J Infect Chemother 25 (2019) 720–726

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

Genetic characteristics and antibiotic resistance of *Haemophilus influenzae* isolates from pediatric patients with acute otitis media after introduction of 13-valent pneumococcal conjugate vaccine in Japan☆

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ARTICLE INFO

Article history:
Received 22 October 2018
Received in revised form
29 January 2019
Accepted 25 March 2019
Available online 12 April 2019

Keywords:
Acute otitis media
Haemophilus influenzae
Genotypic resistance
gBLNAR
Multilocus sequence typing
Quinolone resistance

ABSTRACT

Acute otitis media (AOM) occurs commonly in pediatric populations. We examined resistance genotype, antibiotic susceptibility, quinolone (QL) resistance, and multilocus sequence type (MLST) among *Haemophilus influenzae* isolates causing AOM following introduction of pneumococcal conjugate vaccines in Japan.

The AOM surveillance group included 69 participating otolaryngologists. Causative pathogens isolated from middle ear fluid (MEF) samples collected from 582 children with AOM were identified using both bacterial culture and real-time PCR. *H. influenzae* isolates among these pathogens were characterized by capsular type, resistance genotype, antibiotic susceptibility, QL resistance, and MLST.

In 2016, *H. influenzae* was identified in 319 samples (54.8%), among which 72.4% (n = 231) tested positive by both culture and PCR; remaining *H. influenzae* cases were only PCR-positive. This proportion of *H. influenzae* positivity has increased significantly from 41.2% in 2006 ($p < 0.001$). Among culture-positive strains, genotypic β-lactamase-nonproducing ampicillin (AMP)-resistant (gBLNAR) strains were frequent (63.2%), with β-lactamase-nonproducing AMP-susceptible (gBLNAS) strains accounting for only 24.2%. Susceptibilities of gBLNAR to oral antimicrobials were best for tosufloxacin, followed by cefditoren and tebipenem; MIC_{90}s were 0.031 μg/mL, 0.5 μg/mL, and 1 μg/mL, respectively. In 7 gBLNAR isolates (3.0%), QL susceptibility was low, owing to amino acid substitutions in GyrA and/or ParC. Sequence types identified numbered 107, including 28 that were new.

Prevention of further increases in resistance to antimicrobial agents will require antibiotic selection based on characterization of causative pathogens in clinical practice.

1. Introduction

Alone or in combination, bacteria and respiratory viruses are known to cause acute otitis media (AOM) in pediatric patients [1,2]. *Haemophilus influenzae* is among the leading bacterial causes of AOM at ages below 2 years, along with *Streptococcus pneumoniae* [2–5]. Isolation frequencies in samples from middle ear fluid (MEF) are affected by whether or not bacterial culture is combined with polymerase chain reaction (PCR) of MEF [2,6].

Introduction of heptavalent pneumococcal conjugate vaccine (PCV7) and the subsequent 13-valent pneumococcal conjugate vaccine (PCV13) has decreased incidence of AOM caused by vaccine serotype (VT) pneumococcal infections [7–9]. As a result, *H. influenzae* has displaced *S. pneumoniae* as the most frequent pathogen in AOM [2,4,5,10,11]. *H. influenzae* isolates causing AOM are mostly non-typeable *H. influenzae* (NTHi) [4,10–12]. NTHi readily colonize the mucous membrane of the nasopharynx, where

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https://doi.org/10.1016/j.jiac.2019.03.019
1341-321X/

they promote formation of biofilms [13–16]. Such colonization by NTHi is associated with recurrence and prolonged symptom duration in AOM [13,17–19].

Two well-known mechanisms underlie β-lactam resistance in *H. influenzae*. One is production of β-lactamases that hydrolyze penicillins and related antibiotics. The other is decreased affinity for β-lactam agents of penicillin-binding protein 3 (PBP3), an enzyme important for peptidoglycan synthesis during septum formation in cell division. The latter resistance mechanism involves amino acid (AA) substitutions located near conserved AA motifs in PBP3, as a result of *ftsI* gene mutations [20].

Strains producing β-lactamase, termed β-lactamase-producing ampicillin (AMP)-resistant strains (BLPAR), are isolated frequently from AOM patients in countries where amoxicillin (AMX) and AMX-clavulanic acid (AMC) are most prescribed [21,22]. In contrast, resistant strains with AA substitutions of PBP3, termed β-lactamase-nonproducing AMP-resistant strains (BLNAR), are isolated most frequently in Japan, where oral cephalosporin antibiotics are prescribed in preference to AMP, AMX, and AMC [23–26].

