Novel mechanisms of resistance to β-lactam antibiotics in Haemophilus parainfluenzae: β-lactamase-negative ampicillin resistance and inhibitor-resistant TEM β-lactamases

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Objectives: To determine the mechanisms of resistance to β-lactam antibiotics in clinical isolates of Haemophilus parainfluenzae.

Methods: Twenty clinical isolates of H. parainfluenzae with decreased susceptibility to aminopenicillins were examined and compared with a control group of 20 fully susceptible isolates. In this collection, the presence of amino acid substitutions in the transpeptidase domain of penicillin-binding protein 3 (PBP3), β-lactamase production and the surrounding genetic regions of blaTEM genes in selected isolates were analysed.

Results: Of the 20 non-susceptible isolates, 8 produced TEM β-lactamase (gBLPAR), 7 had mutations in the transpeptidase domain of the ftsI gene related to decreased susceptibility to β-lactams (gBLNAR) and 5 had both resistance mechanisms (gBLPACR). No resistance mechanisms were identified in the susceptible control group (gBLNAS). gBLNAR isolates had MIC90 values 4- to 16-fold higher than gBLNAS isolates for ampicillin, amoxicillin/clavulanic acid, cefuroxime, cefotaxime and cefixime, and the most common PBP3 mutation was Asn526Ser. The additional Ser385Thr substitution (III-like group) may confer decreased susceptibility to cefotaxime, cefixime and aztreonam, as in Haemophilus influenzae. In two β-lactamase-positive isolates without PBP3 mutations, the inhibitor-resistant TEM (IRT) β-lactamases TEM-34 and the novel TEM-182 were detected and carried by a TnA transposon of the Tn2 type; both isolates had an amoxicillin/clavulanic acid MIC of ≥8 mg/L. The TnA transposons of two β-lactamase-positive isolates (TEM-1 and TEM-182) were inserted between the tfc20 and tfc21 genes, typically associated with integrative and conjugative elements in Haemophilus spp.; the TEM-34 IRT β-lactamase was harbour in a ~5.5 kb plasmid.

Conclusions: Clinical isolates of H. parainfluenzae express a variety of aminopenicillin resistance mechanisms, either alone or in combination, including PBP3 modifications, blaTEM-1 and IRT β-lactamase production.

Keywords: PBP3, ftsI, amoxicillin/clavulanic acid resistance, transposon TnA

Introduction

Haemophilus parainfluenzae is a commensal organism of the human upper respiratory tract, but can also cause a variety of infections, such as bacteraemia, meningitis, endocarditis, peritonitis and biliary and genital tract infections. In the genus Haemophilus, the most common mechanism of ampicillin resistance is the production of the TEM-1 β-lactamase; recently, however, reduced susceptibility to ampicillin without β-lactamase production (BLNAR phenotype) has become widespread in Haemophilus influenzae. The BLNAR genotype has been mapped to amino acid substitutions in the transpeptidase domain of penicillin-binding protein 3 (PBP3), defined by either an Arg517His or an Asn526Lys substitution. Although H. parainfluenzae is often resistant to β-lactam antibiotics, its mechanisms of resistance to this class of antibiotics have received little attention. The BLNAR mechanism has been very rarely reported in H. parainfluenzae, and, to our knowledge,
the production of inhibitor-resistant TEM (IRT) β-lactamases has not been described in clinical isolates of Haemophilus spp., although their effect has been studied in artificially produced recombinant strains.9 Accordingly, the aims of this study were to determine the mechanisms of resistance to β-lactam antibiotics in clinical isolates of H. parainfluenzae and to elucidate the impact that β-lactam resistance genotypes have on the antibiotic susceptibility of H. parainfluenzae.

