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Original Article

Predominance of Serogroup 19 CC320/271 among Penicillin-Nonsusceptible *Streptococcus pneumoniae* Isolates after Introduction of the PCV7 Vaccine in Several Regions of Japan

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SUMMARY: Multidrug-resistant *Streptococcus pneumoniae* serogroup 19, including serotypes 19A and 19F, associated with clonal complex 320/271 (CC320/271), has been previously shown to be predominant in many countries after introduction of a 7-valent pneumococcal conjugate vaccine (PCV7). However, in Japan there has been no epidemiological research focused on penicillin-nonsusceptible isolates after this event. Therefore, we aimed to characterize penicillin-nonsusceptible *S. pneumoniae* (PNSSP; penicillin minimum inhibitory concentration [MIC] ≥ 4.0 μg/ml) after the introduction of PCV7 in Japan. Throughout Japan, we collected 1,057 pneumococcal isolates from 2010 to 2014. We then evaluated MICs and performed serotyping, multilocus sequence typing, and sequencing of penicillin-binding protein genes in 51 isolates (penicillin MIC ≥ 2.0 μg/ml). Twenty-three isolates (2.2%) showed penicillin nonsusceptibility (penicillin MIC ≥ 4.0 μg/ml). Serotypes 19F (14 isolates, 60.9%) and 23F (4 isolates, 17.4%), which are covered by the vaccine, were predominant among PNSSP strains. Only 3 isolates belonged to nonvaccine serotype 19A. Among the PNSSP isolates, CC320/271 (16/23 strains, 69.6%) was the most prevalent clone. Moreover, CC320/271 clones showed high MIC values of a third-generation cephalosporin. Thus, we demonstrated clonal predominance of serogroup 19 CC320/271 with strong resistance to β-lactams including a third-generation cephalosporin among PNSSP isolates.

INTRODUCTION

Streptococcus pneumoniae is a common bacterial pathogen and is responsible for a wide range of diseases including otitis media, sinusitis, pneumonia, sepsis, and meningitis (1). This pathogen remains a major cause of pneumonia-associated morbidity and mortality worldwide. Penicillins (PENs) have been used for the treatment of most pneumococcal infections. However, the efficacy of β-lactam antibiotics has decreased due to the emergence and rapid global spread of PEN-resistant *S. pneumoniae* (PRSP) (2). In recent years, the most important event that affected the epidemiology of *S. pneumoniae* was introduction of the pneumococcal conjugate vaccine (PCV). The 7-valent PCV vaccine (PCV7) includes the most common *S. pneumoniae* serotypes that cause pediatric invasive pneumococcal diseases (IPD), specifically serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. This vaccine has contributed to a significant decrease in the incidence of IPD caused by the serotypes included in the vaccine as well as the prevalence of antibiotic resistance in developed countries where PCV7 has been extensively used (3).

In the United States, PCV7 has been applied to vaccinate children since 2000, resulting in individual and herd immunity and a decline in pneumococcal-infection prevalence in this population (3–6). After PCV7 was introduced, the prevalence of serotype 19A pneumococcal strains, which are associated with PEN resistance, increased (5–7). According to a study from the Asian Network for Surveillance of Resistant Pathogens (ANSORP) in 2008–2009, 28.1% of PEN-nonsusceptible *S. pneumoniae* (PNSSP) isolates belonged to serotype 19A in Asian countries (8). Most serotype 19A *S. pneumoniae* isolates belonged to clonal complex 320 (CC320), which originated from the multidrug resistant (MDR) Taiwan19F-14 Pneumococcal Molecular Epidemiology Network clone (a double-locus variant of Taiwan19F-14), in many parts of the world (9,10). According to the ANSORP study, MDR sequence type 320 (ST320) was the most prevalent clone among 19A *S. pneumoniae* isolates from Asian countries (11). In the United States, the emergence of MDR CC320 has contributed to an increase in serotype 19A prevalence after the introduction of PCV7 (5).

