



Clinical and Bacteriologic Analysis of Nontypeable *Haemophilus influenzae* Strains Isolated from Children with Invasive Diseases in Japan from 2008 to 2015

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ABSTRACT *Haemophilus influenzae* type b (Hib) conjugate vaccines have led to dramatic reductions in Hib disease among young children worldwide. Nontypeable *H. influenzae* (NTHi) is now the major cause of invasive *H. influenzae* infections. We investigated the clinical characteristics of invasive NTHi diseases among children in Japan, to clarify the pathogenicity of isolated NTHi strains. The mortality rate was 10.7%, with deaths occurring mainly among children with underlying comorbidities. Biotypes II and III were the most common, and most strains (64.3%) had multiple amino acid substitutions at the Asp-350, Ser-357, Ser-385, and/or Met-377 sites of penicillin-binding protein 3. Two strains were β -lactamase positive and ampicillin-clavulanate resistant. Biofilm indices varied widely, and IS1016 was detected in 10.7% of the strains tested. Moreover, there was wide variation in the characteristics of invasive NTHi strains. NTHi strains, showing great genetic diversity, are responsible for most invasive *H. influenzae* infections in children in the postvaccine era. Continuous monitoring of NTHi strains responsible for invasive diseases in children is important to detect changes in the epidemiology of invasive *H. influenzae* infections in the postvaccine era.

KEYWORDS nontypeable *Haemophilus influenzae*, infection, invasive disease, epidemiology, children, Japan

Haemophilus influenzae is a small Gram-negative coccobacillus that commonly colonizes the human upper respiratory tract and causes both invasive and noninvasive diseases in children (1). It is classified into 7 groups, i.e., 6 chemically distinct polysaccharide capsular groups (serotypes a to f) and 1 group called nonencapsulated or nontypeable *H. influenzae* (NTHi). Among the encapsulated strains, *H. influenzae* type b (Hib) is the most virulent and causes most cases of invasive *H. influenzae* disease (2). In contrast, NTHi strains mainly cause noninvasive diseases (otitis media, bronchitis, and pneumonia) in healthy children, although they can also cause invasive disease, mostly in older adults and people with comorbidities (3). A substantial burden of perinatal invasive NTHi infections has been reported. Although rare, these infections can result in serious morbidity and death among pregnant women and neonates (4). Among neonates, NTHi infections are 10 times more common than Hib infections; most cases are associated with preterm birth and occur in the first week of life (5).

The introduction of Hib conjugate vaccines has dramatically decreased the incidence of invasive Hib disease globally (2, 3, 6, 7). Consequently, invasive *H. influenzae* infections are now mainly caused by NTHi (4, 7–9). Seven surveillance studies of *H.*

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influenzae infections in the post-Hib-vaccine era have shown clear increases in the numbers and incidence rates of invasive NTHi infections (10). Therefore, we should be aware of the virulence potentials of these strains.

In Japan, the Hib vaccine has been available on an optional basis since December 2008, and it was included in the routine immunization program for children under 5 years of age in April 2013. Since the subsidization of the Hib vaccine, the number of invasive Hib disease cases among children has decreased dramatically (11). However, invasive disease caused by NTHi, although infrequent, has been reported. The bacteriologic and epidemiologic characteristics of the NTHi strains responsible for invasive disease are poorly defined. Our study aimed to clarify the clinical impact of invasive NTHi disease in children and to determine the pathogenicity of NTHi strains isolated from patients with invasive disease.

MATERIALS AND METHODS

Bacterial isolates. Between 2008 and 2015, a total of 28 *H. influenzae* strains, isolated from invasive sources such as blood and cerebrospinal fluid (CSF), were collected and sent to the National Institute of Infectious Diseases and Chiba University Hospital from hospitals in 8 distantly located prefectures in Japan. Of those strains, 21 were collected via a population-based surveillance study of invasive *H. influenzae* disease performed in 10 prefectures in Japan, including Chiba prefecture (12). Strains were identified as *H. influenzae* on the basis of colony morphology and dependence on X and V growth factors that do not lyse horse blood cells. The strains also underwent matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) at Chiba University Hospital (MALDI Biotyper Microflex LT; Bruker Daltonics, Bremen, Germany), further confirming them to be *H. influenzae* (13). All strains were stored at -80°C and, prior to analysis, were cultivated overnight at 37°C in 5% CO_2 on BY chocolate agar (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 1% IsoVitalX with 10 $\mu\text{g}/\text{ml}$ hemin and 10 $\mu\text{g}/\text{ml}$ β -NAD (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan). DNA templates of these strains were prepared using a QIAamp DNA minikit (Qiagen, Hilden, Germany), unless otherwise noted.

Clinical data. Patients' clinical data, collected from the treating doctors in each hospital, were deidentified at each hospital before further analysis. The investigation findings required to confirm diagnoses were as follows: (i) meningitis, isolation of *H. influenzae* from CSF samples; (ii) pneumonia, features of consolidation on chest radiographic images and isolation of *H. influenzae* from blood samples; (iii) sinusitis, opacification evident on paranasal sinus radiographic images and isolation of *H. influenzae* from blood samples; (iv) peritonitis, isolation of *H. influenzae* from both blood and ascitic fluid samples. When *H. influenzae* was isolated from a blood sample but the clinical symptoms, examination findings, and investigation results were unable to reveal the site of infection, the patient was categorized as having bacteremia.