Materials and methods

Bacterial isolates and susceptibility testing

A total of 40 epidemiologically unrelated clinical isolates of H. parainfluenzae collected from individual patients were studied. All of them had been submitted to our Haemophilus Reference Laboratory for identification and susceptibility testing from different geographical areas of Spain from 2004 to 2010. Twenty non-consecutive isolates were selected because they had reduced susceptibility to ampicillin, amoxicillin and/or amoxicillin/clavulanic acid. For comparative purposes, we also studied 20 additional unrelated clinical isolates of H. parainfluenzae fully susceptible to aminopenicillins. The clinical sources of the 40 isolates were as follows: genital mucosa (11), urine (11), respiratory secretions (6), peritoneal fluid (5), blood (2) and one each from conjunctiva, biliary fluid, pericardial fluid, abscess and liver biopsy. All isolates were identified as H. parainfluenzae by standard microbiological methods and confirmed by PCR amplification and sequencing of the 16S ribosomal DNA.10

Antibiotic susceptibility testing was determined by the microdilution method following the CLSI guidelines.11 Susceptibility results were interpreted according to the CLSI.11 The β-lactam antibiotics tested were ampicillin, amoxicillin, amoxicillin/clavulanic acid (2:1 ratio), cefuroxime, cefotaxime, cefixime, imipenem and aztreonam. β-Lactamase production was determined by the chromogenic cephalosporin test with nitrocefin as the substrate.12

ftsI and blaTEM sequencing

In all study isolates, the PBP3 transpeptidase domain of the ftsI gene from nucleotide 943 to nucleotide 1750 was amplified. Numbering was based on the ftsI sequence of H. parainfluenzae.13 The following primers were used: para2F 5’CTAGACCGTGATCGATTGATCCT, designed in this study, and paraR2 5’CTACGAGATACCCGGGCGAC (nt 943–1708);7 and paraF 5’CAGCGAGACGTGATGAAAACT with paraR3 5’TTTCTTGTTGGTTCTGC (nt 1025–1750). PCR was carried out as described for H. influenzae.14 H. parainfluenzae T3T1, whose whole genome has been sequenced,13 was used as a reference for the sequence alignment procedures. DNA sequencing of the blaTEM genes and their promoter regions was performed as described previously.3

Genotype definition

Due to the high degree of nucleotide (78%) and amino acid (83%) homology between ftsI and PBPs of H. influenzae Rd KW20 and H. parainfluenzae T3T1,13 we used the same genotype classification as that already used for H. influenzae.13,15 As follows: gBLNAS, β-lactamase-negative isolates that are ampicillin susceptible without resistance mechanisms; gBLNAR, β-lactamase-negative isolates with PBPs amino acid substitutions causing reduced susceptibility to ampicillin (defined by either an Asn526 or an Arg517 substitution); gBLPAR, β-lactamase-positive isolates that are ampicillin resistant without gBLNAR-defining PBPs amino acid substitutions; and gBLPACR, β-lactamase-positive isolates also presenting PBPs amino acid substitutions.

We observed that H. influenzae Rd KW20 and H. parainfluenzae T3T1 have the same PBPs conserved amino acid motifs [Ser-Thr-Val-Lys (STVK), Ser-Ser-Asn (SSN) and Lys-Thr-Gly (KTG)] in which most ftsI mutations have been described. In addition, we followed the same I–III group classification as that already used in H. influenzae.14,16

PCR mapping of blaTEM

Genomic DNA was extracted and used as the template for different PCRs to determine the surrounding genetic regions of blaTEM-1, blaTEM-26, and blaTEM-183. Primers and product sizes are listed in Table S1 (available as Supplementary data at JAC Online). In H. influenzae, TnA transposons, flanked by tfc20 and tfc21 genes, have been described as part of the integrative and conjugative elements (ICEs);17 we explored this possibility in three bla-producing H. parainfluenzae isolates using the PCR primers described in Table S1 (available as Supplementary data at JAC Online). Previously described sequences of Tn1, Tn2 and Tn3 were used for comparative purposes.18

When amplification of the complete ICE structure failed we attempted to amplify the insertion sequence IS26, previously described upstream of the blaTEM gene as part of TnA in Escherichia coli.19 In addition, we also investigated the presence of the small blaTEM-encoding plasmids that have been described recently,18 using the PCR conditions and primers described in Table S1 (available as Supplementary data at JAC Online).

Amplifications were carried out as previously described.14 PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of denaturation (94°C for 45 s), annealing [temperature depending on region (Table S1 available as Supplementary data at JAC Online) for 45 s] and extension (72°C for 45 s) and a final extension at 72°C for 10 min.

