The molecular analysis of quinolone-resistant and nonencapsulated *Streptococcus pneumoniae* after the introduction of new oral fluoroquinolone and pneumococcal conjugate vaccine

（肺炎球菌に対する新規経口薬とワクチン導入後の
問題となるキノロン耐性肺炎球菌と無莢膜型肺炎球菌の検討）

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Contents

1. Abbreviations

AOM	acute otitis media
CC	clonal complex
CFU	colony-forming unit
CLI	clindamycin
CLR	clarithromycin
CLSI	Clinical and Laboratory Standards Institute
CPS	polysaccharide capsule
CTX	cefotaxime
IPD	invasive pneumococcal disease
IPM	imipenem
LVX	levofloxacin
MBA	microtiter biofilm assay
MIC	minimum inhibitory concentration
MLST	multi-locus sequence typing
NCC	null-capsule clade
NESp	nonencapsulated *Streptococcus pneumoniae*
OD	optical density
PBP	penicillin-binding protein
PCG	penicillin G
PCR	polymerase chain reaction
PCV	pneumococcal conjugate vaccine
PFGE	pulse-field gel electrophoresis
PRSP	penicillin-resistant *Streptococcus pneumoniae*
PspK	pneumococcal surface protein K
QRDR	quinolone-resistant determination region
SEM	scanning electron microscopy
ST	sequence type
TFLX	tosufloxacin
THYB	Todd-Hewitt broth plus 0.5% yeast extract
TSA	trypticase soy agar
VAN	vancomycin

2. Abstract

With the aim of controlling pediatric pneumococcal diseases, oral broad-spectrum antibiotics for use against drug-resistant *Streptococcus pneumoniae* and pneumococcal conjugate vaccine (PCV) were recently introduced in Japan. Tosufloxacin (TFLX) is a fluoroquinolone antimicrobial agent. TFLX granules for children were initially released in Japan in 2010 to treat otitis media and pneumonia caused by drug-resistant bacteria, e.g. penicillin-resistant *S. pneumoniae* and beta-lactamase-negative, ampicillin-resistant *Haemophilus influenzae*. The evolution of bacterial resistance since TFLX approval is not well known. On the other hand, the prevalence of nonencapsulated *S. pneumoniae* (NESp) has increased with the introduction of PCV in children; however, the bacteriological characteristics of NESp have not been sufficiently clarified.

To clarify the influence of *S. pneumoniae* derived from children after the introduction of new oral fluoroquinolone and PCV, two studies were conducted.

In the first study, to clarify the influence of quinolones administered to children since their approval, we examined the resistance mechanism of TFLX-resistant *S. pneumoniae* isolated from pediatric patients as well as patient clinical characteristics. TFLX-resistant strains (MIC ≥2 mg/L) were detected among clinical isolates of *S. pneumoniae* derived from children (≤15 years old) between

2010 and 2014. These strains were characterized based on quinolone-resistance determination regions (QRDRs), i.e. *gyrA*, *gyrB*, *parC*, and *parE*. In addition, the antimicrobial susceptibility, serotype, and multilocus sequence type of the strains were determined, pulsed-field gel electrophoresis was performed, and patients clinical characteristics based on medical records were assessed for cases with underling TFLX-resistant strains. Among 1,168 *S. pneumoniae* isolates, two TFLX-resistant strains were detected from respiratory specimens obtained from pediatric patients with frequent exposure to TFLX. Both strains had mutations in the QRDRs of *gyrA* and *parC*. One case exhibited gradual changes in the QRDR during the clinical course.

In the second study, NESp strains isolated from the nasopharyngeal carriage of children from four nursery schools in Japan were analyzed for molecular type, antibiotic susceptibility, and biofilm productivity. A total of 152 putative *S. pneumoniae* strains were identified by optochin-susceptibility analysis, of which 21 were not serotypeable by slide agglutination, quellung reaction, or multiplex PCR. Among these 21 strains, three were *lytA*-negative and, therefore, not *S. pneumoniae*. The remaining 18 strains were positive for *lytA*, *ply*, *pspK*, and bile solubility and were therefore confirmed as NESp. Therefore, the isolation rate of NESp in the *S. pneumoniae* strains in this study was 12.0% (18/149). Molecular-typing analyses classified five strains as two existing sequence types (STs;

ST7502 and ST7786), and 13 strains formed four novel STs. Horizontal spread was suspected, because strains with the same ST were often isolated from the same nursery school. The NESp isolates were generally susceptible to most antimicrobials, with the exception of macrolides; however, all isolates possessed more than one abnormal penicillin-binding protein gene. Furthermore, NESp strains were more effective than encapsulated counterparts at forming biofilms, which showed obvious differences in morphology.

To our knowledge, this is the first study of quinolone-resistant *S. pneumoniae* isolated from children, including clinical data, in Japan. These data may help prevent increases in infections of quinolone-resistant *S. pneumoniae* in children; specifically, the results emphasize the importance of administering fluoroquinolones only in appropriate cases. Furthermore, the data of NESp indicated that NESp strains should be continuously monitored as emerging respiratory pathogens.

3. Introduction

Streptococcus pneumoniae colonizes the upper respiratory tract and can be a major causal pathogen of otitis media, pneumonia, and invasive diseases, such as sepsis and meningitis, in children. With the aim of controlling pediatric pneumococcal diseases, oral broad-spectrum antibiotics against drug-resistant *S. pneumoniae* and pneumococcal conjugate vaccine were recently introduced in Japan.

Penicillin-nonsusceptible *S. pneumoniae* have increased rapidly since around 1990 and are a major problem in many countries [1-3]. Recently, pediatric respiratory infections due to macrolide-resistant *Mycoplasma pneumoniae* and beta-lactamase non-producing ampicillin-resistant *Haemophilus influenzae* have become critical issues in Japan [4,5]. Accordingly, oral fluoroquinolone tosufloxacin (TFLX) was initially released for children in January 2010. Since its introduction, many pediatricians and otolaryngologists prescribed TFLX owing to its clinical effectiveness, good compliance based on a twice-daily dosage, and relatively pleasant taste. Frequent use of TFLX may lead to an increased risk of drug-resistant bacteria. Therefore, the development of quinolone-resistant *S. pneumoniae* in children is a concern in Japan. A decrease in TFLX susceptibility has been observed for *S. pneumoniae* derived from children with community-acquired pneumonia [6]. However, the precise frequency of quinolone-resistant *S.*

pneumoniae in children in Japan is unknown.

Most of *S. pneumoniae* strains have polysaccharide capsule (CPS) and more than 95 different CPS types have been identified. The heptavalent pneumococcal conjugate vaccine (PCV7) was introduced in Japan in February 2010 and switched to 13-valent vaccine (PCV13) in November 2013 for prevention of pediatric pneumococcal diseases. The increased use of the PCV in infants has decreased the total number of invasive pneumococcal disease (IPD) cases; however, the percentage of those caused by non-vaccine serotypes has increased dramatically, suggesting that non-vaccine serotype IPDs have replaced vaccine serotype IPDs [7-9]. A similar change has also been observed in *S. pneumoniae* isolated from nasopharyngeal carriage [10]. Most *S. pneumoniae* are regarded as harboring a CPS; however, the existence of nonencapsulated *S. pneumoniae* (NESp) was recently reported.

NESp is mainly classified into two types based on CPS presence. The first group harbors a non-functional capsular polysaccharide synthesis (*cps*) locus located between the glucan 1,6-a-glucosidase gene (*dexB*) and the oligopeptide ABC-transporter gene (*aliA*). Previous studies found that *cps* becomes non-functional because of mutations in the initiating glucose phosphate transferase (*cpsE*; also identified as *wchA*) [11,12]. These strains are nontypeable with the quellung reaction using pneumococcal antisera, but can be typed by polymerase chain

reaction (PCR). The second group comprises true NESp that completely lacks the cps locus, which is replaced with other gene sequences. The lack of the capsular polysaccharide biosynthesis gene (*cpsA*; also identified as *wzg*) identifies this group, which is divided into four clades based on the presence of three genes encoding pneumococcal surface protein K (*pspK*; also identified as *nspA*) and two oligopeptide-binding lipoproteins (*aliB*-like homologs called *aliC* and *aliD*). These four groups are named null-capsule clade (NCC)1, NCC2, NCC3, and NCC4 [12-14]. NCC1 harbors the gene encoding the virulence factor *pspK*, which possesses basic structure and size variations and is negative for *aliC* and *aliD* [13-16]. NCC2 is *aliC*- and *aliD*-positive, but negative for *pspK*, and NCC3 is *aliD*-positive, but negative for *aliC* and *pspK*. NCC4 contains only transposable elements in the *cps* locus.

For the clinical aspects of NESp, a recent study reported that the NESp rate was 12.5% for streptococcal carriage in children [17]. Because PCVs do not provide immunity against NESp, the appearance of NESp might be related to the wide use of PCVs for children. Additionally, NESp pathogenicity has not been clarified, with previous reports indicating that NESp rarely cause IPD [7]. On the other hand, NESp induces non-invasive diseases, such as otitis media, sinusitis, and conjunctivitis [17-20], and it is expected that respiratory infections caused by NESp will increase along with increases in the NESp carriage rate in children.

Therefore, understanding NESp pathogenicity and drug sensitivity is critically important to formulate effective treatment strategies.

To clarify the influence of *S. pneumoniae* derived from children after the introduction of new oral fluoroquinolone and PCV, two studies were conducted.

In the first study, we analyzed the gene sequences of quinolone resistance-determining regions (QRDRs) in TFLX-resistant *S. pneumoniae* isolated from pediatric patients to determine the mechanism of TFLX resistance. Additionally, we analyzed *S. pneumoniae* isolates obtained from a single patient over time to elucidate the dynamic process by which TFLX resistance is acquired. The frequent use of oral quinolones by children led to changes in the QRDR and an increase in the minimum inhibitory concentration (MIC) of quinolone in *S. pneumoniae*.

In the second study, we characterized NESp strains by multi-locus sequence typing (MLST) and assessed strains for *cps* and *pspK* expression. Additionally, we examined drug sensitivity, mutations in genes encoding penicillin-binding proteins (PBPs), and the presence of macrolide- and quinolone-resistance genes. Moreover, we determined differences in biofilm formation between typeable *S. pneumoniae* and NESp strains by microtiter biofilm assays (MBAs) and scanning electron microscopy (SEM).