Serotyping and biotyping. Serotypes of the *H. influenzae* isolates were determined by slide agglutination testing using antisera (Denka Seiken Co., Ltd., Tokyo, Japan) to the bacterial polysaccharide antigens to identify capsular types a, b, c, d, e, and f. We confirmed the results using a PCR assay, according to the method reported by Falla et al. (14). For these analyses, ATCC strains such as ATCC 9327 (serotype a), ATCC 9334 (serotype b), ATCC 9007 (serotype c), ATCC 9332 (serotype d), ATCC 8142 (serotype e), and ATCC 9833 (serotype f) were used as controls for encapsulated *H. influenzae*. ATCC 35056 was used as a control for NTHi. Biotype analysis was performed using in-house media for urease, indole, and ornithine decarboxylase reactions, as described previously (15). These results were confirmed using a commercial kit (ID Test HN-20 Rapid kit; Nissui Pharmaceutical Company, Ltd., Tokyo, Japan).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed for all *H. influenzae* strains using the broth microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (16). β -Lactamase production was detected using a disc impregnated with nitrocefin (Cefinase paper discs; Nippon Becton Dickinson Company, Ltd., Tokyo, Japan).

The genetic profiles of penicillin-binding protein 3 (PBP-3) were investigated to identify mutations, using PCR and sequence analysis. Briefly, a single colony was used to extract DNA, and PCR was performed using the primers fts11F (GTTTCCCAGTCACGACGTTGTAGTTAATGCGTAACCGTGCAATTAC) and fts11R (TTGTGAGCGGATAACAATTCCACCCTAATGCATAACGAGGATC). The PCR products were then purified and subjected to sequence analysis using the primers SeqftsF (GTTTCCCAGTCACGACGTTGTGTA) and SeqftsR (TTGTGAGCGGATAACAATTTC), using an ABI Prism 3130 genetic analyzer.

The β -lactamase gene TEM-1 was detected using a commercially available kit (Wakunaga Seiyaku Kabushikigaisha, Hiroshima, Japan). Strain Rd KW20, which is known to have no alteration in PBP-3, was used as a control. Genetically β -lactamase-negative ampicillin-resistant (BLNAR) strains were divided into subgroups, i.e., group I/II, with R517H/N526K substitutions, and group III, with M377I, S385T, and L389F substitutions in addition to N526K substitutions. Group II was further divided into subgroups a to d, based on substitutions at amino acids 502 and/or 449. Strains with M377I, S385T, and L389F substitutions and an R517H substitution rather than an N526K substitution were categorized as group III-like, based on the findings of previous studies (17, 18).

Microtiter biofilm assay. The ability to form biofilm was assessed for all 28 strains using a quantitative biofilm assay in a microtiter polystyrene plate, as described previously (19). Briefly, the bacterial cells were grown overnight at 37°C in brain heart infusion broth with 10 $\mu\text{g}/\text{ml}$ hemin and 10 $\mu\text{g}/\text{ml}$ β -NAD. Overnight cultures (5 μl for each strain) were inoculated into a 96-well, flat-bottom,

TABLE 1 PCR primers used for amplification of adhesin genes

Target gene or locus	GenBank accession number(s)	Primer name ^a	Sequence (5' to 3')	Size of amplicon (bp)	Reference or source
<i>hifA</i>	AF020909-1	hifA-F hifA-R	ATGAAAAAACACTWCTTGGTAGC TTATYCGTAAGCAATTKGGAAATC	624–642	Clemans et al. (49)
<i>hmw1A/hmw2A</i>	U08876, AY497552	hmw-F hmw-R	ATGAACAAGATATATCGTCTC CCGTGATTCACAATTTTCAGC	602	This study
<i>hia/hsf</i>	U38617	hia/hsf-F hia/hsf-R	TCCACCGATGCGATTAACGG TGCAACGCCTGTTTTACCTTG	315	This study
<i>Hia</i>	NC_000907	Hia-F Hia-R	CCGAAAGCACAATGGATATGGACG GATAAATCCTGACCTCGTCTC	6,235	Satola et al. (33)
<i>Hap</i>	U11024	hap-F hap-R	GATGATGTCGGGTTTTGCC CACGCCACATTTTGCTGTT	532	This study
Fimbrial gene	L08448	Fimb-F Fimb-R	GAATACCAATGGCTAACTCGCG ATTTCTACACGACGGTCTGGAG	512	This study

^aF and R correspond to the forward and reverse directions, respectively.

microtiter polystyrene plate (Becton Dickinson, Franklin Lakes, NJ) containing 200 μ l of brain heart infusion broth with hemin and β -NAD, at 37°C. After 18 h of incubation, each well was washed with distilled water. Biofilm formation was visualized by staining with 0.5% crystal violet for 5 min. Two hundred microliters of 95% ethanol was added, and the biofilm was quantified in an enzyme-linked immunosorbent assay plate reader (iMark microplate reader; Bio-Rad) at 570 nm. The biofilm index was defined using the average optical density at 570 nm (OD₅₇₀). All strains were tested in triplicate, and the results are reported as the averages of results from three different experiments.