In Japan, vaccination of children under 5 years old with *H. influenzae* type b (Hib) conjugate vaccine and PCV7 was prioritized in November 2010 by the Ministry of Health, Labour and Welfare as the Provisional Fund for the Urgent Promotion of Vaccination. PCV7 was incorporated in the routine immunization in 2013 and then replaced by PCV13 at the end of the same year. These policies are credited with rapid declines in incidence of meningitis due to Hib [27] and invasive pneumococcal diseases (IPD) caused by VT *S. pneumoniae* [28].

Unfortunately, few studies have examined details of changes in bacterial AOM pathogens after PCV13 introduction in Japan [26]. To clarify the impact of Hib conjugate vaccine and PCVs upon etiology of AOM, we helped to organize an AOM surveillance study group (AOM Study Group) based upon voluntary participation of 69 otolaryngologists in private practice throughout our country. As previously described, surveillance results for *S. pneumoniae* showed decreased isolation frequency for VT, with their replacement by NVT. Penicillin-resistant *S. pneumoniae* (PRSP) showed a decrease [29].

In this study we describe characteristics of *H. influenzae* isolates from pediatric patients with AOM after vaccination with PCVs, including frequency of isolation, resistance genotype, antibiotic susceptibilities, quinolone (QL) resistance, and sequence type (ST) by multilocus sequence typing (MLST). These results were compared with pre-PCV findings reported previously by Ubukata et al., in 2006 [30,31].

2. Materials and methods

2.1. Patients and sample collection

Sixty-nine otolaryngologists practicing privately in clinics located in 34 prefectures throughout Japan participated voluntarily in the AOM Study Group between June 2016 and January 2017 (research director: Eriko Mokuno, MD, PhD).

This study was approved by the ethics committee at Hakujikai Memorial Hospital (approval number 17) and Keio University School of Medicine Ethics Committee (approval number 20140432).

After informed consent was obtained from all parents or guardians of eligible patients by otolaryngologists before sampling, subjects with AOM were collected by each otolaryngologist following AOM guidelines established jointly by 3 Japanese otolaryngologic societies in 2015 (http://minds4.jcqhc.or.jp/minds/otitis/CPG_AOM_JPN.pdf). As described previously, sterilization of the external auditory canal was followed by MEF collection by tympanocentesis or by sampling through a spontaneous tympanic membrane perforation (STMP) [29]. All samples were sent immediately to the Department of Infectious Diseases at Keio University School of Medicine, where causative pathogens in MEF were identified by bacterial cultures and comprehensive real-time polymerase chain reaction (real-time PCR) [32]. Information concerning examination results was reported promptly to each referring clinic. In all, MEF samples from 582 patients were found to be suitable.

2.2. Microbiology

For each sample, comprehensive real-time PCR was performed as described previously to identify causative pathogens among 6 microorganisms including *S. pneumoniae* (*lytA* gene), *H. influenzae* (16S rRNA gene), and *Streptococcus pyogenes* (group A streptococcus or GAS; 16S rRNA and *slo* genes) [32]. Additionally, each sample was cultured by spreading 5 μL of sample over each of 4 plates: chocolate II agar, sheep blood agar, mannitol salt agar, and modified Drigalski agar (Nippon Becton Dickinson, Tokyo, Japan). Colonies grown on each agar plate after overnight culture were picked up, and species were identified by routine methods.

2.3. Genotypic β-lactam resistance and serotype

Isolates considered likely to be *H. influenzae* underwent species identification and genotypic β-lactam resistance determination by real-time PCR as we previously devised [33].

All isolates were tested with respect to 6 genes: the *p6* gene, encoding *H. influenzae*-specific P6 membrane protein; the TEM-1 type β-lactamase gene ($bla_{\text{TEM-1}}$); the ROB-1 type β-lactamase gene ($bla_{\text{ROB-1}}$); AA substitution for Asn526 by Lys526 in the *ftsI* gene encoding PBP3, located near the conserved KTG motif (Lys512-Thr513-Gly514); AA substitution for Ser385 by Thr385 in the *ftsI* gene, located near the conserved SSN motif (Ser379-Ser380-Asn381); and the Hib-specific *capB* locus.

Genotypic β-lactam resistance of isolates was classified into 5 categories designated by the prefix "g": β-lactamase-nonproducing, AMP-susceptible (gBLNAS), lacking any resistance factor; β-lactamase-producing, AMP-resistant (gBLPAR), able to produce TEM-1 type or ROB-1 type β-lactamases; β-lactamase-nonproducing, low AMP-resistant (gLow-BLNAR), having AA substitutions of Arg517His (defined as group I) or Asn526 by Lys526 (defined as group II) in the *ftsI* gene; β-lactamase-nonproducing, AMP-resistant (gBLNAR), having 3 AA substitutions for Ser385Thr, Met377Ile, and Leu389Phe in addition to Arg517His or Asn526 by Lys526 (defined as group III) in the *ftsI* gene [20]; and strains with both β-lactamase- production and AA substitution in *ftsI*, termed β-lactamase-producing, AMC-resistant (gBLPACR).

