Journal of Infection and Public Health 12 (2019) 867–872

Contents lists available at ScienceDirect

Journal of Infection and Public Health

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

Assessment of the local clonal spread of *Streptococcus pneumoniae* serotype 12F caused invasive pneumococcal diseases among children and adults

Noriko Nakanishi[a,*], Takeshi Yonezawa[a], Shinobu Tanaka[a], Yuki Shirouzu[b], Yuki Naito[b], Akemi Ozaki[b], Natsuki Hama[a], Akihiro Ijichi[b], Tomotada Iwamoto[a], Ryohei Nomoto[a,*]

[a] *Department of Infectious Diseases, Kobe Institute of Health, 4-6-5 Minatojima-nakamachi, Chuo-ku, Kobe, Hyogo 650-0046, Japan*
[b] *Public Health Center of Kobe City, 6-5-1 Kano-cho, Chuo-ku, Kobe, Hyogo 650-8570, Japan*

ARTICLE INFO

Article history:
Received 26 October 2018
Received in revised form 11 February 2019
Accepted 29 May 2019

Keywords:
Streptococcus pneumoniae
Serotype
Sp12F
Whole-genome sequencing
Single nucleotide polymorphism

ABSTRACT

Background: We conducted active surveillance to elucidate the distribution of *Streptococcus pneumoniae* serotypes causing invasive pneumococcal disease (IPD) and clarified the genetic relatedness among the isolates in Kobe City, Japan.
Methods: Forty-five IPD-causing *S. pneumoniae* strains were analyzed from March 2016 to May 2018 through active surveillance in Kobe City, Hyogo, Japan. Serotypes were determined by multiplex serotyping PCR and the Quellung reaction with pneumococcal antisera. Fourteen Sp12F strains were subjected to whole-genome sequencing (WGS).
Results: Among 45 isolates, the most frequent serotypes were 12F (n = 14, 31%), 24F (n = 5, 11%), and 10A (n = 4, 9%). Multilocus sequence typing (MLST) analysis of 14 isolates of Sp12F divided them into ST4846 (n = 4) and ST6495 (n = 10). WGS showed clonality of the 10 isolates of ST6495, with only 13 single nucleotide polymorphisms in the genomes. Meanwhile, ST4846 strains in Kobe differed from only the outbreak strains of Sp12F ST4846 in Tsuruoka, Japan, reported on 2018.
Conclusions: Serotype monitoring showed Sp12F to be the predominant serotype in Kobe, and WGS revealed the clonal spread of Sp12F ST6495 in this city. Thus, the spread of Sp12F could become a serious public health problem in Japan, warranting thorough monitoring in future.

Introduction

Streptococcus pneumoniae is a clinically important pathogen that causes invasive pneumococcal diseases (IPDs), such as pneumonia, bacteremia, meningitis, and sepsis [1–3]; however, the introduction of vaccines has reduced the incidence of IPDs. In Japan, a heptavalent pneumococcal conjugate vaccine (PCV7) was introduced in 2010, which was replaced by 13-valent PCV (PCV13) in November 2013. For individuals aged 65 years and above, a 23-valent pneumococcal polysaccharide vaccine (PPSV23) was approved in 1988 and was included in routine immunization since October 2014. After the introduction of these vaccines, a nationwide surveillance programme was conducted [4–6]. The introduction of PCV7, followed by that of PCV13, was found to have dramatically reduced the rate of IPD among vaccinated children [6]. However, the incidence of IPD caused by non-PCV13 serotypes has recently been increasing in both children and adults in Japan [4,6]. This is due to serotype replacement, wherein the prevalence of serotypes included in PCV13 decrease, while the serotypes not included in PCV13 increase.

In Israel, an increase in IPD caused by non-PCV13 serotypes of *S. pneumoniae*, namely serotype 12F (Sp12F), was reported on 2018 [7]. Moreover, an outbreak of IPD cases caused by Sp12F was reported in Japan on 2018 [8]. Since Sp12F is well-known as a serotype associated with high morbidity and mortality compared with other serotypes causing IPD [9,10], it is undoubtedly a serotype that should be monitored in the future.

Abbreviations: PCV7, heptavalent pneumococcal conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; PPSV23, 23-valent pneumococcal polysaccharide vaccine; Sp12F, *Streptococcus pneumoniae* serotype 12F; gPSSP, genotypic penicillin-susceptible *S. pneumoniae*; gPSIP, penicillin-intermediate resistant *S. pneumoniae*.

