Molecular Evolution of β-Lactam-Resistant *Haemophilus influenzae*: 9-Year Surveillance of Penicillin-Binding Protein 3 Mutations in Isolates from Japan

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A total of 621 clinical isolates of *Haemophilus influenzae* collected in Japan between 1995 and 2003 were studied for their susceptibilities to several antimicrobial agents, β-lactamase production, and amino acid substitutions in penicillin-binding protein 3 (PBP 3). Over the four study periods (first period, 1995 to 1996; second period, 1997 to 1998; third period, 2000 to 2001; fourth period, 2002 to 2003), the susceptibilities to β-lactam agents decreased and the incidence of isolates with substitutions at positions 377, 385, 389, 517, and/or 526 in PBP 3 increased from 28.8% to 52.0%. Five hundred seventy-one β-lactamase-nonproducing isolates were grouped into 18 classes, based on the pattern of the five mutations in PBP 3. The Asp526Lys substitution led to 6.0-, 4.3-, 2.4-, and 5.4-fold increases in amoxicillin-clavulanic acid, cefdinir, cefditoren, and faropenem resistance, respectively. PBP 3 with multiple substitutions (Met377Ile, Ser385Thr, and/or Leu389Phe) together with Asp526Lys resulted in increased resistance compared to that for PBP 3 with the Asp526Lys substitution alone. These results indicate that mutations at these five positions increased resistance to most β-lactams. Although a significant change in the prevalence of β-lactamase-producing strains was not observed, the proportions of those possessing both PBP 3 alterations and β-lactamase production have slightly increased (from 1.4% to 5.0%). The ROB-1 β-lactamase was rare, but this is the first report of this β-lactamase in Japan.

*Haemophilus influenzae* is an important pathogen that causes community-acquired infections such as pneumonia, otitis media, and meningitis and has become increasingly resistant to β-lactam antibiotics (5, 18). There are two major mechanisms involved in β-lactam resistance, one enzymatic and the other nonenzymatic. The enzymatic resistance mechanism is mainly mediated by the hydrolysis of β-lactams due to the production of TEM-1 β-lactamase (17, 24) and, in some cases, to a ROB-1 β-lactamase (10, 13). The nonenzymatic mechanism involves a decreased affinity of penicillin-binding protein 3 (PBP 3) for β-lactamase (17, 24) and, in some cases, to a ROB-1 β-lactamase (10, 13). The nonenzymatic mechanism involves a decreased affinity of penicillin-binding protein 3 (PBP 3) for β-lactam antibiotics due to amino acid substitutions (17, 23). The β-lactam resistance phenotype mediated by the nonenzymatic mechanism is called “β-lactamase-nonproducing ampicillin resistance (BLNAR)” in *H. influenzae*.

Recently, the isolation frequency of BLNAR strains has been increasing exponentially among clinical isolates from patients with community-acquired infections in Japan (21, 25). A recent report also described an increased prevalence of the BLNAR phenotype in Europe (4). Strains with both resistance mechanisms were also found among clinical isolates, and such strains are termed β-lactamase-producing ampicillin-clavulanic acid-resistant (BLPACR) *H. influenzae* (6, 12, 22).

Studies of β-lactam resistance caused by *H. influenzae* PBP 3 mutations have been reported by several investigators (1, 6, 16, 20, 23). From the genetic analysis of the *ftsI* gene encoding PBP 3 in BLNAR strains, the amino acid substitutions surrounding the conserved KTG (Lys512-Thr-Gly) and SSN (Ser379-Ser-Asn) motifs appear to be responsible for β-lactam resistance. Amino acid substitutions, including asparaginase to lysine at position 526 (N526K) or arginine to histidine at position 517 (R517H), near the KTG motif, were commonly found in isolates with intermediate resistance (MICs, 0.063 to 0.25 μg/ml) to cefotaxime. Additional amino acid substitutions near the SSN motif, such as methionine to isoleucine at position 377 (M377I), serine to threonine at position 385 (S385T), and/or leucine to phenylalanine at position 389 (L389F), were frequently found in isolates with higher levels of resistance (MICs, 1 to 2 μg/ml) to cefotaxime.

