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Multilocus Sequence Typing of *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 *Streptococcus pneumoniae*. PCR amplicons were subjected to *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 *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 *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.

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). *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 *S. pneumoniae* in human populations. *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 *S. pneumoniae*. Multilocus sequence typing (MLST) characterizes bacterial isolates on the basis of the sequences of designated housekeeping genes, and in the case of *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 *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), *Staphylococcus aureus* (9), and *Neisseria meningitidis* (11).

We have developed a new method for MLST of *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 *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 *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 *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-

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TABLE 1. Primers for MALDI-TOF MS MLST of *S. pneumoniae*

Primer	Sequence (5'-3') ^a	Source
aroE_T7F	<u>CAGTAATACGACTCACTATAGGGAGAAGGCTT</u> CCTATTAAGCATTCTATTTCTCCCTTC	CDC
aroE_SP6R	<u>CGATTTAGGTGACACTATAGAAGAGAGGCTACAGGAGAGGATTGGCCATCCATGCC</u> ACACTG	CDC
gdh_T7F	<u>CAGTAATACGACTCACTATAGGGAGAAGGCTATGGACAAACCAGCNAGYTT</u>	mlst.net
gdh_SP6R	<u>CGATTTAGGTGACACTATAGAAGAGAGGCTGCTTGAGGTCCCATRCTNCC</u>	mlst.net
gki_T7F	<u>CAGTAATACGACTCACTATAGGGAGAAGGCTGGCATTGGAATGGGATCACC</u>	mlst.net
gki_SP6R	<u>CGATTTAGGTGACACTATAGAAGAGAGGCTTCTCCCGCAGCTGACAC</u>	mlst.net
recA_T7F	<u>CAGTAATACGACTCACTATAGGGAGAAGGCTGAATGTGTGATTCAATAATCACCTCA</u> AATAGAAGG	CDC
recA_SP6R	<u>CGATTTAGGTGACACTATAGAAGAGAGGCTTGCTGTTTCGATAGCAGCATGGATGGC</u> TTCC	CDC
spi_T7F	<u>CAGTAATACGACTCACTATAGGGAGAAGGCTCGCTTAGAAAGGTAAGTTATGAATTT</u>	CDC
spi_SP6R	<u>CGATTTAGGTGACACTATAGAAGAGAGGCTGTGATTGGCCAGAAGCGGAA</u>	mlst.net
xpt_T7F	<u>CAGTAATACGACTCACTATAGGGAGAAGGCTTTAACTTTTAGACTTTAGGAGGTCT</u> TATG	CDC
xpt_SP6R	<u>CGATTTAGGTGACACTATAGAAGAGAGGCTCGGCTGCTTGCAGTGTTTTTCTTGAG</u>	CDC
ddl_T7F	<u>CAGTAATACGACTCACTATAGGGAGAAGGCTTAAAATCACGACTAAGCGTGTCTGG</u>	CDC
ddl_SP6R	<u>CGATTTAGGTGACACTATAGAAGAGAGGCTAAGTAGTGGGTACATAGACCACTGGG</u>	CDC

^a The underlined portion of the primer indicates the tagged T7 or SP6 promoter region.

terial strains is lacking. This is the first study to report MLST data for *S. pneumoniae* from Papua New Guinea.

MATERIALS AND METHODS

Bacterial isolates. Fifteen *S. pneumoniae* isolates of 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/pmen_table2.html): ST175 (19A), ST41 (19A), ST236 (19F), ST242 (23F), ST173 (23F), ST384 (6B), ST67 (serotype 14), ST289 (serotype 5), ST315 (6B), ST177 (19F), ST273 (6B), ST376 (6A), ST377 (35B), ST63 (15A), and ST388 (23F). We also examined four serogroup 6 isolates, ST176 (6B), ST4240 (6C), ST639 (6D), 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 glycerol 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 the 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*, *recP*, *spi*, *xpt*, 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://spneumoniae.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 Hot-StarTaq 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 deoxynucleoside triphosphate (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 *spi*, 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, Australia) 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 four *in vitro* transcription and base-specific cleavage reactions. *In vitro* transcription with T7 or SP6 RNA polymerase and dCMP or dTMP/dUMP replacing 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 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.