* Corresponding authors.
E-mail addresses: noriko_nakanishi@office.city.kobe.lg.jp (N. Nakanishi), ryohei_nomoto@office.city.kobe.lg.jp (R. Nomoto).

https://doi.org/10.1016/j.jiph.2019.05.019

In spite of introducing PCV13 in 2013, even in Kobe City the number of patients with IPD has gradually increased as well as the global situation described above. Here, we describe the predominance in IPD caused by Sp12F through active surveillance in Kobe City from March 2016 to May 2018. We also compared the whole-genome sequences (WGS) to reveal the genetic relationship of Sp12F isolates.

Methods

Bacterial isolates

We analyzed 45 IPD-causing *S. pneumoniae* strains isolated from blood and/ or cerebrospinal fluid (CSF), which were collected by active surveillance in Kobe City, Hyogo, Japan, from March 2016 to May 2018. Our study was approved by the Ethics Committee of Kobe City. Isolates of all IPD patients consisted of 20 people aged <5 years, five aged 5–15 years, six aged 20–39 years, nine aged 40–64 years, and five aged ≥65 years. All isolates were plated onto Columbia agar with 5% sheep blood overnight.

Serotyping and antimicrobial susceptibility testing of S. pneumoniae *strains*

The serotyping was performed by multiplex PCR using primers and conditions according to the Centers for Disease Control and Prevention (https://www.cdc.gov/streplab/pcr.html). Serotypes were confirmed by the Quellung reaction with pneumococcal antisera (Statens Serum Institut, Copenhagen, Denmark). Because the serotypes 11A and 11E could not be discriminated by the Quellung reaction, they are indicated as serotype 11A/E.

Susceptibilities of the Sp12F isolates to nine antibiotics were determined by using the broth microdilution method according to previous studies [14,15]. PCR for the detection of three penicillin-binding protein (PBP) genes and the macrolide resistance genes *mef*(A) and *erm*(B) was performed as previously described [17–19].

Multilocus sequence typing

Sequence types (STs) of the *S. pneumoniae* strains were determined by the sequences of seven housekeeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*) [16] obtained from the results of WGS. Allelic numbers and STs of the strains were assigned using the pneumococcal multilocus sequence typing (MLST) website PubMLST (https://pubmlst.org/spneumoniae/).

Whole-genome sequencing and phylogenetic analyses

The predominant serogroup Sp12F isolates were analyzed. DNA libraries were constructed using a QIAseq FX DNA Library kit (Qiagen, Hilden, Germany), and paired-end sequences (2 × 300 bp) were generated using the MiSeq system (Illumina, San Diego, CA, USA) with a MiSeq reagent kit v.3 (Illumina). All the generated reads were assembled into contigs using A5-miseq [11]. Gene predictions and annotations were performed with PROKKA [12]. For comparative analysis, a core single nucleotide polymorphism (SNP) matrix and maximum-likelihood phylogenetic tree were generated using kSNP (v. 3.0) with a k-mer size of 31 [13]. The same analysis was also performed using Illumina sequence read data of nine strains responsible for the outbreak in the city of Tsuruoka belonged to ST4846 (Accession nos. DRX114436–DRX114444) [8]. The genomic sequences of the Sp12F strains MT-1 and MT-20, belonging to ST218 and ST220 (Accession nos. LIAD01000001 and LJMW01000001), respectively, and which caused the most outbreaks [10], were obtained from the NCBI database and used as a reference.

Nucleotide sequence accession number

The whole-genome sequencing (WGS) reads are available from the DDBJ/EMBL/GenBank Sequence Read Archive under accession number DRA007245 (Table 1).

Results

Distribution of S. pneumoniae *serotypes*

Among 45 isolates, the most frequent serotypes were 12F (n = 14, 31%), 24F (n = 5, 11%), and 10A (n = 4, 9%) (Fig. 1). In children aged <5 years and 5–15 years and vaccinated with PCV13, non-PCV13 serotypes (except for 3 and 19A, detected from patients vaccinated with PCV7) were detected. Serotype 24F in children aged <5 years and vaccinated with PCV13 was detected only in 2016. Meanwhile, in adults aged ≥20 years, 8 out of 13 serotypes detected were covered by PPSV23. The predominant Sp12F was not covered by PCV7 or PCV13 but was covered by PPSV23. The patients with IPD caused by Sp12F were children aged up to 15 years who had been vaccinated with PCV13, and individuals 20–64 years of age who had not been vaccinated. Sp12F strains were detected from patients from nine different hospitals during the period from May 2017 through May 2018 in Kobe (Table 1).

