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New *Staphylococcus aureus* Genotyping Method Based on Exotoxin (*set*) Genes†

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A variety of methods for genotyping *Staphylococcus aureus* isolates exists: the two most widely used methods are pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Here, we describe a sequence-based genotyping method based on genes encoding *S. aureus* superantigen-like proteins, which belong to a family of exotoxins called staphylococcal exotoxins. The sequences of PCR-amplified internal fragments of three different *set* genes (*set2*, *set5*, and *set7*) of 61 well-characterized clinical methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) isolates and reference strains were compared. Phylogenetic analysis was performed based on single-nucleotide polymorphisms (SNP). The SNP dendrograms of the *set* gene sequences differentiated the 61 isolates into 22 distinct subgroups, designated exotoxin sequence types (ETST), while the standard seven-gene MLST profiles differentiated the same 61 isolates into 19 subgroups. Of the 19 different MLST subgroups, 16 corresponded to 16 distinct ETST groups. However, three MLST subgroups, ST1, ST30, and ST36, were each further separated into more than one ETST subgroup. The exotoxin-based genotyping method was able to discriminate MRSA and MSSA isolates according to their specific epidemiological characteristics. This SNP analysis of the three *set* genes is thus equally or more discriminatory than the seven-gene MLST method, providing a good alternative typing tool for a laboratory that has sequencing capability.

Staphylococcus aureus isolates, especially methicillin-resistant *S. aureus* (MRSA) isolates, are a leading cause of hospital-acquired infections (19) and are increasingly recognized as a cause of variety of community-acquired infections associated with poor hygiene, antimicrobial drug use, wounds, and crowding, as well as those with no obvious underlying medical conditions (2, 4–6, 9, 14–16, 20, 22). Community-acquired MRSA differs from hospital-associated MRSA in several ways, including epidemiologic associations, drug resistance determinants, and putative virulence factors (3, 18, 25, 28). Much of what we know about these differences came from studies based on genotypic characterizations of clinical isolates of *S. aureus*. Hence, simple and affordable methods to subtype *S. aureus* could further contribute to better understanding of the epidemiology of infections caused by this organism.

MRSA is typically genotyped by pulsed-field gel electrophoresis (PFGE), combined with staphylococcal cassette chromosome *mec* (SCC*mec*) typing, or multilocus sequence typing (MLST). While highly discriminating, PFGE is labor-intensive and has poor interlaboratory reproducibility and interpretability (8, 27). Despite its high reproducibility and standardization, MLST is relatively expensive due to the large number of targets that need to be sequenced (26).

Comparative genomic analysis of *S. aureus* strains revealed a high degree of interstrain variation (1, 11–13). One such variable region is called RD13, which, in strains N315 and Mu50, contains exotoxin gene-containing genomic islands SaPIn2 and SaPIm2, respectively (12). Although the RD13 locus is found in all *S. aureus* strains, in the eight reference strains, the RD13 region contains 5 (in strain NCTC6571) to 10 (in strain NCTC 8325) *Staphylococcus* exotoxin-like protein genes (12). These *Staphylococcus* exotoxin-like protein genes are allelic among different strains (1, 24), indicating that they are subject to selective pressures encountered during infection. This led Baba et al. to propose that *S. aureus* isolates may be typed by comparing allelic differences in these *set* genes (allotyping) (1).

Here, we describe one allotyping method based on *set* genes and compared it with the standard MLST method using an epidemiologically well-characterized collection of MRSA and MSSA isolates.

MATERIALS AND METHODS

Bacterial isolates. Sixty-one *S. aureus* isolates were studied: 8 MRSA isolates and 13 MSSA clinical isolates from different neighborhoods in Rio de Janeiro, Brazil (Diagnosticos da America S.A.); 22 MRSA isolates and 11 MSSA isolates from the San Francisco Bay area, California; and 7 reference strains from the Network of Antimicrobial Resistance in *Staphylococcus aureus*, with known genome sequences (NCTC8325, MW2, MSSA476, MRSA252, Mu50, USA300, and N315). The clinical isolates were selected from different geographical regions and clinical manifestations to ensure strain diversity and minimize epidemiologic linkages (Table 1). Isolates from patients with invasive disease were defined as MRSA or MSSA isolates recovered from blood, urine, skin abscess, or soft tissue ulcers.