Detection of adhesin genes. Using PCR assays, NTHi strains were evaluated for possession of the following five adhesin genes: *hmw1A/hmw2A*, *hia*, *hap*, *hifA*, and *p5* (20). The primer sequences used and the PCR conditions are shown in Table 1. Amplification reactions were performed using a TProfessional Basic thermocycler (Biomera GmbH, Göttingen, Germany). The products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized with UV transillumination. Primers were designed from reference strains (as shown in Table 1) by using software (NCBI Primer-BLAST), and the primer sequences were compared with the genome sequences of approximately 10 NTHi strains registered in the NCBI database to confirm that no mutations were found at the primer sites. For the *hmw*, *hia/hsf*, *hap*, and *p5* genes, PCR results, both positive and negative, were confirmed using another set of primers at different sites of the targeted genes (data not shown). R2866 was used as a positive control for the *hia/hsf*, *hap*, *hifA*, and *p5* genes, and R2846 was used as a positive control for *hmw*. Purified water was used as a negative control.

Multilocus sequence typing. PCR of seven housekeeping genes (*adhA*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA*) was performed as described previously (21). The PCR products were then sequenced using an ABI Prism 3130 genetic analyzer, and the multilocus sequence typing (MLST) website (www.mlst.net) was used to determine the allele number and sequence type (ST) of each isolate. New allelic profiles were submitted to the database for assignment. The relatedness of the isolates was determined by constructing a gene tree with the neighbor-joining method, using MEGA7. The eBURST v3 program was used to determine the clonal complex (CC). Strains were considered to be in the same CC when at least five of the seven loci matched.

Screening for the insertion element IS1016. PCR was performed for all 28 strains as described previously (22). Amplification reactions were performed using a MyCycler thermocycler (Bio-Rad).

Whole-genome sequencing and sequence analysis. Fourteen draft genome sequences were generated using a MiSeq system (Illumina, San Diego, CA, USA). Genomic DNA was purified with phenol-chloroform extraction. The Nextera DNA sample preparation kit (Illumina) was used to prepare fragmented genomic DNA libraries. The quantities of the libraries were validated with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a Quant-iT Pico Green double-stranded DNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing was performed with a MiSeq system (Illumina), using MiSeq reagent kit v2 (500 cycles; Illumina), according to the manufacturer's instructions. All Illumina data sets were cleaned using Trimmomatic v0.33 (23). The draft genome sequences were assembled using Platanus v1.2.4 (24), with default parameters. Prokka v1.11 software was used for gene prediction and annotation (25). Homologous genes for IS1016, adhesin genes (*hia/hsf* and *hifA*), *cap* genes (*capA* to *capF*), and *bexDCBA* in *H. influenzae* were searched for using BLASTN (v2.2.28+) or BLASTP (26).

Statistical analysis. Statistical analysis of biofilm index values was performed using the two-tailed chi-square test for independence and the Mann-Whitney *U* test, using SPSS 17.0 (SPSS Japan Inc.). *P* values of <0.05 were considered significant.

Ethical considerations. This study was approved by the Chiba University Ethics Committee (approval no. 666). The invasive *H. influenzae* disease surveillance study involving the 10 prefectures was reviewed and approved by the Mie Hospital Ethics Committee and was conducted according to the principles expressed in the Declaration of Helsinki (12).

Accession number(s). Sequence reads were assembled as contigs by *de novo* assembly. All draft genome sequences were deposited in the DNA Data Bank of Japan under accession numbers BGMN01000001 to BGMN01000048, BGMO01000001 to BGMO01000058, BGMP01000001 to BGMP01000045, BGMQ01000001 to BGMQ01000053, BGMR01000001 to BGMR01000043, BGMS01000001 to BGMS01000024,

TABLE 2 Clinical backgrounds of children with invasive nontypeable *Haemophilus influenzae* diseases in Japan

Strain no.	Yr of isolation	Patient age	Prefecture	Underlying disease(s) ^a	Hib vaccination (no. of doses) ^b	Clinical diagnosis	Disease outcome
1	2008	1 yr	Kagoshima	None	0	Bacteremia	No complications
2	2008	10 yr	Kochi	Reye's syndrome	0	Pneumonia	No complications
3	2010	0 mo	Chiba	None	0	Pneumonia	No complications
4	2010	2 yr	Fukuoka	Seckel syndrome	0	Bacteremia	Died
5	2010	0 mo	Fukuoka	Preterm, LBW	0	Bacteremia	No complications
6	2010	14 yr	Chiba	None	0	Bacteremia	No complications
7	2010	1 yr	Chiba	None	0	CPAOA	Died
8	2010	4 yr	Fukuoka	None	0	Bacteremia	No complications
9	2011	1 mo	Chiba	None	0	Bacteremia	No complications
10	2011	5 yr	Kagoshima	Acute lymphocytic leukemia	0	Bacteremia	No complications
11	2011	1 yr	Chiba	None	1	Pneumonia	No complications
12	2012	1 yr	Nagasaki	Long QT syndrome	3	Bacteremia	Died
13	2012	7 mo	Fukuoka	Infant hepatitis, PA	2	Pneumonia	No complications
14	2012	4 yr	Fukuoka	PA, VSD, major aortopulmonary collateral artery	1	Pneumonia	No complications
15	2012	1 yr	Kagoshima	None	1	Meningitis	No complications
16	2012	7 mo	Fukuoka	None	3	Pneumonia	No complications
17	2012	2 yr	Chiba	None	0	Sinusitis	No complications
18	2012	8 mo	Fukuoka	None	0	Bacteremia	No complications
19	2012	10 yr	Chiba	None	0	Sinusitis	No complications
20	2012	1 yr	Okinawa	None	1	Bacteremia	No complications
21	2012	1 yr	Okinawa	None	4	Bacteremia	No complications
22	2013	8 yr	Chiba	Chromosomal abnormality, hydrocephalus	1	Peritonitis	No complications
23	2013	0 mo	Tokyo	Extremely LBW	0	Bacteremia	No complications
24	2013	11 mo	Tokyo	None	3	Bacteremia	No complications
25	2014	1 yr	Shizuoka	None	4	Pneumonia	No complications
26	2014	4 yr	Chiba	None	1	Pneumonia	No complications
27	2015	3 yr	Chiba	Preterm, LBW, jawless, tracheotomy	4	Pneumonia	No complications
28	2015	11 mo	Chiba	None	3	Pneumonia	No complications

