influenzae* from Norway and other geographical regions, with PBP3 types A and B (and compatible patterns) frequently reported in Europe [22,63,204,247,394,414], we postulated the existence of endemic European rPBP3 clones [464].

To explore this hypothesis, we developed and validated the novel concept of MLST-*ftsI* typing in Study II [465]. By this approach, allele designations for unique 710-bp *ftsI* sequences are added to MLST allelic profiles, and isolates with identical MLST-*ftsI* profiles are considered clonal. MLST-*ftsI* typing was also utilized for epidemiological characterization of the 30 high-rPBP3 strains in Study III [466]. In the

two investigations, encompassing 143 rPBP3 (representing all six genotypes) and 80 sPBP3 isolates, we found that MLST-*ftsI* typing increased the discriminatory power compared with MLST alone and MLST combined with PBP3 types, without compromising consistency with PFGE-based grouping.

Phylogenetic analysis revealed 35 unique *ftsI* alleles in 166 rPBP3 isolates in this project (**Figure 21**). The six low-rPBP3-encoding alleles *lambda-2* (n=35), *zeta* (n=26), *lambda-1* (n=23), *omicron* (n=18), *gamma* (n=6), and *nu-3* (n=5), and the two high-rPBP3 alleles *ftsI-1* (n=10) and *ftsI-2* (n=6), together accounted for 78% of all rPBP3 isolates. Most alleles encoding high-rPBP3 genotypes (*ftsI* types 1-10) clustered on a highly diverging major branch. Alleles encoding group II low-rPBP3 types A (*lambda*), C (*nu*) and D (*omicron*) formed separate clusters on another major branch, whereas *zeta* (type B) and *gamma* (type H) diverged less from the *alpha* cluster, defined by the reference DNA sequence.

The resolution level of MLST-*ftsI* typing compared to PFGE may be illustrated by PFGE cluster 3B, consisting of 12 CC-ST395 isolates with four pulsotypes (**Figure 17**) [465]. MLST-*ftsI* typing identified one group III(-) high-rPBP3 (ST1197) and two group II low-rPBP3 (ST556) isolates and separated them from nine sPBP3 with identical or related pulsotypes. The ST1197 group III(-) isolate in PFGE cluster 3B was the first in a clonal group of ten high-rPBP3 isolates with unique MLST-*ftsI* profiles (CG2) in Study III [466]. PFGE analysis of seven representatives of this clone, isolated from patients in different geographical regions over a period of three years, revealed four related pulsotypes differing by a maximum of five bands.

In this project, MLST-*ftsI* typing was validated with a 710 bp sequence (aa 338-573). Use of longer fragments (e.g. the complete *ftsI* gene) would theoretically increase resolution, whereas shorter sequences would reduce discriminatory power. Notably, a shorter sequence (nt 977-1597, 621 bp) is obtained with the PCR methodology published at http://pubmlst.org/hinfluenzae for detection of PBP3 substitutions in invasive *H. influenzae*, compared to the methodology used in this project (**Table 22**).

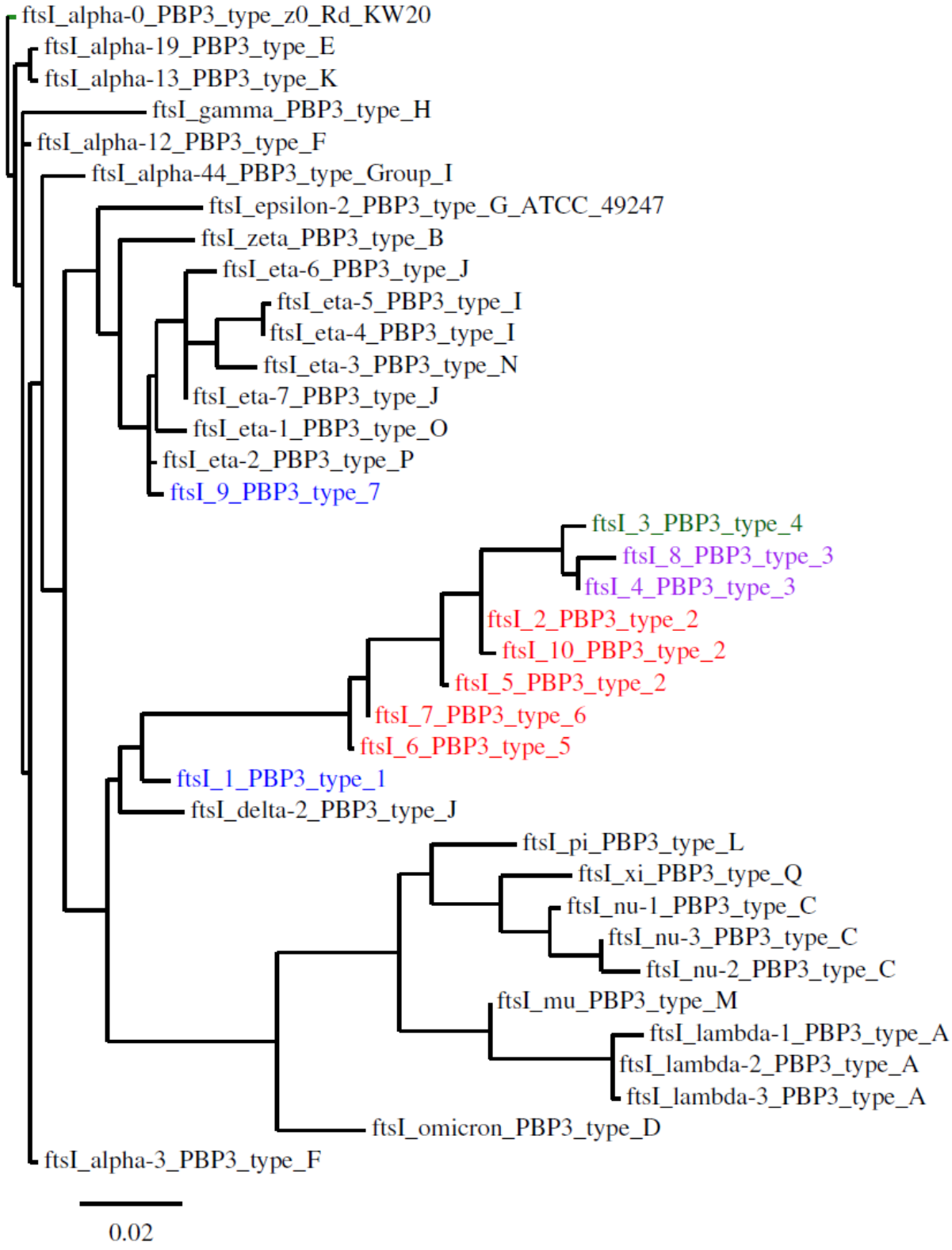


Figure 21 Phylogram showing the genetic diversity of the *ftsI* gene (transpeptidase region, nucleotides 1010-1719) and the correlation between *ftsI* types and PBP3 types in 166 rPBP3 isolates [464-466]. Outgroup and reference sequence (*alpha*-0) is *H. influenzae* Rd KW20 (top). Colours indicate high-rPBP3-encoding alleles: red, III(+); purple, III-like(+); blue, group III(-); green; group III-like (-). The scale is DNA divergence (0.02 = 2%)

Study II showed that the increased rPBP3 prevalence in Norway is due to dissemination of a few clonal groups. In NORM 2007, four clonal groups (MLST-*ftsI* types) accounted for 61% (71/116) of rPBP3 isolates [465]. Two clones carried PBP3 type A encoded by slightly different *ftsI* alleles (ST14 with *lambda*-1 and ST367 with *lambda-2*); one clone consisted of ST396 isolates with PBP3 type B; and one clone comprised ST201 isolates with PBP3 type D. Combined analysis of *ftsI* alleles, MLST and PFGE (**Figure 17**) revealed that the ST14/A and the ST396/B clones corresponded to the two predominating rPBP3 clones (PFGE clusters 1 and 2, respectively) in NORM 2004 [464]; i.e. the clones persisted over a period of at least three years.

MLST-PBP3 typing, i.e. adding the PBP3 type to the MLST allelic profile, may be used as a surrogate approach when *ftsI* sequences are not available. It should be emphasized that MLST-PBP3 typing does not offer the same level of resolution, as identical PBP3 types frequently are encoded by different (although related) *ftsI* alleles (**Figure 21**). However, grouping of three ST12 isolates with PBP3-type A encoded by the slightly different alleles *lambda-1* and *lambda-3* represented the only discrepancy between MLST-*ftsI* typing and MLST-PBP3 typing in this project.

Some of the MLST-PBP3 types observed in this project were identical or compatible to MLST-PBP3 types reported by others (**Table 30**). Of particular notice is that PBP3 type A is frequently linked to ST14 and ST367 in the very limited number of reports on the molecular epidemiology of rPBP3 (chapter 6.3.7). ST14 and ST367 NTHi with type A-compatible substitution patterns have caused invasive disease [146] and pneumonia [394] in Spain. A study on invasive *H. influenzae* in Sweden 2008-2010 [414] identified a cluster of seven NTHi of ST14, ST367 and related STs carrying PBP3 type A (F. Resman, personal communication). A recent report described an outbreak in a Swedish nursing home caused by a ST14 clone with PBP3 type A, affecting 15 individuals including eight residents during one month in 2011 [9].

Finally, studies on invasive *H. influenzae* in Canada during 2000-2006 [458-460] and 2008-2009 [457] revealed an increasing prevalence of rPBP3 NTHi, with PBP3 type A being common in both periods [457,458]. ST14 and ST367, respectively, were the most common STs in NTHi from two different regions and periods [457,459]. PBP3

type A was by far the most frequent substitution pattern in ST14 and also appeared in some ST367 isolates (R. Tsang, personal communication).

Clonal dissemination also contributed strongly to the remarkable number of high-rPBP3 *H. influenzae* identified in Norway 2006-2013 [466]. Five clonal groups (CG1-CG5) comprising 2-10 isolates with identical MLST-*ftsI* types accounted for 22 of the 30 isolates in Study III (**Figure 22**, left). An invasive NTHi isolate with an MLST-PBP3 type compatible with the three CG1 isolates (2006-2007) was identified in Spain (2004-2009) [146], suggesting a widely disseminated high-rPBP3 clone in Europe.

Table 30 Disseminated rPBP3 clones in this project [464-466] and other investigations

Genotype[a]	ST	PBP3[b]	*ftsI*[c]	CG[d]	Country	Period	n[e]	Reference
III(+)	ST836	2	*2*	5	Norway	2013	4	[466]
III(+)	ST159	2	*5*	4	Norway	2013	3	[466]
III(-)	ST1197	1	*1*	2	Norway	2007-2010	10	[466]
III-like(+)	ST422	3	*4*	3	Norway	2011-2013	2	[466]
III-like(-)	ST155	4	*3*	1	Norway	2006-2007	3	[466]
III-like(-)	ST155	(4)	ND	ND	Spain	2004-2009	1	[146]
II	ST14[f]	A	*lambda-1*	NA	Norway	2004	12	[464,465]
II	ST14	A	*lambda-1*	NA	Norway	2007	11	[465]
II	ST14	A	ND	ND	Canada	2000-2009	ND	[457-460][g]
II	ST14	(A)	ND	ND	Spain	2000-2009	1	[394]
II	ST14[g]	A	ND	ND	Sweden	2008-2010	ND	[414][g]
II	ST14	A	ND	ND	Sweden	2011	15	[9]
II	ST367	A	*lambda-2*	NA	Norway	2007	29	[465]
II	ST367	A	ND	ND	Canada	2000-2009	ND	[457-460][g]
II	ST367	(A)	ND	ND	Spain	2000-2009	1	[394]
II	ST367	(A)	ND	ND	Spain	2004-2009	2	[146]
II	ST367[g]	A	ND	ND	Sweden	2008-2010	ND	[414][g]
II	ST396[f]	B	*zeta*	NA	Norway	2004	7	[464,465]
II	ST396	B	*zeta*	NA	Norway	2007	16	[465]
II	ST201	D	*omicron*	NA	Norway	2007	15	[465]

[a] PBP3 resistance genotype according to the modified Ubukata-Osaki system (**Figure 11**)
[b] PBP3 type (**Table 26**). Compatible patterns in brackets
[c] *ftsI* allele (**Figure 21**). ND, no data
[d] Clonal group (defined in [466]). ND, no data; NA, not applicable
[e] Number of isolates with the respective MLST-*ftsI* or MLST-PBP3 profiles. ND, no data
[f] ST assignment based on PFGE pulsotypes identical to isolates of known STs in [465]
[g] Personal communication (see text)

Two clones (CG4 and CG5) were both restricted to single hospitals and isolated within very short time periods (four and 16 days, respectively). CG5 was present in samples from two persons within the same household. Person-to-person transmission of beta-lactam-resistant NTHi has been reported by others, including outbreaks in a respiratory ward [182] and a nursing home [9], and intrafamilial transmission of rPBP3 strains [549]. These observations highlight the importance of hygiene measures in health institutions to prevent nosocomial spread of high-rPBP3 strains.

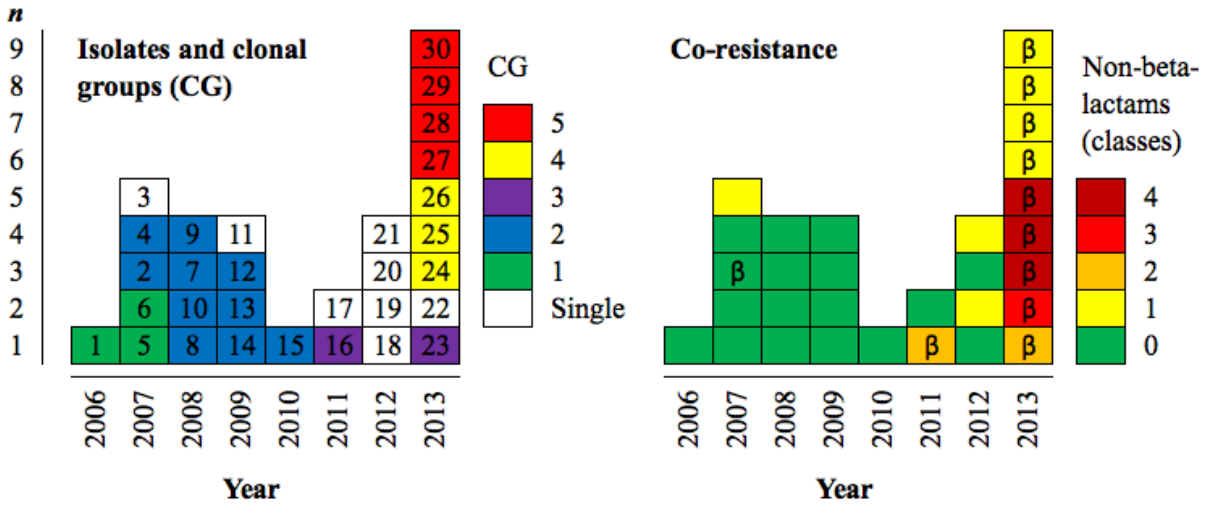


Figure 22 Clonality and co-resistance in high-rPBP3 *H. influenzae* from Norway 2006-2013. Left, clonal groups (by MLST-*ftsI* typing); right, co-resistance to non-beta-lactams (number of classes) and presence of bla_{TEM} (β). From [466]

14.2.8 *Multi-drug resistance (MDR)*

Co-resistance was more frequent than expected in Study III [466]. The proportion of *bla*-positive isolates (37%, 11/30) was more than twice the prevalence in respiratory *H. influenzae* in NORM 2014 (17%, **Table 15**) [345]. Similarly, resistance rates for trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, and ciprofloxacin in Study III were 43% (13/30), 13% (4/30), 13% (4/30), and 10% (3/30), respectively, compared to 19%, 1.3%, 0.9%, and 0.4%, respectively, in NORM 2014 (**Table 16**) [345]. All isolates in Study III had wild-type MICs to rifampicin and macrolides.

There was a remarkable accumulation of MDR *H. influenzae* towards the end of the study period (**Figure 22**, right). Six isolates expressed resistance to two or more groups of non-beta-lactams; all six had third-stage high-rPBP3 genotypes. The resistotypes are presented in **Table 31**.

Table 31 Resistotypes of MDR high-rPBP3 strains from Norway [466]

						Non-beta-lactam susceptibility[c]					
CG[a]	n	ST	PBP3[b]	Period	*bla*	LF	CI	DO	TC	CH	TS
4	3	ST159	III(+)	2013	TEM-1	S[d]	R	I	R	R	R
Sg	1	ST1282	III-like(+)	2013	TEM-1	S	S	I	R	R	R
3	2	ST422	III-like(+)	2011-2013	TEM-1	S[d]	S[d]	S	S	S	R

[a] Clonal group (**Table 29** and **Figure 22**). Sg, singleton
[b] PBP3 resistance genotype according to the modified Ubukata-Osaki system (**Figure 11**)
[c] S/I/R-categorization according to EUCAST MIC breakpoints [111]. LF, levofloxacin; CI, ciprofloxacin; DO, doxycycline; TC, tetracycline; CH, chloramphenicol; TS, trimethoprim-sulfamethoxazole
[d] Increased MIC within the susceptible range

Five isolates had ciprofloxacin MIC above ECOFF (>0.06 mg/L) and amino acid substitutions in the QRDR of GyrA and/or ParC. The tree CG4 isolates had significant substitutions in both proteins (GyrA, S84L and D88N; ParC, S84I) and were categorized as ciprofloxacin-resistant. The two CG3 isolates had only one significant substitution (GyrA, S84L) and quinolone MICs within the susceptible category.

Quinolone resistance in *H. influenzae* is associated with hypermutability [369] (chapter 7.2). It seems likely that hypermutability also increases the ability to acquire PBP3 substitutions by spontaneous mutations, favoring development of strains with both resistance mechanisms. Hypermutable *H. influenzae* are prevalent in cystic fibrosis patients [550]. One CG4 isolate was from sputum of a cystic fibrosis patient; another was from nasopharynx of a patient with primary ciliary dyskinesia. High antibiotic pressure in these patient categories may result in selection of MDR strains.

The CG4 isolates also had $bla_{\text{TEM-1}}$ and were resistant to chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole. This resistance profile suggests the presence of the previously described integrating and conjugative element ICE*Hin1056* [310]

(chapter 7.3). The remarkable resistotype of CG4 might thus be the result of two independent factors: hypermutability, leading to PBP3 and QRDR substitutions, and the acquisition of a mobile genetic element, e.g. ICE*Hin1056*.

To my knowledge, the three CG4 isolates in Study III represent the first ciprofloxacin-resistant high-rPBP3 *H. influenzae* reported outside Japan, and CG4 represent the first clonal group with this particular combination of resistance mechanisms. The resistance mechanisms of this clone will be further characterized in a future study.

14.2.9 *Phylogeny, resistance and pathogenicity*

Associations between PBP3 resistance genotypes, phylogeny and pathogenicity (chapter 6.5), based on available clinical data, were explored in Study II [465]. Multivariate regression analysis of isolates with known hospitalization status (766/795, 96%) showed that increasing age (OR=1.3, p<0.001) and male gender (OR=1.8, p=0.001) were significant independent risk factors for hospitalization. With adjustment for age, gender and beta-lactamase production, there was a borderline significant association between rPBP3 and hospitalization (OR=1.6, p=0.053). Similarly, multivariate analysis of isolates with known site of isolation (768/795, 97%) showed a significant association between rPBP3 and eye infection (OR=2.1, p=0.003) but no association with other localizations. Information about STs was available for study isolates only and thus not included in the regression analysis.

The most prevalent STs were highly diverse with respect to resistance genotypes and clinical characteristics. Notably, ST-specific analysis showed no correlation between rPBP3 and hospitalization, indicating that the association between rPBP3 and pathogenicity suggested by the regression analysis most likely reflects the presence of strain-associated virulence determinants in some of the high-prevalent rPBP3 strains. For instance, all ST396 (n=16) and ST201 (n=15) isolates were rPBP3, and both STs were significantly associated with eye infection (p<0.05).

Some non-significant associations between ST and disease (see [465] for details) were recognizable from previous investigations. ST159 was in most cases isolated from

respiratory tract of hospitalized patients above 50 yrs of age, reflecting the reported association between this ST and infection in COPD patients [322]. Most ST57 isolates were from children below 3 yrs of age, consistent with a previously reported association with AOM [244]. Finally, the highest hospitalization rate was observed for ST14 isolates (all carrying PBP3 type A), in accordance with the potential of this ST to cause pneumonia [394] and invasive disease [414,457,459], and the recently reported ST14/PBP3 type A outbreak in a nursing home in Sweden in 2011, during which one of eight infected residents died and four were hospitalized [9].

These observations are consistent with previous investigations on associations between population structure and disease [244] and distribution of virulence determinants [88,102]. Of particular note is the association between *igaB* and WGS clades II and IV [88], as three of four major low-rPBP3 clones (ST14, ST201 and ST367) and the four most prevalent high-rPBP3 STs (ST155, ST159, ST836 and ST1197) belong to these two clades (**Tables 24-25**).

14.2.10 *Development of rPBP3 strains*

Accumulating evidence suggest that new rPBP3 strains evolve through a combination of point mutations and transformation with exchange of mutant *ftsI* sequences through homologous recombination (chapter 6.3.8). Comparative analyses of *ftsI* sequences (**Figure 21**) and phylogeny in Study II [465] and Study III [466] revealed several examples of identical rPBP3-encoding *ftsI* alleles in phylogenetically unrelated strains (**Table 32**), supporting that HGT contributes to development of rPBP3 strains *in vivo*.

The highly divergent allele *lambda-2*, encoding PBP3 type A, was distributed to five STs, belonging to three separate WGS clades within phylogenetic division I. Similarly, the two high-rPBP3-encoding alleles *ftsI-2* and *ftsI-4* were both distributed to STs assigned to separate WGS clades. Conversely, seven STs hosted more than one PBP3 type. Six ST57 isolates carried four rPBP3 types (A, K, L and N) and the reference sequence (z0).

Considering that *H. influenzae* is naturally competent and transformation is frequent *in vivo* (chapter 3.3), horizontal transfer of complete *ftsI* alleles is a more likely explanation to these observations than independent development of identical, highly mutated alleles through convergence. Recombinational exchange of complete *ftsI* genes has been demonstrated *in vitro* [478]. In another study, the mean length of donor segments was 8.1±4.5 kb [293], suggesting that most homologous recombinational events in *H. influenzae* involve DNA fragments greatly exceeding the length of the *ftsI* alleles in this project (0.71 kb).

The distribution of *ftsI-2* to phylogenetic divisions I and II indicates the absence of restriction barriers preventing exchange of *ftsI* sequences encoding complete transpeptidase regions between the two major phylogenetic groups of *H. influenzae*. This is consistent with previous reports of inter-species recombinational exchange of *ftsI* sequences between *H. influenzae* and *H. haemolyticus* [288,499,565], with no differences between inter- and intra-species transformation frequencies [478].

Table 32 Identical *ftsI* alleles present in isolates of different STs. Data from [465] and [466]

Geno-type[a]	PBP3 type[b]	*ftsI* type[c]	ST [289]	CC [125]	WGS [88]	Division [289]	n
III(+)	2	*2*	ST836	CC-ST245	V	I[d]	4
			ST160	CC-ST160	V	I[e]	1
			ST124	CC-ST124	I	II[d]	1
III-like(+)	3	*4*	ST422	CC-ST422	IV	I[d]	2
			ST1282	CC-ST503	II[d]	I[e]	1
II	A	*lambda-2*	ST367	CC-ST3	V[d]	I[d]	29
			ST12	CC-ST12	V	I	2
			ST1193	CC-ST12	V[d]	I[d]	1
			ST85	CC-ST472	III[d]	I[d]	2
			ST57	CC-ST57	IV	I	1
II	H	*gamma*	ST12	CC-ST12	V	I	4
			ST411	CC-ST422	IV	I[d]	2

[a] According to the modified Ubukata-Osaki system (**Figure 11**)
[b] See **Table 26**
[c] See **Figure 21**
[d] Indirect assignment via another ST assigned to the same CC
[e] Indirect assignment via assignment to WGS clade

Mosaic *ftsI* genes in *H. influenzae*, suggesting recombination with shorter fragments, have been reported previously [478,499,564,565]. A preliminary multiple sequence alignment analysis of *ftsI* sequences in Study II indicated the presence of mosaicism in isolates with group II rPBP3 genotypes. A detailed analysis on the role of intragenic recombination in the development of rPBP3 strains will be carried out in a future study encompassing *ftsI* sequences from isolates of all genotypes.

As the presence of USS/pUSS facilitates DNA uptake (chapter 6.3.8), it seems likely that the frequency of recombinational events in the *ftsI* gene (including events leading to development of rPBP3 strains) correlates to the density of USS/pUSS in the donor sequence. The *ftsI* gene of *H. influenzae* Rd KW20 contains two USS and four pUSS copies (**Figure 12**). Interestingly, several rPBP3-encoding *ftsI* alleles contained additional copies of pUSS. One extra copy was present in allele *zeta*, encoding the frequently occurring low-rPBP3 type B; in seven alleles in cluster *eta*, encoding the infrequent low-rPBP3 types I, J, N, O and P; and in the high-rPBP3-encoding alleles *ftsI-2* and *ftsI-10* (group III(+)), *ftsI-9* (group III(-)), *ftsI-4* and *ftsI-8* (group III-like(+)), and *ftsI-3* (group III-like(-)). Two extra copies were present in three alleles in cluster *nu*, encoding PBP3 type C, and in the infrequently occurring allele *xi* (PBP3 type Q). Transformational studies using rPBP3 *H. influenzae* with different densities of pUSS may clarify the significance of these observations for uptake and recombination of mutant *ftsI* fragments.

As hypothesized in chapter 6.3.8, bacterial fitness and compensatory substitutions may be used in an explanatory model to explain the apparently stepwise and non-random development of rPBP3 strains. A notable observation in this project was that isolates with R517H as the first stage substitution were significantly more frequent ($p<0.0001$) among high-rPBP3 (7/30, 30.4%) versus low-rPBP3 isolates (4/136, 2.9%). A possible explanation for this observation could be that acquisition of the second stage S385T substitution is associated with reduced fitness cost, and that fitness improvement is more prominent in R517H isolates compared to N526K isolates. The impact of distinct PBP3 substitutions on fitness will be investigated in a future study.

14.3 CLINICAL CASE REPORTS

14.3.1 *Case descriptions*

In Study III [466], the efficacy of antimicrobial therapy was evaluated for three hospitalized patients with well-defined infections likely caused by study isolates (patients and isolates 18, 27 and 28; **Figure 22**). All isolates had group III genotypes as defined by the N526K and S385T substitutions; isolates 27 and 28 also possessed the third-stage L389F substitution and were categorized as group III(+) high-rPBP3 (chapter 6.3.4).

Isolate 18 (ST1287) was cultured from the blood of a patient with X-ray confirmed pneumonia, COPD, hypogammaglobulinemia, chronic lymphocytic leukaemia and myasthenia gravis and represents the first reported invasive group III high-rPBP3 *H. influenzae* from Europe (**Table 12**). The strain was categorized as susceptible to cefotaxime (MIC ≤0.12 mg/L) and generally expressed lower MICs to beta-lactams than other (noninvasive) study isolates with comparable resistance genotypes. There was no co-resistance to non-beta-lactams. Accordingly, the patient responded well to initial parenteral therapy with cefotaxime (three days) followed by oral treatment with ciprofloxacin (susceptible, MIC ≤0.03 mg/L).

Isolates 27 and 28 belonged to CG5 (ST836) and were categorized as resistant to amoxicillin-clavulanic acid (MIC = 8 mg/L), ampicillin-sulbactam (MIC = 8 mg/L), cefotaxime (MIC = 1 mg/L), ceftriaxone (MIC = 0.5 mg/L), cefepime (MIC = 2 mg/L) and ceftaroline (MIC = 0.25 mg/L) according to EUCAST clinical breakpoints [111]. The isolates also possessed $bla_{\text{TEM-1}}$ and were trimethoprim-sulfamethoxazole resistant, but susceptible to, including ciprofloxacin (MIC ≤0.03 mg/L) and other non-beta-lactams. Both patients had X-ray confirmed pneumonia (C-reactive protein ≥160 mg/L) and significant co-morbidity. The study isolates were cultured from sputum.

Patient 27 (cardiovascular disease) responded to high-dose cefotaxime therapy (2 g three times a day) after initial treatment with benzylpenicillin. Patient 28 (disseminated cancer) responded to ciprofloxacin after initial piperacillin-tazobactam therapy.

14.3.2 *Discussion*

The first case (patient 18) indicates that the therapeutic efficacy of cefotaxime (in this case used for empirical therapy) in invasive infections caused by high-rPBP3 *H. influenzae* may be predicted by a low MIC value, even in immunocompromized, multimorbid patients. In the two other cases, the isolate was categorized as resistant to the agent used for initial empirical therapy (benzylpenicillin and piperacillin-tazobactam), and therapy was altered to high-dose cefotaxime and ciprofloxacin, respectively.

Benzylpenicillin therapy was associated with increased mortality compared to ampicillin and cefuroxime in a clinical study on *H. influenzae* bacteremia [505], and is definitively not a therapeutic option for infections caused by rPBP3 strains. Clinical breakpoints for benzylpenicillin are not defined by EUCAST [111].

For piperacillin-tazobactam, the picture is less clear. EUCAST recommend that amoxicillin-clavulanic acid resistant *H. influenzae* be reported as resistant to piperacillin-tazobactam and have not defined breakpoints [111]. Previous investigations have shown that PBP3 alterations affect the *in vitro* activity of piperacillin less than the activity of aminopenicillins and extended-spectrum cephalosporins [187,313,436] (chapter 6.3.5). The reports suggest that this agent may be a useful therapeutic alternative in infections caused by high-rpBP3 *H. influenzae* and that the interpretative rule recommended by EUCAST results in overestimation of resistance. Piperacillin-tazobactam MICs were not determined in Study III.

It is not known why patient 27 received cefotaxime therapy despite categorization of isolate 27 as resistant. However, clinical response to high-dose cefotaxime is consistent with CLSI breakpoints (susceptible, ≤2 mg/L) [75], and also with EUCAST PK/PD breakpoints for cefotaxime using a dose of at least 2 g x 3 or more (susceptible, ≤2 mg/L; resistant, >2 mg/L) [111].

Based on these observations, the relevance of EUCAST MIC breakpoints for clinical susceptibility categorization of *H. influenzae* to parenteral extended-spectrum cephalosporins may be questioned. EUCAST breakpoints for this group of agents are identical to or close to ECOFF, whereas CLSI breakpoints largely correspond to

PK/PD breakpoints (**Table 17**). Different breakpoints greatly affect clinical susceptibility categorization of high-rPBP3 *H. influenzae* to extended-spectrum cephalosporins. Resistance rates for the 30 high-rPBP3 isolates in Study III varied from 47% (ceftriaxone) to 97% (cefixime) with EUCAST breakpoints (chapter 14.2.3). For comparison, all isolates were susceptible to ceftriaxone and ceftaroline using the breakpoints recommended by CLSI, whereas only one isolate was categorized as resistant to cefotaxime and cefepime, and six isolates (20%) were categorized as resistant to cefixime.

As EUCAST PK/PD breakpoints indicate, increasing the doses may compensate for reduced susceptibility to extended-spectrum cephalosporins. This is exemplified by the intermediate categories for this group of agents against pneumococci, in which reduced beta-lactam susceptibility is caused by mechanisms very similar to rPBP3 *H. influenzae*. However, by not defining intermediate categories for extended-spectrum cephalosporins, EUCAST state that high-dose therapy with these drugs is not considered an option in infections caused by high-rPBP3 *H. influenzae*.

The cases presented here illustrate that by categorizing most high-rPBP3 *H. influenzae* as resistant to extended-spectrum cephalosporins and piperacillin-tazobactam, EUCAST breakpoints and interpretative rules lead to the use of other agents with less favorable ecological profiles. Clinical data are needed to determine whether these agents are safe therapeutic options in severe infections caused by high-rPBP3 *H. influenzae* with increased MICs, and for determination of clinically relevant breakpoints.

