Genotypic characterization of *Haemophilus influenzae* isolates from paediatric patients in Japan

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Abstract

**Purpose.** β-lactamase-negative ampicillin-resistant (BLNAR) *Haemophilus influenzae* is frequently isolated from respiratory samples and is particularly problematic in Japan. The aim of this study was to characterize circulating isolates of *H. influenzae* genotypically by BLNAR-PCR and multilocus sequence typing (MLST), and to determine any associations between them.

**Methods.** *H. influenzae* isolates (n=191) were collected from paediatric patients (1 month to 12 years old) between 2000 and 2011 for three types of infections: pneumonia (n=61), acute otitis media (AOM) (n=68) and meningitis (n=62). All were characterized for capsular type by agglutination tests, and for β-lactam resistance by real-time PCR. The sequence types (STs) determined by MLST were analysed using *eBURST* v3.

**Results.** Eighty-eight out of 191 (46.1 %) *H. influenzae* isolates were BLNAR by PCR; 37 of 61 (60.7 %) from pneumonia; 33 of 68 (48.5 %) from AOM and 18 of 62 (29.0 %) from meningitis cases. MLST identified 40 and 44 STs among isolates from pneumonia and AOM, respectively. BLNAR were found in singletons such as ST156 in pneumonia, and ST161 and ST396 in AOM. In contrast, eight STs were identified in meningitis, of which seven were genotypically closely related, while ST54 was the most frequent (62.9 %), unlike in the MLST database registrations, where ST6 predominated.

**Conclusion.** Non-typeable *H. influenzae* (NTHi), mostly derived from pneumonia and AOM, were genetically diverse, in contrast to the predominance of *H. influenzae* type b (Hib) among meningitis cases. The associations between certain STs and β-lactam resistance among NTHi were confirmed.

INTRODUCTION

*Haemophilus influenzae*, which causes a variety of respiratory tract infections, is occasionally responsible for serious invasive disease in children and the elderly [1]. *H. influenzae* can be divided into encapsulated strains and noncapsulated (non-typeable) strains; among the former, the six capsular types include types a through f [2, 3]. *H. influenzae* type b (Hib) has been particularly responsible for serious diseases, such as meningitis, epiglottitis and septicaemia [4].

In the USA, the first conjugate Hib vaccine was approved in 1987 and introduced to the vaccination schedule in the early 1990s, achieving an extensive reduction in the annual number of bacterial meningitis cases caused by Hib [5]. The Hib vaccine has now been adopted by over 190 countries worldwide (http://www.who.int/mediacentre/factsheets/fs378/en/), and Hib infection has nearly been eradicated.

Before the introduction of the Hib vaccine in Japan, 54.8 to 67.8 % of bacterial meningitis cases involving patients under 16 years of age were caused by Hib, according to a nationwide surveillance questionnaire study performed from 2001 to 2012 [6]. Hib vaccination of children was approved by the Japanese Ministry of Health, Labour and Welfare in 2007, which was later than in many other countries, introduced on a voluntary basis in 2008, and routinely subsidized by the government from late 2010. Since this initiative, the number of meningitis cases caused by Hib has decreased significantly [7].

In contrast to Hib, non-typeable *H. influenzae* (NTHi) continues to be isolated not only from paediatric, but also from elderly, populations. NTHi colonizing the pharynx is among the causes of acute otitis media (AOM) and upper and lower respiratory tract infections, including sinusitis and...
pneumonia [8]. At Japanese clinics, culture and/or PCR are not always performed to identify the causative pathogens for AOM, unless a patient fails to respond to initial antibiotic treatment, or the treating physician is a participant in a surveillance study. The recurrence of AOM and prolonged treatment are particular problems in Japan, where β-lactamase-negative ampicillin-resistant (BLNAR) isolates are frequently observed [9].

