Multiple clonal types of nonvaccine serotypes constitute β-lactams nonsusceptible pneumococcus isolates from adult pneumonia patients in Japan

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Research Article

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Abstract

Background and objective The global spread of antimicrobial-nonsusceptible *Streptococcus pneumoniae* is a major concern. Molecular epidemiology of those strains in relation to vaccine serotype remains to be explored in Japan. This study aimed to elucidate the distribution of molecular types with the serotypes and antimicrobial susceptibility of pneumococcus strains isolated from adult pneumonia patients.

Methods We enrolled adult pneumonia patients from four sites in Japan between September 2011 and August 2014. *S. pneumoniae* isolates from sputum and blood were analyzed for serotyping by the Quellung reaction and for antimicrobial susceptibility by the agar dilution method and e-test and for multilocus sequence typing.

Results In total, 204 isolates were analyzed from 200 patients with a median age of 72.5 years, of whom 55 (27.5%) patients had received a 23-valent pneumococcal polysaccharide vaccine (PPSV23). We detected 41 clonal complexes (CCs) and 62 sequence types (STs), including 10 new STs: CC/ST 180 of serotype 3 was the most common followed by CC/ST 236 of serotype 19F and CC/ST 99 of serotype 11A; 144 (70.6%) isolates were PPSV23 serotypes; 40 (19.6%) and 121 (59.3%) isolates were b-lactam nonsusceptible (bNS) and multidrug-nonsusceptible strains, respectively. Among the bNS strains, 18 (45%) were nonvaccine serotypes, and 4 CCs (CC236, CC63, CC242, and CC558) comprised of 62.5% of them.

Conclusion Multiple CCs of bNS strains, including nonvaccine serotype are spreading. It is crucial to monitor the antimicrobial susceptibility, serotypes, and molecular types of pneumococci to predict the effectiveness of vaccines in preventing pneumonia by bNS pneumococci strains.

Introduction

The disease burden of adult pneumonia remains high in both low-middle and high-income countries. Pneumonia was the fifth leading cause of death in patients over 65 years old in Japan in 2018 (1). *Streptococcus pneumoniae,* or pneumococcus, is a major pathogen that causes adult pneumonia. It has been estimated that 0.53 million cases of adult pneumococcal pneumonia are treated annually in Japan and its incidence has drastically increased as the proportion of the elderly population has also increased (2, 3). Prevention and effective treatment of pneumococcal pneumonia is of high public health importance in the super-aging society in Japan. More than 95 serotypes of pneumococci are known, and some common serotypes can be prevented by the 13-valent pneumococcal conjugate vaccine (PCV13) and 23-valent pneumococcal polysaccharide vaccine (PPSV23).

Antimicrobial nonsusceptibility of *S. pneumoniae* is a growing concern. In recent studies in Asia, the level of antimicrobial resistance in pneumococcal isolates for penicillin and ceftriaxone (CRO) was 11.9-16.0% and 34.8-38.1%, respectively (4-6). The proportion of isolates that are nonsusceptible to meropenem (MEM) and macrolides has increased in recent years along with the spread of multidrug-resistant (MDR) pneumococcal strains or multidrug- nonsusceptible (MDNS) pneumococcal strains (7-9).

Monitoring the serotype distribution and antimicrobial susceptibility of circulating strains in Japan and understanding their molecular epidemiology is crucial for developing rational prevention and treatment strategies. Multilocus sequence typing (MLST) has revealed molecular epidemiological characteristics of antimicrobial-nonsusceptible pneumococcal strains in various geographical areas (8, 10-13), and identified the distribution of globally circulating strains, which are described as clones of the Pneumococcal Molecular Epidemiology Network (PMEN) (https://www.pneumogen.net/pmen/) (14).

Studies evaluating sequence types (STs) and antimicrobial susceptibility in specific serotypes have been reported (9, 15). However, to our knowledge, no hospital-based studies have investigated the combined distribution of serotypes, STs, and antimicrobial susceptibility of pneumococcal strains from adult pneumonia patients. In this study, we aimed to describe the distribution of pneumococcal strains with their serotypes, antimicrobial nonsusceptibility patterns, and STs among isolates from adult pneumonia patients in Japan.

