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Methicillin-resistant *Staphylococcus aureus* containing the Panton-Valentine leucocidin gene in Germany in 2005 and 2006

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***Objectives*: The aim of this paper is to attribute Panton-Valentine leucocidin (PVL)-positive methicillin-resistant *Staphylococcus aureus* (MRSA) to clonal lineages by molecular typing with special reference to isolates exhibiting *spa* type t008/multilocus sequence type (MLST) ST8 [widely disseminated in the USA as 'community-associated MRSA (caMRSA) USA300'].**

***Methods*: PVL-positive MRSA (n = 117) were detected among 4815 MRSA sent to the German National Reference Laboratory for typing. These isolates were analysed by PFGE, *spa* typing, multilocus sequence typing, grouping of SCC*mec* elements and PCR detection of *arcA*, *msr*(A), *mph*(B) and the ≥6 AT repeat unit in the SACOL0058 sequence.**

***Results*: Among the 117 isolates, 80 exhibited *spa* type t044 (corresponding to MLST ST80) and 23 exhibited *spa* type t008/MLST ST8. Other *spa* types were sporadically represented. Further characterization of isolates exhibiting t008/ST8 by PCR [*arcA*, *msr*(A), *mph*(B), ≥6 AT repeat signature] indicates the arrival of caMRSA 'USA300' in Central Europe.**

***Conclusions*: caMRSA ST80 still predominate; however, caMRSA ST8 exhibiting the characteristics of the 'USA300' clone became the second most frequent. Routine detection of this clone in clinical bacteriology can be easily performed by PCR.**

Keywords: community MRSA, genotyping, molecular markers

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) are a common cause of nosocomial infections worldwide. Hospital-associated MRSA (haMRSA) are often resistant to multiple antibiotic classes, which limits treatment options.[1] Since the end of the 1990s, however, MRSA infections in the general population without the risk factors known for acquisition of haMRSA have been described, first in the USA and later worldwide.[2–4] Community-associated MRSA (caMRSA) are usually less broadly resistant to antibiotics; they often contain the genes *lukS-PV lukF-PV* coding for Panton-Valentine leucocidin (PVL) and SCC*mec* elements of types IV and V. Most of haMRSA are associated with 8 of the 10 major clonal lineages of the *S. aureus* population. caMRSA belong to various other clonal lineages than haMRSA.[4–8] However, only a few of them are widely disseminated and therefore can be regarded as epidemic. In the USA, two lineages, namely multilocus sequence type (MLST) ST1 ('USA400' according to the PFGE pattern) and ST8 ('USA300'), are the most frequent and widely disseminated caMRSA.[9] In Oceania, this is ST30[10] and in Europe ST80.[4,6,11] caMRSA 'USA300' has become the predominant cause of community-onset *S. aureus* skin and soft tissue infections in a number of areas in the USA;[12] there are also first reports of healthcare-associated infections.[13,14] Therefore, timely recognition of this clone in other parts of the world is an essential prerequisite for the prevention of further spread.

caMRSA 'USA300' exhibits MLST ST8. Although PVL-negative methicillin-susceptible *S. aureus* (MSSA) of this clonal lineage are still common among *S. aureus* isolates from colonization and infections,[15] MRSA have evolved from ST8 MSSA at several times and on several occasions by acquisition of SCC*mec* elements of types I, II and IV.[16,17] PVL-negative MRSA ST8, SCC*mec* IV, are particularly epidemic in Irish hospitals.[18] Reliable recognition of caMRSA of ST8 not only requires discrimination from other lineages of caMRSA, but also from nosocomial MRSA, particularly those exhibiting ST8.

Traditional molecular typing methods for the detection of caMRSA ST8 include *Sma*I-macrorestriction patterns, multilocus sequence typing, *spa* sequence typing and PCR demonstration of *lukS-PV lukF-PV*.

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An additional arginine deiminase pathway contained by a chromosomal cassette element adjacent to SCC*mec* IV seems to be specific for caMRSA 'USA300' strains.[19] Furthermore, plasmid-located macrolide resistance genes *msr*(A) and *mph*(B) coding for macrolide efflux and a macrolide phosphotransferase, respectively, have been reported for 'USA300'.[20] These resistance genes are more frequent among coagulase-negative staphylococci and so far are rare among *S. aureus*.[21] A signature AT repeat consisting of $\geq$6 AT repeats in the vicinity of the SCC*mec* integration site has been described as a further characteristic of caMRSA 'USA300'.[22]

Here, we report on types of caMRSA from Germany in 2005 and 2006 with special emphasis on caMRSA of lineage ST8.

