Different Clonal Complexes of Methicillin-Resistant *Staphylococcus aureus* Are Disseminated in the Euregio Meuse-Rhine Region

Ruud H. Deurenberg,1 Cornelis Vink,1 Guy J. Oudhuis,1 Jascha E. Mooij,1 Christel Driessen,1
Guy Coppens,2 Jos Craeghs,3 Els De Brauwer,4 Sebastian Lemmen,5 Hans Wagenvoort,4
Alexander W. Friedrich,6 Jacques Scheres,3 and Ellen E. Stobberingh1*

Department of Medical Microbiology, Maastricht Infection Center (MINC), University Hospital Maastricht, Maastricht, The Netherlands;1 Hospital East-Limbourg, Genk, Belgium;2 General Hospital Vesalius, Tongeren, Belgium;3 Atrium Medical Centre, Heerlen, The Netherlands;4; Universitätsklinikum Aachen, Aachen, Germany;5; and Institute of Hygiene, University of Münster, Münster, Germany6

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The Euregio Meuse-Rhine (EMR) is formed by the border regions of Belgium, Germany, and The Netherlands. Cross-border health care requires infection control measures, in particular since the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) differs among the three countries. To investigate the dissemination of MRSA in the EMR, 152 MRSA isolates were characterized by pulsed-field gel electrophoresis (PFGE), SCCmec typing, and multilocus sequence typing. PFGE revealed major clonal groups A, G, L, and Q, suggesting dissemination of MRSA in the EMR. Group A harbored mainly SCCmec type III and sequence types (STs) 239 and 241. The majority of the strains from group G harbored SCCmec type I and ST8 and ST247, whereas most strains from group L carried either SCCmec type IV or type I. Within group L, ST8 and ST228 were found, belonging to clonal complexes 8 and 5, respectively. Most strains from group Q included SCCmec type II and were sequence typed as ST225. Both ST225-MRSA-II and ST241-MRSA-III were novel findings in Germany. In addition, the SCCmec type of two isolates has not been described previously. One strain was classified as SCCmec type III but harbored the *pls* gene and the *des* region. Another strain was characterized as SCCmec type IV but lacked the *des* region. In addition, one isolate harbored both SCCmec type V and Panton-Valentine leukocidin. Finally, the SCCmec type of the strains was found to be correlated with the antibiotic susceptibility pattern.


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*Staphylococcus aureus* is a potentially pathogenic bacterium that can cause various diseases such as postoperative wound infections and necrotizing pneumonia (20). *S. aureus* has a strong adaptive power to antibiotics. Since the introduction of methicillin in 1959, methicillin-resistant *S. aureus* (MRSA) strains have been isolated, first in the United Kingdom in 1961 and subsequently in other parts of the world. Although most of the MRSA strains are hospital acquired, community-acquired strains (CA-MRSA) have also recently been reported (32).

Resistance of MRSA strains to methicillin is determined by the presence of the *mecA* gene, which encodes the penicillin binding protein 2a. The *mecA* gene is localized on a mobile genetic element, which is designated the staphylococcal chromosomal cassette mec (SCCmec) (3, 14, 21). Currently, five main types of SCCmec (types I to V) are distinguished. SCCmec types I, II, and III are associated with hospital-acquired MRSA, whereas types IV and V are associated with CA-MRSA (12, 15). SCCmec types I, IV, and V exclusively encode resistance to β-lactam antibiotics. By contrast, SCCmec types II and III determine multiresistance, as these cassettes carry both integrated plasmid sequences (e.g., pT181 and pUB110) and transposons (e.g., Tn554) containing drug resistance genes. Besides the resistance genes on SCCmec, *S. aureus* can also carry drug resistance genes on other sites of its chromosome and on plasmids. Also situated on SCCmec are genes responsible for the regulation of the transcription of *mecA*: Δ*mecR1* (on SCCmec types I, IV, and V) and *mecI* and *mecI* (on SCCmec types II and III) (13, 14, 15). For integration into and excision from the chromosome at a specific site (*attBscC*), genes encoding chromosomal cassette recombinases (*ccr*) are located within the SCCmec elements. These genes are designated *ccrA1* and *ccrB1* (in SCCmec type I), *ccrA2* and *ccrB2* (in SCCmec types II and IV), *ccrA3* and *ccrB3* (in SCCmec type III), and *ccrC* (in SCCmec type V) (6, 12, 15).

