Analysis of *Haemophilus influenzae* serotype f isolated from three Japanese children with invasive *H. influenzae* infection

Tadashi Hoshino,¹ Yushi Hachisu,² Takashi Kikuchi,² Shoko Tokutake,¹ Hideyuki Okui,¹ Satoru Kutsuna,¹ Chie Fukasawa,¹ Kei Murayama,³ Asami Oohara,⁴ Hiroyuki Shimizu,⁴ Midori Ito,⁵ Yoshiko Takahashi⁶ and Naruhiko Ishiwada⁷

1 Division of Infectious Diseases, Chiba Children’s Hospital, Chiba, Japan
2 Chiba Prefectural Institute of Public Health, Chiba, Japan
3 Department of Metabolism, Chiba Children’s Hospital, Chiba, Japan
4 Children’s Medical Center, Fujisawa City Hospital, Kanagawa, Japan
5 Department of Pediatrics, Nagoya City West Medical Center, Aichi, Japan
6 Department of Pediatrics, Graduate School of Medicine, Chiba University, Chiba, Japan
7 Department of Infectious Diseases, Medical Mycology Research Center, Chiba University, Chiba, Japan

In Japan, publicly subsidized *Haemophilus influenzae* serotype b vaccines became available in 2011; consequently, the incidence of invasive *H. influenzae* infection in pediatric patients of less than 5 years of age decreased dramatically. In 2013, the first case of *H. influenzae* serotype f (Hif) meningitis in a Japanese infant was reported, and another case of Hif meningitis in a Japanese infant was observed in 2013. We experienced a fatal paediatric case of Hif bacteraemia in 2004; therefore, we conducted an analysis of the three Hif strains isolated from these three Japanese children with invasive Hif infections. All three strains were β-lactamase-non-producing, ampicillin-sensitive strains, with MICs of 1 μg ml⁻¹ or less. However, one of the three strains showed slightly elevated MICs for ampicillin (1 μg ml⁻¹), cefotaxime (0.25 μg ml⁻¹) and meropenem (0.13 μg ml⁻¹). A molecular analysis by multilocus sequence typing identified all three strains as sequence type (ST) 124, which is a predominant invasive Hif strain in many countries. Smal-digested PFGE showed variable DNA fragmentation patterns among the strains, suggesting that some highly virulent strains have originated from a single ST124 clone and caused invasive Hif infections in Japan. Additional studies are needed to determine the factors that have led to the clonal expansion of virulent ST124 strains.

INTRODUCTION

In western countries, the *Haemophilus influenzae* serotype b (Hib) conjugate vaccine was introduced in the 1990s, and the rate of invasive *H. influenzae* infection among children decreased dramatically thereafter. Since then, a shift from Hib to non-typeable *H. influenzae* and encapsulated strains other than Hib (non-b), particularly serotypes a, e and f (Hif), has been observed (Ladhani et al., 2010; MacNeil et al., 2011). Hif is the most common non-b strain in some countries (MacNeil et al., 2011; Ladhani et al., 2012; Giufre et al., 2013); in the USA (MacNeil et al., 2011), England and Wales (Ladhani et al., 2012), the incidence of invasive Hif infection has been steadily increasing in recent years. Multilocus sequence typing (MLST) analysis has revealed that the majority of invasive Hif strains are of sequence type (ST) 124 (Sill et al., 2007; Ladhani et al., 2012; Giufre et al., 2013).

The introduction of the Hib vaccine was delayed until 2008 in Japan and, as a result, *H. influenzae* has been the leading pathogen in invasive paediatric infections since 2000. Publicly subsidized vaccines became available in 2011, and with the subsequent increase in vaccine coverage rate, the incidence of invasive *H. influenzae* infection in paediatric patients less than 5 years old decreased by 84%...
from 2011 to 2013 in Chiba prefecture (Ishiwada et al., 2014).