Methods

Study population and study outline

This study was conducted as a part of a prospective hospital-based study for adult pneumonia in Japan between September 2011 and August 2014 as previously described (2). Patients who fulfilled the following criteria were enrolled in the study: 1) age ≥15 years, 2) symptoms compatible with pneumonia, and 3) new infiltrates present on chest radiography or computed tomography scan films. Patients who developed pneumonia after 48 hours after admission were excluded.

Identification of *S. pneumoniae* and antimicrobial susceptibility testing

Sputum and/or blood samples were collected from the enrolled patients upon their first hospital visit or admission. Quantitative or semiquantitative cultures were performed at each hospital laboratory. We judged *S. pneumoniae* as the causative pathogen of adult pneumonia using the following diagnostic criteria: 1) it grew more than 10^4 CFU/mL for quantitative culture in sputum or its equivalent for semiqualitative culture or positive blood culture; 2) if the sample did not satisfy the culture standard, microscopic Gram stained examination of the sputum scored Geckler group ≥3 (16) and *S. pneumoniae* was the only potential pathogen. The isolates were transported to the Institute of Tropical Medicine at Nagasaki University where the serotype was determined by the Quellung reaction test (Serum Statens Institut, Denmark) and antimicrobial susceptibility to penicillin G (PEN), CRO, and MEM by the agar dilution method, and chloramphenicol (CHL), azithromycin (AZM), tetracycline (TET), levofloxacin (LVX), and sulfamethoxazole and trimethoprim (SXT) by the E-test strip (Sysmex bioMérieux) (17). The susceptibility of isolates to antimicrobial agents was interpreted as susceptible, intermediate, and resistant according to the standards of the Clinical and Laboratory Standards Institute (CLSI) 2014 guidelines (18). We classified the strains that were nonsusceptible to at least one of three b-lactam antimicrobials tested as b-lactam nonsusceptible (bNS) pneumococcal strains. MDNS pneumococcus strains were defined as the isolates nonsusceptible to three or more classes of antimicrobials.

We extracted DNA from isolates and confirmed the pneumococcus identification by *lytA* real-time PCR (19, 20). We performed DNA sequencing for MLST; the details of the methods are described elsewhere (13). Sequence Typing was determined by reference to the PubMLST (https://pubmlst.org/spneumoniae/) (21). We excluded one of the two strains to avoid duplication if they were isolated both from sputum and blood cultures from the same patients, and their serotype and ST were same.

We submitted new ST data to PubMLST and assigned designations. The goeBURST algorithm via PHYLOViZ 2.0 (http://www.phyloviz.net/) was applied to estimate the relationship among the STs and classify the clonal complexes (CCs) (22). In this study, we named each CC according to an ST number of the goeBURST predicted founder, which was defined as the ST with the greatest number of single-locus variants and a smaller number of STs in a group consisting of more than two different STs or a singleton itself. This goeBURST analysis was done on June 5th, 2020.

Analysis

We described characteristics of the enrolled patients with calculated CURB-65 score (23), antimicrobial susceptibility interpretations, and CC distribution by serotypes of isolates. Genetic relationships of ST with allelic profile, CC, and serotype presented as a dendrogram. The dendrogram was generated from a distance matrix among STs by using the unweighted pair group method with arithmetic averages in PHYLOViZ 2.0. The proportions of categorical variables were compared by using the chi-squared test, and the medians of continuous variables were compared by using the Wilcoxon rank sum test.

Ethics

The study was approved by the Institutional Review Boards (IRBs) of the Institute of Tropical Medicine at Nagasaki University, Ebetsu City Hospital, Kameda Medical Center, Chikamori Hospital, and Juzenkai Hospital. The requirement for obtaining written consent from all participants was waived by all IRBs because of the observational nature of the study and with no deviation

from current medical practice. Anonymized data were used for our analyses. All methods were performed in accordance with the relevant guidelines and regulations.