For isolates suspected to have a polysaccharide capsule apart from type b, PCR was carried out again to detect capsule types a, c, d, e, or f.

2.4. Susceptibility testing

Antibiotic susceptibilities of *H. influenzae* isolates to 14 antimicrobial agents were determined by agar dilution methods. Muller-Hinton agar with addition of 0.5% yeast extract and 5% horse blood was used after heat treatment. Each strain was grown on a chocolate II agar plate for 18 h under 5% CO_2 incubation at 37 °C and then adjusted to McFarland turbidity 0.5 followed by 100-fold dilution, after which 5 μL was inoculated onto an agar plate for susceptibility testing using a Steers replicator.

Fourteen antimicrobial agents were assessed: AMP, AMX, tebipenem (TBM), cefdinir (CDR), cefditoren (CDN), cefcapene (CPN), clarithromycin (CLR), azithromycin (AZM), tosufloxacin (TFX),

levofloxacin (LVX), piperacillin-tazobactam (TZP), cefotaxime (CTX), ceftriaxone (CRO), and meropenem (MEM). ATCC49247 and ATCC49766 were tested simultaneously as reference strains.

The oral agents TBM and TFX were approved by the Japanese Ministry of Health, Labour and Welfare only for pediatric infections caused by non-susceptible *S. pneumoniae* and BLNAR. TFX was approved additionally for *Mycoplasma pneumoniae* infection because of the high prevalence of macrolide resistance in our country.

2.5. Determination of quinolone resistance

Seven isolates with apparently reduced susceptibilities to TFX and LVX were identified by nucleotide sequencing performed in the QL-resistance determining region (QRDR) of *gyrA* and *gyrB* genes encoding GyrA and GyrB of subunits of DNA gyrase, and *parC* and *parE* genes encoding ParC and ParE of subunits of DNA topoisomerase IV, respectively [34].

2.6. Multilocus sequence typing

MLST of *H. influenzae* was performed by analyzing 7 housekeeping genes as previously described [35,36]. Each sequence obtained for these housekeeping genes was queried on the MLST database website (https://pubmlst.org/hinfluenzae/), after which a unique allelic profile was assigned as well as an ST. Isolates with novel STs were submitted to the database. Phylogenetic analysis was performed using eBURST v3.1 (http://eburst.mlst.net/).

2.7. Statistical analysis

The chi-squared test or Fisher's exact test was used as appropriate. A p value below 0.05 was considered to indicate statistical significance.

3. Results

3.1. Causative pathogens

Causative bacterial pathogens in samples collected from patients with AOM in the study are shown in Fig. 1, in comparison with findings obtained in 2006 by Ubukata et al. [30] (A, n = 399 in 2006; B, n = 582 in 2016). In 2016, MEF samples were collected by tympanocentesis in 264 patients (45.4%) and through an STMP in 318 (54.6%). In the present study, bacteria were causative in 81.3% of cases, similar to the proportion in 2006 (78.2%).

Among all samples, 46.0% (n = 268) were positive only for *H. influenzae*, compared with 17.4% (n = 101) positive only for *S. pneumoniae* and 7.6% (n = 44) positive for both *S. pneumoniae* and *H. influenzae*. Thus, *H. influenzae* was frequent as either the sole pathogen or as a component of polymicrobial infection (54.8%, n = 319). Compared with findings using the same techniques for AOM samples in 2006, the frequency of *H. influenzae* in the present study increased significantly, from 41.2% to 54.8% ($p < 0.001$), while that of *S. pneumoniae* decreased from 39.4% to 25.5% ($p < 0.001$). Most *H. influenzae* isolates were identified as NTHi, except for 2 strains belonging to serotype *e* or *f*.

The age distribution of *H. influenzae*-positive patients is shown in Supplementary Figure 1: 54.9% were 1 year old, 16.4% were younger than 1 year, 13.2% were 2 years old, and 15.6% were 3 or older.

3.2. Genotypic β-lactam resistance

Table 1 compares genotypic β-lactam resistances in *H. influenzae* isolates from AOM cases in 2006 (n = 112) with those in 2016

Table 1
Comparison of genotypic resistance in *Haemophilus influenzae* isolated from patients with acute otitis media in 2006 and 2016.