Materials and methods

Origin of S. aureus isolates investigated

The German National Reference Center for Staphylococci at the Robert Koch Institute, Wernigerode Branch has established a network based on diagnostic microbiological laboratories that send isolates for typing, all together 145 laboratories in all of Germany. MRSA isolates thought to possibly be caMRSA had been collected and submitted to the reference centre based on the following criteria: (i) originating from infections acquired in the community in the absence of risk factors associated with haMRSA; and (ii) MRSA from hospital-acquired infections (more than 48 h after admission) when associated with deep-seated infections of skin and soft tissue and from necrotizing pneumonia.

All isolates were primarily grown on sheep blood agar and confirmed by standard procedures as *S. aureus*.

Antimicrobial susceptibility testing was performed by broth microdilution according to DIN 58940, Deutsches Institut für Normung.[23]

Molecular typing

For *Sma*I-macrorestriction patterns, the HARMONY protocol as described by Murchan *et al*.[24] was followed. The resulting patterns were analysed using the Bionumerics software (Applied Maths, Ghent, Belgium) for relatedness evaluation and dendrogram generation. Multilocus sequence typing was performed according to Enright *et al*.,[25] except for the use of an alternative forward primer for the tpi amplimer.[26]

spa sequence typing was performed according to the Ridom Staph Type standard protocol (www.ridom.com) and by use of the Ridom Staph Type software package for assigning *spa* types.[27] SCC*mec* elements of types I–V were identified by using a combination of different PCRs, as described previously.[26,28]

PCR experiments

Procedures for DNA extraction and detection of antibiotic resistance genes *mecA*, *erm*(A), *erm*(B) and *erm*(C) by PCR were performed using primers and conditions as reported previously.[29] PCR for the detection of *lukS-PV lukF-PV* was performed using primers as described by Lina *et al*.[30] and conditions as reported previously.[29]

For the detection of *msr*(A), we used the primer pair *msr*(A)-f 5′-GAAGCACTTGAGCGTTCT (1687–1704 in ABO13298) and *msr*(A)-r 5′-CCTTGTATCGTGTGATGT (1887–1869), and for the detection of *mph*(B), we used primers *mph*(B)-f 5′-CATGGAGTAGAATGGGTT (2417–2434 in ABO13298) and *mph*(B)-r 5′-TGGACTTATTGTGGCTGC (2604–2587).

PCR for *arcA* (ACME gene cluster) was performed using primers *arcA*-f 5′-TCAAACTTTGAGAGATGA (169–188, locus SAUSA300-0065 in CP000255) and *arcA*-r (346–329), the cycling scheme according to Strommenger *et al*.[29] and an annealing temperature of 50°C.

PCR for the detection of the SACOL0058 sequence and its AT repeats was performed by use of primers and the cycling scheme as described by Bonnstetter *et al*.,[22] with the exception of an annealing temperature of 55°C.

Results

caMRSA of different clonal lineages and infections caused by them

The number of MRSA encoding PVL that were referred for investigation was 46 among 2497 (1.8%) in 2005 and 71 among 2318 (3.1%) in 2006.

Data on the clinical origin and results of typing are shown in Table 1. Attribution to clonal lineages was deduced from *spa* sequence types using the BURP algorithm. For selected isolates of *spa* types t008 (ST8), t437 (ST59) and t305 (ST617), MLSTs were determined and assigned. For only two patients, a nosocomial acquisition was likely. The isolates mostly originated from deep-seated infections of skin and soft tissue; there were, however, also five cases of pneumonia, two of them with a fatal outcome.

Among the 117 isolates investigated, 80 (68%) were *spa* type t044 (suggesting CC80), 23 isolates (20%) were t008 (suggesting CC8) and 2 non-typeable isolates were found to be ST8. Other clonal lineages were sporadically represented. Besides three isolates exhibiting *spa* type t002 and containing SCC*mec* V, PCR typing indicated SCC*mec* IV elements for all the other isolates investigated. Isolates exhibiting *spa* type t044 originated from various areas of Germany (federal states). The first cases of infections with caMRSA *spa* type t008 were recorded in the West of Germany in 2005, with other cases occurring elsewhere in 2006. Cases associated with isolates of *spa* type t310 were restricted to the South of Germany in 2005–06.

