Multilocus Sequence Typing of \textit{Streptococcus pneumoniae} by Use of Mass Spectrometry^{*}

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Multilocus sequence typing (MLST) is an important tool for the global surveillance of bacterial pathogens that is performed by comparing the sequences of designated housekeeping genes. We developed and tested a novel mass spectrometry-based method for MLST of \textit{Streptococcus pneumoniae}. PCR amplicons were subjected to \textit{in vitro} transcription and base-specific cleavage, followed by analysis of the resultant fragments by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Comparison of the cleavage fragment peak patterns to a reference sequence set permitted automated identification of alleles. Validation experiments using 29 isolates of \textit{S. pneumoniae} revealed that the results of MALDI-TOF MS MLST matched those obtained by traditional sequence-based MLST for 99% of alleles and that the MALDI-TOF MS method accurately identified two single-nucleotide variations. The MALDI-TOF MS method was then used for MLST analysis of 43 \textit{S. pneumoniae} isolates from Papua New Guinean children. The majority of the isolates present in this population were not clonal and contained seven new alleles and 30 previously unreported sequence types.

\textit{Streptococcus pneumoniae} is an important human pathogen that causes respiratory tract infections, bacteremia, and meningitis (17). Worldwide, it is the most common cause of pneumonia and a leading killer of children under the age of 5 years, especially in developing countries (16, 20, 26). \textit{S. pneumoniae} frequently colonizes the nasopharynx of healthy children. Although nasopharyngeal carriage is generally asymptomatic, it is a prerequisite for disease and serves as a reservoir for \textit{S. pneumoniae} in human populations. \textit{S. pneumoniae} is a diverse species, with over 90 immunologically distinct serotypes. The changing prevalences in serotype carriage following vaccination (1, 4, 18) and the emergence of antibiotic resistance (5, 13, 27) highlight the need for increased molecular epidemiology and surveillance of \textit{S. pneumoniae}. Multilocus sequence typing (MLST) characterizes bacterial isolates on the basis of the sequences of designated housekeeping genes, and in the case of \textit{S. pneumoniae}, the sequences of seven housekeeping genes are used (6, 7). Data can be easily uploaded onto a centralized online database (http://spneumoniae.mlst.net/) for comparative analysis. The \textit{S. pneumoniae} database currently contains over 5,000 sequence types.

MLST is a powerful tool for studying the genetic relatedness of clinical isolates (10), investigating the origins of newly identified serotypes, such as serotype 6D (24), and tracking the emergence and spread of multidrug-resistant isolates, such as sequence type 320 (ST320) (23). MLST is typically performed by isolating genomic DNA, amplifying the housekeeping genes by PCR, and sequencing the resultant PCR amplicons. Analysis of sequence data is often a laborious process, as sequence trace data must be manually reviewed, trimmed to the MLST core region, and uploaded to the online database for allele and ST identification. Advances in mass spectrometry (MS) technology have provided an alternative means to analyze nucleotide composition, and MS is increasingly being employed for the genetic characterization of pathogens such as hepatitis B virus (8), \textit{Staphylococcus aureus} (9), and \textit{Neisseria meningitidis} (11).

We have developed a new method for MLST of \textit{S. pneumoniae} that utilizes matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS to identify alleles. Similarly to traditional MLST, seven housekeeping genes are amplified by PCR. Next, they are subject to \textit{in vitro} transcription and base-specific RNA cleavage. Analysis of cleavage fragments by MALDI-TOF MS generates a mass signal pattern that serves as a fingerprint of the sample isolate. Spectral data are then compared to data in a reference library created by \textit{in silico} digestion of known allele types to identify the unknown sample and any possible single-base deviations. The main advantage of the MALDI-TOF MS-based approach for MLST is the speed of data acquisition and the automated analysis. Following the development and assessment of MALDI-TOF MS for MLST of \textit{S. pneumoniae}, this novel method was applied to 43 isolates carried by children in Papua New Guinea, a country where pneumococcal carriage (15) and disease (2, 14) are common but information on the population structure of bac-
TABLE 1. Primers for MALDI-TOF MS MLST of S. pneumoniae