Genotypic resistance	2006		2016		P value[c]
	No.	(%)	No.	(%)	
gBLNAS	46	(41.1)	56	(24.2)	0.002
gBLPAR	0	(0.0)	4	(1.7)	0.308
gLow BLNAR[a]	9	(8.0)	15	(6.5)	0.653
gBLNAR[b]	55	(49.1)	146	(63.2)	0.014
gBLPACR	2	(1.8)	10	(4.3)	0.350
Total (%)	112	(100.0)	231	(100.0)	

[a] gLow BLNAR was defined by real-time PCR to have amino acid substitutions of Arg517His (group I) or Asn526Lys (group II) near the conserved KTG motif of PBP3 according to Ubukata [25] and Kishii [39].
[b] gBLNAR was defined by real-time PCR to have 3 amino acid substitutions of Met377Ile, Ser385Thr, and Leu389Phe near the conserved SSN motif in addition to Arg517His or Asn526Lys (group III).
[c] Analyzed by Fisher's exact test.

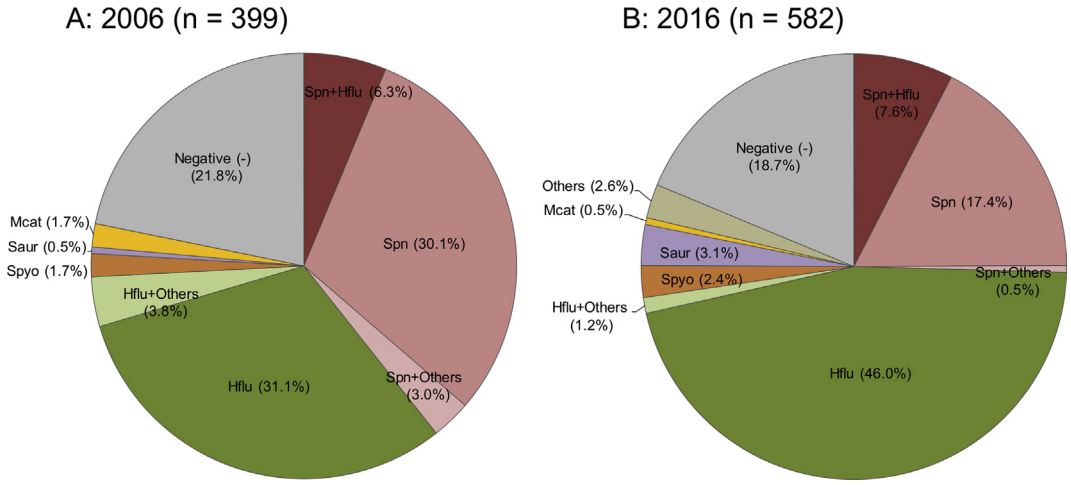

Fig. 1. Comparison of causative bacterial pathogens in middle ear fluid samples from children diagnosed with AOM in 2006 (A, n = 399) and 2016 (B, n = 582). Species of *H. influenzae*, *S. pneumoniae*, and *S. pyogenes* were determined using real-time PCR and bacterial cultures, while other pathogens were identified using only bacterial culture. Abbreviations of bacterial species as follows: *S. pneumoniae*, Spn; *H. influenzae*, Hflu; *S. pyogenes*, Spyo; *Staphylococcus aureus*, Saur; *Moraxella catarrhalis*, Mcat.

isolates (n = 231). Resistance genotypes were determined by real-time PCR to identify the 2 β-lactamase genes, bla_{TEM-1} and bla_{ROB-1}, as well as mutation(s) in the *ftsI* gene, which encodes PBP3.

Isolates representing gBLNAR increased significantly from 49.1% in 2006 to 63.2% in 2016 ($p = 0.014$), while gBLNAS decreased significantly from 41.1% in 2006 to 24.2% in 2016 ($p = 0.002$). In 2016, only 4 strains were identified as gBLPAR producing β-lactamase TEM-1, compared with none in 2006 (no significant change).

3.3. Antimicrobial susceptibility by genotype

Susceptibilities for 227 *H. influenzae* strains (range of minimum inhibitory concentrations [MIC], MIC for 50% of isolates [MIC_{50}], and MIC for 90% of isolates [MIC_{90}]) to 10 oral and 4 parenteral agents are shown in Table 2 and in Supplementary Table 1. gBLPARs (n = 4) were not considered because of small numbers.

In order of most to least active, MIC_{90}s of oral agents for gBLNAR (n = 146) were TFX and LVX (each 0.031 μg/mL); CDN (0.5 μg/mL); TBM (1 μg/mL); AZM (4 μg/mL); and CPN (8 μg/mL). MIC_{90}s of AMP, AMX, CDR, and CLR all were inferior (≥32 μg/mL). MIC_{90}s of parenteral agents for gBLNARs were superior, in the order of TZP (0.25 μg/mL), CRO (0.25 μg/mL), MEM (1 μg/mL), and CTX (2 μg/mL).