Although several studies have shown the considerable genetic diversity of NTHi using multilocus sequence typing (MLST) [10–13], we know of no previous study on the genetic characteristics and β-lactam resistance of H. influenzae from multiple infection sites in Japan.

We therefore examined the relatedness of the genetic characterization by MLST and the β-lactam resistance identified by real-time PCR among circulating isolates of H. influenzae from Japanese paediatric patients diagnosed with pneumonia, AOM, or meningitis, and sought to identify any genotypic differences among isolates from these three diseases.

**METHODS**

**Patients and strains**

Pathogens in pneumonia and AOM cases were isolated from clinical samples at our previous laboratory (Kitasato Institute, Kitasato University). Nasopharyngeal swab or sputum samples from patients diagnosed with pneumonia, and middle ear fluid from patients diagnosed with AOM, were obtained by physicians actively participating in each nationwide surveillance group [14, 15]. Diagnoses of pneumonia were confirmed by treating physicians on the basis of symptoms and clinical findings, blood test results and chest radiography. For AOM, diagnoses were confirmed based on inflammatory signs on inspection of the tympanic membrane [16]. For samples from pneumonia and AOM cases, bacterial cultures were obtained, and comprehensive real-time PCR was conducted to determine the causative pathogens – H. influenzae, Streptococcus pneumoniae, Mycoplasma pneumoniae, Streptococcus pyogenes and others – immediately following sample arrival at our laboratory. Real-time PCR was carried out using a method described previously [17]. Sixty-one H. influenzae isolates from pneumonia were sent from five different clinics and general hospitals in four prefectures in 2005. In AOM, 68 H. influenzae isolates were sent from 27 clinics and hospitals in 15 prefectures in 2008. The patient age for pneumonia ranged from 1 month to 12 years, (median=3 years); 55.9 % of patients were male. In AOM, the ages ranged from 7 months to 8 years (median=2 years); 49.2 % of patients were male.

Meanwhile, H. influenzae isolates from meningitis were collected from multiple medical institutions participating in the Working Group of Nationwide Surveillance for Bacterial Meningitis (1999–2012) based at our laboratory. Among the strains sent from 45 institutions in 20 prefectures, 27 strains sent in 2000 and 35 strains sent in 2011 were selected randomly with the aim of comparing the 2 periods. The patient age ranged from 1 months to 6 years (median=1 year) for 2000 and from 2 months to 4 years (median=1 year) for 2011. The gender of patients was not specified in the available data.

Isolates identified as H. influenzae by PCR targeting the P6 gene [18] and X and V factor testing were subjected to capsular typing by slide agglutination with antisera (Difco Laboratories, Detroit, MI, USA). All isolates from pneumonia and AOM were NTHi, except for three isolates of Hib from AOM, while all isolates from meningitis were identified as Hib, except for a single NTHi.

All strains were stored in 10 % skim milk medium at −80 °C until use. At the time of study, the isolates were cultured overnight on chocolate II agar plates (Nippon Becton-Dickinson, Tokyo, Japan) at 37 °C in an atmosphere containing 5 % CO₂. After incubation, a single colony was picked up from each plate and suspended in lysis solution (25 µl/tube; solution composition as described previously [19]).

**Genotypic β-lactam resistance**

PCR was used for molecular characterization of β-lactam resistance in all strains. Genes amplified were blatem-1, which encodes TEM-1 β-lactamase, and mutated ftsI, which encodes penicillin-binding protein 3 (PBP3). The latter protein mediates septum formation during cell wall synthesis. An H. influenzae PCR detection kit (Wakunaga Pharmaceutical, Osaka, Japan) was used according to the supplier’s instructions. Genotypic (g) resistance patterns were classified into six types: β-lactamase-negative ampicillin (AMP)-susceptible (gBLNAS); β-lactamase-positive AMP-resistant (gBLPAR); β-lactamase-negative AMP-resistant (gBLNAR); β-lactamase-negative AMP intermediate-resistant (gLow-BLNAR); and β-lactamase-positive amoxicillin/clavulanic acid-resistant (gBLPACR-I/gBLPACR-II) [20].