Although the mechanisms of resistance have been revealed in BLNAR strains, the evolution of BLNAR strains has not been clarified. The aim of this study is to clarify the molecular change of PBP 3 amino acid substitutions and β-lactam susceptibilities in clinical isolates. Therefore, we collected a total of 621 clinical isolates between 1995 and 2003 from several Japanese hospitals and investigated the amino acid substitutions in PBP 3 and their correlation with β-lactam susceptibilities.

**MATERIALS AND METHODS**

**Bacterial strains.** A total of 621 clinical isolates were collected between 1995 and 2003 from 122 hospitals in different parts of Japan or from a working group in Japan that collected strains for a postmarketing surveillance of cefditoren pivovil (11). Most strains (＞95%) were isolated from patients with community-acquired respiratory tract infections, such as pneumonia, bronchitis, otitis media, pharyngitis, and sinusitis, in various medical settings. There were only a few isolates from patients with bacteremia, meningitis, and conjunctivitis. These isolates were divided into four groups on the basis of isolation periods. The numbers of isolates tested were 73 for the first period (1995 to 1996), 119 for...
In order to identify the isolates as *H. influenzae*, all isolates were tested for the requirement of /NAD (V factor) and hemin (X factor). The production of /lactamase was detected by the P/Case test (Nissui Seiyaku Co., Ltd. Tokyo Japan), which is one of the modified acidimetry methods.

Antimicrobial susceptibility testing. MICs were determined by the microdilution method recommended by the Clinical and Laboratory Standards Institute (14). For the MIC tests, the prepared medium, which was Mueller-Hinton II broth (Becton Dickinson Company, Sparks, MD) supplemented with 2% defibrinated, heat-treated horse blood plus 15 μg/ml /NAD, was obtained from Eiken Chemical Co., Ltd. (Tokyo Japan). The antibiotics tested were as follows: ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefotiam, ceftazidime, cefotaxime, ceftriaxone, cefaclor, cefpodoxime, cefdinir, cefditoren, faropenem, imipenem, meropenem, azithromycin, and levofloxacin.

PCR and nucleotide sequencing. We determined the DNA sequences of the /ftsI gene encoding PBP 3 for the 621 *H. influenzae* isolates. The full-length /ftsI gene was amplified by PCR with Ex Taq polymerase (Takara Shuzo Co., Ltd.,