^aLBW, low birth weight; PA, pulmonary atresia; VSD, ventricular septal defect; CPAOA, cardiac pulmonary arrest on arrival.

^bNumber of doses received.

BGMT01000001 to BGMT01000039, BGMU01000001 to BGMU01000034, BGMV01000001 to BGMV01000048, BGMW01000001 to BGMW01000083, BGMX01000001 to BGMX01000061, BGMY01000001 to BGMY01000061, BGMZ01000001 to BGMZ01000051, and BGNA01000001 to BGNA01000050.

RESULTS

Clinical characteristics. The patients' clinical characteristics are shown in Table 2. Four patients were neonates, and the other 24 ranged in age from 7 months to 14 years. Excluding the neonates, 33.3% of the patients (8/24 patients) had underlying diseases. Ten patients (35.7%) had pneumonia and only 1 patient, from Kagoshima prefecture, had meningitis. Three (10.7%) patients died, as follows. One patient suffered cardiac arrest on arrival at the hospital. The second patient was known to have long QT syndrome, but it was unclear whether the cause of death was arrhythmia or invasive NTHi infection. The third patient had a fever and depressed level of consciousness on admission; the patient suffered cardiac arrest soon after treatment was started. The other 25 patients, including the patient with meningitis, survived without any complications. Except for the NTHi strain isolated from the CSF, all strains were isolated from blood samples. The strains were isolated from more than one hospital in each prefecture. Some prefectures (for example, Chiba and Tokyo prefectures) are located next to each other, while others are distantly located. We could not find any specific characteristics depending on the area of isolation. Excluding 4 neonates and 4 patients with missing information, 75% of the patients (15/20 patients) were known to use childcare services or were schoolchildren.

Serotyping and biotyping. None of the 28 isolates demonstrated agglutination with capsule-typing antisera. Moreover, all isolates analyzed by PCR lacked both *bexA* and capsule-specific genes (defined to be NTHi in this study). Among the 4 NTHi strains

TABLE 3 Antimicrobial susceptibilities and β -lactamase production of invasive nontypeable *Haemophilus influenzae* strains isolated from children

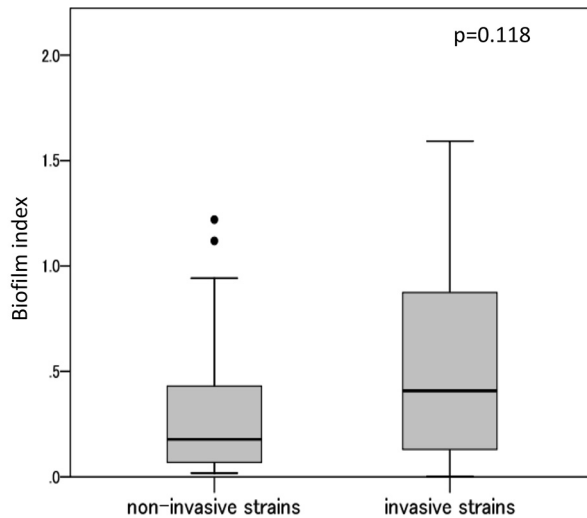
Strain no.	MIC ($\mu\text{g/ml}$) of ^a :							β -Lactamase production ^b	Amino acid substitutions in PBP-3						Group	
	ABPC	AMC	CTX	CRO	MEM	CLR	LVX		D350	S357	M377	S385	L389	R517		N526
1	1	1/0.5	0.5	0.25	≤ 0.06	8	≤ 0.06	–	N	N	I	T	F		K	III
2	1	0.5/0.25	0.25	≤ 0.06	≤ 0.06	4	≤ 0.06	–	N	N		T		H		III-like
3	0.25	0.25/0.12	≤ 0.06	≤ 0.06	≤ 0.06	4	≤ 0.06	–								
4	1	1/0.5	0.5	0.25	0.12	1	≤ 0.06	–	N	N	I	T	F		K	III
5	0.25	0.25/0.12	≤ 0.06	≤ 0.06	≤ 0.06	8	≤ 0.06	–								
6	≤ 0.12	0.25/0.12	≤ 0.06	≤ 0.06	≤ 0.06	4	≤ 0.06	–								
7	0.25	0.5/0.25	≤ 0.06	≤ 0.06	≤ 0.06	8	≤ 0.06	–								
8	≤ 0.12	0.25/0.12	≤ 0.06	≤ 0.06	≤ 0.06	8	≤ 0.06	–								
9	≥ 16	8/4	0.5	0.25	0.25	16	≤ 0.06	+	N	N	I	T	F		K	III
10	2	2/1	0.5	0.25	0.25	8	≤ 0.06	–	N	N	I	T	F		K	III
11	4	8/4	1	0.25	0.25	8	≤ 0.06	–	N	N	I	T	F		K	III
12	2	2/1	1	0.25	≤ 0.06	16	≤ 0.06	–	N	N	I	T	F		K	III
13	1	1/0.5	0.25	0.12	0.12	8	≤ 0.06	–	N	N	I	T	F		K	III
14	1	1/0.5	0.5	0.12	≤ 0.06	8	≤ 0.06	–	N	N	I	T	F	H		III-like
15	0.25	0.5/0.25	≤ 0.06	≤ 0.06	≤ 0.06	4	≤ 0.06	–								
16	0.25	0.5/0.25	≤ 0.06	≤ 0.06	≤ 0.06	8	≤ 0.06	–								
17	1	1/0.5	≤ 0.06	≤ 0.06	0.12	16	≤ 0.06	–							K	II d
18	4	4/2	1	0.25	0.5	32	≤ 0.06	–	N	N	I	T	F		K	III
19	0.25	0.25	≤ 0.06	≤ 0.06	≤ 0.06	8	≤ 0.06	–								
20	2	2/1	0.5	0.25	0.5	8	0.12	–	N	N	I	T	F		K	III
21	2	2/1	0.5	0.12	≤ 0.06	8	≤ 0.06	–	N	N	I	T	F	H		III-like
22	1	1/0.5	0.12	≤ 0.06	0.25	4	≤ 0.06	–	N	N	I	T		H		III-like
23	1	1/0.5	0.12	≤ 0.06	0.25	8	≤ 0.06	–	N	N	I	T		H		III-like
24	1	1/0.5	0.25	≤ 0.06	≤ 0.06	4	≤ 0.06	–	N	N	I	T	F		K	III
25	0.25	0.25/0.12	≤ 0.06	≤ 0.06	≤ 0.06	16	≤ 0.06	–								
26	1	0.5/0.25	0.5	0.25	≤ 0.06	4	≤ 0.06	–	N	N	I	T	F		K	III
27	2	2/1	0.5	0.25	0.25	8	≤ 0.06	–	N	N	I	T	F		K	III
28	16	16/8	0.5	0.25	0.12	8	≤ 0.06	+	N	N	I	T	F		K	III

