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Resman, Fredrik; Ristovski, Mikael; Forsgren, Arne; Kaijser, Bertil; Kronvall, Goran; Medstrand, Patrik; Melander, Eva; Odenholt, Inga; Riesbeck, Kristian

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PO Box 117
221 00 Lund
+46 46-222 00 00

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Increase of ß-lactam resistant invasive *Haemophilus influenzae* in Sweden 1997-2010

Fredrik Resman[1,2], Mikael Ristovski[1], Arne Forsgren[1], Bertil Kaijser[3] , Göran Kronvall[4], Patrik Medstrand[1], Eva Melander[5], Inga Odenholt[2], and Kristian Riesbeck[1#]

Medical Microbiology, Dept. of Laboratory Medicine Malmö, Lund University, Sweden[1], Infectious Diseases Unit, Dept. of Clinical sciences, Malmö, Lund University, Sweden[2] , Institute of Laboratory Medicine, Dept. of Clinical Bacteriology, Gothenburg University, Gothenburg, Sweden[3], Clinical Microbiology – MTC, Karolinska Institute, Karolinska Hospital, Stockholm, Sweden[4], Infection Control, Laboratory Medicine, Skåne County[5]

RUNNING TITLE: ANTIMICROBIAL RESISTANCE OF INVASIVE *HAEMOPHILUS INFLUENZAE*

#Corresponding author: Dr Kristian Riesbeck, Medical Microbiology, Dept. of Laboratory Medicine Malmö, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden. Phone 46-40338494. Fax:46-40336234. E-mail: kristian.riesbeck@med.lu.se

ABSTRACT

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

Keywords: betalactamase, BLNAR, *Haemophilus influenzae*, invasive *Haemophilus* disease, sepsis

INTRODUCTION

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

Resistance to ampicillin in *H. influenzae* was first described in 1974 (17). Ampicillin is in Sweden, as in many other countries, the main drug of choice in proven *H. influenzae* infections and the primary empiric choice in respiratory tract infections,

where *H. influenzae* can be suspected. Ampicillin resistance in *H. influenzae* is now globally widespread with incidences varying from 8-30% in different European countries and North America to more than 50% in some east Asian countries (12,13) The nomenclature of resistant *H. influenzae* is complex, and since definitions vary between different studies and regions, the definitions used by us are outlined in Table 1. Isolates with resistance to ampicillin can be sorted into two main categories; those that carry a β-lactamase, and those that do not. The most common mechanism of β-lactam resistance in *H. influenzae* is by TEM-1 or ROB-1 β-lactamases (7), and such isolates are denoted "β-lactamase positive ampicillin-resistant" (BLPAR). The commonly used term "β-lactamase negative ampicillin resistant" (BLNAR) is used for isolates with ampicillin resistance with no evidence of β-lactamase production. After this definition was established, it was concluded that ampicillin resistance in such isolates was due to key mutations in the *ftsI* gene (encoding for penicillin-binding protein [PBP] 3) that lower the affinity for β-lactams (36). Subsequently, it became clear that some isolates had such mutations, but were not ampicillin resistant according to phenotype testing. Isolates with key mutations in PBP-3 regardless of resistance pheontype are designated as "genomic" BLNAR (gBLNAR), a group of isolates that overlaps, but does not match the BLNAR group (34,36).

Clinical isolates that are susceptible to ampicillin, but resistant to other β-lactams are consequently not included in the BLNAR definition. However, other β-lactam antibiotics than ampicillin are often used empirically in infections where *H. influenzae* can be the pathogen. Due to this, resistance of *H. influenzae* to other β-lactam antibiotics than ampicillin needs to be considered. Since many years, the screening method for identification of β-lactam resistant *H. influenzae* in Sweden has been disc

diffusion tesing for penicillin and cefaclor/loracarbef followed by a nitrocefin resistanct β-lactamase test. Even though penicillin rarely is an alternative for treatment of *H. influenzae* infections, experience suggests that this method is suitable for resistance surveillance, allowing for sensitive monitoring of β-lactam resistance. In this study we refer to the β-lactamase negative isolates with resistance (according to disc diffusion test screening) to any tested β-lactam antibiotic as "β-lactamase negative β-lactam resistant" (BLNBR). This term includes the BLNAR isolates as a subset. Finally, isolates with both a β-lactamase and chromosomally derived resistance are defined as "β-lactamase-positive amoxicillin-clavulanate resistant" (BLPACR).