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>aroE_T7F</td>
<td>CAGAATTACGCTCAGCTCATAATAGGGAAGAGCCTTCTTATTAAGCATCTTTTTCTCTTCCTTCA</td>
<td>CDC</td>
</tr>
<tr>
<td>aroE_Sp6R</td>
<td>CGATTAGTGTACATATAGAAGAGGCTTCTTGTTGAAGCTTTCCTACATCGGCCATCCCTGCCC</td>
<td>CDC</td>
</tr>
<tr>
<td>gdh_T7F</td>
<td>CAGAATTACGCTCAGCTCATAATAGGGAAGAGCCTTCTTATTAAGCATCTTTTTCTCTTCCTTCA</td>
<td>mlst.net</td>
</tr>
<tr>
<td>gdh_Sp6R</td>
<td>CGATTAGTGTACATATAGAAGAGGCTTCTTGTTGAAGCTTTCCTACATCGGCCATCCCTGCCC</td>
<td>mlst.net</td>
</tr>
<tr>
<td>gki_T7F</td>
<td>CAGAATTACGCTCAGCTCATAATAGGGAAGAGCCTTCTTATTAAGCATCTTTTTCTCTTCCTTCA</td>
<td>mlst.net</td>
</tr>
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</tr>
<tr>
<td>recA_T7F</td>
<td>CAGAATTACGCTCAGCTCATAATAGGGAAGAGCCTTCTTATTAAGCATCTTTTTCTCTTCCTTCA</td>
<td>CDC</td>
</tr>
<tr>
<td>recA_Sp6R</td>
<td>CGATTAGTGTACATATAGAAGAGGCTTCTTGTTGAAGCTTTCCTACATCGGCCATCCCTGCCC</td>
<td>CDC</td>
</tr>
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*The underlined portion of the primer indicates the tagged T7 or Sp6 promoter region.*

Bacterial isolates. Fifteen S. pneumoniae isolates from the following sequence types (serotypes are in parentheses) previously typed by sequence-based MLST were provided by the Pneumococcal Molecular Epidemiology Network (PMEN; clones 11 to 26; http://www.sph.emory.edu/PMEN/pen_table2.html): ST175 (19A), ST41 (19A), ST256 (19F), ST240 (23F), ST173 (23F), ST384 (6B), ST67 (serotype 14), ST289 (serotype 5), ST315 (6B), ST177 (19F), ST242 (23F), ST63 (6A), ST377 (35B), ST63 (15A), and ST388 (23F). We also examined four serogroup 6 isolates, ST176 (6B), ST41 (19F), ST240 (6C), and ST473 (6D), from Fijian children that had previously been typed by sequence-based MLST (24). Forty-three S. pneumoniae isolates were obtained from nasopharyngeal swabs taken from Papua New Guinean children aged 6 to 24 months. Children were recruited from the well baby immunization clinic conducted at Lopi Urban Clinic in Goroka, Eastern Highlands Province, Papua New Guinea, during February and March of 2009. Swabs were collected by study nurses and immediately placed in 1 ml of skim milk glucose broth. Samples were transported to the Papua New Guinea Institute of Medical Research, stored at −80°C, and sent to Melbourne, Australia, where analysis was conducted. S. pneumoniae was isolated from swabs, identified by standard methods (19), and serotyped by Quellung reaction, and pure isolates were stored in STGG medium (21) at −80°C until use.