***set* gene allotyping. (i) Design of three exotoxin gene primers.** The sequences of three genes, *set2*, *set5*, and *set7*, from the strains Mu50 and N315, were matched through BLAST analysis with eight publicly available *S. aureus* genome sequences to obtain their consensus gene sequences (COL, NCTC8325, MW2,

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TABLE 1. *S. aureus* isolates from Rio de Janeiro, Brazil, and San Francisco, Calif., and reference strains from the Network of Antimicrobial Resistance in *Staphylococcus aureus* used in this study

Isolate	Yr of isolation	Origin[a]	*S. aureus* type	PFGE type	SCC*mec* type	Gene allotype			ETST[b]	ST[c]
						set2	*set5*	*set7*		
33	2001	RJ	MRSA	A1	IIIa	1	1	1	1	239
39	2001	RJ	MRSA	A1	IIIa	1	1	1	1	239
40	2001	RJ	MRSA	A1	IIIa	1	1	1	1	239
53	2001	RJ	MRSA	A1	IIIa	1	1	1	1	239
59	2001	RJ	MRSA	A1	IIIa	1	1	1	1	239
80	2001	RJ	MRSA	A1	IIIa	1	1	1	1	239
163	2002	RJ	MRSA	A1	IIIa	1	1	1	1	239
130	2002	RJ	MRSA	A1	IIIa	1	1	1	1	239
1307	1999	SF	MRSA	D	IV	2	2	2	2	5
1460	1999	SF	MRSA	D	II	2	2	2	2	5
6211	2004	SF	MRSA	D	IV	2	2	2	2	5
2845	2001	SF	MRSA	D	IV	2	2	2	2	5
Snif46	2002	SF	MSSA			4	8	3	6	1
2209	2001	SF	MRSA	K8/10	IV	4	8	3	6	1
Snif50	2002	SF	MSSA			4	8	7	7	1
2421	2001	SF	MRSA	K	IV	9	10	10	15	1
1573	2000	SF	MRSA	A10	II	6	15	9	10	36
1436	1999	SF	MRSA	A11	II	6	15	9	10	36
1376	1999	SF	MRSA	A	II	6	3	9	14	36
3952	1999	SF	MRSA	C	IV	1	5	1	11	8
3669	2002	SF	MRSA	S	IV	1	5	1	11	8
1424	1999	SF	MRSA	C	IV	1	5	1	11	8
3674	2002	SF	MRSA	S	IV	1	5	1	11	8
6281	2004	SF	MRSA	S	IV	1	5	1	11	8
1372	1999	SF	MRSA	C	IV	1	5	1	11	8
1386	1999	SF	MRSA	PSb	IV	7	6	16	12	87
Snif20	2002	SF	MSSA			10	11	11	21	88
Snif129	2002	SF	MSSA			12	12	12	20	25
Snif30	2002	SF	MSSA			12	12	12	20	25
Snif58	2002	SF	MSSA			13	14	15	19	15
Snif73	2002	SF	MSSA			11	14	14	18	109
Snif142	2002	SF	MSSA			8	14	14	17	20
126	2001	SF	MSSA			7	6	13	16	59
6289	2004	SF	MSSA			7	6	13	16	59
2459	2001	SF	MRSA	B	IV	8	7	16	13	72
1362	1999	SF	MRSA	B	IV	8	7	16	13	72
Snif77	2002	SF	MSSA			8	7	16	13	72
3962	1999	SF	MRSA	Z	IV	6	17	9	22	30
2382	2001	SF	MRSA	Z	IV	6	17	9	22	30
6280	2004	SF	MRSA	Z	IV	6	3	9	14	30
6141	2004	SF	MRSA	Z	IV	6	3	9	14	30
63	2001	RJ	MSSA			2	2	2	2	5
113	2001	RJ	MSSA			2	2	2	2	5
57	2001	RJ	MSSA			3	9	5	5	14
138	2001	RJ	MSSA			3	9	5	5	14
112	2001	RJ	MSSA			5	3	6	8	74
109	2001	RJ	MSSA			5	3	6	8	74
105	2001	RJ	MSSA			4	8	3	6	1
34	2001	RJ	MSSA			2	4	4	3	12
47	2001	RJ	MSSA			2	16	5	4	New
106	2001	RJ	MSSA			9	8	8	9	188
107	2001	RJ	MSSA			4	8	3	6	1
87	2001	RJ	MSSA			4	8	3	6	1
38	2001	RJ	MSSA			2	2	2	2	5
Mu50	1996	Japan	MRSA			2	2	2	2	5
N315	1982	Japan	MRSA			2	2	2	2	5
MSSA476	1998	UK	MSSA			4	8	7	7	1
MW2	1998	US	MRSA			4	8	7	7	1
NCTC8325	1963	US	MRSA			1	5	1	11	8
USA300	1999	US	MRSA			1	5	1	11	8
MRSA252	1997	UK	MRSA			6	3	9	14	36

[a] RJ, Rio de Janeiro, Brazil; SF, San Francisco, California; US, United States; UK, United Kingdom.
[b] Allotype designation based on the three *set* genes.
[c] MLST designation.

