Journal of Microbiology, Immunology and Infection 57 (2024) 107—117

Available online at www.sciencedirect.com

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journal homepage: www.e-jmii.com

Original Article

Identification of pneumococcal serotypes with individual recognition of vaccine types by a highly multiplexed real-time PCR-based MeltArray approach

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Received 17 April 2023; received in revised form 7 September 2023; accepted 24 October 2023
Available online 29 October 2023

KEYWORDS
Streptococcus pneumoniae;
Pneumococcal serotyping;
MeltArray;
Vaccine serotype

Abstract *Background:* Pneumococcus serotyping is important for monitoring serotype epidemiology, vaccine-induced serotypes replacement and emerging pathogenic serotypes. However, the lack of high-resolution serotyping tools has hindered its widespread implementation.
Methods: We devised a single-step, multiplex real-time polymerase chain reaction (PCR)-based MeltArray approach termed PneumoSero that can identify 92 serotypes with individual recognition of 54 serotypes, including all 24 currently available vaccine types. The limit of detection (LOD) and the ability to coexisting serotypes were studied, followed by analytical evaluation using 92 reference pneumococcal strains and 125 non-pneumococcal strains, and clinical evaluation using 471 pneumococcus isolates and 46 pneumococcus-positive clinical samples.
Results: The LODs varied with serotypes from 50 to 100 copies per reaction and 10 % of the minor serotypes were detectable in samples containing two mixed serotypes. Analytical evaluation

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https://doi.org/10.1016/j.jmii.2023.10.008

presented 100 % accuracy in both 92 reference pneumococcal strains and 125 non-pneumococcal strains. Clinical evaluation of 471 pneumococcus isolates displayed full concordance with Sanger sequencing results. The 46 clinical specimens yielded 45 typeable results and one untypeable result. Of the 45 typeable samples, 41 were of a single serotype and four were of mixed serotypes, all of which were confirmed by Sanger sequencing or separate PCR assays.
Conclusion: We conclude that the PneumoSero assay can be implemented as a routine tool for pneumococcal serotyping in standard microbiology laboratories and even in clinical settings.

Introduction

Streptococcus pneumoniae causes various life-threatening diseases, such as community-acquired pneumonia, bacteremia, and meningitis, at different ages.[1–4] Administration of serotype-specific pneumococcal vaccines has proven to be a significant advancement in controlling this deadly organism.[5–7] However, pneumococci have more than 100 serotypes, the distribution of which varies temporally and geographically.[8–10] The use of serotype-specific vaccines, such as 7- and 13-valent pneumococcal conjugate vaccines (PCV7 and PCV13), has dramatically changed serotype epidemiology, resulting in a continuous decline in vaccine serotypes (VTs), but the sustained accumulation of non-vaccine serotypes (NVTs).[10,11] Nowadays, upgraded vaccines, such as 15- and 20-valent pneumococcal conjugate vaccines (PCV15 and PCV20), have been developed.[12] New selective pressures may alter the current equilibrium among pneumococcal carriage isolates and lead to further serotype replacement. Therefore, monitoring serotype distribution is of great significance for evaluating the dynamic coverage of current pneumococcal vaccines for future updating.

Currently, the gold standard for pneumococcal serotyping is the Quellung reaction, which can be used to identify 92 serotypes.[13] More recently, the Quellung reaction has been complemented by alternative detection techniques, among which latex agglutination has been widely adopted because of its speed and simplicity.[14] However, these phenotypic pneumococcal capsular typing methods have obvious limitations: First, the overall procedure is complex and costly because a large supply of pneumococcal antisera is required. Second, result interpretation is subjective and requires experienced personnel. Third, multiple serotypes cannot be detected in one sample.[13] Last but not least, the absolute requisite of this test for live strains renders its application invalidated in cases of culture negative infections. Collectively, these limitations have restricted phenotypic methods only to those well-equipped reference laboratories and also make it unsuitable for a large sample volume.

Following elucidation of the sequence of the capsule biosynthesis gene of *S. pneumoniae*,[15] genotypic serotyping methods have gained popularity in recent years. To date, various multiplex polymerase chain reaction (PCR) approaches using either fluorescent capillary electrophoresis[16] or real-time PCR[17] have been developed. Compared to their phenotypic counterparts, genotyping approaches have many obvious advantages, such as cost-effectiveness, short turnaround time, and ease of use, opening avenues for routine surveillance of pneumococcal serotypes. However, current multiplex PCR approaches are disadvantaged by the small number of targets, which can be alleviated by increasing the reaction number but at the cost of assay throughput. Thus, only the dominant disease-causing serotypes are detected without discrimination between VTs and NVTs.[16–19] Whole-genome sequencing (WGS) can provide thorough sequence-based serotype prediction. Thus far, many pneumococcal serotype prediction algorithms have been developed, such as PneumoCat,[20] SeroBA,[21] and SeroCall.[22] However, the use of WGS platform is still uncommon in developing countries.

