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Original article

Worldwide Lineages of Clinical Pneumococci in a Japanese Teaching Hospital Identified by DiversiLab System

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ABSTRACT

Pneumococcal Molecular Epidemiology Network (PMEN) clones are representatives of worldwide-spreading pathogens. DiversiLab system, a repetitive PCR system, has been proposed as a less labor- and time-intensive genotyping platform alternative to conventional methods. However, the utility and analysis parameters of DiversiLab for identifying worldwide lineages was not established. To evaluate and optimize the performance of DiversiLab for identifying worldwide pneumococcal lineages, we examined 245 consecutive isolates of clinical *Streptococcus pneumoniae* from all age-group patients at a teaching hospital in Japan. The capsular swelling reaction of all isolates yielded 24 different serotypes. Intensive visual observation (VO) of DiversiLab band pattern difference divided all isolates into 73 clusters. Multilocus sequence typing (MLST) of representative 73 isolates from each VO cluster yielded 51 different STs. Among them, PMEN-related lineages accounted for 63% (46/73). Although the serotype of PMEN-related isolates was identical to that of the original PMEN clone in 70% (32/46), CC156-related PMEN lineages, namely Greece^{6B}-22 and Colombia^{23F}-26, harbored various capsular types discordant to the original PMEN clones. Regarding automated analysis, genotyping by extended Jaccard (XJ) with a 75% similarity index cutoff (SIC) showed the highest correlation with serotyping (adjusted Rand's coefficient, 0.528). Elevating the SIC for XJ to 85% increased the discriminatory power sufficient for distinguishing two major PMEN-related isolates of Taiwan^{19F}-14 and Netherlands^{3}-31. These results demonstrated a potential utility of DiversiLab for identifying worldwide lineage of pneumococcus. An optimized parameters of automated analysis should be useful especially for comparison for reference strains by “identification” function of DiversiLab.

1. Introduction

Streptococcus pneumoniae, today, remains a major cause of morbidity and mortality worldwide [1]. Recent advance in the molecular epidemiological analysis has revealed the presence of worldwide clones, as represented by Pneumococcal Molecular Epidemiology Network (PMEN) clones [2]; the transmission of PMEN clones is presumed to affect substantially the epidemiology of pneumococcus. Meanwhile, the ability of pneumococcus to change its capsular type sometimes makes the situation more complicated [3].

In bacterial genotyping, repetitive PCR (rep-PCR) requires less labor and time, and is therefore more useful in practice than other genotyping methods. Recently, the DiversiLab (DL) system (bioMérieux, Marcy l'Etoile, France), a rep-PCR package system in which assay reproducibility is improved, has been developed for a number of bacterial species [4]. Regarding pneumococcus, the performance of the DL system was presumed to be comparable to that of pulsed-field gel electrophoresis by anecdotal reports [5–7].

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In addition to fingerprinting, DL could potentially provide additional information regarding the genetic background of the isolates, by comparing the band patterns with the reference data in the classification mode, leading to identification of the worldwide lineages. Unfortunately, however, the absence of established similarity index cutoff (SIC) calculated by automated algorithms limits application of DL for deciphering pneumococcal lineages.