### TABLE 1. Trends in /lactam susceptibilities

| Agent and period | MIC (µg/ml) | Agent and period | MIC (µg/ml) |
|------------------|-------------|------------------|-------------|
|                 | Geometric mean | 50% | 90% | Range | Geometric mean | 50% | 90% | Range |
| **Ampicillin**   |             |     |     |        |             |     |     |        |
| First            | 0.534       | 0.25 | 2   | 0.25–64 | 1.292       | 1   | 2   | 0.5–8  |
| Second           | 0.895       | 0.5  | 64  | 0.12–64 | 1.833       | 1   | 8   | 0.25–128 |
| Third            | 0.719       | 0.5  | 8   | 0.12–64 | 2.563       | 2   | 32  | 0.5–128 |
| Fourth           | 1.145       | 0.5  | 8   | 0.12–64 | 6.255       | 4   | 128  | 0.25–256 |
| All              | 0.841       | 0.5  | 8   | 0.12–64 | 2.965       | 2   | 64  | 0.25–256 |
| **Ampicillin-clavulanic acid** |             |     |     |        |             |     |     |        |
| First            | 0.678       | 0.5  | 2   | 0.25–4  | 0.027       | 0.03 | 0.06 | 0.008–0.12 |
| Second           | 0.850       | 0.5  | 4   | 0.25–16 | 0.036       | 0.03 | 0.12 | 0.004–2  |
| Third            | 1.317       | 1    | 16  | 0.25–32 | 0.041       | 0.03 | 0.5  | 0.004–2  |
| Fourth           | 1.693       | 1    | 16  | 0.25–64 | 0.048       | 0.03 | 1   | 0.004–2  |
| All              | 1.214       | 0.5  | 16  | 0.25–64 | 0.040       | 0.03 | 0.5  | 0.004–2  |
| **Piperacillin-tazobactam** |             |     |     |        |             |     |     |        |
| First            | 0.030       | 0.03 | 0.12 | 0.008–0.25 | 0.006       | 0.008 | 0.016 | 0.002–0.03 |
| Second           | 0.048       | 0.03 | 0.25 | 0.008–0.5 | 0.008       | 0.008 | 0.016 | 0.002–0.25 |
| Third            | 0.051       | 0.06 | 0.25 | 0.008–1  | 0.012       | 0.008 | 0.12  | 0.002–0.25 |
| Fourth           | 0.034       | 0.03 | 0.25 | 0.004–0.5 | 0.012       | 0.008 | 0.25  | 0.002–0.25 |
| All              | 0.042       | 0.03 | 0.25 | 0.004–1  | 0.010       | 0.008 | 0.12  | 0.002–0.5  |
| **Imipenem**     |             |     |     |        |             |     |     |        |
| First            | 0.500       | 0.5  | 1   | 0.06–4  | 0.130       | 0.12 | 0.25 | 0.06–0.25 |
| Second           | 0.575       | 0.5  | 2   | 0.06–8  | 0.132       | 0.12 | 0.25 | 0.016–1  |
| Third            | 0.809       | 1    | 4   | 0.06–16 | 0.162       | 0.12 | 0.5  | 0.03–2   |
| Fourth           | 0.865       | 1    | 4   | 0.12–8  | 0.108       | 0.12 | 0.25 | 0.03–1   |
| All              | 0.732       | 0.5  | 4   | 0.06–16 | 0.133       | 0.12 | 0.5  | 0.016–2  |
| **Faropenem**    |             |     |     |        |             |     |     |        |
| First            | 0.622       | 0.5  | 2   | 0.12–8  | 0.140       | 0.06 | 0.12 | 0.03–0.5 |
| Second           | 0.870       | 0.5  | 4   | 0.25–8  | 0.132       | 0.12 | 0.25 | 0.016–8  |
| Third            | 1.122       | 1    | 8   | 0.016–16 | 0.221       | 0.12 | 4   | 0.016–16 |
| Fourth           | 1.068       | 1    | 8   | 0.12–16 | 0.293       | 0.12 | 8   | 0.03–16  |
| All              | 0.981       | 1    | 4   | 0.016–16 | 0.197       | 0.12 | 4   | 0.016–16 |
| **Azithromycin** |             |     |     |        |             |     |     |        |
| First            | 1.785       | 2    | 4   | 0.25–4  | 0.355       | 0.25 | 1   | 0.12–2  |
| Second           | 1.965       | 2    | 4   | 0.5–8   | 0.549       | 0.25 | 4   | 0.12–16 |
| Third            | 1.362       | 1    | 2   | 0.12–4  | 0.790       | 0.5  | 8   | 0.06–16 |
| Fourth           | 0.949       | 1    | 2   | 0.25–4  | 1.064       | 0.5  | 8   | 0.12–32 |
| All              | 1.343       | 1    | 4   | 0.12–8  | 0.738       | 0.5  | 8   | 0.06–32 |
| **Levofloxacin** |             |     |     |        |             |     |     |        |
| First            | 0.023       | 0.03 | 0.03 | 0.016–0.03 | 0.018       | 0.016 | 0.03 | 0.004–0.06 |
| Second           | 0.024       | 0.03 | 0.03 | 0.016–0.06 | 0.024       | 0.016 | 0.06 | 0.012–0.5 |
| Third            | 0.026       | 0.03 | 0.03 | 0.008–0.12 | 0.033       | 0.03 | 0.12 | 0.004–0.5 |
| Fourth           | 0.016       | 0.016 | 0.016 | 0.008–0.12 | 0.032       | 0.016 | 0.25 | 0.008–0.5 |
| All              | 0.021       | 0.016 | 0.03 | 0.008–0.12 | 0.029       | 0.03 | 0.12 | 0.002–0.5 |
TABLE 2. Frequencies of isolation of β-lactamase-producing strains