PCR. Genomic DNA was extracted from fresh bacterial cultures using a DNeasy Blood and Tissue kit (Qiagen, Doncaster, Australia). Primer pairs used to amplify aroE, gdh, gki, recA, spi, gki, and ddl (Table 1) were based on those described by the Centers for Disease Control and Prevention (CDC; http://www.cdc.gov/ncidod/biotech/strep/alt-MLST-primers.htm) or Enright and Spratt (7) and listed on the MLST website (http://snpneumoniae.mlst.net/). The primers were tagged with a promoter sequence for T7 or Sp6 RNA polymerase for forward and reverse transcription, respectively (underlined in Table 1). PCRs were performed using approximately 5 ng of genomic DNA, 0.125 U of HotStarTaq DNA polymerase, 1× PCR buffer (Qiagen), 2.0 mM MgCl2, 0.2 μM forward and reverse primers (Sigma-Aldrich, Sydney, Australia), and 200 μM each dNTPs (Qiagen). PCR cycling conditions were a 15-min activation at 95°C, followed by 35 cycles of amplification at 94°C for 30 s, 62°C for 45 s, and 72°C for 60 s and a final extension at 72°C for 10 min. For amplifications of gki, genomic DNA and primer concentrations were increased to 7.5 ng and 2.5 mM, respectively, and the annealing temperature was lowered to 56°C.

MLST by sequencing. Amplicons were sequenced in both directions using capillary separation on an ABI 3730xl DNA analyzer with ABI BigDye Terminator labeling (version 3.1; Australian Genome Research Facility) using primers with only the T7 and Sp6 tag sequences. Contiguous sequences were formed and edited using the Sequencher program (version 4.9; Gene Codes Corporation, Ann Arbor, MI). Core sequences were uploaded to the S. pneumoniae MLST database for allele identification.

iSEQ library creation and simulation experiments. Reference sequence sets were created by downloading allelic data from the S. pneumoniae MLST database and modifying the sequences by adding forward and reverse primers and a stretch of consensus sequence compiled from available GenBank entries (http://www.ncbi.nlm.nih.gov/GenBank/) to fill the gap between primer sequences and the core MLST region. The iSEQ simulation tool (version 1.0) was used to assess the discriminatory power of each tab-delimited text file-formatted reference sequence set by in silico base cleavage and peak analysis (8). In short, the iSEQ simulation tool software performs in silico base-specific cleavages at each C or T of the forward or reverse strands covering all four bases and generating up to four fragment mixtures of a single DNA strand. The mass of individual RNA fragments in each mixture is calculated, and a mass spectrometric peak pattern is simulated. Using an iterative clustering process, individual sequences (in a reference sequence set) are analyzed, and discriminating features such as missing peaks, additional peaks, and peak intensity changes are summarized in a discriminatory power digit (11). On the basis of the sum of discriminatory features, each reference sequence can be grouped into different discriminatory power groups: very weak power (0 to 2 sum of features), weak power (2 to 4 sum of features), medium power (4 to 8 sum of features), and strong power (>8 sum of features).

MLST by mass spectrometry. Following PCR amplification, samples were prepared for MALDI-TOF MS analysis using MassCLEAVE T7/Sp6 kit (Sequenom, USA) reagents and the standard MassCLEAVE protocol (Sequenom, San Diego, CA) as previously described (11). Briefly, PCR amplicons containing T7 and Sp6 promoter sequences were digested with shrimp alkaline phosphatase, transferred into 4 individual wells of a 384-well plate, and subjected to in vitro transcription and base-specific cleavage reactions. In vitro transcription was performed with T7 or Sp6 RNA polymerase and dCMP or dTMP by using the regular nucleoside enabled RNase A to cleave target sequences after U and C residues, respectively (25). The resulting RNA fragments, corresponding to T-forward, T-reverse, C-forward, and C-reverse cleavage patterns, were desalted using an ion-exchange resin, and approximately 10 nl of each reaction mixture was spotted on a 384-sample pad (Spectrochip; Sequenom). Fragments were analyzed on a MALDI linear time of flight mass spectrometer (Sequenom) by using an ion-exchange resin, and approximately 10 nl of each reaction mixture was spotted on a 384-sample pad (Spectrochip; Sequenom). Fragments were analyzed on a MALDI linear time of flight mass spectrometer (Sequenom) by using the RT-Workstation (version 3.4) software package (Sequenom). Data analysis was performed by using iSEQ software (version 1.0; Sequenom), which compares experimental mass fingerprints acquired by MALDI-TOF MS to the in silico mass fingerprints of the reference library sequences to identify the closest-matching allele number and any single-nucleotide variations such as substitutions, insertions, and deletions. Spectral data from the top two matches were reviewed by the operator to confirm correct identification of the closest match. Allele numbers were uploaded to the MLST online database for sequence type identification.
RESULTS