Relationships between susceptibilities and genotypic resistances that affect MICs of β-lactam agents, 4 oral (AMP, AMX, CDR, and TBM) and 4 parenteral (TZP, CTX, CRO, and MEM), are shown as Supplementary Figure 2 and Supplementary Figure 3, respectively. Susceptibility distributions according to genotype differences, especially in gLow BLNAR and gBLNAR, clearly affected cephalosporin agents (mutations of the *ftsI* gene encoding PBP3) rather than penicillin agents (β-lactamase gene acquisition).

Cumulative distributions of MICs of oral agents for gBLNAR are shown in Supplementary Figure 4. Distributions of TFX and LVX were superior, ranging mostly from 0.008 μg/mL to 0.031 μg/mL and followed by CDN and TBM, from 0.031 μg/mL to 1 μg/mL. Results for remaining oral agents were inferior more than 1 μg/mL against gBLNAR.

Table 2
Susceptibilities to 6 oral antimicrobial agents of *Haemophilus influenzae* classified by resistance genotype (n = 227).

Antimicrobial agent and resistance class[a]	Range	MIC (μg/mL) MIC_{50}[b]	MIC_{90}[b]
Ampicillin	0.25->64	2	32
gBLNAS (n = 56)	0.25–0.5	0.5	0.5
gLow BLNAR (n = 15)	0.5–2	1	2
gBLNAR (n = 146)	0.5->64	4	32
gBLPACR (n = 10)	64->64	>64	>64
Amoxicillin	0.5->64	32	>64
gBLNAS	0.5–1	0.5	1
gLow BLNAR	1–16	4	16
gBLNAR	0.5->64	32	>64
gBLPACR	>64	>64	>64
Cefditoren	0.008–1	0.25	0.5
gBLNAS	0.008–0.031	0.016	0.016
gLow BLNAR	0.016–0.25	0.031	0.25
gBLNAR	0.063–1	0.25	0.5
gBLPACR	0.125–1	0.5	1
Tebipenem	0.031–16	0.5	1
gBLNAS	0.063–0.25	0.125	0.125
gLow BLNAR	0.063–1	0.5	0.5
gBLNAR	0.031–4	0.5	1
gBLPACR	0.5–16	1	8
Azithromycin	0.25->64	4	4
gBLNAS	0.25–8	4	4
gLow BLNAR	1–8	4	4
gBLNAR	1–8	4	4
gBLPACR	2–16	4	8
Tosufloxacin	0.004–2	0.016	0.031
gBLNAS	0.004–0.063	0.016	0.016
gLow BLNAR	0.008–0.063	0.016	0.063
gBLNAR	0.008–2	0.016	0.031
gBLPACR	0.016–0.031	0.016	0.031

[a] The "g" prefix indicates genotypic determination of the resistance class. Because of low number, gBLPAR (n = 4) is excluded from the Table.
[b] MIC_{50} and MIC_{90} indicate minimum inhibitory concentrations for 50% and for 90% of isolates, respectively.

3.4. Quinolone resistance

Genetic characteristics of 7 isolates with reduced susceptibilities to 2 QLs, TFX and LVX, are shown in Table 3. MICs ranged from 0.125 μg/mL to 2 μg/mL of TFX and LVX for these isolates, about 8–128 times greater than for the susceptible reference strain.

AA substitutions of Ser84Leu or Asp88Tyr in the QRDR of GyrA were found in 5 isolates showing MICs of TFX and LVX from 0.125 μg/mL to 0.25 μg/mL. Two isolates with higher MICs of TFX and LVX, 1 μg/mL to 2 μg/mL, proved to have mutations in *gyrA* and *parC* genes causing the AA substitutions Ser84Leu in GyrA and Ser84Ile in ParC.

3.5. MLST and resistance genotype

Fig. 2 shows correlations between genetic resistance and phylogenetic analysis of 231 isolates from AOM patients. These isolates represented 107 different STs, showing great genetic diversity and including 28 STs newly identified in the present study. Solid and broken lines link single-locus and double-locus variants, respectively; however, most STs were unconnected. No associations between ST and a gBLNAR resistance genotype, and/or QL resistance, were found; MLST analysis showed a large number of genetic mutations even in housekeeping genes, which contributed to diversity of STs. Additionally, QL resistance emerged in ST3, ST57, ST143, ST160, and ST695, which were registered on the MLST site at a relatively early stage.

4. Discussion

In this study we analyzed molecular epidemiologic characteristics including antibiotic resistance and MLST in *H. influenzae* isolates collected during 2016 by an AOM Surveillance Study Group consisting of 69 private otolaryngologic clinics located throughout Japan. The year of that study period began 3 years after PCV13 introduction.