| β-Lactamase | First period (73)* | Second period (119) | Third period (229) | Fourth period (200) | All periods (621) |
|-------------|-------------------|--------------------|--------------------|---------------------|------------------|
| None        | 68 (93.2)         | 102 (85.7)         | 217 (94.8)         | 184 (92.0)          | 571 (91.9)       |
| TEM-1       | 5 (6.8)           | 16 (13.4)          | 12 (5.2)           | 16 (8.0)            | 49 (7.9)         |
| ROB-1       | 0 (0)             | 1 (0.8)            | 0 (0)              | 0 (0)               | 1 (0.2)          |

* Numbers in parentheses are the total number of isolates recovered in each period.

Kyoto, Japan). The primers used for DNA amplification and sequencing were originally designed on the basis of the DNA sequences of the Rd strain (DDBJ/EMBL/GenBank accession number NC000907), as follows: 5′-CTCGTATCCGTGTACAGCAG-3′ for the sense primer and 5′-GCCAAACGCTTGATGATGAAAC-3′ for the antisense primer. PCR amplification was performed with a PCR system 9700 (Applied Biosystems, Foster City, CA), as follows: 2 min of denaturation at 98°C and 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 3 min. Both strands of the amplified DNA fragments were sequenced by using an Applied Biosystems 3730 DNA analyzer. The deduced amino acid sequences of PBP 3 of the strains tested were compared with the amino acid sequence of PBP 3 from the Rd strain. The changes in the MICs occurred.

Frequency of β-lactamase-producing isolates. The isolation frequencies of β-lactamase-producing H. influenzae isolates for the four periods are shown in Table 2. The incidences of β-lactamase-producing isolates were 6.8, 14.2, 5.2, and 8.0% for the first, second, third, and fourth periods, respectively. Only one strain was positive for the ROB-1 β-lactamase gene, while all others carried a TEM-1 β-lactamase gene.

Molecular survey of amino acid substitutions in PBP 3. Amino acid substitutions in PBP 3 at positions 377, 385, 389, and 526 have been found to be responsible for β-lactam resistance. The frequencies of isolates carrying these mutant PBP 3s are shown in Table 3. No new amino acid substitutions were detected at positions 377, 385, 389, and 517; however, at position 526, one strain had a histidine and not a lysine in place of the asparagine.

For the β-lactamase-nonproducing isolates, the ratio of those without any of the amino acid substitutions listed above steadily declined during the surveillance period (first period,

| Peptide | First period (73) | Second period (119) | Third period (229) | Fourth period (200) | All periods (621) |
|---------|-------------------|--------------------|--------------------|---------------------|------------------|
| Ile     | 65.8              | 53.8               | 47.6               | 45.0                | 50.1             |
| Thr     | 14                | 1.7                | 1.3                | 2.0                 | 1.6              |
| His     | 12.3              | 7.6                | 12.2               | 3.5                 | 8.5              |
| Phe     | 1.4               | 0.4                | 0.4                | 0.5                 | 0.2              |
| Lys     | 13.4              | 2.5                | 1.3                | 1.5                 | 1.6              |
| His     | 0.8               | 2.2                | 3.0                | 0.2                 | 0.2              |
| 0.4     |                    |                    |                    |                     | 0.2              |

