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ORIGINAL ARTICLE

Development and evaluation of MALDI-TOF MS-based serotyping for *Streptococcus pneumoniae*

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Abstract Surveillance of *Streptococcus pneumoniae* serotypes is important for the successful implementation of vaccination strategies to prevent the spread of invasive pneumococcal diseases. The standard method of serotyping of pneumococcal isolates is the phenotypic Neufeld test, which is cost- and labor-intensive. Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been implemented as a rapid, simple and inexpensive method for identifying species. We evaluated the performance of MALDI-TOF MS for serotyping ten major serotypes of *S. pneumoniae* in Japan (serotypes 3, 6B, 15A, 15C, 19A, 19 F, 23A, 24 F, 35B and 38) using the Biotyper and ClinProTools. After optimizing the settings, we validated their serotyping performance for serotypes 3, 15A and 19A using a separate set of isolates that were not used in the creation of the classification algorithms. A total of 574 isolates of *S. pneumoniae* collected from Japanese nationwide surveillance studies were included. Of these, 407 isolates belonged to the ten major serotypes. Biotyper and ClinProTools correctly identified 77.9 % and 84.0 %, respectively, of the ten major serotype isolates. The validation analysis included a total of 113 isolates of the serotypes 3, 15A and 19A isolates. Biotyper and ClinProTools correctly identified 85.0 % and 69.9 % of the validation cohort isolates, respectively. MALDI-TOF MS has the potential to discriminate the ten major *S. pneumoniae* serotypes prevalent in Japan.

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Introduction

Streptococcus pneumoniae is a major human pathogen that causes a variety of diseases, including meningitis, pneumonia, and acute otitis media [1]. Although many countries have recently introduced pneumococcal conjugate vaccines [2–5], the incidence of pneumococcal disease caused by non-vaccine serotypes is increasing, primarily due to clones that have acquired another capsular type via multiple genetic recombination events [1, 6, 7]. For this reason, epidemiological serotyping surveillance is extremely important.

The standard method for serotyping pneumococcal isolates is the Neufeld test [8, 9]. However, the antiserum needed for this test is expensive, and the judgment of the test results requires special skills and experience. Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been implemented for the rapid, accurate and low-cost identification of pathogens including *S. pneumoniae* [10–12]. Several reports have described the use of MALDI-TOF MS for bacterial isolate typing [13–15]. Williamson et al. reported the differentiation of *S. pneumoniae* conjunctivitis outbreak isolates from non-outbreak isolates by MALDI-TOF MS [16]. However, this

study did not evaluate the performance of MALDI-TOF MS for the discrimination of each serotype.

The aim of this study was to evaluate the performance of MALDI-TOF MS for serotyping *S. pneumoniae*.

Material and methods

Bacterial isolates

A total of 574 clinical isolates of *S. pneumoniae* were investigated. Of these, 438 were collected between January 2012 and December 2013 as part of the Pneumocatch surveillance study, a nationwide pediatric invasive pneumococcal disease surveillance program. One hundred and seven hospitals participated in this surveillance study, which included patients under 15 years of age diagnosed with invasive pneumococcal disease, pneumococcal pneumonia or otitis media. The other 136 isolates were collected by the regional community-acquired pneumococcal pneumonia surveillance program in Japan from patients older than 15 years of age between May 2003 and February 2005 [17].

Identification and conventional serotyping

All isolates were identified on the basis of optochin sensitivity and multilocus sequence typing (MLST) as previously described [18]. Sequence types were assigned using the online MLST database (http://pubmlst.org/spneumoniae/). Conventional serotyping was performed using pneumococcal Neufeld antisera and the Pneumotest kit (Statens Serum Institut, Copenhagen, Denmark). The kit comprises 14 pooled antisera (A to I and P to T) that are used to identify the serotype or serogroup with the aid of a chessboard.