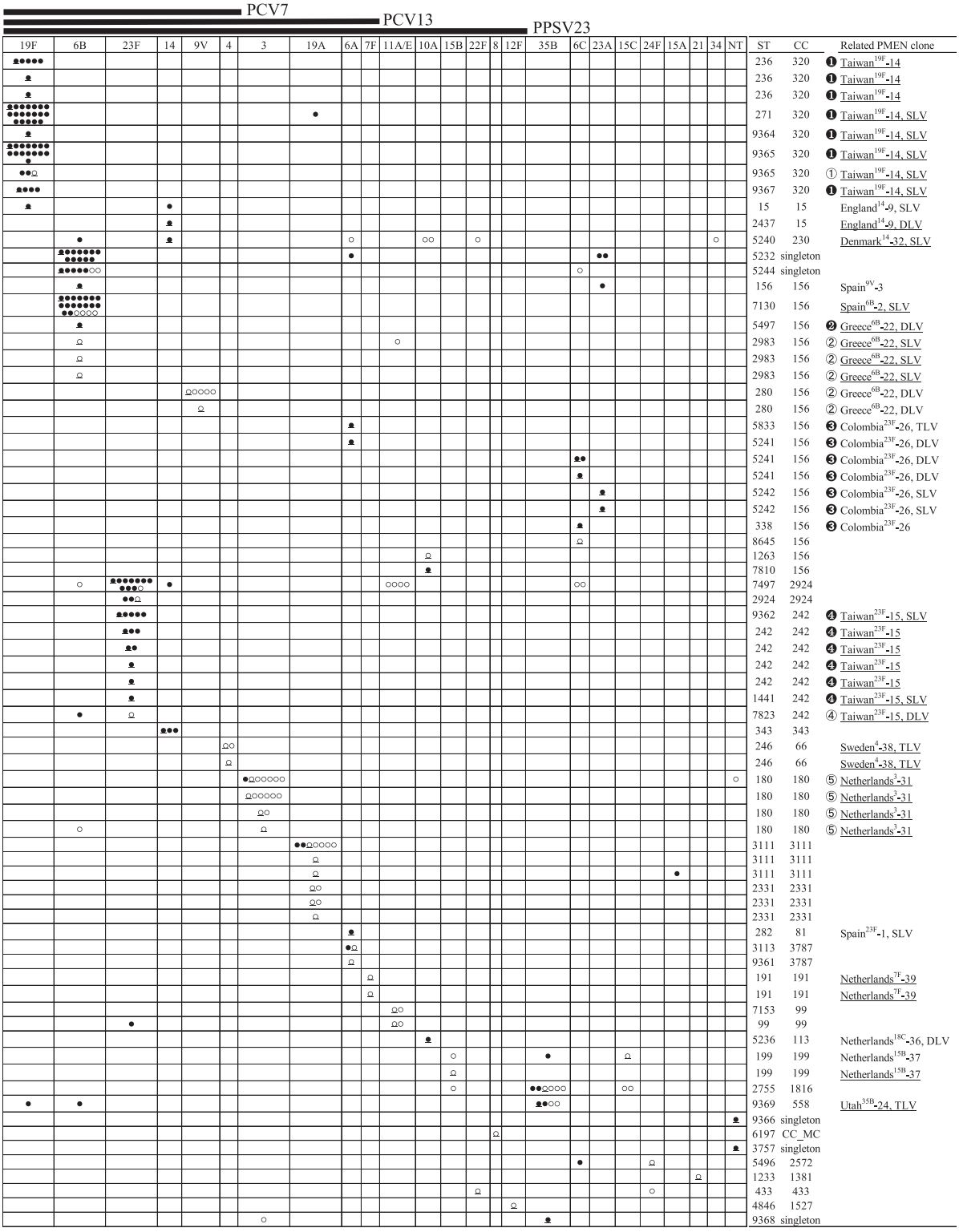

Fig. 1. Distribution of isolates to serotypes and genotypes by intensive visual observation (VO) of DiversiLab. A closed and open circle, represent a penicillin-non-susceptible (minimum inhibitory concentration $\geq$0.12 μg/ml) and penicillin-susceptible isolate, respectively. Regarding the underlined circles, sequence type (ST), its clonal complex (CC), and related Pneumococcal Molecular Epidemiology (PMEN) clones are shown in the right column. The number in the circle represents the five major PMEN-related lineages in Table 1. Underlined PMEN clones represent the serotype of the isolate that was consistent with serotype of the related PMEN clone. PCV, pneumococcal conjugate vaccine; PPSV, pneumococcal polysaccharide vaccine; NT, non-typeable; SLV, single-locus variant; DLV, double-locus variant; TLV, triple-locus variant; CC_MC, CC with multiple candidates for its founder.

