Multilocus Sequence Typing of Methicillin-Resistant *Staphylococcus aureus* from an Area of Low Endemicity by Real-Time PCR

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A protocol for multilocus sequence typing (MLST) of methicillin-resistant *Staphylococcus aureus* (MRSA) was adapted to real-time LightCycler System PCR for efficient and rapid amplification of seven housekeeping genes in the same PCR run and real-time detection of the products. The method was evaluated on a representative and well-characterized collection of clinical MRSA isolates ($n = 57$) obtained from an area of low endemicity. Twenty sequence types (STs) and nine clonal complexes were identified. Combining STs and the staphylococcal cassette chromosome *mec* (SCC*mec*) type identified 27 different genotypes, and type IV SCC*mec* was present in 11 different STs. The presence of the Panton Valentine leukocidin (PVL) genes was found in isolates of four different STs. Eleven different STs were found among the community-acquired as well as among the hospital-acquired MRSA. The genetic heterogeneity was also denoted by pulsed-field gel electrophoresis analysis that showed 24 different pulsotypes among the 57 MRSA isolates. The presence of more than one different type of SCC*mec* in the same ST indicates that the MRSA clones have arisen at several occasions in the same genetic background by independent acquisition of SCC*mec* into methicillin-sensitive strains. This circumstance shows the importance of combining MLST data with SCC*mec*-typing results when investigating the origins of MRSA.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is well known as a nosocomial pathogen that has spread worldwide since it was first described in the early 1960s (16). However, there has recently been a changing epidemiology and emerging problems with MRSA that are disseminating in the community (5, 23) among otherwise healthy people, including children (3). These isolates are described to harbor potential virulence factors, and reports from several parts of Europe describe the spread of clones that are producing the Panton-Valentine leukocidin (PVL) (2, 19, 28, 29).

Acquisition and expression of the gene *mecA* is the genetic event that makes *S. aureus* resistant to methicillin and all other β -lactam antimicrobials. *mecA* encodes an altered penicillin-binding protein, PBP2a (or PBP2'), and it is always carried on the mobile staphylococcal cassette chromosomal *mec* (SCC*mec*) (15, 20). The SCC*mec* element is present in five (I to V) different allotypes and contains a characteristic combination of two essential genetic components, the *mec* gene complex and the *ccr* gene complex (14). The SCC*mec* elements are widely disseminated among coagulase-negative staphylococci, and studies have presented evidence for horizontal transfer between staphylococcal species, although this seems to be a rare event (12, 30).

There are various molecular typing methods available for *S. aureus* but pulsed-field gel electrophoresis (PFGE) is still the gold standard method for investigating outbreaks and local epidemiology. However, PFGE has major disadvantages, including difficulties in comparing the results of different laboratories (8, 25). Multilocus sequence typing (MLST) is used to

identify clones within populations of pathogenic microorganisms and genetic variation is observed by assigning the alleles at each locus directly by nucleotide sequencing the internal fragments of seven housekeeping genes. The sequences of each gene are assigned as distinct alleles, and the sequence type (ST) or allelic profile of each isolate is defined by the alleles at each of the seven housekeeping loci. Isolates with the same allelic profile for six out of seven genes are assigned as members of the same clonal complex (8, 21). A major advantage of MLST is the ability to compare sequence data between laboratories via the MLST website on the Internet.

MLST of large numbers of MRSA from different geographic areas has revealed that there are five major clonal lineages that are internationally dominating (1, 4, 24). However, community-acquired MRSA are found to possess more diverse evolutionary origins compared to nosocomial strains (7, 11, 20, 23, 24), and they are predominantly carrying type IV SCC*mec*.

The aim of the present study was to adapt the MLST protocol for *S. aureus* to real-time PCR amplification of the seven housekeeping genes and to employ the method on a well characterized collection of MRSA isolates from an area of low endemicity.

MATERIALS AND METHODS

Bacterial isolates. A selection ($n = 57$) of representative clinical MRSA isolates ($n = 92$), identified during 1987 to 2004 in Örebro County (population approximately 280,000 inhabitants) were analyzed. Outbreak- or otherwise related isolates (e.g., intrafamily spread), confirmed by PFGE, were excluded. Fifty-one of the 57 isolates have previously been characterized by SCC*mec* typing, PFGE, and the presence of PVL genes (2). Contact tracing has been performed according to the Swedish Communicable Diseases Act since 2000 and essential epidemiological information has been collected and available in a county database since 1997. Isolates were stored at -70°C in preservation medium (yeast extract, horse serum, Trypticase soy broth) at the Department of Clinical Microbiology at Örebro University Hospital.

