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Detection of New Methicillin-Resistant *Staphylococcus aureus* Strains That Carry a Novel Genetic Homologue and Important Virulence Determinants

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In this study, 18 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates harboring staphylococcal cassette chromosome *mec* (SCC*mec*) type XI, recovered in the Dutch-German Euregio, were characterized by DNA microarrays. In contrast to previous data, we found two MRSA strains of different clonal lineages possessing SCC*mec* XI that carried important virulence determinants. The worrisome emergence of such toxigenic MRSA strains raises concerns that MRSA strains with enhanced virulence potential and impaired detectability by standard molecular assays may spread in Europe.

The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in livestock has caused significant concern during the past decade (4, 11).Very recently, the situation has been complicated by the detection of bovine and human MRSA isolates carrying a new staphylococcal cassette chromosome *mec* (SCC*mec*) element (type XI) that leads to phenotypic β-lactam resistance (9, 19). This SCC*mec* element contains a novel *mecA* homologue, first isolated from strain LGA251 and designated $mecA_{LGA251}$, which is not reliably detectable by conventional molecular approaches and PBP2a agglutination tests (9, 19). MRSA isolates with $mecA_{LGA251}$ have been recovered so far in England, Denmark, Germany, and Ireland and mostly belonged to clonal complex 130 (CC130) as determined by multilocus sequence typing (MLST) (5, 9, 12, 19). Other MRSA isolates with SCC*mec* XI were representatives of MLST CC425, CC705, and CC1943 (9). However, an analysis of the virulence potential of MRSA harboring SCC*mec* XI was until now only reported for isolates associated with the CC130 clonal lineage (*S. aureus* protein A gene [*spa*] sequencing types t373, t843, t1736, and t1773), which demonstrate an absence of pyrogenic toxin superantigen (PTSAg)-encoding genes, including those of the toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins, and of exfoliative toxin genes (with the exception of the *edinB* gene) (5, 19).

In this study, we compare the virulence-associated gene content of 18 MRSA isolates from three distinct clonal lineages containing the novel *mecA* homologue $mecA_{LGA251}$.

A total of 18 nonrepetitive MRSA isolates harboring SCC*mec* XI with $mecA_{LGA251}$ were characterized in this study. Among these, 16 isolates were obtained from a previously described collection already characterized by *spa* typing, antimicrobial susceptibility testing, and the presence and orientation of $mecA_{LGA251}$, *mecR1*, *mecI*, *blaZ*, *ccrA*, and *ccrB*, which are related to type XI SCC*mec* (12). The isolates had *spa* types t843 ($n = 11$), t978 ($n = 1$), t1535 ($n = 2$), t1773 ($n = 1$), and t7189 ($n = 1$). The remaining two isolates were newly recovered from nasal swabs of patients in hospitals participating in the project EurSafety Healthnet (www.eursafety.eu) and located in the Dutch-German Euregio Twente-Münsterland. One isolate was recovered on the Dutch side and one isolate on the German side of the border. All isolates were characterized by antimicrobial susceptibility testing by means of the Vitek 2 system (bioMérieux SA, Marcy l'Etoile, France) and the Clinical and Laboratory Standards Institute (CLSI) reference broth microdilution (BMD) method (2). The BMD method was performed with cation-supplemented Mueller-Hinton broth (BBL; BD Diagnostic Systems, Sparks, MD), and the CLSI-recommended reference strain *S. aureus* ATCC 29213 was used as the control with every set of tests. Susceptibility was determined according to standard CLSI breakpoints (3). The isolates were characterized by using the Xpert MRSA-SA assay on a GeneXpert DX system real-time PCR platform (Cepheid, Sunnyvale, CA). DNA microarray analysis was performed using the StaphyType and PM7Plus kits (Alere Technologies GmbH, Jena, Germany). *spa* typing was carried out as described by Aires-de-Sousa et al. (1) using StaphType software version 2.2.1 (Ridom GmbH, Münster, Germany) (10). Clustering of the isolates was conducted by the BURP (based upon repeat pattern) algorithm implemented in StaphType, with the calculated cost between members of a group set as less than or equal to 4 (default value). MLST was performed as described by Enright et al. (7). The MLST groups were defined by the eBURST (based upon related sequence types) method, using eBURSTv3 (eburst.mlst.net/v3/mlst_datasets) (8, 20). Isolates with at least six of the seven alleles being identical were classified in the same clonal complex. Total DNA was extracted from all isolates by enzymatic lysis using the buffers and solutions provided with the StaphyType DNA microarray kit (Alere Technologies GmbH, Jena, Germany) and the Qiagen DNeasy blood and tissue kit (Crawley, West Sussex, United King-

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dom) as described previously (15). The presence of tested genes encoding species markers, *agr* types, and virulence factors, as well as the affiliation to clonal complexes, was determined by using the StaphyType DNA microarray kit as described elsewhere (14, 15).

