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Molecular characterization of methicillin-resistant *Staphylococcus aureus* bloodstream isolates from Croatia

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***Objectives*: The objectives of this study were (i) to investigate the genetic background of methicillin-resistant *Staphylococcus aureus* (MRSA) bloodstream isolates from Croatia and (ii) to monitor the prevalence of Panton-Valentine leucocidin (PVL) and toxic shock syndrome toxin-1 (TSST-1) among these isolates.**

***Methods*: Eighty-two hospital-acquired MRSA bloodstream isolates, collected in 2001 and 2002 in Croatia, were characterized by PFGE, staphylococcal cassette chromosome *mec* (SCC*mec*) typing and multilocus sequence typing (MLST). The presence of genes encoding PVL and TSST-1 was investigated by real-time PCR.**

***Results*: All strains were multiresistant and were distributed among 16 different similarity groups as determined by PFGE. Two of the groups, groups H and K, harboured the majority of the MRSA strains with 52 and 12%, respectively. The predominant SCC*mec* type found among the isolates was type I (89%). Eleven per cent of the strains harboured a modified SCC*mec* type III, which contained, in contrast to the regular type III, an additional *dcs* region. One strain harboured a novel SCC*mec* type, containing the *ccrC* gene in combination with the *mecI* gene, the *dcs* region, the locus between pI258 and Tn*554* (locus E) and the locus between Tn*554* and *orfX* (locus F). MLST showed the presence of ST111-MRSA-I and ST247-MRSA-I among Croatian MRSA isolates. All isolates were negative for both PVL and TSST-1.**

***Conclusions*: These results indicate the emergence of ST111-MRSA-I and ST247-MRSA-I in Croatia among MRSA bloodstream isolates. The virulence factors PVL and TSST-1 were not present among these isolates.**

Keywords: MRSA, PVL, SCC*mec*, TSST-1

Introduction

The resistance of *Staphylococcus aureus* to β-lactam antibiotics is associated with the expression of penicillin-binding protein 2a (PBP2a). This protein is encoded by the *mecA* gene, which is situated on a mobile genetic element, staphylococcal cassette chromosome *mec* (SCC*mec*). Five different SCC*mec* types have been identified in methicillin-resistant *S. aureus* (MRSA) strains. SCC*mec* types I, II and III are mainly found in hospital-acquired MSRA (HA-MRSA), whereas SCC*mec* types IV and V are mainly associated with community-acquired MRSA (CA-MRSA). SCC*mec* contains the *mec* complex (*mecA* and its regulators) and the *ccr* gene complex, which encodes site-specific recombinases, responsible for the mobility of SCC*mec*. Several different *ccr* genes have been identified: *ccrA1* and *ccrB1* in SCC*mec* type I, *ccrA2* and *ccrB2* in SCC*mec* types II and IV, *ccrA3* and *ccrB3* in SCC*mec* type III, *ccrA4* and *ccrB4* in SCC*mec* type IV and *ccrC* in SCC*mec* type V.[1,2]

S. aureus can produce a number of virulence factors. Panton-Valentine leucocidin (PVL) is predominantly associated with severe skin infections and necrotizing pneumonia. PVL, together with SCC*mec* type IV, is suggested to be a marker for CA-MRSA,[2] although there are exceptions.[3,4] Toxic shock syndrome toxin-1 (TSST-1) is a 29.1 kDa superantigen that is encoded by the *tst* gene. The release of TSST-1 into the bloodstream may give rise to a variety of severe clinical conditions, like TSS. TSST-1 is produced by many MRSA strains, particularly in Japanese hospitals.[5]

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Croatia has a MRSA prevalence of 36.7% among bloodstream isolates.[6] Information on the clonal distribution of MRSA in Croatia, however, is not available. Furthermore, nothing is known about the prevalence of PVL and TSST-1 among Croatian MRSA isolates. Therefore, the aim of this study was to determine the clonal distribution of MRSA among bloodstream isolates in Croatia isolated in 2001 and 2002 and to determine the presence of the virulence factors PVL and TSST-1.