Community-acquired infection was defined as identification of MRSA in the

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TABLE 1. Mean T_m for clinical isolates and reference strain calculated for all PCR runs of each gene^a

Gene	No. positive/no. tested	T_m (°C)					
		All PCR runs		Interassay variation		Intra-assay variation	
		Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
<i>arcC</i>	57/57 ^b	83.1 (0.2)	82.6–83.6	83.0 (0.08)	82.9–83.1	83.1 (0.1)	83.0–83.3
<i>aroE</i>	57/57	80.8 (0.3)	80.0–81.4	81.2 (0.05)	81.1–81.2	81.0 (0.1)	80.8–81.2
<i>glpF</i>	57/57	84.6 (0.3)	83.9–85.1	84.6 (0.1)	84.5–84.8	84.9 (0.2)	84.6–85.2
<i>gmk</i>	57/57	80.3 (0.2)	79.8–80.8	80.5 (0.1)	80.3–80.6	80.5 (0.2)	80.3–80.8
<i>pta</i>	57/57	82.9 (0.3)	82.3–83.6	82.7 (0.05)	82.7–82.8	82.9 (0.2)	82.7–83.1
<i>tpi</i>	57/57	82.2 (0.3)	82.1–82.9	82.2 (0.04)	82.2–82.3	82.1 (0.1)	82.0–82.3
<i>ygiL</i>	57/57	83.5 (0.3)	83.2–83.9	83.6 (0.04)	83.6–83.7	83.5 (0.2)	83.2–83.8

^a Interassay variation was tested in five different runs, and intra-assay variation was tested in five different capillaries in the same run.

^b Isolate 00T-231 was amplified with an alternative primer (*arcCF2*), and the T_m was 83.25°C.

outpatient setting or a culture positive for MRSA within 48 h after hospital admission. In addition, the patient had no medical history of MRSA infection or colonization, and no medical history in the last year of hospitalization, admission to a nursing home, dialysis or surgery. The patient had no permanent indwelling catheters or medical devices that pass through the skin. Twenty-nine of 57 (50.9%) MRSA were categorized as community-acquired MRSA, 24 (42.1%) were hospital-acquired MRSA, and four (7.0%) could not be classified due to lack of epidemiological information. Isolates were derived from skin ($n = 46$), nares ($n = 9$), blood ($n = 1$), and urine ($n = 1$).

DNA isolation. All isolates were cultured on blood agar (Columbia II agar, 4.25% wt/vol; horse blood, defibrinated, SVA, 6% wt/vol) and incubated overnight at 37°C. DNA isolation of the reference strain was made with Dynabeads DNA DIRECT system I kit (Dynal Biotech A.S. Oslo, Norway) for higher purity and longer stability. Genomic DNA of the clinical isolates was extracted by denaturing a few colonies suspended in sterile water at 98°C for 15 min and then centrifuged at $13,000 \times g$ for 30 seconds. The supernatant was used as template for amplification in the real-time PCR.

MLST. The conventional procedure for performing MLST in *S. aureus* described by Enright et al. (8) was adapted and optimized for use in the real-time LightCycler System PCR and for amplifying the seven genes in the same PCR program, but in different reactions for each gene. An alternative primer described by Crisostomo et al. (4) was used for amplification of carbamate kinase (*arcCF2*) in one isolate. Primers used in the present study were synthesized by Scandinavian Gene Synthesis (SGS), Köping, Sweden. The strain NCTC 8325 with a characterized allelic profile (ST8 [3-3-1-1-4-4-3]) was used as the reference during optimization of the protocols and was included as a standard DNA control for all amplifications.

The LightCycler FastStart DNA Master SYBR Green I (FastStart Taq DNA polymerase, SYBR Green I, deoxynucleoside triphosphates with dUTP instead of dTTP, reaction buffer, 10 mM MgCl₂) (Roche Diagnostics, Mannheim, Germany) was used. The reactions were performed in glass capillaries at a volume of 20 µl and the cycling conditions were optimized for LightCycler System PCR. These included titration of MgCl₂ and primer concentrations as well as optimizing annealing temperatures and extension times. Each reaction contained 2 µl LightCycler FastStart DNA Master SYBR Green I, 0.5 µM of each primer, 3 mM MgCl₂ and 2 µl DNA template (3 ng). Samples were preincubated for 10 min at 95°C and then subjected to 30 cycles of amplification run according to the following schedule: denaturation at 95°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 24 s.

Characterization of the amplification products was performed by melting curve analysis subsequent to the amplification run. The melting program consisted of one cycle of 95°C for 0 s, 65°C for 15 s and then the temperature was increased at a transition rate of 0.1°C/s to 95°C during continuous fluorescence monitoring. The samples were then cooled at 40°C during 30 s. Melting peaks were derived from the initial melting curves (fluorescence [F_1] versus temperature [T]) by plotting the negative derivative of fluorescence over temperature versus temperature ($-dF_1/dT$ versus T). The specific T_m obtained from all runs of the analyzed isolates ($n = 57$) and the reference strain was used to calculate the mean T_m of each gene. Intra-assay variations were tested by comparing the calculated T_m of reference strain NCTC 8325, (3 ng), in five different capillaries during the same run. Interassay variations were tested with NCTC 8325 (3 ng) by comparing the given T_m in five different runs.