4. Chapter1. Quinolone-resistant strains in *Streptococcus pneumoniae* isolated from pediatric patients

4.1 Materials and methods

4.1.1 *S. pneumoniae* strains

In total, 1,168 clinical isolates of *S. pneumoniae* derived from children (≤15 years old) were isolated at the Chiba Children's Hospital between 2010 and 2014. The specimen categories for *S. pneumoniae* strains are shown in Table 1. Among these isolates, two TFLX-resistant strains (MIC ≥2 mg/L) were analyzed; one strain, referred to as strain 1, was isolated in 2010 and the other strain, referred to as strain 2, was isolated in 2014. Six pneumococcal strains isolated from a single patient who had TFLX-resistant strain 2 were also analyzed (Figure 1). Patient characteristics (i.e. age, sex, underlying disease, previous exposure to TFLX, and history of pneumococcal conjugate vaccination) were retrospectively analyzed based on medical records for patients with a confirmed TFLX-resistant strain. Patient names were de-identified. This study was approved by the Chiba Children's Hospital Ethics Committee (approval number 2016-11-31).

These isolates were stored at -80°C in 10% skim milk until use. Each isolate was grown on trypticase soy agar (TSA) with 5% sheep blood (Nippon Becton

Dickinson Co. Ltd., Tokyo, Japan) for 24 h at 37°C in 5% CO_2. Each isolate was identified as *S. pneumoniae* using an optochin susceptibility test and a bile solubility test. PCR assays targeting the *lytA* gene, which encodes the major pneumococcal autolysin (LytA), were also used to identify *S. pneumoniae*. All strains were susceptible to optochin, bile-soluble, and positive for the *lytA* gene, and were therefore identified as *S. pneumoniae.*

Table 1. Specimen categories of *Streptococcus pneumoniae* strains

Source	Strain numbers (%)
sputum	706 (60.4)
nasopharynx	217 (18.5)
nasal discharge	146 (12.5)
ear discharge/middle ear effusion	33 (2.8)
pharynx/tonsil/gingiva	20 (1.7)
blood	19 (1.6)
eye discharge	12 (1.0)
cerebrospinal fluid	2 (0.1)
others	13 (1.1)
total	1,168 (100.0)

J Infect Chemother 2017;23:218–23, Table 1.

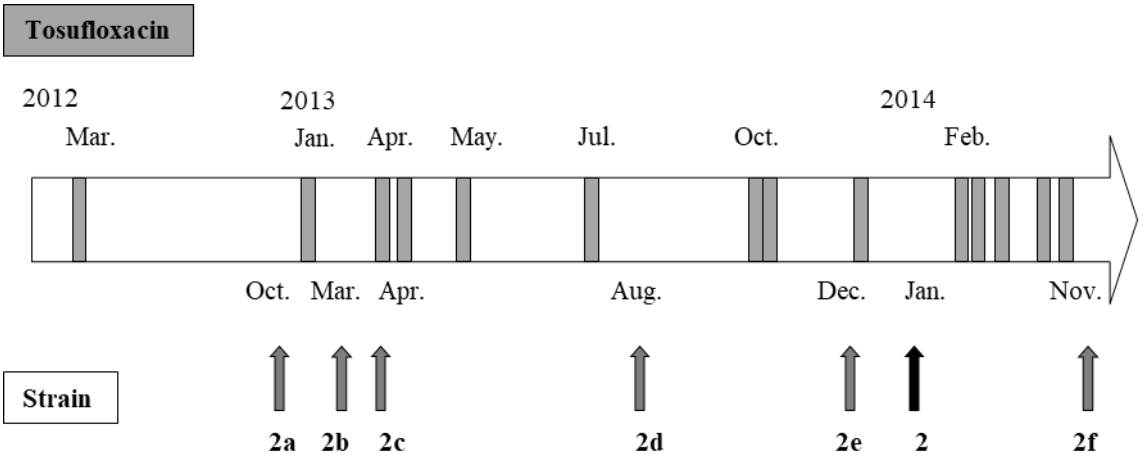


Figure 1. The drug history for a single patient.
Grey bars show the episodes of exposure to quinolones. Arrows show the timing of isolation of each strain. Between the isolation of 2c and 2, TFLX was prescribed in several episodes. ***J Infect Chemother 2017;23:218–23, Figure 1.***

4.1.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) standard method and the MIC was determined. The MIC breakpoints for TFLX were determined in accordance with the recommendations for respiratory infections established by the Japanese Society of Chemotherapy [21].

4.1.3 Capsular serotyping and molecular typing

Serotypes were determined using the slide agglutination reaction with the *S. pneumoniae* antisera 'Seiken' set (Denka Seiken, Tokyo, Japan) and the Quellung reaction using pneumococcal antisera (Statens Serum Institut, Copenhagen,

Denmark).

Molecular typing was performed by MLST as described by Enright et al. [22]. Seven housekeeping genes, i.e. *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*, were amplified by PCR for MLST. The sequencing reaction was carried out using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The Applied Biosystems 3130xl Genetic Analyzer was used for sequencing. Allelic numbers and sequence types (STs) were determined using the MLST website (http://spneumoniae.mlst.net/).

Each isolate was subcultured on TSA with 5% sheep blood (Nippon Becton Dickinson) for 24 h at 37°C in 5% CO_2. Bacterial genomic DNA was extracted from several colonies of each isolate by the boiling method. Extracted DNA was stored at -20°C prior to PCR amplification.

4.1.4 Quinolone resistance-determining region (QRDR) sequencing

The QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* were amplified by PCR. The primers for each loci were synthesized as described by Pan et al. and Balsalobere et al. [23,24]. The primers are described in Table 2 and were generated using KOD FX Neo® (TOYOBO, Osaka, Japan). The sequencing reaction was performed using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed using the Applied Biosystems 3130xl Genetic

Analyzer. The sequences were compared with those of wild-type *S. pneumoniae* R6 using data obtained from GenBank (accession number NC-003098).

Table 2. Primers used for QRDR sequencing

Target gene	Primer sequence (5′ to 3′)	Product size (bp)	References
gyrA	CCGTCGCATTCTCTACGGAATGAATGAATT	380	24
	GCTCCATTAACCAAAAGGTTTGGAA		24
gyrB	TTCTCCGATTTCCTCATG	458	23
	AGAAGGGTACGAATGTGG		23
parC	CAAGACCGGGCTTTGCCAGATATTC	406	24
	TGCTGGCAAGACCGTTGG		23
parE	AAGGCGCGTGATGAGAGC	290	23
	TCTGCTCCAACACCCGCA		23

J Infect Chemother 2017;23:218–23, Table 2.

4.1.5 Detection of other drug-resistance genes

Mutations in genes encoding PBPs (i.e. *pbp1a*, *2x*, and *2b*), the targets of beta-lactams, and the presence of *mef*(A) and *erm*(B), which are associated with resistance to macrolides in *S. pneumoniae*, were detected by PCR using the Penicillin-resistant *S. pneumoniae* Detection Reagent Kit (Wakunaga Pharmaceutical, Osaka, Japan).

4.1.6 Pulse-field gel electrophoresis (PFGE)

The clonal relatedness among isolates was determined by PFGE using the restriction enzyme *Sma*I [25]. The DNA fragments were separated on a 1% agarose gel. Electrophoresis was performed in 0.5× Tris/borate/EDTA buffer at 6 V/cm and at 14°C for 20 h with pulse time of 5.3–34.9 s and linear ramping using the CHEF-DRIII system (Japan Bio-Rad Laboratories, Inc., Tokyo, Japan). A DNA size standard lambda ladder was used as a reference marker.

4.2 Results

4.2.1 Bacteriological analysis and patient clinical characteristics for TFLX-resistant *S. pneumoniae*

Two TFLX-resistant strains (isolated in 2010 and 2014) were detected from 1,168 clinical isolates of *S. pneumoniae*. Accordingly, the frequency of quinolone-resistant *S. pneumoniae* was 0.2%.

The demographic data and antimicrobial susceptibility profiles of the two TFLX-resistant strains are summarized in Table 3. Strain 1 was isolated from the gingiva of a 7-year-old patient with gingivitis. Strain 2 was isolated from the sputum of a 3-year-old patient with pneumonia. With respect to strain 1, several normal bacterial flora were also cultured from gingiva and *S. pneumoniae* could not be conclusively identified as the causative pathogen. However, for strain 2, *S.*

pneumoniae was predominantly cultured from the sputum compared with normal bacterial flora, and was considered the causal pathogen.

Both strains exhibited sensitivity to penicillin G (PCG), but were resistant to macrolides and fluoroquinolones. The MICs of TFLX for the two strains were high, i.e. ≥32 and 8 mg/L, respectively, and those of levofloxacin were both 16 mg/L. The serotype of the two strains was 23F, which is included in PCV7, but they exhibited different STs. The two isolates showed different PFGE profiles.

With respect to clinical background, both cases had an underlying disease and were previously prescribed several courses of oral quinolones. The patient who had strain 1 was prescribed powdered TFLX tablets prior to the approval of TFLX granules for children. TFLX was administered approximately once a month for a 1-year period when the patient had a fever in an outpatient clinic. For the patient who had strain 2, the times at which TFLX was administered are shown in Figure1.

4.2.2 Detection of drug-resistance genes in TFLX-resistant *S. pneumoniae*

The two strains had mutations in the QRDRs of *gyrA* and *parC*. There was a Ser81 mutation in the GyrA QRDR and Ser79 and Asp83 mutations in the ParC QRDR, indicating that both strains had mutations in genes related to tolerance, according to previous functional analyses [26-29]. Several mutations other than Ser79 and Asp83 were also detected in the ParC QRDR. In ParE, no mutations were

observed in strain 1; however, but a mutation was observed at Ile460 in strain 2. Neither of the strains had a mutation in GyrB.

Both strains had mutations in other drug resistance genes, i.e. *pbp1a*, *2x*, and *2b*. Strain 1 had *mef*(A) and strain 2 had both *erm*(B) and *mef*(A).

