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Predominance of staphylococcal cassette chromosome *mec* (SCC*mec*) type IV among methicillin-resistant *Staphylococcus aureus* (MRSA) in a Swedish county and presence of unknown SCC*mec* types with Panton-Valentine leukocidin genes

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an established nosocomial pathogen, but has recently begun to appear in the community. The clones in the community may not have originated in the hospital setting, and are referred to as community-acquired MRSA (CA-MRSA). Resistance to methicillin is mediated by the gene *mecA*, which is carried by the mobile genetic element staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* typing (I–IV) of all clinical isolates of MRSA (n = 92) from 1987 to 2004 in Örebro County, Sweden, was performed by real-time LightCycler PCR to detect the essential genetic components *mecA*, *mecR1*, IS*1272*, *ccrA* and *ccrB*. Forty-one isolates harboured type IV SCC*mec*, of which ten could be classified further as subtype IVa, and 27 as subtype IVc. No isolates belonged to subtype IVb, but four isolates could not be subtyped, and may be examples of novel type IV SCC*mec* subtypes. Thirty-five MRSA isolates, assigned to six different pulsotypes by pulsed-field gel electrophoresis, did not belong to SCC*mec* types I–IV. The Panton-Valentine leukocidin (PVL) genes were identified in two of these pulsotypes. Only *SCCmec* type IV has been associated previously with the PVL toxin, but the results suggest that new PVL-positive clones with novel SCC*mec* types may be arising and disseminating in the community.

Keywords Community-acquired MRSA, LightCycler PCR, *mec*A, methicillin-resistant *Staphylococcus aureus*, Panton-Valentine leukocidin genes, SCC*mec*

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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first described in 1961 [1] and such bacteria have emerged subsequently as a major worldwide nosocomial problem. Strains of epidemic MRSA (EMRSA) are well-adapted to survive, spread and colonise humans in a hospital environment [2], as exemplified in the UK by the EMRSA-15 and EMRSA-16 clones which predominate in UK hospitals and account for >95% of MRSA bacteraemias [3]. Investigators in several countries have reported that the epidemiology of MRSA is changing, and there is growing concern regarding the MRSA strains that are now appearing in the community [4–8]. Community-acquired MRSA (CA-MRSA) refers to strains isolated from individuals who have not recently been in contact with a hospital or long-term care facility, received antibiotic treatment, nor have other risk-factors for MRSA. CA-MRSA causes predominantly skin and soft tissue infections, and has been identified as a significant problem among Australian aborigines [9], intravenous drug users [10,11], prison inmates [12,13] and other categories of individuals [14–16]. The reports of four paediatric deaths caused by a CA-MRSA clone in the USA [5] have attracted the attention of physicians worldwide, and have led to increased interest in finding the origin of CA-MRSA.

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Methicillin-resistance in *S. aureus* is characterised by the expression of an altered penicillin-binding protein, PBP2a (or PBP2′), with decreased affinity for all β-lactam antibiotics, which thereby allows continuous cell-wall assembly. PBP2a is encoded by *mecA* and its regulatory genes, which are located in a mobile genetic element termed staphylococcal cassette chromosome *mec* (SCC*mec*), and incorporated in the chromosome of *S. aureus* at a site-specific location near the origin of replication [17,18]. Cloning and sequencing of SCC*mec* from various MRSA strains has revealed five different allotypes that are designated SCC*mec* types I–V [17–20]. Each of these SCC*mec* elements carries a characteristic combination of two essential genetic components, known as the *mec* gene complex and the cassette chromosome recombinase (*ccr*) gene complex. The *mec* gene complex consists of the insertion sequence IS*431*, together with *mecA* and its regulatory genes *mecI* and *mecR1,* which can be either intact or mutated. Three different *mec* gene complexes (classes A, B and C2) have been described in SCC*mec* types in *S. aureus*. The *ccr* gene complex comprises the genes *ccrA* and *ccrB*, which code for two recombinases that are required for site-specific integration and excision of SCC*mec*. There are at least three allotypes of *ccrA* and *ccrB* (types 1–3) in SCC*mec*, but only type 2 has been shown to be functionally intact [17,21,22]. Most recently, a novel *ccrC* gene complex (or type 5 *ccr*), responsible for recombination events of type V SCC*mec,* was identified in MRSA strain WIS [20]. The SCC*mec* allotypes are classified as I–V, depending on the combination of the *mec* gene complex and the *ccr* gene complex that is present. Further classification of the type IV SCC*mec* is based on differences in the remaining areas of SCC*mec*, which are referred to as junkyard (J) regions. Previously, all isolates collected from various countries have been found to carry subtype IVa or IVb [7], but Ito *et al.* [21] recently identified a novel type IVc SCC*mec* in an isolate (81/108) from an outpatient in Japan.

