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Emergence of *Haemophilus influenzae* with low susceptibility to quinolones and persistence in tosufloxacin treatment

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ABSTRACT

Background: The use of non-β-lactam agents has increased in Japan due to the prevalence of β-lactam-resistant pathogens. This study aimed to clarify the recent trend of antimicrobial susceptibility and molecular epidemiological features in *Haemophilus influenzae*.
Methods: Fifty-seven *Haemophilus influenzae* isolated from a Japanese teaching hospital in 2017 were characterised, and the data were compared with those of a previous study. The MICs were determined using the broth dilution method. Genetic backgrounds were compared by multilocus sequence typing. The bactericidal activity of tosufloxacin at, or near, the therapeutic Cmax was determined in vitro, with susceptible isolates and quinolone low-susceptible isolates by time-kill assay.
Results: The results of the susceptibility tests showed that >90% of isolates were susceptible to cephalosporins and carbapenems, whereas ampicillin-susceptible and clarithromycin-susceptible isolates decreased. Regarding quinolones, low-susceptible isolates were noted in 2017, although all isolates were judged as susceptible. All low-susceptible isolates had an amino acid substitution in GyrA, and two isolates had an additional substitution in ParC. These isolates had different genetic backgrounds. Furthermore, the time-kill kinetic assay using the Cmax of tosufloxacin indicated that the low-susceptible isolates could persist for at least 8 hours.
Conclusions: This study revealed that *Haemophilus influenzae* has demonstrated multidrug low-susceptibility in recent years. The low-susceptible isolates had genetic diversity, meaning that resistance occurred independently.

1. Introduction

Haemophilus influenzae (*H. influenzae*) is a causative pathogen of respiratory infections, otitis media and meningitis, especially in the paediatric field. There are encapsulated (type a–f) and non-encapsulated strains of *H. influenzae*, so-called non-typeable *H. influenzae* (NTHi). Among them, capsular type b (Hib) is highly pathogenic. In many countries, a vaccine targeting capsular type b (Hib vaccine) is given to infants. In Japan, the vaccine has been included in routine vaccinations since 2013. Accordingly, it is known that meningitis caused by Hib has significantly decreased [1]. In contrast, since most noninvasive infections, such as pneumonia, acute otitis media and sinusitis, are caused by NTHi, they have not decreased. In addition, among causative pathogens of acute otitis media, an increase in *H. influenzae* and decrease in *Streptococcus pneumoniae* have been reported [2].

β-lactams have mainly been used for NTHi treatment; however, β-lactamase-non-producing ampicillin-resistant *H. influenzae* (BLNAR), β-lactamase-producing ampicillin-resistant *H. influenzae* (BLPAR), and β-lactamase-producing amoxicillin-clavulanic acid-resistant *H. influenzae* (BLPACR) have already emerged. In particular, it is known that BLNAR makes up a large proportion of *H. influenzae* in Japan, compared with the United States and Europe [3]. β-lactamase-non-producing ampicillin-resistant *H. influenzae* shows less susceptibility to all β-lactams, including cephems. Therefore, non-β-lactam agents, like macrolides andquinolones, have become important as alternative agents. In Japan, azithromycin fine granule was introduced in 2009, and the application of tosufloxacin fine granule was extended to children in 2010. Accordingly, use of these agents has increased in recent

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years. In general, since the use of antimicrobial agents is related to drug resistance, antimicrobial susceptibility may have also changed in recent years. In fact, low susceptibility and resistance to non-β-lactams have already been reported [4]. For example, there are several reports about the decrease in susceptibility to clarithromycin [5–7], and the emergence of quinolone low-susceptible strains with GyrA amino acid substitutions at the 84th residue, which is in the quinolone resistance-determination region [8].

Therefore, this study analysed the antimicrobial susceptibility and molecular epidemiological characteristics of *H. influenzae*, isolated from a Japanese teaching hospital in 2017, in order to investigate the recent trend in antimicrobial susceptibility of *H. influenzae*. Furthermore, it compared these data with previous epidemiological results of the same hospital.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Fifty-seven *H. influenzae* isolates from Yokohama Rosai Hospital isolated from January 2017 to December 2017 were used in this study. In addition, information on patient age, sex, and pre-treatment of antimicrobial agents within 60 days was collected. The median age of patients was 1 year (range, 3 months to 89 years). These isolates were cultured on chocolate agar overnight at 37 °C under 5% CO_2 atmosphere. The capsule type was identified by PCR established by Falla et al. [9]. Subsequently, these isolates were suspended in 10% skim milk and stored at −80 °C until use. For the antimicrobial susceptibility test and time-kill kinetic assay, *H. influenzae* ATCC 49247 was used as a control strain. *H. influenzae* Rd was also used for the antimicrobial susceptibility test.