^aABPC, aminobenzylpenicillin; AMC, amoxicillin-clavulanic acid; CTX, cefotaxime; CRO, ceftriaxone; MEM, meropenem; CLR, clarithromycin; LVX, levofloxacin. ^b–, negative; +, positive.

isolated from neonates, 2 belonged to biotype II and the others were biotypes I and III. Of the other 24 isolates, 10 (41.7%) were biotype II, 9 (37.5%) were biotype III, 4 (16.7%) were biotype V, and 1 (4%) was biotype IV (see Fig. 2).

Antimicrobial susceptibility. The results of antimicrobial susceptibility testing are shown in Table 3. Two strains that showed high levels of aminobenzylpenicillin (ABPC) resistance (MICs of $\geq 16 \mu\text{g/ml}$) were β -lactamase-producing strains. PCR analysis showed that both strains were positive for TEM-1 (data not shown). These strains also showed multiple substitutions in PBP-3 and high resistance to amoxicillin-clavulanate; they were defined as β -lactamase-positive ampicillin-clavulanate-resistant (BLPACR) strains (Table 3). Only 2 strains, strains 11 and 18, were BLNAR strains (MICs of $\geq 4 \mu\text{g/ml}$). Most strains with ABPC MICs of $\geq 1 \mu\text{g/ml}$ had multiple amino acid substitutions in PBP-3 at Asp-350, Ser-357, Ser-385, and/or Met-377, which categorized them in group III or III-like gBLNAR strains (except for strain 17). Strain 17 also had an Ile-449-Val substitution in PBP-3, which categorized it in group II d. Group III and III-like strains had slightly higher MICs for cephalosporin antibiotics than did strains without any substitutions, but none of the strains met the criteria for resistance to cefditoren-pivoxil, cefotaxime (CTX), or ceftriaxone (CRO), as determined by the CLSI (MICs of $\geq 2 \mu\text{g/ml}$). Strains with ABPC MICs of $\leq 0.25 \mu\text{g/ml}$ showed no PBP-3 amino acid substitutions. All strains were sensitive to meropenem and levofloxacin. Twenty-three strains (85.7%) were sensitive to clarithromycin (MICs of $\leq 8 \mu\text{g/ml}$) (16).

Except for the patient with strain 7, who experienced cardiac arrest before arrival at the hospital for initiation of treatment, all of the patients were treated with antibiotics. The majority of the patients were treated with ABPC, ABPC-sulbactam (SBT), CRO, or CTX, which are antibiotics recommended in the Japanese guidelines for bacteremia (50). The patients with BLPACR strains (strains 9 and 28) were both successfully treated with CTX.



	Number of strains	Average biofilm formation	Standard deviation
Non-invasive strains	31	0.334	0.355
Invasive strains	28	0.520	0.431

FIG 1 Comparison of the biofilm indices of invasive and noninvasive nontypeable *H. influenzae* strains. Thirty-one noninvasive NTHi strains were collected from nasal cavities or sputum of children with respiratory infections (these strains were used only for biofilm assays). The boxplot shows the medians and 10th, 25th, 75th, and 90th percentiles; outliers are plotted as individual points. The biofilm index was defined using the average OD₅₇₀ value.

