Isolates of β-lactamase-negative ampicillin-resistant Haemophilus influenzae causing invasive infections in Spain remain susceptible to cefotaxime and imipenem

Silvia García-Cobos1, Margarita Arroyo1, María Pérez-Vázquez1, Belén Aracil1, Noelia Lara1, Jesús Oteo1, Emilia Cercenado2,3 and José Campos1,4*

1Antibiotic Laboratory, Servicio de Bacteriología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; 2Servicio de Microbiología, Hospital General Universitario Gregorio Marañón, Madrid, Spain; 3Department of Medicine, Universidad Complutense, Madrid, Spain; 4Consejo Superior de Investigaciones Científicas, Madrid, Spain

*Corresponding author. Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera Pozuelo a Majadahonda, 28220 Majadahonda, Madrid, Spain. Tel: +34-918223650; Fax: +34-915097966; E-mail: jcampos@isciii.es

Received 19 June 2013; returned 3 July 2013; revised 17 July 2013; accepted 18 July 2013

Objectives: The epidemiology of invasive Haemophilus influenzae has changed in recent years. β-Lactamase-negative ampicillin-resistant (BLNAR) invasive isolates have recently been described in Europe but their clinical significance is unclear. Our main goal was to determine whether invasive H. influenzae remains susceptible to β-lactam antibiotics indicated in the treatment of invasive infections.

Methods: The antibiotic susceptibility of 307 invasive H. influenzae isolates to seven β-lactam antibiotics was determined by microdilution and interpreted by EUCAST and CLSI breakpoints. We also identified the bla genes, the amino acid substitutions in the transpeptidase domain of penicillin-binding protein 3 (PBP3), the molecular epidemiology of invasive BLNAR isolates by PFGE and MLST, and the time–kill curves of two isolates with PBP3 mutations conferring reduced susceptibility to aminopenicillins and cephalosporins.

Results: Of the invasive isolates, 86.6% were non-typeable and 62% were isolated from adults. Decreased susceptibility to β-lactams was due to the BLNAR genotype (gBLNAR; 19.2%) and to β-lactamase production (16.9%). Susceptibility rates to amoxicillin/clavulanic acid, cefotaxime, cefixime and imipenem were greater than 98%. Of 18 gBLNAR non-typeable isolates studied by MLST, 15 different STs were obtained. Amoxicillin and cefotaxime were bactericidal after 2 and 4 h of incubation, respectively.

Conclusions: Invasive H. influenzae disease was mainly due to non-typeable isolates infecting adults, and the most common mechanism of β-lactam resistance was mutations in the transpeptidase domain of PBP3. The gBLNAR non-typeable isolates were genetically diverse. The majority of invasive H. influenzae remained susceptible to third-generation cephalosporins; amoxicillin and cefotaxime were bactericidal in two gBLNAR isolates.

Keywords: PBP3, bacteraemia, β-lactams

Introduction

In the past, Haemophilus influenzae type b (Hib) was a major cause of invasive infections, especially in children <5 years of age;1–3 however, after widespread vaccination against Hib, non-typeable H. influenzae has become predominant among invasive H. influenzae.4–6

Invasive infections due to H. influenzae are usually treated with β-lactam antibiotics including aminopenicillins or cephalosporins. Aminopenicillin resistance in H. influenzae is well documented and may be mediated by β-lactamase production or by alterations in the transpeptidase domain of penicillin-binding proteins (PBPs). Interestingly, β-lactamase-negative ampicillin-resistant (BLNAR) isolates with PBP3 modifications can show decreased susceptibility to both aminopenicillins and cephalosporins.7–9 Some authors10,11 have recently described invasive infections caused by BLNAR H. influenzae in Europe.

The main objective of this study was to determine whether invasive isolates of H. influenzae in Spain remain susceptible to the β-lactam antibiotics indicated in the treatment of invasive infections, such as amoxicillin/clavulanic acid and third-generation cephalosporins. The specific objectives of the study were: (i) to determine the susceptibility of invasive H. influenzae to β-lactam antibiotics according to EUCAST and CLSI (formerly NCCLS) criteria;
(ii) to describe the molecular mechanisms and molecular epidemiology of aminopenicillin-resistant invasive *H. influenzae*, with a special emphasis on BLNR isolates; and (iii) to determine the *in vitro* bactericidal activity of amoxicillin and cefotaxime for isolates of the BLNR genotype (gBLNAR) with decreased susceptibility to aminopenicillins and cephalosporins.

