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A Novel Multiplex Real-Time PCR Assay for Rapid Typing of Major Staphylococcal Cassette Chromosome *mec* Elements

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We describe a novel procedure for rapid typing of the staphylococcal cassette chromosome *mec* element, a molecular marker allowing discrimination between community- and hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Oligonucleotides targeting the recombinase genes were type specific and used to type a collection of 399 MRSA isolates recovered during patient screening at admission. This novel assay constitutes a valuable tool for evaluating the molecular epidemiology of MRSA and adjusting infection control strategies against MRSA.

Staphylococcus aureus is a major pathogen responsible for both nosocomial and community-acquired infections. While the first methicillin-resistant *S. aureus* (MRSA) isolates were reported in the early 1960s (1), endemic strains of MRSA carrying multiple resistance determinants became a worldwide nosocomial problem only in the early 1980s (8). The presence of MRSA in an institution is paralleled by an increased rate of bacteremia or other severe MRSA infections (5). The spread of MRSA in health care centers is difficult to control and requires elaborate infection control guidelines (6, 17, 18). The difficulty of eradicating nosocomial MRSA infections may be explained by (i) the presence of an unknown hidden reservoir of carriers, (ii) the emergence of novel epidemic *S. aureus* clones (2), or (iii) failures in infection control.

Recently, the appearance of endemic community-acquired MRSA (CA-MRSA) has been reported (13, 16, 20). In contrast to hospital-acquired MRSA (HA-MRSA), CA-MRSA is frequently isolated from healthy people. Most of these strains are susceptible to several older but clinically important antibiotics (20). An additional characteristic of CA-MRSA strains potentially explaining their emergence is the carriage of exotoxins (3), in particular Panton-Valentine leukocidin (PVL) (20, 21), which is involved in recurrent skin and soft tissue infections or lethal necrotizing pneumonia (4).

The genetic basis of the methicillin resistance of MRSA isolates is the presence of *mecA*, a gene coding for the low-affinity penicillin-binding protein PBP2′, which is invariably located on a mobile genetic element designated SCC-*mec* (staphylococcal cassette chromosome *mec*) (11), flanked by terminal inverted and direct repeats (9). This element contains two site-specific cassette chromosome recombinases, *ccrA* and *ccrB*, responsible for the precise excision and integration of SCC-*mec* within the bacterial chromosome (11).

Four differently organized SCC-*mec* elements have been characterized (10). Three types of SCC-*mec* elements are typically found in HA-MRSA strains: (i) type I, a 34-kb element that was prevalent in MRSA isolates in the 1960s; (ii) type II, a 53-kb element that was identified in 1982 and is ubiquitous in Japan, Korea, and the United States; and (iii) type III, the largest element at 67 kb, which was identified in 1985 and is prevalent in Germany, Austria, India, and other South Asian and Pacific areas (7, 11). In contrast to HA-MRSA isolates, CA-MRSA isolates generally carry the SCC-*mec* type IV element, which is much smaller than the SCC-*mec* type I, II, and III elements (10, 12). At least four subtypes of the type IV SCC-*mec* element, whose sizes vary from 20 to 24 kb, have been reported (IVa to IVc [10] and IVd [accession number AB097677]).

The molecular composition of the four SCC-*mec* elements reveals key components useful for SCC-*mec* typing (9). Variations in this gene set allowed the identification of four classes of *mecA* gene complexes (7, 10). A second essential region is the *ccr* gene complex mentioned above. Types I and III harbor *ccrA1-B1* and *ccrA3-B3* recombinases, respectively; whereas both types II and IV contain *ccrA2-B2* recombinases, showing some difference in their amino acid sequences (9).

The aim of this study was to develop a novel procedure for rapid typing of the SCC-*mec* element for use as a molecular epidemiological screening assay. Type-specific oligonucleotides targeting the recombinase genes (*ccr*) were selected and validated against reference strains. Finally, the distribution of SCC-*mec* types was determined in a collection of 399 MRSA isolates recovered during systematic screening of patients at hospital admission. An additional objective was to evaluate the possible relationship between the presence of the SCC-*mec* type IV element and that of the gene for PVL.

The study protocol was approved by the institution review board as a Continuous Quality Improvement project. No informed consent was requested.

Strain collection, identification, and antimicrobial susceptibility testing. Three hundred ninety-nine MRSA isolates were obtained from >14,000 patients screened at hospital admission (January to August 2003). Identification was performed on ORSA plates (Oxoid, Basingstoke, United Kingdom). Further identification of MRSA was based on Pastorex

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TABLE 1. List and characteristics of oligonucleotides used in the qPCR and PCR assays