TABLE 2. Synthetic oligonucleotide primers used for *set* allotyping by PCR

Gene targeted	Primer sequences (forward; reverse)[a]	Annealing temp (°C)	Amplicon size (bp)
set2	5′-CAACAGARGYYAATTCAGGTC–3′; 5′-RAYCCTACCATCTTTTGAGAK-3′	52	499
set5	5′-CGATACTACTCAKCASMAAG-3′; 5′-TATTCAACACATCGCCCATG-3′	52	511
set7	5′-TGCGAGYGAACATKAAGCAA-3′; 5′-SRYRTCACYCATRCGRTKWG-3′	52	573

[a] R, G/A; Y, C/T; K, G/T; S, G/C; W, T/A; M, A/C.

MSSA476, MRSA252, Mu50, USA300, and N315). The COL strain lacks *set5* and *set7*, so this strain was not included in the analysis in this study (12). The nucleotide sequence analysis (blastn) was performed with a program provided on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences of the *set* genes were aligned, and we found that *set5* and *set7* genes exhibit the most diversity among the different sequenced reference strains. *set2* is found in all reference strains. Thus, primers were targeted against *set2*, *set5*, and *set7*. The primers were designed to target the most divergent region in these genes. Table 2 summarizes the primer sequences used in this study and shows expected amplicon sizes.

(ii) DNA extraction and PCR. *S. aureus* DNA was prepared for PCR by boiling. Briefly, cells were scraped off an overnight blood agar plate with a sterile loop, washed twice in 1.5 ml of 1 × Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]), resuspended in 0.5 ml of water, and immersed in boiling water for 15 min. The cell debris was pelleted by centrifugation at 8,000 × *g* for 5 min, and the supernatant containing the released DNA was transferred to a fresh microcentrifuge tube. PCR amplification for the three different exotoxins genes (*set2*, *set5*, and *set7*) was performed with the oligonucleotide primers (Sigma-Genosys, Woodlands, TX) listed in Table 2. The PCR mixture (50 μl) contained 0.3 μl primer 1 (100 pmol), 0.3 μl primer 2 (100 pmol), 0.4 μl deoxynucleoside triphosphates (10 mM), 5.0 μl of 10 × thermophilic buffer (Promega Corporation, Madison, Wis.), 0.2 μl *Taq* DNA polymerase (5 U/μl) (Promega Corporation, Madison, Wis.), and 46 μl of distilled water. Finally, 1.0 μl of the template DNA suspension was added to each 0.2-ml reaction tube. The DNA amplification conditions were 1 min of initial denaturation at 94°C; 32 cycles consisting of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C; and a final extension for 5 min at 72°C. The presence of a PCR product was determined by electrophoresis of 6 μl of the reaction product in a 1% agarose gel, in Tris-acetate-EDTA buffer (Tris–acetate–2 mM EDTA [pH 8.3]) at 110 V, visualized under UV light by the Gel Doc 1000 system (Bio-Rad). The size of the PCR products was determined by comparison to a 100-bp DNA molecular weight marker (Promega Corporation, Madison, Wis.). The primers and PCR cycling conditions used for MLST analysis were the same as those described by Enright et al. (10) and are updated on the *S. aureus* MLST website (http://www.mlst.net).

(iii) Sequence analysis. The PCR products were purified by the QIAquick PCR purification kit (QIAGEN Sciences, Maryland) and sequenced in both directions. Sequencing was performed at the University of California—Berkeley sequencing facility. The facility runs a 25-cycle sequencing reaction with the following program: 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 min. The sizes of the amplicons for *set2*, *set5*, and *set7* were 499 bp, 511 bp, and 573 bp, respectively (Table 2). The sequences used to generate a phylogenetic tree consisted of a region shorter than the total amplicon sizes (484 bp, 414 bp and 491 bp for *set2*, *set5*, and *set7*, respectively) (Fig. 1). All sequences reported by the sequencing facility were reviewed visually, and analysis was performed by Seqman and EditSeq (DNA Star/Lasergene, Madison, WI).

(iv) Analysis of gene sequences and genetic relationship among isolates. As is done with MLST nomenclature, for each *set* gene locus, the sequences obtained from all 61 isolates were compared and any new *set* gene sequence was assigned a numerical allotype designation (10). Then, strains with an identical set of allotype numbers assigned to *set2*, *set5*, and *set7* were classified to belong to the same exotoxin sequence type (ETST) and given a numerical designation (Table 1).