In the present study, we aimed to evaluate and optimize the performance of DL for identifying and characterizing worldwide lineage of pneumococci by analyzing consecutively isolated *S. pneumoniae* from all age-group patients in a teaching hospital in Japan.

2. Materials and methods

2.1. Clinical isolates

A total of 245 clinical isolates of *S. pneumoniae* were consecutively collected from Toho University Omori Medical Center in Japan, from January to December during 2009, and stored at −80 °C until use. Isolates were recovered on trypticase soy agar with 5% sheep blood (Becton Dickinson, Sparks Glencoe, MD, USA) overnight in a 5% CO_2 incubator at 35 °C. The identification of *S. pneumoniae* was reconfirmed by bile solubility and optochin-sensitivity tests in the presence of 5% CO_2. This study (No. 24064) was approved by the ethics committee of the Faculty of Medicine, Toho University, Japan.

2.2. Minimum inhibitory concentration (MIC)

The MICs of the antimicrobials, penicillin G (PEN), meropenem (MEM), clarithromycin (CLR), clindamycin (CLI), and levofloxacin (LVX), were determined by the broth microdilution method (dry plate Eiken test; Eiken Chemical Co., Tokyo, Japan). The Clinical and Laboratory Standards Institute (CLSI) breakpoint for each antimicrobial was applied [8], except for PEN (with 0.12–1 μg/ml determined as intermediate and ≥2 μg/ml as resistant). PEN non-susceptibility was defined as MIC ≥0.12 μg/ml.

2.3. Serotyping

Serotypes were identified by capsular swelling reaction using commercial serogroup and serotype specific antisera (Statens Serum Institut, Copenhagen, Denmark).

2.4. Bacterial DNA extraction

Enrichment culture in 0.4 ml of brain heart infusion (Becton–Dickinson) supplemented with 0.5% yeast extract (Becton–Dickinson) for 3 h was performed before inoculation on an agar plate. After overnight incubation, bacterial colonies were suspended in 0.65 ml of sterile normal saline using a sterile disposable T-shaped spreader, collected in a 2 ml tube, and centrifuged at 10,000 g for 1 min. The procedure of collecting the bacterial pellets was performed twice.

Bacterial genomic DNA was extracted using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was quantified using a Biospec-nano spectrophotometer (Shimadzu, Kyoto, Japan) at 260- and 280-nm wavelengths, and adjusted to a working concentration of 35 ng/μl.

2.5. rep-PCR by DL

rep-PCR was performed using a DiversiLab *Enterococcus* kit (BioMérieux) and AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR products were separated using a microfluidics DNA chip device (BioMérieux) in an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA). DNA fingerprint patterns were analyzed using DiversiLab software version 3.4 (BioMérieux) and automated coefficients of Pearson correlation (PC), Kullback-Leibler (KL) and extended Jaccard (XJ) with different similarity cutoffs.

In addition, intensive typing by visual observation (VO), where pairs of even one band difference in band patterns were separately grouped, was performed. Because of practical limitations of the DiversiLab software in handling many samples, we conducted a stepwise comparison by VO as follows: the first-round clustering was executed using a 95% SIC of PC coefficient. Subsequently, samples were split into subgroups consisting of two to 12 samples. Within each subgroup, a one-on-one visual comparison was performed in a round-robin manner. If band patterns were indistinguishable, samples were put together and a representative sample proceeded to the next stage. We finally accomplished a visual comparison of all samples by repeating this procedure using six stages in total.