Table 3. Demographic data and antimicrobial susceptibility profile of two TFLX-resistant strains

Strain No.	Patient Clinical Information					Source	MIC (mg/L)[a]									Mutation in PBP genes[b]	Presence of macrolide resistant genes[c]		Mutation in QRDR[d]				Sero type	ST	PFGE pattern[e]
	Age (yr)	Infection	Previous exposure to quinolones	Underlying disease	Vaccination of PCV		TFLX	LVX	PCG	CTX	IPM	MIN	CLR	CLI	VAN		*erm*(B)	*mef*(A)	ParC	ParE	GyrA	GyrB			
1	7	gingivitis	Yes	congenital neutropenia	PPSV23	gingiva	≥32	16	1	0.5	0.12	4	≥16	≥16	0.25	*pbp1a* + *2x*+*2b*	−	+	S52G S79Y I81M Y82R D83N N91D	none	S81Y	none	23F	242	A
2	3	pneumonia	Yes	West syndrome, chronic respiratory disease	PCV7×1	sputum	8	16	0.25	0.5	＜0.06	4	≥16	≥16	0.25	*pbp1a* + *2x*+*2b*	+	+	S52G S79F N91D	I460V	S81F	none	23F	1437	B

a: TFLX, tosufloxacin; LVX, levofloxacin; PCG, penicillin G; CTX, cefotaxime; IPM, imipenem; MIN, minocycline; CLR, clarithromycin; CLI, clindamycin; VAN, vancomycin

b: Mutations in penicillin-binding protein genes (*pbp1a*, *2x*, and *2b*), the targets of the beta-lactams

c: The presence of the *mef*(A) and *erm*(B) genes was associated with resistance to macrolides ; +, gene was detected; −, gene was not detected

d: A mutation in QRDR was associated with resistance to quinolones

e: Data are displayed in Fig.2.

J Infect Chemother 2017;23:218–23, Table 3.

Table 4. Antimicrobial susceptibility profile of isolates from the patient who had strain 2

Strain No.	Patient Age		Source	MIC (mg/L)[a]									Mutation in PBP genes[b]	Presence of macrolide resistant genes[c]		Mutation in QRDR[d]				Sero type	ST	PFGE pattern[e]
	Yr	Mo		TFLX	LVX	PCG	CTX	IPM	MIN	CLR	CLI	VAN		*erm*(B)	*mef*(A)	ParC	ParE	GyrA	GyrB			
2a	1	11	sputum	＜0.12	1	2	1	0.25	0.12	≥16	≥16	0.5	*pbp1a + 2x+2b*	－	+	none	I460V	none	none	15A	63	C
2b	2	5	sputum	0.25	2	1	0.25	0.25	8	≥16	≥16	0.25	*pbp1a + 2x+2b*	－	+	A115P	I460V	none	none	15A	63	C
2c	2	5	sputum	＜0.12	1	0.25	0.5	＜0.06	8	≥16	≥16	0.25	*pbp1a + 2x+2b*	+	+	none	I460V	none	none	23F	1437	B
2d	2	10	sputum	＜0.12	1	0.25	0.5	0.12	8	≥16	≥16	0.25	*pbp1a + 2x+2b*	+	+	none	I460V	none	none	23F	1437	B
2e	3	1	sputum	0.25	1	0.25	0.5	＜0.06	4	≥16	≥16	0.25	*pbp1a + 2x+2b*	+	+	S52G S79F N91D	I460V	none	none	23F	1437	B
2	**3**	**2**	**sputum**	**8**	**16**	**0.25**	**0.5**	**＜0.06**	**4**	**≥16**	**≥16**	**0.25**	***pbp1a + 2x+2b***	**+**	**+**	**S52G S79F N91D**	**I460V**	**S81F**	**none**	**23F**	**1437**	**B**
2f	4	1	sputum	＜0.12	1	0.5	0.5	0.12	8	1	＜0.06	0.25	*pbp2x+2b*	+	－	none	none	none	none	35B	7809	D

a: TFLX, tosufloxacin; LVX, levofloxacin; PCG, penicillin G; CTX, cefotaxime; IPM, imipenem; MIN, minocycline; CLR, clarithromycin; CLI, clindamycin; VAN, vancomycin

b: Mutations in penicillin-binding protein genes (*pbp1a*, *2x*, and *2b*), targets of the beta-lactams

c: Presence of the *mef* (A) and *erm* (B) genes was associated with resistance to macrolides; +, gene was detected; －, gene was not detected

d: The mutation in QRDR was associated with resistance to quinolones

e: Data are summarized in Fig.2.

J Infect Chemother 2017;23:218–23, Table 4.

4.2.3 Changes in quinolone-resistance genes of *S. pneumoniae* isolated from a single patient before and after the use of TFLX

In the patient who had strain 1, *S. pneumoniae* was not previously detected. However, in the other patient, *S. pneumoniae* was detected several times before and after the detection of strain 2. Therefore, we analyzed *S. pneumoniae* isolates from the patient that became resistant to TFLX during the repeated use of TFLX. Six isolates were obtained before (strain 2a, 2b, 2c, 2d, and 2e) and after (strain 2f) the initial time of isolation of the TFLX-resistant strain (strain 2) (Figure 1). Table 4 summarizes the properties of the 7 isolates, including strain 2.

In ParC, a mutation was detected in 2b and 2e, in addition to strain 2. Strain 2b had an Ala115→Pro mutation, and strain 2e had Ser52→Gly, Ser79→Phe, and Asn91→Asp in ParC. Strain 2e and 2 had the same mutant ParE genotype. In ParE, a mutation was detected in Ile460→Val in strain 2a, 2b, 2c, 2d, and 2e, in addition to strain 2. No isolates other than strain 2 had a mutation in GyrA, and no isolates had a mutation in GyrB.

Figure 2 shows the PFGE profiles of *Sma*I restriction digests. In strain 2a and 2b, the serotype (15A), ST (ST 63), and PFGE pattern were identical. In strain 2c, 2d, 2e, and 2, the serotype (23F) and ST (ST1437) were also

identical, and the PFGE patterns were almost identical, except for a one-band shift in strain 2e and 2. Strain 2f and strain 1 had a distinct pattern. These results show that the particular strain gained quinolone tolerance progressively, with changes noted in strain 2a and 2b, as well as strain 2c, 2d, 2e, and 2.

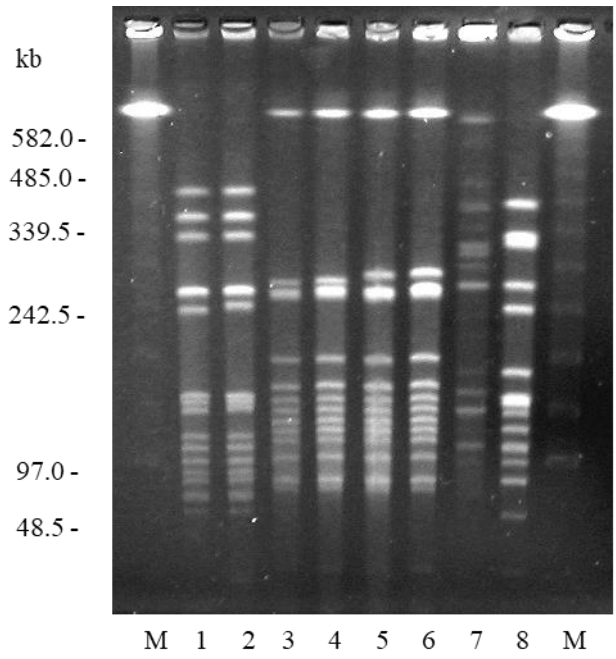


Figure 2. PFGE profiles of *Sma*I restriction digests.
M: DNA ladder, Lane1: strain 2a, Lane2: strain 2b, Lane3: strain 2c, Lane 4: strain 2d, Lane5: strain 2e, Lane6: strain 2, Lane7: strain 2f, Lane8: strain 1
J Infect Chemother 2017;23:218–23, Figure 2.

4.3 Discussion

TFLX is an oral fluoroquinolone developed by Toyama Chemical Co. Ltd.

(Tokyo, Japan) in 1990. The granule form was released for administration to children with otitis media or pneumonia in Japan in January 2010. In Japan, the use of TFLX for children is recommended only for pneumonia when a resistant bacterial infection is suspected and other antimicrobial agents are expected to be ineffective. However, TFLX use in Japan for pneumonia in children from 0 to 14 years old increased since 2010 and reached approximately 30% in 2012 [30]. This increase may be explained by the Mycoplasma epidemic caused by the high rate of macrolide-resistant *M. pneumoniae* in Japan from late 2011 to the end of 2012 [31,32] because TFLX is clinically effective for the treatment of macrolide-resistant *M. pneumoniae* infections[33].

Fluoroquinolone-resistant *S. pneumoniae* has been reported in adults, for whom fluoroquinolones are frequently used to treat a wide range of infections [34-37]. In Japan, Yanagihara et al. examined the rate of levofloxacin-resistant *S. pneumoniae* isolated from adults based on nationwide surveillance. They estimated that the frequency of resistant strains was 1.1% [38]. On the other hand, in children, the frequency of fluoroquinolone-resistant or nonsusceptible strains is very low worldwide, and only a few studies have examined resistant strains in Taiwan, China, Korea, Spain, Belgium, Canada, Russia, and South Africa [39-43]. In Japan,

there has been an increase in the MIC of quinolone in *S. pneumoniae* derived from children with community-acquired pneumonia [6]. Some surveillance has been performed, but fluoroquinolone-resistant strains have not been detected in children [44-46]. In our study, two TFLX-resistant strains were detected from 1,168 clinical isolates of *S. pneumoniae* from pediatric patients. The frequency of quinolone-resistant *S. pneumoniae* was 0.2%. Two patients who had quinolone-resistant *S. pneumoniae* had an underlying disease.

DNA gyrase and topoisomerase IV, the topoisomerase enzymes targeted by the quinolones are each composed of 2 subunits. These subunits are designated GyrA and GyrB, which are encoded by *gyrA* and *gyrB*, for DNA gyrase, and ParC and ParE, which are encoded by *parC* and *parE*, for topoisomerase IV.