Biofilm formation. The biofilm index values of the NTHi strains isolated from patients with invasive diseases are shown in Fig. 1. Heterogeneity was observed, and the biofilm indices varied widely, from 0.006 to 1.592. The average biofilm formation of invasive strains was greater than that of strains isolated from sputum and nasal cavities of patients with noninvasive, community-acquired pneumonia; however, this difference was not statistically significant (Fig. 1).

Prevalence of adhesin genes. The PCR results showed 17 strains as being positive for *hmw* (60.7%) and 13 strains as being positive for *hia/hsf* (46.4%). The PCR products of strains 12, 17, 22, and 23 were shorter than expected. BLAST results were used to confirm that those strains had only partial segments of *hia/hsf*. In terms of the fimbrial gene, most strains (89.3%) possessed the *p5* gene, while only 9 (32.1%) harbored *hifA* (Fig. 2).

MLST. Molecular typing using MLST showed wide variety among the 28 isolates, which had 26 different STs (Table 4). Strains 1, 3, 7, 20, and 27 had new STs, which we submitted as ST-1736, ST-712, ST-1431, ST-1434, and ST-1444, respectively. Strain 10 lacked fuculokinase (*fucK*) and so could not be genotyped. eBURST analysis distributed the 28 isolates into 3 clonal groups, with 11 singletons. The largest clonal group, CC-3, included 9 isolates. CC-3 is also the largest CC of NTHi isolates in the database. Within CC-3, there were 8 STs, including 2 isolates of ST-3 and 1 isolate each of ST-11, ST-14, ST-145, ST-160, ST-436, ST-953, and ST-1017. The other 2 groups included 3 strains each, i.e., CC-107 (ST-107, ST-155, and ST-159) and CC-584 (ST-187, ST-584, and ST-597). Among the 11 singletons, the most frequent ST was ST-84, with 2 isolates. A phylogenetic tree based on MLST relatedness is shown in Fig. 2.

Insertion element IS1016. PCR was performed for all 28 strains; only 3 strains had the insertion element IS1016. In addition, whole-genome sequencing, which was performed for 14 isolates, was used to analyze the existence of IS1016. Of the 14 strains,