2.6. Multilocus sequence typing (MLST)

MLST analysis was performed using methods by Enright et al. [9]. Clustering of each sequence type (ST), as well as the identification of

Table 1
Distribution of genotypes by DiversiLab (DL) of major PMEN-related isolates by multilocus sequence typing.[a]

	MLST		Relation to PMEN clone	Number of isolates	Serotype	PC[a]		KL[a]		XJ[a]	
	CC	ST				97%	95%	95%	93%	85%	75%
(1)	320	236	Taiwan^{19F}-14	3	19F	A, B, C	A, A, B	A, B, C	A, B, C	A, A, B	A, A, B
	320	9365	Taiwan^{19F}-14, SLV	2	19F	A, B	A, A	A, A	A, A	C, C	A, A
	320	271, 9364, 9367	Taiwan^{19F}-14, SLVs	3	19F	A, A, D	A, A, C	A, A, D	A, A, D	A, C, D	A, A, A
(2)	156	2983	Greece^{6B}-22, SLV	3	6B	E, F, F	D, E, E	E, F, F	E, F, F	E, F, G	C, D, D
	156	5497	Greece^{6B}-22, DLV	1	6B	G	E	F	F	F	D
	156	280	Greece^{6B}-22, DLV	2	9V	H, H	F, F	G, G	G, G	H, H	E, E
(3)	156	5242	Colombia^{23F}-26, SLV	2	23A	C, I	B, B	H, I	H, I	I, J	F, F
	156	338	Colombia^{23F}-26	1	6B	J	G	J	J	K	G
	156	5241	Colombia^{23F}-26, DLV	3	6A, 6C	K, L, M	H, H, G	J, K, K	J, K, K	K, K, L	G, G, H
	156	5833	Colombia^{23F}-26, TLV	1	6A	N	I	L	L	M	G
(4)	242	242	Taiwan^{23F}-15	4	23F	O, O, O, O	J, J, J, J	M, M, M, M	M, M, M, M	N, N, N, N	I, I, I, I
	242	1441, 7823, 9362	Taiwan^{23F}-15, SLV or DLV	3	23F	O, P, P	J, J, J	M, N, N	M, M, M	N, O, O	I, I, I
(5)	180	180	Netherlands^{3}-31	4	3	A, Q, R, S	A, K, K, L	A, O, P, Q	A, A, N, O	P, Q, Q, R	A, A, A, J

MLST, multilocus sequence typing; CC, clonal complex; ST, sequence type; PMEN, Pneumococcal Molecular Epidemiology Network; non-S to PEN, non-susceptible to penicillin; PC, Pearson correlation; KL, Kullback-Leibler; XJ, Extended Jaccard; SLV, single-locus variant; DLV, double-locus variant; TLV, triple-locus variant.

[a] One alphabet represents one isolate. Different alphabet represents different clusters divided by the similarity index cutoff for each automated analysis algorithm. Relationship between the serotype and genotype of all isolates in the current study was shown in Fig. 2 and Fig. S3.

its founder, was determined using the program eBURST version 3 [10], and single locus variants were used to define the clonal complexes (CCs).

ST profiles of PMEN clones 1–43 were acquired using the PMEN website (http://www.sph.emory.edu/PMEN/pmen_table1.html). PMEN-related clones were designated as ≤3-locus variants from original PMEN clone. The relationship of STs to CCs and PMEN clones in the present study, estimated by eBURST, is shown in Fig. S1.

2.7. Evaluation and comparison of typing methods

For the evaluation and comparison of typing methods with different parameter sets, Simpson's index and adjusted Rand's index (ARI) were calculated by EpiCompare version 1.0 software (Ridom GmbH, Wuerzburg, Germany) [11–14].

3. Results

3.1. Characteristics of patients and pneumococcal isolates in the current study

A total of 245 isolates were available for the study period. The demographic characteristics of patients, as well as the drug susceptibility of isolates, are shown in Table S1, Table S2 and Fig. S2; A total of 24 different serotypes were identified (Fig. 1); the most prevalent was 19F (n = 51), followed by 6B (n = 49), 23F (n = 29), 3 (n = 17), and 19A (n = 15). The prevalence of penicillin-non-susceptibility was higher among isolates with a PCV7-type capsule (83.4%, 121/145) than those with a PCV13-type (69.5%, 130/187) or a PPSV23-type (63.5%, 127/200) capsule.