Results

Pneumococcal isolates and characteristics of patients

A total of 287 pneumococcal isolates were identified from sputum and blood cultures in this study. Three isolates did not fulfill the microbiologic diagnostic criteria and were excluded. 204 isolates subsequently re-grew on culture, and antimicrobial susceptibility testing was performed. Real-time PCR of *lytA* confirmed that they were pneumococcal isolates. These 204 isolates from 200 adult pneumonia patients which were considered causative pathogens for pneumonia, and their data were processed for analysis (Selection flow is shown in Additional Figure 1). The characteristics of the patients are shown in Table 1. The majority of patients (n=145, 72.5%) were ≥65 years old with a median age of 72.5 years. 159 (79.5%) cases were community-acquired pneumonia (CAP) and mostly mild pneumonia with CURB-65 ≤2. 127 (63.5%) patients were admitted to the hospital, and the mortality rate was 3.5%. Only 55 (27.5%) patients were confirmed to have received PPSV23, and 32 (16.0%) had a course of antimicrobials prior to the enrollment.

Distribution of serotypes and molecular epidemiology

Figure 1 shows the STs, CCs, and serotypes of the isolates. We detected 41 CCs, 62 STs and 2 singletons (STs 7801 and 13216). CC/ST 180 of serotype 3 was the most frequently observed strain (n=45, 22%), followed by CC/ST 236 of serotype 19F (n=12, 6%) and CC/ST 99 of serotype 11A (n=12, 6%). We identified 10 new STs (STs 13216, 13217, 13218, 13219, 13220, 13221, 13302, 13303, 13304, and 13389). In total, 109 (53.4%) isolates belonged to PMEN-type CCs, and 144 (70.6%) isolates were PPSV23-type serotypes.

Antimicrobial susceptibility

The susceptibility patterns for b-lactam antimicrobials, the number of nonsusceptible antimicrobial classes other than b-lactams, and the number of MDNS strains in relation to CCs and PMEN strains are shown in Table 2. Susceptibility for antimicrobials other than b-lactams are also shown in Additional Table 1. In total, 40 (19.6%) isolates (from 39 patients) were categorized as bNS strains. The most common molecular type among bNS strains was CC 236 (n=9, 22.5%), followed by CC63 (n=6, 15%), CC242 (n=5, 12.5%), and CC558 (n=5, 12.5%). In total, 30 (75%) bNS strains were PMEN clones. The remaining 10 (25%) bNS strains, which had 6 CCs and one singleton, have not previously been categorized as PMEN-type CCs. These included CC3111, which was isolated from 3 patients, and CC2756, which was isolated from 2 patients. Similarly, 121 (59.3%) isolates from 119 cases (33 CCs out of the total 41 CCs, 80.5%) were categorized as MDNS strains. Of those, 62.8% (n=76) of MDNS strains were PMEN related. MDNS strains were found among CC180 (n=36/50, 72% of total isolates in the CCs), CC236 (n=11/12, 91.7%), CC63 (n=7/8, 87.5%), CC2755 (n=7/8, 87.5%), and CC242 (n=7/7, 100%) (Table 2). A total of 83 (68.6%) of MDNS strains, in particular CC180 and CC2755, met the MDNS criteria without being bNS strains, whereas almost all bNS strains were MDNS strains.

Comparison of bNS and bS isolates

Figure 2 (A and B) compares the CC distribution by serotype among bS and bNS strains. Out of 40 bNS strains, 22 (55%) were PPSV23 types (serotypes 6B, 11A, 14, 19A, 19F, and 23F), and the remainder belonged to PPSV23 nonvaccine types (serotypes 6A, 15A, 23A, and 35B). We found that bNS strains were significantly associated with serotypes 19F, 15A, and 35B (Additional Table 2, $P<0.05$, respectively) when compared to bS pneumococcal strains.

Serotype 19F strains were mainly CC236 and serotype 15A of CC63, while serotype 35B contained multiple CC strains. Among bNS strains, CC63 and CC558 were exclusively present in the nonvaccine serotype 15A and 35B, respectively (Figure 2B). Among PMEN-related bNS strains (n=30, 75%), 6 (20%) had different serotypes from those of the original PMEN strains. It was

noted that the serotype had shifted from vaccine type to nonvaccine type in four of those six PMEN strains: from CC242/serotype 23F to 23A (n=2) and from CC81/serotype 23F to 6A (n=1) and 15A (n=1) (Figure 1).