Prior to sequencing, the PCR product was purified by High Pure PCR Puri-

fication Kit (Roche Diagnostics) and sequenced with ABI PRISM BigDye Terminator version 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Stockholm, Sweden). Sequencing was performed in 96-well plates and each reaction (10 µl) consisted of 1 µl of purified DNA (2 to 50 ng DNA), and 2 µl of Terminator Ready Reaction mix (A, BigDye terminator labeled with dichloro [R6G], C, BigDye terminator labeled with dichloro[ROX], G, BigDye terminator labeled with dichloro[R110], T, BigDye terminator labeled with dichloro [TAMRA], dATP, dCTP, dTTP, dUTP, AmpliTaq DNA polymerase [FS] with rTth pyrophosphatase, MgCl₂, Tris-HCl buffer [pH 9.0]), and 1.6 pmol primer.

Cycle sequencing was carried out on the GeneAmp PCR System 2700 (Applied Biosystems, Warrington, England) and the same primers initially used for LightCycler System PCR (4, 8). Sequences were confirmed on both strands, by 25 cycles consisting of 96°C denaturation for 10 min, 55°C of annealing for 5 s, 60°C of extension for 4 min, and then cooling at 4°C.

Reactions were precipitated by using 3 M sodium acetate, pH 4.6 and 95% ethanol with a final ethanol concentration of 65%, washed in 70% ethanol, and resuspended in 10 µl formamide (Applied Biosystems, Stockholm, Sweden) before separation on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The nucleotide sequences of the seven loci were analyzed using ABI PRISM AutoAssembler DNA Sequence Assembly 1.4.0 software and were compared to allelic profiles and associated epidemiological data via the MLST website (<http://www.mlst.net>). The allelic profiles were compared by using the program eBURST v.2. (based upon related sequence types), developed by Feil et al. (10) and available on the MLST website.

Determination of SCCmec types. SCCmec typing was performed by real-time LightCycler System PCR to detect the essential genetic components *mecA*, *mecRI*, *IS1272*, *ccrA*, and *ccrB* according to Berglund et al. (2).

Detection of *lukS-PV* and *lukF-PV*. The LightCycler System PCR and SYBR Green I dye were used to detect PVL genes as described previously (17).

Pulsed-field gel electrophoresis. The 57 MRSA isolates were characterized by pulsed-field gel electrophoresis (PFGE) of chromosomal SmaI digests, prepared with the GenePath group 1 reagent kit (Bio-Rad Laboratories, Hercules, CA) and PFGE patterns were obtained with a contour-clamped homogeneous electric field apparatus (GenePath System; Bio-Rad) as described (2).

RESULTS

MLST was successfully performed in all ($n = 57$) isolates by sequencing the seven housekeeping genes following amplification with real-time LightCycler System PCR. Single peaks were observed for all gene products and the mean T_m and intra- as well as interassay variations are given in Table 1.

Twenty STs were identified by MLST and these are summarized in Table 2. The evolutionary origins were examined by the algorithm eBURST, in order to group the received allelic profiles into clonal complexes. Grouping was based on the similarity between sequence types in six of seven loci and singleton STs are defined as not sharing six out of seven loci with any other STs in the data set. Four groups of related