We recently reported a highly multiplexed PCR approach named MeltArray, which allows the detection of dozens of targets in one reaction on a common real-time PCR platform.[23] A distinct feature of MeltArray is that it can resolve targets that differ by a single nucleotide. We speculated that a new pneumococcal serotyping assay with higher throughput and resolution than current assays could be developed using MeltArray. Because of the wide availability and cost-effectiveness of real-time PCR thermocyclers, such an assay would enable large-scale surveillance of the dynamic changes in pneumococcal serotypes. Here, we describe the development and evaluation of this MeltArray-based serotyping assay, termed PneumoSero, for detecting 92 pneumococcal serotypes, with special attention paid to the discrimination between VTs and NVTs.

Methods

Bacterial reference strains and DNA extraction

The 92 pneumococcal reference strains, representing 92 different serotypes, were cultured at the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. The serotypes of all the strains were determined using the Quellung reaction and WGS. A total of 125 non-pneumococcal strains, including 15 streptococci other than pneumococcal strains and 21 non-streptococcal strains, were cultured at the Medical Culture Preservation Center of Xiamen Zhongshan Hospital. These isolates were identified using matrix-assisted laser desorption/ionization time-of-flight mass

spectrometry (MALDI-TOF MS, Bruker Daltonics, Bremen, Germany). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The DNA concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The PneumoSero assay

The PneumoSero assay was designed to detect 92 serotypes in three reactions, including serotypes 1, 2, 3, 4, 5, 6A, 6B, 6C/6D, 7A, 7B/7C/40, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10C, 10F, 11A, 11B/11C, 11D, 11F, 12A/12B, 12F, 13, 14, 15A/15F, 15B, 15C, 16A, 16F, 17A, 17F, 18A/18B, 18C, 18F, 19A, 19B/19C, 19F, 20, 21, 22A, 22F, 23A, 23B, 23F, 24A/24B/24F, 25A/25F/38, 27, 28A/28F, 29, 31, 32A/32F, 33A/37, 33B/33D, 33C, 33F, 34, 35A/35C/42, 35B, 35F/47F, 36, 39, 41A/41F, 43, 44/46, 45, 47A, and 48. Primers and probes were designed in the highly conserved regions of the specific gene for each pneumococcal serotype using Primer Premier 5.0 software (version 5.0, Premier Biosoft, Palo Alto, CA, USA). The specificity of the primer and probe sequences were confirmed using Primer-BLAST of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and all sequences are provided in Tables S1, S2 and S3. For sample detection, 5 μL of DNA template was added to each of the three reaction tubes prefilled with 20 μL of MeltArray master mix (Zeesan Biotech, Xiamen, China) containing the target-specific primers and probes (Tables S1, S2 and S3). The assay was performed using a SLAN 96S real-time PCR instrument (Zeesan Biotech). The program started with decontamination at 50 °C for 2 min and denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 60 s, annealing at 35 °C for 20 min, denaturation at 95 °C for 2 min, hybridization at 45 °C for 2 min, and melting analysis from 45 °C to 95 °C (0.16 °C/s). Fluorescence intensity was measured in five detection channels (FAM, HEX, ROX, Cy5, and Quasar 705) at each step, and the serotype results of the samples were automatically provided by the onboard software (MeltPro Manager V2.0, Zeesan Biotech) according to the interpretation rules as described in the results section.

Whole-genome sequencing

WGS of the reference strains was performed using a HiSeq 2000 sequencing platform (Illumina, San Diego, CA, USA). The resulting short-read whole-genome sequences were assembled and annotated using SPAdes (3.13.0) and Prokka (1.14.6), respectively.[24,25] A DNA fragment of *cps* loci was extracted from the DNA sequence using seqtk (Zheng Li, Boston, MA, USA) and aligned with the *cps* reference sequencing database using BLAST+ (2.6.0). Sequences of *cps* loci were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/). A database of *cps* locus sequences covering 92 pneumococcal serotypes was generated (Table S4).

Four *lytA*-negative clinical isolates were sequenced using the same method as that used for the reference strains. After obtaining the assembled genome sequence, complete species identification was performed using a generic open-access sequencing analysis website (https://pathogen.watch/).

Analytical evaluation of the PneumoSero assay

The limit of detection (LOD) was estimated using 15 random pneumococcal serotypes from different fluorescent channels. Serial dilutions of templates from 5, 50, 100, 5×10^2 to 5×10^3 copies/reaction were detected 20 times at each concentration using the PneumoSero assay. The LOD was defined as the lowest concentration that yielded no more than one negative result in 20 replicates (positive rate >95 %). The reproducibility of the PneumoSero assay was determined by performing nine replicate experiments in three batches (at a concentration of 5×10^2 copies/reaction), from which the three-fold standard deviation (3SD) and coefficient of variation (CV) for each average melting temperature (T_m) value were calculated.