In Japan, PRSP has increased in prevalence rapidly as a cause of respiratory tract infections, acute otitis media, and IPD since the late 1990s (12). Subsequently, PCV7 vaccination was introduced for children in February 2010 via voluntary vaccination and in November 2010 by the Provisional Special Fund for the Urgent Promotion of Vaccination. PCV7 was incorporated into routine vaccination schedules in April 2013 and replaced

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by PCV13 in November 2013. As a result, the estimated rates of PCV7 vaccination for such children were < 10% in 2010, 50–60% in 2011, and 80–90% in 2012 (13). PCV7 vaccination resulted in a rapid decrease in the incidence of IPD infections among children, as in other countries. However, pneumococcal infections caused by non-PCV7 serotypes (19A, 15A, 15B, 15C, and 24) increased in prevalence among children during 2012 (13). In accordance with the observed changes in the predominance of serotype 19A CC320 in cases of infection caused by PRSP, after the introduction of PCV7 in the United States and Asian countries, a similar trend was hypothesized after initiation of PCV7 childhood vaccination in Japan. However, no epidemiological data regarding the prevalence of PRSP after the introduction of PCV7 in Japan have been reported. Molecular epidemiological analysis, data on antibiotic susceptibility, and analysis of resistance genes are crucial for the prevention and treatment of infectious diseases such as drug-resistant *S. pneumoniae*. Therefore, we performed serotyping, multilocus sequence typing (MLST), and sequencing of the penicillin-binding protein (PBP) genes to characterize PNSSP isolated in several regions of Japan after PCV7 was introduced.

MATERIALS AND METHODS

Clinical isolates: In this study, 1,057 clinical isolates of *S. pneumoniae* were collected in several regions of Japan between March 2010 and January 2014 by the Miroku Medical Laboratory, a clinical microbial testing company. These clinical isolates of *S. pneumoniae* were recovered from samples derived from various sites: nasopharynx, 685/1,057 (64.8%); lower respiratory tract, 246/1,057 (23.3%); ear, 87/1,057 (8.2%); eye, 12/1,057 (1.1%); blood, 10/1,057 (0.9%), and several others. The patient age distributions by clinical isolates were as follows: 0–1 year old, 302/1,057 (28.6%); 2–4 years old, 256/1,057 (24.2%); 5–19 years old, 143/1,057 (13.5%); 20–64 years old, 89/1,057 (8.4%); ≥ 65 years old, 211/1,057 (20.0%); and unknown, 56/1,057 (5.3%).

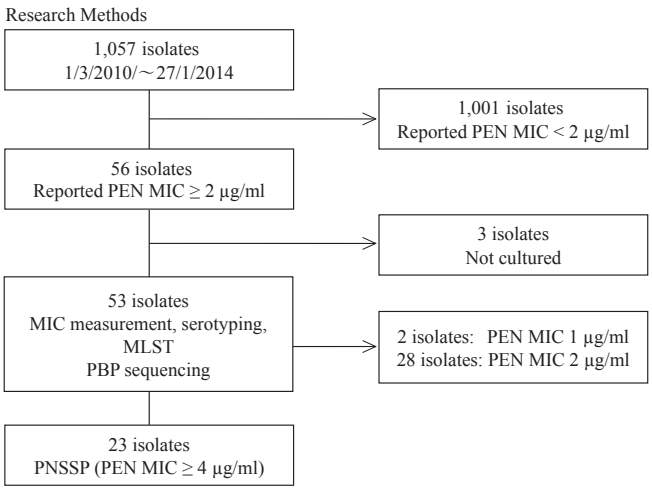

Fig. 1. Flow chart of the research method used in this study. PEN, penicillin; MIC, minimum inhibitory concentration; MLST, multilocus sequence typing; PBP, penicillin binding protein; PSSP, penicillin susceptible *Streptococcus pneumoniae*; PNSSP, penicillin non-susceptible *Streptococcus pneumonia*.