MLST and antibiotic susceptibility of the Sp12F isolates

MLST analysis revealed two different STs among the serogroup 12F. In 14 Sp12F strains, 10 strains belonged to ST6945 (MLST allelic profile: *aroE-gdh-gki-recP-spi-xpt-ddl*: 12-32-111-15-13-429-6), and four strains belonged to ST4846 (12-32-111-1-13-48-6) (Table 1). More strains belonged to ST6945 than to ST4846 in children <10 years old who had been vaccinated with PCV7 or PCV13. However, in adults <65 years old, three and two strains belonging to ST6945 and ST4846, respectively, were detected.

The ST6945 and ST4846 strains were susceptible to penicillin (minimal inhibitory concentration (MIC), 0.15 μg/ml and 0.06 μg/ml, respectively) and cefotaxime (MIC, 0.06 μg/ml) but were resistant to clindamycin (MIC, ≥8 μg/ml) and erythromycin (MIC, ≥8 μg/ml). By PCR, we examined whether the Sp12F strains harbored antibiotic resistance genes. All strains possessed the macrolide resistance gene *erm*(B). We classified the Sp12F strains into the genotypes for *pbp1a*, *pbp2x*, and *pbp2b* genes. All strains belonging to ST6945 were genotypic penicillin-susceptible *S. pneumoniae* (gPSSP) with three normal genes, *pbp1a*, *pbp2x*, and *pbp2b*. In the strains assigned to ST4846, three strains and one strain indicated genotypic penicillin-intermediate resistant *S. pneumoniae* (gPISP) with abnormal *pbp2b* and gPSSP with three normal genes, respectively.

WGS analysis of the Sp12F isolates

An outbreak of Sp12F ST4846 was reported in Tsuruoka City, Yamagata Prefecture, Japan on 2018 [8]. To clarify whether the Sp12F ST6945 strains isolated in a local area of Kobe were of the same clone, and to ascertain the genetic relatedness among the Sp12F ST4846 strains isolated between the cities of Tsuruoka and Kobe, the whole-genome sequences were compared. The strains ST6945 and ST4846 were divided into two clades, and 2100 core SNPs were detected among the Sp12F strains in this study (Fig. 2). Meanwhile, only 13 core SNPs were detected among the 10 strains belonging to ST6945 isolated from patients from different hospitals in Kobe. We surmise that the genetically clonal ST6945 strains circulated in Kobe during the short period from May 2017 to May 2018. Similarly, in our analysis, only 10 core SNPs detected in Tsuruoka strains were reported as causative agents of the outbreak [8],

Table 1
Information and characteristics of the *Streptococcus pneumoniae* serotype 12F strains isolated from IPD patients in Kobe city.