**Materials and methods**

**Study isolates**

The Centro Nacional de Microbiología is a public health and research institution that runs an active national surveillance programme for invasive Haemophilus influenzae infections in Spain. We studied 307 clinical isolates of *H. influenzae* causing individual invasive infections (265 from blood and 42 from CSF) submitted to the Spanish Haemophilus reference laboratory from 70 different hospitals in 30 Spanish geographical regions from 1 January 2000 to 31 October 2009. A total of 112 isolates were from adults (59.6%) and 51 from children (16.6%); for 73 (23.8%) isolates, the age of the patient was unknown. Of the 307 isolates, 67 (21.8%) were sub-

**Susceptibility testing**

Antibiotic susceptibility was determined by the broth microdilution method on *Haemophilus* test medium using microtitre plates (Sensititre Enmizo 14, Trek Diagnostics Inc. Westlake, OH, USA) according to the CLSI guidelines. **11** Susceptibility data were interpreted by CLSI and EUCAST clinical breakpoints. **13,14** The following antibiotics were studied: ampicillin, amoxicillin, amoxicillin/clavulanic acid (2:1 ratio), cefuroxime, cefotaxime, cefixime and imipenem.

**β-Lactamase production** was determined by the chromogenic cephalosporin test with nitrocefin as substrate. **16** *H. influenzae* ATCC 49247 and ATCC 49766 were used as quality control strains as recommended. **13**

**Genotype definition**

Invasive isolates were classified into different genotypes as follows: gBLNAS, β-lactamase-negative isolates that were ampicillin susceptible and lacked detectable mechanisms of resistance to β-lactams; gBLNAR, β-lactamase-negative isolates with PBP3 amino acid substitutions causing reduced susceptibility to ampicillin (defined by either an Asn526Lys or an Arg517His substitution); gBLPAR, reduced susceptibility to ampicillin (defined by either an Asn526Lys or an Ser517His substitution); gBLPACR and gBLNAR, β-lactamase-positive isolates that were ampicillin resistant without gBLNAR-defining PBP3 amino acid substitutions; and gBLPLACR, β-lactamase-positive isolates also presenting PBP3 amino acid substitutions. **17** gBLNAR isolates were classified into different PBP3 mutation patterns as previously described. **7,13**

**PCR and DNA sequencing**

Amplification of the PBP3 transpeptidase domain of the *ftsI* gene from amino acid positions 327 to 540 was carried out as previously described **9** in a total of 162 (52.8%) isolates selected as described below.

**β-Lactamase-negative isolates**

Of the 255 invasive isolates that were β-lactamase negative, *ftsI* was sequenced in 110 (43.1%), comprising 47 with an amoxicillin MIC ≤0.5 mg/L (possible susceptible control group or gBLNAS) **9** and all 63 isolates with an amoxicillin MIC ≥1 mg/L (a group that according to our previous data was suggestive of being gBLNAR). **8**

**β-Lactamase-positive isolates**

*ftsI* was sequenced in all 52 β-lactamase positive isolates; 42 had an amoxicillin/clavulanic acid MIC ≤2 mg/L, suggesting that they were gBLPAR, **8** and 10 had an amoxicillin/clavulanic acid MIC ≥2 mg/L, suggesting that they were gBLPACR. **8**

The presence of *blaTEM*, its promoter region and the *blaOBB* gene was studied in all β-lactamase-positive isolates, as previously described. **7,9**

**Molecular epidemiology**

To elucidate whether invasive gBLNAR isolates from different geographical regions were epidemiologically linked, we analysed them by PFGE and multilocus sequence typing (MLST). PFGE was carried out on 95 selected isolates as previously described. **7,18** 40 of these were capsulated (29 fully susceptible to aminopenicillins, 7 gBLPAR, 2 gBLPACR and 2 gBLNAS) and 55 were non-typeable and gBLNAR. A genetic similarity dendrogram was obtained according to the Dice correlation coefficient with a tolerance level of 1% (InfoQuest FP software version 4.5; Bio-Rad, USA).