TABLE 2. Genetic backgrounds and epidemiological information on MRSA isolates

Isolate	Country	Yr of isolation	ST	MLST profile ^a	CC ^b	SCCmec type ^c	PVL	Origin
01T-796	China	2001	1	1-1-1-1-1-1-1	1	NT	—	Unknown
99T-047	Thailand	1999	1	1-1-1-1-1-1-1	1	IV	—	Community
01T-044	Sweden	2001	5	1-4-1-4-12-1-10	5	IVc	—	Unknown
02T-455	USA	2002	5	1-4-1-4-12-1-10	5	II	—	Hospital
03T-137	Sweden	2003	5	1-4-1-4-12-1-10	5	IVc	—	Community
03T-282	Sweden	2003	5	1-4-1-4-12-1-10	5	II	—	Community
99T-240	Sweden	1999	5	1-4-1-4-12-1-10	5	IVc	—	Unknown
P5747/2002	Sweden	2002	5	1-4-1-4-12-1-10	5	NT	—	Community
02T-773	Sweden	2002	8	3-3-1-1-4-4-3	8	IVc	+	Community
99T-554	Sweden	1999	8	3-3-1-1-4-4-3	8	IVa	—	Community
98T-019	England	1998	22	7-6-1-5-8-8-6	22	IV	—	Hospital
01T-1029	Lebanon	2001	30	2-2-2-2-6-3-2	30	IVa	—	Hospital
04T-295 ^d	Greece	2004	30	2-2-2-2-6-3-2	30	IVc	—	Hospital
04T-691 ^d	Sweden	2004	39	2-2-2-2-2-2-2	30	II	—	Community
00T-648	Sweden	2000	45	10-14-8-6-10-3-2	45	IVa	—	Hospital
01T-384	Sweden	2001	45	10-14-8-6-10-3-2	45	IV	—	Hospital
04T-206	Sweden	2004	45	10-14-8-6-10-3-2	45	IVa	—	Community
04T-265 ^d	Armenia	2004	45	10-14-8-6-10-3-2	45	IV	—	Hospital
04T-490 ^d	Hungary	2004	45	10-14-8-6-10-3-2	45	IVa	—	Community
95T-041	Sweden	1995	45	10-14-8-6-10-3-2	45	IVa	—	Community
98B-249	Sweden	1998	45	10-14-8-6-10-3-2	45	NT	—	Hospital
98PP-013	Sweden	1998	45	10-14-8-6-10-3-2	45	IVa	—	Hospital
98PP-079	Sweden	1998	45	10-14-8-6-10-3-2	45	NT	—	Hospital
99T-299	Sweden	1999	45	10-14-8-6-10-3-2	45	NT	—	Hospital
99T-361	Sweden	1999	45	10-14-8-6-10-3-2	45	NT	—	Hospital
99T-376	Yugoslavia	1999	45	10-14-8-6-10-3-2	45	NT	—	Community
99T-655	Sweden	1999	45	10-14-8-6-10-3-2	45	NT	—	Hospital
96T-105	Sweden	1996	46	10-14-8-6-14-3-2	45	IVc	—	Hospital
00T-005	Yugoslavia	2000	80	1-3-1-14-11-51-10	80	IVc	+	Community
01T-1035	Sweden	2001	80	1-3-1-14-11-51-10	80	IVc	+	Community
01T-936	Sweden	2001	80	1-3-1-14-11-51-10	80	IVc	+	Community
02T-150	Somalia	2002	80	1-3-1-14-11-51-10	80	IVc	+	Community
02T-635	Yugoslavia	2002	80	1-3-1-14-11-51-10	80	IVc	+	Community
02T-884	Lebanon	2002	80	1-3-1-14-11-51-10	80	IVc	+	Community
03T-010	Yugoslavia	2003	80	1-3-1-14-11-51-10	80	IVc	+	Community
03T-498	Sweden	2003	80	1-3-1-14-11-51-10	80	IVc	+	Community
03T-510	Sweden	2003	80	1-3-1-14-11-51-10	80	IVc	+	Community
04T-109	Sweden	2004	80	1-3-1-14-11-51-10	80	IVc	+	Community
04T-181	Lebanon	2004	80	1-3-1-14-11-51-10	80	IVc	+	Community
04T-275 ^d	Algeria	2004	80	1-3-1-14-11-51-10	80	IVc	+	Community
03T-430	Croatia	2003	111	1-4-1-4-46-24-29	—	I	—	Hospital
99T-576	Sweden	1999	125	1-4-1-4-12-1-54	5	IVc	—	Hospital
95T-049	Sweden	1995	140	43-37-48-19-49-26-39	S	IV	—	Community
02T-659	Sweden	2002	150	22-1-14-45-12-53-31	78	NT	—	Community
01T-902	Sweden	2001	152	46-75-49-44-13-68-60	—	NT	+	Community
02T-565	Sweden	2002	152	46-75-49-44-13-68-60	—	NT	+	Community
02T-284	Kazakhstan	2002	154	12-1-1-15-51-1-40	—	NT	+	Community
00T-415	Spain	2000	228	1-4-1-4-12-24-29	—	I	—	Hospital
00T-848	Italy	2000	228	1-4-1-4-12-24-29	—	I	—	Hospital
00T-300	Thailand	2000	239	2-3-1-1-4-4-3	8	III	—	Hospital
92T-556	Thailand	2002	239	2-3-1-1-4-4-3	8	III	—	Hospital
04T-457 ^d	Syria	2004	239	2-3-1-1-4-4-3	8	III	—	Hospital
98T-080	Sweden	1998	239	2-3-1-1-4-4-3	8	III	—	Hospital
FR 071	Sweden	1987	239	2-3-1-1-4-4-3	8	III	—	Community
97T-013	Sweden	1997	241	2-3-1-1-4-4-30	8	III	—	Hospital
00T-231	Israel	2000	247	3-3-1-12-4-4-16	8	I	—	Hospital
03T-812	Sweden	2003	375	19-23-15-2-19-20-42	59	IVa	—	Unknown

^a Allelic profiles are read from left to right and correspond to genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *ygiL*.