There was no significant difference in demographic and clinical characteristics of patients with b-lactam-susceptible (bS) pneumococcal strains compared with patients with bNS pneumococcal strains, except that patients with bNS strains tended to be associated with bronchial asthma (Additional Table 2).

Discussion

This study elucidated the molecular epidemiology of antimicrobial-nonsusceptible pneumococcal strains causing adult pneumonia in Japan in relation to vaccine-serotype. It showed that 19.6% of pneumococcal isolates from adult pneumonia patients in Japan were bNS strains and 59.3% were MDNS strains. Among bNS strains, only a half were PPSV23 serotypes, and 4 CCs out of 14 CCs comprised of 62.5% of bNS strains. There were multiple CCs of bNS strains, which were not covered by PPSV23.

While our findings are similar to other study performed in Japan from 2003 to 2004. It showed that 49 STs, 27 CCs, and 3 singletons with the most common molecular type of CC/ST 180 of serotype 3, followed by CC/ST 236 serotype 19F, CC/ST 242 serotype 23F, the proportion of PPSV23 serotypes was 80.6% and the proportion of PMEN clone CCs was 63.6% (23). Our study showed more diverse STs and CCs, and a lower proportion of vaccine-type serotypes and CCs related to the PMEN than the previous study. The differences might be due to the vaccine availability; the previous study was conducted before the implementation of routine immunization with the 7-valent PCV and PCV13 in children. We could not compare the proportion of bNS strains or other factors because the previous study used an older antimicrobial susceptibility interpretation criterion, and they did not report the susceptibility to MEM.

Among bNS strains, CC236/serotype 19F in vaccine type and CC63/serotype15A and CC558/serotype 35B in nonvaccine type were common. CC 236 of serotype 19F is a PMEN-related strain (Taiwan19F-14), and many reports show that it has successfully spread among children and globally as MDNS strains (24, 25). CC63 of serotype 15A (Sweden15A-25) was reported as a MEM-nonsusceptible strain isolated from children in Japan (9), and CC558 of serotype 35B (single locus variant of Utah35B-24) was reported as one of the emerging nonvaccine serotypes in the United States after PCV introduction (26, 27). Our results indicate that bNS isolates from adult pneumonia patients in Japan consisted mainly PMEN clones, and part of those are epidemic strains in children.

There was no significant difference in clinical characteristics, including mortality, between patients who had bNS strains and those with bS strains. The exception was asthma, which has been found to be a risk factor for penicillin-resistant pneumococcal pneumonia (28) (Additional Table 2). Epidemic and widespread prevalence of nonvaccine type bNS strains might still be a problem, especially in invasive pneumococcal disease, because 95% of bNS strains showed nonsusceptibility for MEM, a potential first choice of antimicrobial if a patient's condition is severe.

We showed that there were many CCs in the MDNS group of isolates in Japan. A number of studies have shown that MDR or MDNS pneumococci are predominantly PMEN-related strains and nonsusceptible to TET, SXT, and macrolides (29-31). The antimicrobials recommended for treatment CAP in the guidelines are b-lactams, macrolides, and fluoroquinolones (32, 33). In addition to nonsusceptibility of pneumococci to macrolides, we should be concerned about b-lactam or fluoroquinolone nonsusceptibility of pneumococci.

There were a few limitations in the study. The first limitation was that some isolates failed to remain viable after transport from the research sites to our laboratory. Changes in during transportation from each hospital to our laboratory may have been a critical factor (34). The second limitation was that we included most of the obtained *S. pneumoniae* strains that were isolated from sputum even if the sputum quality or bacterial load in sputum culture was low (35). We considered that elderly patients could not expectorate good-quality sputum in this clinical situation that might have limited isolation of pneumococci in culture. However, we did a sensitivity analysis for 187 samples with better sputum quality and middle to high bacterial load, and it did

not suggest that the quality of sample had a significant impact on our results (data not shown). And the third limitation was that we used different methods (the agar dilution method and the E-test strip) for antimicrobial susceptibility testing. Although the methods were different, studies have shown a good correlation between them for pneumococcal antimicrobial susceptibility with fluoroquinolones, macrolides, CP, TET, and SXT (36-38).