3.2. Genetic lineages and their relationship to serotypes of the pneumococcal isolates

Genotyping by intensive VO divided all isolates into 73 clusters (Fig. 1). To characterize the lineages of isolates, we performed MLST for one representative isolate from each VO cluster. A total of 73 isolates yielded 51 different STs belonging to 29 CCs. As many as 63% (46/73) of ST-determined isolates belonged to PMEN-related STs. Five major PMEN lineages identified in the present study, namely (1) Taiwan^{19F}-14 (N = 8), (2) Greece^{6B}-22 (N = 6), (3) Colombia^{23F}-26 (N = 7), (4) Taiwan^{23F}-15 (N = 7), and (5) Netherlands^{3}-31 (N = 4), accounted for 70% (32/46) of PMEN-related isolates.

The serotype of PMEN-related isolates was identical to that of the original PMEN clone in 70% (32/46) of PMEN-related isolates. Among major PMEN lineages, serotypes of all isolates related to Taiwan^{19F}-14, Taiwan^{23F}-15, and Netherlands^{3}-31, were identical to serotype of the most closely related PMEN clone (Fig. 1 and Table 1). In contrast, majority of the CC156-related PMEN lineages, namely Greece^{6B}-22 and Colombia^{23F}-26, harbored various capsular types discordant to the original PMEN clones (Fig. 1 and Table 1).

3.3. Optimization of similarity index cutoff value of DL for identifying pneumococcal worldwide lineages

Among automated calculation algorithms for similarity index, the most concordant SIC to serotyping was 75% for XJ (ARC with serotyping, 0.528) (Fig. S3(e)), followed by 93% for KL (0.439) and 95% for PC (0.394) (Table 2). However, these SIC parameters could not distinguish major PMEN lineages Taiwan^{19F}-14 from Netherlands^{3}-31 despite successful discrimination by VO (Fig. S3 and Table 1). The most concordant SICs to VO genotyping for each automated algorithm was 95% for KL (ARC with VO

Table 2
Comparisons of serotyping and genotyping using various analysis parameters of the DiversiLab system and objective coefficients.[a]

	Serotyping	VO	PC(%)				KL (%)					XJ (%)					
			99	97	95	93	99	97	95	93	91	95	90	85	80	75	70
No of Clusters	24	73	169	50	23	12	202	83	44	28	20	152	107	67	43	26	19
SI	0.888	0.967	0.996	0.950	0.879	0.783	0.998	0.959	0.934	0.910	0.872	0.991	0.979	0.966	0.924	0.889	0.848
95% CI	0.868-0.909	0.959-0.974	0.994-0.997	0.938-0.962	0.857-0.900	0.746-0.820	0.997-0.999	0.945-0.974	0.918-0.949	0.892-0.928	0.847-0.896	0.988-0.995	0.972-0.987	0.958-0.974	0.904-0.944	0.865-0.913	0.821-0.875
ARC, compared with serotyping	–	0.312	0.055	0.38	0.394	0.242	0.029	0.32	0.424	0.439	0.37	0.125	0.256	0.364	0.478	0.528	0.435
ARC, compared with VO	0.312	–	0.138	0.462	0.347	0.194	0.087	0.386	0.514	0.482	0.354	0.211	0.392	0.468	0.451	0.375	0.302

[a]VO, visual observation; PC, Pearson Correlation; KL, Kullback-Leibler; XJ, Extended Jaccard; SI, Simpson's index; CI, confidential interval; ARC, adjusted Rand's coefficient.