Sample preparation and MALDI-TOF MS

Bacterial colonies were grown overnight on sheep blood agar and subjected to ethanol-formic acid extraction according to the MALDI Biotyper protocol (Bruker Daltonics GmbH, Bremen, Germany) as described previously [19]. MALDI-TOF MS was performed on a Microflex LT controlled by FlexControl version 3.4 software (Bruker Daltonics GmbH). Mass spectra were acquired by the standard recommended proprietary method utilizing the Biotyper pre-processing standard method and the Biotyper MSP identification standard method. In brief, the laser frequency for ionization was 60 Hz with a 150 ns pulse, the target potential was 20.00 kV, the potential at the second electrode was 18.15 kV, the potential at the subsequence slit was 6.0 kV, and the mass range was 2,000–20,000 *m*/*z*. For automatic sample collection, a standard Biotyper method (MBT_AutoX) was used, and 240 shots in 40 steps were collected to generate the final spectrum. The system was calibrated every 96 spots by analyzing one spot on the steel target plate loaded with the Bruker Bacterial Test Standard (Bruker Daltonics GmbH).

Identification with the Biotyper

Species were identified using MALDI Biotyper version 3.1 (Bruker Daltonics GmbH) and its standard database (Bruker Taxonomy database version 4.0.0.1 [5627]) without supplementation.

Definitions of major, minor and rare serotypes

According to the manufacturer's instructions, a good average can be achieved by creating a main spectrum (MSP) in Biotyper and calculating the cross-validation, the reliability of a calculated model, in ClinProTools of more than 19 mass spectra. Thus, ten major serotypes with more than 19 isolates (serotypes 3, 6B, 15A, 15C, 19A, 19 F, 23A, 24 F, 35B and 38) were defined. To avoid loss of the performance by creating only one MSP for all of the non-major serotype isolates, we separated non-major serotype isolates into seven minor serotypes with 10–19 isolates (serotypes 6A, 6C, 10A, 11A, 15B, 22 F and 23 F) and one rare serotype group including all serotypes with less than ten isolates (serotypes 1, 4, 6D, 7, 9 N, 9 V, 12 F, 14, 18C, 24B, 33 F, 34 and 37). We evaluated the performance of the MALDI-TOF MS method for the detection of the ten major serotypes using Biotyper and ClinProTools.

Biotyper serotyping

We created MSPs of the serotypes using the MSP Creation Standard Method set to the default parameters. Using all mass spectra representing each serotype, we created ten MSPs for each major serotype, seven MSPs for each minor serotype and one MSP for the rare serotype group. Finally, we added these 18 MSPs to the Biotyper database, and all 574 isolates were re-analyzed using this database. The best-match MSP was considered a serotyping result. When minor or rare serotypes were identified, the identification result was regarded as a serotype other than one of the major serotypes and not the corresponding serotype of the MSP. Replicated spots and multiple measurements per spot were not performed because the characteristic MSPs of the same serotype isolates were used to compare serotype groups and not to detect variations among the isolates.

(i) The initial setting

We created 18 MSPs representing ten major serotypes, seven minor serotypes, and one rare serotype group.

(ii) The best setting

We modified the MSP setting to maximize the system's performance (the sum of its sensitivity and specificity) for the serotyping of the ten major serotypes. When an MSP for a major serotype showed poor sensitivity of less than 70 % in the initial setting, we created a new MSP using an additional affiliate serotype other than the major serotypes. For example, a new MSP of serotype 15BC was created by adding the serotype 15B isolates to the major serotype 15C MSP. This modification was performed only when more than half of the affiliated serotype isolates belonged to the sequence types found in the original serotype isolates. When the number of isolates for the creation of an MSP exceeded 100, we created a new MSP by randomly selecting half of the isolates to avoid loss of serotyping performance caused by using a greater number of isolates. Finally, we adopted the best MSP setting, which had the highest sum of overall sensitivity and specificity.

ClinProTools serotyping

We generated models for the classification of the ten major serotypes using ClinProTools with three available algorithms in our institution (the Genetic Algorithm, the Supervised Neural Network Algorithm and the QuickClassifier Algorithm). The Support Vector Machine Algorithm was not available. To generate a serotype-specific model, all isolates of the serotype were used as the target group, and all other isolates were used as the control group.

To calculate overall performance, all 574 isolates were classified using the ten models; each serotype-specific model classified a mass spectrum of a test isolate as positive or negative for that serotype. When an isolate was classified as more than one serotype, we considered the isolate incorrectly classified. With regard to minor or rare serotype isolates, when an isolate was not classified as any of the ten major serotypes, we considered the isolate correctly classified.

(i) The initial setting

The ten models with the highest sums of recognition capability and cross-validation were selected for the identification of each major serotype. Recognition capability, a measure that describes the performance of a calculated model, is calculated as the relative number of data points correctly classified by the model. Cross-validation is a statistical measure of the reliability of a calculated model.