Strain no.	Date of diagnosis (year/month)	Hospital	Isolated location	Sex	Age	Vaccination history[a]	ST[b]	MICs (μg/ml) for[c]:									ATB resistance gene	DDBJ accession no. of read data	β-Lactam resistance
								PCG	MEPM	PAPM	TBPM	CTX	VCM	EM	CLDM	TFLX			
Sp 2017-5	2017-5	A	Blood	Female	3	PCV13 four times	6945	≤0.015	≤0.008	≤0.008	≤0.008	≤0.03	0.25	≥8	≥8	≤0.12	*ermB*	DRX139216	gPSSP
Sp 2017-12	2017-10	B	Blood	Male	37	–	6945	≤0.015	0.015	≤0.008	≤0.008	≤0.03	0.25	≥8	≥8	0.25	*ermB*	DRX139217	gPSSP
Sp 2017-15	2017-12	C	Blood	Female	22	–	4846	0.06	0.03	≤0.008	≤0.008	0.06	0.25	≥8	≥8	≤0.12	*ermB*	DRX139218	gPISP(*pbp2b*)
Sp 2018-4	2017-12	D	Blood	Male	5	PCV7 three times, PCV13 once	6945	≤0.015	0.015	≤0.008	≤0.008	≤0.03	0.25	≥8	≥8	0.25	*ermB*	DRX139219	gPSSP
Sp 2018-5	2017-12	E	Blood	Female	56	–	4846	0.06	0.03	≤0.008	≤0.008	0.06	0.25	≥8	≥8	0.25	*ermB*	DRX139220	gPSSP
Sp 2018-7	2018-1	F	Blood	Female	53	–	6945	≤0.015	≤0.008	≤0.008	≤0.008	≤0.03	0.25	≥8	≥8	0.25	*ermB*	DRX139221	gPSSP
Sp 2018-11	2018-2	A	Blood	Male	6	PCV7 four times	6945	≤0.015	≤0.008	≤0.008	≤0.008	≤0.03	0.25	≥8	≥8	0.25	*ermB*	DRX139222	gPSSP
Sp 2018-16	2018-2	F	Blood	Male	2	PCV13 four times	6945	≤0.015	0.015	≤0.008	≤0.008	≤0.03	0.25	≥8	≥8	0.25	*ermB*	DRX139223	gPSSP
Sp 2018-17	2018-3	A	Blood	Male	4	PCV13 four times	6945	≤0.015	≤0.008	≤0.008	≤0.008	≤0.03	0.5	≥8	≥8	0.25	*ermB*	DRX139224	gPSSP
Sp 2018-19	2018-3	G	Blood	Female	35	–	6945	≤0.015	0.015	≤0.008	≤0.008	≤0.03	0.25	≥8	≥8	0.25	*ermB*	DRX139225	gPSSP
Sp 2018-21	2018-4	A	Blood	Male	1	PCV13 four times	6945	≤0.015	0.015	≤0.008	≤0.008	≤0.03	0.25	≥8	≥8	0.25	*ermB*	DRX139226	gPSSP
Sp 2018-22	2018-5	D	Blood	Male	1	PCV13 four times	4846	0.06	0.03	≤0.008	≤0.008	0.06	0.25	≥8	≥8	0.25	*ermB*	DRX139227	gPISP(*pbp2b*)
Sp 2018-26	2018-5	H	Blood	Female	1	PCV13 four times	6945	≤0.015	0.015	≤0.008	≤0.008	≤0.03	≤0.12	≥8	≥8	0.25	*ermB*	DRX139228	gPSSP
Sp 2018-27	2018-5	I	Blood	Male	2	PCV13 four times	4846	0.06	0.03	≤0.008	≤0.008	0.06	0.25	≥8	≥8	≤0.12	*ermB*	DRX139229	gPISP(*pbp2b*)

[a] PCV7 or PCV13, 7- or 13-valent pneumococcal conjugate vaccine; PPSV23, 23-valent pneumococcal polysaccharide vaccine.
[b] ST, sequence type.
[c] PCG, penicillin G; MEPM, meropenem; PAPM, panipenem; TBPM, tebipenem; CTX, cefotaxime; VCM, vancomycin; EM, erythromycin; CLDM, clindamycin; TFLX, tosufloxacin; LVFX, levofloxacin.

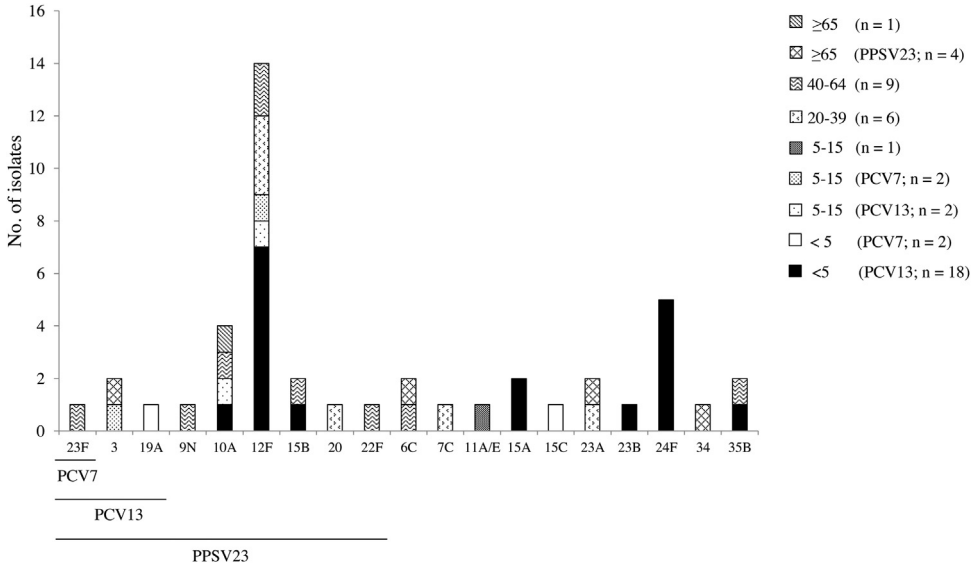

Fig. 1. Distribution of the serotypes of invasive pneumococcal disease (IPD)-causing pneumococcal isolates (n = 45) from IPD patients in Kobe City from March 2016 to May 2018.