^b CC, clonal complex, based on BURST. S, singleton.

^c NT, not typeable.

^d SCCmec, PFGE, and PVL were investigated for this study.

genotypes were identified and in addition 10 singletons. Comparison with all *S. aureus* isolates in the database at mlst.net revealed the presence of nine clonal complexes (CC1, -5, -8, -22, -30, -45, -59, -78, and -80) and one singleton (ST140). Eleven STs (ST22, -39, -46, -111, -125, -140, -150, -154, -241,

-247, and -375) were represented by a single isolate, while ST45 ($n = 13$) and ST80 ($n = 12$) were the most common profiles.

Five sequence types (ST1, ST5, ST8, ST30, and ST45) were represented by at least two isolates each that has been assigned different SCCmec types (or different subtypes of type IV

SCCmec). Combining ST and the SCCmec type, 27 different genotypes were identified in our area. Type IV SCCmec was present in 11 different STs, 1, 5, 8, 22, 30, 45, 46, 80, 125, 140, and 375. Those were clustered into three clonal complexes, and the remaining six STs did not correspond to any group and were defined as singleton STs. If compared to all *S. aureus* isolates in the mlst.net database, one of the profiles (ST140) was defined as a singleton.

Type I SCCmec was found in three different STs (111, 228, and 247), type II in two STs (5 and 39) as well as type III (STs 239 and 241), and isolates with untypeable SCCmec were present in six different STs (1, 5, 45, 150, 152, and 154). In contrast, ST80 ($n = 12$) was associated only with type IVc SCCmec, ST228 ($n = 2$) with type I SCCmec, and isolates with ST239 ($n = 5$) all carried type III SCCmec. ST80 and type IVc SCCmec were predominant among the 30 community-acquired isolates, but there were 10 additional STs represented by a single or few community-acquired MRSA carrying either type II, type III, type IV, or unknown types of SCCmec. The hospital-acquired isolates ($n = 24$) were represented by 11 STs, and ST45 and ST239 were the most frequent. The isolates ($n = 4$) that it was not possible to classify as community-acquired or hospital-acquired MRSA were represented by three STs.

The genes *lukS*-PV and *lukF*-PV were found among isolates in four different STs (8, 80, 152, and 154) whereas ST8 and ST80 all had the type IVc SCCmec but ST152 and ST154 both included isolates that were untypeable for type I to IV SCCmec. These four STs are assigned to different origins according to the database www.mlst.net.

The 57 MRSA isolates were clustered into 24 pulsotypes (Fig. 1) that were indistinguishable, closely related, or possibly related (27). Isolates with ST1, ST8, ST45, ST80, and ST239 were clustered into more than one pulsotype, while two pulsotypes included isolates that were assigned to different STs.

DISCUSSION

In this study, we adapted an existing protocol for MLST (8) to the real-time LightCycler System PCR and amplification of the seven housekeeping genes in the same PCR run. The method was evaluated on a representative material of clinical MRSA ($n = 57$) that was well characterized by SCCmec typing, PFGE, and the presence of PVL genes. MLST was highly discriminatory and identified 20 STs among the 57 isolates, although a much higher diversity was seen when combining MLST data with associated types of SCCmec.

The isolates analyzed could be assigned to nine clonal complexes based on the similarity between sequence types in six of seven loci. CC5 ($n = 7$), CC45 ($n = 14$), and CC80 ($n = 12$) were the predominant clonal complexes represented in Örebro County, Sweden, which was expected since those are widely disseminated international clones (7, 28). ST111 is a single-locus variant of ST228, and those STs were grouped but not defined as a clonal complex. However, by using the less stringent group definition (i.e., five of seven shared alleles) ST228 and ST111 are assigned to CC5. ST152 and ST154 were assigned to different groups, and only one singleton ST (ST140) was identified. There were equal numbers ($n = 11$) of STs among the community-acquired isolates and the hospital-acquired MRSA, which indicates a high diversity of genotypes

and sporadic cases in the hospital environment. Only three STs (5, 45, and 239) were represented by MRSA that were isolated both in the community and among the nosocomial isolates. The different genetic backgrounds of community-acquired MRSA and hospital-acquired MRSA suggest that they are not related and that community-acquired MRSA are not nosocomial isolates that have spread outside the hospital environment.

Enright et al. (7) strongly recommend characterization of MRSA with both MLST and SCCmec typing since isolates with the same ST may carry different SCCmec. By using this approach, four of the five major pandemic clones were recognized in our area. The Brazilian-Hungarian clone (ST239, type III SCCmec) was represented by five isolates, the New York-Japan clone (ST5, type II SCCmec) by two isolates, the pediatric clone (ST5, type IV SCCmec) by three isolates, clone V (ST8, type IV SCCmec) by two isolates, and the Iberian clone (ST247, type I SCCmec) by one isolate.