* Numbers in parentheses are the total number of isolates recovered in each period. bla(−), β-lactam negative; bla(+), β-lactam positive.
were rarely seen (total, 1.4%) in the and 16.5%, respectively. Amino acid substitutions in PBP 3 increased as follows during the four study periods: 0, 5.0, 7.9, L389F substitutions, in addition to the N526K substitution, was observed since the third period. No remarkable change was observed in the frequencies of isolates M377I, S385T, and/or L389F substitution, in addition to the R517H substitution decreased from 12.3 to 3.5% from the first period, 45.0%). Although the prevalence of isolates with the 65.8%; second period, 53.8%; third period, 47.6%; and fourth period, 45.0%). Although the prevalence of isolates with the

**TABLE 4. Amino acid substitutions in PBP 3 and β-lactam susceptibilities**

| Group | Amino acid substitution in PBP 3 | No. of isolates | Geometric mean of MIC (µg/ml) [increase compared to that for group I] |
|-------|---------------------------------|-----------------|------------------------------------------|
|       | 377Met 385Ser 389Leu 517Arg 526Asn|                 |                                          |
| I     | Ile                             | 311             | 0.27 0.48 0.022 0.54                      |
| II    | Ile                             | 10              | 0.27 (1.0) 0.38 (0.8) 0.071 (3.2) 0.22 (0.4) |
| III   | His                             | 53              | 0.60 (2.3) 1.12 (2.3) 0.060 (2.7) 0.43 (0.8) |
| IV    | Ile Thr                         | 10              | 1.23 (4.6) 3.25 (6.8) 0.065 (3.0) 0.76 (1.4) |
| V     | Lys                             | 98              | 1.29 (4.8) 2.87 (6.0) 0.050 (2.3) 1.33 (2.4) |
| VI    | Thr Lys                         | 11              | 2.57 (9.6) 7.05 (14.7) 0.095 (4.4) 3.76 (6.9) |
| VII   | Thr Phe Lys                     | 57              | 5.62 (20.9) 17.21 (35.9) 0.084 (3.8) 2.37 (4.4) |

Amino acid substitutions in PBP 3 and β-lactam resistance. To investigate the correlation between amino acid substitutions and resistance to β-lactam antibiotics without bias from β-lactamases, 571 β-lactamase-nonproducing isolates were chosen and grouped into 18 classes, based on the pattern of the five mutations in PBP 3. Of the 18 classes, 11 did not contain a sufficient number of isolates for significant evaluation. For the seven major classes (number of isolates in each class, >10), the relationship between amino acid substitutions and β-lactam resistance is indicated in Table 4. The M377I substitution (classified as group II) did not result in increased resistance to β-lactams except piperacillin-tazobactam. For the group III isolates with the R517H substitution, the levels of resistance to penicillins and cephalosporins increased 1.7- to 4.3-fold compared with those of the nonmutated group I strains. Addition of the M377I and S385T substitutions (group IV) led to further increases in resistance, particularly to cefpodoxime (17.5-fold), cefotaxime (11.7-fold), and ceftriaxone for the group VII isolates were less than 1 µg/ml, the increases in resistance were 58.3- and 44.5-fold, respectively, compared with those for the group I isolates. Resistance to piperacillin-tazobactam, imipenem, and meropenem was not affected by the addition of M377I, S385T, and L389F substitutions in the N526K background (the increases in the ratios were less than 1.8-fold).

**fsr gene sequence comparisons.** In this study, it became apparent that several BLNAR isolates carried the fsr gene with the same nucleotide mutations. The genetic composition of the fsr gene, especially in five isolates with the M377I, S385T, and L389F substitutions, was of particular interest. The homology of the fsr genes between these isolates and the Rd control strain is shown in Table 5.