The Euregio Meuse-Rhine (EMR) is a region consisting of the Belgian provinces of Limburg and Liège, the German-speaking region of Belgium, the region Aachen in Germany, and the southern part of the Dutch province of Limburg, with an area of 10,478 km². Cross-border patient mobility and free access to health care facilities within the European Union in general, and the EMR in particular, are important issues for patients, doctors, hospitals, sickness funds, and health care insurance companies. Each year, many thousands of the 3.7 million inhabitants of the EMR cross the border to visit a medical specialist or a hospital on the other side of the border. In an official publication of the European Commission (D. Byrne, Maastricht Conference on Cross-Border Health Care, Maastricht, The Netherlands, 8 June 2004), the EMR was therefore recently mentioned as a model region for the European Union in the field of cross-border health care and cross-border cooperation of hospitals and sickness funds. Nevertheless, an
important issue of concern that is related to cross-border health care is the dissemination of multiresistant bacteria. In this regard, it is interesting to note that the three countries forming the EMR differ considerably in prevalence of MRSA isolated in hospitals (23.6%, 13.8%, and 0.6% in Belgium, Germany, and The Netherlands, respectively) (31). Consequently, the cross-border transfer of patients may have an important impact on the dissemination and prevalence of MRSA, in particular in cases where patients are transferred from countries with a relatively high prevalence to a country with a low prevalence. Therefore, we investigated the dissemination of MRSA isolates between hospitals from the EMR during the last 5 years. The MRSA isolates were subjected to pulsed-field gel electrophoresis (PFGE), SCCmec typing, and multilocus sequence typing (MLST) (2). As the presence of Panton-Valentine leukocidin (PVL) genes was suggested to be an important characteristic of CA-MRSA (33), the isolates were also subjected to a PVL-specific real-time PCR.

**MATERIALS AND METHODS**

**Clinical isolates.** One hundred fifty-two isolates of MRSA from individual patients, isolated between December 1999 and February 2004 from five geographically closely related hospitals in the EMR, were included in the study (Table 1). These included two Belgian hospitals (Hospital East-Limbourg, Genk, an 822-bed general hospital, and General Hospital Vesalius, Tongeren, a 355-bed general hospital), one German hospital (Universitätsklinikum Aachen, a tertiary 1,500-bed university hospital), and two Dutch hospitals (Atrium Medical Centre, Heerlen, an 811-bed general hospital, and University Hospital Maastricht, a tertiary 680-bed university hospital). The 152 MRSA isolates included 40 isolates from Belgium, 49 from Germany, and 63 from The Netherlands (Table 1). The isolates from the Belgian and German hospitals represented a random selection of 50% of the clinical isolates. The Dutch isolates were derived from surveillance cultures from patients at risk for MRSA carriage who were admitted to the two Dutch hospitals during the study period. During this period, infections with MRSA were not found in the two Dutch hospitals. All strains were identified as *S. aureus* by catalase and coagulase testing. Meticillin resistance was determined by the disk diffusion test with oxacillin concentration disks, according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (22), and by PCR amplification of the *mec* gene (26).

**Reference strains.** MRSA strains COL, BK2464, ANS46, HDE288, and WIS were used as reference strains for SCCmec types II, III, IV, and V, and *V.* respectively (15, 23). *S. aureus* strain 1206 was used as a positive control for the Tn554 PCR (35). For PFGE, reference strain *S. aureus* Ps 47 was used as a molecular weight marker, whereas MRSA strains BK2464, COL, HDE288, HU25, and PER34 were used as reference strains for the New York/Japan, archaic, pediatric, Brazilian, and Iberian clonal types, respectively (24).

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was performed by microbroth dilution according to the NCCLS guidelines (22) for the following antibiotics: amikacin, amoxicillin, cefazolin, clindamycin, co-trimoxazole, doxycycline, erythromycin, flucloxacillin, gentamicin, penicillin, rifampin, and vancomycin.

**SCCmec typing.** SCCmec typing was essentially carried out as described by Oliveira and de Lencastre (23), in which *mecA* and six different loci on SCCmec (Fig. 1) were amplified by PCR with the following modifications. PCR amplification of *mecA* sequences was carried out with primers *mecA1* and *mecA2* (Sigma Genosys, The Netherlands), resulting in a PCR product of 527 bp instead of 162 bp (26). *mecA* was performed in a volume of 50 µl containing 10 µl of a 0.5 McFarland suspension (1.5 × 10^6 CFU/ml) of the MRSA strain, 0.2 mM of each deoxynucleoside triphosphate (Amersham Biosciences, The Netherlands), 1× PCR buffer (QIAGEN, The Netherlands), 1.25 U HotStarTaq (QIAGEN, The Netherlands), and primers. The primer concentrations used were similar to those previously described (23), except for those of the *mecA* primers, which were 0.6 µM for both *mecA1* and *mecA2. The amplifications were performed on a GeneAmp PCR system, model 9600 (Applied Biosystems, The Netherlands), with the following program: 15 min at 94°C, followed by 30 cycles of 30 s at 94°C,
RESULTS