Interestingly, one community-acquired MRSA with type II SCCmec was isolated in Örebro and is assigned to ST39, an allelic profile that, along with ST30 and the widespread MRSA clone ST36, belongs to CC30. However, all MRSA isolates within this complex have been assigned to ST36, while ST39 has been described as a successful clone of methicillin-susceptible *S. aureus* (9). MRSA with type II SCCmec are described as mainly nosocomial and as carrying multiple resistance genes for non- β -lactam antibiotics (20, 22) and these circumstances makes our community-acquired isolate unique since it has a non-multiresistant nature and was manifested by a clinical significant infection.

The presence of more than one different type of SCCmec in the same ST indicates, in congruence with other studies (1, 7), that the MRSA clones arose at several occasions in the same genetic background by independent acquisition of SCCmec into methicillin-sensitive strains. This circumstance denotes the importance of combining MLST data with the SCCmec typing results when trying to understand the origins of MRSA. About one third of the isolates in our county were not typeable for type I to IV SCCmec (2), and those may represent novel types and are therefore taken in consideration when discussing possible origins of our MRSA. The presence of unknown types of SCCmec in as many as six unrelated STs depicted by eBURST gives rise to suspicion that these elements represent either more than one novel type of SCCmec or perhaps one new type with an enhanced mobility that has generated several clones of MRSA.

A previous study (23) describes nontypeable SCCmec (i.e., not type I to IV) in community-acquired MRSA with ST45 that originated from Australia, and those were found to contain the class C2 *mec* complex, an essential part of SCCmec that is commonly found among coagulase-negative staphylococci (18). The isolates with ST45 and nontypeable SCCmec in our county might carry the same *mec* gene complex, recently described as a part of the type V SCCmec (14). The presence of more than two different types of SCCmec in our isolates with ST45 may indicate that this clone of *S. aureus* has acquired SCCmec on more than one occasion, and it is therefore possible that this ST carries novel types of SCCmec.

The PVL genes are found among isolates in four different STs, two of which (ST8 and ST80) contain type IVc SCCmec

FIG. 1. PFGE patterns of SmaI-digested total chromosomal DNA. Isolate designation, ST, SCCmec type, and presence (+) or absence (-) of the PVL locus are indicated to the right of the dendrogram. Origins of isolates are indicated as CA (community acquired), HA (hospital acquired), or Un (unknown).

and the other two of which (ST152 and ST154) were untypeable for type I to IV SCCmec. The ST80 strains with type IVc SCCmec, fusidic acid resistance, and PVL locus are disseminating in several parts of Europe (28, 31) and this ST appears to be a predominant clone of community-acquired MRSA in Örebro.

ST8 is an ancestral genotype that has created two major

clones of MRSA with either type II or type IV SCCmec (6). ST8 is represented by two community-acquired MRSA in our area, with either a type IVa SCCmec or a type IVc and the PVL locus. ST1 and ST30 are known as community-acquired MRSA and as carrying the PVL locus (23, 26), however, our ST1 and ST30 isolates were not found to carry the PVL genes. The presence of the PVL genes in four genetic backgrounds

among our isolates is, in addition, an indication of the genetic diversity of community-acquired MRSA. Previously, the PVL toxin has only been associated with MRSA carrying the type IV *SCCmec* (19, 23). Therefore, the isolates with untypeable *SCCmec* and PVL genes may represent altered type IV *SCCmec* that are not detectable with the primers used, or they may represent the acquisition of a novel *SCCmec* into PVL-producing lineages of *S. aureus*.

The association between ST type and *SCCmec* is more obvious in hospital isolates with a more multiresistant nature and type I to III *SCCmec*, whereas the type IV *SCCmec* is a promiscuous element found in widely divergent genotypes. Type IV *SCCmec* is considered to have an enhanced ability for horizontal transfer into distantly related clones of *S. aureus* (24), probably due to its smaller size and functional recombinases, which are responsible for site-specific excision and integration of the element (13).

Isolates that are considered not related by PFGE but are assigned to the same genetic background by MLST are well demonstrated by ST45 and ST239, which were clustered into four different pulsotypes. All but one isolate in ST80 were clustered in one pulsotype, which demonstrates a younger or maybe more stable clone of MRSA compared to ST45 and ST239. On the other hand, ST154, which has a totally different allelic profile, is clustered together with ST45 in the same pulsotype. PFGE reflects genetic events that have occurred during a short time period and is an unsurpassed method for analyzing outbreak situations, but long-term epidemiology is better understood by MLST.

LightCycler System PCR for MLST offers the advantage to perform efficient and rapid amplification, and real-time detection of the seven housekeeping genes in the same PCR run. Melting-curve analysis of the products is performed subsequent to every run to increase the specificity of SYBR Green I detection and thus there is no need for time-consuming gel electrophoresis that is often applied when using a conventional protocol. In addition, amplification and detection are performed in a closed system to eliminate carry over contamination. With the LightCycler System PCR thermal cycling is performed using air instead of thermal blocks in a capillary system to ensure efficient heat transfer to the samples, which decreases each PCR cycle to approximately 15 to 20 seconds.