Patients with infections caused by caMRSA t008, PVL-positive, SCCmec IVa and their relationship to the USA

In 2005, a patient suffering from a skin abscess had stayed in the USA for several months. In addition, there were two episodes of infections associated with a US military base. In early 2006, a *lukS-PV lukF-PV*-positive caMRSA isolate exhibiting ST8, t008, SCC*mec* IVa and positive for *lukS-lukF* was obtained from blood culture and tracheal secretion from a 2-month-old child with severe pneumonia. The family belonged to the staff of a US military base. Further investigations of family members revealed vaginal carriage for MRSA exhibiting the same characteristics. In November/December 2006, five staff members of the same military base were affected by skin abscesses. The isolates were ST8, *lukS-PV lukF-PV*-positive and SCC*mec* IVa and showed pulse types as seen in lane 13 of Figure 1. Three isolates were *spa* t008 and two were *spa* non-typeable.

Table 1. Clinical origin and molecular typing of caMRSA from infections in 2005 and 2006 in Germany

			Clonal lineages as deduced from *spa* types via eBURP															
	Number of isolates		t002/ST5 SCC*mec* V		t008/ST8 SCC*mec* IV		t310/ST22 SCC*mec* IV		t021/ST30 SCC*mec* IV		t437/ST59 SCC*mec* IV		t044/ST80 SCC*mec* IV		t355/ST152 SCC*mec* IV		t305/ST617 SCC*mec* IV	
Clinical origin	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006
Abscess (skin, soft tissue)	30	45	2	1	5	9	1	1	–	3	–	1	22	29	–	1	–	–
Furuncle	9	11	–	–	2	1	–	–	–	–	–	–	7	10	–	–	–	–
Diabetic ulcer	1	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Wound infection	1	5	–	–	1	–	–	2	–	1	–	–	–	2	–	–	–	–
Folliculitis	1	2	–	–	–	–	–	–	–	–	–	–	1	1	–	–	–	1
Phlegmonous inflammation	–	1	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–
Pneumonia	1	4	–	–	1	3	–	–	–	–	–	–	–	1	–	–	–	–
Septicaemia	–	1	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–
Nasal colonization	3	2	–	–	–	–	–	–	–	–	–	–	3	2	–	–	–	–
Total	46	71	2	1	10	13	1	3	–	4	–	1	33	47	–	1	–	1

