Contribution of ROB-1 and PBP3 mutations to the resistance phenotype of a β-lactamase-positive amoxicillin/clavulanic acid-resistant Haemophilus influenzae carrying plasmid pB1000 in Italy

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Objectives: Plasmid pB1000 bearing blaROB-1 is responsible for high-level β-lactam resistance in Haemophilus influenzae as well as in Pasteurella multocida and Haemophilus parasuis isolates from Spain. Here, we explore the presence of ROB-1 in Italy and investigate the relative contribution of penicillin-binding protein 3 (PBP3) mutations and ROB-1 to the β-lactam resistance phenotype in H. influenzae.

Methods: The collection of the Italian Reference Laboratory of H. influenzae was investigated for ROB-1-positive isolates between 2004 and 2009. H. influenzae Rd KW20 was used as recipient for pB1000 electroporation and for mutagenesis of the ftsI gene encoding PBP3.

Results: The presence of plasmid pB1000 in a non-typeable H. influenzae isolated in Italy, BB1059, is reported in this work. This strain is not genetically related to the H. influenzae clinical isolates bearing pB1000 described in Spain. The sequence of ftsI from BB1059 revealed several mutations in the predicted amino acid sequence of PBP3. To determine the relative contribution of pB1000 and PBP3 mutations to the β-lactam resistance phenotype of BB1059, H. influenzae Rd KW20 was transformed with ftsI and/or pB1000 from BB1059. β-Lactam resistance profiles revealed the additive effect of pB1000 and PBP3 mutations conferring resistance to β-lactams, including amoxicillin/clavulanic acid and third-generation cephalosporins.

Conclusions: Intra-European spread of plasmid pB1000 among H. influenzae has been shown. The coexistence of plasmid pB1000 and mutations in PBP3 produces an additive resistance phenotype in H. influenzae.

Keywords: plasmid spread, ftsI, cefaclor

Introduction

Haemophilus influenzae is a Gram-negative bacillus usually involved in community-acquired respiratory tract infections, acute otitis media, acute sinusitis and meningitis in both children and adults.1 β-Lactam antibiotics are used for the treatment of diseases caused by this pathogen.1 Resistance to β-lactam antibiotics in H. influenzae is conferred either by production of β-lactamases or by mutations in the ftsI gene encoding penicillin-binding protein 3 (PBP3), or by a combination of both mechanisms. Currently, two different β-lactamases are documented in H. influenzae, ROB-1 and TEM-1. These enzymes confer similar susceptibility profiles, characterized by high-level resistance to ampicillin and other aminopenicillins; in addition, ROB-1 also confers resistance to cefaclor.2 Mutations in PBP3 are responsible for low-level resistance or reduced susceptibility to both β-lactams and combinations of β-lactams and β-lactamases inhibitors.1 Thus, H. influenzae strains bearing both resistance mechanisms are designated as β-lactamase-positive amoxicillin/clavulanic acid-resistant (BLPACR) strains, and are becoming more prevalent.3 However, the specific contribution of each resistance mechanism to the final BLPACR phenotype has not been clarified.

Recently, the presence of plasmid pB1000 bearing blaROB-1 in non-related clinical isolates of H. influenzae has been reported.2 This plasmid was first described in animal Pasteurellaceae.4,5 Previous analyses support the idea that pB1000 has spread among Pasteurellaceae family species in Spain.2 Here, we describe the...
presence of pB1000 in a non-typeable BLPACR H. influenzae iso-
lated in Italy in 2008, showing that pB1000 is present in other
countries in Europe. Further, we characterize the relative con-
tribution of pB1000 and PB3 mutations to the β-lactam resistance
phenotype using an isogenic environment.