In the testing of 277 phenotypically determined MRSA isolates by microarrays using the StaphyType kit, one isolate of *spa* type t5930 did not show a signal from the *mecA* and other probes designed for the detection of the SCC*mec* elements, with the exception of the probe for *ccrA3* recombinase. Furthermore, this isolate was tested by microarrays using the not-yet commercially available PM7Plus kit for confirmation of the presence of SCC*mec* XI by detecting *mecA* and *blaZ* alleles of strains M10 and LGA251. This experiment revealed the presence of SCC*mec* XI in the tested isolate, because *mecA* and *blaZ* alleles of strains M10 and LGA251 were detected.

Using the BURP analysis, we found a single isolate in our collection showing *spa* type t7603 that had not been previously described as $mecA_{LGA251}$ MRSA but which differed from *spa* type t843 only by one repeat. The microarray approach using the StaphyType and PM7Plus kits revealed that this isolate contained SCC*mec* XI, because *mecA* and *blaZ* alleles of strains M10 and LGA251 were detected.

All isolates were *mecA* negative in the GeneXpert MRSA real-time PCR assay. Based on Vitek 2 susceptibility determination, all isolates tested were resistant to penicillin and cefoxitin but susceptible to gentamicin, tobramycin, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin, tetracycline, fosfomycin, nitrofurantoin, fusidic acid, mupirocin, rifampin, and trimethoprim-sulfamethoxazole. Using the CLSI BMD method, we found 2 isolates which displayed an oxacillin MIC in the CLSI susceptible breakpoint range (≤2 μg/ml). These 2 isolates had *spa* types t843 and t7603 with oxacillin MICs of 0.35 and 0.6 μg/ml, respectively. Two other $mecA_{LGA251}$-positive isolates showed oxacillin MICs as low as 3 μg/ml. The remaining isolates displayed an oxacillin MIC in the CLSI resistant breakpoint range (≥4 μg/ml) (Table 1). The BMD results showed that all isolates were resistant to cefoxitin and penicillin G but susceptible to all non-β-lactam antibiotics (Table 1).

For all 18 MRSA isolates included in this study, a BURP analysis was performed, which clustered 16 isolates associated with five *spa* types (t843, t1535, t1773, t7189, and t7603) into the *spa* clonal complex 843/1535 (*spa* CC843/CC1535), while 2 remaining isolates (*spa* types t978 and t5930) were singletons. In previous investigations, isolates of *spa* types t843 and t1773 were characterized by MLST as clonal complex 130 (CC130) (5, 9, 19). Therefore, the isolates of *spa* CC843/CC1535 were attributed to MLST CC130. Furthermore, we characterized the isolates of *spa* types t978 and t5930 by MLST, which exhibited a new sequence type (ST) assigned by the *S. aureus* MLST curator as ST2361 and an allelic profile which matched that of ST599 in the MLST database (saureus.beta.mlst.net), respectively. To determine the genotypes in the whole *S. aureus* MLST database that were closely related to the newly identified STs with SCC*mec* XI, the allelic profiles of ST2361 and ST599 were subjected to an eBURSTv3 analysis. The eBURSTv3 method allocated ST2361 to CC1943, as it differed from ST1943 at only one locus. For ST599, with the exception of ST2179 (single-locus variant of ST599), the eBURSTv3 analysis did not find related STs.

The DNA microarray (Alere Technologies) results for the 18 $mecA_{LGA251}$ MRSA isolates are shown in Table 2. Isolates of *spa* CC843/CC1535 did not carry toxin genes, with the exception of the *edinB* gene. In contrast, other identified MRSA genotypes containing SCC*mec* XI were characterized by the presence of higher number of toxin genes: the t5930 isolate possessed three PTSAg genes (*tst1*, *sec*, and *sel*), while the t978 isolate carried eight PTSAg genes (*tst1*, *sec*, *seg*, *sei*, *sel*, *sen*, *seo*, and *seu*).

So far, MRSA isolates with $mecA_{LGA251}$ have rarely been detected in cases of human infections. However, the identification of such isolates from joint fluids and infected wound sites (12) indicates that they have the ability to cause infections in humans. Therefore, the emergence of strains with novel $mecA_{LGA251}$ harboring numerous toxin genes is worrying, since those isolates are not detectable by either commercially available or in-house nucleic acid detection approaches targeting the classical *mecA* gene. Moreover, a substantial increase in MRSA isolates with $mecA_{LGA251}$ was detected among isolates obtained in Denmark between 2007 and 2010 (9), which raises issues about the necessity of continuous monitoring of these strains to control their resistance to clinically important antimicrobials and the occurrence of new virulence patterns.