Materials and methods

Clinical isolates

Eighty-two MRSA bloodstream isolates were collected from 10 hospitals in seven Croatian cities during 2001 and 2002 as part of the European Antimicrobial Resistance Surveillance System (EARSS) project (Figure 1). All strains were hospital-acquired, i.e. isolated ≥72 h after patient admission to the hospital. Reference strains *S. aureus* ATCC 25923 for PFGE, MRSA COL (SCC*mec* I), BK2464 (SCC*mec* II), ANS46 (SCC*mec* III), HDE288 (SCC*mec* IV) and WIS (SCC*mec* V) for SCC*mec* typing and *ccr* gene complex determination were used. MRSA Cluster 28 was used as a reference strain for PVL and *S. aureus* HT.2004.0349 as the reference strain for TSST-1.[3,5] All strains were identified as *S. aureus* by catalase, coagulase and DNase production.

Susceptibility testing

Susceptibility testing was performed by the broth microdilution method according to the guidelines of the CLSI (formerly NCCLS).[7] The following antimicrobial drugs were tested: azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, linezolid, norfloxacin, oxacillin, penicillin, rifampicin, tetracycline, trimethoprim, trimethoprim/sulfamethoxazole and vancomycin.

SCCmec typing and detection of virulence factors

SCC*mec* typing and the detection of *ccrAB3* and *ccrC* were performed as described previously.[3] The presence of the genes encoding PVL and TSST-1 was investigated by using real-time PCR as described previously.[3,5]

PFGE and multilocus sequence typing

PFGE analyses and multilocus sequence typing (MLST) were performed as previously described.[3]

Results

Susceptibility patterns

All 82 MRSA isolates had MIC values for oxacillin of ≥128 mg/L. All strains were resistant to gentamicin and ciprofloxacin and were susceptible to vancomycin and linezolid. Most of the strains (96%) were resistant to clindamycin, erythromycin and azithromycin. In addition, 36% of the strains were resistant to rifampicin, 32% were resistant to tetracycline, 9% were resistant to chloramphenicol and 0.2% were resistant to trimethoprim/sulfamethoxazole.

SCCmec typing

Seventy-three of the 82 (89%) MRSA strains were found to contain SCC*mec* type I. From nine (11%) strains, the SCC*mec* type was not typeable with the method used.[3] SCC*mec* of these strains was found to harbour *mecA* and loci C, D, E and F. Therefore, these cassettes only differ from SCC*mec* type III in the presence of locus D. To investigate the nature of these cassettes in more detail, the *ccr* genes of these nine strains were characterized. In eight of the nine MRSA strains, the *ccr* gene was identified as *ccrAB3*. Consequently, these strains were classified as harbouring SCC*mec* type III. The other non-typeable strain, strain 79, harboured *ccrC*, which is normally only carried on SCC*mec* type V.