The gene *arcC* was not amplified in one isolate using primers described by Enright et al. (8), possibly due to alterations in the binding site of the primers. Amplification of this isolate (00T-231) was possible using the alternative primer *arcCF2* described by Crisostomo et al. (4). Another advantage of using the real-time LightCycler PCR is that such failure in amplification is observed during the run and is noticed at an early stage of the MLST analysis. Single peaks were seen in all runs and the melting points were specific for each of the seven genes. The PCR conditions were stable and showed minor intra-assay variations as well as interassay variations for all seven genes. The ability to perform amplification of all genes in one PCR run is convenient when a small number of isolates are investigated. MRSA are still uncommon in Sweden (0.8% of the invasive *S. aureus* in 2003 [SWEDRES 2003, a report on Swedish antibiotic utilization and resistance in human medicine]), but increasing, and this method is suitable for a laboratory operating in a low-prevalence area.

To conclude, this protocol for MLST of *S. aureus* adapted to the real-time LightCycler System PCR offers an efficient and rapid amplification of seven housekeeping genes in the same PCR run and real-time detection of the products without gel electrophoresis. This application is suitable and convenient to perform when a small number of isolates of MRSA will be investigated, particularly in an area of low endemicity as in Sweden. MLST of 57 representative clinical MRSA isolates from such an area showed a high heterogeneity, with 20 STs identified and nine clonal complexes. Combining ST and *SCCmec* types distinguished 27 different genotypes. The presence of various *SCCmec* types in the same ST indicates that the MRSA clones occurred on several occasions in the same genetic background by independent insertions of *SCCmec* into methicillin-sensitive strains. This circumstance shows the importance of analyzing MLST data together with the *SCCmec* typing results when investigating the origins of MRSA.

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REFERENCES

1. Aires de Sousa, M., and H. de Lencastre. 2003. Evolution of sporadic isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals and their similarities to isolates of community-acquired MRSA. *J. Clin. Microbiol.* **41**:3806–3815.
2. Berglund, C., P. Mölling, L. Sjöberg, and B. Söderquist. 2005. Predominance of staphylococcal cassette chromosome *mec* (*SCCmec*) type IV among methicillin-resistant *S. aureus* (MRSA) in a Swedish County, and presence of unknown *SCCmec* types with Panton-Valentine leukocidin genes. *Clin. Microbiol. Infect.* **11**:447–456.
3. Centers for Disease Control and Prevention. 1999. Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*—Minnesota and North Dakota, 1997–1999. *JAMA* **282**:1123–1125.
4. Crisostomo, M. I., H. Westh, A. Tomasz, M. Chung, D. C. Oliveira, and H. de Lencastre. 2001. The evolution of methicillin resistance in *Staphylococcus aureus*: similarity of genetic backgrounds in historically early methicillin-susceptible and -resistant isolates and contemporary epidemic clones. *Proc. Natl. Acad. Sci. USA* **98**:9865–9870.
5. Daum, R. S., T. Ito, K. Hiramatsu, F. Hussain, K. Mongkolrattanothai, M. Jamklang, and S. Boyle-Vavra. 2002. A novel methicillin-resistance cassette in community-acquired methicillin-resistant *Staphylococcus aureus* isolates of diverse genetic backgrounds. *J. Infect. Dis.* **186**:1344–1347.
6. Enright, M. C. 2003. The evolution of a resistant pathogen—the case of MRSA. *Curr. Opin. Pharmacol.* **3**:474–479.
7. Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt. 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci. USA* **99**:7687–7692.
8. Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J. Clin. Microbiol.* **38**:1008–1015.
9. Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt. 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol.* **186**:1518–1530.
10. Feil, E. J., J. M. Smith, M. C. Enright, and B. G. Spratt. 2000. Estimating recombinational parameters in *Streptococcus pneumoniae* from multilocus sequence typing data. *Genetics* **154**:1439–1450.
11. Fey, P. D., B. Said-Salim, M. E. Rupp, S. H. Hinrichs, D. J. Boxrud, C. C. Davis, B. N. Kreiswirth, and P. M. Schlievert. 2003. Comparative molecular analysis of community- or hospital-acquired methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **47**:196–203.
12. Hanssen, A. M., G. Kjeldsen, and J. U. Sollid. 2004. Local variants of *Staphylococcal cassette chromosome mec* in sporadic methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative *Staphylococci*: evidence of horizontal gene transfer? *Antimicrob. Agents Chemother.* **48**:285–296.
13. Ito, T., K. Okuma, X. X. Ma, H. Yuzawa, and K. Hiramatsu. 2003. Insights