To determine the ability of the PneumoSero assay to detect mixed serotypes, we first tested a series of simulated samples containing two serotypes (15A and 4) that occurred in two different fluorescent channels (Cy5 and HEX). For this purpose, genomic DNA (10^3 copies/μL) of serotypes 15A and 4 was mixed at different percentages to produce DNA templates containing 0 %, 10 %, 25 %, 50 %, 75 %, 90 %, and 100 % serotype 15A. Additionally, a second series of simulated samples containing two serotypes (34 and 40) occurring in the same fluorescence channel (FAM) were tested similarly as above.

Clinical evaluation of the PneumoSero assay

Clinical evaluation was performed using two collections of clinical isolates. The first collection of 225 isolates was provided by the Respiratory Infectious Diseases Department of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. Pneumococcal isolates were identified according to the World Health Organization manual for colony morphology, optochin sensitivity, and bile solubility.[26] The second collection of 250 isolates was obtained from the Medical Culture Preservation Center of Zhongshan Hospital, Xiamen. All pneumococcal strains were identified by MALDI-TOF MS. DNA was extracted from clinical isolates. DNA extraction and concentration determination of pneumococcal strains were performed according to the procedures used for the reference strains, as described above.

Clinical evaluation of direct clinical samples was carried out using 42 bronchoalveolar lavage fluid (BALF) specimens and four cerebrospinal fluid (CSF) specimens. These samples were confirmed to contain pneumococci due to the presence of *lytA*-CDC and SP2020 detected by PCR.[27] DNA was extracted from the clinical samples using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Sanger sequencing was performed to confirm the serotyping results of the PneumoSero assay. Sanger sequencing was performed on the PCR amplicons, targeting the serotype-specific genes separated by agarose gel electrophoresis.

Statistical analysis

SPSS Statistics (version 23.0, IBM Corporation, Armonk, NY, USA) and GraphPad Prism (version 8.0.0, GraphPad, San

Diego, California, USA) were used for data analysis to calculate the average T_m value, standard deviation, serotype distribution, and coverage rates. The chi-square test or Fisher's exact test was used for comparisons between groups. Statistical significance was set at $P < 0.05$.