(ii) The best setting

We modified the ClinProTools settings to maximize recognition capability for each major serotype; we generated a new model targeting the combined serotype group of the major serotype and its affiliated serotypes in the same manner described for the Biotyper best setting. For models with less than 90 % recognition capability for the control group, we adopted the algorithm that had the highest recognition capability for the control group even if it exhibited a lower sum of the recognition capability and the cross-validation.

Validation cohort analysis

A validation cohort included the three serotypes that corresponded to the largest numbers of isolates (serotypes 3, 15A and 19A). Half of these isolates (n=113) were randomly selected (serotype 3, n=30; serotype 15A, n=27; and serotype 19A, n=56) for the validation cohort. The other 461 isolates, including the remaining half of the three dominant serotype isolates, were used in the creation of the Biotyper MSPs and the ClinProTools models for validation analysis. For Biotyper, the optimal settings (for the 18 MSPs in the database) were used to classify the validation cohort isolates. For ClinProTools, we created three models for serotypes 3, 15A and 19A using the same algorithms as determined for the best setting.

Results

Conventional and Biotyper identification

On the basis of optochin sensitivity and multilocus sequence typing, all of the 574 tested isolates were identified as *S. pneumoniae*. On the basis of MALDI-TOF MS identification, 573 isolates were identified as *S. pneumoniae* by Biotyper software with a score>2.0 (categorized as probable species identification). One isolate was identified as *S. pneumoniae* with a score of 1.982 (categorized as probable genus level). The isolates were comprised of 30 serotypes and 17 untypeable isolates, which did not react with any of the 14 pooled antisera (Table 1). A total of 116 sequence types (STs) was observed. The ten major serotypes, the seven minor serotypes, and the rare serotype group included 407, 106, and 44 isolates, respectively. All 574 isolates were used in the MALDI-TOF MS analysis.

Biotyper serotyping

Table 2 shows the performance of Biotyper serotyping for the ten major serotypes. The use of the ten MSPs for each major serotype resulted in a mean sensitivity and specificity (an average of the sensitivities and specificities of ten major serotype MSPs) of 67.7 % and 98.8 %, respectively, in the initial setting, and 73.5 % and 98.7 %, respectively, in the best setting.

Table 1 Serotypes and sequence types of the 574 isolates used in this study

Serotype category	Serotype	Number of isolates	Sequence type (no.)
Major serotypes	3	60	180 (53), 2808 (1), 2809 (1), 4054 (1), 5234 (1), 7494 (1), 7803 (1), 9336 (1)
	6B	29	2983 (5), 90 (4), 2756 (3), 2923 (3), 902 (2), 9335 (2), 242 (1), 385 (1), 1437 (1), 2924 (1), 2925 (1), 3787 (1), 3931 (1), 4233 (1), 5497 (1), 9333 (1)
	15A	55	63 (36), 9084 (5), 7874 (3), 3111 (2), 292 (1), 1621 (1), 2105 (1), 5246 (1), 5496 (1), 9617 (1), 9643 (1), 9644 (1), 9646 (1)
	15C	29	199 (22), 83 (1), 1531 (1), 3934 (1), 7793 (1), 9643 (1), 9648 (1), 9649 (1)
	19A	113	3111 (81), 2331 (12), 320 (2), 338 (2), 5842 (2), 6429 (2), 9647 (2), 199 (1), 558 (1), 3109 (1), 5237 (1), 5242 (1), 5246 (1), 5496 (1), 7811 (1), 7988 (1), 9334 (1)
	19 F	32	236 (25), 115 (1), 257 (1), 271 (1), 280 (1), 393 (1), 926 (1), 1428 (1)
	23A	21	338 (13), 5242 (5), 63 (1), 180 (1), 3112 (1)
	24 F	23	2572 (11), 5496 (6), 4982 (2), 3111 (1), 5241 (1), 7984 (1), 9621 (1)
	35B	25	558 (14), 2755 (8), 3111 (1), 3118 (1), 7502 (1)
	38	20	6429 (11), 393 (4), 115 (1), 180 (1), 199 (1), 7502 (1), 9336 (1)
Minor serotypes	6A	18	3113 (4), 2756 (3), 63 (2), 5832 (2), 81 (1), 180 (1), 338 (1), 3114 (1), 3115 (1), 3787 (1), 6429 (1)
	6C	14	5832 (3), 242 (2), 2923 (2), 2924 (2), 5241 (2), 63 (1), 6183 (1), 9336 (1)
	10A	11	5236 (8), 558 (1), 3109 (1), 3111 (1)
	11A	18	99 (13), 1012 (2), 62 (1), 166 (1), 393 (1)
	15B	13	199 (6), 1531 (2), 5242 (2), 83 (1), 419 (1), 9622 (1)
	22 F	17	433 (14) , 819 (1), 5236 (1), 7158 (1)
	23 F	15	242 (10), 1437 (4), 3543 (1)
Rare serotypes	1	7	306 (7)
	4	3	246 (1), 695 (1), 3193 (1)
	6D	1	282 (1)
	7	3	191 (2), 236 (1)
	9 N	1	66 (1)
	9 V	6	280 (5), 5231 (1)
	12 F	1	4846 (1)
	14	8	343 (2), 2922 (2), 13 (1), 156 (1), 554 (1), 5240 (1)
	18C	1	3594 (1)
	24B	4	5496 (2), 2572 (1), 2754 (1)
	33 F	2	717 (2)
	34	5	3116 (3), 3111 (1), 7388 (1)
	37	2	447 (2)
Not categorized	Untypeable	17	558 (3), 3111 (3), 3116 (2), 3117 (2), 199 (1), 242 (1), 2924 (1), 3194 (1), 5236 (1), 7019 (1), 9642 (1)
Total		574	