14.4 BETA-LACTAM SUSCEPTIBILITY TESTING

14.4.1 *Broth microdilution MIC*

BMD MIC was used as the reference methodology for MIC determination and susceptibility categorization in this project (chapter 8.2). An overview of beta-lactams tested and corresponding BMD MIC_{50} values is shown in chapter 14.2.3 (**Table 27**). **Table 33** presents BMD MICs for QC reference strains in Study II [465].

Table 33 QC BMD MIC data in Study II [465]. Numbers of readings at various MICs (mg/L) for *H. influenzae* reference strains (**Table 19**). Green, ATCC 49766 (sPBP3); red, ATCC 49247 (rPBP3); blue, ATCC 35056 (*bla* positive). Shadings; QC MIC ranges [75,113]; vertical lines, ECOFFs (**Table 17**)

	BMD MIC (mg/L)													
Agents	**≤0.002**	**0.004**	**0.008**	**0.016**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**
Ampicillin						4	2				3	9		
Ampicillin-sulbactam[a]						1	1		1	1	1	2		
Amoxicillin								4	2		1	6	5	
Amoxicillin-clavulanic acid[b]							1	1		2		2	1	
Piperacillin	6					1	8	2	1					
Piperacillin-tazobactam[a]	2	2				2	1							
Cefuroxime								8			2	2	7	4
Cefotaxime	6	2					3	8	4					
Meropenem					7	1	2	4	9					

[a] *bla* inhibitor concentration fixed at 4 mg/L
[b] *bla* inhibitor concentration fixed at 2 mg/L

Several QC ranges for the sPBP3 strain ATCC 49766 were established by EUCAST in 2014 and were not available when the data were produced and published. A retrospective quality assessment revealed that cefotaxime MIC values for this strain were out of range (too low). Accordingly, cefotaxime MIC_{50} values for clinical sPBP3 isolates in Study II (0.004 mg/L) were two dilutions lower than modal MIC of the wild-type population (0.016 mg/L). Similarly, MIC_{50} values for clinical sPBP3 isolates and piperacillin (with and without tazobactam) were two dilutions lower than modal MIC of the wild-type population (0.004 mg/L versus 0.016 mg/L).

All cefotaxime and piperacillin (with and without tazobactam) MICs for ATCC 49247 (rPBP3) were within the accepted ranges, indicating that the published MIC values for clinical isolates [465] are reliable at ranges close to ECOFF (piperacillin) and the clinical breakpoints (cefotaxime). However, the two agents were excluded from the methodology study (Study IV) [467], as they were considered unsuitable as gold standards for evaluation of Etest and disk diffusion (chapters 14.4.2 and 14.4.3).

Ampicillin and amoxicillin QC MICs were within accepted ranges. However, ampicillin MIC_{50} for clinical *bla* negative sPBP3 isolates (0.25 mg/L, **Figure 18**) was

one dilution lower than modal MIC of the wild-type population according to the EUCAST database (0.5 mg/L). The discrepancy triggered an investigation at the EUCAST Development Laboratory, showing that all distributions in the database had median MICs at 0.25 mg/L, except one large distribution with a median MIC at 0.5 mg/L (G. Kahlmeter, personal communication 2010-06-22). After removal of the distribution from the database, modal ampicillin MIC of the wild type population in the EUCAST database was lowered by one dilution step (**Figure 23**).

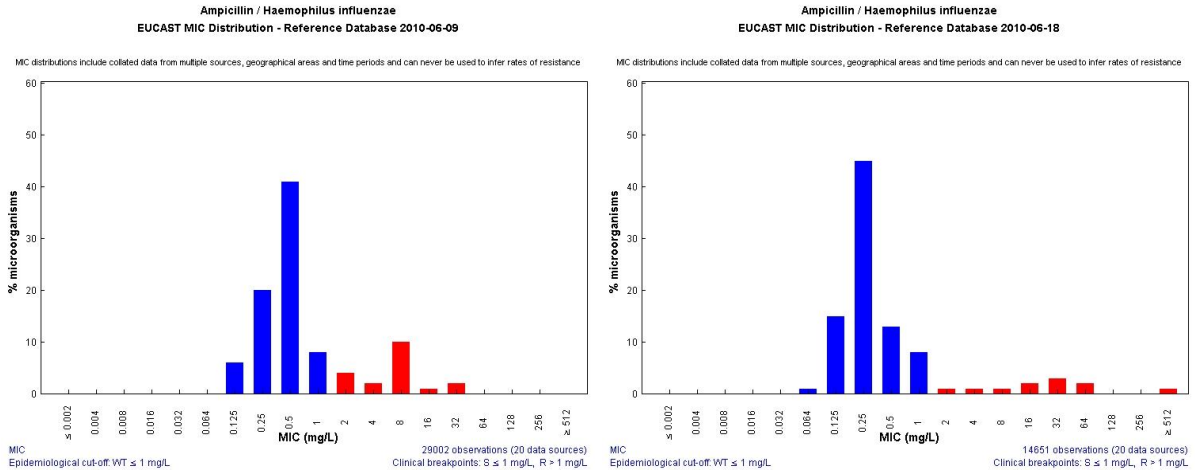


Figure 23 MIC distributions for *H. influenzae* and ampicillin in the EUCAST database (www.eucast.org/MIC_distributions) before (left) and after (right) adjustments in June 2010 (see text). Images provided by G. Kahlmeter

The revised MIC distribution for ampicillin (modal MIC 0.25 mg/L) visualized the slightly higher *in vitro* activity of this agent against *H. influenzae* compared to amoxicillin (0.5 mg/L). This is consistent with different ECOFFs (ampicillin, 1 mg/L; amoxicillin, 2 mg/L). Accordingly, EUCAST MIC breakpoints for amoxicillin (and amoxicillin-clavulanic acid) were changed from 1/1 (S≤/R>, mg/L) to 2/2 in January 2012 (www.eucast.org/ast_of_bacteria/previous_versions_of_documents).

EUCAST and CLSI recommend that ampicillin may be used to infer susceptibility to amoxicillin [75,111]. Before the change in breakpoints, this recommendation implicated a discrepancy between categorization of susceptibility to amoxicillin based on amoxicillin MIC and categorization based on susceptibility to ampicillin. Using current MIC breakpoints for ampicillin and amoxicillin, ‘categorical correlation’ (susceptible to both or resistant to both) in Study IV was 89.6% (138/154), and

amoxicillin MIC was within ±1 dilution of ampicillin MIC +1 dilution ('essential correlation') for 86.4% (133/154) of the isolates [467].

The observations and events referred in this chapter illustrate that MIC data may be misleading even when obtained by reference methodology, and underline that careful QC is crucial to ensure reliable results (chapter 8.2). The contribution to improved quality of the EUCAST database, leading to adjustments in clinical breakpoints for *H. influenzae*, represents one of the main achievements in this project.

14.4.2 *Evaluation of Etest*

In Study IV [467], 104 *bla* negative group II low-rPBP3 and 50 *bla* negative sPBP3 *H. influenzae* from Study II [465] were used to evaluate Etest for categorization to beta-lactams, with BMD MIC interpreted according to EUCAST breakpoints [111] as the gold standard. All Etest MICs for QC strains were within accepted ranges (**Table 34**).

Table 34 QC Etest MIC data in Study IV [467]. Numbers of readings at various MICs (mg/L) for *H. influenzae* reference strains (**Table 19**). Green, ATCC 49766 (sPBP3); red, ATCC 49247 (rPBP3). Shadings; QC MIC ranges [75,113]; vertical lines, ECOFFs (**Table 17**)

	Etest MIC (mg/L)													
Agents	**≤0.002**	**0.004**	**0.008**	**0.016**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**
Ampicillin								5			1	5		
Amoxicillin									5			6		
Piperacillin			2	2	1		1	3	2					
Cefuroxime									4	2		1	3	3
Cefotaxime				6				7						
Meropenem						6	1	4	1					

Etest MICs for the isolates in Study IV are compared to BMD in **Table 35**. Essential and categorical agreement rates were low (<70%) for all agents tested. Consistent with ampicillin and amoxicillin MICs for the sPBP3 QC strain deviating from target by +1 dilution, Etest generally overestimated MIC at lower ranges and underestimated MIC at higher ranges.

Table 35 Susceptibility categorization of *H. influenzae* (*bla* negative, n=154) by Etest (HTM) with BMD MIC (HTM) and EUCAST breakpoints [111] as gold standard. Data from [467]

	BMD MIC (mg/L)												
Etest[a]	0.06	0.125	0.25	0.5	1	2	4	8	16	≥32	n	%	EA[b] / FSR[c]
AMP													
+3	2										2	1.3%	
+2	7	6	3	1							17	11.0%	
+1	2	12	4	10	1	1					30	19.5%	
0			8	3	21	3					35	22.7%	69.5%[b]
-1			2	2	9	26	3				42	27.3%	
-2					1	5	17				23	14.9%	
-3						2[d]	1	2			5	3.2%	
CA	11	18	17	15	31	4	3				99	64.3%	
ME				1	1						2	1.3%	
VME						33	18	2			53	34.4%	88.3%[c]
AMX													
+3		1[d]			2						3	1.9%	
+2		5	8	5	2	2					22	14.3%	
+1		1	4	8	10	1	1	1			26	16.9%	
0				9	8	11	3	9			40	26.0%	63.0%[b]
-1				2	8	4	12	5			31	20.1%	
-2						3	5	14		1	23	14.9%	
-3							2[d]	5[d]	2		9	5.8%	
CA		7	12	24	26	18	4	15		1	107	69.5%	
ME					4	3					7	4.5%	
VME							19	19	2		40	26.0%	66.7%[c]
CXM													
+3		1	2	3[d]	13[d]	1					20	13.0%	
+2			7	4	6	3	1				21	13.6%	
+1		1	10	8	10	3		4			36	23.4%	
0			1	4	12	4		9			30	19.5%	54.5%[b]
-1					5		2	7		4	18	11.7%	
-2								12	5	2	19	12.3%	
-3								4[d]	2	4[d]	10	6.5%	
CA		2	18	12	17	4	1	20	5	9	88	57.1%	
mE			2	4	10	7	2	12	2	1	40	26.0%	
ME				3	19						22	14.3%	
VME								4			4	2.6%	7.1%[c]

(See legend on next page)

Table 35 (previous page)

[a] Difference between Etest (AMP, ampicillin; AMX, amoxicillin; CXM, cefuroxime) and BMD MIC expressed as the number of twofold dilutions by which Etest MIC is higher (positive values) or lower (negative values) than BMD MIC. CA, categorical agreement between Etest MIC and BMD MIC; mE, minor error: intermediate by Etest and susceptible/resistant by BMD MIC, or susceptible/resistant by Etest and intermediate by BMD MIC; ME, major error: resistant by Etest and susceptible by BMD MIC; VME, very major error: susceptible by Etest and resistant by BMD MIC

[b] Essential agreement: proportion of gradient MICs within ±1 dilution of BMD MIC

[c] False susceptible rate: proportion of isolates resistant by BMD MIC categorized as susceptible by Etest (calculated by dividing the number of VMEs by the number of isolates with BMD MICs above the R-breakpoint (breakpoints indicated by vertical lines)

[d] Includes Etest MICs deviating from BMD MIC by more than three dilutions

By Etest, 94.2% (145/154) of the isolates were categorized as ampicillin susceptible, compared to 61.0% (94/154) with reference methodology. On average, Etest underestimated ampicillin MIC by one, two and three dilutions for isolates with BMD MICs of 2 mg/L, 4 mg/L and 8 mg/L, respectively, leading to high VME (34.4%) and FSR (88.3%) for this agent. In other words, Etest wrongly categorized most ampicillin resistant isolates as susceptible.

The results are in accordance with previous reports of underestimation of resistance to ampicillin by Etest [27,149,522] (chapter 8.3). Etest is widely used for routine susceptibility testing, and false categorization of rPBP3 *H. influenzae* as susceptible to aminopenicillins may be clinically important.

The results illustrate that a systematic difference between MIC methodologies of one dilution may greatly affect susceptibility rates when the clinical breakpoints divide the resistant population, which is the case for aminopenicillins and low-rPBP3 *H. influenzae* (chapter 8.1). It should be noted that only one medium (HTM) with MHA from only one manufacturer (**Table 21**) was used in the present evaluation.

Data from Study IV suggest that introduction of a wide intermediate category to avoid division of the rPBP3 population would improve categorical agreement between Etest and BMD MIC (not shown). PK/PD breakpoints defined by EUCAST support an intermediate category encompassing *bla* negative *H. influenzae* with ampicillin MIC

up to 8 mg/L [111]; however, clinical data to support breakpoint changes are lacking [110,139,516,537]. In contrast to EUCAST, CLSI recommend that *H. influenzae* with ampicillin MIC = 2 mg/L are categorized as intermediately susceptible [75]. Replacing EUCAST breakpoints with CLSI breakpoints for ampicillin would not improve categorical agreement (CLSI, 61.7%; EUCAST, 64.3%) or FSR (CLSI, 87.0%; EUCAST, 88.3%). The number of VME would be reduced (CLSI, 13.0%; EUCAST, 34.4%), but mE would be frequent (25.3%) [467].

The poor categorical agreement of cefuroxime Etest was mainly due to a high number of minor errors. Notably, current EUCAST breakpoints define part of the wild-type population (MIC = 2 mg/L) as intermediate susceptible to cefuroxime. Using ECOFF for S/R-categorization, recalculated categorical agreement and FSR by cefuroxime Etest were 67.5% (104/154) and 32.8% (21/64), respectively; i.e. underestimation of resistance was frequent, but less frequent compared to aminopenicillins.

Implications of high FSR by ampicillin, amoxicillin and cefuroxime Etest for test algorithms and interpretation and reporting of results are discussed in chapter 14.4.5.

Etest was also used to test susceptibility to piperacillin (with/without tazobactam), cefotaxime and meropenem. Piperacillin and cefotaxime BMD MICs were found unsuitable as gold standards (chapter 14.4.1) and excluded from the evaluation.

Evaluation of meropenem Etest was valid, but data were not included in the paper [467] for pedagogic reasons (similar test panels for evaluation of Etest and disk diffusion; chapter 14.4.3). Similar to other agents, meropenem Etest overestimated MIC in lower ranges and underestimated MIC at higher ranges (**Figure 24**). Essential agreement with BMD MIC was 77% (118/154), and categorical agreement (with EUCAST meningitis breakpoints) was 84% (130/154). No ME were observed; VME and FSR could not be evaluated as no resistant isolates were included.

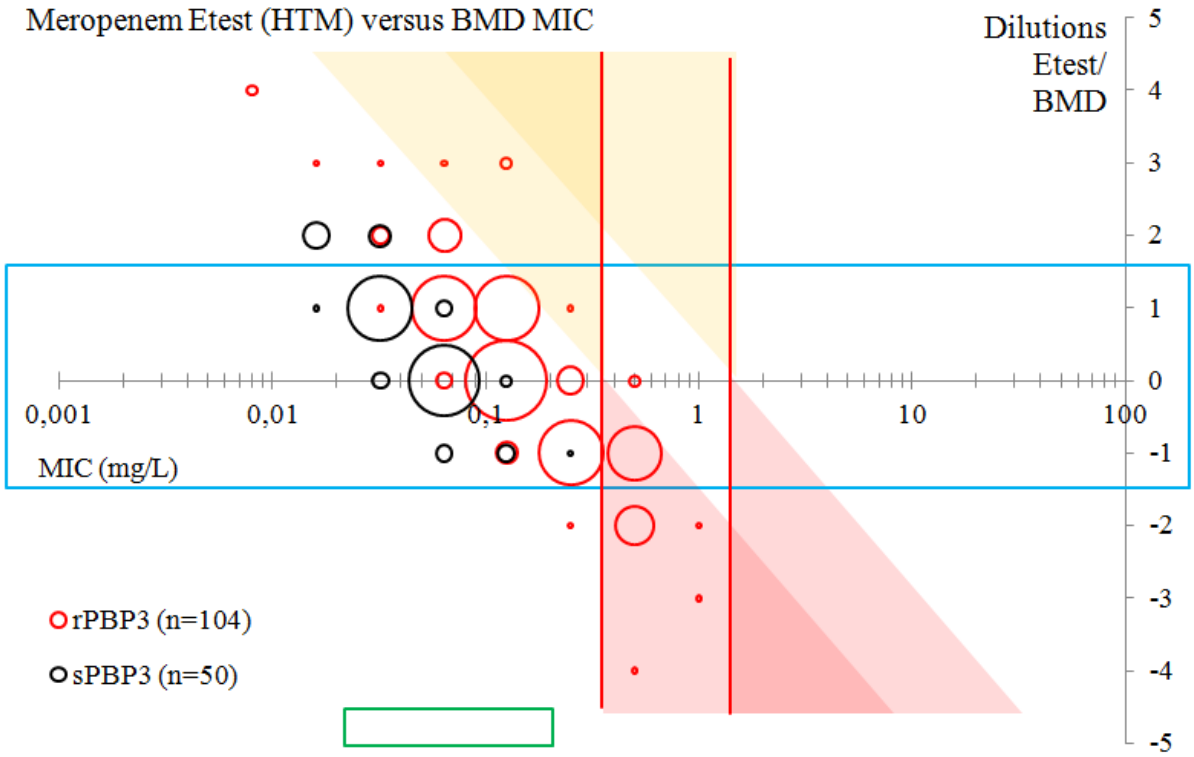


Figure 24 Bubble chart (modified Bland-Altman plot) showing the correlation between Etest and BMD for determination of meropenem MIC in *H. influenzae* (n=154). BMD MICs from [465]. The vertical axis shows the difference between the methods expressed as the number of twofold dilutions by which Etest MIC is higher (positive) or lower (negative) than BMD MIC. Bubble sizes indicate proportions of isolates. Data are presented separately for rPBP3 (red) and sPBP3 (black) isolates. Blue frame, essential agreement (±1 dilution). Red vertical lines, EUCAST breakpoints [111]. Shadings indicate VME (dark red), ME (dark yellow), and mE (light red/yellow). Green frame, QC MIC range (ATCC 49766) [75,113]

14.4.3 *Evaluation of EUCAST disk diffusion*

In Study IV [467], EUCAST disk diffusion [282] was evaluated for susceptibility categorization of *H. influenzae* to aminopenicillins and cefuroxime, using the same test population as for Etest (chapter 14.4.2). All zones for the QC strains NCTC 8468 were within accepted ranges [113].

Categorization was performed according to EUCAST recommendations [111], using standard disks (ampicillin 2 µg, AMP2; cefuroxime 30 µg, CXM30) and breakpoints, and amoxicillin susceptibility inferred from ampicillin. Categorization to ampicillin, amoxicillin and cefuroxime was also performed with alternative disks (amoxicillin-

clavulanic acid 2-1 µg, AMC3; cefuroxime 5 µg, CXM5) and zone breakpoints, and with the benzylpenicillin 1 unit (PG1) disk and EUCAST screening breakpoints [111].

Zone-MIC correlations showed considerable overlapping of susceptible and resistant isolates with AMP2, AMC3, CXM30 and CXM5 (Supplemental Figure S1 [467], see Appendix). The performances of the four disks and PG1 for categorization of susceptibility to ampicillin, amoxicillin and cefuroxime using different sets of zone breakpoints, and a comparative analysis with Etest (chapter 14.4.2), are summarized in **Table 36**. Categorical agreement was poor and VME were frequent with currently recommended disks and breakpoints [111], in particular for aminopenicillins. The AMP2 disk underestimated resistance to ampicillin and categorized 87.0% (134/154) of the isolates as susceptible, compared to 61.0% (94/154) with BMD MIC. Agreement with ampicillin BMD MIC was slightly higher with the AMC3 disk compared to AMP2 (p=0.1081), and VME (p=0.0047) and FSR (p=0.0001) were significantly lower. AMC3 was also superior to ampicillin Etest for categorization to ampicillin.

A minor zone breakpoint adjustment (+2 mm) for AMP2 slightly increased agreement with BMD MIC for categorization to ampicillin (p=0.1593) and amoxicillin (p=0.0464). With adjusted zone breakpoints for AMP2 and AMC3 (+1 mm), there were no significant differences between the disks, and both were superior to Etest for categorization of susceptibility to aminopenicillins. In addition, categorical correlation between AMP2 (ampicillin) and AMC3 (amoxicillin) increased from 79.9% (123/154) with current breakpoints to 90.9% (140/154) with adjusted breakpoints (p=0.0121).

Notably, use of disks and media from different manufacturers may greatly affect the results by disk diffusion. Such variation was not investigated in this project, and a broader evaluation is necessary to decide whether a change in breakpoints is advisable.

Even with adjusted breakpoints, FSR with AMP2 (28.3%) in Study IV was higher than the FSR calculated from zone-MIC correlations published by EUCAST (13.6%) (**Table 18**). Differences in accuracy and precision of the methods used for determination of reference MICs are important contributors to inter-investigator variations in zone-MIC correlations and categorical agreement rates.

Table 36 Susceptibility categorization to ampicillin, amoxicillin and cefuroxime by disk diffusion (EUCAST methodology), with BMD MIC interpreted according to EUCAST breakpoints [111] as gold standard. Beta-lactamase negative isolates (n=154). From [467]

Agent categorized	Disk[a]	Zone breakpoints[b]	FSR[c]			VME[c]			ME[c]		mE[c]		CA[c]		
			Fraction	%[d]		n	%[d]		n	%	n	%	n	%[d]	
Ampicillin	AMP2	16/16[e]	46/60	76.7%	(↓)	46	29.9%	(↓)	6	3.9%	NA	NA	103	66.9%	(↑)
	AMP2	18/18	17/60	28.3%	↓↓	17	11.0%	↓↓	24	15.6%	NA	NA	114	74.0%	(↑)
	AMC3	15/15[f]	24/60	40.0%	↓↓	24	15.6%	↓	15	9.7%	NA	NA	116	75.3%	↑
	AMC3	16/16	17/60	28.3%	↓↓	17	11.0%	↓↓	20	13.0%	NA	NA	118	76.6%	↑
	PG1	12/12[g]	2/60	3.3%	↓↓	2	1.3%	↓↓	45	29.2%	NA	NA	107	69.8%	(↑)
Amoxicillin	AMP2	16/16[e]	47/60	78.3%	(↑)	47	30.5%	(↑)	7	4.5%	NA	NA	101	65.6%	(↓)
	AMP2	18/18	16/60	26.7%	↓↓	16	10.4%	↓	22	14.3%	NA	NA	117	76.0%	(↑)
	AMC3	15/15[f]	25/60	41.7%	↓	25	16.2%	↓	16	10.4%	NA	NA	114	74.0%	(↑)
	AMC3	16/16	17/60	28.3%	↓↓	17	11.0%	↓	20	13.0%	NA	NA	118	76.6%	(↑)
	PG1	12/12[g]	3/60	5.0%	↓↓	3	1.9%	↓↓	46	29.9%	NA	NA	105	68.2%	(↓)
Cefuroxime	CXM30	26/25[e]	15/56	26.8%	↑	15	9.7%	↑	27	17.5%	20	13.0%	93	60.4%	(↑)
	CXM5	20/19	7/56	12.5%	(↓)	7	4.5%	(↑)	32	20.8%	17	11.0%	99	64.3%	(↑)
	PG1	12/12[g]	4/56	7.1%	-	4	2.6%	-	40	26.0%	11	7.1%	99	64.3%	(↑)

[a] AMP2, ampicillin 2 µg; AMC3, amoxicillin-clavulanic acid 2-1 µg; CXM5, cefuroxime 5 µg; CXM30, cefuroxime 30 µg; PG1, benzylpenicillin 1 unit
[b] S≥/R< (mm). The reference is Study IV [467] unless otherwise indicated
[c] FSR, false susceptible rate (proportion of isolates R by BMD MIC categorized as S by disk diffusion); VME, very major error (S by disk diffusion and R by BMD MIC); ME, major error (R by disk diffusion and S by BMD MIC); mE, minor error (S/R by disk diffusion and I by BMD MIC, or I by disk diffusion and S/R by BMD MIC); CA, categorical agreement; NA, not applicable
[d] Comparison with results obtained by Etest (**Table 35**) and calculation of significance levels using chi-square test. ↑, higher; ↓, lower; -, identical; ↑↑/↓↓, $p \leq 0.0001$; ↑/↓, $p \leq 0.05$; (↑)/(↓), tendency, not significant ($p > 0.05$)
[e] EUCAST clinical breakpoint [111]
[f] EUCAST clinical breakpoint for susceptibility categorization to amoxicillin-clavulanic acid [111]
[g] EUCAST screening breakpoint for detection of isolates with beta-lactam resistance mechanisms [111]

I am aware of only one published, independent investigation comparing EUCAST disk diffusion to BMD MIC for susceptibility categorization of *H. influenzae* to beta-lactams (chapter 8.4). Søndergaard *et al.* reported that >50% (7/13 or 8/14) of ampicillin resistant isolates were susceptible with AMP2 [475], consistent with FSR of 76.7% (current breakpoints) and 28.3% (adjusted breakpoints) in Study IV (**Table 36**).

Non-inferiority of AMC3 compared to AMP2 for categorization to aminopenicillins is consistent with previous investigations based on HTM [219]. The MIC-MIC (chapter 14.4.1), MIC-zone and zone-zone correlations observed in the present project suggest that AMP2 and AMC3 may be used interchangeably for categorization of susceptibility to aminopenicillins in *bla* negative *H. influenzae*. Consequently, AMC3 may be used for testing of *bla* positive and *bla* negative isolates for susceptibility to aminopenicillins with and without *bla* inhibitor. Use a single disk irrespective of *bla* production would significantly simplify routine susceptibility testing of *H. influenzae*.

Although the antibacterial activity of clavulanic acid against *H. influenzae* is low (MIC range 25-125 mg/L) [129], the inhibitor component may theoretically have a slight impact on the inhibition zone by testing of *bla* negative isolates with the AMC3 disk, and it needs to be clarified whether separate zone breakpoints are needed for susceptibility categorization of *bla* positive versus *bla* negative isolates.

As BMD MICs for piperacillin for the sPBP3 QC strain were out of range (too low), data for this agent were excluded from Study IV (chapter 14.4.1). However, BMD MICs for the rPBP3 QC strain were within the accepted range (**Table 33**), and overestimation of piperacillin MIC is unlikely. Six rPBP3 isolates included in Study IV had piperacillin BMD MIC above ECOFF (range 0.125-0.25 mg/L); according to current EUCAST zone breakpoints, 6/6 and 4/6 were categorized as susceptible with AMP2 and AMC3, respectively. The results suggest that the disks recommended by EUCAST for categorization of susceptibility to piperacillin and piperacillin-tazobactam are unable to identify *H. influenzae* with increased MIC to these agents.

For cefuroxime, categorical agreement by disk diffusion was poor with both disk potencies and not significantly different from Etest (**Table 36**). FSR and VME rates were significantly higher with CXM30 compared to Etest (and PG1), but there were no

significant differences between Etest and CXM5 or between CXM5 and CXM30. Notably, a considerably higher FSR was obtained with the CXM30 disk in this project (15/56, 26.8%) compared to the MIC-zone correlations registered in the EUCAST database (1/85, 1.2%; **Table 18**).

In conclusion, data from this project suggest that disk diffusion according to current EUCAST recommendations is associated with high frequencies of VME by categorization of susceptibility to aminopenicillins, piperacillin (with/without tazobactam) and cefuroxime. However, there are discrepancies with EUCAST data and additional independent investigations are needed. Strategies to reduce the clinical consequences of VME by routine susceptibility testing are discussed in chapter 14.4.5.

14.4.4 *Evaluation of rPBP3 screening disks*

Different disks and media may be used to screen for beta-lactamase resistance in *H. influenzae* (chapter 8.5). In a 2007 pilot study (presented at the SSAC 2007) [463], we evaluated the pre-EUCAST screening method (PV10 and CEC30 disk; supplemented ISA) with the 46 isolates from Study I [464]. Screening failed to identify 22% (5/23) of the rPBP3 isolates, clearly illustrating the need for improved screening methods. Although not formally part of the present project, the investigation has historical interest; the poster [463] is therefore included in the Appendix section of this thesis.

In Study IV [467], 154 *bla* negative *H. influenzae* from Study II [465] were used to evaluate nine disks by their ability to identify low-rPBP3 isolates. The test population was identical to the population used to evaluate Etest (chapter 14.4.2) and disk diffusion (chapter 14.4.3).

As the first evaluation of PG1, premilinary results from Study IV were used to establish the screening breakpoints introduced in the first NordicAST breakpoint table in 2010 (chapter 8.5). The data were partly (PG1, PV10 and CEC30) presented at the 21st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in 2011 [462]; the poster is included in the Appendix section.

An ROC diagram showing the relative performance of the evaluated disks is presented in **Figure 25**. Genotype-zone correlations are presented in Paper IV [467] (supplemental Figure S3; see Appendix). The calculated test performances are summarized in **Table 37**.