PFGE. All of the MRSA isolates ($n = 30$) were also typed by PFGE, according to the method described by Charlesbois et al. (7). The extracted DNA was digested by SmaI endonuclease and the DNA fragments were resolved in 1% agarose in 0.5× Tris-borate-EDTA buffer for 22 h at 14°C and 6 V/cm in a CHEF DR-II apparatus (Bio-Rad, Hercules, CA).

SCC*mec* typing. SCC*mec* typing was performed on all the MRSA isolates by the PCR method described by Oliveira and de Lencastre. (21). Briefly, SCC*mec* type was determined by multiplex PCR amplifications with the following parameters: predenaturation for 4 min at 94°C; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; postextension for 4 min at 72°C; and soaking at 4°C. PCR products (10 μl) were resolved in a 2% MetaPhor (Cambrex Bioscience, Rockland, ME) agarose gel in 0.5 × Tris-borate-EDTA buffer (Bio-Rad, Hercules, CA) at 100 V and visualized with ethidium bromide.

The phylogenetic trees based on the concatenated sequences of the three gene sequences were reconstructed by neighbor-joining algorithm based on the Kimura 2 parameter, calculated by MEGA, version 3.0 (17). One concatenated sequence from each ETST group was included in the tree. Estimates of the sampling variance were made by bootstrap analysis with 1,000 repetitions.

The work in this study was performed at the Division of Infectious Diseases, School of Public Health, University of California, Berkeley, Calif.

RESULTS

Genotype analysis. By the seven-gene standard MLST genotyping method, the 61 isolates were differentiated into 19 different genotypes (Table 1). Sixteen of them corresponded to 16 distinct ETST genotypes. However, three MLST subgroups, ST1, ST30, and ST36, were each further separated into more than one genotype. The ST1 subgroup containing nine isolates was separated into three ETST subgroups, ETST6, ETST7, and ETST15 (Table 1). The ST30 subgroup containing four isolates was composed of ETST14 and ETST22. The ST36 subgroup containing four isolates was separated into ETST10 and ETST14. The ST36 isolates showed the most heterologous *set7* gene sequence (~10%) among all isolates tested. Even with only one *set* gene (*set5* or *set7*), the 61 isolates were separated into 16 subgroups (Table 1).

The neighbor-joining tree showed a good correlation with results obtained by the exotoxin allotyping and MLST. However, the exotoxin allotyping method showed a higher number of allotypes than did MLST for the 61 isolates tested. The

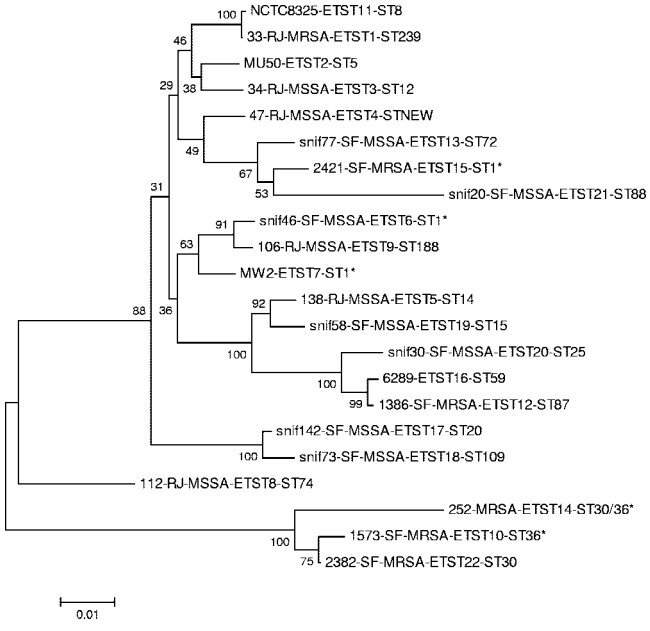

FIG. 1. Phylogenetic analysis based on concatenated sequences of the three *set* genes of *S. aureus*. Each strain is abbreviated with the isolate designation and place of isolation, followed by the MLST (ST) designation. *, MLST subgroup with more than one ETST genotype.