We determined the minimum inhibitory concentration (MIC) of PEN by broth microdilution tests of isolates that were reported to have PEN MIC values ≥ 2 μg/ml by the Miroku Medical Laboratory (n = 56). Three isolates were not cultured, 2 isolates were found to have PEN MIC 1 μg/ml, and 28 isolates 2 μg/ml. Finally, we defined 23 isolates for which the PEN MIC values were ≥ 4 μg/ml as PNSSP and evaluated the MIC values of various antimicrobials for the serotypes and sequence types (STs) and performed analyses of the *pbp* genes (Fig. 1). Isolates were confirmed to be *S. pneumoniae* by polymerase chain reaction analysis of *lytA* (14), and samples were stored at −80 °C in brain heart infusion broth containing 15% glycerol.

Antibiotic susceptibility testing: MIC values were determined by the broth microdilution method described by the Clinical and Laboratory Standards Institute (CLSI) (15). The following antimicrobials were tested: PEN, cefotaxime (CTX), ceftriaxone (CRO), meropenem (MEM), vancomycin (VAN), erythromycin (ERY), and levofloxacin (LVX). MIC breakpoints were defined according to the CLSI criteria (15). We used the PEN breakpoints for nonmeningitis parenteral PEN in all cases.

Serotyping: Pneumococcal isolates (PEN MIC ≥ 2 μg/ml) were serotyped by multiplex polymerase chain reaction (MP-PCR) as described previously (16). If isolates could not be typed by MP-PCR, they were next analyzed by an agglutination method using serotype-specific antisera (obtained from Denkaseiken, Tokyo, Japan) as recommended by the manufacturer. The Quellung reaction carried out with serotype-specific antisera (Statens Serum Institute, Copenhagen, Denmark) served for confirmation when we had to distinguish 19F and 19A strains.

MLST: This testing was performed on all PNSSP strains by means of 7 housekeeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*) as described elsewhere (17). The allele sequences and STs were determined using the *S. pneumoniae* MLST database (http://pubmlst.org/spneumoniae/). New alleles and STs were submitted to the Institute Pasteur MLST databases. To identify phylogenetic relations among STs and to determine the clonal complex, we conducted the eBURST analysis in eBURST version 3 (http://eburst.mlst.net/).

Analyses of *pbp* genes: Because it is known that isolates with PEN MICs of 2 μg/ml also often harbor amino acid substitutions in PBPs, isolates with PEN MICs ≥ 2 μg/ml were subjected to this analysis (n = 51). Genes *pbp*, *pbp1a*, *pbp2b*, and *pbp2x* were amplified and sequenced by the Sanger method. The amino acid sequences of PBP1a, PBP2b, and PBP2x were aligned to the corresponding sequences of *S. pneumoniae* R6 (GenBank accession numbers: AAK99133 for PBP1a, AAL00321 for PBP2b, and AAK99108 for PBP2x). All sequences of *pbp* genes analyzed in this study were deposited in GenBank and EMBL databases through the DNA Data Bank of Japan (DDBJ; accession numbers: LC195227, LC195834–LC195884, LC198078–LC198181).

Ethical statement: This study was focused on bacterial clinical isolates and was completely anonymous, without identifiable information being obtained. According to the ethics guidelines for epidemiological studies

from the Ministry of Health, Labour and Welfare, Japan, ethical approval and written informed consent are not required for this type of study.

RESULTS

Clinical isolates: Among the 1,057 *S. pneumoniae* isolates, 23 (2.2%) were found to be PNSSP (PEN MIC ≥ 4 μg/ml; Fig. 1, Table 1). Of the 23 PNSSP isolates, 11 were isolated from the nasopharynx, 8 from the lower respiratory tract, 3 from ear discharge, and one from blood.

PEN-nonsusceptible isolates: Among all the collected *S. pneumoniae* isolates, 2.2% (n = 23) were PNSSP (PEN MIC ≥ 4 μg/ml). Among the PNSSP isolates, prevalence rates of resistance to CTX (n = 12, 52.2%), CRO (n = 11, 47.8%), MEM (n = 10, 43.5%), and to ERY (n = 23, 100%) were high. Only one isolate was resistant to LVX, and no isolate was nonsusceptible to VAN.