The serotype was determined by Neufeld test using the Pneumotest kit (Statens Serum Institut, Copenhagen, Denmark). Untypeable isolates did not react with any of the 14 pooled antisera

The best setting had an overall sensitivity of 77.9 % and a specificity of 75.4 %, when all ten major serotype isolates and the other non-major serotype isolates were considered as the target and control groups, respectively.

ClinProTools serotyping

Table 3 shows the performance of ClinProTools serotyping for the ten major serotypes. The use of the ten models for each major serotype resulted in a mean sensitivity and specificity (an average of the sensitivities and specificities of ten major serotype models) of 90.0 % and 88.8 %, respectively, in the initial setting and 82.4 % and 99.1 %, respectively, in the best setting. The best setting had an overall sensitivity of 84.0 % and specificity of 82.0 % when all ten major serotype isolates and the other non-major serotype isolates were considered as the target and control groups, respectively.

Table 2 The performance of classification with Biotyper for the detection of ten major serotypes

Setting	Serogroup used for MSP creation or classification	Number of target isolates classified		Number of control isolates classified		Sensitivity (%)	Specificity (%)
		Total	Correctly classified	Total	Correctly classified		
Initial MSPs[a]	3	60	57	514	512	95.0	99.6
	6B	29	11	545	543	37.9	99.6
	15A	55	51	519	517	92.7	99.6
	15C	29	13	545	529	44.8	97.0
	19A	113	64	461	452	56.6	98.0
	19 F	32	25	542	540	78.1	99.6
	23A	21	21	553	539	100	97.5
	24 F	23	16	551	542	69.6	98.4
	35B	25	8	549	545	32.0	99.3
	38	20	14	554	551	70.0	99.5
	Overall	407	280	167	130	68.8	77.8
Best MSPs[b]	3	60	57	514	514	95.0	100
	6B	29	15	545	541	51.7	99.3
	15A	55	51	519	517	92.7	99.6
	15BC[c]	29	21	545	523	72.4	96.0
	19A[d]	113	91	461	444	80.5	96.3
	19 F	32	24	542	539	75.0	99.4
	23A	21	21	553	540	100	97.6
	24BF[e]	23	16	551	547	69.6	99.3
	35B	25	7	549	546	28.0	99.5
	38	20	14	554	551	70.0	99.5
	Overall	407	317	167	126	77.9	75.4

MSPs were created using all isolates of a specific serotype. The best-match MSP was considered a serotyping result

[a] In the initial setting, the average of the sensitivities and specificities of the ten MSPs for each major serotype were 67.7 % and 98.8 %, respectively

[b] In the best setting, the average of the sensitivities and specificities of the ten MSPs for each major serotype were 73.5 % and 98.7 %, respectively