MLST was carried out on a selected sample of 41 isolates (23 capsulated and 18 non-typeable and gBLNAR) representing specific PFGE profiles; internal fragments of the seven housekeeping genes were sequenced using published methods. **19,20** The sequences obtained were submitted to the MLST website **21** for assignment of allele numbers and sequence type (ST).

**Time–kill curves**

Time–kill curve experiments were undertaken using two gBLNAR isolates of the III-like group, **18** one isolated in 2006 and the other in 2009, from distant geographical locations. The III-like group is characterized by Met377Leu and Ser385Thr mutations in the SSN motif **19** (Table S1, available as Supplementary data at JAC Online) and presents reduced susceptibility to aminopenicillins and cefotaxime. **13** The in vitro bactericidal activities were determined according to CLSI-recommended methods. **21**

Time–kill experiments were performed at concentrations 4× the MICs of amoxicillin and cefotaxime for the study isolates. Each time–kill experiment was carried out in triplicate in separate experiments. A decrease of 3 log_{10} cfu/mL in bacterial counts in antimicrobial solution compared with counts for the growth control was considered bactericidal. **22**

**Statistical analysis**

Differences in the prevalence of mechanisms of resistance and antibiotic susceptibility data were assessed by the χ² test. A P value of 0.05 was considered statistically significant.

**Results and discussion**

**Clinical isolates and antibiotic susceptibility**

**Characteristics of the invasive isolates**

Of the 307 invasive isolates, 266 (86.6%) were non-typeable (62% adults, 15.4% children and 22.6% unknown) and 41 (13.4%) were capsulated (17 [6.6%] type f, 15 [5.5%] type b, 6 [2%] type e and 3 [1%] type a). Of the 41 capsulated isolates, there were 21 from adults (nine type b, four type e and eight type f), 10 from children (five type b, three type f and two type a) and 10 from patients of
unknown age. Of the 42 CSF isolates, 37 were non-typeable and 5 were capsulated (three type f, one type b and one type a).

Antibiotic susceptibility

Of the 307 invasive isolates studied, 52 (16.9%) produced b-lactamase (16.4% of isolates from the GMUH and 17% from the remaining participant hospitals; \( P = 1 \)).

The total rate of ampicillin resistance was 19.2% using EUCAST breakpoints and 16.9% using those of the CLSI (Table 1). The rates of resistance to amoxicillin/clavulanic acid were 1.3% (EUCAST) and 0% (CLSI); by the EUCAST criteria, two of nine gBLPACR isolates (22.2%) were resistant to amoxicillin/clavulanic acid. In addition, 99.3% (EUCAST) and 100% (CLSI) of the isolates were susceptible to cefotaxime and cefixime, respectively (Table 1). All isolates were susceptible to imipenem by EUCAST and CLSI breakpoints (Table 1).

The most important discrepancies detected between the EUCAST and CLSI criteria were for cefuroxime; by current CLSI criteria, 99.7% of the invasive isolates were susceptible to cefuroxime and one gBLNAR isolate was intermediate. EUCAST has two different breakpoints for cefuroxime, intravenous and oral; according to

Table 1. Antibiotic susceptibility of 307 invasive Haemophilus influenzae isolates in relation to resistance genotypes according to EUCAST and CLSI clinical breakpoints14,15