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

MATERIALS AND METHODS

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

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

of correct size was not obtained, isolates were subjected to 16SrRNA sequencing. Since not a single isolate of *H. haemolyticus* was identified, the procedure was discontinued in 2010.

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

since the TEM-1 or ROB-1 β-lactamases of *H. influenzae* do not confer resistance to cephalosporins, BLPACR isolates were defined as β-lactamase-positive isolates with resistance to a tested cephalosporin. β-lactam resistant isolates were thereby defined as BLPAR, BLNBR or BLPACR based on results from nitrocephine testing and cefaclor (30 μg)/ loracarbef (10 μg) tests, respectively. E-tests for ampicillin (Biodisk, Solna, Sweden) were performed on all available β-lactam resistant isolates.

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

PBP-3 sequencing. All available isolates that were either defined as BLNBR ("β-lactamase negative β-lactam resistant") or BLPACR ("β-lactamase positive amoxicillin-clavulanate resistant") using the method described above were subjected to an *ftsI*-PCR, amplifying the transmembranous part of PBP-3 using primers 5'–CCTTTCGTTGTTTTAACCGCA–3' and 5'–AGCTGCTTCAGCATCTTG–3' (annealing temperature 52°C), resulting in a product size of 770 bp. All products were sent for sequencing, analysed for amino acid substitutions and compared to the wildtype *H. influenzae* RdKW20 PBP-3 using CLC-DNA Workbench (CLC bio, Aarhus, Denmark).

Multi Locus Sequence Typing (MLST). All available BLNBR and BLPACR isolates were sequence typed using PCR primers and conditions according to the *H. influenzae* protocol described on the MLST webpage (http://haemophilus.mlst.net/). Sequences were trimmed manually, concatenated and aligned using ClustalX (19). A best-fitting nucleotide substitution model was estimated using the Akaike Information Criterion corrected for small sample sizes (AICc) as implemented injModeltest 0.1.0 (25). A neighbor-joining (NJ) tree was constructed in PAUP* v4.0b10 (33) using a the AICc model (HKY+I+G). Support for internal branches was obtained by 1000 bootstrap replicates in PAUP*. The resulting phylogenetic tree was visualized using FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/software/figtree).

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

Statistical analyses. To test the significance of the increase in proprtions of *H. influenzae* β-lactam resistance mechanisms, trend tests using yearly proportions of

each type of resistance as a dependent variable in linear regression analyses were initially performed. These analyses gave significances of the increase and confidence intervals. We had a priori knowledge that the dataset was skewed towards the end of the study period, and considering the fact that the dependant variable was binomial, logistic regressions were also performed on the three datasets. After plotting the three datasets, the assumption of a linear relation of data used in both the linear and logistic regressions could not be assumed for the BLNBR dataset nor the dataset with all ampicillin resistant isolates. The curve fit of these two datasets suggested that a quadratic polynomial regression should be used. For the BLNBR dataset, a cubic equation (a third degree polynomial equation) fit the data almost equally well. For these two datasets, quadratic logistic regressions were performed with centered squared years and centered years used as covariates. Years, and not exact dates were used as timepoints, since we know that there is a seasonal variation in *H. influenzae* disease. The most conservative estimate of significance was used. The data was analysed using PASW Statistics 20.0.

RESULTS

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

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

isolates, since only eight encapsulated strains displayed this characteristic during the study period.