It is a matter of debate as to whether the newly emerging CA-MRSA strains were derived from existing hospital-acquired strains (HA-MRSA) and were transmitted to the community, or whether they evolved independently [4,14,23,24]. It has been proposed that all MRSA are descendants of a single common ancestor which has spread worldwide [25], while an alternative suggestion is that SCC*mec* has been acquired horizontally on several occasions from other staphylococcal species in the non-hospital environment [2,26–28]. Several molecular typing techniques have been used to study large collections of MRSA isolates from different geographical areas, and have revealed relatively few pandemic clones [2,23,26,27,29,30]. In contrast, CA-MRSA strains have been found to comprise at least ten clonal complexes, which indicates that these bacteria originated from *S. aureus* clones with a greater genetic diversity than HA-MRSA strains [2,26]. SCC*mec* types I–IV all occur among healthcare-associated isolates, whereas the type IV SCC*mec* is the predominant element in CA-MRSA [4,18]. There is an obvious association between the five major clonal complexes and the hospital-acquired SCC*mec* types I, II and III. However, the type IV SCC*mec* is found in *S. aureus* isolates with several different genetic backgrounds, is smaller than the other SCC*mec* types, and generally does not contain any additional resistance genes, which may facilitate its mobility. Type IV SCC*mec* contains functional recombinases, and it has been found in many clones, which further suggests that this element is more mobile than the other SCC*mec* types [4,18,29,31]. It is possible that strains with different types of SCC*mec* have evolved from genetic backgrounds with additional virulence traits and the ability to disseminate [29,32].

The aim of this study was to investigate the distribution of SCC*mec* types I–IV among MRSA isolates from Örebro County, Sweden, with special focus on the characterisation of CA-MRSA isolates by LightCycler PCR analysis of the various types of *ccr* and *mec* gene complexes.

MATERIALS AND METHODS

Bacterial isolates

All clinical isolates of MRSA (n = 92) identified between 1987 and 2004 in Örebro County (population *c.*280 000) were analysed. In Sweden, contact tracing is conducted according to the Communicable Diseases Act, and essential epidemiological information has been collected and available in a county database since 2000. Isolates were stored at −70°C at the Department of Clinical Microbiology at Örebro University Hospital.

Community-acquired infection was recorded following identification of MRSA in an outpatient setting, or if a patient was positive for MRSA within 48 h of admission to a hospital, provided that the patient had no medical history of MRSA

infection or colonisation, no hospitalisation, admission to a nursing home, dialysis or surgery during the previous year, and no permanent indwelling catheters or medical devices that penetrated the skin. Using these criteria, 36 (39%) of 92 MRSA isolates were categorised as CA-MRSA, 46 (50%) as HA-MRSA, and ten (11%) could not be categorised because of lack of epidemiological information. Isolates were derived from skin (n = 56), nares (n = 14) and other sites (n = 3), or information concerning the site was not available (n = 19).