Conclusion

We reported the molecular epidemiology of pneumococcal isolates from adult pneumonia patients in four regions of Japan. We found that a high proportion of the isolates were bNS and MDNS strains, and majority of them were related to the PMEN. Multiple CCs of bNS strains, including serotype not covered by PPSV23, are spreading. It is important to monitor the distribution of antimicrobial susceptibility, serotypes, and sequence types of pneumococci to detect the clonal spread of antimicrobial-nonsusceptible pneumococci, particularly nonvaccine serotype bNS strains, such as serotypes 15A and 35B.

Abbreviations List

PCV13, 13-valent pneumococcal polysaccharide conjugate vaccine; PPSV23, 23-valent pneumococcal polysaccharide vaccine; PCV7, 7-valent pneumococcal polysaccharide vaccine; MDR, multidrug-resistant pneumococcal strains; MDNS, multidrug-nonsusceptible pneumococcal strains; MLST, multilocus sequence typing; PMEN, Pneumococcal Molecular Epidemiology Network; STs, sequence types; CC, clonal complex; S, susceptible; I, intermediate; R, resistant; CLSI, Clinical and Laboratory Standards Institute; bNS, b-lactam-nonsusceptible; bS, b-lactam-susceptible; CAP, community-acquired pneumonia; COPD, chronic obstructive pulmonary disease; SLV, single locus variant.

Declarations

Ethics approval and consent to participate: The study was approved by the Institutional Review Boards (IRBs) of the Institute of Tropical Medicine at Nagasaki University, Ebetsu City Hospital, Kameda Medical Center, Chikamori Hospital, and Juzenkai Hospital. The requirement for obtaining written consent from all participants was waived by all IRBs because of the observational nature of the study and with no deviation from current medical practice. Anonymized data were used for our analyses.

Consent for publication: Not applicable

Availability of data and materials: The clinical epidemiology datasets generated and analyzed during the current study are not publicly available due to being a part of the prospective cohort study but are available from the corresponding author on reasonable request. The sequence type datasets generated during the current study are available in the PubMLST repository, https://pubmlst.org/bigsdb?db=pubmlst_spneumoniae_isolates&page=query

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Tables

Table 1. Characteristics of enrolled patients

	Total
Enrolled patients, number (%)	200
Median age (range)	72.5 (18-95)
Sex (male%)	120 (60.0)
CAP (%)	159 (79.5)
Inpatients (%)	127 (63.5)
Past PPSV23 history (%)	
Yes	55 (27.5)
No	110 (55.0)
Unknown	35 (17.5)
Underlying diseases	
Diabetes	46 (23.0)
Hypertension	85 (42.5)
Hyperlipidemia	40 (20.0)
Asthma	27 (13.5)
COPD	44 (22.0)
Preuse of antimicrobials before enrollment (%)	32 (16.0)
Mortality (%)	7 (3.5)
CURB-65 (n, %)	160
≤2	119 (74.4)
≥3	41 (25.6)
Region	
Hokkaido	30 (15.0)
Chiba	126 (63.0)
Kochi	16 (8.0)
Nagasaki	28 (14.0)

CAP: community-acquired pneumonia, PPSV23: 23-valent pneumococcal polysaccharide vaccine, COPD: chronic obstructive pulmonary disease

Table 2. Distribution of clonal complexes, b-lactams, and other classes of antimicrobial-susceptibility, and multidrug nonsusceptible strains