TABLE 1 MICs of β-lactam and non-β-lactam antibiotics for the $mecA_{LGA251}$ MRSA isolates, determined by broth microdilution

Antimicrobial class and agent	MIC range (μg/ml)
β-lactams	
Penicillins	
Penicillin	0.4–8
Oxacillin	0.35–32
Cephalosporins	
Cefoxitin	8–64
Non-β-lactams	
Glycopeptides	
Vancomycin	0.5–1
Teicoplanin	0.5–1
Lincosamides	
Clindamycin	0.125–0.25
Oxazolidinones	
Linezolid	0.125–0.5
Rifamycins	
Rifampin	0.004–0.008
Fosfomycins	
Fosfomycin	1.2–9.6
Folate pathway inhibitors	
Trimethoprim-sulfamethoxazole	0.024–0.48
Aminoglycosides	
Gentamicin	0.2–0.4
Tobramycin	0.256–0.512
Fluoroquinolones	
Ciprofloxacin	0.08–0.16
Levofloxacin	0.2–0.4
Moxifloxacin	0.04–0.08
Macrolides	
Erythromycin	0.025–0.4
Monoxycarbolic acids	
Mupirocin	0.128–0.256
Tetracyclines	
Tetracycline	0.125–1
Nitrofurans	
Nitrofurantoin	2–8
Others	
Fusidic acid	0.064–0.128

TABLE 2 Virulence microarray hybridization profiles of the $mecA_{LGA251}$ MRSA isolates assigned to *spa* CC843/CC1535 complex and *spa* types t978 and t5930

Gene class	Gene(s)	DNA microarray hybridization profile of isolates assigned to[a]: CC843/CC1535	t978	t5930
Species markers	*coa*, *gapA*, *nuc*, *katA*, *spa*	Pos	Pos	Pos
Hemolysins	*hla*, *hlb*, *hld*	Pos	Pos	Pos
Immune evasion	*sak*, *chp*, *scn*	Neg	Neg	Neg
	aur	Pos	Pos	Pos
	splA, *splB*	Pos	Pos	Pos
	splE	Neg/Pos[b]	Pos	Pos
Superantigens	*tst1*	Neg	Pos	Pos
	sea, *seb*, *see*, *seh*	Neg	Neg	Neg
	sec-sel	Neg	Pos	Pos
	sed-sej-ser	Neg	Neg	Neg
	seg-sei-sem-sen-seo-seu	Neg	Pos	Neg
	sek-seq	Neg	Neg	Neg
Exfoliative toxins	*eta*, *etb*, *etd*	Neg	Neg	Neg
	edinA	Neg	Neg	Neg
	edinB	Pos	Neg	Neg
Biofilm	*ica* operon	Pos	Pos	Pos
Capsule		*cap8*	*cap5*	*cap5*
agr type		III	IV	I

[a] Only clinically relevant markers, including exotoxins and resistance genes, are shown. The table does not represent the complete hybridization profile of 334 probes on the array. Pos, positive; Neg, negative.
[b] Two t843 isolates and one t1773 isolate were *splE* positive.

In this paper, we do not provide direct evidence that animals may be reservoirs of the more toxigenic MRSA ST599 and ST1946 identified in this study, since the strains were recovered from asymptomatic human carriers. However, as is typical for *S. aureus* strains from animal origins (13), a lack of the genes *sak*, *chp*, and *scn* that are involved in human immune evasion can show that animals could be the epidemiological source of these strains.

In our study, we found for 2 isolates a lack of association between the presence of $mecA_{LGA251}$ and oxacillin resistance. However, isolates with the novel *mecA* homologue and low-level β-lactam resistance (exhibiting oxacillin MICs of 1.0 μg/ml and 2.0 μg/ml) have been described by Shore and colleagues (19). The discovery of isolates that were both $mecA_{LGA251}$ positive and had oxacillin MICs in the susceptible range of the CLSI breakpoint (≤2 μg/ml) increasingly complicates the detection and confirmation of MRSA. Failure to detect such MRSA isolates could have serious implications in the suboptimal implementation of infection control measures or therapeutic strategies.

In this work, we tested 16 MRSA isolates which were investigated previously (12). In both investigations, oxacillin MICs were determined. In general, oxacillin MICs obtained in the current study were lower than those determined in the previous study (12). However, different antibacterial agent susceptibility testing methods were used in the two investigations. BMD was the method for MIC determination in this study, while previously, the Etest (bioMérieux SA, Marcy-l'Étoile, France) was applied. Very recently, Rennie and colleagues (17) compared the MICs obtained by BMD and Etest, and they concluded that the bioMérieux Etest often reported higher MICs than the CLSI reference BMD method.

Among *S. aureus* isolates carrying the *mecA* gene, the antibiotic susceptibility patterns most similar to those of MRSA isolates harboring SCC*mec* XI can be found in community-associated MRSA (CA-MRSA) strains, which are classically characterized by susceptibility to the majority of non-β-lactam antimicrobial agents (6). A number of published studies described CA-MRSA strains of SCC*mec* IV as susceptible to all non-β-lactam antibiotics (6, 18). Also, some CA-MRSA strains can display low-level oxacillin resistance (16).

The current study showed acquisition of SCC*mec* XI with $mecA_{LGA251}$ by *S. aureus* isolates with additional genotypes (with *spa* types t5930 and t7603), which complicates the identification of MRSA. Thus, DNA-based tests, which have been developed to detect MRSA more rapidly, cannot completely replace phenotypic culture-based MRSA screening tests.

Although most of the MRSA isolates with SCC*mec* XI carried none of the tested virulence factors, the two toxin gene-positive isolates identified in this study showed the genomic plasticity known from "classical" MRSA. This could indicate that we are likely to face further surprises, such as new waves of animal-related MRSA or the emergence of further *mecA* homologues.

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