2.2. Antimicrobial susceptibility test and detection of drug-resistant genes

Based on the standards of the Clinical and Laboratory Standards Institute (CLSI), the minimum inhibitory concentration (MIC) was measured by a broth microdilution method [10]. The following antimicrobial agents were used: amoxicillin (AMX, Sigma-Aldrich Japan, Tokyo, Japan); clavulanic acid (CVA, Santa Cruz Biotechnology, Dallas, TX); ampicillin (AMP, Wako, Osaka, Japan); sulbactam (SBT, Wako); ceftriaxone (CRO, Wako); cefotaxime (CTX, Wako); cefcapene (CFPN, Shionogi, Osaka, Japan); cefditoren (CDN, Meiji Seika pharma, Tokyo, Japan); meropenem (MEM, Wako); tebipenem (TBPM, Meiji Seika); tosufloxacin (TFLX, Tokyo Chemical Industry); clarithromycin (CLR, Tokyo Chemical Industry, Tokyo, Japan); azithromycin (AZM, Tokyo Chemical Industry); levofloxacin (LVX, Tokyo Chemical Industry); and garenoxacin (GRNX, Toyama Chemical, Tokyo, Japan).

Antimicrobial susceptible breakpoints were employed using the following CLSI criteria [10]: AMX-CVA (AMC, ≤ 4/2 μg/mL), AMP (≤ 1 μg/mL), CRO (≤ 2 μg/mL), CTX (≤ 2 μg/mL), MEM (≤ 0.5 μg/mL), CLR (≤ 8 μg/mL), AZM (≤ 4 μg/mL), and LVX (≤ 2 μg/mL). In addition, the breakpoint of the LVX low-susceptible strain was set at ≥ 0.063 based on a previous report [8]. The dataset of clinical isolates from Yokohama Rosai Hospital in 2007 and 2012 was extracted from previous research and employed for comparison [11]. Detection of β-lactamase-producing gene, bla_{TEM-1},bla_{ROB-1}, was performed by PCR as previously described [7,12].

2.3. Analysis on quinolone resistant-determining regions in GyrA and ParC

For all LVX low-susceptible isolates, quinolone resistant-determining regions (QRDRs) were sequenced and amino acid substitutions were estimated using GENETYX ver. 10 (GENETYX, Tokyo, Japan) [8,13].

2.4. Comparison of genetic backgrounds among clinical isolates

The genetic background of all isolates was analysed by multilocus sequence typing (MLST), which was classified by the nucleotide sequences of seven *H. influenzae* housekeeping genes – *adk, atpG, frdB, fucK, mdh, pgi,* and *recA* – according to the method by Meats et al. [14]. The minimum spanning tree was drawn using Bio Numerics ver.7 (Applied Maths, Sin-Martens-Latem, Belgium).

2.5. Time-kill kinetic assay

Bacterial time-kill kinetic assay of tosufloxacin at the concentrations of Cmax (1 μg/mL) and 1/2 Cmax (0.5 μg/ mL) were performed with reference to a previous report [15]. In brief, tested strains were incubated overnight in brain heart infusion broth (Oxoid, Hampshire, UK) supplemented with 15 μg/mL Hemin and 15 μg/mL NAD (sBHI broth) at 37 °C. These cultures were diluted 1:100 with fresh sBHI broth or sBHI broth containing 0.5 or 1 μg/mL of tosufloxacin and statically incubated at 37 °C under ambient air. Before and after 1, 2, 4, 6 and 8 hours of incubation, the cultures were appropriately diluted with phosphate buffered saline (PBS) and plated onto sBHI agar plates. On the following day, the numbers of bacteria were counted. This experiment was repeated to confirm the same trend at least twice on independent days.

Table 1
Antimicrobial susceptibility of *Haemophilus influenzae* clinical isolates.