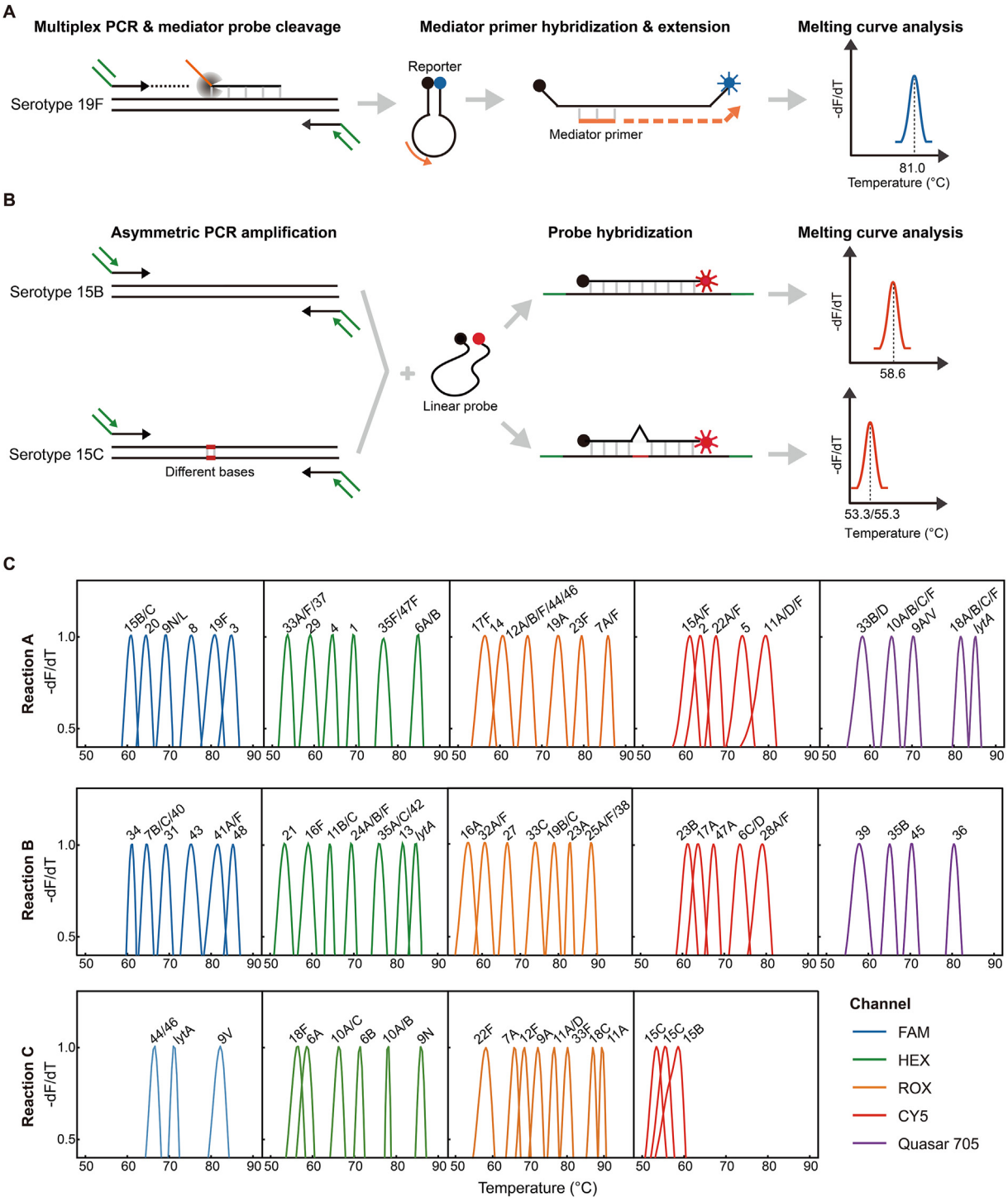

Figure 1. Identification of 92 pneumococcal serotypes using the PneumoSero assay. (A) The working principle of detecting a serotype using a mediator probe. Each serotype (e.g.,19F) is identified at a predefined the melting temperature (T_m) in a detection channel. (B) The working principle of distinguishing between two serotypes (15B and 15C) using a linear probe. Serotype 15B produces a higher temperature melting peak (58.6 °C) whereas 15C produces a lower temperature melting peak (53.3 °C or 55.3 °C). (C) T_m value and fluorescence detection channel for 92 serotypes and the *lytA* gene of *Streptococcus pneumoniae* in three reactions.

Results

Readout of pneumococcal serotypes from the PneumoSero assay

The PneumoSero assay was designed according to the working principle of MeltArray, which is achieved by coupling the 5′-flap endonuclease activity of *Taq* DNA polymerase and multiple annealing sites of the molecular beacon reporters.[23] The 5′ flap endonuclease cleaves a probe specifically into a "mediator" primer, and one molecular beacon reporter allows for the extension of multiple "mediator" primers to produce a series of fluorescent hybrids with different T_ms unique to each target. By choosing a capsule biosynthesis gene sequence corresponding to a specific serogroup or serotype, a pair of primers and a mediator probe can be designed to generate a serotype-specific T_m (Fig. 1A). Because *Taq* DNA polymerase cleaves the first 5′-terminal base in the probe-binding region, a single mutation in the target would move the cleavage site to the next base of the probe, yielding a mediator primer with a different 3′-terminal base and thus, a distinct T_m value. This feature was mainly used to differentiate VTs from NVTs, which often differ by a single nucleotide (Table 1). An exception was the differentiation between serotypes 15B and 15C, which contained eight and seven or nine TA tandem repeats in the *wciZ* gene, respectively. A dual-labeled, self-quenched probe was designed to fully match 15B, but mismatch with serotype 15C, yielding a higher T_m for the former than the latter (Fig. 1B). The finalized PneumoSero assay comprised three reactions: Reaction A, 28 targets; Reaction B, 29 targets; and Reaction C, 18 targets (Fig. 1C). Of the 92 serotypes covered, 54 could be identified at the serotype level and 38 were grouped into 17 serogroups or clusters. Notably, 24 current VTs were all included in the individually identified serotypes. As the interpretation rule, each serotype or serogroup was output by a unique combination of T_m values in three reactions (Fig. 2), and the results were valid only if the *lytA* result was positive.

Analytical evaluation

Using 92 pneumococcal reference strains and 125 non-pneumococcal strains, we evaluated the analytical specificity of the PneumoSero assay. All the 92 reference strains yielded the expected serotypes, none of the 125 non-pneumococcal strains gave valid serotyping results (Fig. 3). One *Streptococcus mitis* strain was interpreted as serotype 45, which was however invalid because of the absence of *lytA*.

The LODs of the PneumoSero assay obtained from the three reactions varied from 50 to 100 genome equivalents per reaction (Table 2). The T_m values had a maximum 3-fold standard deviation (3SD) of 0.72 °C and a relative CV of 0.30 %, and no T_m crosstalk was observed (Table 2). In samples with mixed serotypes, 10 % of the minor serotypes could be detected against a background of 90 % of the major serotypes, regardless of whether the two serotypes were present within identical or different detection channels (Fig. S1).

Clinical evaluation

Of the 475 clinical isolates, 4 invalid results were obtained. WGS confirmed that three of them were *Streptococcus infantis* and one was *Streptococcus oralis*. Of the 471 valid strains, 50 serotypes/groups were obtained, including 23 VTs and 27 NVTs (Fig. 4), fully concordant with the Sanger sequencing results. Higher serotype coverage was found in PCV vaccines than in PPV owing to their inclusion of serotype 6A, the third most frequent serotype found in this study. Despite the relatively low coverage of NVTs (18.5 %), the dominant serotypes, e.g., 15A/F, 34, 11D, and 23A, had higher frequencies than many rare VTs.