**Multilocus sequence typing (MLST)**

MLST was performed for all 191 strains by amplifying and sequencing the following genes: adk, atpG, frdB, fucK, mdh, pgI and recA. Primer sets corresponding to these seven housekeeping genes were constructed with reference to Meats et al. [8]. Because of the initial failure to amplify some required DNA fragments, new primers were designed for atpG-s (5’-GCAAGACTTAATAAAAACGG-3’), frdB-s (5’-GCTAATCCAGATATGAATG-3’) and pgI-s (5’-CTTTCAACATTTGCCAAC-3’). PCR was carried out as previously described [8].

Each sequence obtained for these housekeeping genes was queried on the H. influenzae MLST database website (https://pubmlst.org/hinfluenzae/), and each unique allelic profile as well as sequence type (ST) was assigned accordingly. Phylogenetic analysis was performed using eBURST v3, which is available at http://haemophilus.mlst.net/eburst. The novel STs identified in the study were newly posted to the MLST database website.
RESULTS

Pneumonia

Fig. 1 shows the correlation between the genotypic resistance and phylogenetic analysis of 61 isolates from patients with pneumonia. Sixty-one isolates were classified as either gBLNAS ($n=16$; 26.2 %), gBLNAR ($n=37$; 60.7 %), or gLow-BLNAR ($n=8$; 13.1 %). The isolates represented 40 different STs, of which 15 STs (distributed among 16 isolates) were newly identified in this study. Of the 16 isolates assigned to the 15 novel STs (ST1633–ST1656), 11 (68.8 %) were $\beta$-lactam-resistant. Although ST107 ($n=7$; 11.5 %) and ST57 ($n=6$; 9.8 %) were relatively frequent, the remaining 31 STs (50.8 %) only had one isolate assigned to each. The phylogenetic analysis by eBURST generated 10 clonal groups within the pneumonia isolates. Of these 10 groups, 4 groups (ST54–ST1651, ST411–ST1633, ST393–ST1646 and ST1637–1648) showed 100 % uniformity of $\beta$-lactam resistance type. The remaining groups had varied $\beta$-lactam resistance characterizations. The gBLNAR strains were mostly found in the ST107-ST1634 group ($n=8$) and in singletons such as ST156 ($n=4$). As described below, ST54 was predominant in Hib isolates from meningitis, but ST54 found in pneumonia was NTHi. No association was found between STs and the geographical location, age, or gender of patients.

AOM

Fig. 2 shows the correlation between the genotypic resistance and phylogenetic analysis of 68 isolates from patients with AOM. These isolates were genotypically classified as gBLNAS ($n=29$; 42.7 %), gBLNAR ($n=33$; 48.5 %), gLow-BLNAR ($n=3$; 4.4 %) and gBLPACR-II ($n=3$; 4.4 %). Forty-four different STs were detected, including 8 novel STs (among 11 isolates). gBLNAR strains were found in the ST991–ST1641 group ($n=4$) and in singletons such as ST161 ($n=4$) and ST396 ($n=3$). Three isolates identified as Hib were either ST54 ($n=2$) or ST190 ($n=1$). The clinical severity of infection with these Hib isolates was unknown because of the limitations of the database searching.

Meningitis

Fig. 3 shows the genotypic resistance and phylogenetic analysis of 62 isolates from patients diagnosed with meningitis. These isolates showed a diverse distribution of genotypes.
Fig. 2. Analysis by eBURST of 68 isolates from patients diagnosed with AOM. Blue, gBLNAS; red, gBLNAR; yellow, gLow-BLNAR; green, gBLPACR-II. Singletons encompassing three or more isolates (ST 549, ST161, ST407 and ST396) show uniformity in their classification of β-lactam resistance.