The MSC02023 and MSC02104 isolates had the R517H substitution but not the N526K substitution, and the fsr gene sequences were identical. On the other hand, strains MSC07237, MSC02104, and MSC02149 had the N526K substitution but not the R517H substitution, and the fsr gene sequences were also identical between them. Although the degree of identity of the DNA sequence of the 3’ end (nucleotide positions 1188 to 1833), including the region encoding the R517H and N526K substitutions, was 96.4%, the DNA sequences of their 5’ ends (nucleotide positions 1 to 1187), including the region encoding the M377I, S385T, and L389F substitutions, were identical.

**DISCUSSION**

Over the four study periods, between 1995 and 2003, the prevalence of β-lactam resistance has increased among clinical H. influenzae isolates in Japan. In this study, we characterized

**TABLE 5. Percent identities of the nucleotide sequences of the fsr gene between strain Rd and clinical isolates**

| Strain(s) | % Identity of nucleotide sequences of the fsr gene at the region between positions: |
|-----------|----------------------------------|
|           | 1 and 1187 | 1188 and 1833 |
| Rd        | MSC02023, MSC02169 | MSC07237, MSC02104, MSC02149 | MSC02023, MSC02169 | MSC07237, MSC02104, MSC02149 |
| Rd        | 100.0 97.7 97.7 | 100.0 97.3 95.7 | 100.0 97.3 96.4 | 100.0 97.3 96.4 |
| MSC02023, | 100.0 | 100.0 | 100.0 | 100.0 |
| MSC02104, | 100.0 | 100.0 | 100.0 | 100.0 |
| MSC02149  | 100.0 | 100.0 | 100.0 | 100.0 |
β-lactamase production and identified the PBP 3 mutations in the resistant isolates. Based on our results, the number of β-lactamase-positive isolates remained stable (between 5.2% and 14.3%), and these percentages were equivalent to those in Europe but lower than those in the United States (4, 8, 9).

Most of the β-lactamase-positive isolates remained stable (between 5.2% and 14.3%), and these percentages were equivalent to those in -lactamase-positive isolates. Based on our results, the number of -lactamase production and identified the PBP 3 mutations in additional M377I, S385T, and L389F substitutions. The frequencies of isolates with these substitutions were very high compared with the situation in the United States and European countries (4, 8, 9). However, the levels of decreasing susceptibilities over the four study periods differed for the different β-lactam agents. Therefore, piperacillin-tazobactam and ceftazidime were unique, in that increases in resistance to them appeared to be so small compared with those for the other beta-lactams. In contrast, high-level resistance to cefotaxime, cefepime, and ceftazidime was remarkably frequent. For such reasons, we have reviewed the relationship between amino acid substitutions in PBP 3 and resistance to each β-lactam agent. The antimicrobial activities of piperacillin-tazobactam and ceftazidime were unaffected by the N526K substitution and additional M377I, S385T, and L389F substitutions compared with their effects on other cephalosporins and penicillins. It is interesting that resistance to piperacillin-tazobactam and ceftazidime was strongly influenced by the M377I and the R517H substitutions, respectively. These results suggest that piperacillin and ceftazidime might differ from other β-lactams in their interactions with the mutated PBP 3 binding pockets. With ceftazidime, we think that the carbapenem-resistant side chain at the C-7 position might play a role in the unique interaction.

The influence of amino acid substitutions on the activities of the carbapenems was also different from that on the activities of the cephalosporins. Imipenem and meropenem were affected by the N526K substitution and additional substitutions, but were hardly affected by the R517H substitution and additional substitutions. In our previous study with recombinants of the Rd strand carrying a mutated ftsI gene, resistance to carbapenems could not be explained solely by the five amino acid substitutions in PBP 3 (16). In this study, we found two novel amino acid substitutions near the conserved motifs: valine to isoleucine or alanine at position 329 (Val329I/A) in the conserved Ser327-Thr-Val-Lys motif in five isolates and valine to alanine at position 511 (Val511A) near the KTG motif in six isolates. However, these amino acid substitutions may not be responsible for carbapenem resistance (data not shown). We also found several isolates with atypical amino acid substitutions at positions 517 and 526. For those with both the R517H and the N526K substitutions, the levels of susceptibility to imipenem (MICs, 0.25 to 1 μg/ml) were comparable to those for isolates with the R517H substitution. This finding suggests that high-level resistance would not result even if the R517H and the N526K substitutions overlapped. Another mutant had