Determining the etiology of AOM depends upon accurate AOM diagnosis, appropriate sampling technique and specimen transport, and specifics of bacteriologic testing; our surveillance study followed Japanese AOM guidelines [37] at each of these stages. MEF collected by each clinic was sent without delay to our research laboratory, where real-time PCR was carried out promptly in parallel with bacterial culture. We also compared the 2016 results with AOM data from 2006 that we obtained by identical methods before PCV7 introduction [30,31].

The bacterial isolation rate in the present study (81.3%) was similar to that in 2006 (78.2%). As for pathogens identified, *H. influenzae* increased significantly from 41.2% to 54.8%, while *S. pneumoniae* decreased significantly from 39.4% to 25.5%. Most of the 2016 *H. influenzae* isolates were NTHi, except for 2 strains with capsule types e or f. Changes in causative pathogens are believed to reflect the impact of PCV7 and PCV13 vaccinations; similar results have been reported in the US [3,11,38], the EU [5,10,22,39,40], and other locations [26,41].

As for genotypic β-lactam resistance, the frequency of gBLNAR was higher in 2016, at 63.2% compared with 49.1% in 2006.

Table 3
Characteristics of quinolone-resistant *Haemophilus influenzae* isolated from middle ear fluid samples from patients with acute otitis media.

Strain No.	Patient age	Sequence type (ST)	MIC (μg/mL)[a]							GyrA[b]		ParC[b]	
			AMP	AMX	CDN	TBM	CTX	TFX	LVX	Ser84	Asp88	Ser84	Asn138
Reference strain													
M-072	11 m	1680	0.25	0.5	0.016	0.063	0.016	0.016	0.031	–	–	–	–
Resistant isolates													
M-085	6 y	143	8	32	0.125	1	1	2	1	Leu	–	Ile	Ser
M-146	1 y	695	4	32	0.5	1	1	2	2	Leu	Asn	Ile	–
M-040	1 y	160	4	32	0.5	0.5	2	0.25	0.25	–	Tyr	–	–
M-107	1 y	160	16	32	0.5	0.5	4	0.25	0.25	–	Tyr	–	–
M-150	1 y	160	16	32	1	0.5	8	0.25	0.25	–	Tyr	–	–
M-244	7 m	57	4	32	0.5	1	1	0.25	0.125	Leu	–	–	–
M-526	1 y	3	16	64	0.25	0.5	2	0.125	0.125	–	Tyr	–	–

[a] Abbreviations of antimicrobial agents: AMP, ampicillin; AMX, amoxicillin; CDN, cefditoren; TBM, tebipenem; CTX, cefotaxime; TFX, tosufloxacin; LVX, levofloxacin.
[b] Abbreviations of amino acid residues: Ser, serine; Asp, asparagine acid; Asn, asparagine; Leu, leucine; Ile, isoleucine; Tyr, tyrosine.