Distribution of SCC\textit{mec} types. The SCC\textit{mec} types (Fig. 1) could be determined for 148 of the 152 (97.4\%) clinical isolates of MRSA. Only four of the five different types of SCC\textit{mec} were found (I to IV [Table 1]) with the method described by Oliveira and de Lencastre (23). SCC\textit{mec} types I and IV were predominant, with 29\% and 34\%, respectively, and types II and III were less common, with 12\% and 23\%, respectively. The different SCC\textit{mec} types were not distributed similarly among the three countries from the EMR. In Belgium, SCC\textit{mec} types I and IV predominated, whereas in Germany, the most common types were types II and III. In The Netherlands, the most frequently found types were types I, III, and IV (Table 1).

The SCC\textit{mec} types of 4 (DOM068, DOM083, DOM114, and DOM012) of the 152 MRSA isolates (3\%) could not be determined according to the method described by Oliveira and de Lencastre (23). DOM068 was found to contain loci A, C, D, and E, as well as F (Table 2), which is uncommon to the prototypes of the five known SCC\textit{mec} types, as shown in Fig. 1. Another isolate, DOM083, was also found to have a unique SCC\textit{mec} organization, possessing loci C, D, E, and F (Table 2). The SCC\textit{mec} structure of isolates DOM068 and DOM083 was further characterized by determination of the presence of two other loci from the SCC\textit{mec} cassette, i.e., \textit{ccr} and Tn554. Both strains were found to possess Tn554 as well as \textit{ccrAB3}, indicating that their SCC\textit{mec} cassettes most strongly resemble the type III cassette (Table 2).

The cassette of another strain, DOM114, was found to contain only a single locus from the six loci that were defined by Oliveira and de Lencastre (23), i.e., locus E (Table 2). Since DOM114 was also found to possess the \textit{ccr} gene, its SCC\textit{mec} cassette can be classified as type V (Table 2).

The fourth "nontypeable" isolate, DOM012, lacked all six loci as defined by Oliveira and de Lencastre (23). However, this strain was found to contain \textit{ccrAB2}, which is characteristic for both type II and type IV cassettes. Since the DOM012 cassette did not contain Tn554, it was classified as type IV (Table 2).

PFGE analyses. Each of the MRSA strains was subjected to analysis by PFGE. On the basis of the PFGE patterns, a dendrogram was constructed (Fig. 2). One of the isolates (DOM152) could not be typed due to repeated difficulties with the isolation of DNA from this strain. A total of 32 clonal groups (A to AF) were distinguished, of which four were major clonal groups (A, G, L, and Q). Major clonal group A was closely related to the clonal groups B, C, D, and E. Taken together, these groups included 34 of the 152 MRSA isolates (22\%). A large majority of these 34 strains (88\%), isolated from both Germany and The Netherlands, harbored SCC\textit{mec} type III.

The second major clonal group, group G, contained 26 of the 152 MRSA isolates (17\%), of which 21 (81\%) harbored SCC\textit{mec} type I and 5 (19\%) harbored SCC\textit{mec} type IV. Most isolates from this group originated from Belgium and The Netherlands. Group G was also found to include reference strain COL, representative for the arcahaic clone, which is one of the six major MRSA clones spread worldwide (2). Clonal group F, which was related to group G, comprised only four isolates (3\%), three of which harbored SCC\textit{mec} type IV and one of which harbored SCC\textit{mec} type V. The reference strain for the Iberian clone (2), PER34, was linked to clonal group F.

### Table 2. Results of non-typeable$^a$ SCC\textit{mec} within MRSA isolates

<table>
<thead>
<tr>
<th>Code$^a$</th>
<th>Country</th>
<th>Locus (loci)$^b$</th>
<th>ccr</th>
<th>Tn554</th>
<th>SCC\textit{mec} type</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOM012</td>
<td>The Netherlands</td>
<td>—</td>
<td>ccrAB2</td>
<td>—</td>
<td>IV</td>
</tr>
<tr>
<td>DOM068</td>
<td>Germany</td>
<td>A, C to F</td>
<td>ccrAB3</td>
<td>+</td>
<td>III</td>
</tr>
<tr>
<td>DOM083</td>
<td>Germany</td>
<td>C to F</td>
<td>ccrAB3</td>
<td>+</td>
<td>III</td>
</tr>
<tr>
<td>DOM114</td>
<td>The Netherlands</td>
<td>E</td>
<td>ccrC</td>
<td>—</td>
<td>V</td>
</tr>
</tbody>
</table>

$^a$ All strains were positive for \textit{mecA}.