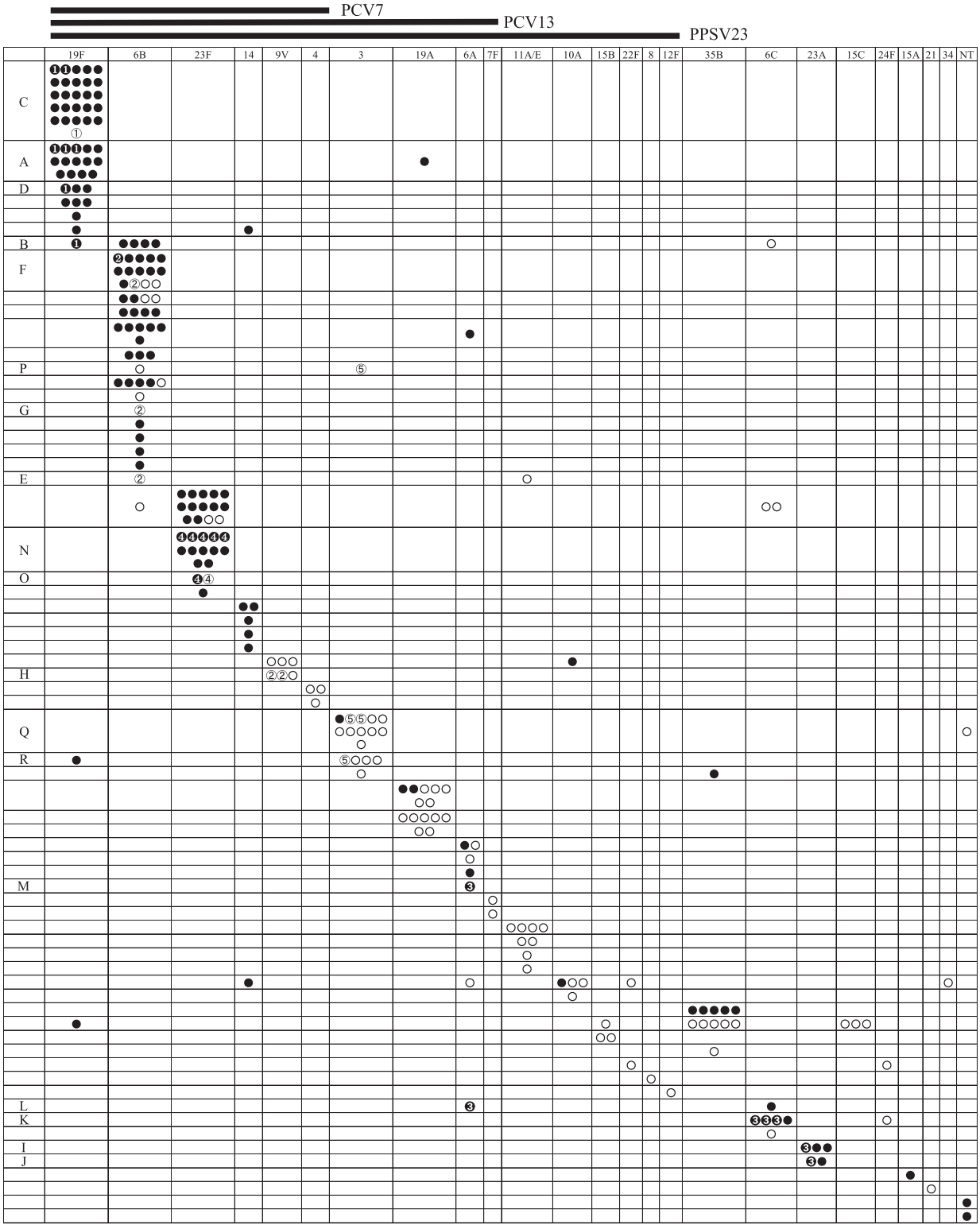

Fig. 2. Distribution of isolates to serotypes and genotypes according to the 85% similarity index cutoff (SIC) for the Extended-Jaccard (XJ) algorithm of DiversiLab. A closed and open circle, represent a penicillin-non-susceptible and penicillin-susceptible isolate, respectively. The number in the circle represents the five major PMEN-related lineages in Table 1 and Fig. 1. The alphabet in the left column represents the cluster name shown in Table 1. PCV, pneumococcal conjugate vaccine; PPSV, pneumococcal polysaccharide vaccine; NT, non-typeable.

genotyping, 0.514), followed by 85% for XJ (0.468) and 97% for PC (0.462) (Table 2). Among them, only 85% SIC for XJ successfully discriminated between Taiwan^{19F}-14 and Netherlands^{3}-31 (Fig. 2, Fig. S3, and Table 1).