- on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. *Drug Resist. Updates* **6**:41–52.
14. Ito, T., X. X. Ma, F. Takeuchi, K. Okuma, H. Yuzawa, and K. Hiramatsu. 2004. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. *Antimicrob. Agents Chemother.* **48**:2637–2651.
 15. Ito, T., Y. Katayama, K. Asada, N. Mori, K. Tsutsumimoto, C. Tiensasitorn, and K. Hiramatsu. 2001. Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **45**:1323–1336.
 16. Jevons, M. P. 1961. Celbenin-resistant staphylococci. *Br. Med. J.* **1**:124–125.
 17. Johnsson, D., P. Mölling, K. Strålin, and B. Söderquist. 2004. Detection of Panton-Valentine leukocidin gene in *Staphylococcus aureus* by LightCycler PCR: clinical and epidemiological aspects. *Clin. Microbiol. Infect.* **10**:884–889.
 18. Katayama, Y., F. Takeuchi, T. Ito, X. X. Ma, Y. Ui-Mizutani, I. Kobayashi, and K. Hiramatsu. 2003. Identification in methicillin-susceptible *Staphylococcus hominis* of an active primordial mobile genetic element for the staphylococcal cassette chromosome *mec* of methicillin-resistant *Staphylococcus aureus*. *J. Bacteriol.* **185**:2711–2722.
 19. Liassine, N., R. Auckenthaler, M. C. Descombes, M. Bes, F. Vandenesch, and J. Etienne. 2004. Community-acquired methicillin-resistant *Staphylococcus aureus* isolated in Switzerland contains the Panton-Valentine leukocidin or exfoliative toxin genes. *J. Clin. Microbiol.* **42**:825–828.
 20. Ma, X. X., T. Ito, C. Tiensasitorn, M. Jamklang, P. Chongtrakool, S. Boyle-Vavra, R. S. Daum, and K. Hiramatsu. 2002. Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. *Antimicrob. Agents Chemother.* **46**:1147–1152.
 21. Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. USA* **95**:3140–3145.
 22. Mongkolrattanothai, K., S. Boyle, M. D. Kahana, and R. S. Daum. 2003. Severe *Staphylococcus aureus* infections caused by clonally related community-acquired methicillin-susceptible and methicillin-resistant isolates. *Clin. Infect. Dis.* **37**:1050–1058.
 23. Okuma, K., K. Iwakawa, J. D. Turnidge, W. B. Grubb, J. M. Bell, F. G. O'Brien, G. W. Coombs, J. W. Pearman, F. C. Tenover, M. Kapi, C. Tiensasitorn, T. Ito, and K. Hiramatsu. 2002. Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *J. Clin. Microbiol.* **40**:4289–4294.
 24. Oliveira, D. C., A. Tomasz, and H. de Lencastre. 2002. Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*. *Lancet Infect. Dis.* **2**:180–189.
 25. Peacock, S. J., G. D. de Silva, A. Justice, A. Cowland, C. E. Moore, C. G. Winearls, and N. P. Day. 2002. Comparison of multilocus sequence typing and pulsed-field gel electrophoresis as tools for typing *Staphylococcus aureus* isolates in a microepidemiological setting. *J. Clin. Microbiol.* **40**:3764–3770.
 26. Robinson, D. A., and M. C. Enright. 2004. Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. *Clin. Microbiol. Infect.* **10**:92–97.
 27. Tenover, F., R. Arbeit, R. Goering, P. Mickelsen, B. Murray, D. Persing, and N. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**:2233–2239.
 28. Vandenesch, F., T. Naimi, M. C. Enright, G. Lina, G. R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M. E. Reverdy, and J. Etienne. 2003. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg. Infect. Dis.* **9**:978–984.
 29. Wannet, W., M. Heck, G. Pluister, E. Spalburg, M. Van Santen, X. Huijsdens, E. Tiemersma, and A. J. de Neeling. 2004. Panton-Valentine leukocidin positive MRSA in 2003: the Dutch situation. *Eur. Surveill.* **9**:3–4. [Online.]
 30. Wisplinghoff, H., A. E. Rosato, M. C. Enright, M. Noto, W. Craig, and G. L. Archer. 2003. Related clones containing SCCmec type IV predominate among clinically significant *Staphylococcus epidermidis* isolates. *Antimicrob. Agents Chemother.* **47**:3574–3579.
 31. Witte, W., C. Bräulke, C. Cuny, B. Strommenger, G. Werner, D. Heuck, U. Jappe, C. Wendt, H. J. Linde, and D. Harmsen. 2005. Emergence of methicillin-resistant *Staphylococcus aureus* with Panton-Valentine leukocidin genes in central Europe. *Eur. J. Clin. Microbiol. Infect. Dis.* **24**:1–5.