Direct pneumococcal serotyping of clinical specimens

The 46 clinical specimens yielded 45 typeable results and one untypeable result. Of the 45 typeable samples, 41 were

Table 1 Genetic differentiation of serotypes within pneumococcal serogroups.

Serogroup	Serotype	Distinguishing genetic features	References
6	6A/B, 6C/D	Gene *wciN* in 6A/B, gene *wciNβ* in 6C/D	18
	6A, 6B	A > G 583 in gene *wciP*	34
7	7F, 7A	A > T 691 in gene *wcwF*	15
9	9A/V, 9N/L	Difference in genes *wzy* and *wzx*	18
	9A, 9V	Frameshift mutation delG 722 in 9A *wcjE* gene	20
	9N, 9L	A > G 790 in gene *wcjB*	35
10	10A, 10B, 10A, 10C	Gene *WcrG* in 10A/B, gene *wcrC* in 10A/C	36,37
11	11A, 11D, 11F	Intact *gct* gene and different codon (N112S) in gene *wcrL*	38
12, 44, 46	12A/B/46, 12F/44	Different gene *wciL* of 12F/44 and 12A/B/46	15
	12F, 44	SNPs in gene *wcxF*	15
15	15B, 15C	Different in TA tandem repeat region of gene *wciZ*	39
18	18A/B/C, 18F	Gene *wcxM* in 18F	35
	18C, 18A/B	G > A 16 in gene *wzd*	15
22	22A, 22F	Gene *wcwC* in 22F	40
33, 37	33A/37, 33F	Frameshift mutation insT 433 in 33F *wcjE* gene	40