[c] The MSP for serotype 15BC was created using all of the 15B and 15C isolates, and all of the serotype 15C isolates were re-identified to evaluate the performance of the MSP. For the serotype 15B isolates, eight out of 13 isolates were correctly identified as serotype 15BC in the best setting

[d] The MSP for serotype 19A in the best MSP setting was created using a random selection of half of the 19A isolates

[e] The MSP for serotype 24BF was created using all of the serotype 24B and 24 F isolates, and all of the serotype 24 F isolates were re-identified to evaluate the performance of the MSP. For the serotype 24B isolates, three out of four isolates were correctly identified as serotype 24BF in the best setting

Validation cohort analysis

Table 4 shows the serotyping performance of Biotyper for the 113 validation cohort isolates. The sensitivities for serotypes 3, 15A and 19A were 96.7 %, 88.9 % and 76.8 %, respectively. The specificities were 100 % each. In total, 85.0 % of the validation cohort isolates were classified correctly.

Table 5 and Table S1 show the serotyping performance of ClinProTools for the validation cohort isolates. The sensitivities for serotypes 3, 15A and 19A were 96.7 %, 81.5 % and 50.0 %, respectively, and the specificities were 100 %, 100 % and 96.5 %, respectively. In total, 69.9 % of the validation cohort isolates were classified correctly.

Discussion

In this study, we assessed the potential of MALDI-TOF MS for serotyping of *S. pneumoniae* using isolates obtained in a Japanese nationwide surveillance study. The performance of MALDI-TOF MS for serotyping was evaluated for the ten major serotypes of *S. pneumoniae* that comprised 70.9 % of our clinical isolates. The detection sensitivities for some major serotypes that were detected with low sensitivity in the initial MSPs or models were improved by optimizing the MSPs or models. In particular, serotype 6B exhibited low sensitivity using either Biotyper or ClinProTools. This low performance may be due to the close relationship among variant subtypes of the serogroup 6 [20] and the small number of isolates used to create the MSP and ClinProTools models. By contrast,

Table 3 The performance of models generated by ClinProTools for the detection of ten major serotypes of *S. pneumoniae*

Setting	Target serotype	Classification algorithm	Peaks (*m*/*z*) used in the model	Cross validation[a] (%)			Recognition capability[b] (%)		
				Overall	Target group	Control group	Overall	Target group	Control group
Initial models[c]	3	GA	2469, 4439, 4539, 6757, 9060	97.2	94.4	100	99.2	98.3	100
	6B	QC	3855	76.0	93.3	58.7	73.7	89.7	57.7
	15A	GA	3255, 4975, 4992, 5049, 10124	91.9	84.7	99.1	96.3	92.3	99.2
	15C	QC	2225, 2809, 3336, 3855, 4200, 4214, 6673, 7709, 8399, 8428	70.4	88.1	52.6	66.5	96.4	36.5
	19A	GA	2809, 5297, 5942, 5005, 9877	87.5	80.2	94.8	94.5	92.0	97.0
	19 F	GA	2970, 3453, 4975, 6497, 6620	89.9	80.3	99.5	93.5	87.5	99.5
	23A	GA	4154, 5863, 6511, 8094, 8399	76.1	53.5	98.7	90.3	81.0	99.6
	24 F	GA	3386, 3486, 4763, 5269, 6160	73.8	47.8	99.7	95.5	91.3	99.6
	35B	GA	2209, 5049, 5284, 5997, 8040	63.3	27.5	99.2	87.7	76.0	99.5
	38	GA	5297, 6271, 6330, 7709, 15016	80.8	62.5	99.0	97.3	95.0	99.6
Best models[d]	6B	GA	3435, 4992, 6893, 6644, 5049	59.7	20.0	99.4	80.9	62.1	99.6
	15BC[e]	SNN	2633, 3081, 3321, 4424, 4568, 4976, 5499, 5916, 6255, 6528, 6908, 7058, 7709, 8116, 8133, 8161, 8812, 9116, 9914, 12316, 13565, 14377	68.9	54.8	83.1	75.8	54.8	96.8
	24BF[f]	GA	6160, 4763, 2985, 3486, 5284	79.9	60.7	99.1	92.6	85.2	100

GA genetic algorithm, *QC* QuickClassifier Algorithm, *SNN* Supervised Neural Network Algorithm