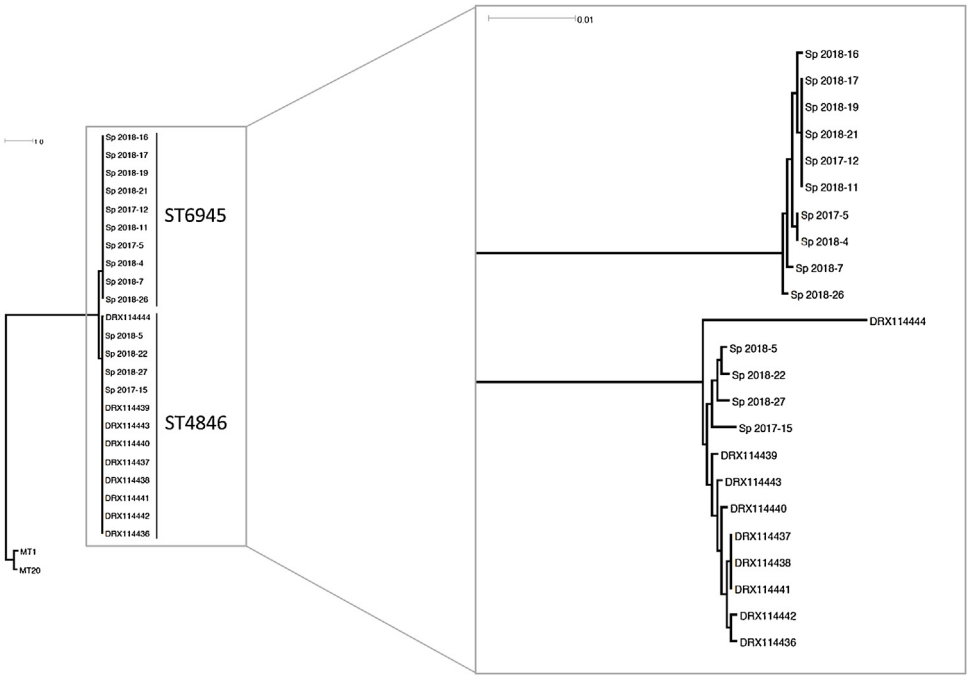

Fig. 2. Phylogenetic tree based on genome-wide single nucleotide polymorphisms among Sp12F strains isolated in Kobe City and strains that caused an outbreak in Tsuruoka City (indicated as accession numbers). A maximum-likelihood tree was constructed using kSNP 3.0. An enlarged view of the ST4846 and ST6945 lineages is shown in the box.

but there were 65 core SNPs in four ST4846 strains in Kobe. Moreover, the ST4846 strains isolated in Kobe were different from the cluster in the phylogenetic SNP tree compared with the strains that caused the outbreak in Tsuruoka (Fig. 2).

Discussion

In this study, 45 IPD-causing *S. pneumoniae* strains were analyzed, and Sp12F was found to be the predominant (31%) serotype in Kobe. Many of the Sp12F strains were isolated from children with IPD, which might be because this serotype is not covered by PCV7 or PCV13. Here, we describe the local clonal spread of Sp12F ST6945 strains during a short period in Kobe.

The 12F serotype is known to be associated with outbreaks of invasive disease, often among adults but occasionally among children as well [20]. In particular, it is well known that the clonal complex CC218 in Sp12F contains the predominant sequence types ST218 and ST220 (a single-allele variant of ST218), which cause large outbreaks and are globally dispersed [9,10]. However, the predominant global Sp12F outbreak clones CC218 are very rare in IPD cases in Japan. The sequence type that caused the first Sp12F outbreak in Japan was ST4846 [8]. In this study, 14 Sp12F strains were classified into two sequence types, ST4846 and ST6945, by MLST. The phylogenetic tree based on WGS showed that these two sequence types are genetically separated from the global outbreak clones ST218 and ST220 (Fig. 2). In Kobe, ST6945 was a predominant sequence type, but in MLST analysis, it demonstrated only a

two-locus (*recP* and *xpt*) difference from ST4846. In fact, in terms of core SNP analysis of the whole-genome sequence, only 2100 SNPs were detected between ST6945 and ST4846, suggesting that these two sequence types are genetically more closely related than with ST218, where core 7643 SNPs was detected. Furthermore, referring to the MLST database (https://pubmlst.org/spneumoniae/), most ST6945 and ST4846 have been registered from Japan, except for two ST6945 strains that had been registered from China and Canada. Therefore, the Sp12F STs ST4846 and ST6945 might be endemic in Japan.