Simulation experiments. The S. pneumoniae MLST online database contains an average of 295 alleles for each of the seven housekeeping genes, arOE, gdh, gki, recP, spi, xpt, and ddl. To determine whether MALDI-TOF analysis of the four cleavage reactions would allow accurate discrimination of allele types, available allele sequences for each gene were downloaded from the MLST database and used for in silico simulation experiments. Figure 1 shows the discriminatory power of individual and combined cleavage reactions for 400 alleles of the ddl gene. When all four cleavage reactions are used, 98.5% of alleles were discriminated with strong power and 1.5% were discriminated with medium power. Simulation experiments for the remaining six MLST genes yielded similar results.

Assessment of MALDI-TOF MS MLST. To assess the suitability of using a mass spectrometry platform for performing MLST of S. pneumoniae, we screened 19 isolates previously characterized by sequencing MLST, considered the “gold standard” method. Of the 15 PMEN isolates examined, MALDI-TOF MS MLST gave the expected results for 101 of 105 alleles. The PCR amplicons of the four discrepant ddl alleles were analyzed by traditional sequencing, and results confirmed the allelic identification obtained by using MALDI-TOF MS, suggesting that the ddl alleles for these four isolates had been mislabeled and they had been correctly identified by MALDI-TOF MS MLST. Distinguishing xpt-6 from xpt-147 required manual interpretation of the spectral data, as the software ranked xpt-147 as the top match but the spectral data matched peak patterns expected for xpt-6, which was ranked as the second-closest match. Sequencing verified the correct identification to be xpt-6. Analysis of four serogroup 6 isolates previously typed by sequence-based MLST gave correct results for three of the four isolates. For the fourth isolate (serotype 6C), two pairs of alleles with extremely similar spectra, alleles gdh-10 and gdh-25 and alleles ddl-369 and ddl-8, were incorrectly ranked by iSEQ software, which ranked the correct allele as the second-best match instead of the best match. Overall, MALDI-TOF MS MLST results matched sequence-based data for 131 of 133 alleles (98.5% concordance). Since these 19 reference isolates represented a limited subset of serotypes and STs, we proceeded to analyze field samples collected from a population with high pneumococcal carriage rates.

MLST analysis of S. pneumoniae carriage isolates from Papua New Guinea. Initially, 10 unknown carriage isolates obtained from Papua New Guinean children were typed by both traditional and MALDI-TOF MS MLST. For all 10, allelic identification by the iSEQ software matched sequencing results. Two new alleles identified by iSEQ were confirmed by traditional sequence analysis: isolate 6563-03-067a had a single-nucleotide variant of spi-187 and a single-nucleotide variant of gdh-3. In total, 29 isolates were typed by both MALDI-TOF MS and sequencing MLST, and iSEQ results matched sequencing data for 201/203 alleles, a 99.0% concordance rate. Following this method of validation, the remaining unknown isolates were typed solely by MALDI-TOF MS, and any new alleles were confirmed by traditional sequencing.