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Genotype</th>
<th>MIC (mg/L)</th>
<th>EUCAST criteria (% isolates)</th>
<th>CLSI criteria (% isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>90%</td>
<td>range</td>
</tr>
<tr>
<td>AMP Total</td>
<td></td>
<td>≤0.25</td>
<td>128</td>
<td>0.25 to ≥256</td>
</tr>
<tr>
<td>gBLPAR</td>
<td></td>
<td>≥256</td>
<td>8 to ≥256</td>
<td>54 (91.5)</td>
</tr>
<tr>
<td>gBlnAR</td>
<td></td>
<td>≥256</td>
<td>8 to ≥256</td>
<td>254 (82.7)</td>
</tr>
<tr>
<td>gBLPACR</td>
<td></td>
<td>≥256</td>
<td>8 to ≥256</td>
<td>58 (98.3)</td>
</tr>
<tr>
<td>AMX Total</td>
<td></td>
<td>≤0.5</td>
<td>128</td>
<td>0.25 to ≥128</td>
</tr>
<tr>
<td>gBLPAR</td>
<td></td>
<td>≥128</td>
<td>8 to ≥128</td>
<td>42 (97.7)</td>
</tr>
<tr>
<td>gBlnAR</td>
<td></td>
<td>≥128</td>
<td>8 to ≥128</td>
<td>58 (98.3)</td>
</tr>
<tr>
<td>gBLPACR</td>
<td></td>
<td>≥128</td>
<td>8 to ≥128</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td>AMC Total</td>
<td></td>
<td>0.5</td>
<td>1</td>
<td>0.25 to 4</td>
</tr>
<tr>
<td>gBLPAR</td>
<td></td>
<td>1</td>
<td>0.5–4</td>
<td>42 (97.7)</td>
</tr>
<tr>
<td>gBlnAR</td>
<td></td>
<td>1</td>
<td>0.5–4</td>
<td>58 (98.3)</td>
</tr>
<tr>
<td>gBLPACR</td>
<td></td>
<td>2</td>
<td>1–4</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td>CTX Total</td>
<td></td>
<td>≤0.03</td>
<td>0.6</td>
<td>≤0.03–0.25</td>
</tr>
<tr>
<td>gBLPAR</td>
<td></td>
<td>≤0.03</td>
<td>≤0.03</td>
<td>43 (100)</td>
</tr>
<tr>
<td>gBlnAR</td>
<td></td>
<td>0.06</td>
<td>≤0.03–0.25</td>
<td>57 (96.6)</td>
</tr>
<tr>
<td>gBLPACR</td>
<td></td>
<td>0.06</td>
<td>≤0.03–0.25</td>
<td>9 (100)</td>
</tr>
<tr>
<td>CFM Total</td>
<td></td>
<td>≤0.03</td>
<td>0.6</td>
<td>≤0.03–0.5</td>
</tr>
<tr>
<td>gBLPAR</td>
<td></td>
<td>≤0.03</td>
<td>≤0.03–0.5</td>
<td>43 (100)</td>
</tr>
<tr>
<td>gBlnAR</td>
<td></td>
<td>0.06</td>
<td>≤0.03–0.5</td>
<td>57 (96.6)</td>
</tr>
<tr>
<td>gBLPACR</td>
<td></td>
<td>0.06</td>
<td>≤0.03–0.5</td>
<td>9 (100)</td>
</tr>
<tr>
<td>IPM Total</td>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>≤0.25–2</td>
</tr>
<tr>
<td>gBLPAR</td>
<td></td>
<td>≤0.25</td>
<td>0.5</td>
<td>≤0.25–2</td>
</tr>
<tr>
<td>gBlnAR</td>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>≤0.25–2</td>
</tr>
<tr>
<td>gBLPACR</td>
<td></td>
<td>0.5</td>
<td>1</td>
<td>0.5–1</td>
</tr>
</tbody>
</table>

S, susceptible; R, resistant; I, intermediate; AMP, ampicillin; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CXM iv, cefuroxime intravenous; CTX, cefotaxime; CFM, cefixime; IPM, imipenem.

\( gBLPAR (n = 43), \) b-lactamase-positive isolates that are ampicillin resistant without gBLNAR-defining PBP3 amino acid substitutions; gBLNAR \( (n = 59), \) b-lactamase-negative isolates with PBP3 amino acid substitutions causing reduced susceptibility to ampicillin; gBLPACR \( (n = 9), \) b-lactamase-positive isolates also presenting PBP3 amino acid substitutions.17

For cefuroxime, the intravenous clinical breakpoints of EUCAST15 and parenteral clinical breakpoints of CLSI14 were considered.