Agent	2007				2012				2017			
	MIC_{50}	MIC_{90}	MIC range	S%	MIC_{50}	MIC_{90}	MIC range	S%	MIC_{50}	MIC_{90}	MIC range	S%
Amoxicillin	–	–	–	–	4	32	0.25–≥64	–	8	≥64	0.5–≥64	–
Amoxicillin-clavulanic acid	8	16	0.5–16	37.5	4	8	0.25–16	58.3	4	16	0.25–16	52.6
Ampicillin	4	8	0.25–8	25.0	4	16	0.25–≥64	25.0	16	≥64	0.25–≥64	8.8
Ampicillin-sulbactam	–	–	–	–	–	–	–	–	8	16	0.25–32	21.1
Ceftriaxone	0.25	0.25	≤0.063–0.25	100	0.25	0.25	≤0.063–0.5	100	0.125	0.25	≤0.063–0.5	100
Cefotaxime	1	2	≤0.063–2	100	0.5	1	≤0.063–2	100	0.5	2	≤0.063–4	94.7
Cefcapene	1	2	≤0.063–4	–	1	2	≤0.063–4	–	2	8	≤0.063–8	–
Cefditren	0.25	0.25	≤0.063–0.5	–	0.125	0.25	≤0.063–0.25	–	0.25	0.5	≤0.063–1	–
Meropenem	0.125	0.5	≤0.063–1	93.8	0.125	0.25	≤0.063–0.5	100	0.25	0.5	≤0.063–1	96.5
Tebipenem	–	–	–	–	0.25	0.5	≤0.063–1	–	0.5	1	≤0.063–2	–
Clarithromycin	8	8	4–8	100	8	16	2–16	79.2	8	32	2–≥64	57.9
Azithromycin	1	2	0.5–2	100	1	4	0.5–4	100	1	2	0.5–16	96.5
Levofloxacin	≤0.063	≤0.063	≤0.063	100	≤0.063	≤0.063	≤0.063	100	0.016	0.032	0.004–1	100
Tosufloxacin	–	–	–	–	≤0.063	≤0.063	≤0.063	–	0.008	0.016	0.004–2	–
Garenoxacin	–	–	–	–	≤0.063	≤0.063	≤0.063	–	0.008	0.032	0.004–≥2	–

S%, percent of susceptible isolates; –, not tested or breakpoint was not defined.

2.6. Statistical analysis

Between-group differences were tested using χ^2 or Fisher's exact tests. $P < 0.05$ was considered as a significant difference.

3. Results

3.1. The recent trend of Haemophilus influenzae antimicrobial susceptibility

To clarify the recent trend in antimicrobial susceptibility of *H. influenzae*, antimicrobial susceptibility of clinical isolates was determined (Table 1). In addition, 5-year comparative analyses were conducted using the data extracted from the same hospital as a previous report [11]. The median age of patients was 1 year in all years. Specifically, AMP-susceptible isolates decreased from 25% in 2012 to 8.8% in 2017 (vs. 2007, $P = 0.099$; vs. 2012, $P = 0.075$). Susceptibility to cephems and carbapenems did not change greatly, and the susceptibility rate was still high.

Regarding non-β-lactams, the susceptibility rate to CLR was 100% in 2007, but it significantly decreased to 79.2% in 2012 and 57.9% in 2017 ($P = 0.001$). Of these, in 2017, six isolates showed resistance to CLR (MIC > 32 μg/mL). Although all isolates showed susceptibility to LVX, four low-susceptible isolates showed LVX MIC of ≥ 0.063 μg/mL. No LVX low-susceptible strains were detected in both 2007 and 2012. All CLR resistant and LVX low-susceptible isolates showed resistance to β-lactams.

3.2. Molecular epidemiological analysis by multilocus sequence typing

To investigate the genetic association among the isolates in 2017, MLST analysis was performed. All 57 strains were divided into 26 sequence types, indicating that these isolates had high diversity (Fig. 1). Sequence type (ST) analyses of CLR resistant (CLR MIC ≥ 32 μg/mL) and LVX low-susceptible isolates showed that all isolates had different STs. These data suggest that there was no specific clone for resistance or low susceptibility, and that each isolate could become resistant or low-susceptible isolates independently.