We examined a total of 43 isolates obtained from 29 swabs by serotyping and MALDI-TOF MS MLST (Table 2). Thirty-nine of these isolates belonged to 23 serotypes, and 4 were nontypeable. The majority of the Papua New Guinean isolates were novel sequence types not previously listed in the MLST database. Only serotypes 6C and 23F displayed clonal alleles. Of the four 6C isolates, one was ST4240, previously identified only in Australia and Fiji (24, 29), and the other three were ST6747, the same as ST4240 except for gdh-8 replacing gdh-25. Three of the four 23F isolates were ST820, found worldwide and associated with antibiotic resistance (3, 22, 28), and the fourth was ST6905, containing a single-nucleotide variation of spi-72. Other serotypes carried by multiple children, such as serotype 14, were more genetically diverse. In one instance, isolates with the same sequence type displayed different serotypes (8 and 9L), evidence of capsular switching. Strain 3108-03-002, a nontypeable isolate, had an identical MLST profile as a serotype 6B isolate (3108-03-116a) obtained from the same swab, suggesting that the nontypeable isolate was a variant of the 6B isolate that produced little or no capsule. For 14 alleles (indicated in Table 2), iSEQ software identified a single-nucleotide change, which was later confirmed by sequencing. Overall, 30 novel sequence types and seven new alleles were identified, and strain information and allele sequences were submitted for entry into the MLST database.

DISCUSSION

MLST by MALDI-TOF MS (MassARRAY iSEQ comparative sequencing) analysis is a novel method for genetic typing of S. pneumoniae that relies on the generation of sequence-specific mass spectra for allele identification. In vitro transcription of PCR products, followed by four base-specific cleavage reactions, generates a distinct set of RNA fragments that can be discerned from closely related alleles. We found a 99.0% concordance rate between this new method and traditional sequencing MLST. Similar to DNA sequencing machinery, a mass spectrometer for MALDI-TOF MS would be best suited for a core facility that serves multiple research groups, whereas sample preparation does not require specialized laboratory
The running cost per isolate is similar for the two methods (approximately US$125 for MALDI-TOF MS versus US$130 for sequencing). Although the MALDI-TOF MS method requires additional sample processing, the speed of data analysis renders the MS-based platform more cost-effective than traditional MLST.

The iSEQ software automatically ranks the closest-matching allele from the in silico database, but in cases of closely matched sequence types, such as xpt-6 and xpt-147, the software prompts the user to manually inspect the spectral data by highlighting closely scoring reference sequences with an asterisk. In this study, manual inspection was recommended for approximately 40% of total alleles. Spectral data are presented in a user-friendly manner, with expected and actual peaks labeled and linked to the corresponding cleavage fragments, and spectra can be transformed into simulated banding patterns for researchers more accustomed to viewing gels. Interpretation of raw spectral data does not require expertise in mass spectrometry and typically takes less than a minute to perform.

Importantly, iSEQ was able to accurately identify single-nucleotide variations within alleles. An electrospray ionization MS method for MLST of S. pneumoniae that relies on primer pairs internal to the core MLST loci (12) would not be suitable for consistent detection of new alleles in a base- and sequence-specific way. Potentially novel single-nucleotide variations should be confirmed by sequencing, as sequencing reaction chromatograms are required for the submission of a new allele to the MLST online database. The iSEQ reference library should be periodically updated to include new additions to the MLST database.

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Our study determined that the MALDI-TOF MS-based
method for MLST of \textit{S. pneumoniae} was both rapid and accurate. The finding that the majority of Papua New Guinean isolates displayed novel sequence types suggests that strains from this geographic region, and presumably other developing countries, are underrepresented in the MLST database. The MLST data presented here provide a population snapshot of \textit{S. pneumoniae} from this geographic region, and presumably other developing regions, and as such are important information for planning vaccination strategies and monitoring serotype replacement following pneumococcal vaccination, as well as identifying the emergence and spread of antibiotic-resistant clones.

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R.J.M. is an employee of Sequenom, Inc.

REFERENCES

ERRATUM

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Volume 49, no. 11, p. 3756–3760, 2011. Page 3757, column 1, last sentence of “PCR” paragraph of Materials and Methods: “genomic DNA and primer concentrations were increased to 7.5 ng and 2.5 mM, respectively” should read “genomic DNA, MgCl₂, and primer concentrations were increased to 7.5 ng, 2.5 mM, and 0.27 μM, respectively.”