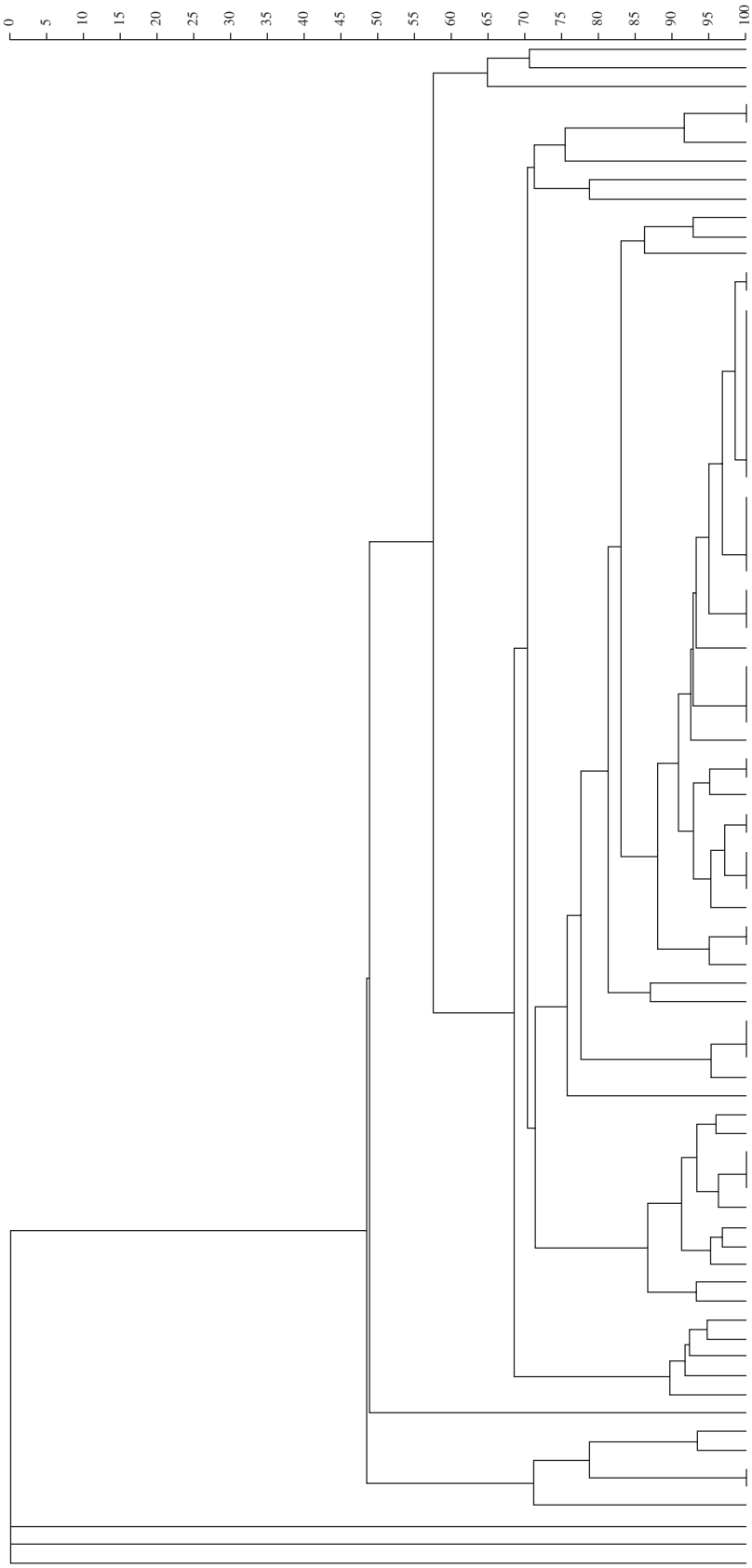
PFGE analyses

Three of the 82 isolates could not be typed using PFGE, either owing to difficulties with lysis of the bacteria or the digestion of the DNA. A total of 16 similarity groups (A to P) were found and two of these, H and K, were classified as major similarity groups (Figure 2). Similarity group H harboured 43 of the 82 (52%) isolates, whereas similarity group K harboured 11 of the 82 (13%) isolates. In both groups H and K, SCC*mec* type I was predominant (i.e. 95 and 82%, respectively). Furthermore, the strains from groups H and K had different susceptibility patterns. The majority (91%) of the strains from group K were resistant to rifampicin and tetracycline, whereas the majority of the strains from group H were susceptible to these antibiotics (84 and 91%, respectively). Strains harbouring SCC*mec* type III were classified into various similarity groups, i.e. groups D (2), H (2), K (2), Q (1) and NT (1).

MLST analyses

Two representative strains from the major similarity groups H and K were characterized by MLST. As shown in Table 1, both strains from group H were identified as ST111, a single locus variant (SLV) of ST228 at the *pta* locus, whereas both strains from group K were classified as ST247. The distribution of these clones in Croatia is presented in Figure 1.