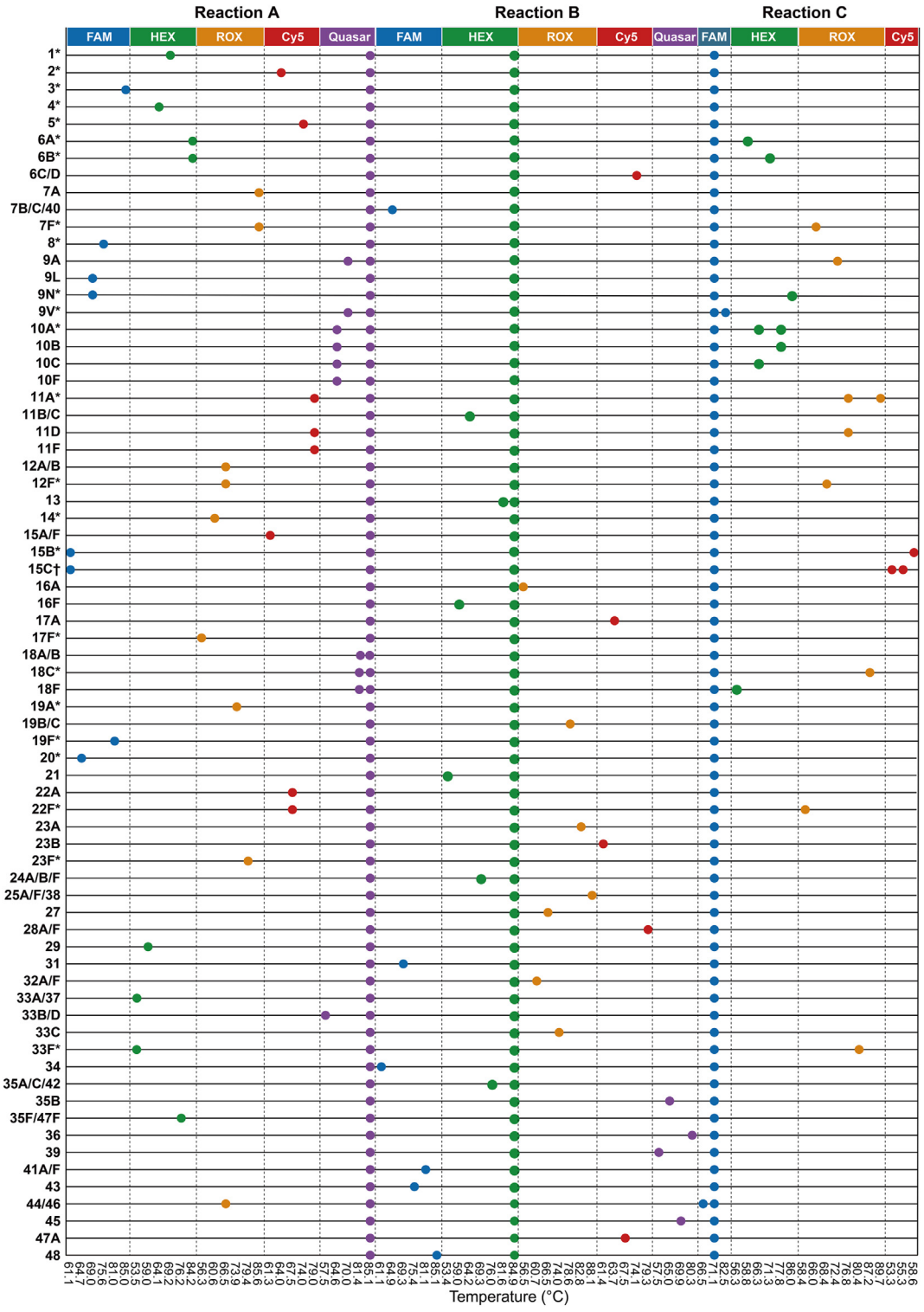

Figure 2. The profile of the melting temperatures (T_ms) of 92 serotypes in three reactions. Each line of the ordinate represents one serogroup or serotype. The colored dots correspond to the melting peak position of the serotype in all three reaction fluorescence channels. The absence of a dot indicates that there is no melting peak at the corresponding position. †The increase in one of the melting peaks with a temperature of 53.3 °C or 55.3 °C in the Cy5 channel of reaction C is interpreted as 15C. *Vaccine serotypes.

of a single serotype and four were of mixed serotypes, all of which were confirmed by Sanger sequencing with the exception of one BALF specimen. This sample contained two serotypes: 19F and 43. Serotype 43 was not detected by sequencing but was confirmed by a singleplex PCR assay. Identical to the clinical isolates, the most common VTs were 19F, 14, 23F, and 6A, and the most dominant NVT was serotype 15A/F (Table S5 and Fig. S2).