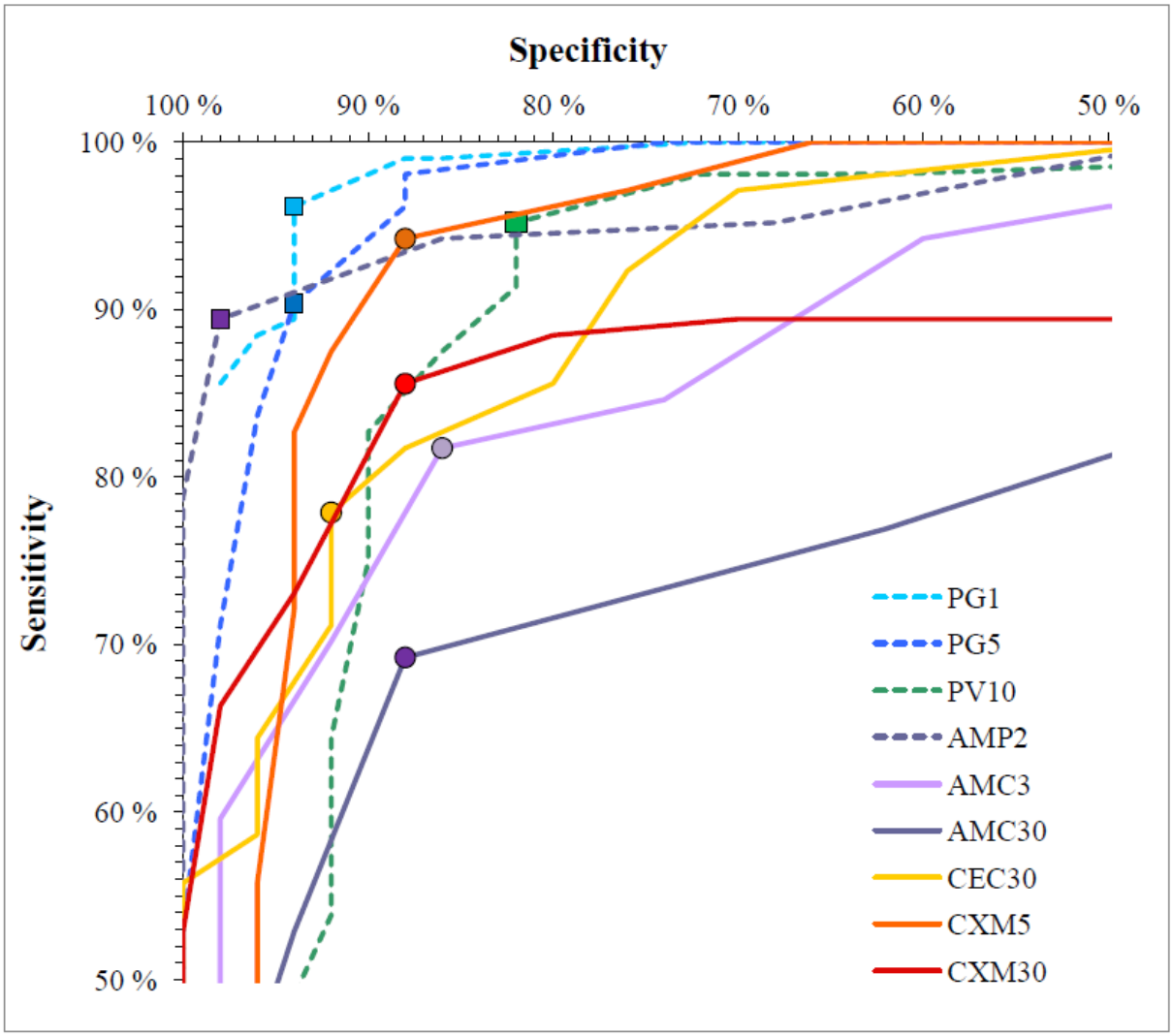


Figure 25 Receiver operating characteristic (ROC) diagram showing the performance of nine beta-lactam disks for detection of the rPBP3 genotype in *H. influenzae* (*bla* negative, n=154). Optimized screening breakpoints (**Table 37**) are indicated for each disk. Solid lines, *bla* stable agents; dashed lines, *bla* susceptible agents. PG1, benzylpenicillin 1 unit; PG5, benzylpenicillin 5 units; PV10, phenoxymethylpenicillin 10 µg; AMP2, ampicillin 2 µg; AMC3, amoxicillin-clavulanic acid 2-1 µg; AMC30, amoxicillin-clavulanic acid 20-10 µg; CEC30, cefaclor 30 µg; CXM5; cefuroxime 5 µg; CXM30, cefuroxime 30 µg. From [467]

Table 37 Screening for penicillin-binding protein 3-mediated beta-lactam resistance (rPBP3) by disk diffusion (EUCAST methodology). Beta-lactamase (*bla*) negative *H. influenzae* (n=154); rPBP3 prevalence in the test population 67.5% (104/154)

Disk[a]	*Bla* stable	Break-point[b]	Sensitivity (%)	Specificity (%)	PPV[c] (%)	NPV[d] (%)	Accuracy[e] (n)	Accuracy[e] (%)
PG1	No	12[f]	96.2	94.0	97.1	92.2	147	95.5
PG5	No	20	90.4	94.0	96.9	82.5	141	91.6
PV10	No	20	95.2	82.0	91.7	89.1	140	90.9
AMP2	No	20	89.4	98.0	98.9	81.7	142	92.2
AMC3	Yes	18	81.7	86.0	92.4	69.4	128	83.1
AMC30	Yes	26	69.2	88.0	92.3	57.9	116	75.3
CEC30	Yes	23	77.9	92.0	95.3	66.7	127	82.5
CXM5	Yes	21	94.2	88.0	94.2	88.0	142	92.2
CXM30	Yes	27	85.6	88.0	93.7	74.6	133	86.4

[a] PG1, benzylpenicillin 1 unit; PG5, benzylpenicillin 5 units; PV10, phenoxymethylpenicillin 10 µg; AMP2, ampicillin 2 µg; AMC3, amoxicillin-clavulanic acid 2-1 µg; AMC30, amoxicillin-clavulanic acid 20-10 µg; CEC30, cefaclor 30 µg; CXM5; cefuroxime 5 µg; CXM30, cefuroxime 30 µg

[b] Optimized screening breakpoints (S≥, mm) with rPBP3 isolates (N526K positive) defined as screening targets (see **Figure 25**)

[c] Positive predictive value

[d] Negative predictive value

[e] Correct assignment to resistance genotype (N526K positive and screening positive, or N526K negative and screening negative)

[f] Identical to the screening breakpoint recommended by EUCAST [111]

The PG1 disk identified rPBP3 isolates with the highest sensitivity and accuracy of all tested disks. EUCAST introduced this disk as first-line screening for beta-lactam resistance in 2012 (www.eucast.org/ast_of_bacteria/previous_versions_of_documents) Validation by EUCAST [112] showed slightly higher sensitivity (98%), specificity (98%) and accuracy (98%) compared to Study IV (96.2%, 94.0%, and 95.5%, respectively).

Somewhat different test performance was reported in a later, independent evaluation of PG1 as screening for the rPBP3 genotype in *H. influenzae* [476]. Using disks and media similar to Study IV, the authors found 91% sensitivity, 99% specificity and 96% accuracy. Diverging results (despite identical screening breakpoints, S≥12 mm [111]) may in part be due to different interpretation of hazy growth [219]. This phenomenon was frequent for rPBP3 isolates in Study IV (in particular with PG1 and CEC30; **Figure 13**); sensitivity was substantially reduced when hazy growth was ignored.

As PG1 is unsuitable for rPBP3 screening in *bla* positive *H. influenzae*, five disks with *bla* stable agents were included in Study IV. The CXM5 disk, evaluated for the first time in this project, categorized isolates according to resistance genotype with the highest accuracy and demonstrated superior test performances compared to the CEC30, CXM30, AMC3 and AMC30 disks previously evaluated by others [147,476]. CXM5 was the only *bla* stable disk with sensitivity and accuracy >90%.

It should be noted that PPV and NPV are prevalence dependent (similar to VME and ME; chapter 8.2), and that the rPBP3 prevalence in the test population (67.5%) was considerably higher than the prevalence in a representative population in Norway (**Figure 19**). When the sensitivity and specificity of a test are known, predictive values at a given prevalence may be calculated using Bayes' theorem [205] (chapter 13.5). The calculated predictive values for the PG1 and CXM5 disks, with the test performances obtained in Study IV (**Table 37**) and the estimated rPBP3 prevalence in the NORM 2014 surveillance population (16.6%, chapter 14.2.5), were as follows:

PG1, PPV = 76.2%, NPV = 99.2%; CXM5, PPV = 61.0%, NPV = 98.7%.

In conclusion, the present and previous investigations indicate that the PG1 disk, with screening breakpoints recommended by EUCAST, reliable detects *H. influenzae* with the rPBP3 genotype. Importantly, all disks with *bla* stable agents evaluated so far have suboptimal test performances. As shown in this project, CXM5 appears to be the most reliable alternative; however, the disk is not a standard EUCAST disk and currently not available from all manufacturers. Further improvements of the screening method for rPBP3 detection in *bla* positive *H. influenzae* are needed.

14.4.5 *Test algorithm*

To my knowledge, Study IV [467] is the first study comparing gradient MIC and EUCAST disk diffusion with reference methodology for susceptibility testing of *H. influenzae* (chapter 8.4). The poor categorical agreement rates and the high frequencies of false susceptible results by categorization of rPBP3 *H. influenzae* to beta-lactams by routine methods are worrisome (chapters 14.4.2 – 14.4.3).

Importantly, the PG1 screening disk detects isolates with acquired resistance mechanisms with high sensitivity and specificity and predictive values (chapter 14.4.4), reducing the need for agent-directed testing to screening-positive isolates. Thus, screening with PG1 should always be performed as a part of susceptibility testing of *H. influenzae* in order to reduce the probability of VME. Isolates that are rPBP3 positive by screening should be reported as ampicillin resistant in cases of meningitis, irrespective of additional test results, analogous to current recommendations for pneumococci positive by screening for beta-lactam resistance [111]. Screening-positive *bla* negative isolates may also be categorized as cefuroxime resistant without further testing.

The results from Study IV indicate that EUCAST disk diffusion is non-inferior to Etest (with HTM) for categorization of susceptibility to ampicillin, amoxicillin and cefuroxime. Correlation data suggest that the AMC3 disk may be used instead of AMP2 for susceptibility categorization of *bla* negative *H. influenzae* to ampicillin. This simplification of agent-directed testing does, however, require validation of zone breakpoints for AMC3 and *bla* negative isolates.

To minimize the clinical consequences of false susceptible results, we suggested adding a comment recommending high-dose aminopenicillin therapy or the use of other agents in severe infections caused by screening-positive isolates categorized as susceptible to aminopenicillins by disk or gradient diffusion [467].

With the exception of replacing AMP2 with AMC3, the recommendations above have been implemented by NordicAST [338].

EUCAST [111] and CLSI [75] recommend that susceptibility to piperacillin and piperacillin-tazobactam is inferred from susceptibility to ampicillin and amoxicillin-clavulanic acid (chapter 8.1). As discussed in chapter 14.3.2, this interpretative rule frequently implicates categorization of *H. influenzae* as resistant to piperacillin and piperacillin-tazobactam, despite retained high *in vitro* activity of the drugs. Conversely, unpublished data from Study IV showed poor correlation between piperacillin MIC and inhibition zones with AMP2 and AMC3, and most isolates with

non-wild-type piperacillin MIC were categorized as susceptible to piperacillin based on susceptibility to aminopenicillins (chapter 14.4.3).

These observations suggest that categorization of susceptibility to piperacillin (with/without tazobactam) should be performed through agent-directed testing and interpretation according to clinical breakpoints.

15 CONCLUSIONS AND FUTURE REMARKS

The beta-lactam resistance epidemiology of *H. influenzae* in Norway has changed considerably during the last decade. When this project was initiated, isolates with non-*bla*-mediated resistance were rare, and few laboratories had established routines for detection of such isolates. Ten years later, the prevalence approaches 20%, and *H. influenzae* strains resistant to extended-spectrum cephalosporins have emerged.

This project was the first to characterize the resistance mechanism in Nordic *H. influenzae* with non-*bla*-mediated resistance. Group II low-rPBP3 isolates accounted for 96% of rPBP3 *H. influenzae* in NORM 2007, and the significantly increased rPBP3 prevalence between 2004 (estimate 5.7%) and 2007 (14.6%) was mainly due to the expansion of four low-rPBP3 clones. Such clones may persist over several years, and a low-rPBP3 ST14 clone capable of causing invasive disease is particularly widespread.

A few (n=13) *bla*-negative isolates with non-wild type beta-lactam susceptibility in Study II lacked rPBP3-defining substitutions, suggesting the existence of additional resistance mechanisms.

Study III showed that high-rPBP3 *H. influenzae* emerged and spread in Norway during the project period. The strain collection is unique outside Japan. Of particular notice is the large number (n=23) of group III isolates, including 12 group III(+)isolates with the additional L389F substitution associated with increased resistance; these genotypes have rarely been reported outside Japan and Korea. An extensively MDR group III(+) high-rPBP3 ST159 strain, resistant to all extended-spectrum cephalosporins tested, and four classes of non-beta-lactams, was isolated from three patients at the same hospital within a period of four days, illustrating the need for hygienic measures to prevent nosocomial spread of MDR *H. influenzae*. The remarkable resistotype of this strain is previously unreported.

The temporal association with significantly increased usage of amoxicillin and extended-spectrum cephalosporins suggests that selective pressure, favouring strains with beta-lactam resistance mechanisms (rPBP3 and *bla*), contributed to the altered

resistance epidemiology of *H. influenzae* in Norway during the 2000s. These observations underline the importance of rational use of antibiotics. In addition, horizontal transfer of rPBP3-encoding *ftsI* gene fragments appears to contribute to evolution of rPBP3 strains *in vivo*. This project was the first to report identical *ftsI* alleles in rPBP3 strains unrelated by housekeeping phylogeny. Mechanisms contributing to the emergence and spread of rPBP3 *H. influenzae* are summarized in **Figure 26**.

The situation calls for improved surveillance of the global molecular epidemiology of resistant *H. influenzae*. The novel MLST-*ftsI* typing approach, developed and validated in this project, is a powerful tool for global surveillance of rPBP3 strains. To ensure safe empirical therapy, *H. influenzae* should be included in regional (EARS-Net) and global (WHO-GLASS) programs for surveillance of antimicrobial resistance in invasive isolates. Notification of *H. influenzae* resistant to extended-spectrum cephalosporins, with molecular characterization at a national reference laboratory, should be considered.

Susceptibility testing of rPBP3 *H. influenzae* and categorization of susceptibility to beta-lactams is challenging, mainly because current clinical breakpoints for aminopenicillins divide the low-rPBP3 population. False susceptibility to aminopenicillins is frequent by disk diffusion and Etest, commonly used in routine laboratories. Breakpoint changes may improve reproducibility of *in vitro* susceptibility testing and agreement with reference methodology, but clinical data to support such changes are insufficient. Future research should include clinical studies evaluating the therapeutic efficacy of aminopenicillins, extended-spectrum cephalosporins and piperacillin-tazobactam in infections caused by rPBP3 *H. influenzae* with different resistance genotypes and MIC levels.

The PG1 screening disk recommended by EUCAST and NordicAST, first evaluated in this project, detects *bla*-negative rPBP3 *H. influenzae* with high sensitivity and specificity. The CXM5 disk was evaluated for the first time in this project and appears to be the best current option for screening of *bla*-positive isolates. Isolates positive by

rPBP3 screening should be reported ampicillin resistant in cases of meningitis irrespective of results by agent-directed testing.

To minimize the clinical consequences of VME, a warning comment should be added for rPBP3 screening-positive isolates susceptible to aminopenicillins by disk diffusion and gradient tests. The recommendations above have been implemented in the NordicAST test algorithm.

Collaboration with other study groups has been established and several new investigations have been initiated as a result of this project. As a EUCAST Network Laboratory, Vestfold Hospital Trust collaborates with the EUCAST Development Laboratory on evaluation and improvement of methods for *in vitro* susceptibility testing of *H. influenzae*. Other projects include improved rPBP3 screening in *bla*-positive isolates and a broad evaluation of gradient tests. In two collaboratory projects with Lund University, imipenem resistance in *H. influenzae* and the characteristics of the widely disseminated and virulent ST14/PBP3 type A clone are investigated.

Collection and characterization of high-rPBP3 *H. influenzae* from Norway is an ongoing project in collaboration with the *Haemophilus* Reference Laboratory at the Norwegian Institute of Public Health and the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-Res). A WGS study with selected isolates from this project (including the MDR-ST159 strain) and more recent isolates with unusual phenotypes is performed in collaboration with K-res and Haukeland University Hospital. The project focuses on MDR and mobile genetic elements, virulence, and novel beta-lactam resistance mechanisms.

Additional future projects include recombinational studies focusing on the correlation between PBP3 substitutions and fitness, and the correlation between USS/pUSS density and uptake and transformation with mutant *ftsI* genes. Finally, the association between beta-lactam resistance and PBP3 substitutions other than the four included in the categorization system will be explored, and the impact of PBP2 substitutions on the susceptibility of *H. influenzae* to piperacillin will be investigated.

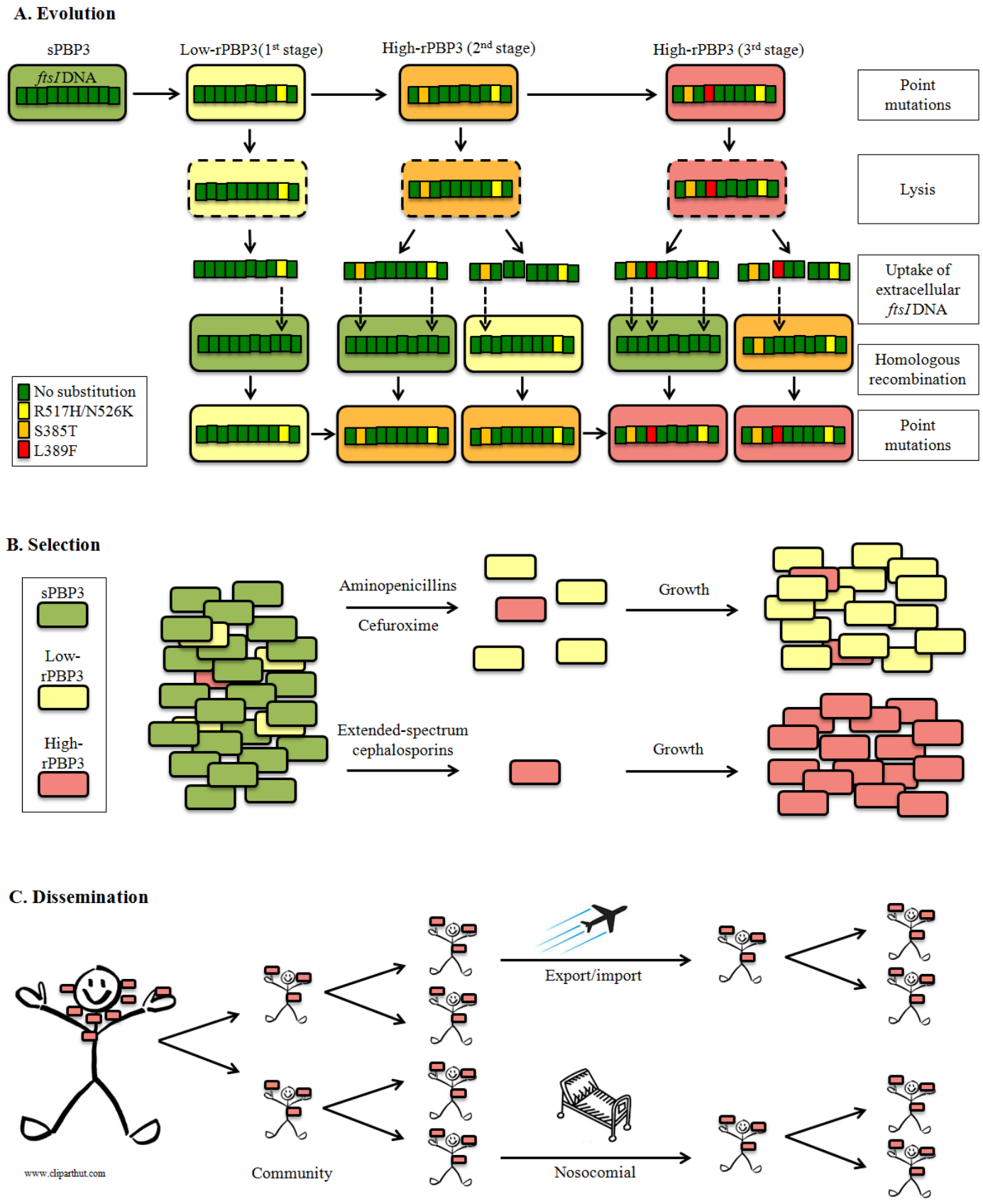


Figure 26 Emergence and spread of rPBP3 *H.influenzae*. A. Evolution of rPBP3 strains by spontaneous point mutations and horizontal transfer of mutant *ftsI* DNA. B. Emergence of low-rPBP3 and high-rPBP3 strains due to selective pressure by beta-lactams. C. Clonal dissemination of rPBP3 strains by person-to-person transmission

REFERENCES

List completed 2015-12-31.

1. Abdeldaim GM, Stralin K, Kirsebom LA, Olcen P, Blomberg J, Herrmann B: Detection of *Haemophilus influenzae* in respiratory secretions from pneumonia patients by quantitative real-time polymerase chain reaction. *Diagn Microbiol Infect Dis* 2009, 64: 366-373.
2. Abdeldaim GM, Stralin K, Olcen P, Blomberg J, Molling P, Herrmann B: Quantitative *fucK* gene polymerase chain reaction on sputum and nasopharyngeal secretions to detect *Haemophilus influenzae* pneumonia. *Diagn Microbiol Infect Dis* 2013, 76: 141-146.
3. Agrawal A, Murphy TF: *Haemophilus influenzae* Infections in the *H. influenzae* Type b Conjugate Vaccine Era. *Journal of Clinical Microbiology* 2011, 49: 3728-3732.
4. Ahren IL, Janson H, Forsgren A, Riesbeck K: Protein D expression promotes the adherence and internalization of non-typeable *Haemophilus influenzae* into human monocytic cells. *Microb Pathog* 2001, 31: 151-158.
5. Ahren IL, Karlsson E, Forsgren A, Riesbeck K: Comparison of the antibacterial activities of ampicillin, ciprofloxacin, clarithromycin, telithromycin and quinupristin/dalfopristin against intracellular non-typeable *Haemophilus influenzae*. *J Antimicrob Chemother* 2002, 50: 903-906.
6. Akerley BJ, Rubin EJ, Novick VL, Amaya K, Judson N, Mekalanos JJ: A genome-scale analysis for identification of genes required for growth or survival of *Haemophilus influenzae*. *Proceedings of the National Academy of Sciences* 2002, 99: 966-971.
7. Anderson RA, Schultz OT: Immunologic study of strains of *Bacillus pfeifferi* isolated from a case of meningitis. *J Exp Med* 1921, 33: 653-666.
8. Anderson R, Wang X, Briere EC, Katz LS, Cohn AC, Clark TA *et al.*: *Haemophilus haemolyticus* Isolates Causing Clinical Disease. *Journal of Clinical Microbiology* 2012, 50: 2462-2465.
9. Andersson M, Resman F, Eitrem R, Drobni P, Riesbeck K, Kahlmeter G *et al.*: Outbreak of a beta-lactam resistant non-typeable *Haemophilus influenzae* sequence type 14 associated with severe clinical outcomes. *BMC Infect Dis* 2015, 15: 581.
10. Andrews JM, BSAC Working Party on Susceptibility Testing ft: BSAC standardized disc susceptibility testing method. *Journal of Antimicrobial Chemotherapy* 2001, 48: 43-57.
11. Arguedas A, Soley C, Kamicker BJ, Jorgensen DM: Single-dose extended-release azithromycin versus a 10-day regimen of amoxicillin/clavulanate for the treatment of children with acute otitis media. *Int J Infect Dis* 2011, 15: e240-e248.
12. Arnold CJ, Garrigues G, St Geme JW, Sexton D: Necrotizing fasciitis caused by *Haemophilus influenzae* serotype f: A case report and review of the literature. *Journal of Clinical Microbiology* 2014.
13. Atkins NA, Kunde DA, Zosky G, Tristram SG: Genotypically defined beta-lactamase-negative ampicillin-resistant isolates of non-typable *Haemophilus influenzae* are associated with increased invasion of bronchial epithelial cells in vitro. *Journal of Medical Microbiology* 2014, 63: 1400-1403.
14. Avadhanula V, Rodriguez CA, Ulett GC, Bakaletz LO, Adderson EE: Nontypeable *Haemophilus influenzae* adheres to intercellular adhesion molecule 1 (ICAM-1) on respiratory epithelial cells and upregulates ICAM-1 expression. *Infect Immun* 2006, 74: 830-838.
15. Baddal B, Muzzi A, Censini S, Calogero RA, Torricelli G, Guidotti S *et al.*: Dual RNA-seq of Nontypeable *Haemophilus influenzae* and Host Cell Transcriptomes Reveals Novel Insights into Host-Pathogen Cross Talk. *MBio* 2015, 6.
16. Bae SM, Lee JH, Lee SK, Yu J, Lee SH, Kang YH: High prevalence of nasal carriage of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* in healthy children in Korea. *Epidemiology & Infection* 2013, 141: 481-489.

17. Bae S, Lee J, Lee J, Kim E, Lee S, Yu J *et al.*: Antimicrobial resistance in *Haemophilus influenzae* respiratory tract isolates in Korea: results of a nationwide acute respiratory infections surveillance. *Antimicrob Agents Chemother* 2010, 54: 65-71.

18. Bailey JK, Pinyon JL, Anantham S, Hall RM: Distribution of the blaTEM gene and blaTEM-containing transposons in commensal Escherichia coli. *Journal of Antimicrobial Chemotherapy* 2011, 66: 745-751.

19. Bajanca-Lavado MP, Simoes AS, Betencourt CR, Sa-Leao R: Characteristics of *Haemophilus influenzae* invasive isolates from Portugal following routine childhood vaccination against *H. influenzae* serotype b (2002-2010). *Eur J Clin Microbiol Infect Dis* 2014, 33: 603-610.

20. Balaban NQ, Gerdes K, Lewis K, McKinney JD: A problem of persistence: still more questions than answers? *Nat Rev Microbiol* 2013, 11: 587-591.

21. Barbé G, Babolat M, Boeufgras JM, Monget D, Freney J: Evaluation of API NH, a new 2-hour system for identification of Neisseria and Haemophilus species and Moraxella catarrhalis in a routine clinical laboratory. *Journal of Clinical Microbiology* 1994, 32: 187-189.

22. Barbosa AR, Giufre M, Cerquetti M, Bajanca-Lavado MP: Polymorphism in *ftsI* gene and beta-lactam susceptibility in Portuguese *Haemophilus influenzae* strains: clonal dissemination of beta-lactamase-positive isolates with decreased susceptibility to amoxicillin/clavulanic acid. *Journal of Antimicrobial Chemotherapy* 2011, 66: 788-796.

23. Barbosa SFC, Hoshino-Shimizu S, Alkmin MdG, Goto H: Implications of *Haemophilus influenzae* Biogroup aegyptius Hemagglutinins in the Pathogenesis of Brazilian Purpuric Fever. *Journal of Infectious Diseases* 2003, 188: 74-80.

24. Barbosa-Cesnik C, Farjo RS, Patel M, Gilsdorf J, McCoy SI, Pettigrew MM *et al.*: Predictors for *Haemophilus influenzae* colonization, antibiotic resistance and for sharing an identical isolate among children attending 16 licensed day-care centers in Michigan. *Pediatr Infect Dis J* 2006, 25: 219-223.

25. Barker BL, Haldar K, Patel H, Pavord ID, Barer MR, Brightling CE *et al.*: Association between pathogens detected using quantitative polymerase chain reaction with airway inflammation in COPD at stable state and exacerbations. *Chest* 2015, 147: 46-55.

26. Barriere SL, Flaherty JF: Third-generation cephalosporins: a critical evaluation. *Clin Pharm* 1984, 3: 351-373.

27. Barry AL, Fuchs PC, Brown SD: Identification of beta-lactamase-negative, ampicillin-resistant strains of *Haemophilus influenzae* with four methods and eight media. *Antimicrob Agents Chemother* 2001, 45: 1585-1588.

28. Bayliss CD, Field D, Moxon ER: The simple sequence contingency loci of *Haemophilus influenzae* and Neisseria meningitidis. *J Clin Invest* 2001, 107: 657-662.

29. Bell SM, Plowman D: Mechanisms of ampicillin resistance in *Haemophilus influenzae* from respiratory tract. *Lancet* 1980, 1: 279-280.

30. Bengtsson S, Drobni P, Drobni M, Resman F, Matuschek E, Kahlmeter G: Beta-lactam resistance caused by alterations in the *ftsI* gene in beta-lactamase negative *H. influenzae* isolates collected over twenty years. Poster P446a. The 25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). 2015.

31. Bergey DH, Harrison FC, Breed RS, Hammer BW, Huntoon FM: *Bergey's Manual of Determinative Bacteriology*, 1 edn. Baltimore, MD: The Williams & Wilkins Co.; 1923.

32. Berndsen MR, Erlendsdottir H, Gottfredsson M: Evolving epidemiology of invasive Haemophilus infections in the post-vaccination era: results from a long-term population-based study. *Clin Microbiol Infect* 2012, 18: 918-923.

33. Bibel DJ, Chen TH: Diagnosis of plaque: an analysis of the Yersin-Kitasato controversy. *Bacteriol Rev* 1976, 40: 633-651.

34. Biberstein EL, White DC: A Proposal For The Establishment Of Two New Haemophilus Species. *Journal of Medical Microbiology* 1969, 2: 75-78.

35. Bigger J: Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *The Lancet* 1944, 244: 497-500.

36. Billal DS, Hotomi M, Yamanaka N: Can the Etest correctly determine the MICs of beta-lactam and cephalosporin antibiotics for beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*? *Antimicrob Agents Chemother* 2007, 51: 3463-3464.

37. Binks MJ, Temple B, Kirkham LA, Wiertsema SP, Dunne EM, Richmond PC *et al*.: Molecular surveillance of true nontypeable *Haemophilus influenzae*: an evaluation of PCR screening assays. *PLoS ONE* 2012, 7: e34083.

38. BioMérieux. Produktinformasjon for Vitek 2 Systems 410818. 2010. BioMérieux.

39. Bolduc GR, Bouchet Vr, Jiang RZ, Geisselsoder J, Truong-Bolduc QC, Rice PA *et al*.: Variability of Outer Membrane Protein P1 and Its Evaluation as a Vaccine Candidate against Experimental Otitis Media due to Nontypeable *Haemophilus influenzae*: an Unambiguous, Multifaceted Approach. *Infection and Immunity* 2000, 68: 4505-4517.

40. Bosch AATM, Biesbroek G, Trzcinski K, Sanders EAM, Bogaert D: Viral and Bacterial Interactions in the Upper Respiratory Tract. *PLoS Pathog* 2013, 9: e1003057.

41. Bouchet V, Huot H, Goldstein R: Molecular Genetic Basis of Ribotyping. *Clin Microbiol Rev* 2008, 21: 262.

42. Boutte CC, Crosson S: Bacterial lifestyle shapes stringent response activation. *Trends Microbiol* 2013, 21: 174-180.

43. Bozdogan B, Tristram S, Appelbaum PC: Combination of altered PBPs and expression of cloned extended-spectrum beta-lactamases confers cefotaxime resistance in *Haemophilus influenzae*. *J Antimicrob Chemother* 2006, 57: 747-749.

44. Brouqui P, Raoult D: Endocarditis due to rare and fastidious bacteria. *Clin Microbiol Rev* 2001, 14: 177-207.

45. Brown KN, Percival A: Penetration of antimicrobials into tissue culture cells and leucocytes. *Scand J Infect Dis Suppl* 1978, 251-260.

46. Bruin JP, Kostrzewa M, van der Ende A, Badoux P, Jansen R, Boers SA *et al*.: Identification of *Haemophilus influenzae* and *Haemophilus haemolyticus* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Eur J Clin Microbiol Infect Dis* 2014, 33: 279-284.

47. Burbach S. Reklassifizierung der Gattung Haemophilus Winslow et al. 1917 auf Grund der DNA-Basensequenzhomologie. Doctoral thesis. 1987. Marburg, Germany, Philipps-Universität Marburg.

48. Burns JL, Mendelman PM, Levy J, Stull TL, Smith AL: A permeability barrier as a mechanism of chloramphenicol resistance in *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1985, 27: 46-54.

49. Burns JL, Smith AL: A major outer-membrane protein functions as a porin in *Haemophilus influenzae*. *J Gen Microbiol* 1987, 133: 1273-1277.

50. Burroughs M, Prasad S, Cabellos C, Mendelman PM, Tuomanen E: The biologic activities of peptidoglycan in experimental *Haemophilus influenzae* meningitis. *J Infect Dis* 1993, 167: 464-468.

51. Burroughs M, Rozdzinski E, Geelen S, Tuomanen E: A structure-activity relationship for induction of meningeal inflammation by muramyl peptides. *J Clin Invest* 1993, 92: 297-302.

52. Burroughs MH, Chang YS, Gage DA, Tuomanen EI: Composition of the peptidoglycan of *Haemophilus influenzae*. *J Biol Chem* 1993, 268: 11594-11598.

53. Bush K, Jacoby GA, Medeiros AA: A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother* 1995, 39: 1211-1233.

54. Busse HJ, Bunka S, Hensel A, Lubitz W: Discrimination of Members of the Family Pasteurellaceae Based on Polyamine Patterns. *International Journal of Systematic Bacteriology* 1997, 47: 698-708.

55. Campos J, Roman F, Georgiou M, Garcia C, Gomez-Lus R, Canton R *et al*.: Long-term persistence of ciprofloxacin-resistant *Haemophilus influenzae* in patients with cystic fibrosis. *Journal of Infectious Diseases* 1996, 174: 1345-1347.

56. Cardines R, Giufre M, Mastrantonio P, gli Atti ML, Cerquetti M: Nontypeable *Haemophilus influenzae* meningitis in children: phenotypic and genotypic characterization of isolates. *Pediatr Infect Dis J* 2007, 26: 577-582.

57. Cardines R, Giufre M, Pompilio A, Fiscarelli E, Ricciotti G, Di BG *et al*.: *Haemophilus influenzae* in children with cystic fibrosis: antimicrobial susceptibility, molecular epidemiology, distribution of adhesins and biofilm formation. *Int J Med Microbiol* 2012, 302: 45-52.