Table 1. Minimum inhibitory concentration (MIC) distributions of each antibiotic for 23 penicillin non-susceptible *Streptococcus pneumoniae* (PNSSP) isolates

	MIC (μg/ml)								
	0.12	0.25	0.5	1	2	4	8	16	≥ 32
Penicillin	0	0	0	0	0	20	3	0	0
Cefotaxime	0	0	0	3	8	4	5	1	2
Ceftriaxone	0	0	0	2	10	7	2	2	0
Meropenem	0	0	13	9	1	0	0	0	0
Vancomycin	0	13	10	0	0	0	0	0	0
Levofloxacin	0	0	3	18	1	0	1	0	0
Erythromycin	0	0	0	0	2	6	2	0	13

Serotype distribution: PNSSP isolates were found to belong to 5 serotypes or serogroups, with 3 dominant serotypes: 19F (n = 14, 60.9%), 23F (n = 4, 17.4%), and 19A (n = 3, 13.0 %). Serotype 19F isolates were detected each year, but all 3 isolates that belonged to 19A were detected in 2013 in infants of age 0 to 2 years (Table 2).

Multilocus ST distribution: The most predominant clonal complex (CC) among PNSSP isolates was CC320/271 (n = 16, 69.6%; Tables 2 and 3). Seven of these strains were ST236, serotype 19F. Three isolates belonged to ST320, which is a double-locus variant of ST236 (Fig. 2), and among these 3 isolates, 2 belonged to 19A, and one to 19F. Two isolates were ST271 serotype 19F. The other 4 strains were various STs that were single-locus variants of ST236. The relation among CC320/271 isolates is shown in Fig. 2. The second most prevalent CC was CC242 (n = 3, 13.0%), and all isolates belonged to ST1435 serotype 23F (Table 2).

Sequences of *pbp1a*, *pbp2b*, and *pbp2x*: In 51 isolates with PEN MIC values ≥ 2 μg/ml (MIC range: 2 to 8 μg/ml), the coding regions of *pbp1a*, *pbp2b*, and *pbp2x* were analyzed. The polymorphisms in amino acid sequences of these PBPs reflect different levels of β-lactam resistance among clinical isolates of *S. pneumoniae*. Table 4 lists the isolates analyzed, their corresponding PEN and CTX MICs, and the amino acid substitutions present in PBP2x, which were near the active-site motifs

Table 2. Sequence types (STs), serotypes, and minimum inhibitory concentrations (MICs) of β-lactams

CC	ST	Isolate	Serotype	MIC (μg/ml)			Isolation year	Patient age (yr)	Source
				PEN	CTX	MEM			
CC320/271	236	NUBL5696	19F	8	8	1	2012	unknown	nose
	236	NUBL1097	19F	8	8	0.5	2011	unknown	nose
	236	NUBL10910	19F	4	8	0.5	2013	4	sputum
	236	NUBL5687	19F	4	2	1	2011	39	sputum
	236	NUBL5690	19F	4	4	0.5	2011	39	nose
	236	NUBL5695	19F	4	16	0.5	2012	68	throat
	236	NUBL7018	19F	4	1	0.5	2012	1	throat
	271	NUBL1088	19F	4	> 16	0.5	2010	unknown	ear
	271	NUBL1085	19F	4	8	1	2010	unknown	ear
	320	NUBL1080	19F	4	4	1	2010	unknown	nose
	320	NUBL5700	19A	4	2	2	2013	0	ear
	320	NUBL5699	19A	4	2	1	2013	1	nose
	926	NUBL1098	19F	4	2	0.5	2011	1	nose
	2993	NUBL5692	19F	4	8	0.5	2011	76	blood
	9027	NUBL5697	19F	4	4	0.5	2012	unknown	sputum
	11941	NUBL10913	19A	8	4	1	2013	2	sputum
CC242	1435	NUBL7022	23F	4	1	0.5	2012	69	sputum
	1435	NUBL7019	23F	4	2	0.5	2012	0	throat
	1435	NUBL1086	23F	4	2	1	2010	1	nose
CC81	282	NUBL10911	6C/6D	4	2	1	2013	3	sputum
Single	3176	NUBL5703	23F	4	1	0.5	2013	88	sputum
CC3787	4845	NUBL5689	19F	4	2	0.5	2011	1	nose
CC230	2756	NUBL5688	6A/6B	4	> 16	1	2011	75	sputum

CC, clonal complex; Single, singleton; PEN, penicillin; CTX, cefotaxime; MEM, meropenem.