Fifty-six isolates (18.2%) had a cefuroxime MIC of 2 mg/L (EUCAST current breakpoints: \( S \leq 1 \) mg/L, \( R > 2 \) mg/L).15

One gBLPAR isolate (2.3%) had a cefuroxime MIC of 2 mg/L.

Forty gBLNAR isolates (67.8%) had a cefuroxime MIC of 2 mg/L.

Seven gBLPACR isolates (77.8%) had a cefuroxime MIC of 2 mg/L.
the intravenous breakpoint, 243 isolates (79.2%) were susceptible, 56 were non-susceptible (18.2%) and 8 were resistant (2.6%, all gBLNAR) (Table 1).

No differences in the antibiotic susceptibility rates were found between the consecutive invasive isolates submitted by the GMUH and the remaining isolates submitted by the other participants.

Mechanisms of resistance to aminopenicillins

MICs in relation to resistance mechanisms

Known mechanisms of resistance were found in the majority of isolates with an amoxicillin/clavulanic acid MIC ≥ 1 mg/L, including all isolates with MICs of 2–4 mg/L, which were essentially gBLNAR. All isolates with cefuroxime MICs of 4–8 mg/L, considered resistant by current EUCAST breakpoints but susceptible or intermediate by CLSI, were also gBLNAR. In addition, most isolates with a cefotaxime MIC of 2 mg/L also had identifiable resistance mechanisms, and were mostly gBLNAR and to a lesser extent gBLPAR. Approximately 10% of the isolates with a cefotaxime MIC of 1 mg/L also were gBLNAR (data not shown).

The vast majority of the study isolates had cefotaxime MICs ≤ 0.06 mg/L; only two isolates (0.7%) had an MIC of 0.25 mg/L for this antibiotic (both from the III-like group) (Table S1, available as Supplementary data at JAC Online) and were considered to be susceptible by CLSI or resistant by EUCAST. This III-like group is described here for the first time in invasive isolates in Europe.

There were 192 isolates with an amoxicillin MIC ≤ 0.5 mg/L, of which 47 were analysed by sequencing the ftsI gene; 43 of the 47 (91.4%) were identified as gBLNAS. Of the 63 isolates with an amoxicillin MIC ≥ 1 mg/L, 54 (85.7%) were identified as gBLNAR. In addition, of the 42 β-lactamase-positive isolates with an amoxicillin/clavulanic acid MIC < 2 mg/L, 39 (92.9%) were identified as gBLPAR, and of the 10 isolates with an amoxicillin/clavulanic acid MIC > 2 mg/L, 6 (60%) were identified as gBLPACR.

Overall, 197 (64.2%) of the 307 invasive isolates were amoxicillin-susceptible (BLNAS), 52 (26.4%) of which were confirmed by ftsI sequencing (gBLNAS). Sixty-eight (22.15%) of the 307 isolates had PBP3 mutations, of which 59 were gBLNAR (19.2%) and 9 were gBLPACR (2.9%). Finally, 43 of the 307 isolates were gBLPAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%). The percentage of isolates with PBP3 mutations found in this study is among the highest published10,11 and the gBLNAR (14%).

Capsule production and mechanisms of resistance

Capsulate isolates produced β-lactamase more frequently than did non-typeable isolates (24.5% versus 15.8%). In contrast, PBP3 mutations were less frequent in capsulate isolates than in non-typeable isolates (9.8% versus 24%).

PBP3 mutation patterns and genotypes

Invasive gBLNAR isolates belonged to the following groups: 42.4% to group IIC, 37.3% to group IIB, 13.6% to group IIA and 3.4% each to groups I and III-like (the two group III-like isolates had MICs of cefotaxime and cefixime of 0.25 and 0.5 mg/L, respectively). Of the gBLPACR isolates, 7 (77.8%) were group IIB and 2 (22.2%) were group IIC (Table S1, available as Supplementary data at JAC Online).

The frequencies of these gBLNAR groups are similar to those previously described by us for respiratory isolates.18 In Sweden, the most frequent group previously found in invasive isolates was shown to be IIb,11 which is also one of the most common groups in this study.