58. Carlone GM, Sottnek FO, Plikaytis BD: Comparison of outer membrane protein and biochemical profiles of *Haemophilus aegyptius* and *Haemophilus influenzae* biotype III. *Journal of Clinical Microbiology* 1985, 22: 708-713.

59. Carrico JA, Pinto FR, Simas C, Nunes S, Sousa NG, Frazao N *et al*.: Assessment of Band-Based Similarity Coefficients for Automatic Type and Subtype Classification of Microbial Isolates Analyzed by Pulsed-Field Gel Electrophoresis. *Journal of Clinical Microbiology* 2005, 43: 5483-5490.

60. Cavaliere R, Ball JL, Turnbull L, Whitchurch CB: The biofilm matrix destabilizers, EDTA and DNaseI, enhance the susceptibility of nontypeable *Hemophilus influenzae* biofilms to treatment with ampicillin and ciprofloxacin. *MicrobiologyOpen* 2014, 3: 557-567.

61. Centers for Disease Control and Prevention: *Haemophilus influenzae* type b. Epidemiology and prevention of vaccine-preventable diseases. http://www.cdc.gov/vaccines/pubs/pinkbook/hib.html. In *The Pink Book*. 12 edition Atlanta, GA: Centers for Disease Control and Prevention; 2012:87-100.

62. Cerquetti M, Giufre M, Cardines R, Mastrantonio P: First characterization of heterogeneous resistance to imipenem in invasive nontypeable *Haemophilus influenzae* isolates. *Antimicrob Agents Chemother* 2007, 51: 3155-3161.

63. Cherkaoui A, Diene SM, Emonet S, Renzi G, Francois P, Schrenzel J: Ampicillin-resistant *Haemophilus influenzae* isolates in Geneva: serotype, antimicrobial susceptibility, and beta-lactam resistance mechanisms. *Eur J Clin Microbiol Infect Dis* 2015.

64. Cherry JD, Sheenan CP: Bacteriologic Relapse in *Haemophilus influenzae* Meningitis. *N Engl J Med* 1968, 278: 1001-1003.

65. Choi J, Cox AD, Li J, McCready W, Ulanova M: Activation of innate immune responses by *Haemophilus influenzae* lipooligosaccharide. *Clin Vaccine Immunol* 2014, 21: 769-776.

66. Chopra I, Roberts M: Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001, 65: 232-260.

67. Christensen H, Kuhnert P: International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of Pasteurellaceae: Minutes of the meetings, 25 August 2011, Elsinore, Denmark. *International Journal of Systematic and Evolutionary Microbiology* 2012, 62: 257-258.

68. Chung A, Perera R, Brueggemann AB, Elamin AE, Harnden A, Mayon-White R *et al*.: Effect of antibiotic prescribing on antibiotic resistance in individual children in primary care: prospective cohort study. *BMJ* 2007, 335: 429.

69. Claesson B, Trollfors B, Ekstrom-Jodal B, Jeppsson PH, Lagergard T, Nylen O *et al*.: Incidence and prognosis of acute epiglottitis in children in a Swedish region. *Pediatr Infect Dis* 1984, 3: 534-538.

70. Claesson BA: Epidemiology of invasive *Haemophilus influenzae* type b disease in Scandinavia. *Vaccine* 1993, 11 Suppl 1: S30-S33.

71. Clairoux N, Picard M, Brochu A, Rousseau N, Gourde P, Beauchamp D *et al*.: Molecular basis of the non-beta-lactamase-mediated resistance to beta-lactam antibiotics in strains of *Haemophilus influenzae* isolated in Canada. *Antimicrob Agents Chemother* 1992, 36: 1504-1513.

72. Clementi C, Murphy T: Nontypeable *Haemophilus influenzae* invasion and persistence in the human respiratory tract. *Frontiers in Cellular and Infection Microbiology* 2011, 1.

73. Clementi CF, Håkansson AP, Murphy TF: Internalization and Trafficking of Nontypeable *Haemophilus influenzae* in Human Respiratory Epithelial Cells and Roles of IgA1 Proteases for Optimal Invasion and Persistence. *Infection and Immunity* 2014, 82: 433-444.

74. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard - ninth edition. CLSI document M07-A9. Wayne, PA, USA. 2012.

75. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing, twenty-third informational supplement. CLSI document M100-S23. Wayne, PA, USA. 2013.

76. Coleman SJ, Auld EB, Connor JD, Rosenman SB, Warren GH: Relapse of *Hemophilus influenzae* type b meningitis during intravenous therapy with ampicillin. *J Pediatr* 1969, 74: 781-784.

77. Collins S, Ramsay M, Slack MP, Campbell H, Flynn S, Litt D *et al*.: Risk of invasive *Haemophilus influenzae* infection during pregnancy and association with adverse fetal outcomes. *JAMA* 2014, 311: 1125-1132.

78. Collins S, Litt DJ, Flynn S, Ramsay ME, Slack MPE, Ladhani SN: Neonatal Invasive *Haemophilus influenzae* Disease in England and Wales: Epidemiology, Clinical Characteristics, and Outcome. *Clinical Infectious Diseases* 2015.

79. Connor TR, Corander J, Hanage WP: Population subdivision and the detection of recombination in non-typable *Haemophilus influenzae*. *Microbiology* 2012, 158: 2958-2964.

80. Coulton JW, Mason P, Dorrance D: The permeability barrier of *Haemophilus influenzae* type b against beta-lactam antibiotics. *J Antimicrob Chemother* 1983, 12: 435-449.

81. Crosby AW: *America's Forgotten Pandemic: The Influenza of 1918*. Cambridge University Press; 2003.

82. Cunningham Aed, Williams Ped: *The Laboratory Revolution in Medicine*. Cambridge University Press; 1992.

83. Dabernat H, Delmas C: Epidemiology and evolution of antibiotic resistance of *Haemophilus influenzae* in children 5 years of age or less in France, 2001-2008: a retrospective database analysis. *Eur J Clin Microbiol Infect Dis* 2012, 31: 2745-2753.

84. Dabernat H, Delmas C, Seguy M, Pelissier R, Faucon G, Bennamani S *et al*.: Diversity of beta-lactam resistance-conferring amino acid substitutions in penicillin-binding protein 3 of *Haemophilus influenzae*. *Antimicrob Agents Chemother* 2002, 46: 2208-2218.

85. Dalhoff A: Global fluoroquinolone resistance epidemiology and implictions for clinical use. *Interdiscip Perspect Infect Dis* 2012, 2012: 976273.

86. Darras-Joly C, Lortholary O, Mainardi JL, Etienne J, Guillevin L, Acar J: Haemophilus Endocarditis: Report of 42 Cases in Adults and Review. *Clinical Infectious Diseases* 1997, 24: 1087-1094.

87. Davis DJ, Pittman M, Griffitts J: Hemagglutination by the Koch-Weeks bacillus (*Hemophilus aegyptius*). *J Bacteriol* 1950, 59: 427-431.

88. De Chiara M, Hood D, Muzzi A, Pickard DJ, Perkins T, Pizza M *et al*.: Genome sequencing of disease and carriage isolates of nontypeable *Haemophilus influenzae* identifies discrete population structure. *Proceedings of the National Academy of Sciences* 2014, 111: 5439-5444.

89. de Gier C, Kirkham LA, Norskov-Lauritsen N: Complete deletion of the fucose operon in *Haemophilus influenzae* is associated with a cluster in MLSA-phylogenetic group II related to *Haemophilus haemolyticus*: implications for identification and typing. *Journal of Clinical Microbiology* 2015.

90. De Groot R, Chaffin DO, Kuehn M, Smith AL: Trimethoprim resistance in *Haemophilus influenzae* is due to altered dihydrofolate reductase(s). *Biochem J* 1991, 274 (Pt 3): 657-662.

91. de Rosa R, Labedan B: The evolutionary relationships between the two bacteria Escherichia coli and *Haemophilus influenzae* and their putative last common ancestor. *Mol Biol Evol* 1998, 15: 17-27.

92. Deza G, Martin-Ezquerra G, Gomez J, Villar-Garcia J, Supervia A, Pujol RM: Isolation of *Haemophilus influenzae* and *Haemophilus parainfluenzae* in urethral exudates from men with acute urethritis: a descriptive study of 52 cases. *Sex Transm Infect* 2015.

93. Dimopoulou ID, Kartali SI, Harding RM, Peto TEA, Crook DW: Diversity of antibiotic resistance integrative and conjugative elements among haemophili. *Journal of Medical Microbiology* 2007, 56: 838-846.

94. Doern GV, Jorgensen JH, Thornsberry C, Preston DA, Tubert T, Redding JS *et al*.: National collaborative study of the prevalence of antimicrobial resistance among clinical isolates of *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1988, 32: 180-185.

95. Drawz SM, Bonomo RA: Three decades of beta-lactamase inhibitors. *Clinical Microbiology Reviews* 2010, 23: 160-201.

96. Eagle H: A Paradoxical Zone Phenomenon in the Bactericidal Action of Penicillin in Vitro. *Science* 1948, 107: 44-45.

97. Ecevit IZ, McCrea KW, Pettigrew MM, Sen A, Marrs CF, Gilsdorf JR: Prevalence of the hifBC, hmw1A, hmw2A, hmwC, and hia genes in *Haemophilus influenzae* Isolates. *Journal of Clinical Microbiology* 2004, 42: 3065-3072.

98. El-Halfawy OM, Valvano MA: Antimicrobial Heteroresistance: an Emerging Field in Need of Clarity. *Clinical Microbiology Reviews* 2015, 28: 191-207.

99. Enne VI, King A, Livermore DM, Hall LM: Sulfonamide resistance in *Haemophilus influenzae* mediated by acquisition of sul2 or a short insertion in chromosomal folP. *Antimicrob Agents Chemother* 2002, 46: 1934-1939.

100. ERICSSON H, TUNEVALL G, WICKMAN K: The paper disc method for determination of bacterial sensitivity to antibiotics. Relationship between the diameter of the zone of inhibition and the minimum inhibitory concentration. *Scand J Clin Lab Invest* 1960, 12: 414-422.

101. Ericsson HM, Sherris JC: Antibiotic sensitivity testing. Report of an international collaborative study. *Acta Pathol Microbiol Scand B Microbiol Immunol* 1971, 217: Suppl.

102. Erwin AL, Sandstedt SA, Bonthuis PJ, Geelhood JL, Nelson KL, Unrath WCT *et al.*: Analysis of genetic relatedness of *Haemophilus influenzae* isolates by multilocus sequence typing. *Journal of Bacteriology* 2008, 190: 1473-1483.

103. Euba B, Moleres J, Viadas C, Barberan M, Caballero L, Grillo MJ *et al.*: Relationship between Azithromycin Susceptibility and Administration Efficacy for Nontypeable *Haemophilus influenzae* Respiratory Infection. *Antimicrob Agents Chemother* 2015, 59: 2700-2712.

104. European Centre for Disease Prevention and Control (ECDC). External quality assurance scheme for *Haemophilus influenzae* - 2011. 2013. Stockholm, ECDC.

105. European Centre for Disease Prevention and Control (ECDC). External quality assurance scheme for *Haemophilus influenzae* - 2012. 2013. Stockholm, ECDC.

106. European Centre for Disease Prevention and Control (ECDC). Surveillance of invasive bacterial diseases in Europe 2011. 2013. Stockholm, ECDC.

107. European Committee on Antimicrobial Susceptibility Testing (EUCAST): Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clinical Microbiology and Infection* 2003, 9: ix-xv.

108. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Compliance of manufacturers with EUCAST guidelines. 25 September 2014. www.eucast.org/antimicrobial_susceptibility_testing/compliance_of_manufacturers. 25-9-2014.

109. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Media preparation for EUCAST disk diffusion testing and for determination of MIC values by the broth microdilution method. Version 4.0. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Version_4/Media_preparation_v_4.0_EUCAST_AST.pdf. 2014.

110. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Organism-agent combinations for which good clinical data supporting the breakpoints are not available http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/Organism-agents_needing_clinical_data_20140501.pdf. 1-5-2014. EUCAST.

111. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Version 5.0, 2015. http://www.eucast.org. 2015.

112. European Committee on Antimicrobial Susceptibility Testing (EUCAST). *Haemophilus influenzae*. Benzylpenicillin 1 unit as screen for beta-lactam resistance. Version 3.2. February 2015. http://www.eucast.org/ast_of_bacteria/calibration_and_validation/. 2015.

113. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Routine and extended internal quality control as recommended by EUCAST. Version 5.0, valid from 2015-01-09. www.eucast.org. 2015.

114. Eutsey RA, Hiller NL, Earl JP, Janto BA, Dahlgren ME, Ahmed A *et al*.: Design and validation of a supragenome array for determination of the genomic content of *Haemophilus influenzae* isolates. *BMC Genomics* 2013, 14: 484.

115. Euzeby JP: List of Bacterial Names with Standing in Nomenclature: a Folder Available on the Internet. *International Journal of Systematic Bacteriology* 1997, 47: 590-592.

116. Evans NM, Bell SM, Smith DD: New satellitism test for isolation and identification of *Haemophilus influenzae* and *Haemophilus parainfluenzae* in sputum. *Journal of Clinical Microbiology* 1975, 1: 89-95.

117. Evers S, Di PK, Meyer M, Langen H, Fountoulakis M, Keck W *et al*.: Mechanism-related changes in the gene transcription and protein synthesis patterns of *Haemophilus influenzae* after treatment with transcriptional and translational inhibitors. *Proteomics* 2001, 1: 522-544.

118. Eyler JM: The state of science, microbiology, and vaccines circa 1918. *Public Health Rep* 2010, 125 Suppl 3: 27-36.

119. Falla TJ, Crook DW, Brophy LN, Maskell D, Kroll JS, Moxon ER: PCR for capsular typing of *Haemophilus influenzae*. *Journal of Clinical Microbiology* 1994, 32: 2382-2386.

120. Farrar WE, Jr., O'Dell NM: Beta-lactamase activity in ampicillin-resistant *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1974, 6: 625-629.

121. Farrell DJ, Flamm RK, Jones RN, Sader HS: Spectrum and potency of ceftaroline tested against leading pathogens causing community-acquired respiratory tract infections in Europe (2010). *Diagn Microbiol Infect Dis* 2013, 75: 86-88.

122. Farrell DJ, Flamm RK, Sader HS, Jones RN: Ceftobiprole activity against over 60,000 clinical bacterial pathogens isolated in Europe, Turkey, and Israel from 2005 to 2010. *Antimicrob Agents Chemother* 2014, 58: 3882-3888.

123. Farrell DJ, Morrissey I, Bakker S, Buckridge S, Felmingham D: Global distribution of TEM-1 and ROB-1 beta-lactamases in *Haemophilus influenzae*. *J Antimicrob Chemother* 2005, 56: 773-776.

124. Feil EJ, Holmes EC, Bessen DE, Chan MS, Day NP, Enright MC *et al*.: Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. *Proc Natl Acad Sci U S A* 2001, 98: 182-187.

125. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG: eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004, 186: 1518-1530.

126. Fenger MG, Ridderberg W, Olesen HV, Norskov-Lauritsen N: Low occurrence of 'non-haemolytic *Haemophilus haemolyticus*' misidentified as *Haemophilus influenzae* in cystic fibrosis respiratory specimens, and frequent recurrence of persistent *H. influenzae* clones despite antimicrobial treatment. *Int J Med Microbiol* 2012, 302: 315-319.

127. Fernaays MM, Lesse AJ, Sethi S, Cai X, Murphy TF: Differential genome contents of nontypeable *Haemophilus influenzae* strains from adults with chronic obstructive pulmonary disease. *Infect Immun* 2006, 74: 3366-3374.

128. Fink DL, Green BA, St Geme JW: The *Haemophilus influenzae* Hap autotransporter binds to fibronectin, laminin, and collagen IV. *Infect Immun* 2002, 70: 4902-4907.

129. Finlay J, Miller L, Poupard JA: A review of the antimicrobial activity of clavulanate. *J Antimicrob Chemother* 2003, 52: 18-23.

130. Flamm RK, Sader HS, Jones RN: Ceftaroline activity tested against contemporary Latin American bacterial pathogens (2011). *Braz J Infect Dis* 2014, 18: 187-195.

131. Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR *et al*.: Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 1995, 269: 496-512.

132. Fleming A: On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*. *Bull World Health Organ* 1929, 79: 780-790.

133. Fleming A. Penicillin. Nobel Lecture, December 11, 1945. 1945.

134. Fleury C, Resman F, Rau J, Riesbeck K: Prevalence, distribution and transfer of small beta-lactamase-containing plasmids in Swedish *Haemophilus influenzae*. *Journal of Antimicrobial Chemotherapy* 2014, 69: 1238-1242.

135. Fluit AC, Florijn A, Verhoef J, Milatovic D: Susceptibility of European beta-lactamase-positive and -negative *Haemophilus influenzae* isolates from the periods 1997/1998 and 2002/2003. *J Antimicrob Chemother* 2005, 56: 133-138.

136. Forbes KJ, Bruce KD, Ball A, Pennington TH: Variation in length and sequence of porin (ompP2) alleles of non-capsulate *Haemophilus influenzae*. *Mol Microbiol* 1992, 6: 2107-2112.

137. Forsgren A, Riesbeck K: Protein D of *Haemophilus influenzae*: A Protective Nontypeable *H. influenzae* Antigen and a Carrier for Pneumococcal Conjugate Vaccines. *Clinical Infectious Diseases* 2008, 46: 726-731.

138. Frickmann H, Christner M, Donat M, Berger A, Essig A, Podbielski A *et al*.: Rapid discrimination of *Haemophilus influenzae*, *H. parainfluenzae*, and *H. haemolyticus* by fluorescence in situ hybridization (FISH) and two matrix-assisted laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) platforms. *PLoS ONE* 2013, 8: e63222.

139. Fuchs PC, Barry AL: Interpretive criteria for susceptibilities of *Haemophilus influenzae* to ampicillin, amoxicillin, and amoxicillin-clavulanic acid. *J Clin Microbiol* 1994, 32: 2846-2850.

140. Fuchs PC, Barry AL, Brown SD: Influence of variations in test methods on susceptibility of *Haemophilus influenzae* to ampicillin, azithromycin, clarithromycin, and telithromycin. *J Clin Microbiol* 2001, 39: 43-46.

141. Fung WWM, O'Dwyer CoA, Sinha S, Brauer AL, Murphy TF, Kroll JS *et al*.: Presence of Copper- and Zinc-Containing Superoxide Dismutase in Commensal *Haemophilus haemolyticus* Isolates Can Be Used as a Marker To Discriminate Them from Nontypeable *H. influenzae* Isolates. *Journal of Clinical Microbiology* 2006, 44: 4222-4226.

142. Gadsby NJ, McHugh MP, Russell CD, Mark H, Conway MA, Laurenson IF *et al*.: Development of two real-time multiplex PCR assays for the detection and quantification of eight key bacterial pathogens in lower respiratory tract infections. *Clin Microbiol Infect* 2015, 21: 788.

143. Galan JC, Morosini MI, Baquero MR, Reig M, Baquero F: *Haemophilus influenzae* bla(ROB-1) mutations in hypermutagenic deltaampC Escherichia coli conferring resistance to cefotaxime and beta-lactamase inhibitors and increased susceptibility to cefaclor. *Antimicrob Agents Chemother* 2003, 47: 2551-2557.

144. Gallaher T, Wu S, Webster P, Aguilera R: Identification of biofilm proteins in non-typeable *Haemophilus influenzae*. *BMC Microbiology* 2006, 6: 65.

145. Galli J, Calo L, Ardito F, Imperiali M, Bassotti E, Fadda G *et al*.: Biofilm formation by *Haemophilus influenzae* isolated from adeno-tonsil tissue samples, and its role in recurrent adenotonsillitis. *Acta Otorhinolaryngol Ital* 2007, 27: 134-138.

146. Garcia-Cobos S, Arroyo M, Perez-Vazquez M, Aracil B, Lara N, Oteo J *et al*.: Isolates of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* causing invasive infections in Spain remain susceptible to cefotaxime and imipenem. *J Antimicrob Chemother* 2014, 69: 111-116.

147. Garcia-Cobos S, Arroyo M, Perez-Vazquez M, Aracil B, Oteo J, Campos J: Evaluation of the EUCAST disc diffusion susceptibility testing method for *Haemophilus influenzae* based on the resistance mechanism to beta-lactam antibiotics. *J Antimicrob Chemother* 2013, 68: 159-163.

148. Garcia-Cobos S, Campos J, Lazaro E, Roman F, Cercenado E, Garcia-Rey C *et al*.: Ampicillin-resistant non-beta-lactamase-producing *Haemophilus influenzae* in Spain: recent emergence of clonal isolates with increased resistance to cefotaxime and cefixime. *Antimicrob Agents Chemother* 2007, 51: 2564-2573.

149. Garcia-Cobos S, Campos J, Roman F, Carrera C, Perez-Vazquez M, Aracil B *et al*.: Low beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* strains are best detected by testing amoxicillin susceptibility by the broth microdilution method. *Antimicrob Agents Chemother* 2008, 52: 2407-2414.

150. Garcia-Cobos S, Moscoso M, Pumarola F, Arroyo M, Lara N, Perez-Vazquez M *et al*.: Frequent carriage of resistance mechanisms to beta-lactams and biofilm formation in *Haemophilus influenzae*

causing treatment failure and recurrent otitis media in young children. *Journal of Antimicrobial Chemotherapy* 2014.

151. Georgiou M, Munoz R, Roman F, Canton R, Gomez-Lus R, Campos J *et al*.: Ciprofloxacin-resistant *Haemophilus influenzae* strains possess mutations in analogous positions of GyrA and ParC. *Antimicrob Agents Chemother* 1996, 40: 1741-1744.

152. Georgopapadakou NH: Penicillin-binding proteins and bacterial resistance to beta-lactams. *Antimicrob Agents Chemother* 1993, 37: 2045-2053.

153. Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ *et al*.: Opinion: Re-evaluating prokaryotic species. *Nat Rev Microbiol* 2005, 3: 733-739.

154. Gilsdorf JR: What the pediatrician should know about non-typeable *Haemophilus influenzae*. *J Infect* 2015, 71 Suppl 1: S10-S14.

155. Gilsdorf JR, McCrea KW, Marrs CF: Role of pili in *Haemophilus influenzae* adherence and colonization. *Infect Immun* 1997, 65: 2997-3002.

156. Girgis NI, Abu el Ella AH, Farid Z, Woody JN, Haberberger RL, Jr., el MM *et al*.: Parenteral aztreonam in the treatment of *Haemophilus influenzae* type b meningitis in Egyptian children. *Scand J Infect Dis* 1988, 20: 111-112.

157. Giufre M, Cardines R, Accogli M, Pardini M, Cerquetti M: Identification of *Haemophilus influenzae* clones associated with invasive disease a decade after introduction of *H. influenzae* serotype b vaccination in Italy. *Clin Vaccine Immunol* 2013, 20: 1223-1229.

158. Giufre M, Cardines R, Caporali MG, Accogli M, D'Ancona F, Cerquetti M: Ten years of Hib vaccination in Italy: prevalence of non-encapsulated *Haemophilus influenzae* among invasive isolates and the possible impact on antibiotic resistance. *Vaccine* 2011, 29: 3857-3862.

159. Giufre M, Cardines R, Accogli M, Cerquetti M: Neonatal Invasive *Haemophilus influenzae* Disease and Genotypic Characterization of the Associated Strains in Italy. *Clinical Infectious Diseases* 2015.

160. Gkentzi D, Slack MP, Ladhani SN: The burden of nonencapsulated *Haemophilus influenzae* in children and potential for prevention. *Curr Opin Infect Dis* 2012, 25: 266-272.

161. Glover WA, Suarez CJ, Clarridge JE, III: Genotypic and phenotypic characterization and clinical significance of 'Haemophilus quentini' isolated from the urinary tract of adult men. *J Med Microbiol* 2011, 60: 1689-1692.

162. Gohler AK, Kokpinar O, Schmidt-Heck W, Geffers R, Guthke R, Rinas U *et al*.: More than just a metabolic regulator--elucidation and validation of new targets of PdhR in Escherichia coli. *BMC Syst Biol* 2011, 5: 197.

163. Gold AJ, Lieberman E, Wright HT, Jr.: Bacteriologic relapse during ampicillin treatment of *Hemophilus influenzae* meningitis. *J Pediatr* 1969, 74: 779-781.

164. Goldstein FW, Acar JF: Epidemiology of antibiotic resistance in *Haemophilus influenzae*. *Microb Drug Resist* 1995, 1: 131-135.

165. Goodgal SH, Mitchell MA: Sequence and uptake specificity of cloned sonicated fragments of *Haemophilus influenzae* DNA. *J Bacteriol* 1990, 172: 5924-5928.

166. Gotoh K, Qin L, Watanabe K, Anh DD, Huong PT, Anh NT *et al*.: Prevalence of *Haemophilus influenzae* with resistant genes isolated from young children with acute lower respiratory tract infections in Nha Trang, Vietnam. *J Infect Chemother* 2008, 14: 349-353.

167. Guo L, Zhang J, Xu C, Zhao Y, Ren T, Zhang B *et al*.: Molecular characterization of fluoroquinolone resistance in Haemophilus parasuis isolated from pigs in South China. *Journal of Antimicrobial Chemotherapy* 2011, 66: 539-542.

168. Haag AM, Taylor SN, Johnston KH, Cole RB: Rapid identification and speciation of Haemophilus bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Mass Spectrom* 1998, 33: 750-756.

169. Hagiwara E, Baba T, Shinohara T, Nishihira R, Komatsu S, Ogura T: Antimicrobial resistance genotype trend and its association with host clinical characteristics in respiratory isolates of *Haemophilus influenzae*. *Chemotherapy* 2012, 58: 352-357.

170. Hallstrom T, Riesbeck K: *Haemophilus influenzae* and the complement system. *Trends Microbiol* 2010, 18: 258-265.

171. Haltalin KC, Smith JB: Reevaluation of ampicillin therapy for *Hemophilus influenzae* meningitis. An appraisal based on a review of cases of persistent or recurrent infection. *Am J Dis Child* 1971, 122: 328-336.

172. Hanage WP, Fraser C, Spratt BG: Sequences, sequence clusters and bacterial species. *Philosophical Transactions of the Royal Society B: Biological Sciences* 2006, 361: 1917-1927.

173. Hara H, Nishimura Y, Kato J, Suzuki H, Nagasawa H, Suzuki A *et al.*: Genetic analyses of processing involving C-terminal cleavage in penicillin-binding protein 3 of Escherichia coli. *Journal of Bacteriology* 1989, 171: 5882-5889.

174. Hariadi NI, Zhang L, Patel M, Sandstedt SA, Davis GS, Marrs CF *et al.*: Comparative Profile of Heme Acquisition Genes in Disease-Causing and Colonizing Nontypeable *Haemophilus influenzae* and *Haemophilus haemolyticus*. *Journal of Clinical Microbiology* 2015.

175. Harrison LH, da Silva GA, Pittman M, Fleming DW, Vranjac A, Broome CV: Epidemiology and clinical spectrum of Brazilian purpuric fever. Brazilian Purpuric Fever Study Group. *J Clin Microbiol* 1989, 27: 599-604.

176. Harrison LH, Simonsen V, Waldman EA: Emergence and Disappearance of a Virulent Clone of *Haemophilus influenzae* Biogroup aegyptius, Cause of Brazilian Purpuric Fever. *Clinical Microbiology Reviews* 2008, 21: 594-605.

177. Harrison OB, Brueggemann AB, Caugant DA, van der Ende A, Frosch M, Gray S *et al.*: Molecular typing methods for outbreak detection and surveillance of invasive disease caused by Neisseria meningitidis, *Haemophilus influenzae* and Streptococcus pneumoniae, a review. *Microbiology* 2011, 157: 2181-2195.

178. Hasegawa K, Chiba N, Kobayashi R, Murayama SY, Iwata S, Sunakawa K *et al.*: Rapidly increasing prevalence of beta-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae* type b in patients with meningitis. *Antimicrob Agents Chemother* 2004, 48: 1509-1514.

179. Hasegawa K, Kobayashi R, Takada E, Ono A, Chiba N, Morozumi M *et al.*: High prevalence of type b beta-lactamase-non-producing ampicillin-resistant *Haemophilus influenzae* in meningitis: the situation in Japan where Hib vaccine has not been introduced. *J Antimicrob Chemother* 2006, 57: 1077-1082.

180. Hasegawa K, Yamamoto K, Chiba N, Kobayashi R, Nagai K, Jacobs MR *et al.*: Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the United States. *Microb Drug Resist* 2003, 9: 39-46.

181. Hashida K, Shiomori T, Hohchi N, Muratani T, Mori T, Udaka T *et al.*: Nasopharyngeal *Haemophilus influenzae* carriage in Japanese children attending day-care centers. *J Clin Microbiol* 2008, 46: 876-881.

182. Hekker TA, van der Schee AC, Kempers J, Namavar F, van AL: A nosocomial outbreak of amoxycillin-resistant non-typable *Haemophilus influenzae* in a respiratory ward. *J Hosp Infect* 1991, 19: 25-31.

183. Henriksen SD: Theodor Thjøtta 1885 - 1955: An Appreciation. *International Bulletin of Bacteriological Nomenclature and Taxonomy* 1955, 5: 175-177.

184. Heuer H, Smalla K: Horizontal gene transfer between bacteria. *Environ Biosafety Res* 2007, 6: 3-13.

185. Hill T, Nordstrom KJ, Thollesson M, Safstrom TM, Vernersson AK, Fredriksson R *et al.*: SPRIT: Identifying horizontal gene transfer in rooted phylogenetic trees. *BMC Evol Biol* 2010, 10: 42.

186. Hinz R, Zautner AE, Hagen RM, Frickmann H: Difficult identification of *Haemophilus influenzae*, a typical cause of upper respiratory tract infections, in the microbiological diagnostic routine. *Eur J Microbiol Immunol (Bp)* 2015, 5: 62-67.

187. Hirakata Y, Ohmori K, Mikuriya M, Saika T, Matsuzaki K, Hasegawa M *et al.*: Antimicrobial activities of piperacillin-tazobactam against *Haemophilus influenzae* isolates, including beta-lactamase-negative ampicillin-resistant and beta-lactamase-positive amoxicillin-clavulanate-resistant isolates, and mutations in their quinolone resistance-determining regions. *Antimicrob Agents Chemother* 2009, 53: 4225-4230.

188. Hodge W, Ciak J, Tramont EC: Simple method for detection of penicillinase-producing Neisseria gonorrhoeae. *J Clin Microbiol* 1978, 7: 102-103.

189. Hogg J, Hu F, Janto B, Boissy R, Hayes J, Keefe R *et al*.: Characterization and modeling of the *Haemophilus influenzae* core and supragenomes based on the complete genomic sequences of Rd and 12 clinical nontypeable strains. *Genome Biology* 2007, 8: R103.

190. Honigsbaum M: *A History of the Great Influenza Pandemics: Death, Panic and Hysteria, 1830 - 1920*. New York: I.B.Tauris; 2014.

191. Hotomi M, Fujihara K, Billal DS, Suzuki K, Nishimura T, Baba S *et al*.: Genetic characteristics and clonal dissemination of beta-lactamase non-producing ampicillin-resistant (BLNAR) *Haemophilus influenzae* isolated from the upper respiratory tract in Japan. *Antimicrobial Agents and Chemotherapy* 2007, 51: 3969-3976.

192. Hotomi M, Sakai KF, Billal DS, Shimada J, Suzumoto M, Yamanaka N: Antimicrobial resistance in *Haemophilus influenzae* isolated from the nasopharynx among Japanese children with acute otitis media. *Acta Otolaryngol* 2006, 126: 130-137.

193. Huisman O, D'Ari R, Gottesman S: Cell-division control in Escherichia coli: specific induction of the SOS function SfiA protein is sufficient to block septation. *Proc Natl Acad Sci U S A* 1984, 81: 4490-4494.