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

The gene variant *bla*(TEM-1P[del]) dominates among BLPAR isolates. All identified ß-lactamase positive isolates (BLPAR or BLPACR) (Table 1) that were available for further analysis (*n*=33) were resistant to ampicillin (the MIC for ampicillin ranged from 4 to 256 mg/L). The corresponding β-lactamase gene was defined by PCR in 30 out of 33 isolates, and we found that *bla*(TEM-1) dominated (*n*=29). Only one isolate carrying the *bla*(ROB-1) gene was found. The gene product encoding for TEM-1 was detected in two variants, resulting in different DNA products using the same primer pair (Fig. 2A). After sequencing, it was clear that the larger product (600 base pairs; bp) represented the wild-type *bla*(TEM-1) gene, whereas the smaller product represented a *bla*(TEM-1) with a 135bp deletion in the promotor region. This corresponded to the *bla*(TEM-1P[del]) gene priorly described in Spain by Molina and colleagues (20). In our clinical collection, the variant *bla*(TEM-1P[del]) dominated during the study period (18 had the *bla*(TEM-1P[del])

gene, whereas 11 isolates carried the wild-type *bla*(TEM-1)). The median MIC for ampicillin, however, was the same for the two identified *bla*(TEM) gene variants. Finally, we found three β-lactamase positive (as revealed by nitrocephine testing) ampicillin-resistant *H. influenzae* isolates from 2009 and 2010 that were negative for both *bla*(TEM) and *bla*(ROB) genes using the described primers.

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

A marked increase of β-lactamase-negative ß-lactam resistant isolates was seen from 2007 and onwards (Fig. 2B), with consistent yearly proportions above 10%. In the

years 1997 and 1998, the proportion of BLNBR isolates was relatively high, but from a very limited number of isolates. This makes the data from these years less reliable and more difficult to interpret. For comparison, the definitions were also adjusted to the definition used in the national surveillance programme described earlier, which only includes isolates resistant to both penicillin and cefaclor/loracarbef in the BLNBR group. From 2007 and onwards, we observed consistently higher proportions of β-lactamase negative β-lactam resistant invasive isolates than the proportions seen in the national surveillance data of nasopharyngeal isolates, where numbers never reached 5%.

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

DISCUSSION

This study identifies an increase in proportions of β-lactam resistance among invasive *H. influenzae* isolates in Sweden during the years 1997-2010. The proportions of β-lactam resistant isolates reached 30% in the final years of the study period. The observed increase was not mainly due to an increase of β-lactamase producing isolates, but among these a *bla*(TEM-1) variant with a promotor deletion dominated (*i.e., bla*(TEM-1P[del])). The increase was mainly due to a recent rise in β-lactamase negative β-lactam resistant (BLNBR) isolates. Since such isolates have a potential for resistance to multiple antibiotics (34), the observation is of concern. Not all of the BLNBR isolates displayed true BLNAR phenotypes, but most isolates were resistant to multiple β-lactam antibiotics. Our study also confirms a strong, but not perfect correlation between BLNAR-defining amino acid substitutions and the ampicillin resistance phenotype established in earlier studies (2,9,31,36). However, it is evident that other mechanisms than PBP-3 mutations or β-lactamase production contribute to β-lactam resistance in *H. influenzae*. A few such mechanisms, including disrupted repression of the acrR efflux pump, have been suggested (15).

Since the study outline is retrospective, our study has limitations. Not all isolates were available for detailed study, and since the absolute numbers of *H. influenzae* were limited, the statistical calculations as well as the indications from the MLST analysis should be interpreted with caution. Furthermore, the reliability of the disk diffusion method for defining precise levels of betalactam-resistance in *H. influenzae* has been questioned. However, as a primary screening method for resistance in clinical isolates, followed by a detailed examination, the disk diffusion method was considered

suitable. Previous reports that have studied clonal relations of resistant *H. influenzae* have used PFGE (9,32), and PFGE is a common method for studying clonal relations in local outbreaks with a limited geographical distribution. Even though all methods have limitations, we believe that MLST is advantageous with its benefits of a high resolution power and the possibility of international comparisons.