| Geometric mean of MIC (μg/ml) [increase compared to that for group I] |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Meropenem | Faropenem | Cefotaxime | Ceftaxime | Ceftazidime | Cefaclor | Cefepime | Cefditoren |
| 0.053 | 0.48 | 1.11 | 0.017 | 0.0045 | 0.093 | 3.1 | 0.070 | 0.306 | 0.015 |
| 0.045 (0.9) | 0.40 (0.8) | 0.47 (0.4) | 0.007 (0.4) | 0.0023 (0.5) | 0.040 (0.4) | 1.5 (0.5) | 0.030 (0.4) | 0.154 (0.5) | 0.018 (1.2) |
| 0.054 (1.0) | 1.08 (2.5) | 1.87 (1.7) | 0.039 (2.3) | 0.0110 (2.5) | 0.333 (3.6) | 13.3 (4.3) | 0.206 (2.9) | 0.949 (3.1) | 0.034 (2.2) |
| 0.091 (1.7) | 2.14 (4.5) | 13.00 (11.7) | 0.140 (8.3) | 0.0396 (8.9) | 0.406 (4.4) | 27.9 (9.1) | 1.231 (17.5) | 3.482 (11.4) | 0.040 (2.6) |
| 0.176 (3.5) | 2.60 (5.4) | 7.19 (6.5) | 0.064 (3.8) | 0.0173 (3.9) | 0.109 (1.2) | 22.6 (7.4) | 0.303 (4.3) | 1.327 (4.3) | 0.036 (2.4) |
| 0.300 (5.6) | 4.54 (9.5) | 32.00 (28.5) | 0.263 (15.7) | 0.0732 (16.4) | 0.281 (3.0) | 43.9 (14.3) | 2.269 (32.3) | 6.022 (21.6) | 0.114 (7.4) |
| 0.306 (5.8) | 5.42 (11.3) | 80.63 (72.6) | 0.976 (58.3) | 0.1984 (44.5) | 0.299 (3.2) | 104.1 (33.9) | 6.748 (96.1) | 10.975 (35.8) | 0.248 (16.1) |
a histidine instead of a lysine substitution at position 526 (N526H), in addition to β-lactamase production. This isolate showed a slightly lower imipenem MIC compared with those for N526K strains and β-lactamase production (data not shown).

In Japan, the expanded-spectrum oral cephalosporins and amoxicillin-clavulanic acid have commonly been used to treat community-acquired respiratory tract infections caused by *H. influenzae* and *S. pneumoniae*. The recent prevalence of the strains carrying the mutated *ftsI* gene may be attributed to the frequent use of these agents. Compared to the β-lactams, levofloxacin, one of the fluoroquinolones, still shows potent activity against *H. influenzae*, including β-lactam-resistant strains (Table 1). However, a recent report described the outbreak of fluoroquinolone-resistant *H. influenzae* that had been associated with the clinical use of levofloxacin (15). Additional measures should be taken in order to ensure that oral β-lactam antibiotics continue to work against BLNAR strains. From the results of this study, we found that susceptibility to cefditoren may be responsible for a different binding interaction with the mutated PBP 3s.

In summary, our study confirms the problematic prevalence of β-lactam-resistant strains of *H. influenzae* in Japan. The increase in the numbers of these isolates correlated well with the spread of strains with PBP 3 mutations. Amino acid substitutions, such as N526K or R517H, together with additional M377I, S385T, and L389F substitutions in PBP 3 brought about reduced susceptibilities to most of the β-lactam agents tested. However, piperacillin-tazobactam, cefazidime, and carbenapenem were not as strongly affected by these mutations.

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