Of the 407 isolates comprising the ten major serotypes, 134 isolates (32.9 %) were classified correctly in the initial models setting, and 342 isolates (84.0 %) were classified correctly in the best models setting. For the GA, we performed *k*-nearest neighbor classification using *k* values of 3, 5 and 7. For the SNN, we adopted 99×100 cycles as the upper limit to allow this algorithm to be used with a large number of mass spectra. For the QC, the default settings were applied

[a] Cross-validation is a statistical measure of the reliability of a calculated model and is expressed as the normalized value of the relative prediction capability

[b] Recognition capability is a measure describing the performance of a calculated model. The measure is calculated for a determined model as the relative number of data points correctly classified by the model and is equal to the sensitivity

[c] In the initial setting, the mean sensitivity and specificity of the ten MSPs for each major serotype were 90.0 % and 88.8 %, respectively

[d] In the best models setting, the models for serotypes 6B, 15BC and 24BF were generated in addition to the initial models for serotypes 3, 15A, 19A, 19 F, 23A, 35B and 38. The mean sensitivity and specificity of the ten MSPs for each major serotype were 82.4 % and 99.1 %, respectively

[e] The model for serotype 15BC was generated using all of the 15B and 15C isolates, and all of the serotype 15C isolates were re-identified to evaluate the performance of the model. For the serotype serotype 15B isolates, seven out of 13 isolates were correctly identified as serotype 15BC in the best setting

[f] The model for serotype 24BF was generated using all of the 24B and 24 F isolates, and all of the serotype 24 F isolates were re-identified to evaluate the performance of the model. For the serotype serotype 24B isolates, two out of four isolates were correctly identified as serotype 24BF in the best setting

serotype 3 does not contain variant subtypes, and an adequate number of 60 isolates was used. Greater than 95 % sensitivity was achieved using Biotyper and ClinProTools. The results also suggest that creating MSP or ClinProTools models for all

Table 4 The performance of Biotyper-based classification for the detection of serotypes 3, 15A and 19A in the validation cohort

Target serotype	Control serotypes	Number of target isolates classified		Number of control isolates classified		Sensitivity (%)	Specificity (%)
		Total	Correctly classified	Total	Correctly classified		
3	15A, 19A	30	29	83	83	96.7	100
15A	3, 19A	27	24	86	86	88.9	100
19A	3, 15A	56	43	57	57	76.8	100

A validation cohort (n=113) was prepared by randomly selecting half of the isolates of serotypes 3, 15A and 19A. Using the other isolates (n=461), we created MSPs for each of the ten major serotypes, the seven minor serotypes and one rare serotype group according to the best settings shown in Table 2. All 18 MSPs were added to the Biotyper database, and the 113 validation cohort isolates were classified. The best-match MSP was considered as the serotyping result. In total, 96 (85.0 %) out of the 113 tested isolates were classified correctly

Table 5 The performance of ClinProTools model-based classification for the detection of serotype 3, 15A and 19A in the validation cohort

Target serotype (total number of isolates)	Number of isolates classified into each model				Sensitivity (%)	Specificity (%)
	3	15A	19A	Not 3, 15A or 19A		
3 (30)	29	0	0	1	96.7	100
15A (27)	0	24[a]	2[a]	3	81.5[a]	100
19A (56)	0	0	28	28	50.0	96.5

A validation cohort (n=113) was prepared by randomly selecting half of the serotype 3, 15A and 19A isolates. Using the other isolates (n=461), we generated ClinProTools models for the detection of serotypes 3, 15A and 19A. Using these models, we analyzed the 113 isolates in the validation cohort. When an isolate was classified into more than one serotype, we considered the isolate to be incorrectly classified. In total, 79 (69.9 %) out of the 113 tested isolates were classified correctly

[a] Twenty-four isolates out of 27 serotype 15A isolates were identified as serotype 15A using the ClinProTools model for serotype 15A. Two isolates out of these 24 isolates were also identified as serotype 19A using the ClinProTools model for serotype 19A. Consequently, 22 isolates out of 27 serotype 15A isolates were correctly identified as serotype 15A. Thus, the ClinProTools model for serotype 15A had a sensitivity of 81.5 % (22/27)

closely related serotypes and increasing the number of isolates of each serotype by collecting more isolates may improve the detection performance of the method for individual serotypes, thus permitting high-performance MALDI-TOF MS serotyping without the use of the Neufeld test. Meanwhile, MALDI-TOF MS generally detects housekeeping proteins such as ribosomal proteins [10] and does not detect capsular polysaccharides or related proteins. Therefore, the identification of the serotypes in this study may be caused by the differences in housekeeping proteins associated with the major sequence types in the serotype. Consequently, we should proceed to clarify the performance of MALDI-TOF MS in discriminating the sequence types of *S. pneumoniae*.