Conjugation and plasmid detection

Conjugation was attempted with two donor H. parainfluenzae isolates carrying TEM-182 and TEM-34 β-lactamases; kanamycin-resistant H. influenzae MAP and E. coli HB101 were used as recipients. The strains were grown to an optical density of 0.6 at 600 nm. Then, 2 mL of each of the donor and recipient cultures was added to 10 mL of fresh medium, mixed and incubated overnight at 37°C with 5% CO2 without shaking. Putative transconjugants were selected on Haemophilus Test Medium (H. influenzae MAP) and on Mueller–Hinton (E. coli HB101) agar plates containing ampicillin (10 mg/L) and kanamycin (10 mg/L).

Results and discussion

Antimicrobial susceptibility in relation to resistance genotype

Of the 40 isolates tested, 20 were gBLNAS, 7 were gBLNAR, 8 were gBLPAR and 5 were gBLPACR. gBLNAR isolates had MIC90 values 4- to 16-fold higher than gBLNAS isolates for ampicillin, amoxicillin/clavulanic acid, cefuroxime, cefotaxime and cefixime (Table 1). Based upon CLSI breakpoints,11 gBLNAS isolates were 100% susceptible to all tested antibiotics, but four out of the seven gBLNAR isolates (57.1%), were ampicillin non-susceptible and two were cefuroxime non-susceptible (28.6%) (data not shown).

All 13 β-lactamase-positive isolates were ampicillin and amoxicillin resistant; 3 of them (2 gBLPAR and 1 gBLPACR) were also amoxicillin/clavulanic acid resistant (MIC ≥8/4 mg/L). All 40 isolates were susceptible to cefotaxime, cefixime, imipenem and aztreonam (Table 1).
Table 1. Amino acid substitutions identified in the ftsI gene of the 40 *H. parainfluenzae* clinical isolates and susceptibility to eight β-lactam antibiotics according to the resistance class of each isolate

<table>
<thead>
<tr>
<th>Genotype (group)</th>
<th>No. of isolates</th>
<th>near the SSN motif</th>
<th>surrounding the KTG motif</th>
<th>Genotype (group)</th>
<th>No. of isolates</th>
<th>near the SSN motif</th>
<th>surrounding the KTG motif</th>
<th>MIC_{90} (mg/L)</th>
<th>AMP</th>
<th>AMX</th>
<th>AMC</th>
<th>CXM</th>
<th>CTX</th>
<th>CFM</th>
<th>IPM</th>
<th>ATZ</th>
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<tr>
<td>gBLNAS 1</td>
<td>Ala-343 Ser-385 Glu-398 Ile-414 Ile-442 Val-488 Val-511 Asn-526 Ala-530 Val-562 Thr-574</td>
<td>0.25 0.5 0.25 0.25 0.03 0.03 0.25 0.12</td>
<td></td>
<td>gBLNAS 1 (IIa)</td>
<td>Ala Lys Ala</td>
<td>2 2 2 4 0.06 0.06 0.25 0.12</td>
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<td>2</td>
<td>Val</td>
<td></td>
<td></td>
<td>gBLNAS 3 (IIa)</td>
<td>Val Ile Ser Ser</td>
<td>1 2 2 1 0.03 0.03 0.12 0.12</td>
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<td>4</td>
<td>Val</td>
<td>Ile</td>
<td></td>
<td>gBLNAS 1 (IIa)</td>
<td>Val Ile Ser Ser</td>
<td>2 4 4 4 0.06 0.06 0.25 0.12</td>
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<tr>
<td>11</td>
<td>Val</td>
<td>Ile</td>
<td></td>
<td>gBLNAS 1 (III-like)</td>
<td>Thr Phe Ala Ser Ile</td>
<td>4 4 4 16 0.5 0.05 0.5 0.5</td>
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<td>4</td>
<td>Val</td>
<td>Asp</td>
<td></td>
<td>gBLNAS 1 (III-like)</td>
<td>Thr Phe Ala Lys Ile</td>
<td>8 4 4 16 0.5 0.5 0.5 0.5</td>
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<td>20 (total)</td>
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<td></td>
<td>gBLNAS 7 (1 TEM-182)</td>
<td>Val Ile</td>
<td>≥256 ≥128 16 0.5 0.06 0.12 0.5 0.12</td>
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<tr>
<td>gBLNAR 7 (1 TEM-182)</td>
<td>Val</td>
<td>Ile</td>
<td></td>
<td>gBLNAR 1 (TEM-34)</td>
<td>Val Val Ile</td>
<td>≥256 ≥128 8 1 0.03 0.03 0.5 0.12</td>
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<td>8 (total)</td>
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<td>gBLNAR 8 (total)</td>
<td>Val Val Ile</td>
<td>≥256 ≥128 16 1 0.06 0.12 0.5 0.12</td>
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<td>gBLPAR 2 (IIa)</td>
<td>Ala Ser</td>
<td></td>
<td></td>
<td>gBLPACR 2 (IIa)</td>
<td>Ala Ser Ser</td>
<td>≥256 ≥128 8 4 0.03 0.06 0.5 0.12</td>
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<tr>
<td>3 (IIa)</td>
<td>Val</td>
<td>Ile</td>
<td></td>
<td>gBLPACR 3 (IIa)</td>
<td>Val Ser Ser</td>
<td>≥256 ≥128 2 2 0.03 0.03 0.5 0.12</td>
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<td>5 (total)</td>
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<td></td>
<td>gBLPACR 5 (total)</td>
<td>Val Ser Ser</td>
<td>≥256 ≥128 8 4 0.03 0.06 0.5 0.12</td>
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AMP, ampicillin; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CXM, cefuroxime; CTX, cefotaxime; CFM, cefixime; IPM, imipenem; ATZ, aztreonam.
gBLNAS, β-lactamase-negative isolates that are ampicillin susceptible without resistance mechanisms; gBLNAR, β-lactamase-negative isolates with PBP3 amino acid substitutions causing reduced susceptibility to ampicillin; gBLPAR, β-lactamase-positive isolates that are ampicillin resistant without gBLNAR-defining PBP3 amino acid substitutions; and gBLPACR, β-lactamase-positive isolates also presenting PBP3 amino acid substitutions.