194. Iino Y, Yoshida N, Kato T, Kakizaki K, Miyazawa T, Kakuta H: Clinical effects of clarithromycin on persistent inflammation following *Haemophilus influenzae*-positive acute otitis media. *Acta Oto-Laryngologica* 2015, 135: 217-225.

195. International Bulletin of Bacteriological Nomenclature and Taxonomy. I Generi e le Specie delle Batteriacee 1889 with Bibliography of Trevisan's Publications on Systematic Bacteriology: File No. 56. 2[1], 11-44. 1-1-1952.

196. Inzana TJ, Johnson JL, Shell L, Moller K, Kilian M: Isolation and characterization of a newly identified Haemophilus species from cats: "Haemophilus felis". *J Clin Microbiol* 1992, 30: 2108-2112.

197. Inzana TJ: Identification of Phase-Variable Genes That May Contribute to Nontypeable *Haemophilus influenzae* Nasopharyngeal Colonization in Humans Contributes to Our Understanding of Specific Host-Pathogen Interactions. *Journal of Infectious Diseases* 2013, 208: 713-716.

198. Ishak N, Tikhomirova A, Bent S, Ehrlich G, Hu F, Kidd S: There is a specific response to pH by isolates of *Haemophilus influenzae* and this has a direct influence on biofilm formation. *BMC Microbiology* 2014, 14: 47.

199. Ishino F, Jung HK, Ikeda M, Doi M, Wachi M, Matsuhashi M: New mutations fts-36, lts-33, and ftsW clustered in the mra region of the Escherichia coli chromosome induce thermosensitive cell growth and division. *J Bacteriol* 1989, 171: 5523-5530.

200. Jacobs MR, Bajaksouzian S, Windau A, Appelbaum PC, Lin G, Felmingham D *et al*.: Effects of Various Test Media on the Activities of 21 Antimicrobial Agents against *Haemophilus influenzae*. *Journal of Clinical Microbiology* 2002, 40: 3269-3276.

201. Jalalvand F, Littorin N, Su YC, Riesbeck K: Impact of immunization with Protein F on pulmonary clearance of nontypeable *Haemophilus influenzae*. *Vaccine* 2014, 32: 2261-2264.

202. Jalalvand F, Riesbeck K: *Haemophilus influenzae*: recent advances in the understanding of molecular pathogenesis and polymicrobial infections. *Curr Opin Infect Dis* 2014, 27: 268-274.

203. Jansen WT, Verel A, Beitsma M, Verhoef J, Milatovic D: Longitudinal European surveillance study of antibiotic resistance of *Haemophilus influenzae*. *J Antimicrob Chemother* 2006, 58: 873-877.

204. Jansen WT, Verel A, Beitsma M, Verhoef J, Milatovic D: Surveillance study of the susceptibility of *Haemophilus influenzae* to various antibacterial agents in Europe and Canada. *Curr Med Res Opin* 2008, 24: 2853-2861.

205. Jekel JF, Katz DL, Elmore JG: *Epidemiology, biostatistics, and preventive medicine*, 2 edn. W. B. Saunders Company; 2001.

206. Johnsen PJ, Townsend JP, Bohn T, Simonsen GS, Sundsfjord A, Nielsen KM: Factors affecting the reversal of antimicrobial-drug resistance. *Lancet Infect Dis* 2009, 9: 357-364.

207. Johnson N: *Britain and the 1918-19 Influenza Pandemic: A Dark Epilogue*. Taylor & Francis; 2006.

208. Jordens JZ, Slack MPE: *Haemophilus influenzae*: Then and now. *Eur J Clin Microbiol Infect Dis* 1995, 14: 935-948.

209. Jorgensen JH, Ferraro MJ: Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin Infect Dis* 2009, 49: 1749-1755.

210. Jorgensen JH, Howell AW, Maher LA: Quantitative antimicrobial susceptibility testing of *Haemophilus influenzae* and Streptococcus pneumoniae by using the E-test. *J Clin Microbiol* 1991, 29: 109-114.

211. Jorgensen JH, Redding JS, Maher LA, Howell AW: Improved medium for antimicrobial susceptibility testing of *Haemophilus influenzae*. *Journal of Clinical Microbiology* 1987, 25: 2105-2113.

212. Juhas M, Power PM, Harding RM, Ferguson DJ, Dimopoulou ID, Elamin AR *et al*.: Sequence and functional analyses of Haemophilus spp. genomic islands. *Genome Biol* 2007, 8: R237.

213. Jurcisek JA, Bakaletz LO: Biofilms formed by Nontypeable *Haemophilus influenzae* in vivo contain both double-stranded dna and type IV pilin protein. *J Bacteriol* 2007, 189: 3868-3875.

214. Kaczmarek FS, Gootz TD, Dib-Hajj F, Shang W, Hallowell S, Cronan M: Genetic and molecular characterization of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* with unusually high resistance to ampicillin. *Antimicrob Agents Chemother* 2004, 48: 1630-1639.

215. Kahlmeter G: Defining antibiotic resistance-towards international harmonization. *Ups J Med Sci* 2014, 119: 78-86.

216. Kais M, Spindler C, Kalin M, Ortqvist A, Giske CG: Quantitative detection of Streptococcus pneumoniae, *Haemophilus influenzae*, and Moraxella catarrhalis in lower respiratory tract samples by real-time PCR. *Diagn Microbiol Infect Dis* 2006, 55: 169-178.

217. Kanazawa K, Nouda H, Sunagawa M: Structure-activity relationships of carbapenem compounds to anti-*Haemophilus influenzae* activity and affinity for penicillin-binding proteins. Effect of 1 beta-methyl group and C-2 side chain. *J Antibiot (Tokyo)* 1997, 50: 162-168.

218. Karlowsky JA, Critchley IA, Blosser-Middleton RS, Karginova EA, Jones ME, Thornsberry C *et al*.: Antimicrobial surveillance of *Haemophilus influenzae* in the United States during 2000-2001 leads to detection of clonal dissemination of a beta-lactamase-negative and ampicillin-resistant strain. *J Clin Microbiol* 2002, 40: 1063-1066.

219. Karpanoja P, Nissinen A, Huovinen P, Sarkkinen H: Disc diffusion susceptibility testing of *Haemophilus influenzae* by NCCLS methodology using low-strength ampicillin and co-amoxiclav discs. *J Antimicrob Chemother* 2004, 53: 660-663.

220. Kaur R, Casey JR, Pichichero ME: Relationship with original pathogen in recurrence of acute otitis media after completion of amoxicillin/clavulanate: bacterial relapse or new pathogen. *Pediatr Infect Dis J* 2013, 32: 1159-1162.

221. Kaur R, Chang A, Xu Q, Casey JR, Pichichero ME: Phylogenetic relatedness and diversity of non-typable *Haemophilus influenzae* in the nasopharynx and middle ear fluid of children with acute otitis media. *J Med Microbiol* 2011, 60: 1841-1848.

222. Kaur R, Czup K, Casey JR, Pichichero ME: Correlation of nasopharyngeal cultures prior to and at onset of acute otitis media with middle ear fluid cultures. *BMC Infect Dis* 2014, 14: 640.

223. Kawai F, Clarke TB, Roper DI, Han GJ, Hwang KY, Unzai S *et al*.: Crystal structures of penicillin-binding proteins 4 and 5 from *Haemophilus influenzae*. *J Mol Biol* 2010, 396: 634-645.

224. Khan W, Ross S, Rodriguez W, Controni G, Saz AK: *Haemophilus influenzae* type B resistant to ampicillin. A report of two cases. *JAMA* 1974, 229: 298-301.

225. Kilian M: A rapid method for the differentiation of Haemophilus strains. The porphyrin test. *Acta Pathol Microbiol Scand B Microbiol Immunol* 1974, 82: 835-842.

226. Kilian M: A Taxonomic Study of the Genus Haemophilus, with the Proposal of a New Species. *Journal of General Microbiology* 1976, 93: 9-62.

227. Kilian M: Genus III. *Haemophilus*. In *Bergey's Manual of Systematic Bacteriology. The Proteobacteriaceae. Part B. The Gammaproteobacteria*. *Volume II*. 2 edition. Edited by Brenner DJ, Krieg NR, Staley JT, Garrity GM. New York, NY: Springer; 2005:883-904.

228. Kilian M: The genus *Haemophilus*. In *Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*. *Volume II*. 1 edition. Edited by Starr MP, Stolp H, Truper HG, Balows A, Schlegel HG. New York: Springer; 1981:1371-1382.

229. Kilian M: *Haemophilus*. In *Manual of Clinical Microbiology*. *Volume 1*. 8 edition. Edited by Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH. Washington, D.C.: ASM Press; 2003:623-635.

230. Kim IS, Ki CS, Kim S, Oh WS, Peck KR, Song JH *et al*.: Diversity of ampicillin resistance genes and antimicrobial susceptibility patterns in *Haemophilus influenzae* strains isolated in Korea. *Antimicrob Agents Chemother* 2007, 51: 453-460.

231. Kishii K, Chiba N, Morozumi M, Hamano-Hasegawa K, Kurokawa I, Masaki J *et al*.: Diverse mutations in the *ftsI* gene in ampicillin-resistant *Haemophilus influenzae* isolates from pediatric patients with acute otitis media. *J Infect Chemother* 2010, 16: 87-93.

232. Kitasato University School of Medicine. About Shibasaburo Kitasato. (http://web.med.kitasato-u.ac.jp/en/about/#anc04, last accessed 2014-10-13). 2014. Kitasato University School of Medicine.

233. Kitasato S: II.-On the Influenza Bacillus and the Mode of Cultivating It. *Br Med J* 1892, 1: 128.

234. Klein RD, Luginbuhl GH: Ampicillin-induced morphological alterations of *Haemophilus influenzae* type b. *Antimicrob Agents Chemother* 1977, 11: 559-562.

235. Koch R: An Address on Bacteriological Research. *Br Med J* 1890, 2: 380-383.

236. Koch R: Bericht über die Thätigkeit der deutschen Cholerakomissionen in Aegypten und Ostindien. *Wien Med Wochenschr* 1883, 33: 1548-1551.

237. Kong KF, Schneper L, Mathee K: Beta-lactam antibiotics: from antibiosis to resistance and bacteriology. *APMIS* 2010, 118: 1-36.

238. Kroll JS: The genetics of encapsulation in *Haemophilus influenzae*. *J Infect Dis* 1992, 165 Suppl 1: S93-S96.

239. Kroll JS, Moxon ER, Loynds BM: An ancestral mutation enhancing the fitness and increasing the virulence of *Haemophilus influenzae* type b. *J Infect Dis* 1993, 168: 172-176.

240. Kubota T, Higa F, Kusano N, Nakasone I, Haranage S, Tateyama M *et al*.: Genetic analyses of beta-lactamase negative ampicillin-resistant strains of *Haemophilus influenzae* isolated in Okinawa, Japan. *Jpn J Infect Dis* 2006, 59: 36-41.

241. Kuo SC, Chen PC, Shiau YR, Wang HY, Lai JF, Huang W *et al*.: Levofloxacin-Resistant *Haemophilus influenzae*, Taiwan, 2004-2010. *Emerg Infect Dis* 2014, 20: 1386-1390.

242. Kussell E, Kishony R, Balaban NQ, Leibler S: Bacterial Persistence: A Model of Survival in Changing Environments. *Genetics* 2005, 169: 1807-1814.

243. LaClaire LL, Tondella ML, Beall DS, Noble CA, Raghunathan PL, Rosenstein NE *et al*.: Identification of *Haemophilus influenzae* Serotypes by Standard Slide Agglutination Serotyping and PCR-Based Capsule Typing. *Journal of Clinical Microbiology* 2003, 41: 393-396.

244. LaCross NC, Marrs CF, Gilsdorf JR: Population structure in nontypeable *Haemophilus influenzae*. *Infection, Genetics and Evolution* 2013, 14: 125-136.

245. Ladhani S, Slack MP, Heath PT, von GA, Chandra M, Ramsay ME: Invasive *Haemophilus influenzae* Disease, Europe, 1996-2006. *Emerg Infect Dis* 2010, 16: 455-463.

246. Ladhani SN, Collins S, Vickers A, Litt DJ, Crawford C, Ramsay ME *et al*.: Invasive *Haemophilus influenzae* serotype e and f disease, England and Wales. *Emerg Infect Dis* 2012, 18: 725-732.

247. Lam T-T, Claus H, Elias J, Frosch M, Vogel U: Ampicillin resistance of invasive *Haemophilus influenzae* isolates in Germany 2009-2012. *International Journal of Medical Microbiology* 2015.

248. Langereis JD, de Jonge MI: Invasive Disease Caused by Nontypeable *Haemophilus influenzae*. *Emerg Infect Dis* 2015, 21.

249. Langereis JD, Hermans PWM: Novel concepts in nontypeable *Haemophilus influenzae* biofilm formation. *FEMS Microbiol Lett* 2013, 346: 81-89.

250. Langford PR, Sheehan BJ, Shaikh T, Kroll JS: Active Copper- and Zinc-Containing Superoxide Dismutase in the Cryptic Genospecies of Haemophilus Causing Urogenital and Neonatal Infections Discriminates Them from *Haemophilus influenzae* Sensu Stricto. *Journal of Clinical Microbiology* 2002, 40: 268-270.

251. Latham R, Zhang B, Tristram S: Identifying *Haemophilus haemolyticus* and *Haemophilus influenzae* by SYBR Green real-time PCR. *J Microbiol Methods* 2015, 112: 67-69.

252. Lavoie GY, Bergeron MG: Influence of four modes of administration on penetration of aztreonam, cefuroxime, and ampicillin into interstitial fluid and fibrin clots and on in vivo efficacy against *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1985, 28: 404-412.

253. Lay JO: MALDI-TOF mass spectrometry of bacteria*. *Mass Spectrom Rev* 2001, 20: 172-194.

254. Leaves NI, Dimopoulou I, Hayes I, Kerridge S, Falla T, Secka O *et al*.: Epidemiological studies of large resistance plasmids in Haemophilus. *J Antimicrob Chemother* 2000, 45: 599-604.

255. Leclercq R, Canton R, Brown DF, Giske CG, Heisig P, MacGowan AP *et al*.: EUCAST expert rules in antimicrobial susceptibility testing. *Clin Microbiol Infect* 2013, 19: 141-160.

256. Ledeboer NA, Doern GV: *Haemophilus*. In *Manual of Clinical Microbiology*. *Volume 1*. 10 edition. Edited by Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW. Washington, DC: ASM Press; 2011:588-602.

257. Lehmann KB, Neumann R: *Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik*. München: 1896.

258. Lioliou EE, Kyriakidis DA: The role of bacterial antizyme: From an inhibitory protein to AtoC transcriptional regulator. *Microb Cell Fact* 2004, 3: 8.

259. Lister S: A filter-passing micro-organism associated with epidemic influenza. *The South African Medical Record* 1922, 20: 434.

260. Livermore DM, Brown DF: Detection of beta-lactamase-mediated resistance. *J Antimicrob Chemother* 2001, 48 Suppl 1: 59-64.

261. Loeb MR, Zachary AL, Smith DH: Isolation and partial characterization of outer and inner membranes from encapsulated *Haemophilus influenzae* type b. *Journal of Bacteriology* 1981, 145: 596-604.

262. Loos BG, Bernstein JM, Dryja DM, Murphy TF, Dickinson DP: Determination of the epidemiology and transmission of nontypable *Haemophilus influenzae* in children with otitis media by comparison of total genomic DNA restriction fingerprints. *Infection and Immunity* 1989, 57: 2751-2757.

263. LPSN. List of prokaryotic names with standing in nomenclature. Genus *Haemophilus*. http://www.bacterio.net/haemophilus.html (last accessed 26.03.2014). 2014.

264. Lucas TJ: An evaluation of 12 methods for the demonstration of penicillinase. *J Clin Pathol* 1979, 32: 1061-1065.

265. Lwoff A, Lwoff M: Role physiologique de l'hemine pour *Haemophilus influenzae* Pfeiffer. *Ann Inst Pasteur (Paris)* 1937, 59: 129-136.

266. Lwoff A, Lwoff M: Studies on Codehydrogenases. II--Physiological Function of Growth Factor "V". *Proceedings of the Royal Society of London Series B - Biological Sciences* 1937, 122: 360-373.

267. Ma C, Redfield RJ: Point mutations in a peptidoglycan biosynthesis gene cause competence induction in *Haemophilus influenzae*. *J Bacteriol* 2000, 182: 3323-3330.

268. Maaroufi Y, De Bruyne JM, Heymans C, Crokaert F: Real-Time PCR for Determining Capsular Serotypes of *Haemophilus influenzae*. *Journal of Clinical Microbiology* 2007, 45: 2305-2308.

269. Machka K, Braveny I, Dabernat H, Dornbusch K, Van DE, Kayser FH *et al*.: Distribution and resistance patterns of *Haemophilus influenzae*: a European cooperative study. *Eur J Clin Microbiol Infect Dis* 1988, 7: 14-24.

270. MacLean RC, Vogwill T: Limits to compensatory adaptation and the persistence of antibiotic resistance in pathogenic bacteria. *Evolution, Medicine, and Public Health* 2015, 2015: 4-12.

271. Mahlen SD, Clarridge JE: Evaluation of a Selection Strategy Before Use of 16S rRNA Gene Sequencing for the Identification of Clinically Significant Gram-Negative Rods and Coccobacilli. *American Journal of Clinical Pathology* 2011, 136: 381-388.

272. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R *et al*.: Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* 1998, 95: 3140-3145.

273. Mak GC, Ho PL, Tse CWS, Lau SKP, Wong SSY: Reduced Levofloxacin Susceptibility and Tetracycline Resistance in a Clinical Isolate of Haemophilus quentini Identified by 16S rRNA Sequencing. *Journal of Clinical Microbiology* 2005, 43: 5391-5392.

274. Makover SD, Wright R, Telep E: Penicillin-binding proteins in *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1981, 19: 584-588.

275. Malouin F, Bryan LE: Modification of penicillin-binding proteins as mechanisms of beta-lactam resistance. *Antimicrob Agents Chemother* 1986, 30: 1-5.

276. Malouin F, Bryan LE: *Haemophilus influenzae* penicillin-binding proteins 1a and 3 possess distinct and opposite temperature-modulated penicillin-binding activities. *Antimicrob Agents Chemother* 1988, 32: 498-502.

277. Malouin F, Parr TR, Jr., Bryan LE: Identification of a group of *Haemophilus influenzae* penicillin-binding proteins that may have complementary physiological roles. *Antimicrob Agents Chemother* 1990, 34: 363-365.

278. Malouin F, Schryvers AB, Bryan LE: Cloning and expression of genes responsible for altered penicillin-binding proteins 3a and 3b in *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1987, 31: 286-291.

279. Markowitz SM: Isolation of an ampicillin-resistant, non-beta-lactamase-producing strain of *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1980, 17: 80-83.

280. Marti S, Puig C, de la Campa AG, Tirado-Velez JM, Tubau F, Domenech A *et al*.: Identification of *Haemophilus haemolyticus* in clinical samples and characterization of their mechanisms of antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* 2015.

281. Matic V, Bozdogan B, Jacobs MR, Ubukata K, Appelbaum PC: Contribution of beta-lactamase and PBP amino acid substitutions to amoxicillin/clavulanate resistance in beta-lactamase-positive, amoxicillin/clavulanate-resistant *Haemophilus influenzae*. *J Antimicrob Chemother* 2003, 52: 1018-1021.

282. Matuschek E, Brown DF, Kahlmeter G: Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clin Microbiol Infect* 2014, 20: O255-O266.

283. Maughan H, Redfield RJ: Extensive variation in natural competence in *Haemophilus influenzae*. *Evolution* 2009, 63: 1852-1866.

284. Mazloum HA, Kilian M, Mohamed ZM, Said MD: Differentiation of *Haemophilus aegyptius* and *Haemophilus influenzae*. *Acta Pathol Microbiol Immunol Scand B* 1982, 90: 109-112.

285. McCann JR, Sheets AJ, Grass S, St.Geme JW: The Haemophilus Cryptic Genospecies Cha Adhesin Has at Least Two Variants That Differ in Host Cell Binding, Bacterial Aggregation, and Biofilm Formation Properties. *Journal of Bacteriology* 2014, 196: 1780-1788.

286. McColm AA, Sowa MA, Ryan DM: Evaluation of ceftazidime, ampicillin and chloramphenicol in experimental *Haemophilus influenzae* type b meningitis. *Journal of Antimicrobial Chemotherapy* 1984, 13: 437-445.

287. McCracken GH, Sakata Y, Olsen KD: Aztreonam therapy in experimental meningitis due to *Haemophilus influenzae* type b and Escherichia coli K1. *Antimicrob Agents Chemother* 1985, 27: 655-656.

288. McCrea KW, Xie J, LaCross N, Patel M, Mukundan D, Murphy TF *et al*.: Relationships of nontypeable *Haemophilus influenzae* strains to hemolytic and nonhemolytic *Haemophilus haemolyticus* strains. *J Clin Microbiol* 2008, 46: 406-416.

289. Meats E, Feil EJ, Stringer S, Cody AJ, Goldstein R, Kroll JS *et al*.: Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. *Journal of Clinical Microbiology* 2003, 41: 1623-1636.

290. Medeiros AA, Levesque R, Jacoby GA: An animal source for the ROB-1 beta-lactamase of *Haemophilus influenzae* type b. *Antimicrob Agents Chemother* 1986, 29: 212-215.

291. Melby K, Leinslie T, Hagen N, Vorland LH: Antibiotic susceptibility pattern and capsular types of *Haemophilus influenzae* recovered from clinical specimens in northern Norway. *Acta Pathol Microbiol Immunol Scand B* 1983, 91: 383-387.

292. Melhus A, Janson H, Westman E, Hermansson A, Forsgren A, Prellner K: Amoxicillin treatment of experimental acute otitis media caused by *Haemophilus influenzae* with non-beta-lactamase-mediated resistance to beta-lactams: aspects of virulence and treatment. *Antimicrob Agents Chemother* 1997, 41: 1979-1984.

293. Mell JC, Shumilina S, Hall IM, Redfield RJ: Transformation of natural genetic variation into *Haemophilus influenzae* genomes. *PLoS Pathog* 2011, 7: e1002151.

294. Mell JC, Hall IM, Redfield RJ: Defining the DNA uptake specificity of naturally competent *Haemophilus influenzae* cells. *Nucleic Acids Research* 2012, 40: 8536-8549.

295. Melnyk AH, Wong A, Kassen R: The fitness costs of antibiotic resistance mutations. *Evol Appl* 2015, 8: 273-283.

296. Mendelman PM, Chaffin DO: PBP profiles of *Haemophilus influenzae*, *H. aegyptius*, and the *H. influenzae* biogroup aegyptius associated with Brazilian Purpuric Fever. *Diagn Microbiol Infect Dis* 1989, 12: 445-447.

297. Mendelman PM, Chaffin DO, Clausen C, Stull TL, Needham C, Williams JD *et al*.: Failure to detect ampicillin-resistant, non-beta-lactamase-producing *Haemophilus influenzae* by standard disk susceptibility testing. *Antimicrob Agents Chemother* 1986, 30: 274-280.

298. Mendelman PM, Chaffin DO, Kalaitzoglou G: Penicillin-binding proteins and ampicillin resistance in *Haemophilus influenzae*. *J Antimicrob Chemother* 1990, 25: 525-534.

299. Mendelman PM, Chaffin DO, Krilov LR, Kalaitzoglou G, Serfass DA, Onay O *et al*.: Cefuroxime treatment failure of nontypable *Haemophilus influenzae* meningitis associated with alteration of penicillin-binding proteins. *J Infect Dis* 1990, 162: 1118-1123.

300. Mendelman PM, Chaffin DO, Musser JM, De GR, Serfass DA, Selander RK: Genetic and phenotypic diversity among ampicillin-resistant, non-beta-lactamase-producing, nontypeable *Haemophilus influenzae* isolates. *Infect Immun* 1987, 55: 2585-2589.

301. Mendelman PM, Chaffin DO, Stull TL, Rubens CE, Mack KD, Smith AL: Characterization of non-beta-lactamase-mediated ampicillin resistance in *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1984, 26: 235-244.

302. Mendelman PM, Doroshow CA, Gandy SL, Syriopoulou V, Weigen CP, Smith AL: Plasmid-mediated resistance in multiply resistant *Haemophilus influenzae* type b causing meningitis: molecular characterization of one strain and review of the literature. *J Infect Dis* 1984, 150: 30-39.

303. Mendelman PM, Henritzy LL, Chaffin DO, Lent K, Smith AL, Stull TL *et al*.: In vitro activities and targets of three cephem antibiotics against *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1989, 33: 1878-1882.

304. Mendelman PM, Serfass DA: The penicillin binding proteins of the genus Haemophilus. *J Med Microbiol* 1988, 27: 95-98.

305. Mercer KLN, Weiss DS: The Escherichia coli Cell Division Protein FtsW Is Required To Recruit Its Cognate Transpeptidase, FtsI (PBP3), to the Division Site. *Journal of Bacteriology* 2002, 184: 904-912.

306. Meyler KL, Meehan M, Bennett D, Cunney R, Cafferkey M: Development of a diagnostic real-time polymerase chain reaction assay for the detection of invasive *Haemophilus influenzae* in clinical samples. *Diagn Microbiol Infect Dis* 2012, 74: 356-362.

307. Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN: SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. *Science* 2004, 305: 1629-1631.

308. Miyazaki S, Fujikawa T, Kanazawa K, Yamaguchi K: In vitro and in vivo activities of meropenem and comparable antimicrobial agents against *Haemophilus influenzae*, including beta-lactamase-negative ampicillin-resistant strains. *J Antimicrob Chemother* 2001, 48: 723-726.

309. Mizrahi A, Cohen R, Varon E, Bonacorsi S, Bechet S, Poyart C *et al*.: Non typable-*Haemophilus influenzae* biofilm formation and acute otitis media. *BMC Infectious Diseases* 2014, 14: 400.

310. Mohd-Zain Z, Turner SL, Cerdeno-Tarraga AM, Lilley AK, Inzana TJ, Duncan AJ *et al*.: Transferable Antibiotic Resistance Elements in *Haemophilus influenzae* Share a Common Evolutionary Origin with a Diverse Family of Syntenic Genomic Islands. *Journal of Bacteriology* 2004, 186: 8114-8122.

311. Molina JM, Cordoba J, Monsoliu A, Diosdado N, Gobernado M: [*Haemophilus influenzae* and betalactam resistance: description of bla TEM gene deletion]. *Rev Esp Quimioter* 2003, 16: 195-203.

312. Morel P, Reverdy C, Michel B, Ehrlich SD, Cassuto E: The role of SOS and flap processing in microsatellite instability in Escherichia coli. *Proc Natl Acad Sci U S A* 1998, 95: 10003-10008.

313. Morikawa Y, Kitazato M, Mitsuyama J, Mizunaga S, Minami S, Watanabe Y: In vitro activities of piperacillin against beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *Antimicrob Agents Chemother* 2004, 48: 1229-1234.

314. Moriyama S, Hotomi M, Shimada J, Billal DS, Fujihara K, Yamanaka N: Formation of biofilm by *Haemophilus influenzae* isolated from pediatric intractable otitis media. *Auris Nasus Larynx* 2009, 36: 525-531.

315. Mouton JW, Brown DF, Apfalter P, Canton R, Giske CG, Ivanova M *et al*.: The role of pharmacokinetics/pharmacodynamics in setting clinical MIC breakpoints: the EUCAST approach. *Clin Microbiol Infect* 2012, 18: E37-E45.

316. Moxon ER: Bacterial variation, virulence and vaccines. *Microbiology* 2009, 155: 997-1003.

317. Munson EL, Doern GV: Comparison of three commercial test systems for biotyping *Haemophilus influenzae* and *Haemophilus parainfluenzae*. *J Clin Microbiol* 2007, 45: 4051-4053.

318. Murphy TF, Kirkham C, Jones MM, Sethi S, Kong Y, Pettigrew MM: Expression of IgA Proteases by *Haemophilus influenzae* in the Respiratory Tract of Adults With Chronic Obstructive Pulmonary Disease. *J Infect Dis* 2015, 212: 1798-1805.

319. Murphy T, Kirkham C: Biofilm formation by nontypeable *Haemophilus influenzae*: strain variability, outer membrane antigen expression and role of pili. *BMC Microbiology* 2002, 2: 7.

320. Murphy TF: Vaccines for Nontypeable *Haemophilus influenzae*: the Future Is Now. *Clinical and Vaccine Immunology* 2015, 22: 459-466.

321. Murphy TF, Brauer AL, Sethi S, Kilian M, Cai X, Lesse AJ: *Haemophilus haemolyticus*: A Human Respiratory Tract Commensal to Be Distinguished from *Haemophilus influenzae*. *Journal of Infectious Diseases* 2007, 195: 81-89.

322. Murphy TF, Lesse AJ, Kirkham C, Zhong H, Sethi S, Munson RS: A clonal group of nontypeable *Haemophilus influenzae* with two IgA proteases is adapted to infection in chronic obstructive pulmonary disease. *PLoS ONE* 2011, 6: e25923.

323. Murphy T: Current and future prospects for a vaccine for nontypeable *Haemophilus influenzae*. *Curr Infect Dis Rep* 2009, 11: 177-182.

324. Mushtaq S, Warner M, Cloke J, Afzal-Shah M, Livermore DM: Performance of the Oxoid M.I.C.Evaluator Strips compared with the Etest assay and BSAC agar dilution. *Journal of Antimicrobial Chemotherapy* 2010, 65: 1702-1711.

325. Musser JM, Barenkamp SJ, Granoff DM, Selander RK: Genetic relationships of serologically nontypable and serotype b strains of *Haemophilus influenzae*. *Infect Immun* 1986, 52: 183-191.

326. Musser JM, Kroll JS, Granoff DM, Moxon ER, Brodeur BR, Campos J *et al*.: Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. *Rev Infect Dis* 1990, 12: 75-111.

327. Musser JM, Kroll JS, Moxon ER, Selander RK: Clonal population structure of encapsulated *Haemophilus influenzae*. *Infect Immun* 1988, 56: 1837-1845.

328. Musser JM, Kroll JS, Moxon ER, Selander RK: Evolutionary genetics of the encapsulated strains of *Haemophilus influenzae*. *Proc Natl Acad Sci U S A* 1988, 85: 7758-7762.

329. Nakamura S, Yanagihara K, Araki N, Yamada K, Morinaga Y, Izumikawa K *et al*.: Efficacy of clarithromycin against experimentally induced pneumonia caused by clarithromycin-resistant *Haemophilus influenzae* in mice. *Antimicrob Agents Chemother* 2010, 54: 757-762.

330. Nakamura S, Yanagihara K, Morinaga Y, Izumikawa K, Seki M, Kakeya H *et al*.: Multiplex real-time polymerase chain reaction for rapid detection of beta-lactamase-negative, ampicillin-resistant *Haemophilus influenzae*. *Diagn Microbiol Infect Dis* 2009, 64: 64-69.

331. Nakamura S, Yanagihara K, Seki M, Izumikawa K, Higashiyama Y, Miyazaki Y *et al*.: Clinical characteristics of pneumonia caused by &b.beta;-lactamase negative ampicillin resistant *Haemophilus influenzae* (BLNAR). *Scandinavian Journal of Infectious Diseases* 2007, 39: 521-524.

332. National Library of Medicine. Profiles in Science. The Oswald T. Avery Collection. http://profiles.nlm.nih.gov/CC (last accessed 26.03.2014). 2013. U.S. National Library of Medicine.

333. Needham CA: *Haemophilus influenzae*: antibiotic susceptibility. *Clin Microbiol Rev* 1988, 1: 218-227.

334. Nelson JD: Should ampicillin be abandoned for treatment of *Haemophilus influenzae* disease? *JAMA* 1974, 229: 322-324.