CCs	PEN		CRO		MEM		No. of bNS strains (n)	No. of NS antimicrobials classes other than b-lactams (n)					MDNS strains (n)	Total (n)	Related PMEN clones
	S (n)	NS (n)	S (n)	NS (n)	S (n)	NS (n)		0	1	2	3	4			
180	50		50		50			3	2	9	34	2	36	50	Netherlands3-31
99	14		14		14					10	4		4	14	
236	12		9	3	3	9	9			2	10		11	12	Taiwan19F-14
433	9		9		9			1	5		3		3	9	
199	8		8		8			1		4	2	1	3	8	Netherlands15B-37
63	6	2	8		2	6	6			7	1		7	8	Sweden15A-25
2755	8		8		8					1	7		7	8	
242	7		6	1	3	4	5				7		7	7	Taiwan23F-15
3111	6	1	7		4	3	3			5	2		5	7	
338	6		6		6					5	1		1	6	Colombia23F-26
280	5		5		5			2	2	1				5	
15	5		4	1	5		1			5			1	5	SLV England14-9
5236	5		5		5					4	1		1	5	
558	5		5			5	5		2	3			3	5	SLV Utah35B-24
2331	5		5		5					3	2		2	5	
5241	4		4		4				3		1		1	4	
62	4		4		4					3	1		1	4	
2756	4		3	1	2	2	2		1	1	2		2	4	
90	3		3		3						2	1	3	3	Spain6B-2
Singleton	3		3		2	1	1			2		1	2	3	
2924	3		3		3					2	1		1	3	
2572	3		3		3						3		3	3	
81	1	2		3		3	3					3	3	3	Spain23F-1
2923	3		3		3				1		2		2	3	
5832	2		2		2				1		1		1	2	
2758	2		2		2						2		2	2	
5238	1		1			1	1					1	1	1	
343	1		1		1							1	1	1	
246	1		1		1					1				1	
7494	1		1		1					1				1	
902	1		1			1	1				1		1	1	
3113		1		1		1	1				1		1	1	
156		1		1		1	1				1		1	1	Spain9V-3
3116	1		1		1				1					1	
191	1		1		1					1				1	Netherlands7F-39
3117	1		1		1			1						1	
6433	1		1		1						1		1	1	
3594	1		1		1							1	1	1	
7800	1		1		1				1					1	
1591		1		1		1	1					1	1	1	
4745	1		1		1						1		1	1	
5233	1		1		1					1				1	

Total	196	8	192	12	166	38	40	8	19	71	94	12	121	204	

Bold CCs indicate β-lactam-nonsusceptible (bNS) strains, CC: clonal complex, PEN: penicillin G, CRO: ceftriaxone, MEM: meropenem, S: susceptible,

NS: nonsusceptible, bNS: β-lactam-nonsusceptible strains, MDNS: multidrug-nonsusceptible strains, PMEN: Pneumococcal Molecular Epidemiology Network