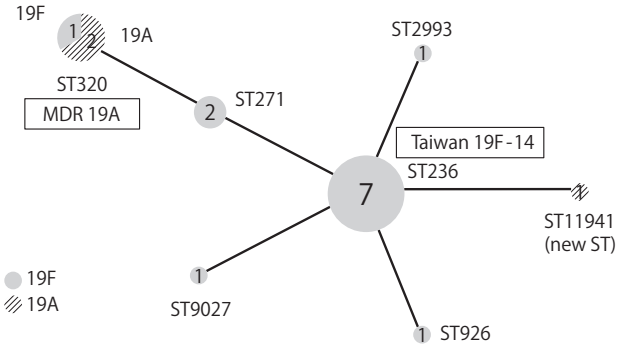

Fig. 2. eBURST of clonal complex (CC)320/271 isolates. eBURST was performed using multilocus sequence typing data. The eBURST analysis was used to connect 2 sequence types (STs) with one allelic profile difference by one line. The distances for the lines between 2 STs do not reflect the genetic distances between 2 STs. This figure does not show all STs that belong to CC320/271. MDR, multi-drug resistance.

of the transpeptidase domains or have been reported to confer resistance to β-lactams.

All 51 isolates with PEN MIC values ≥ 2 μg/ml had amino acid substitutions T371A/S in the STMK motif of PBP1a and 4 consecutive amino acid substitutions, T574SQF to NTGY, which contribute to resistance (18). There were no characteristic amino acid substitutions in highly resistant isolates or certain CCs. Eighteen isolates that belonged to CC320/271 were 99% identical to each other among the CC320/271 strains when PBP1a was analyzed, and only 2 isolates harbored a single-amino acid substitution in PBP1a.

In PBP2b from 51 clinical isolates, amino acid substitutions T451A/S and E481G, which have been shown to occur in PBP2b of many drug-resistant clinical isolates (19), were detected in almost all our isolates. Nineteen amino acid substitutions in PBP2b between residues 566 and 646, which might contribute to high CTX resistance,

Table 3. Minimum inhibitory concentration (MIC) range, MIC_{50}, and MIC_{90} for each clonal complex among 23 penicillin non-susceptible *Streptococcus pneumoniae* (PNSSP) isolates

CC	No. of isolates (%)	PEN MIC (μg/ml)			CTX MIC (μg/ml)			MEM MIC (μg/ml)		
		Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
CC320/271	16 (69.6)	4.0–8.0	4	8	1.0– > 16	4	16	0.25–2	0.5	1
CC242	3 (13.0)	4	4	4	1.0–2.0	2	2	0.5–1	0.5	1
Other	4 (17.4)	4.0–8.0	4	4	1.0– > 16	2	> 16	0.25–1	1	1

CC, clonal complex; No, number; PEN, penicillin; CTX, cefotaxime; MEM, meropenem.

Table 4. Distribution of nucleotide and amino acid substitutions in PBP2x from clinical isolates of *Streptococcus pneumoniae*