335. Neu HC, Aswapokee N, Aswapokee P, Fu KP: HR 756, a New Cephalosporin Active Against Gram-Positive and Gram-Negative Aerobic and Anaerobic Bacteria. *Antimicrob Agents Chemother* 1979, 15: 273-281.

336. Noel GJ, Hoiseth SK, Edelson PJ: Type b capsule inhibits ingestion of *Haemophilus influenzae* by murine macrophages: studies with isogenic encapsulated and unencapsulated strains. *J Infect Dis* 1992, 166: 178-182.

337. Noguchi H, Cohen M: Bacteriological and clinical studies of an epidemic of Koch-Weeks bacillus conjunctivitis associated with cell inclusion conjunctivitis. *J Exp Med* 1915, 22: 304-318.

338. Nordic Committee on Antimicrobial Susceptibility Testing (NordicAST). Brytningspunktttabeller for tolkning av MIC-verdier og sonediametre. Versjon 5.0, 2015. 1-1-2015.

339. NORM/NORM-VET. Consumption of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (2000). Tromsø / Oslo, Norway. http://www.unn.no/getfile.php/UNN%20INTER/Fagfolk/www.antibiotikaresistens.no/Dokumenter/normnormvet2009999996.pdf. 2001.

340. NORM/NORM-VET. Consumption of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (2001). Tromsø / Oslo, Norway. http://www.unn.no/getfile.php/UNN%20INTER/Fagfolk/www.antibiotikaresistens.no/Dokumenter/normnormvet2009999997.pdf. 2002.

341. NORM/NORM-VET. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (2004). Tromsø / Oslo, Norway. http://www.unn.no/getfile.php/UNN%20INTER/Fagfolk/www.antibiotikaresistens.no/Dokumenter/normnormvet2005.pdf. 2005.

342. NORM/NORM-VET. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (2007). Tromsø / Oslo, Norway. http://www.unn.no/getfile.php/UNN%20INTER/Fagfolk/www.antibiotikaresistens.no/NORM%202008/NORM%20NORM-VET%202007.pdf. 2008.

343. NORM/NORM-VET. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (2011). Tromsø / Oslo, Norway. http://www.unn.no/getfile.php/UNN%20INTER/Fagfolk/www.antibiotikaresistens.no/NORM%202012/NORM%20NORM-VET%202011.pdf. 2012.

344. NORM/NORM-VET. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (2013). Tromsø / Oslo, Norway. http://www.unn.no/getfile.php/UNN%20INTER/Fagfolk/www.antibiotikaresistens.no/NORM_VET_2013/NORM%20NORM-VET%202013.pdf. 2014.

345. NORM/NORM-VET. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (2014). Tromsø / Oslo, Norway. http://www.unn.no/getfile.php/UNN%20INTER/Fagfolk/www.antibiotikaresistens.no/NORM_VET_2014/NORM_NORM-VET_2014.pdf. 2015.

346. Norrby SR: Neurotoxicity of carbapenem antibiotics: consequences for their use in bacterial meningitis. *Journal of Antimicrobial Chemotherapy* 2000, 45: 5-7.

347. Nørskov-Lauritsen N: Detection of cryptic genospecies misidentified as *Haemophilus influenzae* in routine clinical samples by assessment of marker genes *fucK*, *hap*, and *sodC*. *J Clin Microbiol* 2009, 47: 2590-2592.

348. Nørskov-Lauritsen N, Bruun B, Andersen C, Kilian M: Identification of haemolytic Haemophilus species isolated from human clinical specimens and description of Haemophilus sputorum sp. nov. *Int J Med Microbiol* 2012, 302: 78-83.

349. Nørskov-Lauritsen N, Overballe MD, Kilian M: Delineation of the species *Haemophilus influenzae* by phenotype, multilocus sequence phylogeny, and detection of marker genes. *J Bacteriol* 2009, 191: 822-831.

350. Nørskov-Lauritsen N, Ridderberg W, Erikstrup LT, Fuursted K: Evaluation of disk diffusion methods to detect low-level beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *APMIS* 2011, 119: 385-392.

351. Nørskov-Lauritsen N: Classification, Identification, and Clinical Significance of Haemophilus and Aggregatibacter Species with Host Specificity for Humans. *Clinical Microbiology Reviews* 2014, 27: 214-240.

352. Nørskov-Lauritsen N, Bruun B, Kilian M: Multilocus sequence phylogenetic study of the genus Haemophilus with description of Haemophilus pittmaniae sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 2005, 55: 449-456.

353. Nørskov-Lauritsen N, Kilian M: Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. *International Journal of Systematic and Evolutionary Microbiology* 2006, 56: 2135-2146.

354. Norwegian Institute of Public Health. Årsrapport for 2014. Invasive infeksjoner. Oslo, Norway. http://www.fhi.no/dokumenter/8b6b5e8a95.pdf. 2015.

355. Obaid NA, Jacobson GA, Tristram S: Relationship between clinical site of isolation and ability to form biofilms in vitro in nontypeable *Haemophilus influenzae*. *Can J Microbiol* 2015, 61: 243-245.

356. Okamoto H, Miyazaki S, Tateda K, Ishii Y, Yamaguchi K: In vivo efficacy of telithromycin (HMR3647) against Streptococcus pneumoniae and *Haemophilus influenzae*. *Antimicrob Agents Chemother* 2001, 45: 3250-3252.

357. Olsen I, Dewhirst FE, Paster BJ, Busse H-J: Family I, *Pasteurellaceae*. In *Bergey's Manual of Systematic Bacteriology. The Proteobacteriaceae. Part B. The Gammaproteobacteria*. *Volume II*. 2 edition. Edited by Brenner DJ, Krieg NR, Staley JT, Garrity GM. New York, NY: Springer; 2005:851-856.

358. Omikunle A, Takahashi S, Ogilvie CL, Wang Y, Rodriguez CA, St Geme JW *et al*.: Limited genetic diversity of recent invasive isolates of non-serotype b encapsulated *Haemophilus influenzae*. *J Clin Microbiol* 2002, 40: 1264-1270.

359. Orstavik I, Odegaard K: A simple test for penicillinase production in Staphylococcus aureus. *Acta Pathol Microbiol Scand B Microbiol Immunol* 1971, 79: 855-856.

360. Osaki Y, Sanbongi Y, Ishikawa M, Kataoka H, Suzuki T, Maeda K *et al*.: Genetic approach to study the relationship between penicillin-binding protein 3 mutations and *Haemophilus influenzae* beta-

lactam resistance by using site-directed mutagenesis and gene recombinants. *Antimicrob Agents Chemother* 2005, 49: 2834-2839.

361. Otsuka T, Komiyama K, Yoshida K, Ishikawa Y, Zaraket H, Fujii K *et al*.: Genotyping of *Haemophilus influenzae* type b in pre-vaccination era. *J Infect Chemother* 2012, 18: 213-218.

362. Papazisi L, Ratnayake S, Remortel BG, Bock GR, Liang W, Saeed AI *et al*.: Tracing phylogenomic events leading to diversity of *Haemophilus influenzae* and the emergence of Brazilian Purpuric Fever (BPF)-associated clones. *Genomics* 2010, 96: 290-302.

363. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA: Carbapenems: past, present, and future. *Antimicrob Agents Chemother* 2011, 55: 4943-4960.

364. Park C, Kim KH, Shin NY, Byun JH, Kwon EY, Lee JW *et al*.: Genetic diversity of the *ftsI* gene in beta-lactamase-nonproducing ampicillin-resistant and beta-lactamase-producing amoxicillin-/clavulanic acid-resistant nasopharyngeal *Haemophilus influenzae* strains isolated from children in South Korea. *Microb Drug Resist* 2013, 19: 224-230.

365. Parkinson AJ, Bruce MG, Zulz T: International Circumpolar Surveillance, an Arctic network for the surveillance of infectious diseases. *Emerg Infect Dis* 2008, 14: 18-24.

366. Parr TR, Bryan LE: Mechanism of resistance of an ampicillin-resistant, beta-lactamase-negative clinical isolate of *Haemophilus influenzae* type b to beta-lactam antibiotics. *Antimicrob Agents Chemother* 1984, 25: 747-753.

367. Pearson T, Okinaka RT, Foster JT, Keim P: Phylogenetic understanding of clonal populations in an era of whole genome sequencing. *Infect Genet Evol* 2009, 9: 1010-1019.

368. Perez-Losada M, Browne EB, Madsen A, Wirth T, Viscidi RP, Crandall KA: Population genetics of microbial pathogens estimated from multilocus sequence typing (MLST) data. *Infect Genet Evol* 2006, 6: 97-112.

369. Perez-Vazquez M, Roman F, Garcia-Cobos S, Campos J: Fluoroquinolone resistance in *Haemophilus influenzae* is associated with hypermutability. *Antimicrob Agents Chemother* 2007, 51: 1566-1569.

370. Perez-Vazquez M, Roman F, Aracil B, Canton R, Campos J: Laboratory Detection of *Haemophilus influenzae* with Decreased Susceptibility to Nalidixic Acid, Ciprofloxacin, Levofloxacin, and Moxifloxacin Due to gyrA and parC Mutations. *Journal of Clinical Microbiology* 2004, 42: 1185-1191.

371. Pfaller MA, Farrell DJ, Sader HS, Jones RN: AWARE Ceftaroline Surveillance Program (2008GÇô2010): Trends in Resistance Patterns Among Streptococcus pneumoniae, *Haemophilus influenzae*, and Moraxella catarrhalis in the United States. *Clinical Infectious Diseases* 2012, 55: S187-S193.

372. Pfeifer Y, Meisinger I, Brechtel K, Grobner S: Emergence of a multidrug-resistant *Haemophilus influenzae* strain causing chronic pneumonia in a patient with common variable immunodeficiency. *Microb Drug Resist* 2013, 19: 1-5.

373. Pfeiffer R: I.-Preliminary Communication on the Exciting causes of Influenza. *Br Med J* 1892, 1: 128.

374. Pfeiffer R: Die Aetiologie der Influenza. *Zeitschr f Hygiene* 1893, 13: 357-386.

375. Philpott-Howard J, Williams JD: Increase in antibiotic resistance in *Haemophilus influenzae* in the United Kingdom since 1977: report of study group. *Br Med J (Clin Res Ed)* 1982, 284: 1597-1599.

376. Philpott-Howard J, Williams JD: Activity of cephalosporin antibiotics against *Haemophilus influenzae*. *Scand J Infect Dis Suppl* 1983, 39: 109-111.

377. Pickering J, Binks MJ, Beissbarth J, Hare KM, Kirkham LA, Smith-Vaughan H: A PCR-high-resolution melt assay for rapid differentiation of nontypeable *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Clin Microbiol* 2014, 52: 663-667.

378. Pickering J, Richmond PC, Kirkham LA: Molecular tools for differentiation of nontypeable *Haemophilus influenzae* from *Haemophilus haemolyticus*. *Frontiers in Microbiology* 2014, 5.

379. Piot P, Van DE, Colaert J: In vitro activity of ceftazidime (GR 20263) and other beta-lactam antibiotics against *Haemophilus influenzae*. *Infection* 1983, 11 Suppl 1: S32-S34.

380. Pittman M: Variation and type specificity in the bacterial species *Hemophilus influenzae*. *J Exp Med* 1931, 53: 471-492.

381. Pittman M: A classification of the hemolytic bacteria of the genus Haemophilus: *Haemophilus haemolyticus* Bergey et al. and Haemophilus parahaemolyticus nov spec. *J Bacteriol* 1953, 65: 750-751.

382. Pittman M, Davis DJ: Identification of the Koch-Weeks bacillus (*Hemophilus aegyptius*). *Journal of Bacteriology* 1950, 59: 413-426.

383. Powell M: Antimicrobial resistance in *Haemophilus influenzae*. *J Med Microbiol* 1988, 27: 81-87.

384. Powell M, Livermore DM: Selection and transformation of non-beta-lactamase-mediated insusceptibility to beta-lactams in *Haemophilus influenzae*: lack of cross-resistance between carbapenems and other agents. *J Antimicrob Chemother* 1990, 26: 741-747.

385. Powell M, Seetulsingh P, Williams JD: In-vitro susceptibility of *Haemophilus influenzae* to meropenem compared with imipenem, five other beta-lactams, chloramphenicol and ciprofloxacin. *J Antimicrob Chemother* 1989, 24 Suppl A: 175-181.

386. Powell M, Williams JD: In vitro activities of aztreonam, imipenem, and amoxycillin-clavulanate against ampicillin-resistant *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1987, 31: 1871-1873.

387. Power P, Bentley S, Parkhill J, Moxon E, Hood D: Investigations into genome diversity of *Haemophilus influenzae* using whole genome sequencing of clinical isolates and laboratory transformants. *BMC Microbiology* 2012, 12: 273.

388. Price EP, Sarovich DS, Nosworthy E, Beissbarth J, Marsh RL, Pickering J *et al.*: *Haemophilus influenzae*: using comparative genomics to accurately identify a highly recombinogenic human pathogen. *BMC Genomics* 2015, 16: 1-10.

389. Pritchett IW, Stillman EG: The occurrence of *Bacillus influenzae* in throats and saliva. *The Journal of Experimental Medicine* 1919, 29: 259-266.

390. Prymula R, Hanovcova I, Splino M, Kriz P, Motlova J, Lebedova V *et al.*: Impact of the 10-valent pneumococcal non-typeable *Haemophilus influenzae* Protein D conjugate vaccine (PHiD-CV) on bacterial nasopharyngeal carriage. *Vaccine* 2011, 29: 1959-1967.

391. Prymula R, Kriz P, Kaliskova E, Pascal T, Poolman J, Schuerman L: Effect of vaccination with pneumococcal capsular polysaccharides conjugated to *Haemophilus influenzae*-derived protein D on nasopharyngeal carriage of Streptococcus pneumoniae and *H. influenzae* in children under 2 years of age. *Vaccine* 2009, 28: 71-78.

392. Prymula R, Peeters P, Chrobok V, Kriz P, Novakova E, Kaliskova E *et al.*: Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable *Haemophilus influenzae*: a randomised double-blind efficacy study. *Lancet* 2006, 367: 740-748.

393. Prymula R, Schuerman L: 10-valent pneumococcal nontypeable *Haemophilus influenzae* PD conjugate vaccine: Synflorix. *Expert Rev Vaccines* 2009, 8: 1479-1500.

394. Puig C, Calatayud L, Marti S, Tubau F, Garcia-Vidal C, Carratala J *et al.*: Molecular epidemiology of nontypeable *Haemophilus influenzae* causing community-acquired pneumonia in adults. *PLoS ONE* 2013, 8: e82515.

395. Puig C, Grau I, Marti S, Tubau F, Calatayud L, Pallares R *et al.*: Clinical and molecular epidemiology of *Haemophilus influenzae* causing invasive disease in adult patients. *PLoS ONE* 2014, 9: e112711.

396. Puig C, Tirado-Velez JM, Calatayud L, Tubau F, Garmendia J, Ardanuy C *et al.*: Molecular characterization of fluoroquinolone resistance in nontypeable *Haemophilus influenzae* clinical isolates. *Antimicrob Agents Chemother* 2015, 59: 461-466.

397. Puig C, Domenech A, Garmendia J, Langereis JD, Mayer P, Calatayud L *et al.*: Increased Biofilm Formation by Nontypeable *Haemophilus influenzae* Isolates from Patients with Invasive Disease or Otitis Media versus Strains Recovered from Cases of Respiratory Infections. *Applied and Environmental Microbiology* 2014, 80: 7088-7095.

398. Pumarola F, Mares J, Losada I, Minguella I, Moraga F, Tarrago D *et al*.: Microbiology of bacteria causing recurrent acute otitis media (AOM) and AOM treatment failure in young children in Spain: Shifting pathogens in the post-pneumococcal conjugate vaccination era. *International Journal of Pediatric Otorhinolaryngology* 2013, 77: 1231-1236.

399. Qin L, Kida Y, Ishiwada N, Ohkusu K, Kaji C, Sakai Y *et al*.: The relationship between biofilm formations and capsule in *Haemophilus influenzae*. *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy* 2014, 20: 151-156.

400. Quellec SL, Gaillot O, Chotel F, Freydiere AM, Laurent F, Vandenesch F *et al*.: Septic arthritis caused by noncapsulated *Haemophilus influenzae*. *J Clin Microbiol* 2013, 51: 1970-1972.

401. Quentin R, Goudeau A, Wallace RJ, Smith AL, Selander RK, Musser JM: Urogenital, maternal and neonatal isolates of *Haemophilus influenzae*: identification of unusually virulent serologically non-typable clone families and evidence for a new Haemophilus species. *Journal of General Microbiology* 1990, 136: 1203-1209.

402. Quentin R, Ruimy R, Rosenau A, Musser JM, Christen R: Genetic identification of cryptic genospecies of Haemophilus causing urogenital and neonatal infections by PCR using specific primers targeting genes coding for 16S rRNA. *Journal of Clinical Microbiology* 1996, 34: 1380-1385.

403. Reddington K, Schwenk S, Tuite N, Platt G, Davar D, Coughlan H *et al*.: Comparison of Established Diagnostic Methodologies and a Novel Bacterial smpB Real-Time PCR Assay for Specific Detection of *Haemophilus influenzae* Isolates Associated with Respiratory Tract Infections. *Journal of Clinical Microbiology* 2015, 53: 2854-2860.

404. Reddy MS, Bernstein JM, Murphy TF, Faden HS: Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. *Infect Immun* 1996, 64: 1477-1479.

405. Redfield RJ: sxy-1, a *Haemophilus influenzae* mutation causing greatly enhanced spontaneous competence. *J Bacteriol* 1991, 173: 5612-5618.

406. Redfield RJ, Cameron AD, Qian Q, Hinds J, Ali TR, Kroll JS *et al*.: A novel CRP-dependent regulon controls expression of competence genes in *Haemophilus influenzae*. *J Mol Biol* 2005, 347: 735-747.

407. Regelink AG, Dahan D, Möller LVM, Coulton JW, Eijk P, van Ulsen P *et al*.: Variation in the Composition and Pore Function of Major Outer Membrane Pore Protein P2 of *Haemophilus influenzae* from Cystic Fibrosis Patients. *Antimicrob Agents Chemother* 1999, 43: 226-232.

408. Rennie RP, Brosnikoff C, Shokoples S, Reller LB, Mirrett S, Janda W *et al*.: Multicenter evaluation of the new Vitek 2 Neisseria-Haemophilus identification card. *J Clin Microbiol* 2008, 46: 2681-2685.

409. Rennie RP, Turnbull L, Brosnikoff C, Cloke J: First comprehensive evaluation of the M.I.C. Evaluator device compared to Etest and CLSI reference dilution methods for antimicrobial susceptibility testing of clinical strains of anaerobes and other fastidious bacterial species. *J Clin Microbiol* 2012, 50: 1153-1157.

410. Rennie RP, Ibrahim KH: Antimicrobial Resistance in *Haemophilus influenzae*: How Can We Prevent the Inevitable? Commentary on Antimicrobial Resistance in *H. influenzae* Based on Data from the TARGETed Surveillance Program. *Clinical Infectious Diseases* 2005, 41: S234-S238.

411. Resman F, Ristovski M, Ahl J, Forsgren A, Gilsdorf JR, Jasir A *et al*.: Invasive disease caused by *Haemophilus influenzae* in Sweden 1997-2009; evidence of increasing incidence and clinical burden of non-type b strains. *Clin Microbiol Infect* 2011, 17: 1638-1645.

412. Resman F, Svensjo T, Unal C, Cronqvist J, Brorson H, Odenholt I *et al*.: Necrotizing myositis and septic shock caused by *Haemophilus influenzae* type f in a previously healthy man diagnosed with an IgG3 and a mannose-binding lectin deficiency. *Scand J Infect Dis* 2011, 43: 972-976.

413. Resman F: *Invasive disease by Haemophilus influenzae in Sweden in the era of the H. influenzae type b vaccine.* Malmö: Lund University; 2012.

414. Resman F, Ristovski M, Forsgren A, Kaijser B, Kronvall G, Medstrand P *et al*.: Increase of beta-lactam-resistant invasive *Haemophilus influenzae* in Sweden, 1997 to 2010. *Antimicrob Agents Chemother* 2012, 56: 4408-4415.

415. Ridderberg W, Fenger MG, Norskov-Lauritsen N: *Haemophilus influenzae* may be untypable by the multilocus sequence typing scheme due to a complete deletion of the fucose operon. *J Med Microbiol* 2010, 59: 740-742.

416. Rittenbury MS: How and why aztreonam works. *Surg Gynecol Obstet* 1990, 171 Suppl: 19-23.

417. Rivers TM: *Bacillus Hemoglobinophilus canis* (Friedberger) (*Hemophilus canis* emend.). *Journal of Bacteriology* 1922, 7: 579-581.

418. Roberts MC, Swenson CD, Owens LM, Smith AL: Characterization of chloramphenicol-resistant *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1980, 18: 610-615.

419. Roca AI, Cox MM, Brenner SL: The RecA Protein: Structure and Function. *Critical Reviews in Biochemistry and Molecular Biology* 1990, 25: 415-456.

420. Rogall T, Flohr T, Böttger EC: Differentiation of Mycobacterium species by direct sequencing of amplified DNA. *Journal of General Microbiology* 1990, 136: 1915-1920.

421. Roier S, Leitner DR, Iwashkiw J, Schild-Prüfert K, Feldman MF, Krohne G *et al*.: Intranasal Immunization with Nontypeable *Haemophilus influenzae* Outer Membrane Vesicles Induces Cross-Protective Immunity in Mice. *PLoS ONE* 2012, 7: e42664.

422. Ronander E, Brant M, Eriksson E, Morgelin M, Hallgren O, Westergren-Thorsson G *et al*.: Nontypeable *Haemophilus influenzae* adhesin protein E: characterization and biological activity. *J Infect Dis* 2009, 199: 522-531.

423. Rubach MP, Bender JM, Mottice S, Hanson K, Weng HY, Korgenski K *et al*.: Increasing incidence of invasive *Haemophilus influenzae* disease in adults, Utah, USA. *Emerg Infect Dis* 2011, 17: 1645-1650.

424. Rubin LG, Medeiros AA, Yolken RH, Moxon ER: Ampicillin treatment failure of apparently beta-lactamase-negative *Haemophilus influenzae* type b meningitis due to novel beta-lactamase. *Lancet* 1981, 2: 1008-1010.

425. Rubin LG, Mendelman PM, Rakita RM, Rosen H: Virulence of non-beta-lactamase-mediated ampicillin-resistant *Haemophilus influenzae*. *FEMS Microbiol Lett* 1991, 68: 27-31.

426. Sá-Leäo R, Nunes S, Brito-Avo A, Alves CR, Carrico JA, Saldanha J *et al*.: High Rates of Transmission of and Colonization by Streptococcus pneumoniae and *Haemophilus influenzae* within a Day Care Center Revealed in a Longitudinal Study. *Journal of Clinical Microbiology* 2008, 46: 225-234.

427. Sacchi CT, Alber D, Dull P, Mothershed EA, Whitney AM, Barnett GA *et al*.: High level of sequence diversity in the 16S rRNA genes of *Haemophilus influenzae* isolates is useful for molecular subtyping. *J Clin Microbiol* 2005, 43: 3734-3742.

428. Sader HS, Farrell DJ, Flamm RK, Jones RN: Antimicrobial susceptibility of Gram-negative organisms isolated from patients hospitalised with pneumonia in US and European hospitals: results from the SENTRY Antimicrobial Surveillance Program, 2009-2012. *Int J Antimicrob Agents* 2014, 43: 328-334.

429. Sader HS, Flamm RK, Jones RN: Antimicrobial activity of ceftaroline and comparator agents tested against bacterial isolates causing skin and soft tissue infections and community-acquired respiratory tract infections isolated from the Asia-Pacific region and South Africa (2010). *Diagn Microbiol Infect Dis* 2013, 76: 61-68.

430. Saha SK, Darmstadt GL, Baqui AH, Islam N, Qazi S, Islam M *et al*.: Direct detection of the multidrug resistance genome of *Haemophilus influenzae* in cerebrospinal fluid of children: implications for treatment of meningitis. *Pediatr Infect Dis J* 2008, 27: 49-53.

431. Saikia KK, Das BK, Bewal RK, Kapil A, Arora NK, Sood S: Characterization of nasopharyngeal isolates of type b *Haemophilus influenzae* from Delhi. *Indian J Med Res* 2012, 136: 855-861.

432. Sakai A, Hotomi M, Billal DS, Yamauchi K, Shimada J, Tamura S *et al*.: Evaluation of mutations in penicillin binding protein-3 gene of non-typeable *Haemophilus influenzae* isolated from the nasopharynx of children with acute otitis media. *Acta Otolaryngol* 2005, 125: 180-183.

433. Sakata Y, McCracken GH, Thomas ML, Olsen KD: Pharmacokinetics and therapeutic efficacy of imipenem, ceftazidime, and ceftriaxone in experimental meningitis due to an ampicillin- and

chloramphenicol-resistant strain of *Haemophilus influenzae* type b. *Antimicrob Agents Chemother* 1984, 25: 29-32.

434. San Millan A, Garcia-Cobos S, Escudero JA, Hidalgo L, Gutierrez B, Carrilero L *et al*.: *Haemophilus influenzae* clinical isolates with plasmid pB1000 bearing blaROB-1: fitness cost and interspecies dissemination. *Antimicrob Agents Chemother* 2010, 54: 1506-1511.

435. San Millan A, Giufre M, Escudero JA, Hidalgo L, Gutierrez B, Cerquetti M *et al*.: Contribution of ROB-1 and PBP3 mutations to the resistance phenotype of a beta-lactamase-positive amoxicillin/clavulanic acid-resistant *Haemophilus influenzae* carrying plasmid pB1000 in Italy. *J Antimicrob Chemother* 2011, 66: 96-99.

436. Sanbongi Y, Suzuki T, Osaki Y, Senju N, Ida T, Ubukata K: Molecular evolution of beta-lactam-resistant *Haemophilus influenzae*: 9-year surveillance of penicillin-binding protein 3 mutations in isolates from Japan. *Antimicrob Agents Chemother* 2006, 50: 2487-2492.

437. Sanchez L, Pan W, Vinas M, Nikaido H: The acrAB homolog of *Haemophilus influenzae* codes for a functional multidrug efflux pump. *Journal of Bacteriology* 1997, 179: 6855-6857.

438. Sanchez L, Puig M, Fuste C, Loren JG, Vinas M: Outer membrane permeability of non-typable *Haemophilus influenzae*. *J Antimicrob Chemother* 1996, 37: 341-344.

439. Sanders CC: Comparative activity of mezlocillin, penicillin, ampicillin, carbenicillin, and ticarcillin against gram-positive bacteria and *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1981, 20: 843-846.

440. Sanders DY, Garbee HW: Failure of response to ampicillin in *Hemophilus influenzae* meningitis. *Am J Dis Child* 1969, 117: 331-333.

441. Santana-Porto EA, Oliveira AA, da-Costa MR, Pinheiro A, Oliveira C, Lopes ML *et al*.: Suspected Brazilian purpuric fever, Brazilian Amazon region. *Emerg Infect Dis* 2009, 15: 675-676.

442. Satola SW, Collins JT, Napier R, Farley MM: Capsule gene analysis of invasive *Haemophilus influenzae*: accuracy of serotyping and prevalence of IS1016 among nontypeable isolates. *J Clin Microbiol* 2007, 45: 3230-3238.

443. Satola SW, Schirmer PL, Farley MM: Complete sequence of the cap locus of *Haemophilus influenzae* serotype b and nonencapsulated b capsule-negative variants. *Infect Immun* 2003, 71: 3639-3644.

444. Schaar V, Nordstrom T, Morgelin M, Riesbeck K: Moraxella catarrhalis outer membrane vesicles carry beta-lactamase and promote survival of Streptococcus pneumoniae and *Haemophilus influenzae* by inactivating amoxicillin. *Antimicrob Agents Chemother* 2011, 55: 3845-3853.

445. Schaar V, Uddback I, Nordstrom T, Riesbeck K: Group A streptococci are protected from amoxicillin-mediated killing by vesicles containing beta-lactamase derived from *Haemophilus influenzae*. *J Antimicrob Chemother* 2014, 69: 117-120.

446. Scheld WM, Brodeur JP, Gratz JC, Foresman P, Rodeheaver G: Evaluation of aztreonam in experimental bacterial meningitis and cerebritis. *Antimicrob Agents Chemother* 1983, 24: 682-688.

447. Schleupner CJ, Engle JC: Clinical evaluation of cefotaxime for therapy of lower respiratory tract infections. *Antimicrob Agents Chemother* 1982, 21: 327-333.

448. Scriver SR, Walmsley SL, Kau CL, Hoban DJ, Brunton J, McGeer A *et al*.: Determination of antimicrobial susceptibilities of Canadian isolates of *Haemophilus influenzae* and characterization of their beta-lactamases. Canadian Haemophilus Study Group. *Antimicrob Agents Chemother* 1994, 38: 1678-1680.

449. Sekiya Y, Eguchi M, Nakamura M, Ubukata K, Omura S, Matsui H: Comparative efficacies of different antibiotic treatments to eradicate nontypeable *Haemophilus influenzae* infection. *BMC Infect Dis* 2008, 8: 15.

450. Seng P, Drancourt M, Gouriet Fdr, La Scola B, Fournier PE, Rolain JM *et al*.: Ongoing Revolution in Bacteriology: Routine Identification of Bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. *Clinical Infectious Diseases* 2009, 49: 543-551.

451. Serfass DA, Mendelman PM, Chaffin DO, Needham CA: Ampicillin resistance and penicillin-binding proteins of *Haemophilus influenzae*. *J Gen Microbiol* 1986, 132: 2855-2861.

452. Serruto D, Spadafina T, Scarselli M, Bambini S, Comanducci M, H+Âhle S *et al*.: HadA is an atypical new multifunctional trimeric coiled-coil adhesin of *Haemophilus influenzae* biogroup aegyptius, which promotes entry into host cells. *Cellular Microbiology* 2009, 11: 1044-1063.

453. Sethi S, Murphy TF: Infection in the Pathogenesis and Course of Chronic Obstructive Pulmonary Disease. *N Engl J Med* 2008, 359: 2355-2365.

454. Sevillano D, Giménez MJ, Cercenado E, Cafini F, Gené A, Alou L *et al*.: Genotypic versus phenotypic characterization, with respect to beta-lactam susceptibility, of *Haemophilus influenzae* isolates exhibiting decreased susceptibility to beta-lactam resistance markers. *Antimicrob Agents Chemother* 2009, 53: 267-270.

455. Shackelford PG, Bobinski JE, Feigin RD, Cherry JD: Therapy of *Haemophilus influenzae* Meningitis Reconsidered. *N Engl J Med* 1972, 287: 634-638.

456. Sham LT, Butler EK, Lebar MD, Kahne D, Bernhardt TG, Ruiz N: Bacterial cell wall. MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. *Science* 2014, 345: 220-222.

457. Shuel M, Hoang L, Law DKS, Tsang R: Invasive *Haemophilus influenzae* in British Columbia: non-Hib and non-typeable strains causing disease in children and adults. *International Journal of Infectious Diseases* 2011, 15: e167-e173.

458. Shuel ML, Tsang RSW: Canadian beta-lactamase negative *Haemophilus influenzae* isolates showing decreased susceptibility toward ampicillin have significant penicillin binding protein 3 mutations. *Diagnostic Microbiology and Infectious Disease* 2009, 63: 379-383.

459. Sill ML, Law DKS, Zhou J, Skinner S, Wylie J, Tsang RSW: Population genetics and antibiotic susceptibility of invasive *Haemophilus influenzae* in Manitoba, Canada, from 2000 to 2006. *FEMS Immunology & Medical Microbiology* 2007, 51: 270-276.

460. Sill ML, Tsang RSW: Antibiotic susceptibility of invasive *Haemophilus influenzae* strains in Canada. *Antimicrob Agents Chemother* 2008, 52: 1551-1552.