Figures

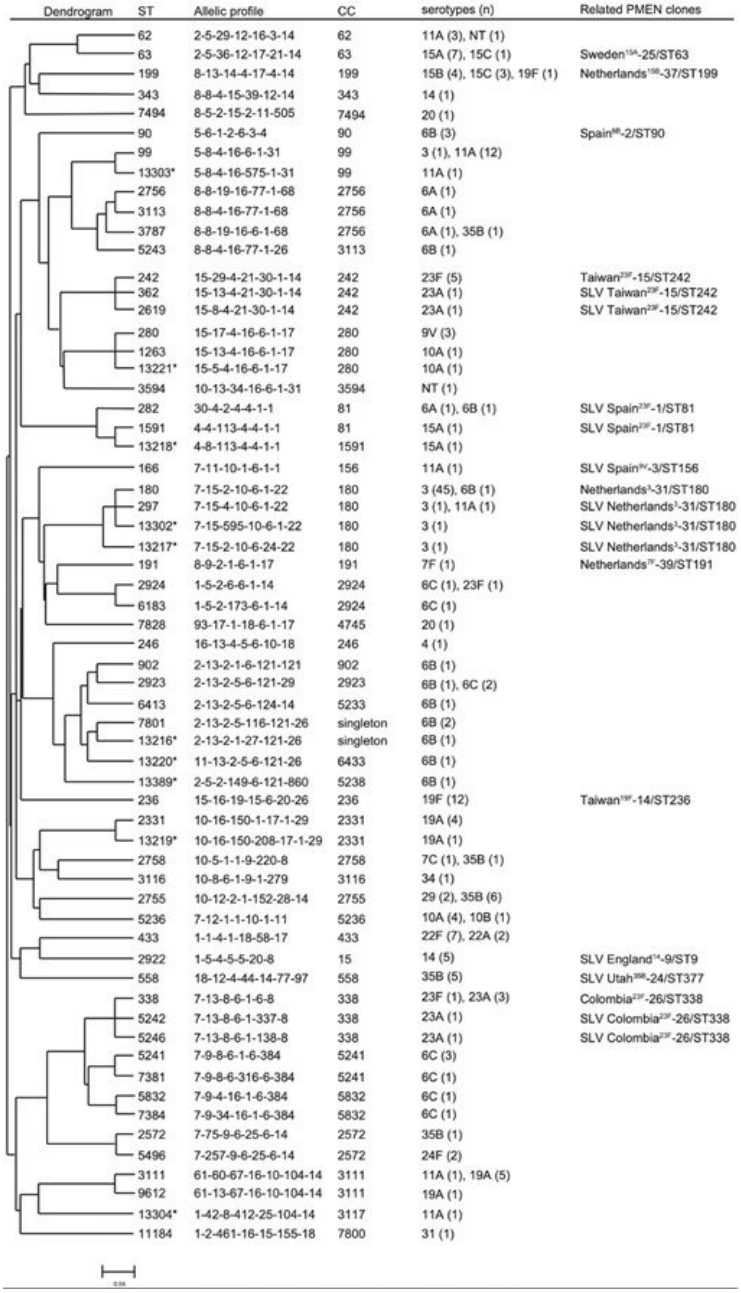

Figure 1

Distribution of clonal complexes, sequence types, and serotypes in this study. * new STs and alleles that were found in the study, ST: sequence type, CC: clonal complex, PMEN: Pneumococcal Molecular Epidemiology Network, SLV: single locus variant

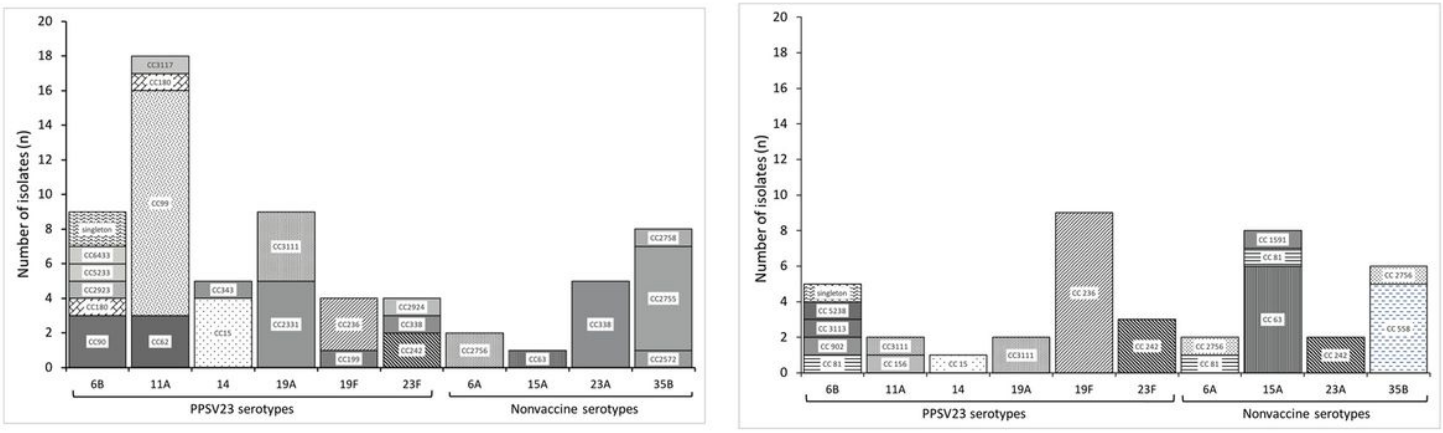

Figure 2

Distribution of clonal complexes in β-lactams susceptible strains and nonsusceptible strains in serotypes with β-lactams-nonsusceptible strains. A. Distribution of CCs in β-lactams-susceptible strains by serotypes. B. Distribution of CCs in β-lactams-nonsusceptible strains by serotypes. CC: clonal complex, PPSV23: 23-valent pneumococcal polysaccharide vaccine

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFigure1.pdf
- AdditionalTable1Word.docx
- AdditionalTable2Word.docx