Resistance to fluoroquinolones in *S. pneumoniae* usually occurs by a stepwise process [47]. Low-level quinolone resistance is caused by mutations in the QRDR of one of the subunits and resistance progresses to a high level via additional mutations in the QRDR of the second target.

According to previous reports, topoisomerase IV, mainly ParC, is the primary target of trovafloxacin, levofloxacin, norfloxacin, and ciprofloxacin, whereas the primary target of gatifloxacin and sparfloxacin is DNA gyrase, mainly GyrA [23,48,49]. In this study, the Ser81 mutation in GyrA in both

strains, Ser79 mutation in ParC in both strain 1 and 2, and Asp83 mutation in ParC in strain 1 were related to tolerance according to the results of previous reports [26-29]. The Ile460→Val mutation in ParE was previously observed in clinical *S. pneumoniae* isolates with low-level fluoroquinolone resistance [50].

We also observed changes in the QRDRs within a clone until it gained resistance to TFLX. Recently, Chan et al. reported that an *S. pneumoniae* strain isolated from the single patient gained quinolone-resistance during the clinical course, but there are no other similar reports [51]. For patients with a history of TFLX exposure with a TFLX-resistant strain, the number of quinolone-resistant mutations in pneumococcal strains increased over a short period of time after TFLX administration. Prescriptions were not provided for intravenous quinolone therapy or other quinolone oral medications. The successive administration of TFLX initially led to mutations in ParC and subsequently in GyrA. After TFLX-resistant strain 2 was detected, the patient was treated with cefotaxime and panipenem. This suggests that the primary target of TFLX is ParC and the secondary target is GyrA, similar to the mechanism of levofloxacin (LVX) resistance. Subsequently, TFLX-resistant *S. pneumoniae* was not detected in this patient and another quinolone-sensitive serotype was detected. Yokota et al. reported the

mutational patterns for quinolone-resistant *S. pneumoniae* derived from adults. Three of the seven quinolone-resistant strains in this study had mutations in only GyrA, and not ParC [34]. Several quinolone agents are used for adult population. Differences in the usage of quinolone agents between adults and children may explain the different mutation patterns among age groups.

We studied fluoroquinolone-resistant strains, but future studies should also consider non-susceptible strains. Some sensitive strains have mutations that confer resistance, and these strains may easily gain high levels of resistance via new mutations.

The frequency of quinolone-resistant *S. pneumoniae* is still low, but an increase in quinolone use may lead to the emergence of quinolone-resistant strains in children. Importantly, to prevent an increase in quinolone-resistant strains and to ensure the continued effectiveness of antimicrobial agents in children, TFLX should only be used in appropriate cases after antimicrobial susceptibility is examined.

5. Chapter2. Molecular typing, antibiotic susceptibility, and biofilm production in nonencapsulated *S. pneumoniae* isolated from children

5.1 Materials and methods

5.1.1 *S. pneumoniae* strains

S. pneumoniae strains were isolated from nasopharyngeal or nasal-swab specimens collected from healthy children (0–1-years old) who were newly enrolled into four nursery schools in Tokyo, Japan, from October 2012 to March 2015. The three nursery schools (A,B, and C) are located in the same region, east of Tokyo, and the other one (D) is located in a different region, west of Tokyo. The two regions are approximately 30 km apart in straight-line distance. Parents provided written informed consent, and this study was approved by the Ethical Committee of Medical Mycology Research Center, Chiba University (Permission No. 2017-12). In total, 11 children were targeted in 2012, 39 in 2013, and 37 in 2014. There were no duplicate specimens for children enrolled in 2012; however, in 2013 and 2014, specimens were collected three times per child per year in general (April-June, September-November, January-March), the total number of specimens was 231. In total, 152 nasopharyngeal carriage isolates were identified by optochin-susceptibility test and were stored in 10% skim milk at −80°C until analysis. Isolates were propagated on TSA with 5% sheep blood (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan) and Todd-Hewitt broth plus 0.5%

yeast extract (THYB) for 18 h at 37°C in 5% CO_2. Bacterial genomic DNA was extracted using a MORA-EXTRACT kit (Kyokuto Pharmaceuticals, Tokyo, Japan) and stored at −20°C. Isolates were serotyped by slide agglutination reactions with the *S. pneumoniae* antisera ('Seiken' set; Denka Seiken, Tokyo, Japan), quellung reactions with pneumococcal antisera (Statens Serum Institute, Copenhagen, Denmark), and multiplex PCR [52]. Further characterization was performed by bile solubility testing and PCR analysis for pneumococcal autolysin (*lytA*) and the intracellular toxin pneumolysin (*ply*). Polysaccharide cell-wall composition was also assessed using ~10^8 CFU/mL bacterial solution and three rapid antigen-detection kits [BinaxNOW pneumococcal antigen kit (Alere, Inc., Waltham, MA, USA); RAPIRAN *S. pneumoniae* and RAPIRAN *S. pneumoniae* HS (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan)] [53,54].

5.1.2 Analysis of capsule-phase variants and molecular typing

Presence of *cpsA*, *pspk*, *aliC*, and *aliD* was determined by PCR amplification as previously described [13]. The PCR products were purified with FastGene Gel/PCR extraction kit (NIPPON Genetics, Tokyo, Japan) and subjected to sequence analysis with a Big Dye Terminator version 3.1 cycle sequencing

kit on an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). The repeat number of the α-helical repeat structure A(E)(E)EEA/TKR/QK contained in *pspK* was counted, and the *pspk* sequences of all strains were registered in GenBank (accession numbers LC258112–LC258129).

MLST was used to analyze seven housekeeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*) by PCR, as previously described [8]. PCR products were purified with a FastGene Gel/PCR extraction kit (NIPPON Genetics) and subjected to sequence analysis with a Big Dye Terminator version 3.1 cycle sequencing kit on an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems). Allelic numbers and STs were determined using an MLST database (http://spneumoniae.mlst.net/).

The relatedness of the isolates was determined by constructing a gene tree with the neighbor-joining method using MEGA7.

5.1.3 Antimicrobial susceptibility and drug-resistance gene analysis

Antimicrobial-susceptibility testing was performed by broth microdilution according to guidelines of the CLSI (for 22 h at 35°C in ambient air) to determine MIC. *S. pneumoniae* ATCC 49619 was used as reference strain for

the antibiotic susceptibility test. Three strains were not grown in ambient air, but rather under 5% CO_2 for the antibiotic susceptibility test. Antimicrobial agents employed in this study included PCG, CTX, IPM, CLR, CLI, VAN, TFLX, and LVX. The MIC breakpoints for TFLX were determined according to recommendations for respiratory infections established by the Japanese Society of Chemotherapy [7].

Mutations in genes encoding PBPs (i.e., *pbp1a*, *pbp2x*, and *pbp2b*) and the presence of the macrolide-resistance genes *mef*(A) and *erm*(B) were assessed by PCR with the penicillin-resistant *S. pneumoniae* detection reagent kit (Wakunaga Pharmaceutical, Osaka, Japan). The QRDRs of gyrase (*gyr*)*A*, *gyrB*, *parC*, and *parE* were amplified and sequenced as described previously in Chapter1.

5.1.4 In vitro biofilm-formation assays

Biofilm formation was assessed by microtiter biofilm assay (MBA). To compare NESp strains with encapsulated strains, encapsulated strains were isolated from nasopharyngeal carriage and from otorrhea or middle-ear effusion with acute otitis media (AOM). Isolates were precultured on TSA in 5% sheep blood at 37°C in 5% CO_2 for 24 h, followed by isolation of single

colonies, which were subcultured on the same medium. On the third day, cultures were transferred to THYB overnight, and 5 μL of the bacterial solution (OD600=0.15-0.65) was added to the wells of 96-well polystyrene microplates (As One Corporation, Osaka, Japan) along with 200 μL THYB and incubated for 18 h at 37°C under 5% CO_2 overnight. Plates were washed three times with water, stained with 100 μL of 0.5% crystal violet for 5 min, and washed another three times. After drying, 200 μL of 95% ethanol was added to each well, and absorbance at 570 nm was measured with SunriseTM microplate reader (Tecan Japan Co. Ltd., Kanagawa, Japan). The *pspK*-deletion mutant MNZ1131 (derived from wild-type MNZ11 and belonging to NCC1) served as a control [13,55].

5.1.5 Scanning electron microscopy (SEM)

The morphology of biofilms produced by strains 15P200 (NESp strain) and 15P255 (serotype 24F) was examined by SEM, as previously described [56]. A 1 × 1.5 cm polystyrene slide was placed in a culture dish, and cells were cultured in THYB for 18 h at 37°C in 5% CO_2. Specimens were fixed with 2.5% glutaraldehyde, post-fixed with 1% OsO_4, dehydrated with graded ethanol, and transferred with t-butyl alcohol. The samples were freeze-dried

with a VFD-21S t-butyl alcohol freeze-drying apparatus (Vacuum Device Co. Ltd., Mito, Japan), coated with platinum-palladium with an E-102 ion spatter (Hitachi High Technologies, Tokyo, Japan), and observed under an S-3400N scanning electron microscope (Hitachi High Technologies, Tokyo, Japan) at 10 kV.

5.1.6 Statistical analysis

Statistical analysis was performed using JMP software (v7 for Windows; https://www.jmp.com/en_us/home.html). The Mann-Whitney *U* test was used to analyze differences among diagnostic groups.

5.2 Results

5.2.1 *S. pneumoniae* strains

In total, 152 presumptive *S. pneumoniae* strains were detected in the nasopharyngeal carriage isolates by optochin-susceptibility testing. Twenty-one strains were nontypeable by slide agglutination and quellung reactions, and of these, 18 strains were positive for *lytA* and the intracellular toxin *ply*

expression and bile solubility, indicative of *S. pneumoniae.* The remaining three strains were *lytA*-negative, and one was also bile-insoluble and, therefore, not *S. pneumoniae* and excluded from further analyses. All 18 nontypeable strains were confirmed as *S. pneumoniae* according to three rapid antigen-detection kits.