461. Simmons LA, Foti JJ, Cohen SE, Walker GC: The SOS Regulatory Network. *Ecosal Plus* 2008, 2008.

462. Skaare D, Anthonisen IL, Hannisdal A, Jenkins A, Kahlmeter G, Lia A *et al*.. Comparison of two disk diffusion-based screening methods for detection and classification of beta-lactam resistance in *Haemophilus influenzae*. Poster 752. The 21st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). 2011. Milan, Italy.

463. Skaare D, Anthonisen IL, Lia A, Tveten Y, Kristiansen BE. PBP-mediated resistance in *Haemophilus influenzae*: evaluation of the recommended disc diffusion screening method. Poster. The 24th Annual Meeting of the Scandinavian Society of Antimicrobial Chemotherapy (SSAC). 2007. Tampere, Finland.

464. Skaare D, Allum AG, Anthonisen IL, Jenkins A, Lia A, Strand L *et al*.: Mutant *ftsI* genes in the emergence of penicillin-binding protein-mediated beta-lactam resistance in *Haemophilus influenzae* in Norway. *Clin Microbiol Infect* 2010, 16: 1117-1124.

465. Skaare D, Anthonisen IL, Caugant DA, Jenkins A, Steinbakk M, Strand L *et al*.: Multilocus sequence typing and *ftsI* sequencing: a powerful tool for surveillance of penicillin-binding protein 3-mediated beta-lactam resistance in nontypeable *Haemophilus influenzae*. *BMC Microbiol* 2014, 14: 131.

466. Skaare D, Anthonisen IL, Kahlmeter G, Matuschek E, Natås OB, Steinbakk M *et al*.: Emergence of clonally related multidrug resistant *Haemophilus influenzae* with penicillin-binding protein 3-mediated resistance to extended-spectrum cephalosporins, Norway, 2006 to 2013. *Euro Surveill* 2014, 19: 6-18.

467. Skaare D, Lia A, Hannisdal A, Tveten Y, Matuschek E, Kahlmeter G *et al*.: *Haemophilus influenzae* with non-beta-lactamase-mediated beta-lactam resistance: easy to find but hard to categorize. *J Clin Microbiol* 2015, 53: 3589-3595.

468. Skoczynska A, Kadlubowski M, Wasko I, Fiett J, Hryniewicz W: Resistance patterns of selected respiratory tract pathogens in Poland. *Clin Microbiol Infect* 2007, 13: 377-383.

469. Slinger R, Chan F, Ferris W, Yeung SW, St Denis M, Gaboury I *et al*.: Multiple combination antibiotic susceptibility testing of nontypeable *Haemophilus influenzae* biofilms. *Diagn Microbiol Infect Dis* 2006, 56: 247-253.

470. Smith AL: Antibiotic resistance in *Haemophilus influenzae*. *Pediatr Infect Dis* 1983, 2: 352-355.

471. Smith HO, Tomb JF, Dougherty BA, Fleischmann RD, Venter JC: Frequency and distribution of DNA uptake signal sequences in the *Haemophilus influenzae* Rd genome. *Science* 1995, 269: 538-540.

472. Smith W, Andrewes CH, Laidlaw PP: A virus obtained from influenza patients. *The Lancet* 1933, 222: 66-68.

473. Smith-Vaughan HC, Chang AB, Sarovich DS, Marsh RL, Grimwood K, Leach AJ *et al*.: Absence of an Important Vaccine and Diagnostic Target in Carriage- and Disease-Related Nontypeable *Haemophilus influenzae*. *Clinical and Vaccine Immunology* 2014, 21: 250-252.

474. Sobhanifar S, King DT, Strynadka NC: Fortifying the wall: synthesis, regulation and degradation of bacterial peptidoglycan. *Current Opinion in Structural Biology* 2013, 23: 695-703.

475. Søndergaard A, Petersen MT, Fuursted K, Nørskov-Lauritsen N: Detection of N526K-substituted penicillin-binding protein 3 conferring low-level mutational resistance to beta-lactam antibiotics in *Haemophilus influenzae* by disc diffusion testing on Mueller-Hinton agar according to EUCAST guidelines. *J Antimicrob Chemother* 2012, 67: 1401-1404.

476. Sondergaard A, Petersen MT, Fuursted K, Norskov-Lauritsen N: Detection of N526K-substituted penicillin-binding protein 3 conferring low-level mutational resistance to beta-lactam antibiotics in *Haemophilus influenzae* by disc diffusion testing on Mueller-Hinton agar according to EUCAST guidelines. *J Antimicrob Chemother* 2012, 67: 1401-1404.

477. Søndergaard A, San MA, Santos-Lopez A, Nielsen SM, Gonzalez-Zorn B, Nørskov-Lauritsen N: Molecular organization of small plasmids bearing blaTEM-1 and conferring resistance to beta-lactams in *Haemophilus influenzae*. *Antimicrob Agents Chemother* 2012, 56: 4958-4960.

478. Søndergaard A, Witherden EA, Nørskov-Lauritsen N, Tristram SG: Interspecies Transfer of the Penicillin-Binding Protein 3-Encoding Gene *ftsI* between *Haemophilus influenzae* and *Haemophilus haemolyticus* Can Confer Reduced Susceptibility to beta-Lactam Antimicrobial Agents. *Antimicrob Agents Chemother* 2015, 59: 4339-4342.

479. Sönksen UW, Christensen JJ, Nielsen L, Hesselbjerg A, Hansen DS, Bruun B: Fastidious Gram-Negatives: Identification by the Vitek 2 Neisseria-Haemophilus Card and by Partial 16S rRNA Gene Sequencing Analysis. *Open Microbiol J* 2010, 4: 123-131.

480. Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ: Displaying the relatedness among isolates of bacterial species -- the eBURST approach. *FEMS Microbiol Lett* 2004, 241: 129-134.

481. Spratt BG: Penicillin-binding Proteins and the Future of Beta-Lactam Antibiotics: The Seventh Fleming Lecture. *Journal of General Microbiology* 1983, 129: 1247-1260.

482. Spratt BG: Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the Internet. *Current Opinion in Microbiology* 1999, 2: 312-316.

483. St Geme JW, Falkow S, Barenkamp SJ: High-molecular-weight proteins of nontypable *Haemophilus influenzae* mediate attachment to human epithelial cells. *Proc Natl Acad Sci U S A* 1993, 90: 2875-2879.

484. St Geme JW, Kumar VV, Cutter D, Barenkamp SJ: Prevalence and distribution of the hmw and hia genes and the HMW and Hia adhesins among genetically diverse strains of nontypeable *Haemophilus influenzae*. *Infect Immun* 1998, 66: 364-368.

485. Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, K+ñmpfer P, Maiden MCJ *et al*.: Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology* 2002, 52: 1043-1047.

486. Starner TD, Zhang N, Kim G, Apicella MA, McCray PB, Jr.: *Haemophilus influenzae* forms biofilms on airway epithelia: implications in cystic fibrosis. *Am J Respir Crit Care Med* 2006, 174: 213-220.

487. Straker K, Wootton M, Simm AM, Bennett PM, MacGowan AP, Walsh TR: Cefuroxime resistance in non-beta-lactamase *Haemophilus influenzae* is linked to mutations in *ftsI*. *J Antimicrob Chemother* 2003, 51: 523-530.

488. Stralin K, Backman A, Holmberg H, Fredlund H, Olcen P: Design of a multiplex PCR for Streptococcus pneumoniae, *Haemophilus influenzae*, Mycoplasma pneumoniae and Chlamydophila pneumoniae to be used on sputum samples. *APMIS* 2005, 113: 99-111.

489. Strouts FR, Power P, Croucher NJ, Corton N, van TA, Quail MA *et al*.: Lineage-specific virulence determinants of *Haemophilus influenzae* biogroup aegyptius. *Emerg Infect Dis* 2012, 18: 449-457.

490. Sugita G, Hotomi M, Sugita R, Kono M, Togawa A, Yamauchi K *et al*.: Genetic characteristics of *Haemophilus influenzae* and Streptococcus pneumoniae isolated from children with conjunctivitis-otitis media syndrome. *J Infect Chemother* 2014, 20: 493-497.

491. Summers AO: Genetic linkage and horizontal gene transfer, the roots of the antibiotic multi-resistance problem. *Anim Biotechnol* 2006, 17: 125-135.

492. Sunakawa K, Farrell DJ: Mechanisms, molecular and sero-epidemiology of antimicrobial resistance in bacterial respiratory pathogens isolated from Japanese children. *Ann Clin Microbiol Antimicrob* 2007, 6: 7.

493. Surette MG, Miller MB, Bassler BL: Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production. *Proc Natl Acad Sci U S A* 1999, 96: 1639-1644.

494. Sweetman WA, Moxon ER, Bayliss CD: Induction of the SOS regulon of *Haemophilus influenzae* does not affect phase variation rates at tetranucleotide or dinucleotide repeats. *Microbiology* 2005, 151: 2751-2763.

495. Swords WE: Nontypeable *Haemophilus influenzae* biofilms: role in chronic airway infections. *Front Cell Infect Microbiol* 2012, 2: 97.

496. Swords WE: Quorum signaling and sensing by nontypeable *Haemophilus influenzae*. *Front Cell Infect Microbiol* 2012, 2: 100.

497. Sykes RB, Matthew M, O'Callaghan CH: R-factor mediated beta-lactamase production by *Haemophilus influenzae*. *J Med Microbiol* 1975, 8: 437-441.

498. Szelestey BR, Heimlich DR, Raffel FK, Justice SS, Mason KM: *Haemophilus* Responses to Nutritional Immunity: Epigenetic and Morphological Contribution to Biofilm Architecture, Invasion, Persistence and Disease Severity. *PLoS Pathog* 2013, 9: e1003709.

499. Takahata S, Ida T, Senju N, Sanbongi Y, Miyata A, Maebashi K *et al*.: Horizontal gene transfer of *ftsI*, the gene encoding penicillin-binding protein 3, in *Haemophilus influenzae*. *Antimicrob Agents Chemother* 2007, 51: 1589-1595.

500. Takala AK, Pekkanen E, Eskola J: Neonatal *Haemophilus influenzae* infections. *Arch Dis Child* 1991, 66: 437-440.

501. Targowski S, Targowski H: Characterization of a Haemophilus paracuniculus isolated from gastrointestinal tracts of rabbits with mucoid enteritis. *J Clin Microbiol* 1979, 9: 33-37.

502. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH *et al*.: Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995, 33: 2233-2239.

503. Thjøtta T, Avery OT: Studies on bacterial nutrition: II. Growth accessory substances in the cultivation of hemophilic bacilli. *The Journal of Experimental Medicine* 1921, 34: 97-114.

504. Thomas J, Pettigrew M: Multilocus sequence typing and pulsed field gel electrophoresis of otitis media causing pathogens. In *Auditory and Vestibular Research*. 493 edition. Edited by Sokolowski B. New York: Humana Press Inc.; 2009:179-190.

505. Thonnings S, Ostergaard C: Treatment of Haemophilus bacteremia with benzylpenicillin is associated with increased (30-day) mortality. *BMC Infect Dis* 2012, 12: 153.

506. Thöny-Meyer L: Biogenesis of respiratory cytochromes in bacteria. *Microbiology and Molecular Biology Reviews* 1997, 61: 337-376.

507. Thornsberry C, Baker CN, Kirven LA, Swenson JM: Susceptibility of ampicillin-resistant *Haemophilus influenzae* to seven penicillins. *Antimicrob Agents Chemother* 1976, 9: 70-73.

508. Thornsberry C, Kirven LA: Ampicillin resistance in *Haemophilus influenzae* as determined by a rapid test for beta-lactamase production. *Antimicrob Agents Chemother* 1974, 6: 653-654.

509. Thornsberry C, Kirven LA: Antimicrobial susceptibility of *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1974, 6: 620-624.

510. Tinguely R, Seiffert SN, Furrer H, Perreten V, Droz S, Endimiani A: Emergence of Extensively Drug-Resistant *Haemophilus parainfluenzae* in Switzerland. *Antimicrob Agents Chemother* 2013, 57: 2867-2869.

511. Tomic V, Dowzicky MJ: Regional and global antimicrobial susceptibility among isolates of Streptococcus pneumoniae and *Haemophilus influenzae* collected as part of the Tigecycline Evaluation and Surveillance Trial (T.E.S.T.) from 2009 to 2012 and comparison with previous years of T.E.S.T. (2004-2008). *Ann Clin Microbiol Antimicrob* 2014, 13: 52.

512. Tormo A, Ayala JA, de Pedro MA, Aldea M, Vicente M: Interaction of FtsA and PBP3 proteins in the Escherichia coli septum. *J Bacteriol* 1986, 166: 985-992.

513. Török ME, Peacock SJ: Rapid whole-genome sequencing of bacterial pathogens in the clinical microbiology laboratoryGÇöpipe dream or reality? *Journal of Antimicrobial Chemotherapy* 2012, 67: 2307-2308.

514. Trees DL, Morse SA: Chancroid and Haemophilus ducreyi: an update. *Clinical Microbiology Reviews* 1995, 8: 357-375.

515. Trepod CM, Mott JE: Elucidation of Essential and Nonessential Genes in the *Haemophilus influenzae* Rd Cell Wall Biosynthetic Pathway by Targeted Gene Disruption. *Antimicrob Agents Chemother* 2005, 49: 824-826.

516. Tristram S, Jacobs MR, Appelbaum PC: Antimicrobial resistance in *Haemophilus influenzae*. *Clin Microbiol Rev* 2007, 20: 368-389.

517. Tristram SG: Effect of extended-spectrum beta-lactamases on the susceptibility of *Haemophilus influenzae* to cephalosporins. *J Antimicrob Chemother* 2003, 51: 39-43.

518. Tristram SG, Burdach JG: Effect of cloned inhibitor-resistant TEM beta-lactamases on the susceptibility of *Haemophilus influenzae* to amoxicillin/clavulanate. *J Antimicrob Chemother* 2007.

519. Tristram SG, Littlejohn R, Bradbury RS: blaROB-1 presence on pB1000 in *Haemophilus influenzae* is widespread, and variable cefaclor resistance is associated with altered penicillin-binding proteins. *Antimicrob Agents Chemother* 2010, 54: 4945-4947.

520. Tristram SG, Nichols S: A multiplex PCR for beta-lactamase genes of *Haemophilus influenzae* and description of a new blaTEM promoter variant. *J Antimicrob Chemother* 2006, 58: 183-185.

521. Tristram SG, Pitout MJ, Forward K, Campbell S, Nichols S, Davidson RJ: Characterization of extended-spectrum beta-lactamase-producing isolates of *Haemophilus parainfluenzae*. *J Antimicrob Chemother* 2008, 61: 509-514.

522. Tristram SG: A comparison of Etest, M.I.C.Evaluator strips and CLSI broth microdilution for determining beta-lactam antimicrobial susceptibility in *Haemophilus influenzae*. *Journal of Antimicrobial Chemotherapy* 2008, 62: 1464-1466.

523. Tristram SG, Franks LR, Harvey GL: Sequences of small blaTEM-encoding plasmids in *Haemophilus influenzae* and description of variants falsely negative for blaTEM by PCR. *Journal of Antimicrobial Chemotherapy* 2012, 67: 2621-2625.

524. Trottier S, Stenberg K, Svanborg-Eden C: Turnover of nontypable *Haemophilus influenzae* in the nasopharynges of healthy children. *Journal of Clinical Microbiology* 1989, 27: 2175-2179.

525. Tsang RS, Bruce MG, Lem M, Barreto L, Ulanova M: A review of invasive *Haemophilus influenzae* disease in the Indigenous populations of North America. *Epidemiol Infect* 2014, 142: 1344-1354.

526. Tsang RS, Sill ML, Skinner SJ, Law DK, Zhou J, Wylie J: Characterization of invasive *Haemophilus influenzae* disease in Manitoba, Canada, 2000-2006: invasive disease due to non-type b strains. *Clin Infect Dis* 2007, 44: 1611-1614.

527. Turner K, Hanage W, Fraser C, Connor T, Spratt B: Assessing the reliability of eBURST using simulated populations with known ancestry. *BMC Microbiology* 2007, 7: 30.

528. Typas A, Banzhaf M, Gross CA, Vollmer W: From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nat Rev Microbiol* 2012, 10: 123-136.

529. Ubukata K, Chiba N, Hasegawa K, Shibasaki Y, Sunakawa K, Nonoyama M *et al*.: Differentiation of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* from other *H. influenzae strains* by a disc method. *J Infect Chemother* 2002, 8: 50-58.

530. Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y *et al*.: Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *Antimicrob Agents Chemother* 2001, 45: 1693-1699.

531. Ubukata K: Problems associated with high prevalence of multidrug-resistant bacteria in patients with community-acquired infections. *J Infect Chemother* 2003, 9: 285-291.

532. Ubukata K, Chiba N, Morozumi M, Iwata S, Sunakawa K: Longitudinal surveillance of *Haemophilus influenzae* isolates from pediatric patients with meningitis throughout Japan, 2000-2011. *J Infect Chemother* 2013, 19: 34-41.

533. Uemura Y, Qin L, Gotoh K, Ohta K, Nakamura K, Watanabe H: Comparison study of single and concurrent administrations of carbapenem, new quinolone, and macrolide against in vitro nontypeable *Haemophilus influenzae* mature biofilms. *J Infect Chemother* 2013, 19: 902-908.

534. Vachon V, Lyew DJ, Coulton JW: Transmembrane permeability channels across the outer membrane of *Haemophilus influenzae* type b. *J Bacteriol* 1985, 162: 918-924.

535. Valleron A-J, Cori A, Valtat S, Meurisse S, Carrat F, Boelle PY: Transmissibility and geographic spread of the 1889 influenza pandemic. *Proceedings of the National Academy of Sciences* 2010, 107: 8778-8781.

536. van den Bergh MR, Spijkerman J, Swinnen KM, Francois NA, Pascal TG, Borys D *et al*.: Effects of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine on nasopharyngeal bacterial colonization in young children: a randomized controlled trial. *Clin Infect Dis* 2013, 56: e30-e39.

537. Van Eldere J, Slack MP, Ladhani S, Cripps AW: Non-typeable *Haemophilus influenzae*, an under-recognised pathogen. *Lancet Infect Dis* 2014, 14: 1281-1292.

538. van Heijenoort J: Lipid Intermediates in the Biosynthesis of Bacterial Peptidoglycan. *Microbiology and Molecular Biology Reviews* 2007, 71: 620-635.

539. van Ketel RJ, de Wever B, van Alphen L: Detection of *Haemophilus influenzae* in cerebrospinal fluids by polymerase chain reaction DNA amplification. *Journal of Medical Microbiology* 1990, 33: 271-276.

540. van Schilfgaarde M, Eijk P, Regelink A, van Ulsen P, Everts V, Dankert J *et al*.: *Haemophilus influenzae*localized in epithelial cell layers is shielded from antibiotics and antibody-mediated bactericidal activity. *Microbial Pathogenesis* 1999, 26: 249-262.

541. Vega R, Sadoff HL, Patterson MJ: Mechanisms of ampicillin resistance in *Haemophilus influenzae* type B. *Antimicrob Agents Chemother* 1976, 9: 164-168.

542. Vicente M, Gomez MJ, Ayala JA: Regulation of transcription of cell division genes in the Escherichia coli dcw cluster. *CMLS, Cell Mol Life Sci* 1998, 54: 317-324.

543. Vik E, Rusten L, Falk ES, Melby K: [Multiresistant *Haemophilus influenzae*. Beta-lactamase producing, chloramphenicol and tetracycline resistant *Haemophilus influenzae* as a cause of septicemia]. *Tidsskr Nor Laegeforen* 1986, 106: 1119-1120.

544. Vollmer W, Joris B, Charlier P, Foster S: Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev* 2008, 32: 259-286.

545. Wallace RJ, Jr., Baker CJ, Quinones FJ, Hollis DG, Weaver RE, Wiss K: Nontypable *Haemophilus influenzae* (biotype 4) as a neonatal, maternal, and genital pathogen. *Rev Infect Dis* 1983, 5: 123-136.

546. Walsh C: *Antibiotics: Actions, Origins, Resistance*. ASM Press; 2003.

547. Wang WB, Jiang T, Gardner S: Detection of homologous recombination events in bacterial genomes. *PLoS ONE* 2013, 8: e75230.

548. Wang X, Mair R, Hatcher C, Theodore MJ, Edmond K, Wu HM *et al*.: Detection of bacterial pathogens in Mongolia meningitis surveillance with a new real-time PCR assay to detect *Haemophilus influenzae*. *Int J Med Microbiol* 2011, 301: 303-309.

549. Watanabe H, Hoshino K, Sugita R, Asoh N, Watanabe K, Oishi K *et al*.: Possible high rate of transmission of nontypeable *Haemophilus influenzae*, including beta-lactamase-negative ampicillin-resistant strains, between children and their parents. *J Clin Microbiol* 2004, 42: 362-365.

550. Watson ME, Jr., Burns JL, Smith AL: Hypermutable *Haemophilus influenzae* with mutations in mutS are found in cystic fibrosis sputum. *Microbiology* 2004, 150: 2947-2958.

551. Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI *et al*.: Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *International Journal of Systematic Bacteriology* 1987, 37: 463-464.

552. Webster P, Wu S, Gomez G, Apicella M, Plaut AG, Geme III JWS: Distribution of Bacterial Proteins in Biofilms Formed by Non-typeable *Haemophilus influenzae*. *Journal of Histochemistry & Cytochemistry* 2006, 54: 829-842.

553. Weeks JE: The bacillus of acute conjunctival catarrh, or 'pink eye'. *Arch Ophthalmol* 1886, 114: 1510-1511.

554. Weiss DS, Chen JC, Ghigo JM, Boyd D, Beckwith J: Localization of FtsI (PBP3) to the Septal Ring Requires Its Membrane Anchor, the Z Ring, FtsA, FtsQ, and FtsL. *Journal of Bacteriology* 1999, 181: 508-520.

555. Westman E, Lundin S, Hermansson A, Melhus A: Beta-lactamase-producing nontypeable *Haemophilus influenzae* fails to protect Streptococcus pneumoniae from amoxicillin during experimental acute otitis media. *Antimicrob Agents Chemother* 2004, 48: 3536-3542.

556. Wilcox KW, Smith HO: Isolation and characterization of mutants of *Haemophilus influenzae* deficient in an adenosine 5'-triphosphate-dependent deoxyribonuclease activity. *J Bacteriol* 1975, 122: 443-453.

557. Williams BJ, Golomb M, Phillips T, Brownlee J, Olson MV, Smith AL: Bacteriophage HP2 of *Haemophilus influenzae*. *J Bacteriol* 2002, 184: 6893-6905.

558. Williams JD, Andrews J: Sensitivity of *Haemophilus influenzae* to antibiotics. *Br Med J* 1974, 1: 134-137.

559. Williams JD, Moosdeen F: Antibiotic resistance in *Haemophilus influenzae*: epidemiology, mechanisms, and therapeutic possibilities. *Rev Infect Dis* 1986, 8 Suppl 5: S555-S561.

560. Winslow CE, Broadhurst J, Buchanan RE, Krumwiede C, Rogers LA, Smith GH: The Families and Genera of the Bacteria: Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types. *J Bacteriol* 1917, 2: 505-566.

561. Winter LE, Barenkamp SJ: Antibodies to the HMW1/HMW2 and Hia adhesins of nontypeable *Haemophilus influenzae* mediate broad-based opsonophagocytic killing of homologous and heterologous strains. *Clin Vaccine Immunol* 2014, 21: 613-621.

562. Witherden EA, Kunde D, Tristram SG: An evaluation of SNP-based PCR methods for the detection of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *J Infect Chemother* 2012, 18: 451-455.

563. Witherden EA, Kunde D, Tristram SG: PCR screening for the N526K substitution in isolates of *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother* 2013, 68: 2255-2258.

564. Witherden EA, Tristram SG: Prevalence and mechanisms of beta-lactam resistance in *Haemophilus haemolyticus*. *J Antimicrob Chemother* 2013, 68: 1049-1053.

565. Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A: Role of inter-species recombination of the *ftsI* gene in the dissemination of altered penicillin-binding-protein-3-mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. *Journal of Antimicrobial Chemotherapy* 2014, 69: 1501-1509.

566. Witherden EA, Montgomery J, Henderson B, Tristram SG: Prevalence and genotypic characteristics of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* in Australia. *Journal of Antimicrobial Chemotherapy* 2011, 66: 1013-1015.

567. Woolfrey BF, Enright MA: Ampicillin killing curve patterns for ampicillin-susceptible nontypeable *Haemophilus influenzae* strains by the agar dilution plate count method. *Antimicrob Agents Chemother* 1990, 34: 1079-1087.

568. Woolfrey BF, Gresser-Burns ME, Lally RT: Ampicillin killing curve patterns of *Haemophilus influenzae* type b isolates by agar dilution plate count method. *Antimicrob Agents Chemother* 1987, 31: 1711-1717.

569. World Health Organization (WHO). Disease surveillance and burden. http://www.who.int/immunization/monitoring_surveillance/burden/en. 2014. WHO.

570. World Health Organization (WHO). Global immunization data. July 2014. www.who.int/immunization/monitoring_surveillance/global_immunization_data.pdf?ua=1. 2014. WHO.

571. World Health Organization (WHO). Global Antimicrobial Resistance Surveillance System (GLASS). Technical Meeting on the Early Implementation Phase. http://apps.who.int/iris/bitstream/10665/204117/1/WHO_OHE_PED_AMR_2016.1_eng.pdf. 2016.

572. Wu S, Li X, Gunawardana M, Maguire K, Guerrero-Given D, Schaudinn C *et al*.: Beta- lactam antibiotics stimulate biofilm formation in non-typeable *Haemophilus influenzae* by up-regulating carbohydrate metabolism. *PLoS ONE* 2014, 9: e99204.

573. Yang CJ, Chen TC, Wang CS, Wang CY, Liao LF, Chen YH *et al*.: Nosocomial outbreak of biotype I, multidrug-resistant, serologically non-typeable *Haemophilus influenzae* in a respiratory care ward in Taiwan. *J Hosp Infect* 2010, 74: 406-409.

574. Yeo SF, Akalin E, Arikan S, Auckenthaler R, Bergan T, Dornbusch K *et al*.: Susceptibility testing of *Haemophilus influenzae*--an international collaborative study in quality assessment. *J Antimicrob Chemother* 1996, 38: 363-386.

575. Yow MD: Ampicillin in the treatment of meningitis dueto *Hemophilus influenzae*: An appraisal after 6 years of experience. *The Journal of Pediatrics* 1969, 74: 848-852.

576. Zachariah J: A randomized, comparative study to evaluate the efficacy and tolerability of a 3-day course of azithromycin versus a 10-day course of co-amoxiclav as treatment of adult patients with lower respiratory tract infections. *J Antimicrob Chemother* 1996, 37 Suppl C: 103-113.

577. Zapun A, Contreras-Martel C, Vernet T: Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiology Reviews* 2008, 32: 361-385.

578. Zerva L, Biedenbach DJ, Jones RN: Reevaluation of interpretive criteria for *Haemophilus influenzae* by using meropenem (10-microgram), imipenem (10-microgram), and ampicillin (2- and 10-microgram) disks. *J Clin Microbiol* 1996, 34: 1970-1974.

579. Zhang B, Kunde D, Tristram S: *Haemophilus haemolyticus* is infrequently misidentified as *Haemophilus influenzae* in diagnostic specimens in Australia. *Diagn Microbiol Infect Dis* 2014, 80: 272-273.

580. Zhu B, Xiao D, Zhang H, Zhang Y, Gao Y, Xu L *et al*.: MALDI-TOF MS distinctly differentiates nontypable *Haemophilus influenzae* from *Haemophilus haemolyticus*. *PLoS ONE* 2013, 8: e56139.

581. Zinnemann K: The Ups and Downs of the Influenza Bacillus. *The University of Leeds Review* 1973, 16: 126-145.

582. Zwahlen A, Kroll JS, Rubin LG, Moxon ER: The molecular basis of pathogenicity in *Haemophilus influenzae*: comparative virulence of genetically-related capsular transformants and correlation with changes at the capsulation locus cap. *Microb Pathog* 1989, 7: 225-235.

583. Zwahlen A, Winkelstein JA, Moxon ER: Participation of complement in host defense against capsule-deficient *Haemophilus influenzae*. *Infect Immun* 1983, 42: 708-715.

APPENDIX: PAPERS AND POSTERS

Paper I

Clin Microbiol Infect, 2010

ORIGINAL ARTICLE BACTERIOLOGY

Mutant *ftsI* genes in the emergence of penicillin-binding protein-mediated β-lactam resistance in *Haemophilus influenzae* in Norway

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Abstract

The most important mechanism for β-lactam resistance in β-lactamase-negative ampicillin-resistant (BLNAR) isolates of *Haemophilus influenzae* is the alteration of penicillin-binding protein 3 (PBP3) as a result of *ftsI* gene mutations. The present study aimed to map PBP3 alterations and to determine the correlation to β-lactam resistance in respiratory tract isolates of *H. influenzae* in Norway, as well as assess the contribution of clonal spread to the emergence of PBP3-mediated resistance. Twenty-three β-lactamase negative respiratory tract isolates with resistance to penicillins and 23 susceptible control isolates were examined by determination of β-lactam MICs, *ftsI* sequencing and molecular typing by pulsed-field gel electrophoresis (PFGE). Ampicillin MIC ranges in the resistant group and the control group were 1–2 mg/L and 0.125–0.5 mg/L, respectively. All isolates in the resistant group had the PBP3 substitution Asn526 → Lys and were thus categorized as group II low-BLNAR. No control isolate met the genetic BLNAR (gBLNAR) criteria. The PBP3 substitution patterns corresponded well to those observed in previous European studies. Eighty-three percent (19/23) of the resistant isolates belonged to two clones, demonstrating the capability of low-BLNAR strains of clonal dissemination. Combined analysis of *ftsI* DNA sequences and PFGE patterns revealed distinctly different *ftsI* alleles in genetically indistinguishable isolates and identical copies of the same *ftsI* allele in unrelated isolates. A possible explanation of this observation is the recombinational exchange of *ftsI* alleles. This phenomenon, as well as the possibility of endemic European gBLNAR strains, should be further investigated.

Keywords: BLNAR, clonality, *H. influenzae*, PBP3, recombination
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10.1111/j.1469-0691.2009.03052.x

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Introduction

Resistance to aminopenicillins in *Haemophilus influenzae* is most commonly caused by plasmid-mediated β-lactamases, of which two types, TEM-1 [1] and ROB-1 [2], have been characterized. The most important β-lactam resistance mechanism in β-lactamase-negative ampicillin-resistant isolates (BLNAR) is alteration of penicillin-binding protein 3 (PBP3), leading to decreased affinity to penicillins and cephalosporins [3], as a result of *ftsI* gene mutations resulting in amino acid substitutions in the transpeptidase domain of PBP3 [4].

The originally phenotypic term BLNAR is also part of a genetically based classification system for *H. influenzae* isolates with PBP3-mediated β-lactam resistance. According to Ubukata *et al.* [4], isolates with the substitutions Arg517 → His or Asn526 → Lys near the Lys-Thr-Gly (KTG) motif are designated group I and group II BLNAR, respectively, whereas isolates possessing the substitutions Met377 → Ile, Ser385 → Thr and Leu389 → Phe near the Ser-Ser-Asn (SSN) motif in addition to Asn526 → Lys are designated group III BLNAR. A system for subgrouping of group II isolates according to amino acid substitution patterns was introduced by Dabernat *et al.* [5]. Simplifications of the classification system have been suggested by Hasegawa *et al.* [6], who defined isolates with only KTG-near substitutions as low-BLNAR and isolates with both KTG- and SSN-near substitutions as BLNAR, as well as by Hotomi *et al.* [7], who introduced the term genetic BLNAR (gBLNAR) as a common description for group I, II and III BLNAR isolates.