β-Lactamase production

All β-lactamase-positive isolates were of the TEM-1 type (n = 52, 16.9%) with the following promoters: 33 were Pdel (63.5%), 10 were Pp (9.2%), 8 were PPaPB (15.4%), and 1 was 2Pp (1.9%). The Pdel promoter was also found to be predominant in Sweden, although the authors named it β-lactamase-TEM-1.11

Molecular epidemiology

PFGE profiles

All 41 capsule strains studied by PFGE, regardless of the resistance mechanism, were grouped into five large PFGE clusters corresponding to isolates of type a, type b, type e and two type f profiles (data not shown).

In contrast, the 55 non-typeable gBLNAR isolates studied were grouped into 38 PFGE profiles, 28 of which were composed of single unique isolates and 10 of which had two to four isolates (Figure S1, available as Supplementary data at JAC Online). In general, the 10 gBLNAR clusters were constituted by isolates originating from different geographical areas (Figure S1, available as Supplementary data at JAC Online).

MLST profiles

MLST was determined in 23 capsule strains (11 type f, 11 type b and 1 type e). Type b isolates belonged to three different STs (6, 190 and 662), and type f isolates to seven STs (106, 124, 973, 1124, 1125, 1126 and 1127), four of which are first described in this study (1124, 1125, 1126 and 1127); the type e isolate was ST18.

MLST was also carried out in 18 gBLNAR non-typeable isolates selected on the basis of previous PFGE profiles; they comprised 15 different STs (107, 140, 155, 201, 367, 949, 1113, 1114, 1115, 1117, 1118, 1120, 1121, 1122, 1123), nine of which are first described in this study (1113, 1114, 1115, 1117, 1118, 1120, 1121, 1122, 1123). The majority of STs obtained from isolates belonging to the same PFGE cluster were either single-locus or double-locus variants (Figure S1, available as Supplementary data at JAC Online). As seen in another study, the majority of BLNAR non-typeable isolates had different ST profiles.11

According to PFGE and MLST data, only one cluster of four gBLNAR isolates was identified, all of which had the same PBP3 mutations (IIc group). Each of these came from distant geographical areas and belonged to STs 949 (two isolates), 1122 (a double-locus variant of ST 949) and 1123 (a single-locus variant of ST 949) (Figure S1, available as Supplementary data at JAC Online).

Time–kill curves

The bactericidal activities of amoxicillin (4×MIC) and cefotaxime (4×MIC) were determined in two individual gBLNAR III-like isolates (Figure 1a). Amoxicillin was bactericidal after the first 2 h of
incubation for both gBLNAR isolates, and cefotaxime was bactericidal after 4 h for the same two isolates (Figure 1b).

In summary, we have shown in this study that the gBLNAR mechanism of antibiotic resistance was the most common found in invasive infections caused by \textit{H. influenzae} in Spain, followed by \(\beta\)-lactamase production. gBLNAR isolates were genetically very diverse and showed very little clonal spread. The vast majority of invasive isolates, regardless of their mechanism of resistance to \(\beta\)-lactam antibiotics, were susceptible to amoxicillin/clavulanic acid, cefotaxime, cefixime and imipenem, both by EUCAST and CLSI clinical breakpoints. In addition, kill-curve experiments demonstrated that amoxicillin and cefotaxime were bactericidal against two gBLNAR isolates with decreased susceptibility to aminopenicillins and third-generation cephalosporins. However, the MICs of \(\beta\)-lactam for our gBLNAR invasive isolates were generally low; precaution is advised when treating this kind of pathogen with \(\beta\)-lactam antibiotics.

\section*{Acknowledgements}
We thank all the hospital participants for submitting isolates for the study.

\section*{Funding}
This study was supported by the Ministerio de Economía y Competitividad, Instituto de Salud Carlos III (FIS PI 12/00780); Plan Nacional de I+D+i 2008-2011 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD12/00015)—co-financed by European Development Regional Fund “A way to achieve Europe” ERDF. S. García-Cobos is recipient of a research contract from Fondo de Investigación Sanitaria (CA09/00031).

\section*{Transparency declarations}
None to declare.

\section*{Supplementary data}
Table S1 and Figure S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

\section*{References}


MLST. http://www.mlst.net/ (6 June 2013, date last accessed).