3.3. Quinolones low-susceptible mechanism

All four LVX low-susceptible isolates also showed low-susceptibility to TFLX and GRNX (MIC ≥ 0.063 μg/mL). Four strains with low susceptibility to quinolones were analysed by DNA sequencing of quinolone target genes to estimate amino acid substitutions in QRDRs. Three isolates were isolated from paediatric patients. All low-susceptible isolates had an amino acid substitution (Ser84Leu) in GyrA (Table 2). In addition, strains with LVX MIC of 0.5 μg/mL and 1 μg/mL had an additional amino acid substitution (Glu88Lys and Ser84Ile, respectively) in ParC.

3.4. Bactericidal effect of therapeutic concentration of tosufloxacin on paediatric patient-isolated quinolones low-susceptible isolates

To estimate the therapeutic effect of TFLX (the only respiratory quinolone approved for use in pneumonia and otitis media in children, except for norfloxacin) on LVX low-susceptible paediatric isolates (2017-Y11 and 2017-Y28), time-kill kinetic assay was performed (Fig. 2). The Cmax after administration of TFLX 6 mg/kg (standard dosage approved by the Health, Labour, and Welfare Ministry, Japan) was 0.96 ± 0.30 μg/mL and $T_{1/2}$ was 3.8 ± 0.5 h [16,17]. Therefore, the TFLX concentration for this assay was set to 1 μg/mL (Cmax) and 0.5 μg/mL (1/2 Cmax). When cultured in the presence of TFLX, the ATCC 49247 strain decreased in a time-dependent manner, and the bacterial number reduced below the detection limit after a 6 h-incubation. In contrast, all low-susceptible isolates gradually decreased, and these isolates remained even after an 8 h-incubation. 2017-Y28, which only had a substitution in GyrA, decreased to approximately 1/1000 after 8 h at Cmax. Conversely, 2017-Y11 that had substitutions in both ParC and GyrA decreased to approximately 1/10 after 8 h at Cmax. Moreover, Y11 increased at 1/2 Cmax.

3.5. Relationship between patient background and antimicrobial susceptibility

To evaluate the relationship between low susceptibility or resistance and the patient background, this study statistically

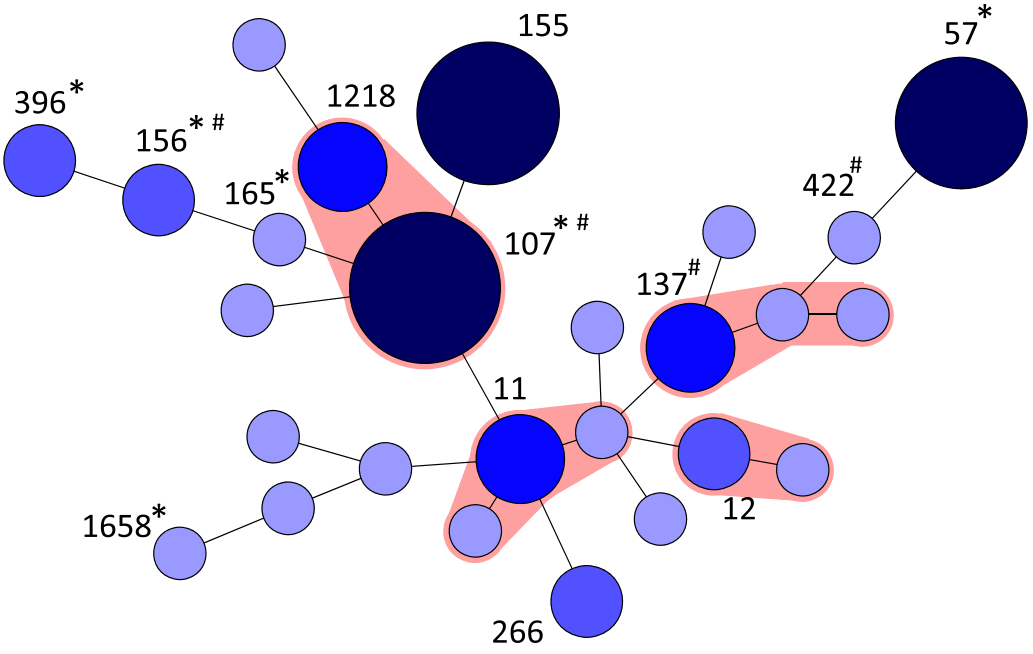

Fig. 1. Minimum spanning tree analysis of sequence type (ST). The node sizes relate to the number of isolates, and the colour border around each node indicates clonal complex (CC). *, including clarithromycin resistant clone (n = 6); #, including levofloxacin low-susceptible clone (n = 4).