TABLE 3. Comparison of exotoxin (ETST) and MLST sequence types of the *S. aureus* isolates by resistance profile (colonization and invasive disease source)

Source	MRSA			MSSA		
	Isolate	ETST[a]	ST[b]	Isolate	ETST[a]	ST[b]
Colonization	33	1	239	113	2	5
	39	1	239	34	3	12
	40	1	239	47	4	New
	80	1	239	112	8	74
	1460*	2	5	106	9	188
	3952*	11	8	38	2	5
	3962*	22	30	Snif46*	6	1
				Snif77*	13	72
				Snif142*	17	20
				Snif58*	19	15
				Snif73*	18	109
				Snif129*	20	25
				Snif20*	21	88
				Snif30*	20	25
				Snif50*	7	1
Invasive disease	53	1	239	63	2	5
	59	1	239	138	5	14
	130	1	239	109	8	74
	1307*	2	5	105	6	1
	2209*	6	1	107	6	1
	1573*	10	36	57	5	14
	1436*	10	36	87	6	1
	3669*	11	8	126*	16	59
	1424*	11	8	6289*	16	59
	3674*	11	8			
	1386*	12	87			
	1362*	13	72			
	2382*	22	30			
	6281*	11	8			
	6280*	14	30			
	6211*	2	5			
	6141*	14	30			
	1376*	14	36			
	1372*	11	8			
	2459*	13	72			
	2421*	15	1			
	2845*	2	5			
Unknown	163	1	239			

[a] Allotype designation based on three *set* genes.
[b] MLST designation. *, San Francisco isolate.

MLST method generated 19 genotypes, while the exotoxin allotyping generated 22 genotypes (Fig. 1). The nucleotide sequences of all of the isolates are shown in the supplemental data.

PFGE. The PFGE analysis of 30 MRSA isolates differentiated them into 14 genotypes. ETST differentiated the same set of MRSA isolates into 10 genotypes, while the MLST generated 8 distinct subtypes.

SCC*mec*. The SCC*mec* typing of 30 MRSA isolates generated three distinct types. The combination of SCC*mec* with MLST differentiated these MRSA isolates into eight subtypes.

Epidemiological analysis. The 13 MSSA isolates from Rio de Janeiro were separated into seven MLST groups and seven corresponding ETST subgroups. All eight MRSA isolates from Rio de Janeiro belonged to one distinct MLST group and one ETST group. Thus, there was greater heterogeneity among MSSA isolates than MRSA isolates from Rio de Janeiro. The 22 MRSA isolates from San Francisco were separated into 7 MLST subgroups and 10 ETST subgroups.

Of 21 Brazilian clinical *S. aureus* isolates, 10 came from nasal swabs, 10 came from invasive disease sources, and 1 had an unknown source (Table 3). MLST and ETST each separated the nasal colonization isolates into six corresponding subgroups. Two of the six nasal MSSA isolates belonged to the same ST and ETST genotypes (ETST2 [ST5] and ETST8 [ST74]) found among 10 invasive disease isolates. The most common genotype among the Brazilian MSSA isolates was ETST6 (ST1), accounting for 3 of 13 Brazilian MSSA isolates, and they were isolated only from invasive disease sources. On the other hand, there was only one genotype among the Brazilian MRSA isolates (ETST1 [ST239]), and they were found in both colonization and invasive disease sources.

Among the San Francisco isolates, 12 were from colonization sources and 21 were from invasive disease sources. All of the genotypes among the MRSA colonization isolates were shared among MRSA invasive disease isolates (Table 3). ETST2 (ST5) and ETST6 (ST1) genotypes were found among both United States and Brazilian isolates.

DISCUSSION

The multiple *set* genes in *S. aureus* and their sequence heterogeneity provided an opportunity to target them for genotyping clinical isolates of *S. aureus*. The discrimination of 61 isolates based on three exotoxin genes agreed well with that obtained by the standard MLST genotyping method. Sequences of the isolates in the exotoxin gene-based phylogenetic tree clustered into either identical or more discriminatory groupings than those based on MLST. However, when a subset of the isolates (MRSA) was tested by PFGE, the three-exotoxin-gene-based allotyping method was not as discriminating as the PFGE-typing method. The 30 MRSA isolates were comprised of 14 distinct PFGE types, compared to 10 ETST genotypes.

Nevertheless, since the *set* gene allotyping is based on only three gene targets, it may serve as an affordable and quick typing method for epidemiological assessment of *S. aureus* infections, such as during outbreak investigations or hospital- or community-based surveillance, especially if the outbreak or hospital or community infections were caused by one of the strains (ST1, ST30, or ST36) found to be further separable by ETST. This allotyping method was indeed able to identify and distinguish recognized international clones of MRSA (ST1 and ST36) and identify common genotypes in two widely separate geographic regions. Thus, the method shows both epidemiologic and typing system concordance with the established MLST method (23).

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