4. Discussion

In the current study, we demonstrated the application of DL system for providing a cross-sectional portrait and characterization of the clinical pneumococci in a Japanese teaching hospital. The presence of substantial proportion of the worldwide lineages, partly shown by previous reports in Japan [15–20], was clearly shown in the current study involving consecutive all age-group patients in more exhaustive manner.

Although there were several reports demonstrating the favorable utility of DL as a rapid (within several hours) and less labor-intensive genotyping method for predicting serotypes or genetic traits of *Salmonella enterica* [21–23], *Esherichia coli* [24], and methicillin-resistant *Staphylococcus aureus* (MRSA) [25], information regarding pneumococcus was insufficient; Two reports demonstrated the utility of DL for estimating the clonality within isolates belonging to specific serotypes [5,7]. Meanwhile, although Harrington et al. reported the performance of DL was comparable to PFGE by analyzing 100 isolates belonging to 12 serotypes, genetic traits were not estimated by other genotyping methods such as MLST [6]. Therefore, the current study demonstrates for the first time the robust comparison of DL with serotypes and MLST genotypes by using various consecutive pneumococcal isolates.

Among ST-determined isolates, serotypes were conserved from the original PMEN clone in the majority of clinical pneumococci, including three out of five major PMEN lineages, as expected. This trend should be favorable in presuming serotypes from genotyping by DL. Moreover, serotype can reflect drug susceptibility, since the prevalence of penicillin non-susceptibility was higher among isolates with the PCV-7-type compared to those with the PCV-13-type capsule (Fig. 1). In contrast, a certain part of isolates identified as CC156-related PMEN lineages, namely England^{14}-9 and Netherlands^{15B}-37, might had experienced capsular switch [3]. Since CC156 includes multiple PMEN clones (Fig. S1), this lineages might be highly various and therefore need attention when presuming capsular types from genotyping.

Importantly, this is the first report regarding optimization of automated analyses of pneumococci by DL; this information is especially crucial for searching the most related reference strains by classification mode, where users cannot compare band patterns directly. A robust validation and comparison of DL algorithms, including newly developed KL and XJ, by determining objective parameters, was not previously been performed. Based on the results of the current study, users could select XJ with a SIC of 75% for discriminatory power comparable to serotyping. Meanwhile, the importance of VO estimation of the subtle band differences, suggested in MRSA [25,26], was not clearly shown to outweigh the labor and potential subjectivity. Rather, elevating the SIC for XJ to 85% appeared practically beneficial, as demonstrated for successful discrimination between Taiwan^{19F}-14 and Netherlands^{3}-31.

Our study has several limitations. For instance, the prediction performance of the serotypes or genetic lineages was imperfect; however, their estimation would be substantially beneficial as additional information. Our data may be less influential on current clinical practice because subjects in this study were identified just before the introduction of the PCV; nevertheless, for the optimization of analysis parameters, well-characterized isolates are advantageous. A study of reproducibility in a systematic manner was also not a focus of the current study; in regards primarily to the "classification" function, users should be aware of the concern regarding intra-laboratory reproducibility [27,28]. Since resource-intensive MLST was not performed for all isolates, the true proportion of PMEN-related lineages was not estimated; nevertheless, clusters seemed characterized sufficiently by analyzing the representative isolates.

In conclusion, the current study added the usefulness of DL for identification of worldwide lineages, and partly at least, their serotypes, of clinical pneumococci within as short as several hours. Optimal cutoffs of the newly developed automated analysis algorithms for DL are proposed. Further research is needed on the validity of the findings of the current study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jiac.2016.03.007.

Conflict of interest

The authors declare no conflict of interest.

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