Remarkably high prevalence of fts I gene mutations in Haemophilus influenzae isolates from upper respiratory tract infections in children of the Sapporo district, Japan

Abstract
Recently, the frequency of isolation of beta-lactamase-negative ampicillin resistant (BLNAR) strains of Haemophilus influenzae in Japanese children has been increasing rapidly. Drug resistance in BLNAR strains is associated with mutations of the fts I gene, which encodes penicillin-binding protein 3. In the otolaryngological field, only a few reports have been available concerning fts I gene mutations in BLNAR. We investigated the prevalence of fts I gene mutations, by polymerase chain reaction (PCR) genotyping, in H. influenzae isolates from the upper respiratory tracts of children in the Sapporo district, Japan. When the isolates were classified according to PCR genotyping, 34 (44.2%) of 77 isolates were beta-lactamase-negative ampicillin-sensitive (g-BLNAS), 8 (10.4%) were g-low-BLNAR, 30 (39.0%) were g-high-BLNAR, 2 (2.6%) were beta-lactamase-positive ampicillin-resistant (g-BLPAR), and 3 (3.9%) were beta-lactamase-positive ampicillin/clavulanic acid-resistant (g-high-BLPACR). Mutations in the fts I gene were generally parallel to ampicillin susceptibility, and were frequently observed in children who were 7 years or younger. Of the beta-lactams tested, cefditoren showed the strongest inhibition of H. influenzae isolates, and it inhibited g-BLNAR and g-BLPACR. This study revealed a remarkably high prevalence of fts I gene mutations (g-BLNAR and g-BLPACR) in our district. Furthermore, a regional difference in the prevalence of fts I gene mutations was observed even at the district level.

Key words
Haemophilus influenzae · Beta-lactamase-negative ampicillin-resistant (BLNAR) · Drug resistance · Upper respiratory tract infection · Children · fts I

Introduction
Haemophilus influenzae is one of the most frequent pathogens in the upper respiratory tracts of children. Although the bacterium is usually sensitive to beta-lactams, beta-lactamase-negative ampicillin-resistant (BLNAR) strains have been increasing recently in Japan.1,2 Especially in children, the frequency of isolation of BLNAR strains is rapidly increasing, and problems caused by BLNAR strains are frequently emerging in Japan.3,4

BLNAR strains acquire drug resistance by mutations in the fts I gene encoding penicillin-binding protein (PBP) 3, which is associated with septal peptidoglycan synthesis.5,6 The amino acid substitutions N526K or R517H, and S385T, are observed in the fts I gene of BLNAR strains. One amino acid substitution, N526K or R517H, alone is observed in the fts I gene of low-BLNAR strains, which show lower ampicillin resistance than BLNAR strains.4,7

Only a few reports are available concerning the prevalence of fts I gene mutations in the otolaryngological field. In order to understand the current status of BLNAR in the Sapporo district, Japan, we investigated the frequency of fts I gene mutations and susceptibility to antibiotics in H. influenzae isolates obtained from upper respiratory tract infections in children.

Patients, materials, and methods

Patients and bacterial isolates

We evaluated 77 clinical isolates of H. influenzae obtained from the upper respiratory tracts of 77 children (33 females and 44 males, from 1 month to 11 years old; median age, 3 years) with upper respiratory tract infections, in four cities.
(Sapporo, Ebetsu, Takikawa, and Date) in Hokkaido Prefecture, Japan (Fig. 1). The isolates were collected between January 2004 and December 2004.

Antimicrobial susceptibility tests

The minimum inhibitory concentrations (MICs) of the isolates to antibiotics were determined by the broth micro-dilution method, according to the standard methods of the Clinical Laboratory Standards Institute (CLSI, formerly the NCCLS). The antibiotics used in this study were ampicillin (Meiji Seika, Tokyo, Japan), penicillin G (Meiji Seika), cefaclor (Shionogi, Osaka, Japan), cefpodoxime (Sankyo, Tokyo, Japan), cefdinir (Astellas, Osaka, Japan), and cefditoren (Meiji Seika). These antibiotics are frequently used for upper respiratory tract infections in Japan.