Genotyping by pulsed-field gel electrophoresis (PFGE) is useful for epidemiological typing of *H. influenzae* [8]. However, previous studies have indicated that gBLNAR isolates in

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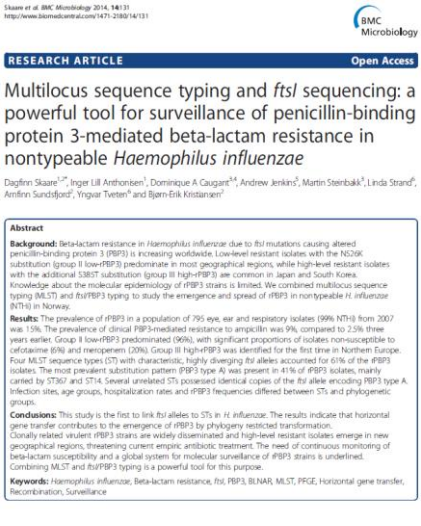
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.01630-15.

Paper II

BMC Microbiol, 2014



Skaare *et al. BMC Microbiology* 2014, **14**:131
http://www.biomedcentral.com/1471-2180/14/131

BMC Microbiology

RESEARCH ARTICLE Open Access

Multilocus sequence typing and *ftsI* sequencing: a powerful tool for surveillance of penicillin-binding protein 3-mediated beta-lactam resistance in nontypeable *Haemophilus influenzae*

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Abstract

Background: Beta-lactam resistance in *Haemophilus influenzae* due to *ftsI* mutations causing altered penicillin-binding protein 3 (PBP3) is increasing worldwide. Low-level resistant isolates with the N526K substitution (group II low-PBP3) predominate in most geographical regions, while high-level resistant isolates with the additional S385T substitution (group III high-rPBP3) are common in Japan and South Korea. Knowledge about the molecular epidemiology of rPBP3 strains is limited. We combined multilocus sequence typing (MLST) and *ftsI*/PBP3 typing to study the emergence and spread of rPBP3 in nontypeable *H. influenzae* (NTHi) in Norway.

Results: The prevalence of rPBP3 in a population of 795 eye, ear and respiratory isolates (99% NTHi) from 2007 was 15%. The prevalence of clinical PBP3-mediated resistance to ampicillin was 9%, compared to 2.5% three years earlier. Group II low-rPBP3 predominated (96%), with significant proportions of isolates non-susceptible to cefotaxime (6%) and meropenem (20%). Group III high-rPBP3 was identified for the first time in Northern Europe. Four MLST sequence types (ST) with characteristic, highly diverging *ftsI* alleles accounted for 61% of the rPBP3 isolates. The most prevalent substitution pattern (rPBP3 type A) was present in 41% of rPBP3 isolates, mainly carried by ST366 and ST14. Several unrelated STs possessed identical copies of the *ftsI* allele encoding PBP3 type A. Infection sites, age groups, hospitalization rates and rPBP3 frequencies differed between STs and phylogenetic groups.

Conclusions: This study is the first to link *ftsI* alleles to STs in *H. influenzae*. The results indicate that horizontal gene transfer contributes to the emergence of rPBP3 by phylogeny restricted transformation. Clonally related virulent rPBP3 strains are widely disseminated and high-level resistant isolates emerge in new geographical regions, threatening current empiric antibiotic treatment. The need of continuous monitoring of beta-lactam susceptibility and a global system for molecular surveillance of rPBP3 strains is underlined. Combining MLST and *ftsI*/rPBP3 typing is a powerful tool for this purpose.

Keywords: *Haemophilus influenzae*, Beta-lactam resistance, *ftsI*, PBP3, BLNAR, MLST, PFGE, Horizontal gene transfer, Recombination, Surveillance

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Paper III

Euro Surveill, 2014

SURVEILLANCE AND OUTBREAK REPORTS

Emergence of clonally related multidrug resistant *Haemophilus influenzae* with penicillin-binding protein 3-mediated resistance to extended-spectrum cephalosporins, Norway, 2006 to 2013

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Citation style for this article:
Skaare D, Anthonisen IL, Kahlmeter G, Matuschek E, Natås OB, Steinbakk M, Sundsfjord A, Kristiansen BE. Emergence of clonally related multidrug resistant *Haemophilus influenzae* with penicillin-binding protein 3-mediated resistance to extended-spectrum cephalosporins, Norway, 2006 to 2013. Euro Surveill. 2014;19(49):pii=20981. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20981

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Resistance to cephalosporins in *Haemophilus influenzae* is usually caused by characteristic alterations in penicillin-binding protein 3 (PBP3), encoded by the *ftsI* gene. Resistance to extended-spectrum cephalosporins is associated with high-level PBP3-mediated resistance (high-rPBP3), defined by the second stage S385T substitution in addition to a first stage substitution (R517H or N526K). The third stage L389F substitution is present in some high-rPBP3 strains. High-rPBP3 *H. influenzae* are considered rare outside Japan and Korea. In this study, 30 high-rPBP3 isolates from Norway, collected between 2006 and 2013, were examined by serotyping, multilocus sequence typing (MLST), *ftsI* sequencing, detection of beta-lactamase genes and minimum inhibitory concentration (MIC) determination. MICs were interpreted according to clinical breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Respiratory isolates predominated (proportion: 24/30). The 30 isolates included one serotype f isolate, while the remaining 29 lacked polysaccharide capsule genes. Resistance to extended-spectrum cephalosporins (cefixime, 29 isolates/30 isolates; cefepime, 28/30; cefotaxime, 26 /30; ceftaroline, 26/30; ceftriaxone, 14/30), beta-lactamase production (11/30) and co-resistance to non-beta-lactams (trimethoprim-sulfamethoxazole, 13/30; tetracycline, 4/30; chloramphenicol, 4/30; ciprofloxacin, 3/30) was frequent. The N526K substitution in PBP3 was present in 23 of 30 isolates; these included a blood isolate which represents the first invasive S385T+N526K isolate reported from Europe. The L389F substitution, present in 16 of 30 isolates, coincided with higher beta-lactam MICs. Non-susceptibility to meropenem was frequent in S385T+L389F+N526K isolates (8/12). All 11 beta-lactamase positive isolates were TEM-1. Five clonal groups of two to 10 isolates with identical MLST-*ftsI* allelic profiles were observed, including the first reported high-rPBP3 clone with TEM-1 beta-lactamase and co-resistance to ciprofloxacin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole. Prior to this study, no multidrug resistant high-rPBP3 *H. influenzae* had been reported in Norway. Intensified surveillance of antimicrobial resistance is needed to guide empiric therapy.

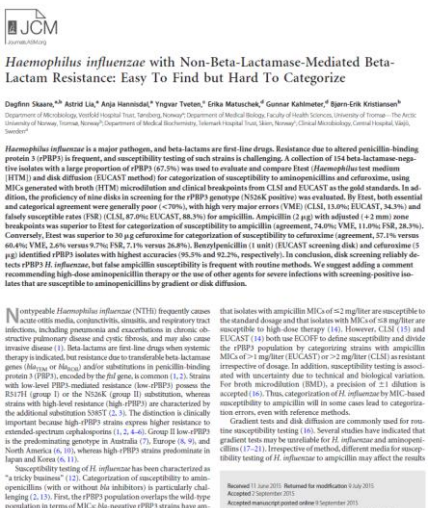
Introduction

Haemophilus influenzae colonises the respiratory tract in humans and infection causes a wide range of conditions, including acute otitis media, arthritis, conjunctivitis, epiglottitis, meningitis, respiratory tract infections, septicaemia and sinusitis [1]. Encapsulated isolates of serotype b (Hib) have the highest potential for invasive disease but the introduction of Hib vaccines has dramatically reduced the burden of disease. Thus, the vast majority of infections, both invasive and non-invasive, are currently caused by nontypeable (i.e. lacking polysaccharide capsule) *H. influenzae* (NTHi) and non-b serotypes, mainly serotype f (Hif) [1-3].

Due to the global emergence of beta-lactamase (TEM-1 and ROB-1) producing strains in the 1970s, empiric therapy for severe disease caused by *H. influenzae* was altered from ampicillin to cephalosporins [4]. Development of resistance to extended-spectrum cephalosporins has forced a second shift in empiric treatment to carbapenems in some geographical areas [4]. The European Committee on Antimicrobial

Paper IV

J Clin Microbiol, 2015



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***Haemophilus influenzae* with Non-Beta-Lactamase-Mediated Beta-Lactam Resistance: Easy To Find but Hard To Categorize**

Dagfinn Skaare,[a,b] Astrid Lia,[a] Anja Hannisdal,[a] Yngvar Tveten,[a] Erika Matuschek,[d] Gunnar Kahlmeter,[d] Bjørn-Erik Kristiansen[b]
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Haemophilus influenzae* is a major pathogen, and beta-lactams are first-line drugs. Resistance due to altered penicillin-binding protein 3 (rPBP3) is frequent, and susceptibility testing of such strains is challenging. A collection of 154 beta-lactamase-negative isolates with a large proportion of rPBP3 (67.5%) was used to evaluate and compare Etest (*Haemophilus* test medium [HTM]) and disk diffusion (EUCAST method) for categorization of susceptibility to aminopenicillins and cefuroxime, using MICs generated with broth (HTM) microdilution and clinical breakpoints from CLSI and EUCAST as the gold standards. In addition, the proficiency of nine disks in screening for the rPBP3 genotype (N526K positive) was evaluated. By Etest, both essential and categorical agreement were generally poor (<70%), with high very major errors (VME) (CLSI, 13.0%; EUCAST, 34.3%) and falsely susceptible rates (FSR) (CLSI, 87.0% EUCAST, 88.3%) for ampicillin. Ampicillin (2 μg) with adjusted (+2 mm) zone breakpoints was superior to Etest for categorization of susceptibility to ampicillin (agreement, 74.0%; VME, 11.0%; FSR, 28.3%). Conversely, Etest was superior to 30 μg cefuroxime for categorization of susceptibility to cefuroxime (agreement, 57.1% versus 60.4%; VME, 2.6% versus 9.7%; FSR, 7.1% versus 26.8%). Benzylpenicillin (1 unit) (EUCAST screening disk) and cefuroxime (5 μg) identified rPBP3 isolates with highest accuracies (95.5% and 92.2%, respectively). In conclusion, disk screening reliably detects rPBP3 *H. influenzae*, but false ampicillin susceptibility is frequent with routine methods. We suggest adding a commpicillin recommending high-dose aminopenicillin therapy or the use of other agents for severe infections with screening-positive isolates that are susceptible to aminopenicillins by gradient or disk diffusion.

Nontypeable *Haemophilus influenzae* (NTHi) frequently causes acute otitis media, conjunctivitis, sinusitis, and respiratory tract infections, including pneumonia and exacerbations in chronic obstructive pulmonary disease and cystic fibrosis, and may also cause invasive disease (1). Beta-lactams are first-line drugs when systemic therapy is indicated, but resistance due to transferable beta-lactamase genes (bla_{TEM} or bla_{ROB}) and/or substitutions in penicillin-binding protein 3 (PBP3), encoded by the *ftsI* gene, is common (1, 2). Strains with low-level PBP3-mediated resistance (low-rPBP3) possess the R517H (group I) or the N526K (group II) substitution, whereas strains with high-level resistance (high-rPBP3) are characterized by the additional substitution S385T (2, 3). The distinction is clinically important because high-rPBP3 strains express higher resistance to extended-spectrum cephalosporins (1, 2, 4–6). Group II low-rPBP3 is the predominating genotype in Australia (7), Europe (8, 9), and North America (6, 10), whereas high-rPBP3 strains predominate in Japan and Korea (6, 11).

Susceptibility testing of *H. influenzae* has been characterized as "a tricky business" (12). Categorization of susceptibility to aminopenicillins (with or without *bla* inhibitors) is particularly challenging (2, 13). First, the rPBP3 population overlaps the wild-type population in terms of MICs; *bla*-negative rPBP3 strains have ampicillin MICs of 0.5 to 16 mg/liter (1, 2, 13), whereas the epidemiological cutoff (ECOFF) value is ≤1 mg/liter (www.eucast.org/mic_distributions_ecoffs). Second, the clinical relevance of current breakpoints is debated (1, 2, 13). Ampicillin breakpoints from the Clinical and Laboratory Standards Institute (CLSI) were originally set to distinguish between *bla*-positive and *bla*-negative strains (13). Non-species-related (pharmacokinetic-pharmacodynamic [PK/PD]) breakpoints calculated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) suggest that isolates with ampicillin MICs of ≤2 mg/liter are susceptible to the standard dosage and that isolates with MICs of >8 mg/liter are susceptible to high-dose therapy (14). However, CLSI (15) and EUCAST (14) both use ECOFF to define susceptibility and divide the rPBP3 population by categorizing strains with ampicillin MICs of >1 mg/liter (EUCAST) or >2 mg/liter (CLSI) as resistant irrespective of dosage. In addition, susceptibility testing is associated with uncertainty due to technical and biological variation. For broth microdilution (BMD), a precision of ±1 dilution is accepted (16). Thus, categorization of *H. influenzae* by MIC-based susceptibility to ampicillin will in some cases lead to categorization errors, even with reference methods.

Gradient tests and disk diffusion are commonly used for routine susceptibility testing (16). Several studies have indicated that gradient tests may be unreliable for *H. influenzae* and aminopenicillins (17–21). Irrespective of method, different media for susceptibility testing of *H. influenzae* to ampicillin may affect the results

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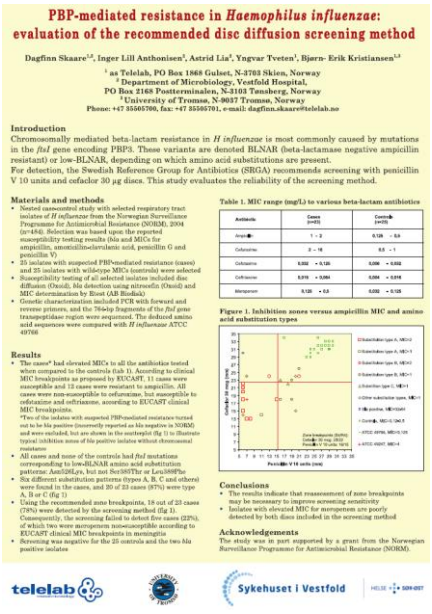
Poster

SSAC, 2007



PBP-mediated resistance in *Haemophilus influenzae*: evaluation of the recommended disc diffusion screening method

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Introduction

Chromosomally mediated beta-lactam resistance in *H influenzae* is most commonly caused by mutations in the *ftsI* gene encoding PBP3. These variants are denoted BLNAR (beta-lactamase negative ampicillin resistant) or low-BLNAR, depending on which amino acid substitutions are present.
For detection, the Swedish Reference Group for Antibiotics (SRGA) recommends screening with penicillin V 10 units and cefaclor 30 μg discs. This study evaluates the reliability of the screening method.

Materials and methods

- Nested case-control study with selected respiratory tract isolates of *H influenzae* from the Norwegian Surveillance Programme for Antimicrobial Resistance (NORM), 2004 (n=494). Selection was based upon the reported susceptibility testing results (bla and MICs for ampicillin, amoxicillin+clavulanic acid, penicillin G and penicillin V).
- 25 isolates with suspected PBP-mediated resistance (cases) and 25 isolates with wild-type MICs (controls) were selected
- Susceptibility testing of all selected isolates included disc diffusion (Oxoid), bla detection using nitrocefin (Oxoid) and MIC determination by Etest (AB Biodisk)
- Genetic characterization included PCR with forward and reverse primers, and the 784bp fragments of the *ftsI* gene transpeptidase region were sequenced. The deduced amino acid sequences were compared with *H influenzae* ATCC 49766

Table 1. MIC range (mg/L) to various beta-lactam antibiotics

Antibiotic	Cases (n=25)	Controls (n=25)
Ampicillin	1 → 2	0.125 → 0.5
Cefotaxime	2 → 16	0.5 → 1
Cefotaxime	0.032 → 0.125	0.008 → 0.032
Ceftriaxone	0.016 → 0.064	0.004 → 0.016
Meropenem	0.125 → 0.5	0.032 → 0.125

Results

- The cases[*] had elevated MICs to all the antibiotics tested when compared to the controls (tab 1). According to clinical MIC breakpoints as proposed by EUCAST, 13 cases were susceptible and 12 cases were resistant to ampicillin. All cases were non-susceptible to cefuroxime, but susceptible to cefotaxime and ceftriaxone, according to EUCAST clinical MIC breakpoints.
- [*]Two of the isolates with suspected PBP-mediated resistance turned out to be bla positive (incorrectly reported as bla negative in NORM) and were excluded, but are shown in the scatterplot (fig 1) to illustrate typical inhibition zones of bla positive isolates without chromosomal resistance
- All cases and none of the controls had *ftsI* mutations corresponding to low-BLNAR amino acid substitution patterns: Asn526Lys, but not Ser385Thr or Leu389Phe
- Six different substitution patterns (types A, B, C and others) were found in the cases, and 20 of 23 cases (87%) were type A, B or C (fig 1)
- Using the recommended zone breakpoints, 18 out of 23 cases (78%) were detected by the screening method (fig 1). Consequently, the screening failed to detect five cases (22%), of which two were meropenem non-susceptible according to EUCAST clinical MIC breakpoints in meningitis
- Screening was negative for the 25 controls and the two bla positive isolates

Figure 1. Inhibition zones versus ampicillin MIC and amino acid substitution types

Conclusions

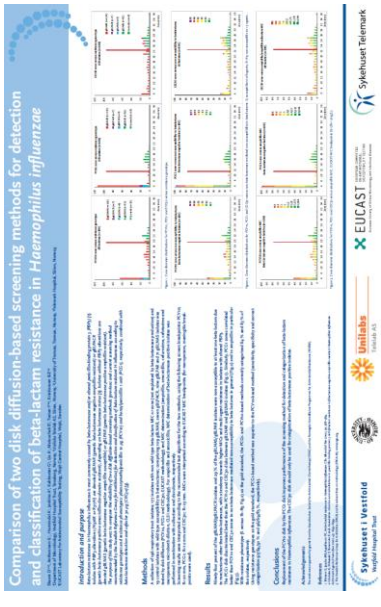
- The results indicate that reassessment of zone breakpoints may be necessary to improve screening sensitivity
- Isolates with elevated MIC for meropenem are poorly detected by both discs included in the screening method

Acknowledgements
The study was in part supported by a grant from the Norwegian Surveillance Programme for Antimicrobial Resistance (NORM).

telelab Sykehuset i Vestfold

Poster

ECCMID, 2011



Comparison of two disk diffusion-based screening methods for detection and classification of beta-lactam resistance in *Haemophilus influenzae*

Sykehuset Telemark
EUCAST
Unilabs
Sykehuset i Vestfold

PBP-mediated resistance in *Haemophilus influenzae*: evaluation of the recommended disc diffusion screening method

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Introduction

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For detection, the Swedish Reference Group for Antibiotics (SRGA) recommends screening with penicillin V 10 units and cefaclor 30 µg discs. This study evaluates the reliability of the screening method.

Materials and methods

- Nested case-control study with selected respiratory tract isolates of *H influenzae* from the Norwegian Surveillance Programme for Antimicrobial Resistance (NORM), 2004 (n=484). Selection was based upon the reported susceptibility testing results (*bla* and MICs for ampicillin, amoxicillin-clavulanic acid, penicillin G and penicillin V)
- 25 isolates with suspected PBP-mediated resistance (cases) and 25 isolates with wild-type MICs (controls) were selected
- Susceptibility testing of all selected isolates included disc diffusion (Oxoid), *bla* detection using nitrocefin (Oxoid) and MIC determination by Etest (AB Biodisk)
- Genetic characterization included PCR with forward and reverse primers, and the 764-bp fragments of the *ftsI* gene transpeptidase region were sequenced. The deduced amino acid sequences were compared with *H influenzae* ATCC 49766

Results

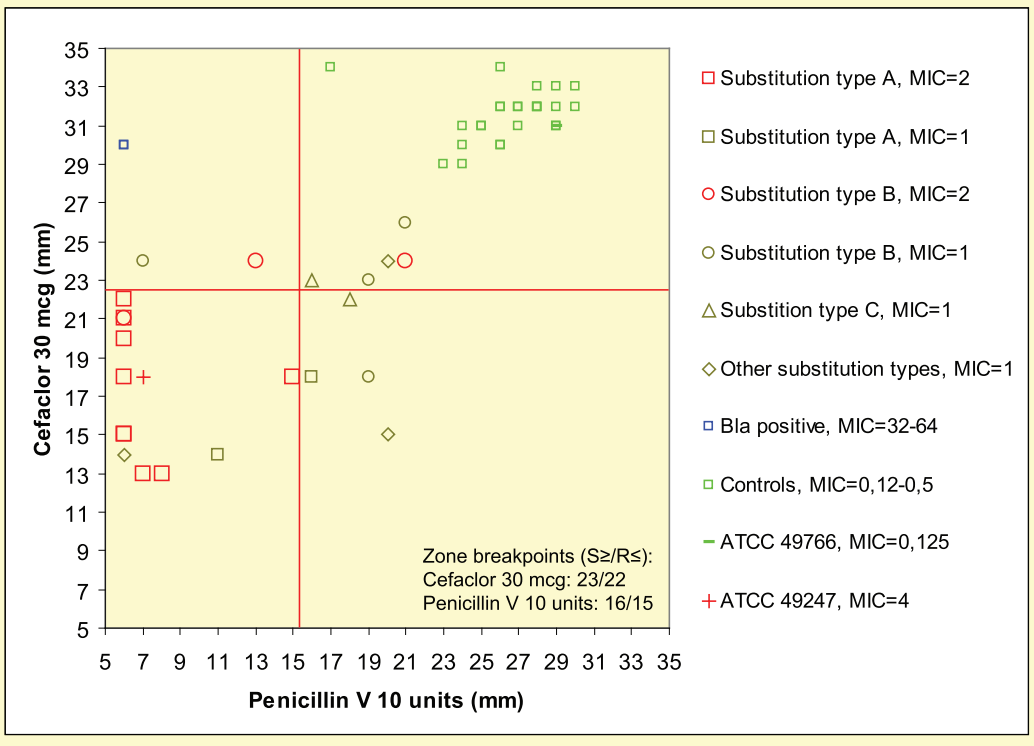
- The cases* had elevated MICs to all the antibiotics tested when compared to the controls (tab 1). According to clinical MIC breakpoints as proposed by EUCAST, 11 cases were susceptible and 12 cases were resistant to ampicillin. All cases were non-susceptible to cefuroxime, but susceptible to cefotaxime and ceftriaxone, according to EUCAST clinical MIC breakpoints.

  *Two of the isolates with suspected PBP-mediated resistance turned out to be *bla* positive (incorrectly reported as *bla* negative in NORM) and were excluded, but are shown in the scatterplot (fig 1) to illustrate typical inhibition zones of *bla* positive isolates without chromosomal resistance
- All cases and none of the controls had *ftsI* mutations corresponding to low-BLNAR amino acid substitution patterns: Asn526Lys, but not Ser385Thr or Leu389Phe
- Six different substitution patterns (types A, B, C and others) were found in the cases, and 20 of 23 cases (87%) were type A, B or C (fig 1)
- Using the recommended zone breakpoints, 18 out of 23 cases (78%) were detected by the screening method (fig 1). Consequently, the screening failed to detect five cases (22%), of which two were meropenem non-susceptible according to EUCAST clinical MIC breakpoints in meningitis
- Screening was negative for the 25 controls and the two *bla* positive isolates

Table 1. MIC range (mg/L) to various beta-lactam antibiotics

Antibiotic	Cases (n=23)	Controls (n=25)
Ampicillin	1 - 2	0,125 - 0,5
Cefuroxime	2 - 16	0,5 - 1
Cefotaxime	0,032 - 0,125	0,008 - 0,032
Ceftriaxone	0,016 - 0,064	0,004 - 0,016
Meropenem	0,125 - 0,5	0,032 - 0,125

Figure 1. Inhibition zones versus ampicillin MIC and amino acid substitution types



Conclusions

- The results indicate that reassessment of zone breakpoints may be necessary to improve screening sensitivity
- Isolates with elevated MIC for meropenem are poorly detected by both discs included in the screening method

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Comparison of two disk diffusion-based screening methods for detection and classification of beta-lactam resistance in *Haemophilus influenzae*

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Introduction and purpose

Beta-lactam resistance in Haemophilus influenzae *is most commonly caused by beta-lactamase and/or altered penicillin-binding protein 3 (PBP3) [1]. Isolates with PBP3 alterations (N526K or R517H) are denoted gBLNAR (genetic beta-lactamase negative ampicillin resistant) or gBLPACR (genetic beta-lactamase positive amoxicillin-clavulanate resistant), depending on beta-lactamase status [2]. Isolates without PBP3 alterations are denoted gBLNAS (genetic beta-lactamase negative ampicillin susceptible) or gBLPAR (genetic beta-lactamase positive ampicillin resistant).*
The purpose of this study was to compare the reliability of two disk diffusion-based screening methods (previous and current screening method recommended by the Swedish Reference Group for Antibiotics) for detection and classification of beta-lactam resistant H influenzae *according to resistance genotypes and resistance phenotypes: phenoxymethylpenicillin 10 µg (PCV10) and benzylpenicillin 1 unit (PCG1), respectively, combined with beta-lactamase detection and cefaclor 30 µg (CEC30) [3].*

Methods

A collection of 196 respiratory tract isolates; 177 isolates with non-wild type beta-lactam MIC or zone (not explained by beta-lactamase production) and 19 control isolates with wild type susceptibility to beta-lactams; comprising 109 gBLNAR, seven gBLPACR, nine gBLPAR and 71 gBLNAS isolates was tested by disk diffusion (PCV10, PCG1 and CEC30; EUCAST methodology) and MIC determination (ampicillin, amoxicillin, cefuroxime, cefotaxime and meropenem; microbroth dilution, CLSI methodology). For ampicillin and amoxicillin, MIC determination of beta-lactamase positive isolates was performed in combination with sulbactam and clavulanate, respectively.
Screening results were interpreted according to the recommended test algorithms for the two methods, using the following screen breakpoints: PCV10; R<20 mm, PCG1; R<12 mm and CEC30; R<23 mm. MICs were interpreted according to EUCAST MIC breakpoints (for meropenem, meningitis break-points were used).

Results

Ninety-four percent of the gBLNAR/gBLPACR isolates and 29 % of the gBLNAS/gBLPAR isolates were non-susceptible to at least one beta-lactam due to mechanisms other than beta-lactamase, with a tendency towards higher MICs and multi-agent resistance in isolates with altered PBP3.
The PCG1 disk discriminated better than the PCV10 and CEC30 disks between gBLNAR and gBLNAS isolates (Fig.1). Similarly, PCG1 zones correlated better than PCV10 and CEC30 zones to non-beta-lactamase mediated non-susceptibility to beta-lactams in general (Fig.2) and to ampicillin in particular (Fig.3).
With resistance phenotype (S versus R1-R5; Fig.2) as the gold standard, the PCG1- and PCV10-based methods correctly categorized 84 % and 83 % of the isolates, respectively.
Using resistance genotypes as the gold standard, the PCG1-based method was superior to the PCV10-based method (sensitivity, specificity and correct categorization 97/84/91 % and 96/69/85 %, respectively).

Conclusions

Replacement of the PCV10 disk by the PCG1 disk improves performance of the screening method for detection and categorization of beta-lactam resistance in *Haemophilus influenzae*. The CEC30 disk should only be used for categorization of beta-lactamase positive isolates.

Acknowledgements

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References

1. Tristram S, Jacobs MR, Appelbaum PC. Antimicrobial resistance in *Haemophilus influenzae*. Clin Microbiol Rev 2007; 20: 368–389.
2. Ubukata K, Shibasaki Y, Yamamoto K et al. Association of amino acid substitutions in penicillin-binding protein 3 with β-lactam resistance in β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob Agents Chemother 2001; 45: 1693-1699.
3. The Swedish Reference Group for Antibiotics (SRGA) and its subcomittee on methodology (SRGA-M). www.srga.org

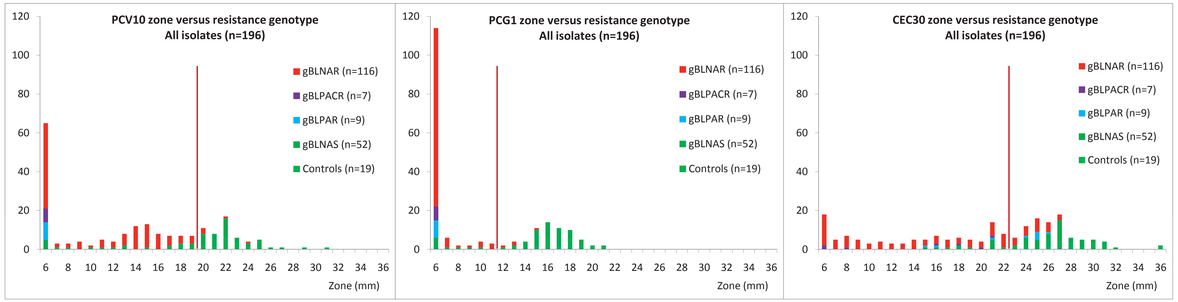

Figure 1. Zone diameter distributions for PCV10, PCG1 and CEC30 versus resistance genotype.

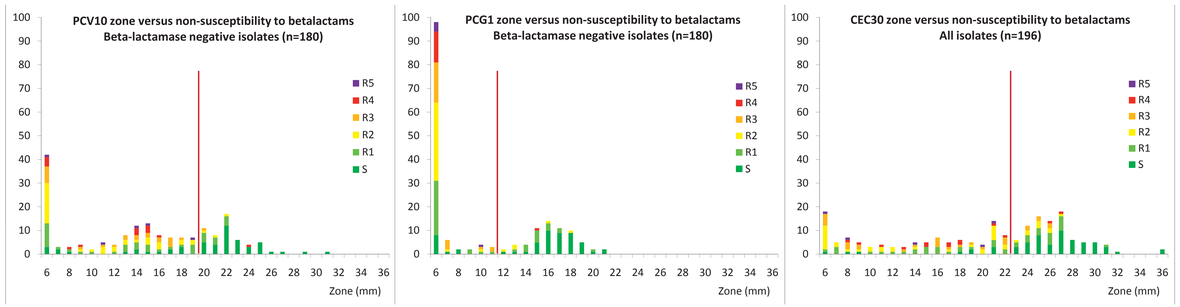

Figure 2. Zone diameter distributions for PCV10, PCG1 and CEC30 versus non-beta-lactamase mediated non-susceptibility to beta-lactams. S; susceptible to all agents, R1-R5; non-susceptible to 1-5 agents.

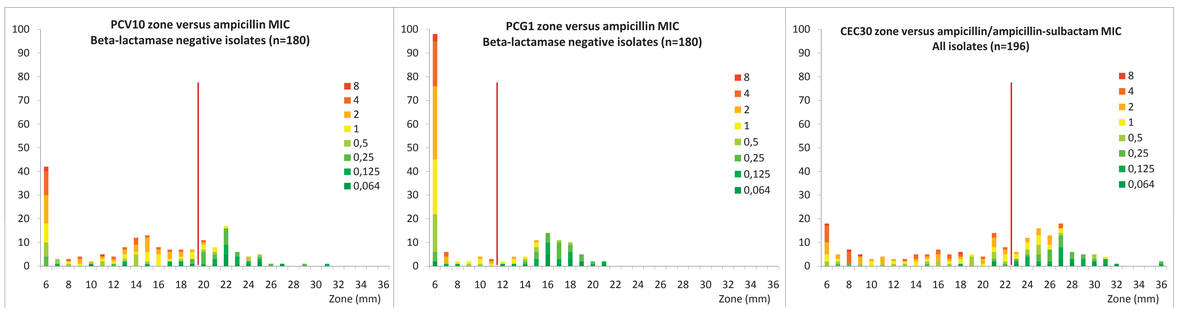

Figure 3. Zone diameter distributions for PCV10, PCG1 and CEC30 versus ampicillin MIC. EUCAST MIC breakpoints S≤1/R>1 (mg/L).

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