Acquisition of antimicrobial resistance is often thought to imply a fitness cost and thereby theoretically reduce bacterial fitness and virulence. However, evidence points to that antimicrobial resistance in Gram-negative bacteria can be linked to a higher degree of virulence (27), possibly due to co-carriage of resistance and virulence genes. The explanation for the increase of the proportion of resistant invasive *H. influenzae* isolates is likely to be multifactorial. Selection pressure from liberal use of antibiotics on upper airway infections can be a contributing factor, and there is support for this mechanism in earlier reports (8). Moreover, a contribution of the spread of dominant clones of *H. influenzae* with antimicrobial resistance should be considered. Such patterns have been suggested in earlier studies (12,16). The MLST results from the present study of invasive isolates suggests a spread of one BLNAR clone with close genetic relation, but the absolute number of isolates was too small to fully conclude this as a fact. Two observations strengthening this indication is that the cluster comprised of isolates from all three geographical areas of the study, and all of the isolates of this cluster had identical PBP-3 sequences. Among the BLPAR isolates, the reason for the spread and domination of the *bla*(TEM-1 P[del]) variant needs further investigation.

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

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

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

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FIGURE LEGENDS

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

FIG. 2. Two variants of *bla*(TEM-1), and a steep increase of β-lactamase negative invasive isolates with a cluster of BLNAR isolates were identified. A) The agarose gel shows an example of a *bla*(TEM-1) PCR result from four different invasive NTHi strains with β-lactamase production.. The lanes are from the left to the right; molecular weight standard, negative control, the clinical NTHi isolates KR553, KR225, KR655 and KR656. Sequencing revealed that the products of KR553 and KR655 are *bla*(TEM-1) wild-type, whereas the products of KR225 and KR656 are representative of the *bla*(TEM-1 P[del]). B) A recent increase of NTHi isolates with a

β-lactamase resistant phenotype. The absolute numbers of invasive β-lactamase negative β-lactam resistant (BLNBR) isolates in 1997-2010, sorted by resistance phenotype, are shown. The black bars show BLNAR isolates, the white bars show isolates resistant to penicillin and a cephalosporine. The striped grey bars show isolates resistant to penicillin only, while the checked bars show isolates resistant to only a cephalosporin or a carbapenem. C) A neighbor-joining phylogenetic tree was constructed based on concatenated MLST-sequences from all available invasive BLNBR isolates. The BLNAR isolates are indicated in red colour. Isolates with penicillin and cephalosporin (PcV/ceph) resistance are indicated in blue colour, while isolates with sole penicillin (PcV only) resistance are shown in black text. The prefix letter of the isolate name indicates the laboratory where the isolate was isolated; G=Gothenburg, S=Stockholm, M=Malmö, or L=Lund. Clusters of >70% bootstrap support are indicated with their bootstrap values, and one cluster of seven gBLNAR/BLNAR isolates, including isolates from all three geographical areas of the study, is indicated by an asteriks.

TABLE 1. Study definitions of the different types of β-lactam resistant invasive *H. influenzae*. The BLNAR and gBLNAR groups have substiantial overlap, and are both subsets of the BLNBR group.

Abbreviation	Name	Study definition	*n*
BLPAR	β-lactamase positive ampicillin resistant[1]	Resistance to penicillin according to disk diffusion testing using SRGA[2] breakpoints for the study period. Nitrocephine positive.	45
BLNAR	β-lactamase negative ampicillin resistant	MIC for ampicillin ≥ 2mg/L Nitrocephine negative.	11[3]
gBLNAR	genomic β-lactamase negative ampicillin resistant	The following substitutions in PBP-3[4]. Genotype I: Arg517His. Genotype II: Asn526Lys Genotype III: Met377Ile, Ser385Thr, Leu389Phe and Asn 526Lys. Nitrocephine negative.	16[3]
BLNBR	β-lactamase negative β-lactam resistant	Resistance to one or more tested ß-lactam antibiotic (penicillin, ampicillin, cephalosporin or a carbapenem) according to SRGA breakpoints. Nitrocephine negative.	43
BLPACR	β-lactamase positive amoxicillin-clavulanate resistant	Resistance to ampicillin or penicillin and a tested cephalosporin using SRGA breakpoints. Nitrocephine positive.	3

[1] All studied isolates that were nitrocephine positive had MIC for ampicillin ≥ 2 mg/L.

[2] Swedish Reference Group for Antibiotics.

[3]Numbers are out of 465 tested isolates, but since only a portion of isolates were available for E-test and sequencing, the number of BLNAR and gBLNAR are defined from fewer isolates, and are not comparable to the other numbers.

[4] Penicillin binding protein 3.