$^b$ According to the classification scheme of Oliveira and de Lencastre (23).
FIG. 2. Dendrogram of the 152 clinical MRSA isolates and five reference clones. The five columns on the right represent MRSA isolate code, country of origin, year of isolation, SCCmec type, and clonal group, respectively. NT, not typeable.
The third major clonal group, group L, included 26 of the 152 MRSA isolates (17%). Of these, 18 (69%) contained SCCmec type IV, 6 (23%) contained SCCmec type I, 1 (4%) contained SCCmec type II, and 1 (4%) contained SCCmec type III. Most of the strains were isolated in Belgium and The Netherlands. None of the reference strains were linked to this clonal group.

The fourth major group, group Q, contained 16 of the 152 MRSA isolates (11%). Ten (63%) of these carried SCCmec type II, whereas five (31%) contained SCCmec IV and one (6%) contained SCCmec type I. All except one of the strains from this group were isolated in either Germany or The Netherlands. Group Q also included reference strain BK2464, which is a representative of the New York/Japan clone (2).

**MLST analyses.** To investigate which major MRSA clones from which clonal complexes are disseminated in the EMR, two representative strains from each major clonal PFGE group were subjected to MLST. As shown in Table 3, MLST identified six different sequence types (ST8, -225, -228, -239, -241, and -247) that belonged to two clonal complexes (CC5 and CC8). In major clonal group A, two different STs, which both belong to CC5, were found: (i) ST239-MRSA-III, a single-locus variant (SLV) of ST8 and representative for the Brazilian clone, and (ii) ST241-MRSA-II, an SLV of ST239-MRSA-III (at locus yqiL). The two STs that were found in major clonal group G are different SLVs of a single ST, i.e., ST250; ST247-MRSA-I is an SLV at the gmk locus, and ST8-MRSA-A is an SLV at the yqiL locus of ST5 (10).

**Prevalence of PVL.** Only 2 (DOM103 and DOM114) of the 152 MRSA isolates (1.3%) contained PVL. Although both strains were isolated in The Netherlands in 2003, they differed in both SCCmec type and PFGE type. Strain DOM103 was classified within major clonal group L, harboring SCCmec type IV, whereas strain DOM114 was classified within group F, carrying SCCmec type V (Fig. 2).

**Correlation between SCCmec type and antibiotic susceptibility pattern.** The antibiotic resistance patterns for the SCCmec types are presented in Table 4. Only the non-β-lactam antibiotics are presented in this table, since all MRSA strains were resistant to the four β-lactam antibiotics tested, i.e., amoxicillin, cefazolin, flucloxacillin, and penicillin. To investigate if a correlation exists between the SCCmec type and the antibiotic susceptibility pattern of MRSA isolates, canonical discriminant analyses were performed. As shown in Fig. 3, the SCCmec types were centered around the four group centroids, indicating that SCCmec type and susceptibility pattern were indeed correlated. More specifically, the antibiotic susceptibility pattern had a predictive value of 84.1% for SCCmec type I, 83.3% for SCCmec type II, 85.7% for SCCmec type III, and 86.3% for SCCmec type IV (Table 5). As shown in Table 4 and Fig. 3, the correlation is more pronounced for SCCmec types II, III, and IV than for SCCmec type I.

**DISCUSSION**

Monitoring the dissemination of MRSA in the EMR is important, since known and novel MRSA clones may spread from...
country to country through cross-border patient care. In particular, the spread of MRSA harboring either SCCmec type II or type III, which encodes multiresistance, could pose a serious threat to health care facilities.

In this study, 152 MRSA strains were characterized, isolated in hospitals from the EMR between 1999 and 2004. Typing of the strains by PFGE revealed four major clonal groups, suggesting dissemination of MRSA in the EMR. The strains that were classified within major clonal group A and within the closely related, minor clonal groups B to E comprised 22% of all isolates. Two representative strains from group A were also typed by MLST and were classified as ST239-MRSA-III and ST241-MRSA-III. Both STs formed within major clonal group A and within the closely related minor clonal groups B to E. Although these clonal complexes were more closely related to each other than they are to other clonal complexes (19), the finding of strains from different MLST clonal complexes within a single PFGE clonal group was novel for S. aureus. Nevertheless, this finding was in line with previous reports that demonstrated the higher discriminatory power of MLST over PFGE for bacterial species other than S. aureus, such as Vibrio cholerae, Salmonella, and Listeria monocytogenes (17, 18, 28). Both ST8-MRSA-IV and ST228-MRSA-I were found previously in the EMR countries: ST8-MRSA-IV in Germany and The Netherlands and ST228-MRSA-I in Belgium and Germany (10, 36).