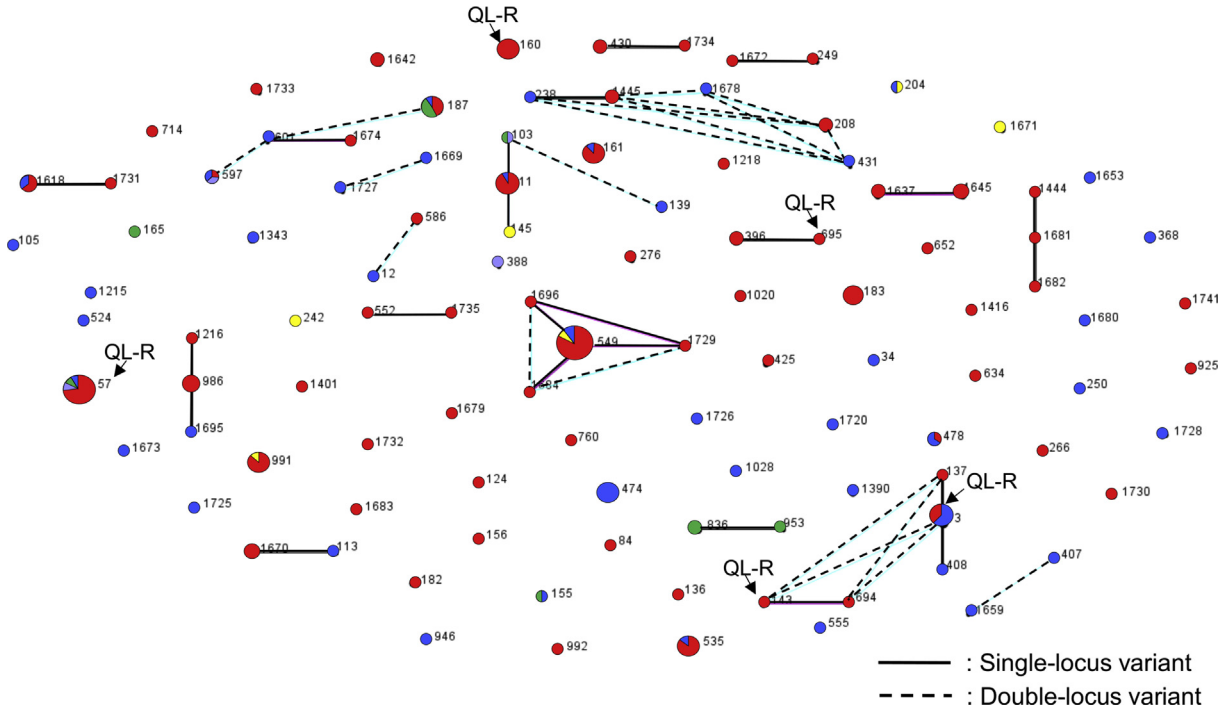

Fig. 2. Analysis by eBURST of 231 *H. influenzae* strains from patients with AOM. Numbers of isolates assigned to each ST are depicted by relative sizes of circles, and distances between circles indicate degree of difference between STs. The distribution of genotypic β-lactam resistance in each ST is shown by colors of circles as follows: blue, gBLNAS; purple, gBLPAR; red, gBLNAR; yellow, gLow-BLNAR; and green, gBLPACR. One hundred seven STs were identified. Solid and dotted lines indicate single-locus and double-locus variants, respectively. QL-R indicates quinolone resistance related to amino acid substitutions in GyrA and/or ParC enzymes.

However, β-lactamase-producing *H. influenzae* accounted for only 1.7% and gBLNAS had a relatively low frequency, 24.2%. In countries where AMX and AMC are predominant first-choice antibiotics for AOM, the frequency of gBLPAR strains producing TEM-1 or ROB-1 is relatively high [21,22,42], while gBLNAR is infrequent [42–44] except to Spain [45]. However, most gBLNAR represent gLow BLNAR having mainly AA substitutions for Asn526 by Lys526 or Arg517His in the *ftsI* gene encoding PBP3 that was defined as group II or group I by Ubukata et al. [20]. In contrast, in our country where oral cephalosporin agents are preferred for AOM complicating respiratory infections, the frequency of gBLNAR belonged to group III remains very high [24,25]. Two factors contributing to resistance problems in Japan are that susceptibilities to oral cephalosporin antibiotics are affected more by mutation(s) in the *ftsI* gene than susceptibilities to AMP [20,46], and bioavailabilities of oral cephalosporins are relatively inferior; the C_{max} obtained from a dose of 6 mg/kg is usually 1 μg/mL or less.

Based upon correlation with clinical outcomes for resistant organisms described above, 2 oral agents (TBM, a carbapenem antibiotic, and TFX, a QL agent) were governmentally approved in Japan for limited use in pediatric patients with pneumonia or AOM infection by non-susceptible *S. pneumoniae* or BLNAR. These 2 agents have different characteristics: TBM shows excellent antimicrobial activity (MIC, 0.002–0.125 μg/mL) against penicillin-nonsusceptible *S. pneumoniae* isolates [47], while TFX shows superior activity (MIC, ≤0.063–0.125 μg/mL) against gBLNAR isolates [48].

The plasma C_{max} of TBM after oral administration of 6 mg/kg was high, at 5.3 μg/mL ± 1.6 μg/mL (mean ± SD) after 0.7 h (T_{max}), maintaining good concentrations after entering MEF (1.2 μg/mL ± 1.6 μg/mL) [49]. The C_{max} of TFX after the same dose as TBM was low, at 0.8 μg/mL ± 0.2 μg/mL after 0.9 h (T_{max}) [50]; MEF concentrations were not reported. This may indicate that wide empirical use of TFX in pediatric patients accelerates not only

selection for QL resistance but also selection for multiple mutations within the *H. influenzae* genome. In fact, strains of QL-resistant *H. influenzae* identified in this study were found to have AA substitutions in respective QRDR regions of GylA and/or ParC, resulting in decreased susceptibilities to TFX and LVX in these strains. Such QL-resistant *H. influenzae* already has been reported by other investigators [34,51]. Limitations on clinical use of QL agents in children will be needed to avoid promotion of QL resistance.