Notably, using a reduced number of isolates for MSP creation for serotype 19A improved the performance of the MSP. This finding indicates that using too many isolates to create an MSP may reduce its performance. Clearly, using too few isolates to generate an MSP reduces its performance; therefore, we should determine the appropriate standard number of isolates to be used for MSP creation.

The validation cohort analysis revealed similarly good performance between Biotyper and ClinProTools for the detection of serotypes 3 and 15A compared to the analyses using the same set of isolates for the creation of the classification algorithms and the classification of the isolates, strengthening our findings regarding MALDI-TOF MS-based serotyping. However, lower performances were observed for serotype 19A. These results suggest that the performance of MALDI-TOF MS-based serotyping may depend on the serotypes examined.

Biotyper is standard software used in species identification. Thus, Biotyper serotyping can be performed without any additional procedures after acquiring mass spectra. The data analysis software ClinProTools is used after the acquisition of spectra, and its use requires some additional time. Although no additional running cost is incurred, an initial software cost of €13,300 is required because ClinProTools is not included in the standard package of the MALDI-TOF MS system used in the clinical laboratory. We believe that Biotyper-based serotyping is an easy, simple method, although the use of the ClinProTools-based method could be considered if the laboratory already possesses ClinProTools.

The limitations of this study should be noted. First, not all serotypes of *S. pneumoniae* were investigated for MALDI-TOF MS performance in this study. However, for the ten major serotypes that are prevalent in Japan, MALDI-TOF MS exhibited promising performance. Using more isolates for all serotype isolates from all regions across the globe would further improve the performance of MALDI-TOF MS. Second, we used the same isolates for the creation of MSPs and for the creation of ClinProTools models, except for the serotypes 3, 15A and 19A, for the evaluation of the performance of the system. To evaluate the performance of MALDI-TOF MS serotyping more accurately, further prospective evaluations of other isolates are needed. Third, in the present study, we did not perform the replicated spots and multiple measurement per spot during the creation of MSPs. Further evaluations using multiple spectra for each isolate may refine the performance of MALDI-TOF MS-based serotyping.

In conclusion, we have demonstrated that MALDI-TOF MS has the potential to discriminate the ten major serotypes of *S. pneumoniae* that are prevalent in Japan. Further prospective validation studies are needed to evaluate the utility of MALDI-TOF MS-based *S. pneumoniae* serotyping for effective serotype surveillance to facilitate the development of vaccination strategies.

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All other authors declare no conflicts of interest.