TABLE 2. Amino acid substitutions in PBP-3 of 36 invasive β-lactamase negative β-lactam resistant (BLNBR) *H. influenzae* isolates. All gBLNAR variants are highlighted in grey.

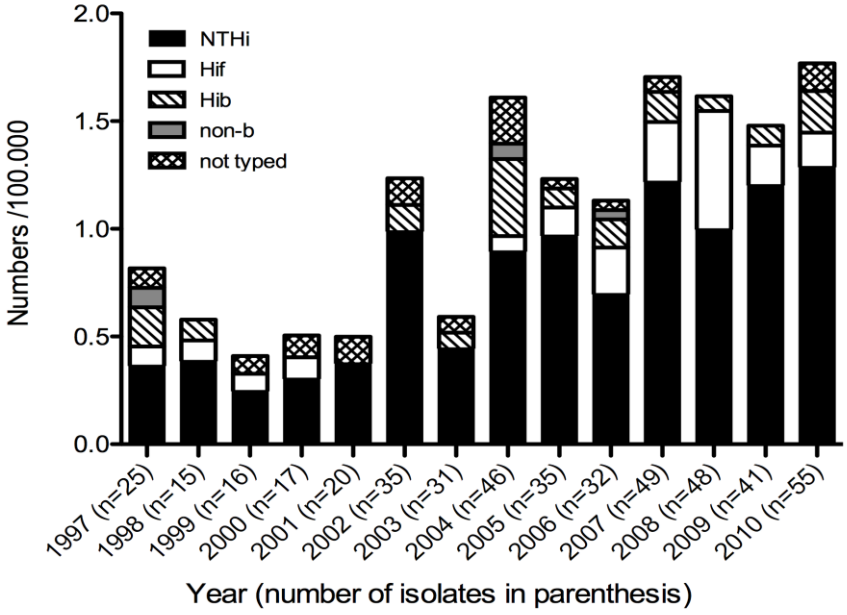
BLNAR genotype	*n*	Amino acid substitutions															Ampicillin MIC[1] (range; mg/L)
		Asp 350	Ala 368	Asp 373	Met 377	Ala 395	Ala 437	Ile 449	Ile 475	Gly 490	Ala 502	Arg 517	Asn 526	Ala 530	Val 547	Asn 569	
I	1											His					1
IIb	8	Asn			Ile						Val		Lys		Ile	Ser	0.5-4
IIb	2	Asn			Ile					Glu	Val		Lys		Ile	Ser	2
IIb	1			Asn		Gly					Val		Lys		Ile	Ser	2
IIb	1				Ile						Val		Lys		Ile		8
IId	2							Val					Lys		Ile	Ser	2
II	1	Asn								Glu			Lys	Ser			0,5
-[2]	3	Asn													Ile	Ser	0.25-0,5
-	2	Asn					Ser								Ile	Ser	0.5
-	2			Asn													0.5
-	1								Leu								1
-	2		Thr														0.25-0,5
-	3														Ile		0.25
-	7	No substitution[3]															0.25-256

[1] MIC (Minimal Inhibitory Concentration) was determined by E-test.

[2] "-" indicates that the isolate is not a gBLNAR.

[3] Two strains produced β-lactamases (BLPACR), hence the broad MIC range.

A


2.0
1.5
1.0
0.5
0.0
Numbers /100.000
NTHi
Hif
Hib
non-b
not typed
1997 (n=25)
1998 (n=15)
1999 (n=16)
2000 (n=17)
2001 (n=20)
2002 (n=35)
2003 (n=31)
2004 (n=46)
2005 (n=35)
2006 (n=32)
2007 (n=49)
2008 (n=48)
2009 (n=41)
2010 (n=55)
Year (number of isolates in parenthesis)