Primer or probe name	Sequence (5′→3′)	Length (bases)	5′ dye	EMBL accession no.	Reference
ccrB type I				AB033763	10
F_*ccrB* Type I	TTTGGCACGTAATACTTCCGATT	23			
R_*ccrB* Type I	AAAATTCAACATTTTGGCGATGA	23			
P_*ccrB* Type I[a]	ACTTACAATAGTCGAAGAAC	20	FAM		
ccrB type II				D86934	10
F_*ccrB* Type II	AACGAGACGTGCCCAAGAAG	20			
R_*ccrB* Type II	CATCAGTTCATGTTTACTATTAGGTATTTTGTC	33			
P_*ccrB* Type II[a]	ATTTGCCGCTGGGCT	15	VIC		
ccrB type III				AB037671	10
F_*ccrB* Type III	ACAATCCACAGTCATTACAT	20			
R_*ccrB* Type III	AGTTACGACTTTCTGTTTCA	20			
P_*ccrB* Type III[b]	ACAAGTCACCGAACGCATCCAACAAA	26	FAM		
ccrB type IV				AB097677, AB096217, AB063172, AB063173	10[c]
F_*ccrB* Type IV	GAACAGACCTGAGCTCCAACGT	22			
R_*ccrB* Type IV	TCGGTTTGTTTTGTAGATCATAACACA	27			
P_*ccrB* Type IV[a]	ATGCAAAAGAAGGCAATAT	19	TET		
PVL				X72700	19
F_*pvl*	AAAATGCCAGTGTTATCCAGAGGTA	25			
R_*pvl*	TTTGCAGCGTTTTGTTTTCG	20			
P_*pvl*[b]	CTTCAATCCAGAATTTATTGGTGT	24	FAM		
Degenerate PCR primers for *ccrA* consensus					
F_*ccrA*	AYRAAYCAACAATCBYTNNCAGC	23			
R_*ccrA*	TTNANNGGTTCATTTTTGAAATAGAT	26			

[a] Minor groove binder probes with nonfluorescent quencher bound to the 3′ end (Applied Biosystems).
[b] Taqman probes with a 3′ TAMRA quencher (Eurogentec).
[c] The sequence of SCC-*mec* type IVd has not been published but is available under accession number AB097677.

agglutination (Bio-Rad, Reinach, Switzerland), DNase reaction on agar, and growth on Mueller-Hinton oxacillin plates (6 μg of oxacillin per ml, in accordance with NCCLS guidelines [14]). MRSA identification was confirmed with the Vitek 2 identification and susceptibility testing cards for gram positives (bioMérieux, Marcy l'Etoile, France).

Sequence analysis, primers, and probe selection. Type I, II, and III *ccrB* genes (see Table 1 for accession numbers) were aligned with ClustalW to localize divergent regions, allowing the design of type-specific oligonucleotides with the PrimerExpress 2.0 software (PE Biosystems, Foster City, Calif.). The four type IV *ccrB* subtype sequences were aligned to localize homologous regions. The derived consensus sequence was then aligned against the type I, II, and III sequences to localize nonhomologous regions to design type IV-specific primers and probes. The reference strains used to optimize primer and probe concentrations for each SCC-*mec* type were *S. aureus* COL, N315, and MW2, for types I, II, and IV, respectively. The type III strain was a clinical isolate obtained from the Clinical Microbiology Laboratory of the University of Geneva Hospitals. The sequence of the F component of PVL was used to select specific primers and probes, yielding an amplicon of 75 nucleotides between positions 2707 and 2783. Selected oligonucleotide specificity was tested by using the reference strain (ATCC 49775) and the four fully sequenced isolates as negative (N315, COL, and Mu50) and positive (MW2) controls.

Bacterial lysis. Genomic DNA was extracted from one colony suspended in 200 μl of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA). One hundred milligrams of glass beads (diameter, 100 μm; Schieritz and Hauenstein, Arlesheim, Switzerland) was added to the suspension, and bacteria were lysed by vortexing at maximum power for 45 s. The liquid phase was cleared of beads and bacterial debris by centrifugation, and a 5-μl aliquot was used for quantitative PCR (qPCR) assays.

Nucleic acid detection by qPCR and analysis. Each analysis was performed in triplicate; the nucleic acids from the reference strains were simultaneously assayed in each run. Conditions for the amplification on the SDS 7700 (Applied Biosystems) were as follows: t_1, 2 min at 50°C; t_2, 10 min at 95°C; t_3, 15 s at 95°C; t_4, 1 min at 60°C (t_3 and t_4 were repeated 30 times). The volume of the PCR mixture (Eurogentec, Seraing, Belgium) was 20 μl and contained all primers and probes (Table 1) at 100 and 50 nM, respectively. Default analysis parameters were used with the SDS 1.9 software; the standard deviation of fluorescence values recorded from cycles 3 to 15 was multiplied by 10 to define the cycle threshold line. Cycle thresholds were derived from the intercept between this line and the signal obtained during the qPCR. A reaction was considered positive when fluorescence levels exceeded the detection threshold between cycles 15 and 30.

