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Multilocus Sequence Typing of *Staphylococcus aureus* with DNA Array Technology

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A newly developed oligonucleotide array suited for multilocus sequence typing (MLST) of *Staphylococcus aureus* strains was analyzed with two strain collections in a two-center study. MLST allele identification for the first strain collection fully agreed with conventional strain typing. Analysis of strains from the second collection revealed that chip-defined MLST was concordant with conventional MLST. Array-mediated MLST data were reproducible, exchangeable, and epidemiologically concordant.

Multilocus sequence typing (MLST) was introduced as a microbial typing method that generates a portable, binary output (14). The sequences of internal fragments of several housekeeping genes are determined for each isolate, thereby defining specific alleles for each locus. The method is highly discriminatory, and allelic profiles are sufficiently stable. Complete genomes of both eukaryotic and prokaryotic pathogens and hosts have been sequenced. Based on these data, high-density oligonucleotide arrays have been developed in parallel. Applications for array technology include resequencing of clinically relevant genes, monitoring quantitative changes in gene expression (5), unraveling the organization and control of genetic pathways and genetic locus-specific typing (9). The introduction of high-throughput systems, such as GeneChip technology (Affymetrix, Santa Clara, Calif.), is a promising methodology for assessing genetic diversity (11). Affymetrix systems have been introduced in microbiological research before (16). The method is based on hybridization of target nucleic acid to large numbers of oligonucleotides synthesized in situ on a small glass substrate (8). In the present research project, two centers determined the feasibility of a *Staphylococcus* GeneChip for MLST of *Staphylococcus aureus* (7).

Bacterial strains. The 50 *S. aureus* strains used for the present study originated from two separate strain collections. The first stock culture collection consisted of 20 methicillin-resistant *S. aureus* (MRSA) strains that had been well characterized by multiple pheno- and genotyping systems (17, 18) (see Table 2). The second stock culture collection comprised 30 *S. aureus* strains (20% MRSA), selected from the MLST database collection (http://www.mlst.net), with known multilocus sequence types (see Table 3). This strain collection was sent to both research centers, which were blinded to the strain identities.

Probe array design and tiling strategy. The *Staphylococcus* DNA array identifies sequence variation in seven MLST targets by using the 4L tiling strategy described previously (16). Briefly, for every base interrogated within the reference sequence, four probes of equal length are synthesized on the chip. Those four probes are identical except at the interrogation position (centrally located within the probe). Each base is determined by comparing the signal intensities of the labeled target for the four probes. A comprehensive database of allele reference sequences of the seven different housekeeping genes (http://www.mlst.net) was utilized to design the array. Table 1 summarizes the number of alleles for each gene at the time this chip was designed. Probe redundancy was eliminated by synthesizing probes shared by two or more allele reference sequences only once on the array.

Target preparation. For DNA isolation, bacteria were grown overnight at 37°C. Three to five individual colonies were suspended in TEG (25 mM Tris, 10 mM EDTA, 50 mM glucose) buffer containing lysostaphin and incubated at 37°C for 1 h. Staphylococcal DNA was extracted with a QIAamp DNA minikit (Qiagen, Westburg, Leusden, The Netherlands) according to the manufacturer's protocol. DNA was stored at −20°C until use. DNA was added to the PCR mixture. The specific primers targeting the seven housekeeping genes were defined by Enright et al. (7) with exception of the of the *arcC* forward primer (4). Multiplex PCR was performed in a GeneAmp PCR system 9700 (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Two different protocols were used for labeling of the PCR products (1). A conventional labeling strategy, based on transcription with incorporation of fluorescein-dUTP, was used for strain collection 1. In vitro-transcribed RNA was chemically fragmented (2). A newly developed DNA chemical labeling technique (13) (bioMérieux, Marcy l'Etoile, France) was used for strain collections 1 and 2. The labeling reaction mixture contained PCR product and labeling reagent (meta-biotin phenylmethyl diazomethyl). DNA was chemically fragmented and subsequently purified (QIAquick nucleotide removal kit; Qiagen).