Fig. 3. Analysis by eBURST of 62 isolates from patients diagnosed with meningitis. A single NTHi from this group was identified as ST474. The STs of all Hib are identical at five or more of seven loci, indicating one clonal group. Orange, gBLPAR; blue, gBLNAS; red, gBLNAR; yellow, gLow-BLNAR; purple, gBLPACR-I; green, gBLPACR-II.
for β-lactam resistance: gBLNAS (21.0% in 2000, n=10; 2011, n=3); gBLPAR (16.1% in 2000, n=10; 2011, n=0); gBLNAR (29.0% in 2000, n=4; 2011, n=14); gLow-BLNAR (25.8% in 2000, n=9; 2011, n=7); gBLPACR-I (3.2% in 2000, n=2; 2011, n=0); and gBLPACR-II (4.8% in 2000, n=0; 2011, n=3). Only eight STs, including two novel STs, were found among these isolates. ST54 was the most common (n=39; 62.9%), followed by ST190 (n=12; 19.4%). Excluding an NTHi identified as ST474, the seven STs among all Hib were identical with other STs at five or more of the seven loci, placing them in the same clonal group. No clinical implications were evident for the single patient from whom the only NTHi (ST474) was isolated, since limited information was available.

As of 23 May 2017, the H. influenzae MLST database website had 689 Hib isolates, for which 7 clonal groups had been identified by eBURST. The largest clonal group of these 7 groups is depicted in Fig. S1 (available in the online version of this article); this group consists of 151 different STs, including all of the STs identified in our study. The eBURST diagram placed ST6, including the largest number of isolates overall, at the centre of the group, while it appeared that the other STs might be derived from ST6. ST54, the most frequently detected ST in our study, was a double-locus variant derived from ST6, which has been reported in the USA as well as Japan according to the database.

### Table 1. Number of H. influenzae isolates in various STs according to clinical diagnosis

<table>
<thead>
<tr>
<th>Sequence type (ST)</th>
<th>No. of isolates</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pneumonia</td>
<td>Acute otitis media</td>
</tr>
<tr>
<td>6</td>
<td>3 3</td>
<td>3</td>
</tr>
<tr>
<td>54</td>
<td>2 2</td>
<td>39 43</td>
</tr>
<tr>
<td>57</td>
<td>6 1</td>
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<td>95</td>
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<tr>
<td>107</td>
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<td>7</td>
</tr>
<tr>
<td>142</td>
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<td>3</td>
</tr>
<tr>
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</tr>
<tr>
<td>1642</td>
<td>34 33</td>
<td>4 71</td>
</tr>
<tr>
<td>Total no.</td>
<td>61 68</td>
<td>62 191</td>
</tr>
</tbody>
</table>

STs encompassing two or fewer isolates are included under ‘others’. Blank spaces indicate the absence of isolates from that disease.

#### Comparison of STs by disease

STs represented by a total number of three or more isolates from pneumonia, AOM and/or meningitis are summarized in Table 1. STs with two or fewer isolates are combined in an ‘others’ category. MLST showed genetic diversity, with 79 different STs in 191 strains, especially among NTHi from pneumonia and AOM.

#### DISCUSSION

In this study, we analysed the molecular characteristics of H. influenzae obtained from three major diseases among children, as well as the phylogenetic relatedness of β-lactam resistance. Phylogenetic analysis of isolates from patients with pneumonia and AOM showed high diversity, in agreement with previous reports [12, 13, 21]. On the other hand, the phylogenetic analysis of isolates from patients with meningitis, which were all Hib except for a single NTHi, showed a limited number of STs and considerable relatedness between STs.