Table 2
Molecular feature of levofloxacin low-susceptible isolate.

Strain	Clinical department	Patient age (year)	ST	MIC (μg/mL)		QRDR substitution	
				Levofloxacin	Tosufloxacin	GyrA	ParC
ATCC49247	–	–	nt	0.008	0.004	None	None
Y11	Paediatrics	1	156	0.5	0.5	Ser84Leu	Glu88Lys
Y28	Paediatrics	4	137	0.125	0.25	Ser84Leu	None
Y31	Paediatrics	3	107	0.125	0.125	Ser84Leu	None
Y55	Respiratory medicine	86	422	1	2	Ser84Leu	Ser84Ile

QRDR, quinolone resistance-determining region; -, no data; nt, not tested; None, no substitution.

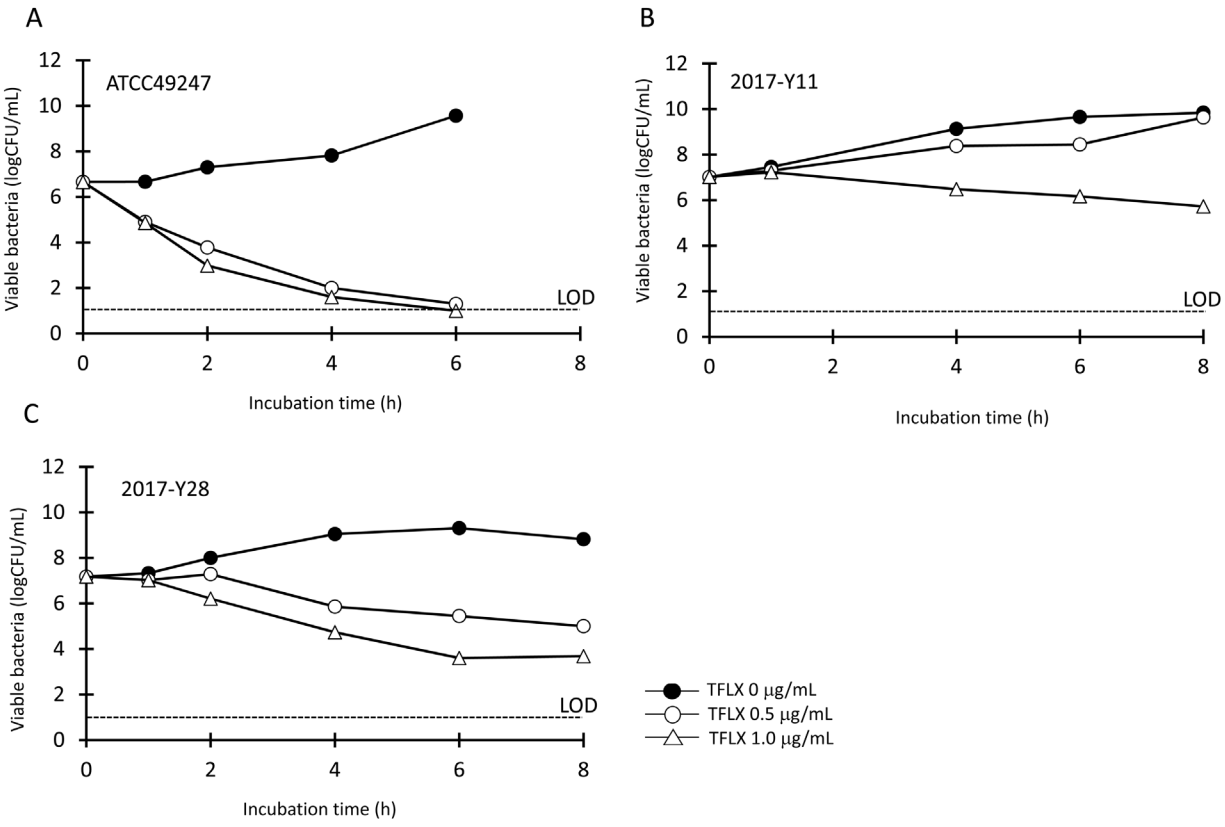

Fig. 2. Time-kill kinetic assay of quinolone low-susceptible isolates. Each isolate was inoculated into sBHI with or without tosufloxacin. Bacterial CFUs were monitored at each time point. LOD, the limit of detection 20 CFU/mL; A, ATCC49247; B, 2017-Y11; C, 2017-Y28; TFLX, tosufloxacin.