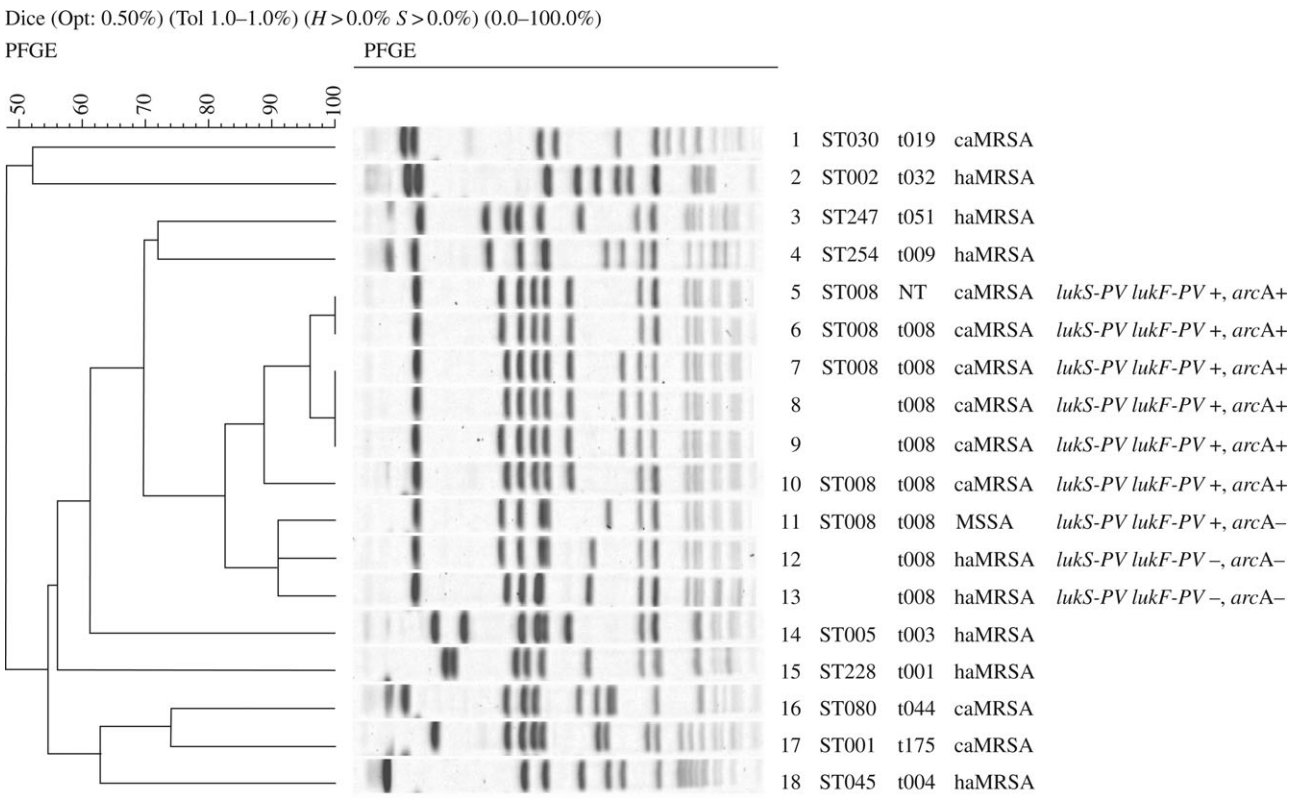

Figure 1. *Sma*I-macrorestriction patterns of MRSA and MSSA of lineage ST8 in comparison with major clonal lineages of epidemic haMRSA and caMRSA.

Introduction of caMRSA t008 into hospitals

During 2005, two patients in different hospitals were admitted for surgical treatment of abscesses. MRSA exhibiting the same characteristics, as those given earlier, were later isolated from post-operative wound infections from other patients who were in the same ward at the same time, suggesting that nosocomial transmission had occurred.

Further characterization of isolates exhibiting spa type t008/MLST ST8

All of the 23 isolates were phenotypically resistant to oxacillin (MIC $\geq$ 4 mg/L) and to erythromycin. Three isolates were also resistant to clindamycin and harboured *erm*(C) in addition to *msr*(A) and *mph*(B). PCR analysis indicated the presence of an SCC*mec* IVa element.

As shown in Figure 1, *Sma*I-macrorestriction patterns of MRSA t008/ST8 form a separate cluster when compared with major clonal lineages of haMRSA and caMRSA. MRSA ST8, SCC*mec* VIa, PVL-positive exhibit patterns that are similar to each other, but differ from those of PVL-negative MRSA by more than three fragments.

PCR for markers characteristic for caMRSA 'USA300'

Although PVL-positive MRSA exhibiting ST8 but lacking other characteristics of 'USA300' have not been reported so far, a rapid identification of this particular clone by PCR is important. Therefore, we tested isolates of other clonal lineages of caMRSA as well as representative isolates of haMRSA clonal lineages for the presence of *arcA*, *msr*(A), *mph*(B) and $\geq$6 AT repeats in the SACOL0058 sequence (Table 2). The number of $\geq$6 AT repeats in the SACOL0058 sequence seems to be characteristic for *S. aureus* of the ST8 clonal lineage. PCR for the ACME-associated *arcA* gene was positive in 21 of 23 PVL-positive MRSA of t008/ST8, but also for 3 from 12 PVL-negative MRSA of this clonal lineage. This marker was not detected in isolates affiliated to other clonal lineages of haMRSA and caMRSA. Macrolide resistance genes *msr*(A) and *mph*(B) have not been found in isolates from other clonal lineages with the exception of one MRSA isolate of lineage ST5.