5.2.2 Analysis of capsule-phase variants and molecular typing

Table 5 shows the number of NESp strains among 149 isolates based on the nursery school and survey year. Eighteen NESp strains (12.0%) among 149 isolates were *cpsA*-negative, and all NESp strains expressed *pspK*, which contained 11 to 33 repeats of the α-helical repeat structure A(E)(E)EEA/TKR/QK that contained the novel repeat regions AEEEEAKRK and AEEETKQK (Table 6). GenBank accession numbers of each strain are shown in Table 6. All NESp strains were negative for both *aliC* and *aliD*, and based on this finding, all 18 strains were classified as NCC1. Additionally, MLST analysis classified the isolates into six STs (Table 7). Five strains were classified as either ST7502 or ST7786, whereas the remaining 13 strains were classified into four new STs. ST11973, ST11974, and ST11975 consisted of known allelic profiles, whereas

ST11976 contains the new allelic profile in *aroE*. ST 7786, ST7502, ST 11975, and ST11976 belonged to clonal complex (CC) 7786, and ST11974 belonged to CC1106. CC1106 strains registered in the MLST database were all nontypeable or of serotype 14, whereas ST11973 was a singleton. Figure3 shows the neighbor-joining tree based on the *pspk* gene and the concatenation of seven selected MLST fragments.

Table 5. The number of nonencapsulated strains among 149 nasopharyngeal carriage isolates of *S. pneumoniae*

survey year	Nursery school(nonencapsulated strains/total)				Total
	A	B	C	D	(%)
2012.10～2013.3	0/2	0/0	0/1	0/5	0/8 (0.0)
2013.4～2014.3	3/11	3/21	0/18	0/21	6/71 (8.4)
2014.4～2015.3	0/12	2/12	4/10	6/36	12/70 (17.1)
					18/149 (12.0)

J Infect Chemother 2019, (in press), Table 1.

Table 6. Analysis of capsule phase variants of 18 nonencapsulated strains of *S. pneumoniae*

Strain	bile solubility test	*lytA*	*ply*	*cpsA*	*pspK*	*aliC*	*aliD*	repeat number of the α-helical repeat structure	GenBank accession no.
15P223*	+	+	+	−	+	−	−	11	LC258112
15P245*	+	+	+	−	+	−	−	33	LC258113
15P246	+	+	+	−	+	−	−	11	LC258114
15P232	+	+	+	−	+	−	−	10	LC258115
15P235	+	+	+	−	+	−	−	10	LC258116
15P237	+	+	+	−	+	−	−	10	LC258117
15P286	+	+	+	−	+	−	−	9	LC258118
15P303	+	+	+	−	+	−	−	9	LC258119
15P281	+	+	+	−	+	−	−	9	LC258120
15P291	+	+	+	−	+	−	−	9	LC258121
15P292	+	+	+	−	+	−	−	9	LC258122
15P293	+	+	+	−	+	−	−	9	LC258123
15P192**	+	+	+	−	+	−	−	30	LC258124
15P195***	+	+	+	−	+	−	−	28	LC258125
15P196	+	+	+	−	+	−	−	28	LC258126
15P200**	+	+	+	−	+	−	−	27	LC258127
15P203***	+	+	+	−	+	−	−	28	LC258128
15P205	+	+	+	−	+	−	−	28	LC258129

*, **, *** are isolated from the same child.

J Infect Chemother 2019, (in press), Table 2.

Table 7. Molecular typing of 18 nonencapsulated strains of *S. pneumoniae*

Strain	*aroE*	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	ST	Nursery school	Detected time
15P223*	8	29	9	15	77	155	31	7786	A	2013.4
15P245*	8	29	9	15	77	155	31	7786	A	2013.9
15P246	8	29	9	15	77	155	31	7786	A	2013.9
15P232	8	29	9	15	77	155	260	11975	B	2013.9
15P235	8	29	9	15	77	155	260	11975	B	2013.9
15P237	8	29	9	15	77	155	260	11975	B	2013.9
15P286	8	29	9	15	77	155	26	7502	B	2014.9
15P303	8	29	9	15	77	155	26	7502	B	2015.1
15P281	368	29	9	15	77	155	31	11976	C	2014.9
15P291	368	29	9	15	77	155	31	11976	C	2015.1
15P292	368	29	9	15	77	155	31	11976	C	2015.1
15P293	368	29	9	15	77	155	31	11976	C	2015.1
15P192**	1	5	4	29	2	3	50	11973	D	2015.1
15P195***	8	29	41	15	17	12	31	11974	D	2015.1
15P196	8	29	41	15	17	12	31	11974	D	2015.1
15P200**	1	5	4	29	2	3	50	11973	D	2015.3
15P203***	8	29	41	15	17	12	31	11974	D	2015.3
15P205	8	29	41	15	17	12	31	11974	D	2015.3

*, **, *** are isolated from the same child.

J Infect Chemother 2019, (in press), Table 3.

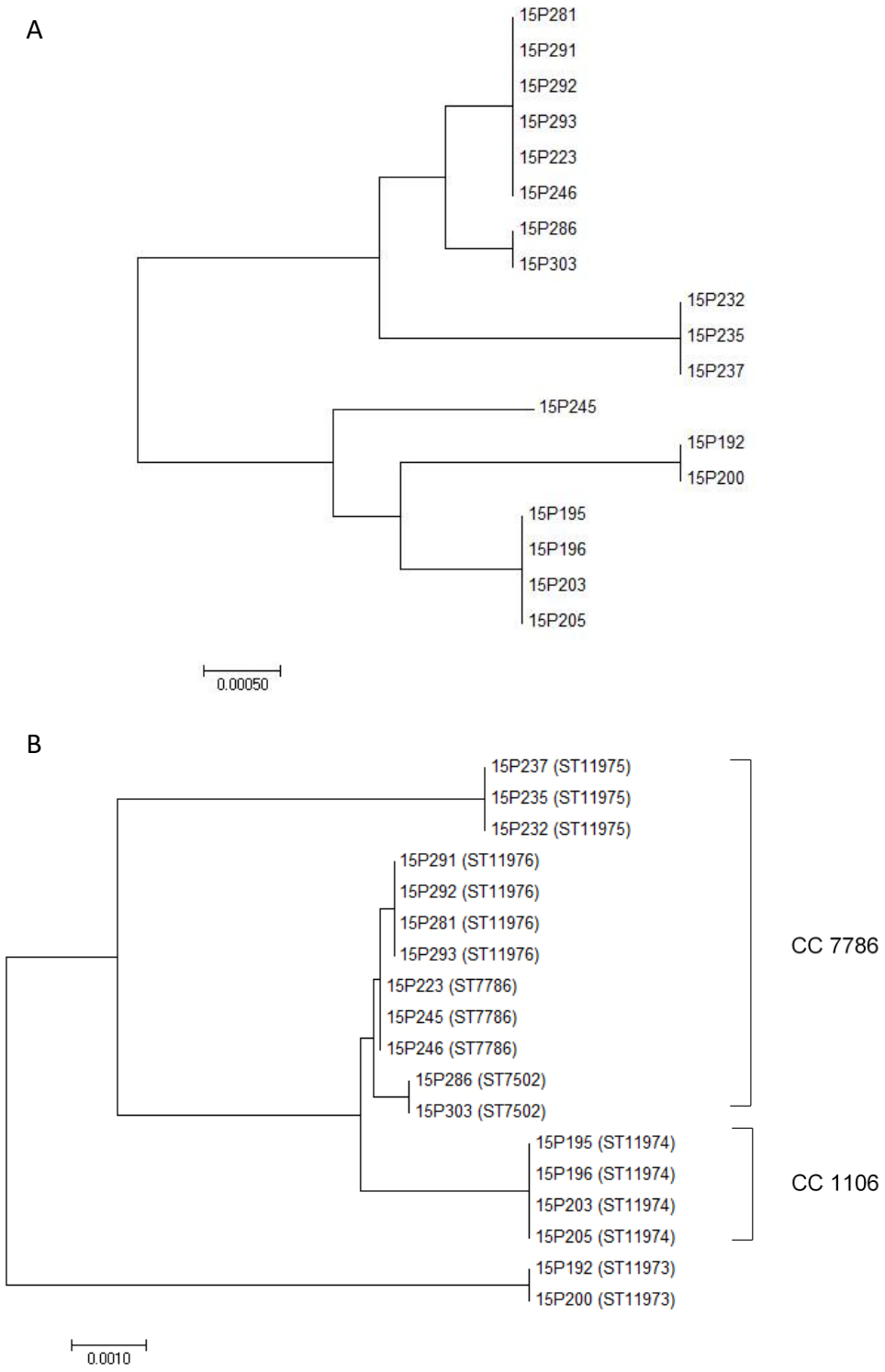


Figure 3. Neighbor-joining tree based on *pspK* fragments and the concatenation of seven selected MLST fragments. (A) Neighbor-joining tree based on *pspK* fragments. (B) Neighbor-joining tree based on the concatenation of seven selected MLST fragments. The dendrogram shows the genetic relatedness among nonencapsulated strains. ***J Infect Chemother 2019, (in press), Figure 1.***

5.2.3 Antimicrobial-susceptibility testing and detection of drug-resistance genes

Table 8 shows the results of antimicrobial-susceptibility testing and drug-resistance genes, as well as MIC values for seven antimicrobial agents. To determine MICs according to antimicrobial-susceptibility testing by broth microdilution, strains were cultured at 35°C in ambient air, whereas strains 15P291, 15P292, and 15P293 were grown under 5% CO_2.

Four strains showed reduced susceptibility to PCG (0.12–0.25 mg/L), and 11 strains showed resistance to CLR (≥1mg/L). The MICs of IPM, TFLX, and LVX for all strains each showed susceptibility at ≤0.06 mg/L, ≤0.12 mg/L, and ≤2 mg/L, respectively. All strains possessed at least one mutation in a PBP, with PBP mutations varying between STs. With respect to macrolide-resistant gene expression, all strains were positive for *erm*(B), whereas only the two ST7502 strains were *mef*(A)-positive. For quinolone resistance, the QRDRs of DNA gyrase (*gyrA* and *parC*) and topoisomerase IV (*parC* and *parE*) were amplified and sequenced, with multiple mutations detected. Six strains harbored a Lys137→Asn mutation in ParC, and 16 strains harbored an Ile460→Val mutations in ParE. No strains harbored mutations in *gyrA* or *gyrB*, and no strains harbored mutations in both the QRDRs of *gyrA* and

parC related to high levels of quinolone resistance.