Probe array hybridization and analysis. Hybridization of the probe arrays was performed with a GeneChip fluidics station (Affymetrix, St. Clara, Calif.). The fluorescein-labeled RNA fragments were diluted in hybridization buffer, incubated, and washed. The chemically labeled, fragmented DNA was dena-

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TABLE 1. GeneChip MLST database for the seven housekeeping genes

Gene (product)	Sequence length (bp)	No. of alleles considered for GeneChip design
arcC (carbamate kinase)	456	20
aroE (shikimate dehydrogenase)	456	33
glpF (glycerol kinase)	465	18
gmk (guanylate kinase)	429	18
pta (phosphate acetyl transferase)	474	19
tpi (triosephosphate isomerase)	402	25
yqiL (acetylcoenzyme A acetyltransferase)	516	29

tured, hybridized with the probe array, and washed, and the hybrids were stained with streptavidin–R-phycoerythrin (Dako, Trappes Cedex, France). The probe array was washed again. Fluorescent signal emitted by the hybrids was detected at 530 nm (fluorescein) or 570 nm (phycoerythrin) by using a GeneArray scanner (Agilent, Palo Alto, Calif.). Probe array fluorescence intensities, base call scores, sequence determinations, and reports were generated by functions available on the GeneChip software (Affymetrix). The percentage base-right score was determined by the percentage homology between the experimentally derived sequence and the distinct reference sequence tiled on the array.

Table 2 summarizes typing results from both centers obtained for the first *S. aureus* collection. Labeling of the DNA samples was achieved by transcription with incorporation of fluorescein-dUTP (Table 2, method A). Overall, a relatively low base call score (range, 54.6 to 99.6%; average, 86.7%; data not shown) was observed in both centers, resulting in discrepant allele identification for the strains in both centers. MLST probing of strains W1 to W5, defined as identical strains, resulted in identical sequence types. The closely related strains W6 to W10 were classified as identical with MLST probing. The sequence types of epidemiologically unique strains W11 to W20 were diverse except for strains W13 and W16. A new direct labeling protocol (method B) was used in one center for retyping the first strain collection with the MLST probing approach, and no discrepant results were observed between centers. Moreover, the query sequences were highly correct, as reflected by the high base-right scores (average score, 98.7%; range, 83.5 to 100%).

The same chemical labeling protocol was applied for the second strain collection in both centers. The results of MLST probing and conventional sequence typing are outlined in Table 3. The vast majority of the query sequences matched perfectly with the allele type of the reference sequence from the GeneChip database, as shown by a high base-right score in both centers (average scores in centers 1 and 2, 99.2 and 99.6%, respectively; data not shown). In center 1, only one discordant result (strain 19) was observed. The reason was that the C residue normally present at position 249 of the *glpF* allele 6 fragment was misinterpreted as a G residue in the derived sequence. This led to a shift from *glpF* allele 6 to 16. Since *tpi* allele type 49, as obtained for strain 21 by conventional MLST, was not present in the GeneChip database, both centers misclassified this gene fragment (*tpi* allele type 3). The difference between the alleles is a replacement of a C with a G residue, that refers to alleles 3 and 49, respectively, at nucleotide position 158 of the *tpi* gene fragment (MLST website [http://www.mlst.net]). The probing results showed a G residue on

TABLE 2. Comparison of MLST GeneChip results obtained from two centers with conventional pheno- and genotyping data for *S. aureus* strain collection 1[a]