S. aureus was identified by conventional microbiological methods, including DNase and coagulase tests. Antibiotic susceptibility testing was performed by disk diffusion and Etests (AB Biodisk, Solna, Sweden), as recommended by the Swedish Reference Group for Antibiotics (SRGA) and the SRGA Subcommittee on Methodology (SRGA-M) (http://www.srga.org). The following antibiotics were tested: oxacillin/cefoxitin, fusidic acid, clindamycin, ciprofloxacin, erythromycin, gentamicin, rifampicin and vancomycin. Chloramphenicol, trimethoprim-sulphamethoxazole and mupirocin were also tested with selected isolates. Resistance to oxacillin was measured by Etest, with resistance defined as an MIC of >1.0 mg/L according to the SRGA. Isolates were verified as MRSA by detecting the *nuc* and *mecA* genes (see below).

Six international MRSA strains characterised previously were used as controls for SCC*mec* typing: reference strains 8/6-3P (JCSC1978), CA05 (JCSC1968), MR108 and JCSC4469 were kindly provided by T. Ito and K. Hiramatsu (Department of Bacteriology, Juntendo University, Tokyo, Japan), and strains M307 (E-MRSA3) and 98/514 (Helsinki VII, E20), were obtained from the HARMONY collection [33]. Strain RS12 (ATCC 29213), which lacks *mecA*, was used as a negative control.

DNA isolation

All isolates were cultured on blood agar and incubated overnight at 37°C. DNA was isolated from the reference strains with the Dynabeads DNA DIRECT System I kit (Dynal Biotech, Oslo, Norway) for higher purity and longer stability. Genomic DNA was extracted from clinical isolates by denaturing a few colonies suspended in sterile water at 98°C for 15 min and then centrifuging at 13 000 *g* for 30 s. The supernatant was used as template for amplification in a real-time PCR in the LightCycler System (Roche Diagnostics, Mannheim, Germany) and specific binding of SYBR Green I fluorescent dye to the resulting double-stranded DNA.

Detection of *nuc* and *mecA*

The isolates were verified as MRSA by using a multiplex PCR adapted for the LightCycler and SYBR Green I to detect the *nuc* (forward primer, 5′-GCGATTGATGGTGATACGGTT-3′; reverse primer, 5′-AGCCAAGCCTTGACGAACTAAAGC-3′) and *mecA* (forward primer, 5′-GCAATCGCTAAAGAACTAAG-3′; reverse primer, 5′-GGGACCAACATAACCTAATA-3′) genes [34]. Samples were preincubated for 10 min at 95°C and then subjected to 35 cycles of amplification comprising 95°C for 10 s, 55°C for 5 s and 72°C for 8 s. The melting programme comprised one cycle of 95°C for 0 s (hold time) and 58°C for 1 min, followed by an increase in temperature at a transition rate of 0.1°C/s to 95°C while continuously monitoring fluorescence.

Determination of SCC*mec* type

The *ccr* gene complex and the *mec* gene complex were detected in separate PCRs. The primers used to identify SCC*mec* types I–IV (Table 1) were as reported previously [7,17,35], with the exception of IVc1, IVc2, and clB. The class A *mec* gene complex was identified by use of the mcR2 and mcR5 [7] primers, which are specific for the penicillin-binding domain of *mecR1*. Primers clB (this study) and IS4 [35] correspond to the nucleotide sequence of IS*1272*, which is a component of the class B *mec* complex. The *ccr* type was determined by combining the common primer for *ccrB* (β2) [17] with specific primers for *ccrA1* (α2), *ccrA2* (α3) and *ccrA3* (α4) [17]. All