5.2.4 In vitro biofilm formation

Biofilm formation by the NESp isolates was examined by MBA (Figure 3A). The MBA value of MNZ1131 was high, similar to that for other NESp strains. Figure 3B shows encapsulated strains isolated from nasopharyngeal carriage, and Figure 3C shows encapsulated strains isolated from otorrhea or middle-ear effusion with AOM. Notably, NESp strains tended to be more effective in generating biofilms than encapsulated strains, regardless of the source ($p < 0.001$; Mann-Whitney *U* test) (Figure 4).

Table 8. Antimicrobial susceptibility testing and detection of drug-resistance genes

Strain	ST	Mutation in PBP genes[a]	Presence of macrolide resistant genes[b]		Mutation in QRDR[c]				MIC (mg/L)[d]							
			erm(*B*)	*mef*(*A*)	ParC	ParE	GyrA	GyrB	PCG	CTX	IPM	CLR	CLI	VAN	TFLX	LVX
15P223*	7786	*pbp2x*	+	−	none	I460V	none	none	≤0.06	0.12	≤0.06	0.25	≤0.06	0.25	≤0.12	1
15P245*	7786	*pbp2x*	+	−	none	I460V	none	none	≤0.06	0.12	≤0.06	0.12	≤0.06	0.25	≤0.12	0.5
15P246	7786	*pbp2x*	+	−	none	I460V	none	none	≤0.06	0.12	≤0.06	0.12	≤0.06	0.25	≤0.12	0.5
15P232	11975	*pbp1a + 2x + 2b*	+	−	none	I460V	none	none	≤0.06	≤0.06	≤0.06	0.12	0.12	≤0.12	≤0.12	0.25
15P235	11975	*pbp1a + 2x + 2b*	+	−	none	I460V	none	none	≤0.06	≤0.06	≤0.06	0.5	8	≤0.12	≤0.12	0.5
15P237	11975	*pbp1a + 2x + 2b*	+	−	none	I460V	none	none	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.12	≤0.12	1
15P286	7502	*pbp1a + 2x + 2b*	+	+	none	I460V	none	none	0.25	0.12	≤0.06	>8	>8	0.25	≤0.12	1
15P303	7502	*pbp1a + 2x + 2b*	+	+	none	I460V	none	none	0.12	0.12	≤0.06	>8	>8	0.25	≤0.12	1
15P281	11976	*pbp2x*	+	−	none	I460V	none	none	≤0.06	≤0.06	≤0.06	0.25	≤0.06	0.25	≤0.2	0.5
15P291[e]	11976	*pbp2x*	+	−	none	I460V	none	none	≤0.06	0.25	≤0.06	>8	>8	0.25	≤0.12	1
15P292[e]	11976	*pbp2x*	+	−	none	I460V	none	none	≤0.06	0.25	≤0.06	>8	>8	0.50	≤0.12	1
15P293[e]	11976	*pbp2x*	+	−	none	I460V	none	none	≤0.06	0.12	≤0.06	>8	>8	0.25	≤0.12	1
15P192**	11973	*pbp2x*	+	−	K137N	none	none	none	0.25	0.25	≤0.06	2	>8	0.25	≤0.12	1
15P195***	11974	*pbp2x + 2b*	+	−	K137N	I460V	none	none	≤0.06	0.12	≤0.06	>8	>8	0.25	≤0.12	1
15P196	11974	*pbp2x + 2b*	+	−	K137N	I460V	none	none	≤0.06	0.25	≤0.06	>8	>8	0.25	≤0.12	1
15P200**	11973	*pbp2x*	+	−	K137N	none	none	none	0.25	0.25	≤0.06	1	>8	0.25	≤0.12	1
15P203***	11974	*pbp2x + 2b*	+	−	K137N	I460V	none	none	≤0.06	0.25	≤0.06	>8	>8	0.25	≤0.12	1
15P205	11974	*pbp2x + 2b*	+	−	K137N	I460V	none	none	≤0.06	0.12	≤0.06	8	>8	≤0.12	≤0.12	0.25

a: Mutations in penicillin-binding protein genes (*pbp1a*, *2x*, and *2b*), the targets of the β-lactams.

b: The presence of the *mef*(A) and *erm*(B) genes was associated with resistance to macrolides; +, gene was detected; −, gene was not detected

c: The mutation in QRDR was associated with resistance to quinolones.

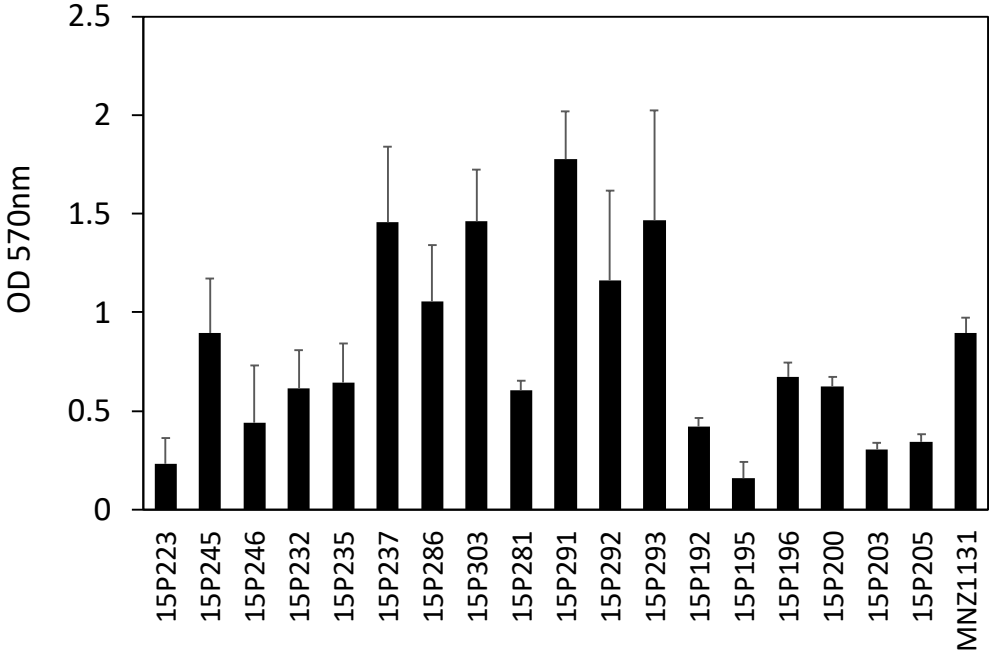
d: PCG, penicillin G; CTX, cefotaxime; IPM, imipenem; CLR, clarithromycin; CLI, clindamycin; VAN, vancomycin; TFLX, tosufloxacin; LVX, levofloxacin

e: Strains 15P291,15P292, and 15P293 were grown under 5% CO_2 because they could not grow under aerobic culture.

*, **, *** are isolated from the same child.

J Infect Chemother 2019, (in press), Table 4.

A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1.5
1
0.5
0
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15P246
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15P303
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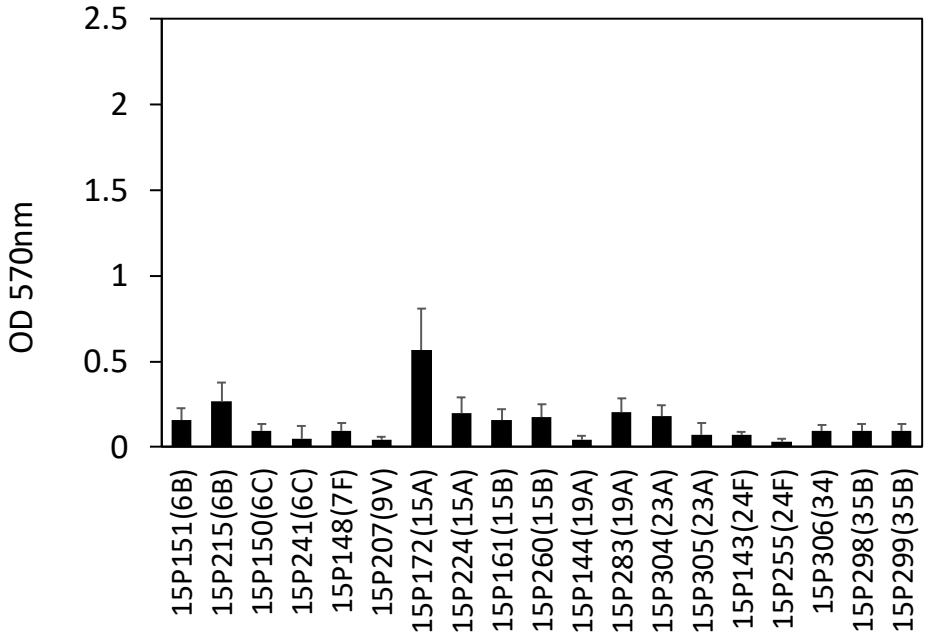
15P203
15P205
MNZ1131