Strain code	Result of conventional technique					MLST StaphChip result					
						Center 1				Center 2 (labeling method A)	
						Labeling method A		Labeling method B			
	PFGE	TAR916/ SHIDA type	RAPD	Phage type	Binary type	Allelic profile	ST	Allelic profile	ST	Allelic profile	ST
W1	A	A	AAA	77	001010011111	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247
W2	A	A	AAA	77	001010011111	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247
W3	A	A	AAA	77	001010011111	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247
W4	A	A	AAA	77	001010011111	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247
W5	A	A	AAA	77	001010011111	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247
W6	B	B	BBB	NT	000110011111	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45
W7	B1	B	BBB	95	000010001111	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45
W8	B2	B	BBB	80	000010001111	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45
W9	B3	B	BBB	80	000010011111	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45
W10	B4	B	BBB	NT	000110011111	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45	10-14-8-6-10-3-2	45
W11	C	A	CAA	75, 77, 84, 84a, 994	001111011111	3-32-**6**-1-4-4-3	**Unknown**	3-32-1-1-4-4-3	81	3-32-1-1-4-4-3	81
W12	D	C	DCC	6, 81	011111011111	1-4-1-4-12-1-10	5	1-4-1-4-12-1-10	5	1-4-1-4-12-1-10	5
W13	E	D	EAD	6, 47, 77, 83a, 85, 994	001110011111	2-3-1-1-4-4-3	239	2-3-1-1-4-4-3	239	2-3-1-1-4-4-3	239
W14	F	E	FDE	85	000010011111	1-1-1-1-1-1-1	1	1-1-1-1-1-1-1	1	1-1-1-1-1-1-1	1
W15	G	F	E′CF	85	001011011111	1-4-1-4-12-1-29	**Unknown**	1-4-1-4-12-24-29	228	1-4-1-4-12-24-29	228
W16	H	G	GAD	6, 47, 66	011110011111	2-3-1-1-4-4-3	239	2-3-1-1-4-4-3	239	2-3-1-1-4-4-3	239
W17	I	H	HAE	75, 77	101110011111	3-3-1-1-4-4-16	**250**	3-3-1-12-4-4-16	247	3-3-1-12-4-4-16	247
W18	J	A	HAA	47, 54, 75, 77, 84, 85, 812	000110011111	3-1-1-1-1-5-3	97	3-1-1-1-1-5-3	97	3-1-1-1-1-5-3	97
W19	K	I	IBG	29, 52, 77	000110011111	2-2-2-2-6-3-2	30	2-2-2-2-6-3-2	30	2-2-2-2-6-3-2	30
W20	L	J	FAI	29	110110011111	1-4-1-4-12-24-29	228	1-4-1-4-12-24-29	228	1-4-1-4-12-24-29	228

[a] Abbreviations: PFGE, pulsed-field gel electrophoresis; TAR916 SHIDA, transposon 916, Shine Dalgamo; RAPD, randomly amplified polymorphic DNA analysis (ERIC2, AP1, and AP7 primers); ST, sequence type; NT, not typeable. Bold indicates a major discrepant result.

TABLE 3. GeneChip results obtained from *S. aureus* strain collection 2 in two centers, compared with "classic" MLST data (7)[a]