Marked diversity of ST was evident in phylogenetic analysis performed for all *H. influenzae* isolates from AOM, involving 107 STs including 28 new STs, while STs such as ST3, ST57, and ST549 remained relatively numerous. Similar high diversity has been noted in previous reports [25,36,52,53]. No associations between STs and gBLNAR or between STs and QL resistance were found. One factor contributing to ST diversity may be extensive colonization of the upper respiratory tract by NTHi, promoting formation of biofilms [13–16]. This characteristic property also is associated with recurrences and treatment failure in NTHi infections [13,14,54]. Additionally, such colonization tends to expose NTHi to a variety of oral antimicrobial agents used for treatment, selecting for bacterial cells with mutations that confer resistance.

Morphologic changes in gBLNAR observed by phase-contrast microscopy and electron microscopy following exposure to antimicrobial agents such as AMX and TBM resulted in time-dependent lysis of large, abnormal-appearing spheroplasts. gBLNAR cells exposed to CDN had an abnormal filamentous appearance, while gBLNAR cells exposed to TFX appeared to be killed without lysis after mild filamentous change [55]. After exposure to various antimicrobial agents, the abnormal but intact *H. influenzae* cells can revert to normal morphology [56]. Such properties tend to promote genetic diversity in this microorganism.

In conclusion, appropriate choice of antimicrobial agents based on accurate laboratory diagnosis by methods such as multiplex real-time PCR to characterize causative pathogens is important in curtailing spread of resistance.

Conflicts of interest

Among the authors, Drs. Ubukata, Tajima, and Iwata have received a speaker's honoraria from Pfizer Japan (Ubukata and Iwata), Meiji Seika Pharma (Ubukata, Tajima, and Iwata), Taisho Toyama Pharmaceutical (Ubukata and Iwata), Japan Vaccine (Tajima and Iwata), and Merck Sharp and Dohme (MSD) (Tajima and Iwata), respectively. The other authors declare that they have no conflict of interest.

Funding

This study was provided a financial support by Meiji Seika Pharma (Tokyo, Japan) (grant No.2015–#22). Meiji had no role in study design, data collection and interpretation, or the decision to submit this work for publication.

Acknowledgements

The authors also thank their staff members, Madoka Naito and Shinji Masuyoshi, for their helpful assistance.

We are very grateful to the otolaryngologists in the AOM Surveillance Study Group: Mikiya Inagaki MD, Kiyoaki Kamakazu MD, Yoshihiro Atago MD, Shinichi Kakurai MD, Junichi Iwamoto MD, Shinji Usui MD, Seizou Ooyama MD, Sumio Sugano MD, Hisashi Kuroda MD, Yousuke Kamide MD, Michihiro Kamide MD, Yasuko Murakawa MD, Satoru Kaieda MD, Ippei Kawaziri MD, Akimitsu Kawai MD, Yasutake Kikuchi MD, Seiji Sawaki MD, Koujin Kyou MD, Shigeru Kimura MD, Yasuko Kimura MD, Yutaka Kimura MD, Hideyuki Sashiyou MD, Hirohide Yoneya MD, Kazumi Kosaka MD, Mototane Komeda MD, Katsuhiko Kobayashi MD, Yudzuru Sakaida MD, Shoichi Sawada MD, Tatsuaki Satoh MD, Masami Satoh MD, Toshiaki Shimizu MD, Osamu Kimura MD, Jun Shimada MD PhD, Yasutaka Hori MD, Rinya Sugita MD PhD, Yoshinobu Sugita MD, Akihiro Uchizono MD, Kazuhiro Soeda MD, Tomizou Tabuchi MD, Chiharu Matsuoka MD, Chiaki Suzuki MD, Hideki Chiba MD, Yasuhiro Tsuboi MD PhD, Michio Tomiyama MD, Kouji Nakano MD, Tsutomu Nakazawa MD, Akira Fukumoto MD, Akiko Fukumoto MD, Mitsuyoshi Nagura MD PhD, Ikuo Nagayama MD, Mutsumi Satoh MD, Katsuhiko Nakamura MD, Kenji Noguchi MD, Kyoko Shiiba MD, Kenichirou Nogami MD, Hideki Matsuda MD PhD, Emiko Shiba MD, Keishi Hirabayashi MD, Masaaki Hiyoshi MD, Shigeo Yamagishi MD, Chika Ozu MD, Jun Maruyama MD, Shigenori Matsubara MD, Mitsuko Suetake MD, Motoaki Miyashita MD, Akira Mogami MD, Mikio Yamaguchi MD, Hideyuki Yamaoka MD, Atsushi Yuta MD PhD, Takuma Yoshikawa MD, Meiwa Toyofuku MD, Sachiko Mori MD PhD, and Mitsuaki Inagaki MD.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiac.2019.03.019.

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