analysed the patient background information, including: patient age, sex, and presence or absence of pre-treatment by antimicrobial agents. There was no significant association between antimicrobial susceptibility and patient background (Data not shown).

4. Discussion

In this study, *H. influenzae* isolated from a Japanese teaching hospital was analysed by antimicrobial susceptibility and molecular epidemiological characteristics. Focusing on trends in antimicrobial susceptibility, proportions of AMP- and CLR-susceptible isolates decreased in 2017. All isolates showed susceptibility to quinolones, according to breakpoints defined by CLSI, suggesting that quinolones would be important options for the treatment of *H. influenzae*. However, LVX low-susceptible isolates harbouring an amino acid substitution in the target molecule of quinolone were found. The CLR resistant and LVX low-susceptible isolates also showed resistance to β-lactams, suggesting that multidrug low-susceptible *H. influenzae* gradually increased.

In Japan, TFLX fine granules have been used for children since 2010 because β-lactam-resistant pathogens were found to be prevalent in paediatric respiratory infections. In addition, the TFLX use further increased due to the epidemic of macrolide-resistant *Mycoplasma pneumoniae* that occurred in 2012 [18]. In *Streptococcus pneumoniae*, it was reported that resistant isolates emerged after the introduction of TFLX [19]. This suggested that the increase in quinolone usage could have contributed to the emergence of resistant and/or low-susceptible isolates. The results of MLST also supported that these quinolone low-susceptible *H. influenzae* could independently emerge. Therefore, quinolone usage in medical practice might have led to mutation acquisition.

Among the respiratory quinolones, the breakpoints of TFLX have not been defined. Therefore, to estimate the therapeutic effect of TFLX, the time-kill kinetic curve in the presence of 1 μg/mL and 0.5 μg/mL of tosufloxacin, which were Cmax and 1/2 Cmax, respectively [16,17], were compared between control strain and paediatric quinolone low-susceptible isolates. When tested at the Cmax, susceptible strains decreased below the detection limit,

whereas both isolates harbouring an amino acid substitution in GyrA and substitutions in GyrA and ParC persisted even after 8 h, indicating that the therapeutic effect of TFLX was insufficient to treat low-susceptible isolates, despite recognition as being 'susceptible' targets to a similar agent LVX by routine susceptibility test.

Taken together, this study showed that *H. influenzae* has become resistant to β-lactams and also to other agents. Furthermore, quinolone low-susceptible *H. influenzae* has emerged, and these isolates may survive at the therapeutic Cmax of tosufloxacin, although these were judged to be susceptible to LVX. Therefore, the use and dosage of antimicrobial agents, especially quinolones, should be reconsidered.

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None.

Competing interests

None.

Ethical approval

This study was approved by research ethics committees at both the Tokyo University of Pharmacy and Life Sciences and Yokohama Rosai Hospital (Case no. 16-29 and 28-66, respectively).