References

1. Mehr S, Wood N (2012) *Streptococcus pneumoniae*–a review of carriage, infection, serotype replacement and vaccination. Paediatr Respir Rev 13(4):258–264
2. Pichon B, Ladhani SN, Slack MP, Segonds-Pichon A, Andrews NJ, Waight PA, Miller E, George R (2013) Changes in molecular epidemiology of *Streptococcus pneumoniae* causing meningitis following introduction of pneumococcal conjugate vaccination in England and Wales. J Clin Microbiol 51(3):820–827
3. Ampofo K, Pavia AT, Chris S, Hersh AL, Bender JM, Blaschke AJ, Weng HY, Korgenski KE, Daly J, Mason EO, Byington CL (2012) The changing epidemiology of invasive pneumococcal disease at a tertiary children's hospital through the 7-valent pneumococcal conjugate vaccine era: a case for continuous surveillance. Pediatr Infect Dis J 31(3):228–234
4. Tan TQ (2012) Pediatric invasive pneumococcal disease in the United States in the era of pneumococcal conjugate vaccines. Clin Microbiol Rev 25(3):409–419
5. Parra EL, Ramos V, Sanabria O, Moreno J (2014) Serotype and genotype distribution among invasive *Streptococcus pneumoniae* isolates in Colombia, 2005–2010. PLoS One 9(1):e84993
6. Hu FZ, Eutsey R, Ahmed A, Frazao N, Powell E, Hiller NL, Hillman T, Buchinsky FJ, Boissy R, Janto B, Kress-Bennett J, Longwell M, Ezzo S, Post JC, Nesin M, Tomasz A, Ehrlich GD (2012) In vivo capsular switch in *Streptococcus pneumoniae*–analysis by whole genome sequencing. PLoS One 7(11):e47983
7. Sabharwal V, Stevenson A, Figueira M, Orthopoulos G, Trzcinski K, Pelton SI (2014) Capsular switching as a strategy to increase pneumococcal virulence in experimental otitis media model. Microbes Infect 16(4):292–299
8. Lund E, Rasmussen P (1966) Omni-serum. A diagnostic Pneumococcus serum, reacting with the 82 known types of Pneumococcus. Acta Pathol Microbiol Scand 68(3):458–460
9. Satzke C, Turner P, Virolainen-Julkunen A, Adrian PV, Antonio M, Hare KM, Henao-Restrepo AM, Leach AJ, Klugman KP, Porter BD, Sa-Leao R, Scott JA, Nohynek H, O'Brien KL (2013) Standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*: updated recommendations from the World Health Organization Pneumococcal Carriage Working Group. Vaccine 32(1):165–179
10. Clark AE, Kaleta EJ, Arora A, Wolk DM (2013) Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clin Microbiol Rev 26(3):547–603
11. Dubois D, Segonds C, Prere MF, Marty N, Oswald E (2013) Identification of clinical *Streptococcus pneumoniae* isolates among other alpha and nonhemolytic streptococci by use of the Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system. J Clin Microbiol 51(6):1861–1867
12. Ikryannikova LN, Filimonova AV, Malakhova MV, Savinova T, Filimonova O, Ilina EN, Dubovickaya VA, Sidorenko SV, Govorun VM (2013) Discrimination between *Streptococcus pneumoniae* and *Streptococcus mitis* based on sorting of their MALDI mass spectra. Clin Microbiol Infect 19(11):1066–1071
13. Matsumura Y, Yamamoto M, Nagao M, Tanaka M, Machida K, Ito Y, Takakura S, Ichiyama S (2014) Detection of extended-spectrum-beta-lactamase-producing *Escherichia coli* ST131 and ST405 clonal groups by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 52(4):1034–1040
14. Nakano S, Matsumura Y, Kato K, Yunoki T, Hotta G, Noguchi T, Yamamoto M, Nagao M, Ito Y, Takakura S, Ichiyama S (2014) Differentiation of *vanA*-positive *Enterococcus faecium* from *vanA*-negative *E. faecium* by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Int J Antimicrob Agents 44(3):256–259
15. Mencacci A, Monari C, Leli C, Merlini L, De Carolis E, Vella A, Cacioni M, Buzi S, Nardelli E, Bistoni F, Sanguinetti M, Vecchiarelli A (2013) Typing of nosocomial outbreaks of *Acinetobacter baumannii* by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 51(2):603–606
16. Williamson YM, Moura H, Woolfitt AR, Pirkle JL, Barr JR, Carvalho Mda G, Ades EP, Carlone GM, Sampson JS (2008) Differentiation of *Streptococcus pneumoniae* conjunctivitis outbreak isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74(19):5891–5897
17. Isozumi R, Ito Y, Ishida T, Osawa M, Hirai T, Ito I, Maniwa K, Hayashi M, Kagioka H, Hirabayashi M, Onari K, Tomioka H, Tomii K, Gohma I, Imai S, Takakura S, Iinuma Y, Ichiyama S, Mishima M (2007) Genotypes and related factors reflecting macrolide resistance in pneumococcal pneumonia infections in Japan. J Clin Microbiol 45(5):1440–1446
18. Enright MC, Spratt BG (1998) A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology 144(Pt 11):3049–3060
19. Griffin PM, Price GR, Schooneveldt JM, Schlebusch S, Tilse MH, Urbanski T, Hamilton B, Venter D (2012) Use of matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify vancomycin-resistant enterococci and investigate the epidemiology of an outbreak. J Clin Microbiol 50(9):2918–2931
20. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, Nahm MH (2007) Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. J Clin Microbiol 45(4):1225–1233