Strain code	MLST		StaphChip allelic profile[a]	
	ST	Allelic profile	Center 1	Center 2
1	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
2	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
3	59	19-23-15-2-19-20-15	19-23-15-2-19-20-15	19-23-15-2-19-20-15
4	36	2-2-2-2-3-3-2	2-2-2-2-3-3-2	2-2-2-2-3-3-2
5	15	13-13-1-1-12-11-13	13-13-1-1-12-11-13	13-13-1-1-12-11-13
6	22	7-6-1-5-8-8-6	7-6-1-5-8-8-6	7-6-1-5-8-8-6
7	97	3-1-1-1-1-5-3	3-1-1-1-1-5-3	3-1-1-1-1-5-3
8	1	1-1-1-1-1-1-1	1-1-1-1-1-1-1	1-1-1-1-1-1-1
9	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
10	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
11	40	2-2-2-2-6-2-2	2-2-2-2-6-2-2	2-2-2-2-6-2-2
12	7	5-4-1-4-4-6-3	5-4-1-4-4-6-3	5-4-1-4-4-6-3
13	9	3-3-1-1-1-1-10	3-3-1-1-1-1-10	3-3-1-1-1-1-10
14	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
15	31	2-2-2-3-6-3-2	2-2-2-3-6-3-2	2-2-2-3-6-3-2
16	12	1-3-1-8-11-5-11	1-3-1-8-11-5-11	1-3-1-8-11-5-11
17	25	4-1-4-1-5-5-4	4-1-4-1-5-5-4	4-1-4-1-5-5-4
18	37	2-2-2-2-15-3-2	2-2-2-2-15-3-2	2-2-2-2-15-3-2
19	121	6-5-6-2-7-14-5	6-5-**16**-2-7-14-5	6-5-6-2-7-14-5
20	45	10-14-8-6-10-3-2	10-14-8-6-10-3-2	10-14-8-6-10-3-2
21	57	2-2-2-2-6-49-2	2-2-2-2-6-**3**-2	2-2-2-2-6-**3**-2
22	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
23	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
24	45	10-14-8-6-10-3-2	10-14-8-6-10-3-2	10-14-8-6-10-3-2
25	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
26	32	2-12-2-2-6-3-2	2-12-2-2-6-3-2	2-12-2-2-6-3-2
27	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
28	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
29	30	2-2-2-2-6-3-2	2-2-2-2-6-3-2	2-2-2-2-6-3-2
30	38	2-2-10-2-3-3-2	2-2-10-2-3-3-2	2-2-10-2-3-3-2

[a] ST, sequence type. The number of errors per allele was two for center 1 and one for center 2. Bold indicates a major discrepant result.

this position, and for that reason, the sequence should have been classified as allele type 49.

The MLST technique is based on the sequence analysis of internal fragments of bacterial housekeeping genes (14). MLST not only has been applied to molecular characterization of a variety of pathogenic microorganisms (2, 7) but also has been used for population genetics purposes (4). MLST results are electronically transferable between different centers, permitting the establishment of international databases via the Internet (3, 14).

The microbiological importance of high-density DNA probe array technology has been demonstrated in *Mycobacterium* species identification and antibiotic resistance determination (16) and identification of *agr*- and *sarA*-regulated *S. aureus* genes by transcription profiling (6). Diverse elements that have been identified in the staphylococcal genome can be addressed as potential targets for the development of probes (10, 14, 15, 18–20) and scanned for genetic variability by using DNA chips. The release of seven *S. aureus* whole-genome sequences (1, 12) (The Institute for Genomic Research, University of Oklahoma, Sanger Center, Trinity College, and the Wellcome Trust Centre for Epidemiology and Infectious Diseases) generated a large number of additional nucleic acid targets and, most probably, additional candidate loci for the epidemiological characterization of MRSA.

The present study describes the application of DNA probe arrays for MLST-based *S. aureus* strain discrimination (7). Oligonucleotide probes, immobilized on the GeneChip, scan every single nucleotide of these target sequences, identify the matching allele of each housekeeping gene, and, finally, define the allelic profile of each isolate. The feasibility of the GeneChip array was determined using two separate strain collections. In the first phase of the study, amplified and transcribed DNA of a well-characterized set of MRSA strains was labeled with a classic fluorochrome. The probing data obtained from two centers confirmed the epidemiological relatedness of the strains, as defined with pheno- and genotyping data. However, single mismatches of the query sequences with the reference sequence were detected, which led to differences in allele identification, mostly in combination with suboptimal hybridization signals. Overall, this resulted in nonoptimal reproduction between centers, although the epidemiological relatedness of the strains was established correctly. The second phase of this study involved the implementation of a new labeling technique. This approach resulted in excellent reproducibility of the data when the two centers were considered and showed agreement with the conventional MLST data.

In conclusion, MLST using high-density DNA arrays is reproducible, exchangeable, and epidemiologically concordant and is validated by conventional MLST. This technique provides an adequate tool for high-throughput genotyping of *S. aureus*, especially in national reference centers, where rigorous quality control procedures can be implemented, allowing the efficient tracking of staphylococcal clones locally, nationally, and internationally.

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