Figure 1. Map of Croatia showing the 10 hospitals [CHC Zagreb (1), CH Dubrava Zagreb (2), CH 'F. Mihaljevic' Zagreb (3), GH Sv. Duh Zagreb (4), GH Koprivnica (5), GH Sisak (6), GH Slav. Brod (7), GH Pula (8), GH Zadar (9) and CHC Split (10)]. Open squares indicate ST111-MRSA-I, whereas filled squares indicate ST247-MRSA-I.



1	GH Zadar	2002	A	I
1	GH Zadar	2002	B	I
3	CHC Zagreb	2002	C	I
4	CHC Zagreb	2002	D	III
5	CHC Zagreb	2002	D	I
6	CH Dubrava Zagreb	2002	D	III
7	CHC Split	2002	E	I
8	CHC Zagreb	2001	F	I
9	CHC Zagreb	2002	G	I
10	GH Zadar	2002	H	I
11	CHC Split	2002	H	I
12	CHC Zagreb	2002	H	I
13	CHC Split	2002	H	I
14	CHC Split	2002	H	I
15	CHC Split	2002	H	I
16	CHC Zagreb	2002	H	I
17	CHC Zagreb	2002	H	I
18	CHC Zagreb	2002	H	I
19	CHC Zagreb	2001	H	I
20	CHC Zagreb	2001	H	I
21	GH Sv.Duh Zaoreb	2001	H	I
22	CHC Split	2001	H	I
23	CHC Zagreb	2002	H	I
24	GH Sv.Duh Zagreb	2002	H	I
25	CHC Zagreb	2002	H	I
26	CHC Split	2002	H	I
27	CHC Zagreb	2002	H	I
28	CHC Zagreb	2002	H	I
29	GH Kaprivnica	2002	H	I
30	GH Kaprivnica	2001	H	I
31	GH Zadar	2002	H	I
32	CHC Split	2002	H	I
33	CHC Zagreb	2001	H	I
34	CHC Zagreb	2002	H	I
35	CHC Zagreb	2002	H	I
36	CHC Zagreb	2002	H	III
37	GH Zadar	2002	H	III
38	GH Zadar	2002	H	I
39	CHC Zagreb	2002	H	I
40	GH Pula	2002	H	I
41	CHC Split	2002	H	I
42	CHC Split	2002	H	I
43	CHC Split	2002	H	I
44	CHC Zagreb	2001	H	I
45	CH Dubrava Zagreb	2001	H	I
46	CHC Zagreb	2002	H	I
47	CHC Zagreb	2002	H	I
48	CH "F.Mihaljevic" Zagreb	2002	H	I
49	GH Zadar	2002	H	I
50	CHC Split	2001	H	I
51	GH Slav. Brod	2002	H	I
52	GH Slav. Brod	2001	H	I
53	CHC Zagreb	2002	I	I
54	CHC Zagreb	2002	I	I
55	CHC Zagreb	2002	I	I
56	CHC Zagreb	2002	I	I
57	CH Dubrava Zagreb	2002	J	I
58	CHC Zagreb	2002	K	I
58	CHC Zagreb	2002	K	I
60	CHC Split	2001	K	I
61	GH Sv.Duh Zagreb	2002	K	I
62	CHC Zagreb	2002	K	III
63	CHC Zagreb	2002	K	I
64	GH Sv.Duh Zagreb	2002	K	I
65	CHC Zagreb	2002	K	I
66	CHC Zagreb	2002	K	I
67	GH Slav.Brod	2002	K	I
68	CHC Zagreb	2002	K	III
69	GH Sisak	2001	L	I
70	GH Koprivnioa	2001	L	I
71	CHC Zagreb	2002	L	I
72	CHC Zagreb	2002	L	I
73	CHC Zagreb	2002	L	I
74	CHC Split	2002	M	I
75	CHC Zagreb	2002	N	I
76	CHC Zagreb	2001	N	I
77	CHC Zagreb	2001	O	I
78	CHC Zagreb	2001	O	I
79	CHC Split	2002	P	NT
80	GH Zadar	2002	NT	III
81	CHC Zagreb	2002	NT	I
82	CHC Zagreb	2002	NT	III

Figure 2. Dendrogram of the 82 MRSA isolates. The five columns on the right represent MRSA isolate code, centre, year of isolation, clonal group and SCC*mec* type, respectively. NT, not typeable.