Materials and methods

Bacterial strains and culture conditions

H. influenzae BB1059 was isolated from the CSF of a 44-year-old male
with meningitis admitted to the University Hospital San Raffaele, Milan,
Italy in March 2008. H. influenzae Rd KW20 strain (a β-lactamase-
negative ampicillin-susceptible (BLNAS) strain) was used as recipient
strain for transformation. BB1060, BB1061 and BB1062 are β-lactama-
se-negative ampicillin-resistant (BLNAR), β-lactamase-positive
ampicillin-resistant (BLPAR) and BLPACR strains obtained from the trans-
formation of H. influenzae Rd KW20 strain with ftsI, pB1000 and both
elements, respectively, from BB1059 (Table 1). Strains were cultured as
previously described.5

Antimicrobial susceptibility testing

Screening was determined by Etest (AB Biodisk, Solna, Sweden) using
Haemophilus test medium (HTM). Antimicrobial susceptibility of
H. influenzae BB1059 was isolated from the CSF of a 44-year-old male
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Screening was determined by Etest (AB Biodisk, Solna, Sweden) using
Haemophilus test medium (HTM). Antimicrobial susceptibility of
H. influenzae strains was determined by broth microdilution in microtitre
plates (SensiSitre Emiza 14; Trek Diagnostics, Inc., Westlake, OH, USA)
and to cefaclor with in-house microtitre plates according to the CLSI
guidelines.6

DNA analysis and transformation experiments

PCR, plasmid DNA extraction, automated sequencing, sequence analysis
and pB1000 PCR mapping and stability analysis were performed as
described previously.5 Primers used for ftsI amplification were ftsI-fw
(5′-CGATACCTTGGAGCCAGGTTC-3′) and ftsI-rev (5′-TGATCTTGTTGCTGC
CCTAGT-3′). The genetic relatedness of H. influenzae isolates was deter-
mined by PFGE.7 H. influenzae Rd KW20 electrocompetent cell prep-
aration and plasmid electroporation was carried out as previously
specified.8 Transformation of the ftsI gene from BB1059 into H. influen-
zea Rd KW20 was performed essentially according to Barcak et al.9 1 µg of
purified ftsI PCR product from BB1059 was mixed with 2 mL of early log-
phase cells in HTM and grown overnight at 37°C with 5% CO2. Transfor-
mants were selected on chocolate agar plates containing 0.01 mg/L
cefotaxime.

Results and discussion

BLPACR high-level cefaclor-resistant clinical isolate
bearing pB1000 in Italy

One hundred and one invasive H. influenzae strains and 58 isolates
from patients with cystic fibrosis were collected between 2004
and 2009 by the Italian Reference Laboratory for H. influenzae.
All were screened for the presence of ROB-1. Twenty-one strains
(21/159, 13.2%) were high-level resistant to amoxicillin (MIC
>128 mg/L). One of them (1/159, 0.6%), BB1059, also showed
resistance to cefaclor (MIC >4 mg/L). The presence of the
举办了 transformation on the H. influenzae Rd KW20 strain with ftsI, pB1000 and both
elements, respectively, from BB1059 (Table 1). Strains were cultured as
previously described.5

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mants were selected on chocolate agar plates containing 0.01 mg/L
cipenam, and to cefaclor with in-house microtitre plates according to the CLSI

guidelines.6

Table 1. β-Lactam resistance genotype and phenotype of H. influenzae strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>name</th>
<th>description</th>
<th>Genotype</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>pB1000</td>
<td>AMP</td>
</tr>
<tr>
<td>BB1059</td>
<td>wt (BLPACR)</td>
<td>+</td>
<td>+</td>
<td>128</td>
</tr>
<tr>
<td>Rd KW20</td>
<td>Rd KW20 (BLNAS)</td>
<td>−</td>
<td>−</td>
<td>0.25</td>
</tr>
<tr>
<td>BB1060</td>
<td>Rd KW20 (BLNAR)</td>
<td>+</td>
<td>+</td>
<td>0.5</td>
</tr>
<tr>
<td>BB1061</td>
<td>Rd KW20 (BLPAR)</td>
<td>+</td>
<td>−</td>
<td>256</td>
</tr>
<tr>
<td>BB1062</td>
<td>Rd KW20 (BLPACR)</td>
<td>+</td>
<td>+</td>
<td>512</td>
</tr>
</tbody>
</table>

AMC, amoxicillin/clavulanic acid (2:1 ratio); AMP, ampicillin; AMX, amoxicillin; CEC, cefaclor; CTX, cefotaxime; CXM, cefuroxime; wt, wild-type; Rd KW20, H. influenzae Rd KW20 strain.

aStrain carrying ftsI from BB1059, encoding PB3 with amino acid substitutions compared with Rd KW20: I449V; N526K; V547I; N569S; and A586S.