Sp12F ST6945 strains predominated over ST4846 strains in our study. WGS analyses showed that 10 strains belonging to ST6945, which varied by 13 core SNPs, were genetically identical. Compared with the 10 core SNPs detected among the outbreak strains of Tsuruoka, clonal Sp12F ST6945 strains might be circulating even though they were isolated at different hospitals in Kobe. In contrast, 65 core SNPs were detected among four ST4846 strains isolated in Kobe, suggesting sporadic infection caused by strains that were genetically different from the Tsuruoka outbreak strains. Chiba et al. reported that 12F was the major IPD-causing serotype in adults in Japan from 2006 to 2007, and most of the serotype 12F strains were gPISP, with a *pbp2b* gene alteration [21]. It has been reported that this epidemic had caused by the Sp12F ST220, which has known as globally outbreak [22]. Therefore, it suggested that the predominance of IPD cased Sp12F from 2017 have no correlation with the endemic in 2006–2007, and caused by endemic Sp12F STs, ST6945 and ST4846.

12F has been reported to be rarely carried in the nasopharynx of healthy persons and to have high invasive disease potential [20]. In our study, Sp12F strains were isolated from adults <60 years old without the underlying disease. The Sp2017-12 strain was isolated from parent with suspected infection from their child who exhibited pharyngitis symptoms. Unfortunately, because we could not investigate nasopharyngeal colonization by *S. pneumoniae* in the child, the details of the infection route could not be determined. Nonetheless, our main findings taken together might also highlight the hyperinvasiveness of Sp12F.

Conclusions

In conclusion, we found a spread in the incidence of Sp12F-causing IPD from May 2017 to May 2018 through active surveillance. We found the clonal spread of Sp12F ST6945 in Kobe during this brief period. Therefore, the predominance in Sp12F IPD could become a serious public health problem in Japan. The PPSV23 vaccine includes the 12F polysaccharide, but PCV7 and PCV13 do not. Therefore, continued monitoring of endemic serotypes causing severe IPD is needed for the development of future vaccination strategies.

Authors' contributions

NN, RN designed the study methods and wrote the first draft of the manuscript.

NN, TY, ST, NH collect the data.

NN, RN analyzed the data.

YS, YN, AO, AI collected samples.

TI contributed to the writing of the manuscript.

Funding

This work was supported by a commissioned project for Research on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development, AMED (grant number JP18fk0108019 and JP19fk0108103), and JSPS KAKENHI grant number JP19K10594.

Conflict of interest

None declared.

Ethical approval

This study was approved by the Kobe City Review Board; Ref. 1423-1.

Acknowledgment

We are grateful to Dr. Bin Chang at the National Institute of Infectious Diseases, Japan, for her advice regarding this study.

References

[1] O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet 2009;374:893–902.

[2] Pallarés R, Liñares J, Vadillo M, Cabellos C, Manresa F, Viladrich PF, et al. Resistance to penicillin and cephalosporin and mortality from severe pneumococcal pneumonia in Barcelona, Spain. N Engl J Med 1995;333:474–80.

[3] Ishiwada N, Kurosaki T, Terashima I, Kohno Y. The incidence of pediatric invasive pneumococcal disease in Chiba prefecture, Japan (2003–2005). J Infect 2008;57:455–8.

[4] Fukusumi M, Chang B, Tanabe Y, Oshima K, Maruyama T, Watanabe H, et al. Invasive pneumococcal disease among adults in Japan, April 2013 to March 2015: disease characteristics and serotype distribution. BMC Infect Dis 2017;17(1):2, http://dx.doi.org/10.1186/s12879-016-2113-y.