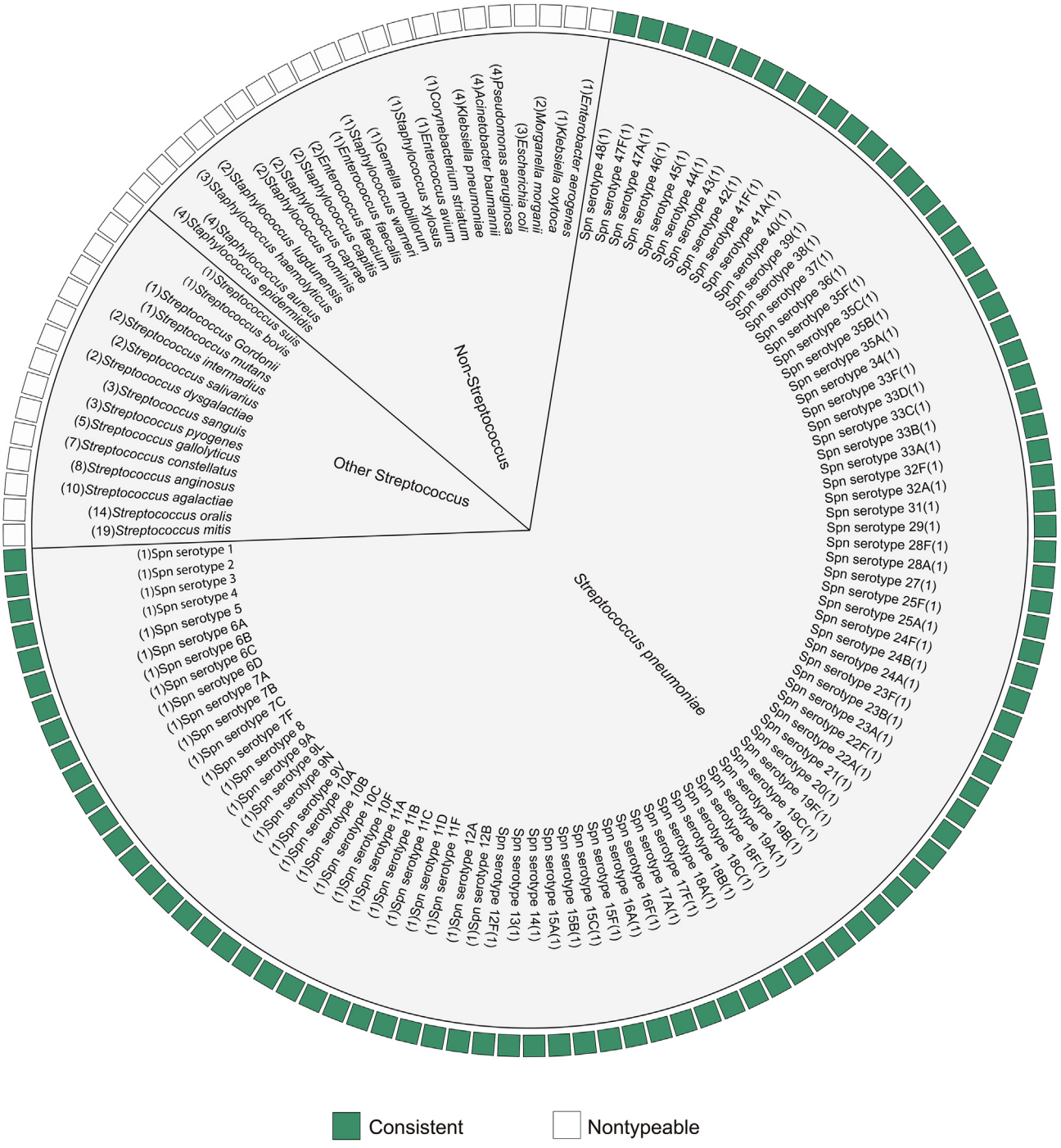

Figure 3. Assay performance on 92 pneumococcal reference strains covering 92 serotypes, 79 other *Streptococcus* strains, and 46 non-*Streptococcus* strains. The number after each species or serotype represents the number of strains. The results of the pneumococcal serotyping assay were compared to those of the Quellung reaction and whole-genome sequencing. The green square indicates that the serotyping results were consistent. The blank square indicates that the strain cannot be serotyped. Spn: *S. pneumoniae*.

Discussion

Concerns over uncovered serotypes, accompanied by the implementation of PCV, warrant close surveillance of pneumococcal serotypes in the population. A high-throughput and cost-effective molecular serotyping approach with wide coverage of serotypes, including both VTs and NVTs, is thus in high demand. However, the large number of closely related serotypes with similar sequences poses challenges to the development of molecular assays. The PneumoSero assay represents the first melting-analysis-based approach for pneumococcal serotyping that takes

Table 2 Reproducibility of melting temperatures (T_ms) and limits of detection for 17 target loci.

Serotype/group	Reaction	Channel	T_m (°C) (Mean ± 3SD)	CV (%)	Limit of detection (copies/reaction)
9N/L	A	FAM	69.22 ± 0.17	0.08	50
6A/B	A	HEX	84.34 ± 0.72	0.28	50
12A/B/F/44/46	A	ROX	66.56 ± 0.45	0.22	100
5	A	Cy5	73.88 ± 0.31	0.14	50
10A/B/C/F	A	Quasar 705	64.79 ± 0.50	0.26	100
lytA	A	Quasar 705	85.09 ± 0.31	0.12	100
7B/C/40	B	FAM	64.77 ± 0.59	0.30	100
21	B	HEX	53.51 ± 0.22	0.14	50
16A	B	ROX	56.53 ± 0.23	0.13	100
6C/D	B	Cy5	73.87 ± 0.33	0.15	50
45	B	Quasar 705	69.96 ± 0.32	0.15	100
lytA	B	HEX	84.89 ± 0.24	0.09	100
44/46	C	FAM	66.45 ± 0.27	0.13	50
6A	C	HEX	58.81 ± 0.38	0.21	50
22F	C	ROX	68.48 ± 0.20	0.10	100
15C	C	Cy5	55.38 ± 0.33	0.20	100
lytA	C	FAM	71.12 ± 0.38	0.18	100

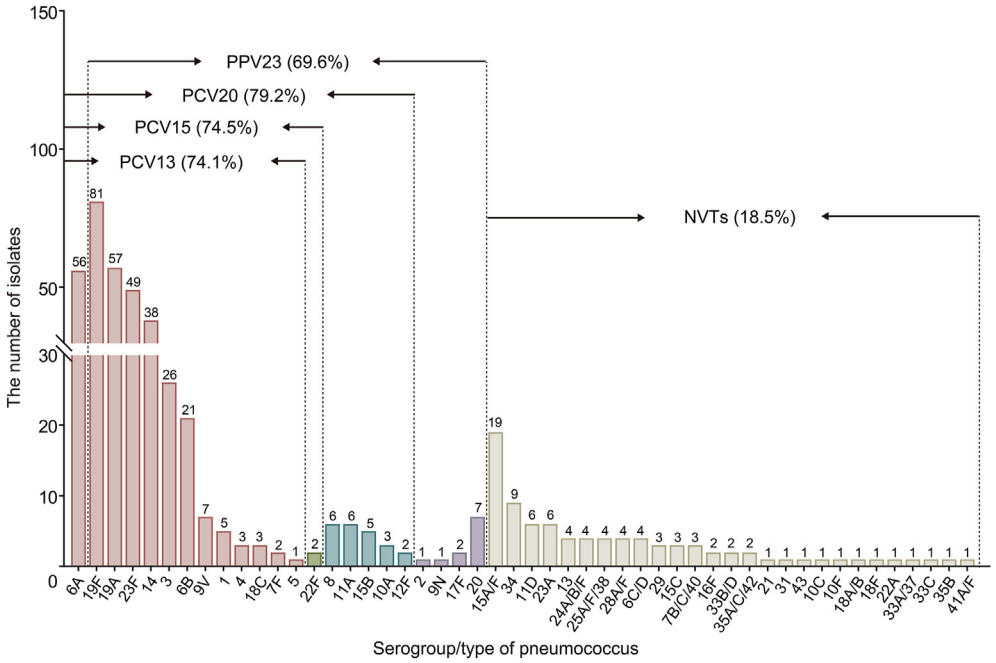

Figure 4. The serotype distribution and vaccine coverage of 471 pneumococcal isolates.