"Numbering according to *H. parainfluenzae* T3T1 sequence."^{3,15}

"The IRT β-lactamase TEM-182 belongs to these seven gBLPAR isolates."
Of the 13 β-lactamase-positive isolates, 11 were identified as possessing TEM-1; one isolate, which caused conjunctivitis in a child, had the IRT β-lactamase TEM-34 (Met69Val) and in another isolate, which caused bacteraemia in a 76-year-old patient, a novel IRT β-lactamase, TEM-182, with three amino acid substitutions (Met69Ile, Trp165Ile and Arg275Leu) was identified—amino acid substitutions at these three positions are typically linked to IRT β-lactamase enzymes. The TEM-34- and TEM-182-producing isolates had an amoxicillin/clavulanic acid MIC of 8 mg/L (TEM-34) and 16 mg/L (TEM-182), without gBLNAR-defining PBP3 amino acid mutations.

Nine of the 13 β-lactamase-positive isolates (69.2%) had the Pdel promoter (including the TEM-182-producing isolate), 2 (15.4%) had the Pa/Pb promoter and 2 (15.4%) had the Prpt promoter (including the TEM-182-producing isolate). These promoter regions have also been described in Haemophilus influenzae, Pdel and Prpt promoters being associated with higher levels of resistance to amoxicillin.

Mapping of blaTEM

Attempts to demonstrate the ability of the isolates to undergo conjugation were not successful. Further, we showed that the blaTEM-1, blaTEM-34 and blaTEM-182 genes were located in a TnA family transposon (Figure 1). blaTEM-1 and blaTEM-182 were carried in a TnA transposon of the Tn2 group, as previously identified in E. coli, but had a 135 bp deletion between the resolvase gene tnpR and blaTEM associated with the Pdel promoter (Figure 1a). In addition, these two transposons were inserted between genes tfe20 and tfe21, as reported in Haemophilus spp. ICEs.