Commonly colonizing the upper respiratory tract [2], NTHi is likely to be exposed to multiple oral antibiotics, such as β-lactams, macrolides and quinolones. In Japan, third-generation cephalosporins have been widely prescribed for the treatment of community-acquired respiratory tract infections, while amoxicillin/clavulanate is the first-line therapy in the USA and Europe [1, 18]. With exposure to multiple antibiotics, especially third-generation oral cephalosporins, NTHi continues to undergo selection, as demonstrated by the diverse STs of NTHi. Particularly in Japan, when mutation occurs in the ftsI gene encoding penicillin-binding protein 3, the strain is likely to acquire β-lactam resistance, which leads to an increase in the number of BLNAR strains [22, 23]. Most cepham antibiotics have shorter half-lives and less post-antibiotic effect than other classes of antibiotics. Because clinical effectiveness is sometimes not fully achieved, BLNAR strains surviving the selection pressure from cephalosporins are able to colonize the respiratory tract and then be transmitted from person to person, causing recurring disease, such as AOM.

Unlike NTHi, Hib isolated from patients diagnosed with meningitis is not ordinarily an indigenous pathogen, and has much less potential exposure to oral antibiotics. Only 3 to 5% of children were colonized with Hib in the pre-Hib vaccine era, and the rate has fallen to nearly zero after the initiation of Hib vaccine [2]. Although resistant Hib mutants are also selected by pressure from antibiotic agents, the frequency of mutation seems to be lower than for NTHi. Upon comparing the STs of strains isolated from patients with meningitis in 2000 and in 2011, ST54 continued to be the most frequently detected ST in both years. Hib is therefore believed to have mutations in genes such as ftsI, but far fewer involving housekeeping genes, as evidenced by the fact that there is far less diversity of STs.

In this study, we found 14 novel allelic profiles (pneumonia, 9; AOM, 4; meningitis, 1) and 25 novel STs (pneumonia, 15;
AOM, 8; meningitis, 2) that we newly registered in the MLST database. This pattern also suggests that mutations were more frequent among NTHi than Hib. Presently, with Hib conjugate vaccine being in wide use, we noted a drastic reduction in the incidence of life-threatening Hib infections. The Hib vaccination rate within the Japanese paediatric population in 2014 was greater than 98 % according to the Japanese Ministry of Health, Labour, and Welfare (http://www.mhlw.go.jp/topics/bcg/other/5.html). We now rarely see Hib infections in Japan. However, non-type b \textit{H. influenzae} and NTHi remain important causative pathogens, as expected. The capsule is a virulence factor that is important in evading host immune defences. NTHi is therefore considered to be less virulent. NTHi nonetheless causes local respiratory infections, such as AOM in children, bronchitis or sinusitis in adults, or pneumonia in individuals with comorbidities [24]. Andersson et al. reported an invasive \textit{H. influenzae} outbreak involving elderly residents as well as staff members at a long-term care facility in Sweden [25]. All strains except for one were identified as NTHi belonging to ST14. This outbreak resulted in the hospitalization of some elderly residents for pneumonia and bacterial bronchitis, with one fatality. The Swedish report thus suggests that certain NTHi strains possess characteristics conveying high virulence.

Other studies [26, 27] have reported BLNAR strains in clusters of ST14 and ST367. Although our study only included one ST14 isolate and no ST367 isolates, we noted a similar relatedness of \(\beta\)-lactam resistance to ST and clonal group.

One limitation of our study was the small number of isolates detected in each ST; nearly 50 % of isolates in both the pneumonia group and the AOM group had no other isolate in their ST. Another limitation was the fact that we studied isolates from different years, because surveillances were carried out separately for pneumonia and AOM, with a 3-year gap. While a larger number of isolates from the same year would be desirable, the comparison of isolates associated with different clinical diagnoses in this study showed reasonable variation in genetic characteristics.

In conclusion, Hib vaccination has nearly eradicated life-threatening diseases caused by Hib in developed countries where Hib vaccine is widely used; however, prolonged treatment and the recurrence of diseases caused by resistant non-type b or non-typeable \textit{H. influenzae} strains underscore the need to develop effective vaccines against NTHi and non-type b infections.

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