Isolate	MIC (μg/ml)		Identity (%)	Amino acid substitution								
	PEN	CTX		337STMK	366I	369A	371I	378E	384R	395SSN	400M	546LKSGT	595Y
NUBL5698 (15A/15F), 7017 (15A/15F), 7025 (15A/15F)	2	0.5	89	- A - -	-	-	T	-	G	- - -	-	V- - - -	-
NUBL1094 (6A/6B), 7023 (6A/6B)	2	1	94	- A - -	-	-	T	D	G	- - -	-	V- - - -	-
NUBL1081 (6A/6B), 1082 (6A/6B), 1092 (6A/6B),1096 (6A/6B), 7020 (23F)	2	1	91	- A - -	-	-	T	D	G	- - -	-	V- - - -	-
NUBL7026 (19F)	2	1	91	- A - -	-	-	T	-	G	- - -	-	V- - - -	-
NUBL1095 (35B), 10912 (35B), 10378 (35B)	2	1	90	- A - -	-	-	T	-	G	- - -	-	V- - - -	-
NUBL5689 (19F), 7024 (15A/15F)	2–4	0.5–2	91	- A - -	-	-	T	-	G	- - -	-	V- - - -	-
NUBL1083 (19F), 1091 (19F), 1098 (19F), 7018 (19F), 7021 (6A/6B), 10916 (19F)	2–4	0.5–2	88	- A - -	-	-	T	D	G	- - -	-	V- - - -	-
NUBL1087 (14), 1093 (6A/6B), 5691 (6C/6D), 5693 (6C/6D), 5694 (15B/15C), 10911 (6C/6D), 10915 (6A/6B), 10917 (6A/6B)	2–4	1–2	90	- A - -	-	-	T	D	G	- - -	-	V- - - -	-
NUBL1086 (23F), 1089 (23F), 7019 (23F), 7022 (23F)	2–4	1–2	88	- A - -	-	-	T	D	G	- - -	-	V- - - -	-
NUBL5703 (23F)	4	1	90	- A - -	-	-	T	D	G	- - -	-	V- - - -	-
NUBL5699 (19A), 5700 (19A)	4	2	90	- A - -	-	-	T	-	G	- - -	-	V- - - -	-
NUBL5687 (19F)	4	2	88	- A - -	-	-	T	D	G	- - -	-	V- - - -	-
NUBL1080 (19F)	4	4	90	- A - -	-	-	T	-	G	- - -	-	V- - - -	-
NUBL5690 (19F), 5697 (19F)	4	4	88	- A F -	-	-	T	-	G	- - -	-	V- - - -	-
NUBL5692 (19F)	4	8	89	- A F -	-	-	T	-	G	- - -	-	V- - - -	-
NUBL10910 (19F)	4	8	88	- A F -	-	-	T	-	G	- - -	-	V- - - -	-
NUBL1085 (19F)	4	8	88	- A F -	-	-	T	A	G	- - -	T	V- - - -	F
NUBL5695 (19F)	4	16	87	- A F -	M	V	T	G	G	- - -	T	V- - - -	I
NUBL1088 (19F)	4	> 16	84	- A F -	M	V	T	G	G	- - -	T	V- - - -	I
NUBL5688 (6A/6B)	4	> 16	84	- A F -	-	-	T	A	G	- - -	T	V- - - -	L
NUBL10913 (19A)	8	4	89	- A F -	-	-	T	A	G	- - -	T	V- - - -	-
NUBL1097 (19F), 5696 (19F)	8	8	89	- A F -	-	-	T	-	G	- - -	T	V- - - -	-

MIC, minimum inhibitory concentration; PEN, penicillin; CTX, cefotaxime.
Isolates with numbers that are underlined belong to CC320/271.
Serotypes are shown in parentheses following isolate names.
Published sequence of penicillin-susceptible *S. pneumoniae* R6 (GenBank accession number: AAK9910) was used for comparison of identity and position numberingof amino acids.

were identified in several strains (20). However, these 19 amino acid substitutions did not necessarily correlate with the degree of CTX resistance (CTX MICs 2–8 μg/ml). Three isolates that belonged to CC320 (3/3), one isolate belonging to CC271 (1/2), and one isolate that belonged to the singleton (ST11941, serotype 19A) possessed these amino acid substitutions at the C-terminal end of PBP2b. A duplication in the region encoding 3 amino acid residues S428WY was detected in PBP2b of one isolate (NUBL1092; PEN MICs 2 μg/ml, CTX MIC 1 μg/ml, serotype 6A/B, ST5232) (21). No amino acid substitution was specific to CC320/271.