advantage of the multiplex capacity and high resolution of MeltArray. The combined specificity of the 5′-flap endonuclease and probe hybridization enables the successful detection of subtle sequence variations, which are difficult, if not impossible, for conventional approaches that solely rely on amplicon size or probe hybridization.[28,29] The established assay covered 92 serotypes, 54 of which could be identified at the serotype level, including all 24 current VTs. To the best of our knowledge, this is the largest number of individually recognized serotypes. In particular, all 24 VTs were identified for the first time at the serotype level, which has not been accomplished using existing PCR-based assays.[16–19,28,29]

Despite an in-depth understanding of capsule biosynthesis gene sequences in the determination of pneumococcal serotypes, little is known about the genetic and antigenic capsular diversity among the vast array of commensal streptococcal strains that represent multiple diverse species. As seen in our study, a *S. mitis* strain was detected as serotype 45 by the PneumoSero assay despite being negative for *lytA*. Although the inclusion of the *lytA* gene improves the specificity of this technique to exclude false positives in other *Streptococcus* spp., the detection of genes encoding polysaccharide capsular antigens, that are not actually being expressed by the bacteria may be a limitation of all *S. pneumoniae* molecular serotyping

methods.[30] In contrast, the possibility of phenotypical false positive reactions of the Quellung test due to other *Streptococcus* spp. is uncommon in practice.

The robustness of the PneumoSero assay was seen by the concordance with Sanger sequencing in the clinical evaluation. Of note, the seven most common serotypes obtained from the 471 clinical isolates, 19F, 19A, 6A, 23F, 14, 3, and 6B, were fully identical to a recent study from China.[31] Also, the four most common VTs (19F, 14, 23F, and 6A) were identical to those found in 46 clinical specimens. Serotype 15A/F was the most common NVT in both clinical isolates and specimens. Such consistencies observed in different studies across varied sample types further confirmed the accuracy of the PneumoSero assay.

Compared with Quellung reaction, which requires pure culture isolates, the direct use of clinical samples without culture is another advantage of the PneumoSero-assay. Direct serotyping of clinical samples not only removes the need for the culturing step, but may also detect culture-negative samples. Additionally, direct serotyping can detect samples containing mixed serotypes, which constitute a challenge in pneumococcus serotyping. Of the 46 clinical specimens, four were observed to contain mixed serotypes, which may, however, become a single serotype after culture and isolation; thus, they cannot truly reflect the serotype distribution. Moreover, the adaptability of the PneumoSero assay to clinical samples promotes access to clinical settings with limited resources, thereby facilitating its widespread application.

The PneumoSero assay has a higher throughput than many current systems, such as electrophoresis-based assays, which require post-PCR manipulation, and real-time PCR, which requires multiple reactions. As a closed-tube detection system, the assay can be completed in a single step following the addition of template DNA. The entire assay takes 2.5 h with less than 20 min of hands-on time when the template DNA is ready. Using this protocol, 32 samples could be processed in one batch on a standard 96-well real-time PCR thermocycler, which is widely available in standard testing laboratories following the COVID-19 pandemic. The average cost of testing per strain for the assay was approximately US$3. Thus, the PneumoSero assay can be immediately implemented as a screening tool for pneumococcal serotypes in standard microbiology laboratories without investing in additional infrastructure, operational procedures, or expertise.

There are several limitations of the PneumoSero assay. First, although the pneumococcal serotyping assay has a higher serotype resolution than other multiplex PCR assays, some genetically related serotypes are still indistinguishable. It remains necessary to identify these genetically related serotypes by combining our assay with the Quellung reaction and/or WGS. This limitation could be solved after the complete elucidation of all serotype-specific sequences. Second, as a molecular serotyping method, the plasticity of the pneumococcus such as capsular transformation or point mutations could easily result in serotype misclassification.[32] As suggested by Jauneikaite et al., the combination of phenotyping and genotyping methods would be the best method for monitoring and evaluating the impact of pneumococcal vaccines.[33]

In conclusion, serotyping tools should be synchronously updated along with the global implementation of new PCVs covering an increasing number of serotypes. In this context, the PneumoSero assay is appropriate, considering its wide serotype coverage, high resolution, ease of use, cost-effectiveness, and accessibility to real clinical samples.

Funding

This work was supported by grants from the National Key R&D Program of China (2022YFC2704904), National Key R&D Program of China (2021YFC1200200), Fujian University Industry-University-Research Joint Innovation Project (2021Y4001), Xiamen Major Science and Technology Projects (3502Z20231025).

Ethics statement

Samples were obtained with written informed consent under a study protocol approved by the Ethics Committee of Xiamen University School of Medicine.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

We thank Prof. Gang Liu and Dr. Binglin Jian of Beijing Children's Hospital for their help with this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.10.008.