In the isolate expressing blaTEM-34, the amplification of tfe20 and tfe21 (usually present in the ICEs of Haemophilus spp.) failed and the transposase tnpA gene was partially amplified (127 bp) (Figure 1b). This truncated tnpA gene was followed by complete copies of tnpR, blaTEM-34 and an inverted repeat region (Figure 1b). A Tn2 transposon group was identified according to the region of variation that distinguishes between transposons. Between tnpR and blaTEM, a 54 bp insertion associated with the Prpt promoter was found (Figure 1b). An IS26-TnA configuration was not identified because IS26 could not be amplified (Table S1 available as Supplementary data at JAC Online). However, we investigated the presence of a replication protein (Rep) and a mobilization protein (Mob) previously described up-stream of the blaTEM gene in small plasmids, and we found the same structure as that described for plasmid pLFH64 (Figure 1b).

ftsI mutation patterns

As in H. influenzae, both gBLNAR and gBLPACR H. parainfluenzae had amino acid changes at the Asn526 position of ftsI, either

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**Figure 1.** Structures of transposons carrying TEM-182 and TEM-1 (a) and TEM-34 (b) β-lactamases. Horizontal arrows show the orientations of genes, black boxes indicate the inverted terminal repeats (IR) and the grey box indicates the region of variation that distinguishes the transposon type. tnpA, transposase gene; tnpR, resolvase gene.
Asn526Ser (the most frequent) or Asn526Lys (Table 1); the latter is commonly found in gBLNAR H. influenzae.\textsuperscript{14,16} In a previous study Asn526 was replaced by histidine in \textit{H. parainfluenzae},\textsuperscript{7} these three amino acid substitutions (Asn526Ser, Asn526Lys and Asn526His) could be similar in terms of polarity and hydrophobicity.

Also, similar to \textit{H. influenzae}, two gBLNAR isolates were classified as group III-like because they had the Ser385Thr substitution that in \textit{H. influenzae} confers higher cefotaxime and cefixime MICs (Table 1).\textsuperscript{14}

The ValS11Ala substitution in \textit{ftsI} has been associated with a 16-fold increase in the amoxicillin MIC for gBLNAR \textit{H. influenzae} isolates from Japan.\textsuperscript{21} This mutation was present in five of our \textit{H. parainfluenzae} isolates; three of them were gBLNAR, with an amoxicillin MIC of 2–4 mg/L, and two were gBLPACR with an amoxicillin/clavulanic acid MIC of 8 mg/L (Table 1). Five of the 13 \beta-lactamase-positive isolates also presented the Asn526Ser substitution and were therefore classified as gBLPACR; they had an amoxicillin/clavulanic acid MIC of 2–8 mg/L (Table 1).

Only one previous study has reported \textit{ftsI} changes in two \textit{H. parainfluenzae} isolates, namely Ala343Val (which we detected in our gBLNAS isolates), Ser385Thr and Asn526His,\textsuperscript{7} but these two isolates also produced an extended-spectrum \beta-lactamase and the direct impact of the PBP3 mutations was not investigated.

The possibility of horizontal gene transfer of \textit{ftsI} in \textit{H. influenzae} has been suggested.\textsuperscript{22} In this study gBLNAR \textit{H. parainfluenzae} was isolated from a variety of anatomical sources, suggesting that an independent acquisition of \textit{ftsI} mutations may have occurred after selective antibiotic pressure. Supporting this hypothesis is the finding that the amino acid substitution Asn526Ser was predominant among our \textit{H. parainfluenzae} isolates, in contrast to Asn526Lys commonly found in \textit{H. influenzae}.

**Conclusions**

In summary, we describe a collection of clinical \textit{H. parainfluenzae} isolates with reduced susceptibility to \beta-lactam antibiotics due not only to TEM-1 \beta-lactamase production but also to two novel resistance mechanisms: PBP3 mutations and the production of IRT \beta-lactamas. This is the first description of IRT \beta-lactamase production in the genus \textit{Haemophilus}.

**Nucleotide sequence accession numbers**

The complete DNA sequence for \textit{bla}_{TEM-182}, its transposon and the small plasmid harbouring TEM-34, described in this study, appear in the GenBank nucleotide sequence database under accession numbers HQ317449, KC292504 and KC292503, respectively. Sequences of the \textit{ftsI} gene representing several mutation patterns have also been deposited in GenBank (accession numbers KC208504 to KC208510).

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**Supplementary data**

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

**References**


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