In PBP2x, all the isolates analyzed in this study possessed T338A amino acid substitutions, adjacent to the active site, S337. Amino acid substitutions L364F, I371T, R384G, and L546V were also detected in all the isolates analyzed here (19). The combination of T338A/M339F and M400T was detected in relatively highly resistant isolates (PEN MICs 4–8 μg/ml, CTX MICs 4 to >16 μg/ml) (22). Two isolates highly resistant CTX (NUBL1088 [PEN MICs 4 μg/ml, CTX MICs > 16 μg/ml, serotype 19F, ST271] and NUBL5695 [PEN MICs 4 μg/ml, CTX MIC 16 μg/ml, serotype 19F, ST236]) harbored new and unique amino acid substitutions, specifically: I366M, A369V, E378G, and Y595I.

DISCUSSION

β-Lactams including PEN are first-line drugs for pneumococcal diseases; therefore, it is important to characterize PRSP in detail. After the introduction of PCV7, the emergence of MDR serotype 19A CC320 has been reported in a number of studies (5,8,23). ST320 is a double-locus variant of the originally described Taiwan19F-14 clone, ST236, isolated at a Taiwanese hospital in 1997 (24) and has probably undergone a capsular switch to nonvaccine serotype 19A. In Japan, however, there has been no epidemiological research focusing on PEN-nonsusceptible isolates after the introduction of PCV7. Especially, molecular epidemiological data based on MLST analysis are quite limited. Therefore, we focused on PEN-nonsusceptible pneumococcal isolates after the introduction of PCV7 in Japan and characterized them.

In our study, PNSSP isolates (PEN MIC ≥ 4 μg/ml) were detected at a frequency of 2.2% (23/1,057). According to data from the Japan Nosocomial Infections Surveillance (JANIS) report, 2.6% (709/26,932) of *S. pneumoniae* isolates from nonmeningitis samples from 745 healthcare institutions in Japan in 2013, and 2.5% (670/27,206) of *S. pneumoniae* isolates from nonmeningitis samples from 883 healthcare institutions in 2014 were nonsusceptible to PEN (PEN MIC ≥ 4 μg/ml; see http://www.nih-janis.jp). The reported prevalence rates (2.5–2.6%) of PNSSP among clinical isolates are the same as the isolation rate revealed in the present study (2.2%). Therefore, although the invasive sample number in this study is small and this study involves specimen bias and regional bias, it is likely that the clinical samples in this study may reflect the state of affairs on *S. pneumoniae* in Japan. A limitation of this study is that clinical information about the clinical isolates is limited, and most clinical isolates might have been recovered from colonized patients. Therefore, the results of this study may be slightly different from the results of studies on clinical isolates recovered from patients with invasive diseases.

Our data clearly indicate the significant prevalence of serogroup 19 CC320/271 among PNSSP isolates (PEN MIC ≥ 4 μg/ml) in Japan after the introduction of PCV7, as observed in other countries. In addition, the CC320/271 strains identified in our study tend to be highly resistant to β-lactams, in particular a third-generation cephalosporin. A similar tendency was reported in Hong Kong (20). That report showed that the high prevalence rates of β-lactam nonsusceptibility in 2011 in Hong Kong were due mainly to the clonal expansion of serotype 19F ST271, which has strong resistance to CTX. Therefore, this tendency may be common in East Asian countries.

Amino acid substitutions surrounding the active site of the transpeptidase domains in PBP1a, PBP2b, and PBP2x have been implicated in resistance to β-lactams (19). In our study, there were no amino acid substitutions specific to CC320/271 in PBPs and no characteristic amino acid substitutions in PBP1a and PBP2b that may account for the increased resistance to the third-generation cephalosporin in *S. pneumoniae*. However, in PBP2x, CC320/271 harbors several amino acid substitutions that are likely to be important for high resistance to CTX, e.g., M339F, M400T, and Y595F. The role of some of these substitutions such as M339F and M400T in decreased affinity of PBP2x for cephalosporins has been studied (22,25), and the combination of substitutions at these key amino acid positions may explain the high MIC values toward CTX. According to the crystal structure of PBP2x (26), M339 is next to the active-site lysine residue K340 in the catalytic motif SXXK (where X is an unspecified amino acid residue) spanning positions 337 to 340. M400T is close to Ser395, which is involved in hydrogen bonding with a β-lactam. Y595F is next to Ser596, which does not directly interact with the antibiotic molecule and is believed to affect the overall structure of the protein. In addition, we found new amino acid substitutions in the highly CTX-resistant isolates (CTX MIC ≥16 μg/ml). These isolates possess substitutions I366M, A369V, and Y595I in PBP2x. Therefore, the amino acid substitutions in PBP2x may be responsible for the resistance to third-generation cephalosporins.