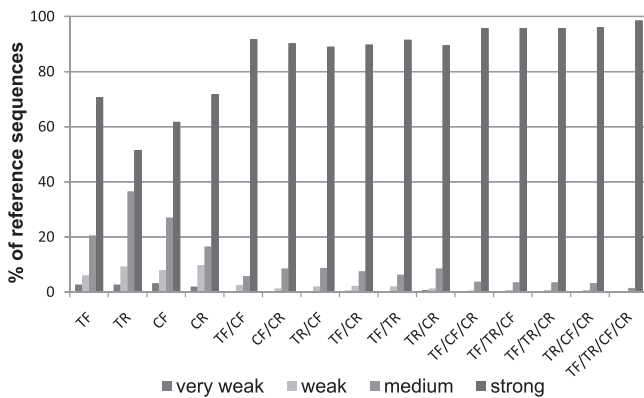


FIG. 1. Simulation results of 400 *ddl* alleles downloaded from the online MLST database. The *x* axis displays the combination of cleavage reactions (T forward [TF], T reverse [TR], C forward [CF], and C reverse [CR]), and the *y* axis displays the percentage of alleles discriminated with very weak, weak, medium, or strong power. For example, if all four cleavage reactions are used, 98.5% of alleles are discriminated with strong power, whereas if the only the T-forward reaction is used, 70.75% of alleles are discriminated with strong power.

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 attributes. 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

TABLE 2. Serotypes and MLST types of *S. pneumoniae* isolates recovered from Papua New Guinean children

Sample	Serotype	Allele no.							MLST sequence type
		<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>	
5133-12-001	6A	2	8	6	16	15	6	5	6746 ^a
2254-05-001	6A	2	8	4	131	6	1	11	4274
3108-03-116a	6B	8	5	244	1	6	277	59	6942 ^a
5877-11-006	6C	93	25	4	15	17	20	8	4240
7704-10-001	6C	93	8	4	15	17	20	8	6747 ^a
7856-08-001	6C	93	8	4	15	17	20	8	6747 ^a
8448-08-081	6C	93	8	4	15	17	20	8	6747 ^a
1522-14-011	8	15	15	8	16	6	20	17	6748 ^a
5877-11-001	8	12	10	6	16	6	6	11	6749 ^a
5158-02-004	9L	12	10	6	16	6	6	11	6749 ^a
8308-08-001	10A	7	25	29	4	48	20	28	6750 ^a
6563-03-067a	13	7	320 ^a	9	16	285 ^a	1	18	6903 ^a
2099-05-001	14	1	164	4	5	6	1	18	6925 ^a
3811-06-001	14	1	2	133	5	6	1	28	6751 ^a
3274-01-027	14	1	2	4	5	6	1	176	6752 ^a
5033-02-001	15A	7	13	4	16	15	1	17	3058
9916-13-004	15B	7	119	312 ^a	4	6	103	14	6926 ^a
8448-08-007	15C	7	119	312 ^a	4	6	103	11	6927 ^a
1522-14-002	16F	8	8	312 ^a	8	6	103	245	6928 ^a
1621-08-002	16F	1	226	4	5	9	224	216	6753 ^a
4197-09-001	18F	5	31	8	1	9	11	31	291
3274-01-001	19A	1	10	9	16	6	1	11	6913 ^a
7576-16-001	19A	215 ^a	12	19	8	6	38	120	6929 ^a
6563-03-016	19A	215 ^a	12	19	8	6	38	120	6929 ^a
3274-01-002a	19A	215 ^a	12	19	8	6	38	469 ^a	6920 ^a
8203-01-008	19F	7	8	312 ^a	131	5	7	11	6904 ^a
907-10-001	19F	7	119	19	16	14	14	28	6930 ^a
546-11-001	21	7	2	4	15	6	1	18	6754 ^a
3477-06-003	23A	13	9	9	16	6	20	11	6755 ^a
3477-06-001	23A	1	2	4	5	6	1	31	6756 ^a
1489-07-001	23F	10	13	53	1	72	38	31	802
872-11-001	23F	10	13	53	1	286 ^a	38	31	6905 ^a
6563-03-001	23F	10	13	53	1	72	38	31	802
2371-03-001	23F	10	13	53	1	72	38	31	802
658-13-001	33	7	60	19	1	6	14	70	6757 ^a
4990-01-002	34	45	13	6	12	9	14	11	6758 ^a
872-11-007	35B	216 ^a	10	312 ^a	131	6	7	11	6921 ^a
3477-06-038	35F	1	5	1	50	6	1	18	6931 ^a
1621-08-001	45	8	119	312 ^a	66	14	103	28	6932 ^a
3108-03-002	NT ^b	8	5	244	1	6	277	59	6924 ^a
6084-12-001	NT	7	25	4	4	48	20	28	6759 ^a
5033-02-015	NT	93	25	4	15	17	20	8	4240
7704-10-004	NT	1	13	4	5	9	17	7	6760 ^a

^a Newly identified by this study.
^b NT, nontypeable.

equipment. 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 pat-

terns 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.

Our study determined that the MALDI-TOF MS-based

method for MLST of *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 the *S. pneumoniae* sequence types carried by children in Papua New Guinea prior to widespread pneumococcal vaccination, 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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ERRATUM

Multilocus Sequence Typing of *Streptococcus pneumoniae* by Use of Mass Spectrometry

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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.”