In 2013, the first case of Hif meningitis in a Japanese infant was reported (Oohara et al., 2014). To the best of our knowledge, there were no reports of invasive Hif infection in Japan before this case. In Japan, active surveillance of paediatric invasive H. influenzae infection, supported by the Ministry of Health, Labour and Welfare, has been conducted in 10 of 47 prefectures, including Chiba prefecture, since 2007, and the serotype of the invasive strain has been determined. Invasive H. influenzae infection in prefectures other than the 10 prefectures under surveillance has also been reported officially since April 2013, and serotyping is performed if needed. From these surveillance efforts, another case of Hif meningitis in a Japanese infant was also observed in 2013. Before the introduction of the Hib vaccine, we had already experienced a fatal case of paediatric invasive Hif infection in 2004. Therefore, in this report, we performed molecular analysis of the three invasive Hif strains isolated from these three patients using MLST and PFGE.

METHODS

H. influenzae strain 04-008 was isolated from a blood culture of a 3-year-old boy with mitochondrial disease who died of bacteraemia in 2004 (Murayama et al., 2009). Strain 13-017 was isolated from a blood culture of a 7-month-old previously healthy infant girl who developed meningitis in 2013 (Oohara et al., 2014). Strain 13-018 was isolated from a cerebrospinal fluid culture of a 5-month-old, previously healthy infant boy who developed meningitis in 2013. The strains were stored at −80°C immediately after isolation and then grown on chocolate II agar (Nippon Becton Dickinson). Strains were identified based on their growth requirements for haemin and NAD (X and V factors, respectively). Serotyping was carried out by standard slide agglutination capsule serological testing using antisera (Denka Seiken), as described by the manufacturer. PCR molecular capsule typing was performed using primers for bexA- and serotype f-specific genes, as described previously (Falla et al., 1994). For antimicrobial susceptibility testing, the MIC was measured by the broth microdilution method, in compliance with the Clinical and Laboratory Standards Institute (CLSI, 2012). Sensitivity to ampicillin (AMP), cefotaxime (CTX) and meropenem (MEP) was tested, and β-lactamase production was evaluated by the nitrocefin method using a cefinase disk (Nippon Becton Dickinson).

MLST analysis was carried out according to a previously described method (Meats et al., 2003). Internal fragments of seven housekeeping genes (adk, atpG, frdB, fucK, mdh, pgi and recA), each of approximately 350–500 bp, were amplified by PCR. The sequences were submitted to the MLST website (http://haemophilus.mlst.net) for allele and ST assignments. A genetic examination was performed by PFGE according to a previously described procedure (Mitsuda et al., 1999). Bacterial genomic DNA was digested using the restriction enzyme Smal (Takara Bio) and was loaded onto 1 % agarose gels. Electrophoresis was performed in 0.5 × Tris/borate/EDTA buffer at 6 V cm⁻¹ and 14 °C for 22 h with pulse times of 5–50 s, ramped linearly using the CHEF-DR III system (Bio-Rad Laboratories). A genetic similarity dendrogram was generated by the unweighted pair group method with arithmetic mean using BioNumerics version 5 software (Applied Maths). Similarity coefficients were calculated using Pearson’s correlation.

RESULTS

All three isolates were identified as serotype f by the slide agglutination test and molecular capsule typing with bexA- and serotype f-specific primers. None of the strains produced β-lactamase, and all three were sensitive to AMP (MIC ≤1 μg ml⁻¹). Thus, all three strains were classified as β-lactamase-non-producing, AMP-sensitive strains. Although 04-008 and 13-017 had low MICs for AMP (0.25 μg ml⁻¹), CTX (≤0.03 μg ml⁻¹) and MEP (≤0.06 μg ml⁻¹), 13-018 had high MICs of 1, 0.25 and 0.13 μg ml⁻¹, respectively (Table 1). The MLST analysis showed that the three strains had identical allelic profiles, leading to their classification as ST124 (Table 1). However, Smal-digested PFGE revealed non-identical DNA fragmentation patterns, with a similarity level of 0.72 or more for 04-008, 13-017 and 13-018 (Fig. 1).