B

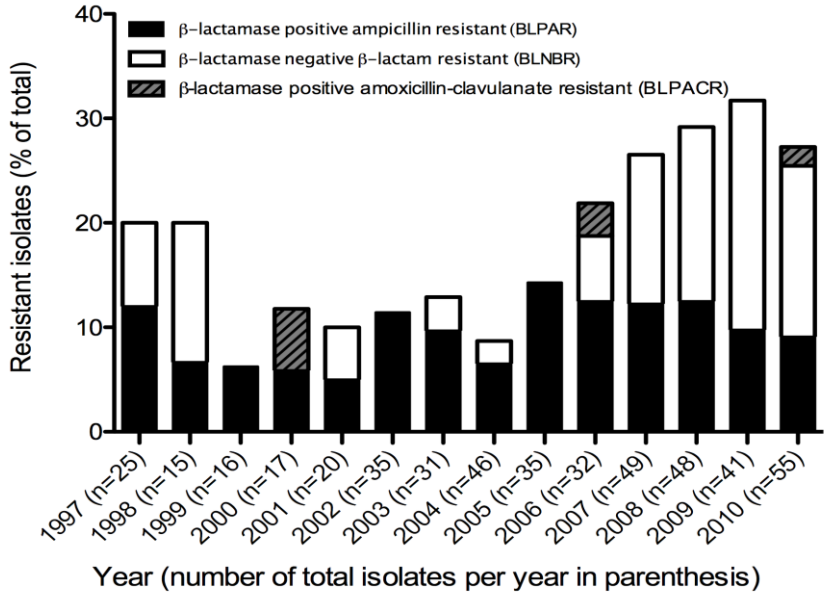
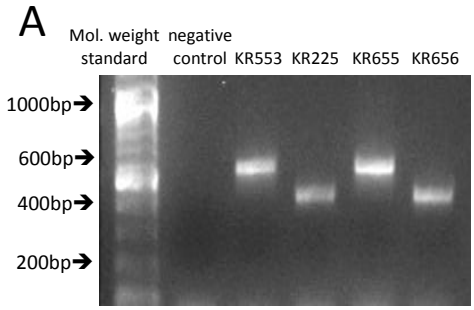
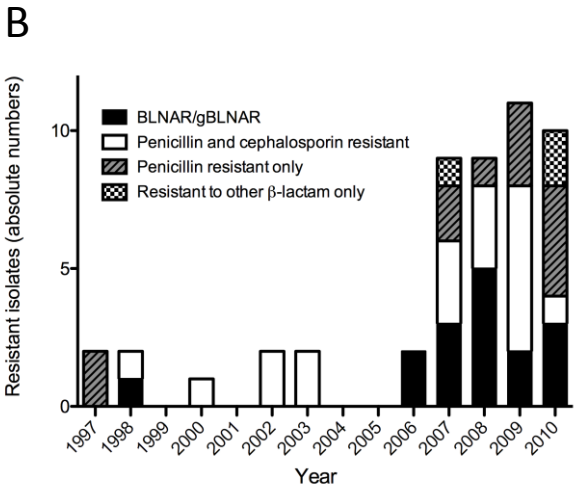
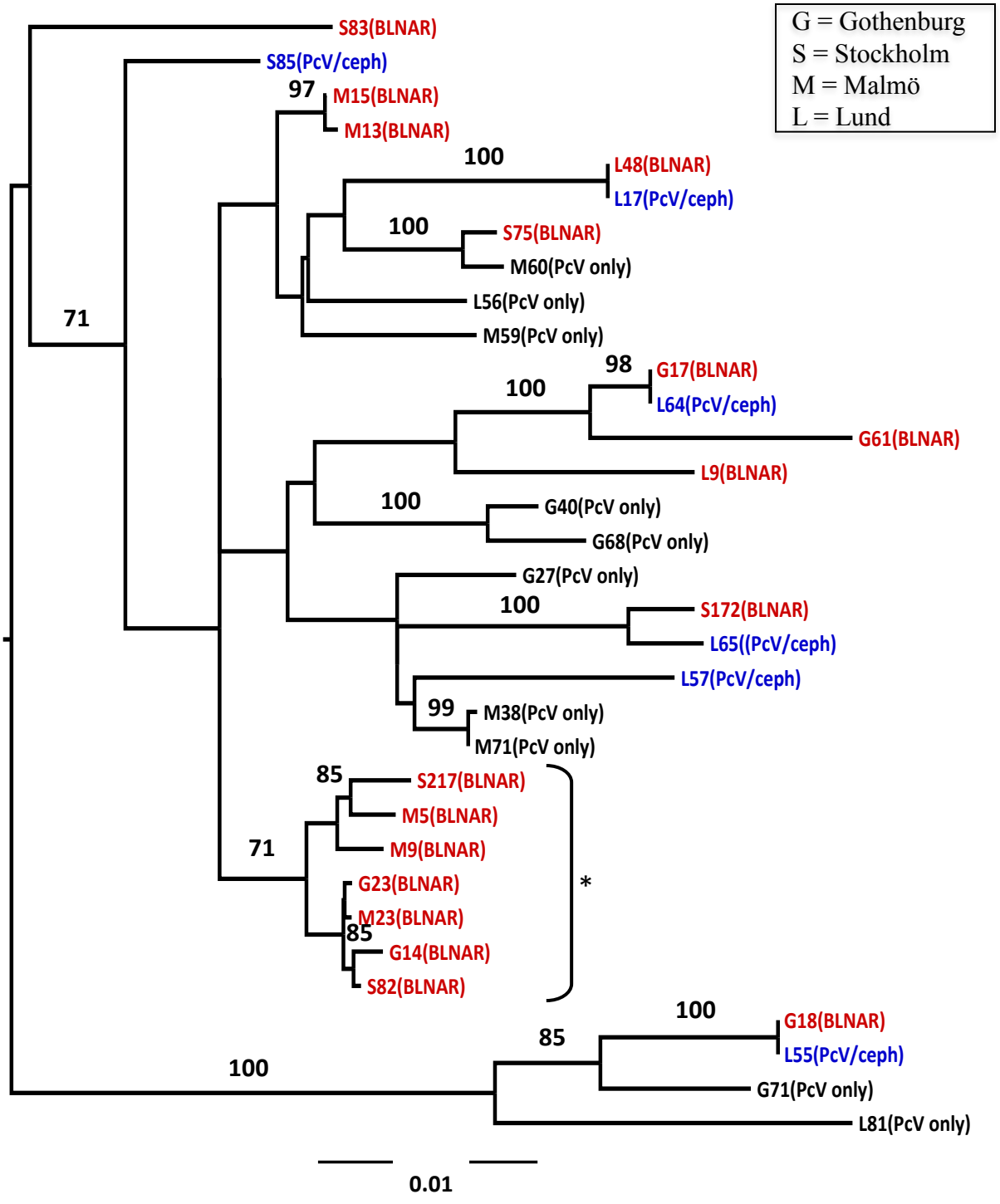