References

[1] Davis S, Feikin D, Johnson HL. The effect of *Haemophilus influenzae* type B and pneumococcal conjugate vaccines on childhood meningitis mortality: a systematic review. BMC Public Health 2013;13(Suppl 3):S21.
[2] Ubukata K, Morozumi M, Sakuma M, Takata M, Mokuno E, Tajima T, et al. Etiology of acute otitis media and characterization of pneumococcal isolates after introduction of 13-valent pneumococcal conjugate vaccine in Japanese children. Pediatr Infect Dis J 2018;37:598–604.
[3] Morozumi M, Chiba N, Okada T, Sakata H, Matsubara K, Iwata S, et al. Antibiotic susceptibility in relation to genotype of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Mycoplasma pneumoniae* responsible for community-acquired pneumonia in children. J Infect Chemother 2013;19:432–40.
[4] Pettigrew MM, Tsuji BT, Gent JF, Kong Y, Holden PN, Sethi S, et al. Effect of fluoroquinolones and macrolides on eradication and resistance of *Haemophilus influenzae* in chronic obstructive pulmonary disease. Antimicrob Agents Chemother 2016;60:4151–8.
[5] Bae S, Lee J, Lee J, Kim E, Lee S, Yu J, et al. Antimicrobial resistance in *Haemophilus influenzae* respiratory tract isolates in Korea: results of a nationwide acute respiratory infections surveillance. Antimicrob Agents Chemother 2010;54:65–71.
[6] Seyama S, Wajima T, Nakaminami H, Noguchi N. Clarithromycin resistance mechanisms of epidemic β-lactamase-nonproducing ampicillin-resistant *Haemophilus influenzae* strains in Japan. Antimicrob Agents Chemother 2016;60:3207–10.
[7] Wajima T, Seyama S, Nakamura Y, Kashima C, Nakaminami H, Ushio M, et al. Prevalence of macrolide-non-susceptible isolates among β-lactamase-negative ampicillin-resistant *Haemophilus influenzae* in a tertiary care hospital in Japan. J Glob Antimicrob Resist 2016;6:22–6.
[8] Seyama S, Wajima T, Yanagisawa Y, Nakaminami H, Ushio M, Fujii T, et al. Rise in *Haemophilus influenzae* with reduced quinolone susceptibility and development of a simple screening method. Pediatr Infect Dis J 2017;36:263–6.
[9] Falla TJ, Crook DW, Brophy LN, Maskell D, Kroll JS, Moxon ER. PCR for capsular typing of *Haemophilus influenzae*. J Clin Microbiol 1994;32:2382–6.
[10] CLSI. Performance standards for antimicrobial susceptibility testing; Twenty-Fifth informational supplement. Document M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.
[11] Shiro H, Sato Y, Toyonaga Y, Hanaki H, Sunakawa K. Nationwide survey of the development of drug resistance in the pediatric field in 2000-2001, 2004, 2007, 2010, and 2012: evaluation of the changes in drug sensitivity of Haemophilus influenzae and patients' background factors. J Infect Chemother 2015;21:247–56.
[12] Seyama S, Wajima T, Suzuki M, Ushio M, Fujii T, Noguchi N. Emergence and molecular characterization of *Haemophilus influenzae* harbouring *mef*(A). J Antimicrob Chemother 2017;72:948–9.
[13] Georgiou M, Munoz R, Roman F, Canton R, Gomez-Lus R, Campos J, et al. Ciprofloxacin-resistant *Haemophilus influenzae* strains possess mutations in analogous positions of GyrA and ParC. Antimicrob Agents Chemother 1996;40:1741–4.
[14] Meats E, Feil EJ, Stringer S, Cody AJ, Goldstein R, Kroll JS, et al. Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol 2003;41:1623–36.
[15] Morozumi M, Okada T, Tajima T, Ubukata K, Iwata S. Killing kinetics of minocycline, doxycycline and tosufloxacin against macrolide-resistant *Mycoplasma pneumoniae*. Int J Antimicrob Agents 2017;50:255–7.
[16] Fukuda Y, Takahata M, Mitsuyama J. Pharmacodynamic evaluation of tosufloxacin against *Streptococcus pneumoniae* in an in vitro model simulating serum concentration. J Infect Chemother 2006;12:1–8.
[17] Sunakawa K, Iwai N, Iwata S, Ouchi K, Sakata H, Suzuki K, et al. Population pharmacokinetics and pharmacodynamics of tosufloxacin granules in pediatric infectious diseases. Jpn J Chemother 2010;58:69–77.
[18] Tanaka T, Oishi T, Miyata I, Wakabayashi S, Kono M, Ono S, et al. Macrolide-resistant *Mycoplasma pneumoniae* infection, Japan, 2008-2015. Emerg Infect Dis 2017;23:1703–6.
[19] Takeuchi N, Ohkusu M, Hoshino T, Naito S, Takaya A, Yamamoto T, et al. Emergence of quinolone-resistant strains in *Streptococcus pneumoniae* isolated from paediatric patients since the approval of oral fluoroquinolones in Japan. J Infect Chemother 2017;23:218–23.