Table 1. Sequences of primers used to detect the components of SCC*mec*

Primer	Nucleotide sequence 5′-3′	Position (bp)	Accession number[a]	Reference
ccr gene complex[b]				
β2	ATTGCCTTGATAATAGCCITCT	25539–25522 (*ccr* 1)	AB033763	[17]
		11738–11720 (*ccr* 2)	AB063172	
		7276–7258 (*ccr* 3)	AB037671	
α2	AACCTATATCATCAATCAGTACGT	24845–24868 (*ccr*A1)	AB033763	[17]
α3	TAAAGGCATCAATGCACAAACACT	10802–10825 (*ccr*A2)	AB063172	[17]
α4	AGCTCAAAAGCAAGCAATAGAAT	5486–5508 (*ccr*A3)	AB037671	[17]
mec gene complex				
class A				
mcR2	CGCTCAGAAATTTGTTGTGC	22758–22777	AB037671	[7]
mcR5	CAGGGAATGAAAATTATTGGA	23076–23056		[7]
class B				
IS4	ACAATCTGTATTCTCAGGTCGT	14846–14867	AB063172	[35]
clB	CGATTGGCATTTGCTTCAAT	15088–15069		This study
SCC*mec*				
subtype IVa				
4a1	TTTGAATGCCCTCCATGAATAAAAT	4726–4750	AB063172	[7]
4a2	AGAAAAGATAGAAGTTCGAAAGA	5183–5161		[7]
subtype IVb				
4b1	AGTACATTTTATCTTTGCGTA	2266–2286	AB063173	[7]
4b2	AGTCATCTTCAATATCGAGAAAGTA	3259–3235		[7]
subtype IVc				
IVc1	CCAGAGAAATGTGGTGTTTT	8923–8942	AB096217	This study
IVc2	GCCCTACATGAATCATCAAA	9128–9109		This study

[a]The accession number is available in the GenBank™ database (http://www.ncbi.nlm.nih.gov). [b]The type of *ccr* gene complex is determined by using primer β2 (the common primer for three types of *ccrB*) and one of the primers α2, α3, or α4 (to reflect the three allotypes of *ccrA*).

isolates were analysed with the primers for each gene complex in separate reactions. Further subtyping was performed on the isolates that were classified as SCC*mec* type IV by examining nucleotide differences in the junkyard region (J1). Primers used to identify the SCC*mec* subtypes were as follows: 4a1 and 4a2 [7] for subtype IVa; 4b1 and 4b2 [7] for subtype IVb; and IVc1 and IVc2 (this study) for subtype IVc. All primers were synthesised by Scandinavian Gene Synthesis (Köping, Sweden).

All PCR reagents, except primers and template DNA, were provided in the LightCycler FastStart DNA Master SYBR Green I kit (Roche). Each reaction contained 2 μL of SYBR Green I and 2 μL of DNA template (1–40 ng) and was performed in a LightCycler glass capillary in a total volume of 20 μL. Cycling conditions were optimised for each LightCycler PCR, including $MgCl_2$ and primer concentrations, annealing temperatures and extension times. The final optimised conditions are summarised in Table 2.

Amplification products were characterised by melting curve analysis to increase the sensitivity and specificity of SYBR Green I detection. The melting programme comprised one cycle of 95°C for 0 s (hold time) and 65°C for 15 s, followed by an increase in temperature at a transition rate of 0.1°C/s to 95°C, while continuously monitoring fluorescence. The samples were then cooled to 40°C over a period of 30 s. Melting peaks were derived from the initial melting curves (fluorescence (F_1) vs. temperature (T)) by plotting ($-dF_1/dT$ vs. T). The specific melting temperatures (T_m) obtained from all runs of the isolates analysed (n = 92) and reference strains (n = 6) were used to calculate the mean T_m for each gene (Table 3).