Table 1. Typing results of major similarity groups H and K

Code	Major similarity group	SCC*mec* type	MLST profile	ST
13	H	I	1-4-1-4-46-24-29	111
46	H	I	1-4-1-4-46-24-29	111
63	K	I	3-3-1-12-4-4-16	247
65	K	I	3-3-1-12-4-4-16	247

ST, sequence type.

PVL and TSST-1 analyses

The genes encoding virulence factors PVL and TSST-1 were not detected in any of the investigated strains.

Discussion

In this study, the clonal distribution of MRSA isolates in Croatia was described. Most of the isolates (89%) were found to possess SCC*mec* type I. Eight of the nine strains that did not contain SCC*mec* type I carried a modified SCC*mec* type III. In contrast to a normal type III cassette, the cassette of these strains harboured locus D (*dcs* region), which is usually only found in SCC*mec* types I, II and IV.[3] An MRSA strain with a similar SCC*mec* element (HSA10) has previously been described by Aires de Sousa and de Lencastre.[4]

The single remaining 'non-SCC*mec* type I' strain from this study (strain 79) was found to harbour *ccrC*, together with loci C (*mecI*, normally found in SCC*mec* II and III), D, E (a locus between pI258 and Tn*554*, which is specific for SCC*mec* type III) and F (a locus between Tn*554* and *orfX*, which is specific for SCC*mec* type III).[3] Since *ccrC* is usually only found in combination with locus E in SCC*mec* type V,[1,3] MRSA strain 79 is likely to harbour a novel SCC*mec* element. Although this novel element shows many similarities with the SCC*mec* type III element, it lacks the type III-specific *ccrAB3* gene. Further investigation into the exact structure of this element is currently underway. An encounter of two *S. aureus* strains harbouring different SCC*mec* elements could have led to the formation of a novel, not previously described, SCC*mec* element, probably through homologous recombination.[1]

Typing of the MRSA strains by PFGE revealed 16 similarity groups (A to P), two of which, H and K, were classified as major similarity groups. These results, together with the SCC*mec* typing, indicated that the distribution of particular MRSA genotypes was not restricted to specific hospitals or cities in Croatia. A total of 52% of the MRSA strains was classified within major similarity group H and 95% of these strains harboured SCC*mec* type I. ST111, found by MLST analyses of two representative strains from group H, was previously found in one strain (AB-903627/02) from Norway (http://www.mlst.net), and one strain harbouring SCC*mec* type I from the Czech Republic.[8,9] ST111-MRSA-I could have evolved from ST228-MRSA-I, the Southern Germany clone, since ST111 is an SLV of ST228 at the *pta* locus. ST228-MRSA-I has been found in Belgium, Denmark, Germany, Italy, Slovenia and Spain.[8] Thus far, ST111-MRSA-I has only been found in various European countries and not in other parts of the world.

The second major similarity group, group K, contained 13% of the MRSA isolates; 82% of the strains from this group harboured SCC*mec* type I. ST247-MRSA-I from group K is similar to the Iberian clone, one of the major MRSA clones currently isolated in European countries, such as the Czech Republic, Germany, Italy, Poland, Slovenia and Switzerland. ST247 is classified in clonal complex (CC) 8.[2,8]

PVL and TSST-1 were not detected in any of the isolates investigated. Similarly, a previous study reported only a single PVL-positive isolate among 86 bloodstream isolates.[10] These results indicate that these virulence factors are not necessarily associated with MRSA strains causing MRSA bloodstream infections.

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Transparency declarations

None to declare.

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