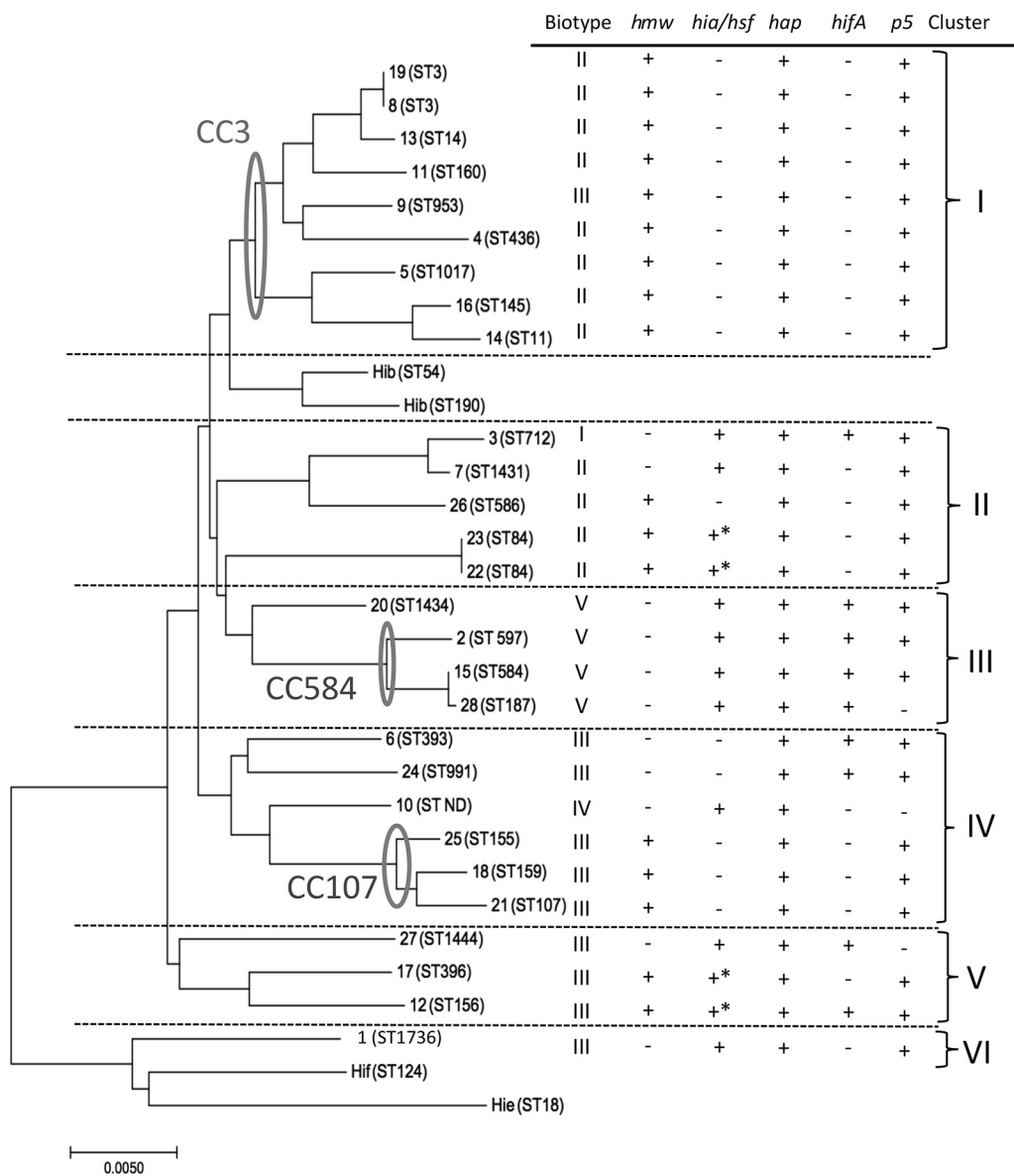


FIG 2 Genetic relatedness among invasive nontypeable *H. influenzae* isolates. The dendrogram shows the relationships of the sequence types of the 28 invasive NTHi strains. Including the biotypes and PCR results for the adhesin genes, the strains were divided into six clusters. The PCR products of strains 12, 17, 22, and 23 were shorter than expected. BLAST results were used to confirm that those strains had only partial segments of *hia/hsf*.

2 were positive for IS1016 and were also PCR positive. All 14 strains were negative for the *cap* and *bex* genes.

DISCUSSION

NTHi display wide genetic diversity (27–29). To date, attempts to identify a potential virulence factor that is present in NTHi strains isolated from patients with invasive infections but not from those with noninvasive NTHi infections have been unsuccessful. It is important to understand the clinical characteristics of patients with such infections, to determine the virulence potential of NTHi strains isolated from invasive diseases. Cases of NTHi meningitis are rare in children, but bacteremia and bacteremic pneumonia are more common (29). Our study confirmed that most invasive NTHi infections were bacteremia without a focus of infection, followed by bacteremic pneumonia.

Three children (10.7%) in our study died, probably from NTHi infections; however, most children without underlying disease(s), including the patient with meningitis,

TABLE 4 Sequence types and allelic profiles of the invasive nontypeable *Haemophilus influenzae* isolates

Strain no.	Sequence type ^a	Allele						
		<i>adk</i>	<i>atpG</i>	<i>frdB</i>	<i>fucK</i>	<i>mdh</i>	<i>pgi</i>	<i>recA</i>
1	1736	68	43	38	7	95	165	18
2	597	11	33	7	1	7	41	29
3	712	63	54	102	1	17	31	10
4	436	40	1	1	10	15	1	5
5	1017	1	1	1	14	9	7	13
6	393	42	2	38	41	104	55	10
7	1431	63	54	165	1	17	31	10
8	3	1	1	1	1	1	1	5
9	953	153	11	18	18	62	1	5
10	ND	139	2	16	ND	62	40	4
11	160	40	1	1	14	1	59	3
12	156	26	2	15	7	22	56	3
13	14	5	1	1	1	1	2	5
14	11	1	8	1	14	9	14	13
15	584	5	33	7	1	26	41	29
16	145	1	8	1	14	22	14	13
17	396	10	2	15	8	26	61	3
18	159	33	8	16	16	17	2	29
19	3	1	1	1	1	1	1	5
20	1434	35	1	22	36	7	14	29
21	107	33	8	16	16	49	2	3
22	84	29	7	13	1	45	13	1
23	84	29	7	13	1	45	13	1
24	991	63	50	22	15	10	40	3
25	155	16	8	16	16	30	1	3
26	586	116	1	1	13	17	25	16
27	1444	26	11	104	7	26	5	42
28	187	5	33	7	32	26	41	29

^aND, not determined.

recovered from invasive NTHi infections without complications. Although neonates with perinatal NTHi infections often have serious comorbidities, those children also recovered well. This could be because of easy access to hospitals in Japan and the early initiation of appropriate antibiotic treatment. NTHi strains are known to be a cause of sudden unexpected infant death (30). Strain 7 was cultured from postmortem samples. Because the NTHi isolate was the only bacterium isolated from that patient, NTHi infection was thought to be the cause of death in that case.

Biotyping is a way to categorize the phenotypes of *H. influenzae* strains by their enzyme activity. Biotypes II and III, the two major NTHi biotypes found in a healthy respiratory tract, accounted for 79.2% of the NTHi isolates in our study. These results were similar to those of several studies from Japan that showed that 77 to 95% of the *H. influenzae* strains isolated mainly from nonsterile sites of respiratory tract infections were either biotype II or biotype III (31, 32). All of the IS1016-positive strains were biotype III, a unique finding, compared with those that have been reported (33). Furthermore, although NTHi biotype IV is most commonly isolated from neonates (34), other biotypes were detected in our study.

There are two known mechanisms by which *H. influenzae* acquires resistance to aminopenicillins, namely, production of β -lactamase and a change in PBP-3. Among ABPC-resistant strains, β -lactamase production (TEM-1 or ROB-1) is common in other countries (35–37); however, Japan is known to have a high percentage of gBLNAR strains with PBP-3 substitutions that are encoded by the *ftsI* gene (38, 39). The *ftsI* gene encodes an essential transpeptidase that catalyzes cross-linking of the peptidoglycan septal cell wall during cell division (40). Only 2 strains (6.7%) were BLNAR, which was relatively low, compared to other studies from Japan that showed that 30 to 40% of *H. influenzae* strains isolated mainly from nonsterile sites were BLNAR (38, 41). In our study, any mutation in the *ftsI* gene yielded 4- to 8-fold resistance to ABPC. PBP-3 substitutions