MRSA strains from the fourth major clonal group, group Q, harbored mainly SCCmec type II. Two representatives from this group were both typed as ST225-MRSA-II, an ST belonging to CC5. Although ST225-MRSA-II has previously been found in the United States (29), the finding of this ST in Germany is novel. Since ST225-MRSA-II is an SLV at the ipi locus of strain ST5-MRSA-II, which was previously found in Belgium (7), ST225-MRSA-II may be derived from ST5-MRSA-II. Recent studies reported the finding of ST225-MRSA-IV and ST45-MRSA-IV in Belgium and ST45-MRSA-I and ST45-MRSA-IV in The Netherlands (7, 34). These STs, however, were not found in this study.

For 4 of the 152 MRSA isolates (3%) the SCCmec type could not be determined using the method described by Oliveira and de Lencastre (23). The percentage of nontypeable SCCmec cassettes was low compared to that from other studies, in which 10 to 15% could not be typed (4, 11). Since two of the nontypeable MRSA strains (DOM068 and DOM083) could not be determined using the method described by Oliveira and de Lencastre (23), it was not possible to classify these strains. Strains with an organization of SCCmec loci similar to that for DOM068 and DOM083, however, were not found in this study.

Another nontypeable strain, DOM012, was found to contain only a single SCCmec locus apart from mecA. This locus, ccrAB3, was present in the prototype cassettes of both type II and type IV. However, since strain DOM012 possessed neither mecI (locus C) nor Tn554, its gene cassette had a higher similarity with the cassette of type IV than with that of type II. Also, the resistance to β-lactam antibiotics found in line with SCCmec type IV. We therefore concluded that strain DOM012 carries SCCmec type IV but lacks locus D.

The low prevalence of PVL was in accordance with previous studies (8, 27). Both strains (DOM103 and DOM114) were very likely not related, as they were classified within different

### Table 5: Correlation between the SCCmec types and the antibiotic susceptibility patterns of the 152 MRSA strains

<table>
<thead>
<tr>
<th>SCCmec type</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>NT</th>
<th>Total (no. [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>37 (84.1)</td>
<td>0 (0)</td>
<td>2 (4.5)</td>
<td>5 (11.4)</td>
<td>0 (0)</td>
<td>44 (100)</td>
</tr>
<tr>
<td>II</td>
<td>0 (0)</td>
<td>15 (83.3)</td>
<td>1 (5.6)</td>
<td>1 (5.6)</td>
<td>1 (5.6)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>III</td>
<td>3 (8.6)</td>
<td>1 (2.9)</td>
<td>30 (85.7)</td>
<td>1 (2.9)</td>
<td>0 (0)</td>
<td>35 (100)</td>
</tr>
<tr>
<td>IV</td>
<td>0 (0)</td>
<td>7 (13.7)</td>
<td>0 (0)</td>
<td>44 (86.3)</td>
<td>0 (0)</td>
<td>51 (100)</td>
</tr>
<tr>
<td>NT</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>1 (25)</td>
<td>2 (50)</td>
<td>4 (100)</td>
</tr>
</tbody>
</table>

*Predicted from susceptibility pattern.

NT, not typeable.
clonal groups and harbored different SCCmec types. DOM114 is to our knowledge the first reported PVL-positive MRSA strain carrying SCCmec type V.

Although antibiotic resistance in S. aureus can also be determined by sequences other than SCCmec, a correlation of approximately 85% was found between antibiotic susceptibility pattern and SCCmec type. Rapid identification of the SCCmec type of MRSA isolates by PCR could therefore be useful to predict the antibiotic susceptibility pattern of isolates and, consequently, guide the choice of antibiotics used for treatment. Hence, the identification of the SCCmec type of clinical isolates might contribute to prevent the unnecessary use of vancomycin, which is needed only in case of MRSA isolates harboring SCCmec type II or III.

In summary, MRSA strains belonging to clonal complexes 5 and 8 were disseminated in the EMR and several “new” types were found: both ST225-MRSA-II and ST241-MRSA-III were novel findings in the German part of the EMR. Furthermore, one strain was classified as SCCmec type III, but contained the pIs gene and the des region, and another strain was characterized as SCCmec type IV, but lacked the dcs region. One isolate harboring both SCCmec type V and PVL was found.

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