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Figure 1. Genetic similarity dendrogram of plasmid pB1000. Strain numbers and species are indicated. The dendrogram was constructed using MEGA software, version 3.1, using the neighbour-joining grouping procedure with Kimura two-parameter distance measurement. Bar, genetic distance of 0.001. Note that pB1000 sequences from H. influenzae form a separate cluster (shaded in grey).

Supplementary data at JAC Online. These results show that clonal dissemination is not responsible for the spread of pB1000, and suggest that the plasmid is transmitted horizontally among Pasteurellaceae.

Genetic analysis of pB1000

Plasmid pB1000 bearing blaROB-1 has been previously described in the swine pathogens Haemophilus parasuis and Pasteurella multocida, and in H. influenzae. PFGE analysis has proved that the presence of pB1000 in an H. influenzae clinical isolate in Italy is not due to clonal spread (Figure S1), as was the case for H. parasuis, but to plasmid dissemination, as described in P. multocida. To study the molecular epidemiology of this replicon the complete nucleotide sequence of pB1000 from BB1059 was obtained and compared with previously existing sequences from H. parasuis, P. multocida and H. influenzae. PB1000 nucleotide identity ranged between 98.8% and 100%. The genetic distances between the plasmids were determined (Figure 1). The results suggest that pB1000 has been circulating separately in human H. influenzae isolates and animal Pasteurellaceae in Europe in recent years, after an apparent transmission event between them.

Relative contribution of pB1000 and PBP3 mutations to the β-lactam resistance phenotype

β-Lactam resistance phenotypes of BLPACR strains have been widely studied in clinical isolates. Notwithstanding this, the precise contribution of each resistance mechanism to the final phenotype in BLPACR H. influenzae is unclear. Comparing the resistance profile of clinical isolates bearing the two mechanisms with those of strains carrying each mechanism separately is not an accurate method, as the different genetic environment of each isolate might bias the interpretation of the results. Here, H. influenzae Rd KW20 has been used as an isogenic environment to study the relative contribution of pB1000, carrying blaROB-1, and PBP3 mutations to the β-lactam resistance phenotype of BB1059. Thus, Rd KW20 gave rise to strains showing BLNAR (BB1060), BLPAR (BB1061) and BLPACR (BB1062) genotypes by substitution of the ftsI gene with that from BB1059, by transformation with pB1000 or by combination of both modifications, respectively (Table 1). Sequence analysis confirmed genetic replacement of the chromosomal ftsI gene, and plasmid preparation and PCR mapping were used to corroborate the presence of pB1000. PFGE of every isogenic strain presented an identical pattern (Figure S1, lanes 1–4). A detailed β-lactam resistance profile of each genotype was determined (Table 1). In BB1060, mutated PBP3 confers a one or two dilution increase in the MICs of aminopenicillins, amoxicillin/clavulanic acid and cephalosporins compared with the parental strain. BB1061, bearing pB1000, showed a high-level resistance to aminopenicillins and cefaclor, and a one dilution increase in the MICs of third-generation cephalosporins and amoxicillin/clavulanic acid. Finally, BB1062 showed the highest level of resistance. Interestingly, the β-lactam resistance profile of BB1062 was the result of adding BB1060 and BB1061 resistance levels for every β-lactam tested except for cefaclor, where the MIC remains at 64 mg/L for both BB1061 and BB1062. These data demonstrate an additive contribution of pB1000 and PBP3 mutations to the BLPACR phenotype. Hence, this combined strategy entails an adaptive advantage as it confers resistance or reduced susceptibility to a broader range of β-lactam antimicrobials than each mechanism separately.

Nucleotide sequence accession number

The nucleotide sequence of pB1000 from BB1059 has been deposited in GenBank under the accession number HM470204.

Acknowledgements

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