DISCUSSION

There is little information about the antimicrobial susceptibility of Hif. A study conducted in Spain reported that 24.5 % of Hif strains (12 of 49) were AMP resistant and produced β-lactamase (Campos et al., 2003). None of the three strains in this report produced β-lactamase, and all were susceptible to AMP. However, 13-018 showed slightly elevated MICs for AMP, CTX and MEP. β-Lactamase-non-producing, AMP-resistant strains have mutations in the ftsI gene, which reduces the binding affinity of penicillin-binding protein 3 for antimicrobial agents, and also show reduced susceptibility to various types of β-lactams, including cephalosporins and carbapenem (Hasegawa et al., 2006), the main therapeutic agents for invasive H. influenzae infection. The increase in β-lactamase-non-producing, AMP-resistant

Table 1. Antimicrobial susceptibilities and results of MLST of seven housekeeping genes in three Hif strains isolated from paediatric invasive infections

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year</th>
<th>MIC (μg ml⁻¹)</th>
<th>No. housekeeping gene alleles</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AMP</td>
<td>CTX</td>
<td>MEP</td>
</tr>
<tr>
<td>04-008</td>
<td>2004</td>
<td>0.25</td>
<td>≤0.03</td>
<td>≤0.06</td>
</tr>
<tr>
<td>13-017</td>
<td>2013</td>
<td>0.25</td>
<td>≤0.03</td>
<td>≤0.06</td>
</tr>
<tr>
<td>13-018</td>
<td>2013</td>
<td>1</td>
<td>0.25</td>
<td>0.13</td>
</tr>
</tbody>
</table>
strains among Hib in the pre-Hib vaccine era in Japan (Ubukata et al., 2013) suggests that studies on antimicrobial susceptibility should also include Hib strains.

All of the three Hif strains isolated in this study were classified as ST124 by the MLST method. Previous studies have shown that invasive encapsulated *H. influenzae* strains have limited genetic diversity, whereas invasive non-typeable *H. influenzae* strains are much more heterogeneous (Omikunle et al., 2002; Gifre et al., 2013). Moreover, most invasive infections arising from non-b strains are caused by a limited number of circulating clones that are inherently more pathogenic than those more frequently associated with non-invasive diseases (Omikunle et al., 2002; Shuel et al., 2011). Hif may represent this trend, as the majority of invasive Hif strains are classified as ST124, including 90% (89 of 99) of strains isolated in England and Wales between 2001 and 2010 (Ladhani et al., 2012), 88.9% (8 of 9) of strains isolated in Italy from 2009 to 2011 (Gifre et al., 2013) and all five cases in Manitoba, Canada, between 2000 and 2006 (Sill et al., 2007). Although rare, other STs, such as ST598 (Meats et al., 2003; Gifre et al., 2013), ST967, ST973, ST864 (Ladhani et al., 2012) and ST1127 (http://haemophilus.mlst.net), which are different from ST124 only at one of seven genomic MLST loci, also cause invasive Hif diseases. It is therefore likely that the clonal complex of ST124 has already exhibited virulence associated with invasive infection before diverging into each ST, and humans became more susceptible to ST124 than to other STs after divergence.

While 04-008 was isolated in 2004 in Chiba prefecture, 13-017 and 13-018 were isolated in 2013 in Kanagawa and Aichi prefectures, respectively, which are located 300 km apart. Thus, the three strains had no obvious epidemiological connection. PFGE analysis revealed non-identical DNA fragment patterns for 04-008, 13-017 and 13-018, precluding horizontal dissemination by a single clone. Invasive non-b strains are assumed to arise from the evolutionary divergence of ancestral strains via selection of specific virulent phenotypes (Omikunle et al., 2002). Hence, some highly virulent strains probably exist that originated from a single ST124 clone and spread to various areas, including Japan, causing invasive Hif infections. Alternatively, several ST124 clones may have been brought to Japan from other countries. Further studies are required to determine how clonal expansion of virulent ST124 strains occurred.

**ACKNOWLEDGEMENTS**

This work was supported by research grants from the Ministry of Health, Labour and Welfare of Japan (H24-Sinko-Ippan-003) and the Ministry of Education, Culture, Sports, Science, and Technology of Japan (no. 26461564).

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