Of the 399 MRSA isolates, 10 failed to produce signals with any of the four qPCR primers and probes. We amplified by PCR (with *Pfu* from Stratagene, La Jolla, Calif.) and sequenced a portion of the SCC-*mec* elements in both directions with an ABI Prism 3100 sequencer (Applied Biosystems). The SCC-*mec* type was then determined by sequence comparison with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

TABLE 2. Antimicrobial susceptibilities of 398[a] MRSA isolates recovered at hospital admission

Drug(s)	% of isolates susceptible		
	Type I (n = 348)	Type II (n = 7)	Type IV (n = 43)
Penicillin	0	0	0
Oxacillin	0	0	0
Ciprofloxacin	3	20	49
Gentamicin	1	76	93
Erythromycin	9	0	63
Clindamycin	10	50	85
Fusidic acid	99	85	99
Trimethoprim-sulfamethoxazole	99	100	100
Amikacin	1	29	30
Vancomycin	100	100	100

[a] The type III strain is not shown in this table.

Assay specificity and robustness. The cycle thresholds obtained with the multiplex qPCR assay for reference strains representative of each SCC-*mec* type showed the expected type-specific signals. Specific signals were recorded with each reference strain in a range of 20 to 21 cycles, whereas no cross-reactive signal from alternative SCC-*mec* types was detected within 30 cycles. The robustness of the qPCR assay was assessed by sequencing amplicons generated in control strains and 15 clinical isolates (from type I, II, or IV) with degenerate PCR primers (Table 1), yielding a >1.4-kb amplicon. Determined sequences were analyzed by BLAST, and the results were compared to the type attributed by the rapid qPCR assay. BLAST results obtained for sequencing experiments matched perfectly the SCC-*mec* types, as determined by the multiplex qPCR assay for all of the strains tested.

This report describes a novel multiplex qPCR assay that permits rapid and robust determination of SCC-*mec* types and the presence of the gene for PVL in a few hours. To date, the only procedures allowing SCC-*mec* typing have relied on PCR amplification and separation of synthesized products (15), a time-consuming and labor-intensive strategy. The method described here is sensitive and rapid, allowing the testing of 100 samples per day in triplicate at a reasonable reagent cost of $6 to $7 per sample.

A total of 399 strains collected during systematic screening of patients at hospital admission were analyzed for SCC-*mec* typing, the presence of a PVL-related gene, and antimicrobial susceptibility profiles. The overwhelming majority of MRSA isolates belonged to the type I cassette (87%, n = 348), whereas the type II cassette was rarely identified (2%, n = 7) and the type III cassette was marginally detected (0.2%, n = 1). The type IV cassette was recovered in 11% of the isolates (n = 43). Unexpectedly, the prevalence of the gene for PVL was low (9.3%) in this group.

Each group of SSC-*mec* type revealed a characteristic antimicrobial susceptibility pattern (Table 2). A large majority of MRSA isolates was found to harbor the ancestral SCC-*mec* type I element (10), which has been reported to be devoid of antibiotic resistance genes except for the methicillin locus (10). Despite the absence of other antibiotic resistance determinants on the type I SCC-*mec*, a large majority of these strains were resistant to erythromycin, ciprofloxacin, gentamicin, and amikacin. All strains harboring the SCC-*mec* type II element containing Tn*554* were resistant to erythromycin. In contrast, type IV strains were still susceptible to several drugs, such as gentamicin, clindamycin, and trimethoprim-sulfamethoxazole.

The multiple antibiotic resistance determinants displayed by the majority of type I isolates suggest several episodes of genetic material exchange besides acquisition of the SCC-*mec* element. Determination of SCC-*mec* element origin could contribute to our understanding of the ability of strains to acquire genetic material providing selective advantages. A recent study reported type I recombinase homologues on a SCC-like element carried by a methicillin-susceptible strain of *S. hominis* (11). This homologue, lacking the *mec* determinant, may represent an ancestral prototype of SCC-*mec* elements transferred between staphylococcal species.

Strains carrying the SCC-*mec* type IV element were the second predominant group in our study, an unexpected high frequency suggesting rapid spread of CA-MRSA in the population. Two previous reports indicated that SCC-*mec* type IV clinical isolates were frequently PVL toxin producers (20, 21). In our study, only 9.3% of SCC-*mec* type IV isolates harbored the PVL locus. The low prevalence of PVL in our SCC-*mec* type IV isolates may be related to the study design targeting on-admission carriers with asymptomatic colonization. Linkage of the two markers could not be used as a predictive indicator in our population. A recent study of U.S. isolates (3) associated the high pathogenicity of CA-MRSA strains with the presence of a superantigen(s), a parameter to be investigated in our collection.

In summary, MRSA isolates carrying SCC-*mec* type I and IV elements were major contributors to the population observed in our setting. An important finding was the relatively high proportion of type IV CA-MRSA isolates in our on-admission population. This could indicate the rapid emergence of a new MRSA lineage(s) (3) with particular fitness for spread in the community. Particular attention should be paid to the early detection of CA-MRSA strains in hospitals because of their potential for easy transmission of the type IV SCC-*mec* element to nosocomial methicillin-susceptible *S. aureus* isolates. An additional risk is the threat of transmission of the PVL toxin to nosocomial MRSA and methicillin-susceptible *S. aureus*. Further epidemiological and molecular typing studies are needed to document CA-MRSA carriage and infection rates and implement adequate infection control guidelines for this emerging pathogen.

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