Discussion

As reported from other European countries[4,6,8,11] caMRSA ST80 still predominate among PVL-positive MRSA of community onset. caMRSA affiliated to clonal lineages ST1, ST5, ST9, ST22, ST30 and ST59 are less frequent, but were already observed in Germany in 2005[11] and were also reported from other European countries. The first PVL-positive caMRSA ST8, t008 were recorded in Germany in early 2005, and 22 further cases followed until the end of 2006. Interestingly, caMRSA ST8 had already been reported from the Netherlands in 2003.[31]

Because of the typical pattern of characteristics, there is no doubt that the 23 PVL-positive MRSA ST8 are a progeny of caMRSA 'USA300', and further importation from the USA is likely to occur. However, PVL-positive caMRSA ST8 have already been reported from other European countries, particularly Belgium, Denmark, the Netherlands[6,8,32,33] and Ireland.[34] There are also reports on PVL-positive MRSA ST8 from Japan[35] and from Hong Kong.[36] Although we have to assume that these isolates also represent 'USA300', an independent evolution of *lukS-PV lukF-PV*-containing MRSA of lineage ST8

Table 2. PCR detection of characteristics for discrimination of caMRSA ST8 'USA300' from other lineages of MRSA in clonal complex 8 and from other lineages of caMRSA and major clonal lineages of haMRSA

				PCR demonstration					
	spa type	Corresponding MLST	Number of isolates	*mecA*	*lukS-PV lukF-PV*	*arcA*	*msr*(A)	*mph*(B)	$\geq$6 AT repeats in SACOL0058
Clonal complex CC8	t008	8	21	+	21	19	21	21	21
	−	8	2	+	2	2	2	2	2
	t008	8	12	−	−	−	−	−	12
	t008	8	12	+	−	3	−	−	12
	t009	254	8	+	−	−	−	−	8
	t036	254	8	+	−	−	−	−	8
	t051	247	8	+	−	−	−	−	8
	t037	239	6	+	−	−	−	−	6
Other caMRSA lineages	t175	1	1	+	1	−	−	−	−
	t002	5	8	+	8	−	1	−	−
	t310	22	8	+	8	−	−	−	−
	t021	30	6	+	6	−	−	−	−
	t437	59	3	+	3	−	−	−	−
	t044	80	50	+	50	−	−	−	−
	t355	152	4	+	4	−	−	−	−
Major haMRSA lineages	t002	5	12	+	−	−	−	−	−
	t001	228	12	+	−	−	−	−	−
	t022, t032	22	15	+	−	−	−	−	−
	t003	225	15	+	−	−	−	−	−
	t004	45	15	+	−	−	−	−	−

cannot be excluded as suggested by a report from the UK on caMRSA of this clonal lineage containing *lukS-PV lukF-PV*, SCC*mec* I and surprisingly the superantigen determinants *sec* and *tst*.[37] PVL-negative MSSA ST8 are widely disseminated among nasal carriers in the community. ST8 MRSA emerged in the early 1970s ('ancestral MRSA' of clonal complex CC8[17]), and they still exist in German hospitals (26 nosocomial and 3 community isolates among 2318 MRSA sent to the authors' laboratory in 2006).

To date, ST8 caMRSA do not appear to be widely disseminated in Europe. This might be due to differences with respect to predisposing conditions among the communities in Europe and in the USA, where an association between non-white race and soft-tissue infections caused by caMRSA was established.[12] However, the reasons for this association are unclear, and there might be confounding factors not recorded (e.g. HIV status).

Apart from the cluster of infections in a US military base and the two cases for which nosocomial transmission was likely, the other 14 cases were sporadic. Intrafamilial transmission as already reported from the USA[38] and from the Netherlands[39] was observed in one case only.

Nevertheless, reliable, early detection in the clinical bacteriological laboratory is necessary and an essential prerequisite to prevent further dissemination. This can be achieved by PCR detection of marker genes that are characteristic for caMRSA 'USA300' such as *arcA* and *msr*(A). Although not frequently, *spa* type t008 can be found in isolates from other clonal lineages of clonal complex CC8. Moreover, 2 of 23 isolates of PVL-positive MRSA ST8 described here were non-typeable by *spa* typing. Therefore, PCR for $\geq$6 AT repeats in SACOL0058 is useful to confirm the attribution of PVL-positive MRSA to lineage ST8. The observation of some *arcA*-negative isolates among PVL-positive MRSA ST8 and *arcA*-positive isolates among PVL-negative MRSA ST8 means that PCR for the presence of *msr*(A) is useful as an additional marker.

However, care must be exercised when using molecular epidemiological markers on transferable genetic elements, as events such as loss or acquisition of these elements by other clonal lineages, e.g. *arcA* in ST5-MRSA-SCC*mec* II from the USA,[40] could mislead.

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Transparency declarations

None to declare.

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