B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2
1.5
1
0.5
0
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15P215(6B)
15P150(6C)
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15P148(7F)
15P207(9V)
15P172(15A)
15P224(15A)
15P161(15B)
15P260(15B)
15P144(19A)
15P283(19A)
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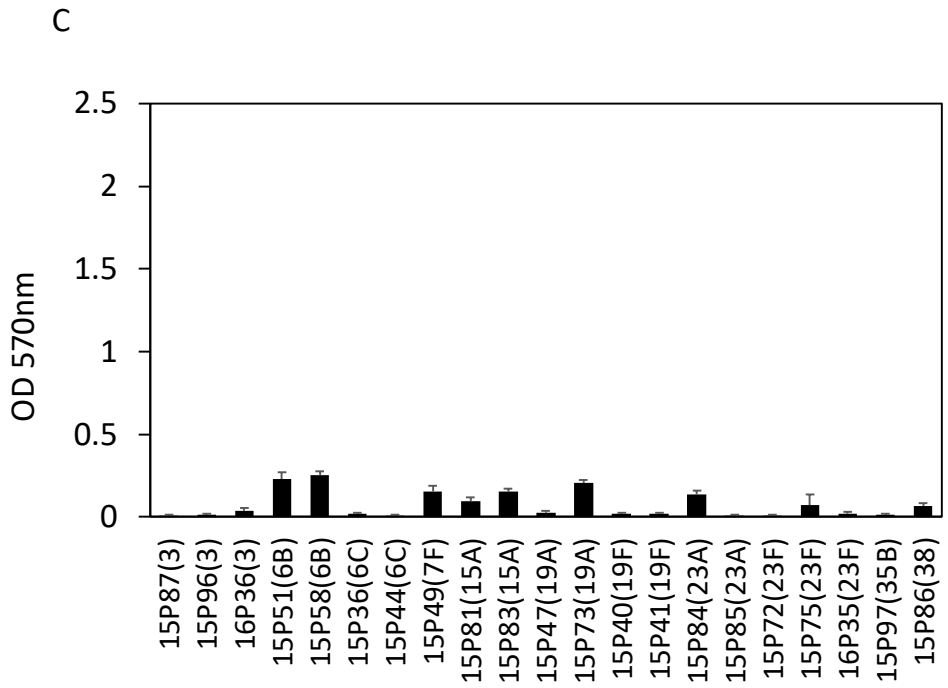
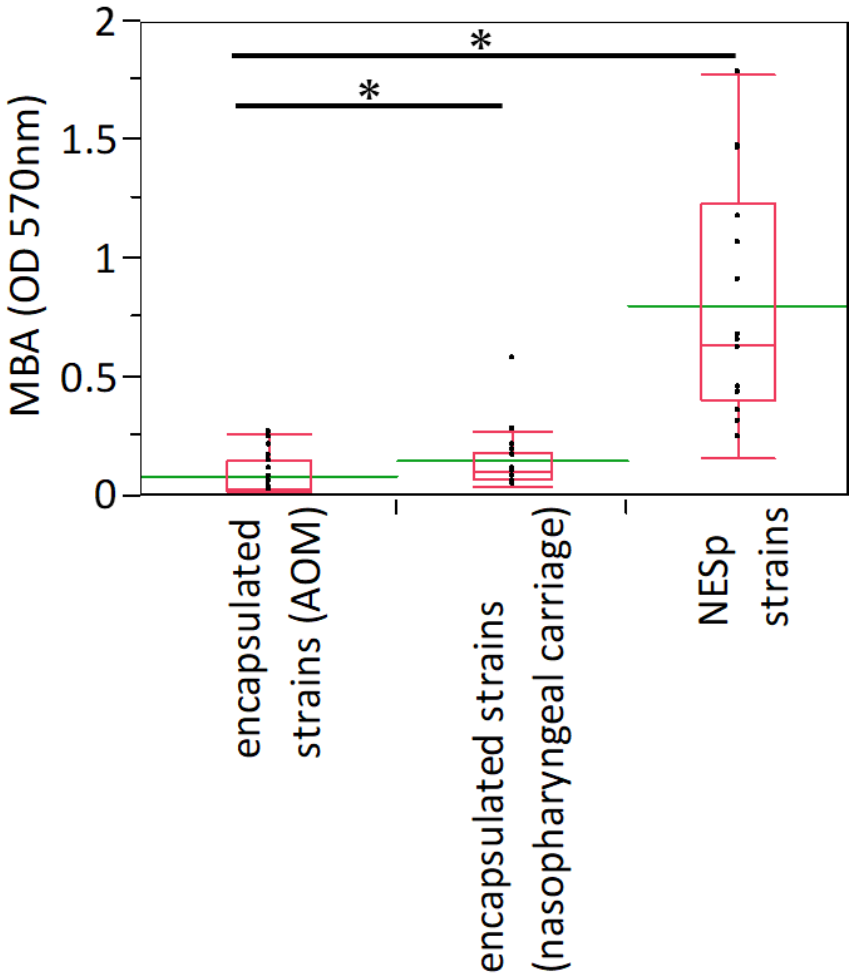


Figure 3. In vitro biofilm formation measured by a microtiter biofilm assay. (A) The MBA of NESp strains, (B) encapsulated strains isolated from nasopharyngeal carriage, and (C) encapsulated strains isolated from otorrhea or middle-ear effusion with AOM. The capsular serotypes are denoted in parenthesis. ***J Infect Chemother 2019, (in press), Figure 1S.***



	Number of strains	Average biofilm formation	Standard deviation
encapsulated strains (AOM)	21	0.0705	0.0809
encapsulated strains (nasopharyngeal carriage)	19	0.1419	0.1217
NESp strains	18	0.7962	0.4898

Figure 4. In vitro biofilm formation measured by a microtiter biofilm assay.
Biofilm productivities of NESp strains, encapsulated strains isolated from nasopharyngeal carriage, and encapsulated strains isolated from otorrhea or middle- ear effusion with acute otitis media. Boxplot shows median and 10th, 25th, 75th, and 90th percentiles. Green lines show the average for each group. NESp strains tended to be more effective in generating biofilms than encapsulated strains, regardless of the source (*p < 0.001). ***J Infect Chemother 2019, (in press), Figure 2.***

5.2.5 Biofilm morphology analysis by SEM

We compared biofilm formation of the NESp strain with that of the encapsulated strain. Figure 5 shows SEM images of the various biofilms. Interestingly, NESp strain 15P200 attached to the plate and formed a chain and clump, and clustered to form a bacterial mass (Figure 5A–C) along with mature biofilm formation (Figure 5C). Conversely, 15P255 (serotype 24F), a low-level MBA-encapsulated strain (Figure 3B), failed to form a biofilm and existed in single, independent, planktonic form (Figure 5D and E).

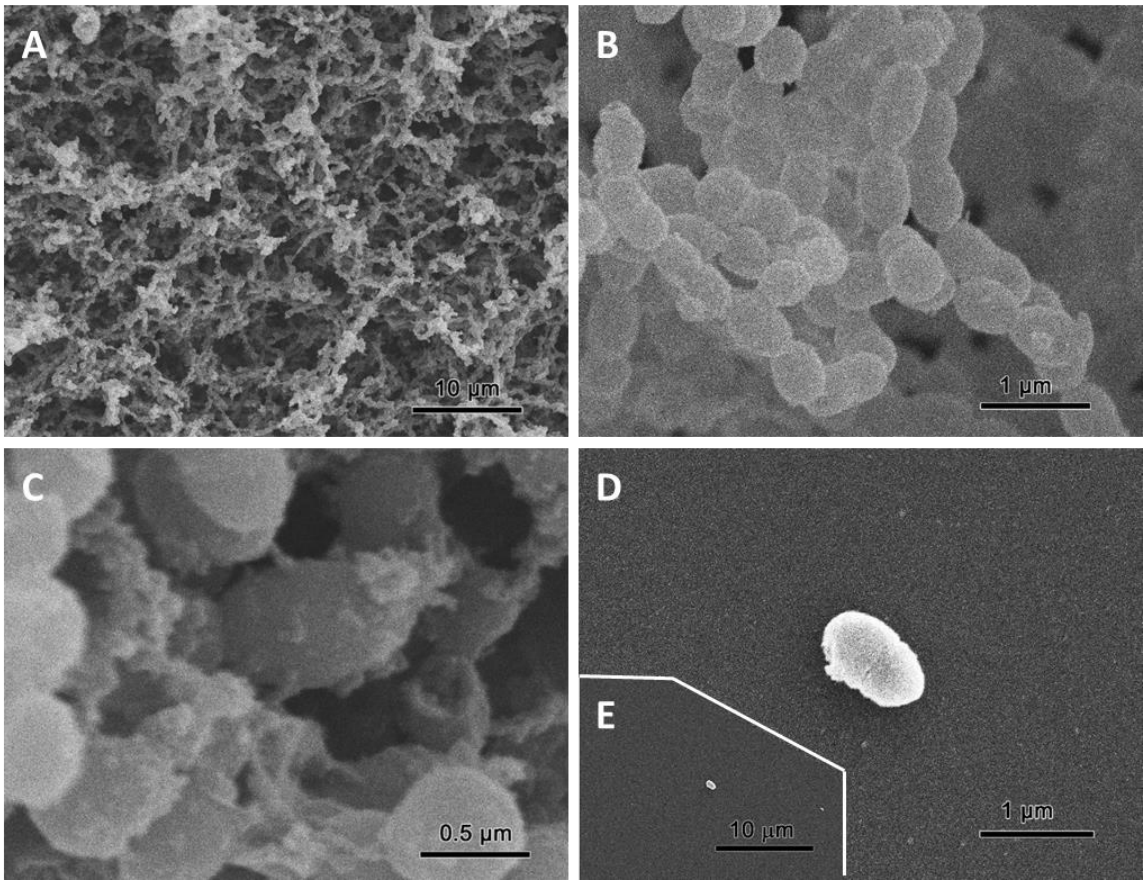


Figure 5. Visualization of biofilm morphology by SEM. (A–C) Strain 15P200 (NESp) was observed attaching to the plate and forming a chain and clump, and biofilms. The arrows show the biofilm structure. (D, E) Strain 15P255 (serotype 24F) was not observed forming a biofilm, but rather existed as a single, independent, and planktonic entity.
NESp, nonencapsulated *S. pneumoniae*; SEM, scanning electron microscopy; ***J Infect Chemother 2019, (in press), Figure 3.***

5.3 Discussion

To our knowledge, this is the first report of bacterial characterization of NESp strains regarding their antibiotic susceptibility, possession of drug-resistance genes, and biofilm productivity. Nontypeable *S. pneumoniae strains* in AOM or carriage were reported from many countries after the introduction of PCV, especially PCV13; however, most reports identified nontypeable strains by the quellung reaction, thereby making it unclear whether these strains were true NESp. The prevalence of true NESp in the nasopharyngeal carriage of children has increased relative to that of encapsulated strains following the recent introduction of PCV in Portugal [10,57]. *PspK*-positive NESp was recently isolated from sinus cultures from children with chronic adenoiditis undergoing a adenoidectomy in the United States [58]. These strains are also found throughout several Asian countries and often isolated from the sputum, sinus aspirate, and middle ear [15]. In Japan, the rate of IPD caused by NESp is low (~0.2–0.3%) according to a population-based surveillance of pediatric IPD [7]. On the other hand, NESp was responsible for 4.7% and 9.5% of AOM and acute rhinosinusitis cases by *S. pneumoniae*, respectively [18]. Additionally, NESp was detected in 12.5% of nasopharyngeal samples from healthy children [18].