Polymerase chain reaction (PCR)

Beta-lactam resistance genes in *H. influenzae* isolates were evaluated by PCR, using six sets of primers, as previously reported by Hasegawa et al. The primers were: P6 primers to amplify the *p6* gene, which encodes P6 outer membrane protein specific for *H. influenzae*; serotype b primers to amplify the gene encoding the serotype b capsule; TEM-1 primers to amplify a part of the *bla* 

\text{TEM-1} \text{ gene, which encodes TEM-1 beta-lactamase; ROB-1 primers to amplify a part of the } 

\text{ROB-1} \text{ gene, which encodes ROB-1 beta-lactamase; PBP3-S primers to amplify the } 

\text{fts I} \text{ gene which has Asn at amino acid residue 526 (intact } 

\text{fts I} \text{ gene); and PBP3-BLN primers to amplify the } 

N526K \text{ and S385T amino acid substitutions in the } 

\text{fts I} \text{ gene (highly mutated } 

\text{fts I} \text{ gene). The reaction profile of the PCR was 30 cycles at 94°C for 15 s, 30 cycles at 55°C for 15 s, and 30 cycles at 72°C for 15 s. PCR products were separated by electrophoresis in 3% agarose gel and visualized by ethidium bromide staining and UV light illumination.

On the basis of the PCR results, the isolates were classified, as previously reported, isolates that were intact *fts I*-positive and highly mutated *fts I*-negative were classified as beta-lactamase-negative ampicillin-sensitive (g-BLNAS); isolates that were intact *fts I*-negative and highly mutated *fts I*-negative were classified as g-low-BLNAR; isolates that were intact *fts I*-negative and highly mutated *fts I*-positive were classified as g-high-BLNAR; isolates that were *bla* 

\text{TEM-1} \text{-positive or } 

\text{BLNAR} \text{ genotype were classified as g-high- } 

\text{BLNAR} \text{; isolates that were beta-lactamase-positive amoxicillin-resistant (g-BLPAR); isolates that were beta-lactamase-positive with g-low-BLNAR genotype were classified as low beta-lactamase-positive amoxicillin-clavulanic acid-resistant (g-low-BLPACR); and isolates that were beta-lactamase-positive with g-high- } 

\text{BLNAR} \text{ genotype were classified as g-high-BLPACR.}

Frequency of *fts I* gene mutations and drug susceptibility

Of the 77 isolates, 36 (46.8%) had the intact *fts I* gene, whereas 41 (53.2%) had *fts I* gene mutations. Eleven isolates (14.3%) had the serotype b capsule gene. Of the 41 isolates having *fts I* gene mutations, 33 (80.5%) had a highly mutated *fts I* gene. Five (6.5%) of the 77 isolates had the *bla* 

\text{TEM-1} \text{ gene. Three isolates (3.9%) had both the highly mutated } 

\text{fts I} \text{ gene and the } 

\text{BLNAR} \text{ genotype. The } 

\text{BLNAR} \text{ was not detected in any of the isolates.}

When the results were classified according to the PCR findings (Fig. 2), 34 (44.2%) of the 77 isolates were g-BLNAS, 8 (10.4%) were g-low-BLNAR, 30 (39.0%) were g-high-BLNAR, 2 (2.6%) were g-BLPAR, and 3 (3.9%) were g-high-BLPACR. g-low-BLPACR was not detected.

When the results were investigated in relation to ampicillin susceptibility, the prevalence of *fts I* gene mutations was generally parallel to the ampicillin MIC (Fig. 3). While 39 (95.1%) of the 41 isolates with ampicillin MIC \(\geq 1\) \mu g/ml, had *fts I* gene mutations (g-low-BLNAR, g-high-BLNAR, and g-BLPACR) only 2 of the 36 isolates (5.6%) with ampicillin MIC \(\geq 0.5\) \mu g/ml had *fts I* gene mutations. There was a significant difference between these frequencies (\(P < 0.001; \chi^2\) test with Yates correction). Of the isolates with ampicillin MIC \(\geq 2\) \mu g/ml, all had *fts I* gene mutations and/or beta-lactamase. The g-high-BLNAR strain showed high resistance (ampicillin MIC \(\geq 2\) \mu g/ml), whereas most of the g-low-BLNAR strains showed an ampicillin MIC of 1 \mu g/ml. Three g-high-BLPACR isolates also showed high resistance; 1 (33.3%) showed an ampicillin MIC of 2 \mu g/ml (twice the breakpoint value), and 2 (66.7%) showed ampicillin MICs \(\geq 8\) \mu g/ml.

As shown in Fig. 4, almost all isolates of the genetically resistant strains were detected in children who were 7 years or younger. In children who were 8 years or older, the total number of strains was small and there was only one resis-