40
30
20
10
0
Resistant isolates (% of total)
β-lactamase positive ampicillin resistant (BLPAR)
β-lactamase negative β-lactam resistant (BLNBR)
β-lactamase positive amoxicillin-clavulanate resistant (BLPACR)
1997 (n=25)
1998 (n=15)
1999 (n=16)
2000 (n=17)
2001 (n=20)
2002 (n=35)
2003 (n=31)
2004 (n=46)
2005 (n=35)
2006 (n=32)
2007 (n=49)
2008 (n=48)
2009 (n=41)
2010 (n=55)
Year (number of total isolates per year in parenthesis)

FIG. 2A and B. Resman *et al.*





C


G = Gothenburg
S = Stockholm
M = Malmö
L = Lund
S83(BLNAR)
S85(PcV/ceph)
97
M15(BLNAR)
M13(BLNAR)
100
L48(BLNAR)
L17(PcV/ceph)
100
S75(BLNAR)
M60(PcV only)
L56(PcV only)
71
M59(PcV only)
98
G17(BLNAR)
100
L64(PcV/ceph)
G61(BLNAR)
L9(BLNAR)
100
G40(PcV only)
G68(PcV only)
G27(PcV only)
100
S172(BLNAR)
L65((PcV/ceph)
L57(PcV/ceph)
99
M38(PcV only)
M71(PcV only)
85
S217(BLNAR)
M5(BLNAR)
71
M9(BLNAR)
G23(BLNAR)
M23(BLNAR)
85
G14(BLNAR)
S82(BLNAR)
*
100
G18(BLNAR)
85
L55(PcV/ceph)
100
G71(PcV only)
L81(PcV only)
0.01