Table 3. Melting temperatures (mean T_m) for positive clinical isolates (n = 92) and reference strains (n = 6)

Assay	Sample	No. positive/ No. tested	Mean T_m °C (SD)
ccr 1	Reference	5/5	81.3 (0.3)
	Clinical isolates	5/92	80.9 (0.1)
ccr 2	Reference	10/10	81.2 (0.7)
	Clinical isolates	44/92	81.5 (0.4)
ccr 3	Reference	7/7	82.2 (0.3)
	Clinical isolates	8/92	81.9 (0.2)
Class A *mec* complex	Reference	6/6	75.9 (0.5) and 80.0 (0.5)[a]
	Clinical isolates	11/92	75.7 (0.4) and 79.9 (0.2)[a]
Class B *mec* complex	Reference	4/4	77.3 (0.1)
	Clinical isolates	46/92	77.1 (0.2)
Subtype IVa	Reference	10/10	77.9 (0.2)
	Clinical isolates	10/41[b]	77.9 (0.2)
Subtype IVb	Reference	10/10	78.5 (0.4)
	Clinical isolates	0/41[b]	No positive isolates
Subtype IVc	Reference	4/4	74.5 (0.2)
	Clinical isolates	27/41[b]	74.1 (0.2)

[a]An additional peak was seen in all runs. [b]Only isolates with a type IV SCC*mec* (n = 41) were differentiated further.

Initial verification

The identities of the PCR products were confirmed initially by gel electrophoresis and sequencing of the amplification products from the reference strains. Before sequencing, the products were purified with a High Pure PCR Purification Kit (Roche). Sequencing was performed with an ABI PRISM BigDye Terminator v.3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Stockholm, Sweden) and a GeneAmp PCR System 2700 (Applied Biosystems), using the same primers as for the LightCycler PCRs. Both strands were sequenced. Reaction products were purified using a Qiagen DyeEx 2.0 Spin kit (VWR International, Stockholm, Sweden) and then separated on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The nucleotide sequences were analysed using ABI PRISM AutoAssembler DNA Sequence Assembly 1.4.0 software, and were then compared with sequence databases at the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) with the basic local alignment search tool BLAST (http://www.ncbi.nlm.nih.gov). In addition, the presence of subtype IVc in a clinical isolate was verified initially by sequencing the product amplified when using the above primers and comparing the sequenced region with the BLAST database at NCBI.

Detection of *luk*S-PV and *luk*F-PV

The LightCycler and SYBR Green I dye were used to detect PVL genes by PCR as described previously [36].

Pulsed-field gel electrophoresis

The 92 MRSA isolates were characterised by pulsed-field gel electrophoresis (PFGE) of chromosomal *Sma*I digests, prepared with the GenePath Group 1 Reagent Kit (Bio-Rad Laboratories, Hercules, CA, USA). PFGE patterns were obtained with a contour-clamped homogeneous electric field apparatus (GenePath System; Bio-Rad) run for 20 h with agarose 1% w/v gels in 0.5 × Tris-borate buffer at 6 V/cm with a switching time of 5.3–34.9 s. Differences in band patterns were analysed with Molecular Analyst Fingerprinting software v.1.6 (Bio-Rad) using the Dice coefficient and unweighted pair grouping by

Table 2. LightCycler parameters used to detect the *ccr* and *mec* complexes[a]

	Cycle conditions				Cycle programme data		
Reaction	$MgCl_2$ (mM)	Primers (μM)		Cycles	Denat	Ann	Ext
ccr 1	3	α2 (0.5)	β2 (0.5)	30	95 °C, 10 s	59 °C, 5 s	72 °C, 28 s
ccr 2	3	α3 (0.5)	β2 (0.7)	30	95 °C, 10 s	55 °C, 5 s	72 °C, 38 s
ccr 3	4	α4 (0.3)	β2 (0.7)	35	95 °C, 10 s	59 °C, 5 s	72 °C, 70 s
Class A *mec* complex	3	mcR2 (0.3)	mcR5 (0.3)	25	95 °C, 10 s	61 °C, 5 s	72 °C, 13 s
Class B *mec* complex	5	IS4 (0.3)	clB (0.5)	30	95 °C, 10 s	56 °C, 5 s	72 °C, 10 s
Subtype IV a	4	4a1 (0.5)	4a2 (0.3)	30	95 °C, 10 s	51 °C, 5 s	72 °C, 18 s
Subtype IV b	4	4b1 (0.3)	4b2 (0.5)	30	95 °C, 10 s	51 °C, 5 s	72 °C, 40 s
Subtype IV c	3	IVc1 (0.7)	IVc2 (0.3)	25	95 °C, 10 s	50 °C, 5 s	72 °C, 9 s