[5] Nakano S, Fujisawa T, Ito Y, Chang B, Suga S, Noguchi T, et al. Serotypes, antimicrobial susceptibility, and molecular epidemiology of invasive and non-invasive *Streptococcus pneumoniae* isolates in paediatric patients after the introduction of 13-valent conjugate vaccine in a nationwide surveillance study conducted in Japan in 2012–2014. Vaccine 2016;34:67–76.

[6] Suga S, Chang B, Asada K, Akeda H, Nishi J, Okada K, et al. Nationwide population-based surveillance of invasive pneumococcal disease in Japanese children: effects of the seven-valent pneumococcal conjugate vaccine. Vaccine 2015;33:6054–60.

[7] Rokney A, Ben-Shimol S, Korenman Z, Porat N, Gorodnitzky Z, Givon-Lavi N, et al. Emergence of *Streptococcus pneumoniae* serotype12F after sequential introduction of 7- and 13-valent vaccines, Israel. Emerg Infect Dis 2018;24:453–61.

[8] Ikuse T, Habuka R, Wakamatsu Y, Nakajima T, Saitoh N, Yoshida H, et al. Local outbreak of *Streptococcus pneumoniae* serotype 12F caused high morbidity and mortality among children and adults. Epidemiol Infect 2018;146:1793–6.

[9] Robinson DA, Turner JS, Facklam RR, Parkinson AJ, Breiman RF, Gratten M, et al. Molecular characterization of a globally distributed lineage of serotype 12F *Streptococcus pneumoniae* causing invasive disease. J Infect Dis 1999;179:414–22.

[10] Deng X, Peirano G, Schillberg E, Mazzulli T, Gray-Owen SD, Wylie JL, et al. Whole-genome sequencing reveals the origin and rapid evolution of an emerging outbreak strain of *Streptococcus pneumoniae* 12F. Clin Infect Dis 2016;62:1126–32.

[11] Coil D, Jospin G, Darling AE. A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. Bioinformatics 2015;31:587–9.

[12] Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014;30:2068–9.

[13] Gardner SN, Slezak T, Hall BG. kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. Bioinformatics 2015;31:2877–8.

[14] Chang B, Nariai A, Sekizuka T, Akeda Y, Kuroda M, Oishi K, et al. Capsule switching and antimicrobial resistance acquired during repeated *Streptococcus pneumoniae* pneumonia episodes. J Clin Microbiol 2015;53:3318–24.

[15] Chang B, Morita M, Lee KI, Ohnishi M. Whole-genome sequence analysis of *Streptococcus pneumoniae* strains that cause hospital-acquired pneumonia infections. J Clin Microbiol 2018;56:e01822-17, http://dx.doi.org/10.1128/JCM.01822-17.

[16] Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology 1998;144:3049–60.

[17] Ubukata K, Iwata S, Sunakawa K. In vitro activities of new ketolide, telithromycin, and eight other macrolide antibiotics against *Streptococcus pneumoniae* having *mefA* and *ermB* genes that mediate macrolide resistance. J Infect Chemother 2003;9:221–6.

[18] Ubukata K, Chiba N, Hasegawa K, Kobayashi R, Iwata S, Sunakawa K. Antibiotic susceptibility in relation to penicillin-binding protein genes and serotype distribution of *Streptococcus pneumoniae* strains responsible for meningitis in Japan, 1999 to 2002. Antimicrob Agents Chemother 2004;48:1488–94.

[19] Chiba N, Kobayashi R, Hasegawa K, Morozumi M, Nakayama E, Tajima T, et al. Antibiotic susceptibility according to genotype of penicillin-binding protein and macrolide resistance genes, and serotype of *Streptococcus pneumo-*

niae isolates from community-acquired pneumonia in children. J Antimicrob Chemother 2005;56:756–60.

[20] Zulz T, Wenger JD, Rudolph K, Robinson DA, Rakov AV, Bruden D, et al. Molecular characterization of *Streptococcus pneumoniae* serotype 12F isolates associated with rural community outbreaks in Alaska. J Clin Microbiol 2013: 511402–7.

[21] Chiba N, Morozumi M, Sunaoshi K, Takahashi S, Takano M, Komori T, et al. Serotype and antibiotic resistance of isolates from patients with invasive pneumococcal disease in Japan. Epidemiol Infect 2010;138:61–8.

[22] Rakov AV, Ubukata K, Robinson DA. Population structure of hyperinvasive serotype 12F, clonal complex 218 *Streptococcus pneumoniae* revealed by multilocus boxB sequence typing. Infect Genet Evol 2011;8:1929–399.