In this study, the most predominant serotype is vaccine serotype 19F, even though PCV7 had already been introduced in Japan. Nonvaccine serotype 19A, which became predominant globally after the introduction of PCV7, was still a minor serotype between 2010 and 2014 on the basis of our data. In addition, the major ST was ST236 (which is regarded as the source strain) and not ST320, which had been predominant among serotype 19A isolates in Asian countries (11). A recent report out of Hong Kong describes the clonal expansion of serotype 19F ST271 (which has high resistance to β-lactams and is a single-locus variant of ST236) after the introduction of PCV7 (20). In South East Asia, isolates of the predominant serotype 19F were recently reported to be ST236 in Malaysia and ST271 in Beijing, China (27,28). Consequently, even in Asian countries, the most prevalent clone may have regional characteristics. Our results show that vaccine-covered serotype 19F remained the predominant serotype in PNSSP isolates

even after the introduction of PCV7. Because studies out of European countries suggest that 19F is slower to disappear than other PCV serotypes (29), we should continue to monitor 19F in the future.

Regarding the prevalence of serotype 19F ST236 before the introduction of PCV7 in Japan, research articles concerning pneumococcal isolates from children with meningitis in Japan (30) and an article about pneumococcal isolates from adult community-acquired pneumonia (31) are relevant here. In these studies, the prevalence of ST236 before PCV7 introduction and the high prevalence rate of PEN resistance among these clones were reported. Therefore, 19F ST236 clones were already present before the introduction of PCV7 in Japan and remained PNSSP isolates after the introduction of PCV7. However, because our study period starts immediately after vaccine introduction and because the estimated rates of PCV7 vaccination were still low in 2010–2011, the full effect of herd immunity was not observed in terms of PNSSP. In a Korean hospital, PEN-nonsusceptible CC271 has been the most prevalent clonal complex both before and after PCV7 introduction. After PCV7 introduction, however, the frequency of detection of serotype 19F ST236/271 among isolates decreased, whereas the prevalence of serotype 19A ST320 increased (32). Based on our data, serotype 19A ST320 was still a minor clone, whereas 19F ST236 clone was predominant among PNSSP isolates. However, serotype 19A ST320 clones were identified in 2013 in infants that might have been vaccinated with PCV7. Because PCV7 was replaced by PCV13 in November 2013, the frequency of isolation of PEN-nonsusceptible serotype 19A ST320 may have increased before the introduction of PCV13, as observed in other countries. Subsequently, as suggested by a few recent publications concerning the era after the introduction of PCV13, the frequency of 19A might have decreased in Japan after the introduction of PCV13.

The CC320/271 clone remains predominant in many Asian countries (8). In Japan, PCV7 vaccination of children $<$ 5 years of age began at the end of 2010. After the introduction of PCV7, there were dramatic reductions in the number of IPD cases caused by vaccine types such as 6B, 19F, and 23F and increases in the prevalence of pneumococcal infections caused by serotype 19A and other nonvaccine serotypes (13). However, our data suggest that serotype 19F ST236 was the predominant clone among PNSSP strains and has persisted even after the introduction of the PCV7 vaccine. Therefore, it will be necessary to monitor the trend in serogroup 19 CC320/271, which has high resistance to β-lactams, even after the switch to expanded-spectrum conjugate vaccines.

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Conflict of interest None to declare.

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