are categorized into several groups. Strains with low-level resistance possess the R517H (group I) or N526K (group II) substitution. Group III is defined as having M377I, S385T, and L389F substitutions, in addition to N526K; this is clinically important, as this group shows higher levels of resistance to extended-spectrum cephalosporins (42). In our study, strains with PBP-3 substitutions were mainly categorized as group III (Table 3), which is a distinctive feature of gBLNAR strains in Japan (38, 39). The higher levels of resistance to cephalosporins among these strains should be taken into account when choosing antibiotics to treat patients with invasive *H. influenzae* infections in Japan. Another study showed that resistance to cephalosporins is dependent on the PBP-3 substitutions R517H, N526K, S385T, and L389F (43). In our study, strains with S385T, L389F, and R517H or N526K substitutions showed more than 2- to 4-fold resistance to cephalosporins. Strains 14 and 21, both with L389F, showed higher levels of cephalosporin resistance than did strains 22 and 23, which did not have this mutation. None of the patients, including those who died (patients 4 and 7), were considered to have been treated with ineffective antibiotics, to which the isolated NTHi strains were resistant.

MLST of the isolates from our study showed wide diversity, except for ST-3 and ST-84, which had 2 strains each. We found no relationship between ST and the geographic region or clinical presentation of the patients. However, using the phylogenetic tree and including biotypes and PCR results for the adhesin genes, these strains were divided into six clusters. Cluster I strains were CC-3 and mainly biotype II, with positive *hmw* and negative *hia/hsf* results for all strains. The *hmw* gene is known to be highly expressed in NTHi strains but is generally absent in encapsulated strains, while *hia* is found in many non-Hib encapsulated strains and NTHi strains that lack *hmw* (20). In contrast, isolates in cluster III were all positive for *hia/hsf* and negative for *hmw*, also with biotype V, an uncommon biotype for NTHi strains, which may be one of the characteristics of invasive NTHi strains in children (33). None of the isolates belonged to CC-6, which is known to contain Hib strains and is detected worldwide (<http://haemophilus.mlst.net>). Several studies have indicated that ST-14 has a PBP-3 type A pattern (D350N, M377I, A502V, N526K, V547I, and N569S) (18). In our study, however, our ST-14 strain (strain 13) had S385T. Moreover, our study showed a high frequency of group III gBLNAR strains, despite their STs. Interestingly, strain 10 lacked *fucK*. Fuculokinase is one of several enzymes involved in the fucose pathway; fucose is used as an energy and carbon source in bacteria. *H. influenzae* isolates that lack *fucK* have been reported (44). In the report, all isolates were NTHi; the prevalence of *fucK*-negative strains was 2%. The strains were obtained from sterile sites (blood and CSF) and belonged to various biotypes. For encapsulated *H. influenzae* strains, the capsule and the genes surrounding the *cap* locus are thought to be the major determinants of virulence. In division I Hib strains, the *cap* locus is flanked by direct repeats of an insertion element known as *IS1016* (45). Whole-genome analysis revealed that 2 strains encoded *IS1016*. Including the other 14 strains that underwent PCR analysis to investigate the existence of *IS1016*, 3 strains (10.7%) were positive for *IS1016*. Although *capa* to *capf* and *bexA* to *bexD* were completely absent in these strains, the existence of *IS1016* suggests that these strains are genetically more closely related to encapsulated strains.

Many microorganisms form biofilms as protection against bactericidal agents, bacteriophages, and host clearance mechanisms (46). We used a microtiter biofilm assay as an indicator of biofilm formation. We did not visually observe biofilm formation, but the data suggest the potential of each strain for biofilm formation (47). A previous report showed increased biofilm formation among the NTHi strains collected from patients with invasive disease or otitis media, compared with NTHi isolates from patients with community-acquired pneumonia or chronic obstructive airway disease or from healthy colonized subjects (48). We also found higher biofilm index values for isolates from patients with invasive diseases than for isolates from patients with respiratory tract infections, but the difference was not statistically significant.

We collected strains from 8 (of 47) prefectures in Japan and included data from the nationwide population-based surveillance study on invasive *H. influenzae* disease in Japan, which includes 10 prefectures (Hokkaido, Fukushima, Niigata, Chiba,

Mie, Okayama, Kouchi, Fukuoka, Kagoshima, and Okinawa) (12); only 6 prefectures had cases of invasive NTHi infections during the study period. The populations of the 10 prefectures account for approximately 20% of the Japanese population. This is the only study to have evaluated the trends in invasive *H. influenzae* infections in Japanese children after introduction of the Hib vaccine.

In conclusion, we revealed the bacteriologic characteristics of NTHi strains responsible for invasive *H. influenzae* diseases among children in Japan, and we described the clinical characteristics of children with such infections. Although most patients recovered without complications, the mortality rate of invasive NTHi disease was 10.7%. Invasive NTHi strains in Japan have peculiar PBP-3 substitutions that result in low penicillin and cephalosporin sensitivity, suggesting the need for a new vaccine to prevent invasive NTHi diseases or suggesting that other antibiotic classes that are effective against ABPC-resistant or BLNAR strains be used to treat such infections in Japan. Moreover, continuous monitoring of the NTHi strains responsible for invasive diseases in children is important to detect changes in the epidemiology of invasive *H. influenzae* infections in the post-Hib vaccine era.

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We report no potential conflicts of interest.

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