Here, the rate of NESp was also 12.0% (18/149) among healthy children in nursery school, which was consistent with previous reports [18]. All strains were *pspK*-positive and classified as NCC1, with the prevalence of these strains increasing annually in nursery school. The evaluation was difficult because the number of specimens was small in 2012; however, in 2013 and 2014, the total detection rates and the number of nursery schools that detected NESp strains clearly increased. There is a possibility that the prevalence of NESp strains is not only increasing in nursery schools but also in the entire region, and there is concern that it will increase further in the future. MLST analysis revealed that 5/18 (27.7%) of isolates were classified as either ST7502 or ST7786, which were previously reported in the Japanese MLST database [18]; however, these STs have not been identified in studies from other countries [13,15,58,59]. Notably, ST groupings tended to associate with specific nursery schools, indicative of horizontal spread. Furthermore, several strains were isolated from the same children at different time points. These data suggested that NESp could be a carriage occurring over several months.

Molecular analysis of *pspK* demonstrated that the number of canonical repeat regions varied from 11 to 33 in our NESp isolates. A(E)EEAKR/QK is an α-helical structure with a highly charged LPXTG motif and a YPT motif that

binds the human polymeric immunoglobulin receptor, which is an established epithelial pneumococcal receptor [13,60]. In the present study, several strains contained AEEEEAKRK or AEEETKQK in novel repeat regions. Previous reports, mostly from Asian countries, indicated that strains often harbor from three to 38 repeats [13,15]. Interestingly, repeat variation is also observed between isolates belonging to the same ST, as well as between subsequent isolations from the same child. It is likely that these children might be consistently colonized, suggesting that this region is highly subject to genomic insertions or deletions.

Antimicrobial susceptibilities of NESp in our study indicated that all NESp isolates showed susceptibility to IPM and TFX; however, 22.2% (4/18) of the strains showed reduced susceptibility to PEN (0.12–0.25 mg/L). Furthermore, all NESp strains possessed at least one mutation in a PBP. Mutations in *pbp1a*, *pbp2b*, and *pbp2x* play important roles in the development of resistance to PEN and cephalosporin by *S. pneumoniae.* Specifically, this includes 9/18 (50.0%) strains harboring mutated *pbp2x*, 5/18 (27.8%) strains harboring mutated pbp1a + pbp2x + pbp2b, and 4/18 (22.2%) strains harboring mutated *pbp2x* + *pbp2b*. In terms of macrolide resistance, 11/18 (61.1%) strains were clarithromycin-resistant (≥1mg/L). Several strains were not grown in ambient air, therefore, these strains were

cultured under 5% CO_2. A previous study showed that the MICs of macrolides and ketolides for *S. pneumoniae* under CO_2 tended to be 1- or 2-fold dilutions higher than those under ambient air due to lower pH [61]. All strains were positive for the macrolide-resistant *erm*(B) gene. In terms of quinolone resistance, 6/18 (33.3%) strains harbored a Lys137→Asn mutation in ParC, 16/18 (88.9%) strains harbored an Ile460→Val in ParE, and there were no strains with mutations in GyrA or GyrB. Resistance to fluoroquinolones in *S. pneumoniae* usually occurs by a stepwise process [47]. Low-level quinolone resistance is caused by mutations in the QRDR of one of the subunits, with resistance progressing to a high level via additional mutations in the QRDR of the second target. Mutations in Ser81 in GyrA, Ser79 in ParC, or Asp83 in ParC are major mutations previously reported in quinolone-resistant *S. pneumoniae* [26]; however, these mutations were not found in this study. Mutations at Lys137→Asn in ParC and Ile460→Val in ParE are not known to contribute to fluoroquinolone resistance generally, but there is a report suggesting that the Lys137→Asn mutation in ParC affects the primary step in the development of high-level quinolone resistance [62,63]. No strains found in this study harbored mutations in both the QRDRs of *gyrA* and *parC*.

The prevalence of penicillin-resistant *S. pneumoniae* (PRSP) strains, such as

serotypes 6B and 19F, decreased after PCV7, whereas serotypes 19A and 15A increased. After replacement with PCV13, serotype 19A strains decreased, but increases were observed in serotype 15A and 35B, which were not targeted by PCV13 and became problematic as drug-resistant strains [7,64-66]. NESp is also capable of becoming a drug-resistant serotype; therefore, it is critical to continue monitoring drug susceptibility in NESp strains.

Because of its simplicity, requiring only the use of basic laboratory materials, the microtiter plate assay is widely used as the method for studying biofilm formation. This assay tests the ability of bacteria to adhere to the plastic surface of a microtiter plate; crystal violet is used to stain the biofilm. Findings made by using microtiter plate assays should be confirmed by microscopy or another method. SEM is frequently used to observe the detailed structure of biofilms at the nanometer-scale level, but live biofilms cannot be observed by SEM. Biofilm observation methods employing fluorescent imaging methods that use confocal laser scanning microscopy and fluorescent protein expression systems have become the mainstream in biofilm studies for real time imaging of biofilms. [67]

There are few studies regarding *S. pneumoniae* biofilm formation [68-70]; however, biofilm production in NESp strains has not been investigated

previously. The presence of capsules hinders biofilm development [69]. Consistently, nontypeable *H. influenzae* strains were more effective at generating biofilms than their encapsulated type b counterparts [71]. In the present study, NESp strains were more effective at forming biofilms than encapsulated strains, independent of the isolation source (e.g., carriage or AOM). Moreover, MBA analysis of MNZ1131 (Δ*pspK*) suggested that the presence of *pspK* did not affect the MBA, which can be an issue with encapsulated stains. Furthermore, SEM imaging revealed an obvious difference in the biofilms produced by NESp versus capsulated strains. Biofilms prevent neutrophil phagocytosis, inhibit the efficacy of antimicrobial agents, and lead to refractory infection. NESp represent a potential causative agent of chronic/recurrent otitis media, as shown using the chinchilla model [72]; therefore, biofilm production by NESp might relate to chronic/recurrent otitis media.

There are several limitations of this study. First, the NESp strains were isolated from the nasopharyngeal carriage of healthy children and not patients with infections; therefore, the characterized strains might not be clinically relevant. Additionally, samples were collected from a limited area, irrespective of population, which might limit their widespread application. Furthermore, the pathogenicity of the strains such as complement sensitivity

was not evaluated. Further studies are needed in order to reveal the pathogenicity of NESp.

In summary, our study identified that the rate of NESp from nasopharyngeal carriage in healthy children was >10%. Many NESp strains were detected as drug resistant through the presence of genetic mutations, and biofilm production was substantially greater than that observed in encapsulated strains. These results suggest that infections due to NESp might represent an important clinical issue in the near future. Therefore, monitoring and investigation of the pathogenicity and drug sensitivity of NESp are extremely important for the development of treatment and prevention strategies.

6. Conclusions

We studied the influence of the introduction of new oral fluoroquinolone and PCV for children on *S. pneumoniae*.

The frequency of quinolone-resistant *S. pneumoniae* among children was 0.2%, still low. However, some sensitive strains with mutations that confer resistance may easily gain high levels of resistance via new mutations. Therefore, an increase in quinolone use may lead to the emergence of quinolone-resistant strains in children. To prevent an increase in quinolone-resistant strains and to ensure the continued effectiveness of antimicrobial agents in children, TFLX should only be administered in appropriate cases

after antimicrobial susceptibility is examined.

On the other hand, the rate of NESp from nasopharyngeal carriage in healthy children was >10%. Many NESp strains were detected as drug resistant through the presence of genetic mutations, and more effective than encapsulated strains at forming biofilms. NESp strains should be further studied to reveal the pathogenicity and continuously monitored as emerging respiratory pathogens.

7. Acknowledgements

This Doctoral degree has been a dream since I started research at the Department of Infectious Diseases, Medical Mycology Research Center, Chiba University. This PhD study has been a long journey and it would have not been possible without the considerable support of my boss, staff, collaborators, and family.

- Professor Dr. Naoki Shimojo, professor Dr. Hiroshi Shirasawa, and professor Dr. Koichiro Tatsumi , for their useful suggestions.
- Professor Dr. Katsuhiko Kamei, for giving advices on my research.
- Assistant Professor Dr. Naruhiko Ishiwada, for constant support, for expert guidance, and patience throughout my study.
- Professor Dr. Moon H Nahm, for giving expert advice and kindly supplying strains.

・ Dr. Misako Ohkusu and Dr. Sachiko Naito, for unstinted technical support and ever-kind encouragement.
・ Dr. Tomoko Yamamoto and Dr. Akiko Takaya, for lending their expert knowledge and advising me on my research.
・ Dr. Masashi Yamaguchi, for expert technical support.
・ Dr. Hoshino Tadashi, Dr. Noriyuki Wada, and Dr. Satoko Kurosawa for kindly supplying strains, collecting data, and giving advice.
・ The participants of the Division of Laboratory Medicine and Clinical Genetics, Chiba University Hospital, and Chiba Children's Hospital, particularly Akiko Miyabe, Hiroko Sato, and Mari Sato, for kind support.

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主論文

Emergence of quinolone-resistant strains in *Streptococcus pneumoniae* isolated from paediatric patients since the approval of oral fluoroquinolones in Japan.

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Journal of Infection and Chemotherapy 23(4):218-223 (2017)

DOI: 10.1016/j.jiac.2019.02.007

公表済

副論文

Molecular typing, antibiotic susceptibility, and biofilm production in nonencapsulated *Streptococcus pneumoniae* isolated from children in Japan.

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Journal of Infection and Chemotherapy

DOI: 10.1016/j.jiac.2016.12.012

2019 年 2 月受理、印刷中