[a]Typing of SCC*mec* was performed in eight separate reactions. The PCR programme starts with a preincubation for 10 min at 95 °C to activate the FastStart polymerase, followed by amplification under different conditions for each gene. The fluorescence was measured at the end of each extension step for real-time visualisation.

mathematical averaging (UPGMA), with a 1% band tolerance and 0.5% optimisation, followed by final visual examination and interpretation as recommended by Tenover *et al.* [37].

RESULTS

Table 4 summarises the results of the SCC*mec* typing. This analysis indicated that among the 92 MRSA isolates studied, five (5.4%) had SCC*mec* type I, three (3.3%) had type II, eight (8.7%) had type III, and 41 (44.6%) had type IV. The 41 isolates with type IV SCC*mec* could be further differentiated into ten (24.4%) isolates with subtype IVa, and 27 (65.9%) isolates with subtype IVc. No isolates with subtype IVb were detected, and the remaining four (9.8%) isolates had an unknown subtype of type IV SCC*mec*. Thirty-five (38.0%) of the 92 isolates were not typable with the primers used in this study for PCR amplification of the *ccr* or *mec* gene complexes.

Isolates with type IV SCC*mec* usually had lower oxacillin MICs (85% had an MIC < 256 mg/L; median 8, range 1.5 - >256) and were rarely resistant to any of the other antibiotics tested. However, most (20/27) of the isolates with type IVc SCC*mec* were resistant or showed decreased sensitivity to fusidic acid, and sometimes also to ciprofloxacin and gentamicin. Most (27/35) of the isolates with unknown SCC*mec* types were resistant to gentamicin, and one isolate was resistant to rifampicin and another to trimethoprim-sulphamethoxazole. Moreover, five of the 35 untypable isolates had higher oxacillin MICs (>256 mg/L), while the other untypable isolates were less resistant (MICs of 1.5–4 mg/L), although one isolate had an oxacillin MIC of 48 mg/L.

Most (66%) of the 41 isolates with type IV SCC*mec* were CA-MRSA, although this group also included eight HA-MRSA isolates; in comparison, only two of 27 isolates with SCC*mec* subtype IVc were HA-MRSA. Sixteen (40%) of the 41 type IV SCC*mec* isolates had a known origin outside Sweden (Somalia, Yugoslavia, Lebanon or Spain). Of the 35 isolates with untypable SCC*mec*, 25 (71.4%) were HA-MRSA, including 20 isolates from a local outbreak in a neonatal intensive care unit (ICU) and five other isolates that were linked indirectly to the same outbreak. Only five (14.3%) of the 35 SCC*mec*-untypable isolates had a known origin outside Sweden (China and Kazakhstan), while the remaining isolates of unknown SCC*mec* type comprised sporadic CA-MRSA isolates.

PFGE analysis showed that the ten isolates with subtype IVa SCC*mec* could be divided into four pulsotypes (Fig. 1), and that pulsotype D included six of these ten isolates. The 27 isolates with SCC*mec* subtype IVc clustered into five pulsotypes (Fig. 1), and most (20/27) of these isolates were indistinguishable or closely related (pulsotype F). The four isolates with a type IV SCC*mec* that could not be further subtyped belonged to four different pulsotypes (Fig. 1). The PFGE results also showed that the 35 isolates with a non-typable SCC*mec* were grouped into six pulsotypes (Fig. 1), and that most (26/35; pulsotype E) were indistinguishable or closely related; all except one of these were linked directly or indirectly to an MRSA outbreak at a neonatal ICU.

None of the isolates with type I, II or III SCC*mec* carried the genes for PVL toxin. However, 22 (81.5%) of 27 isolates with type IVc SCC*mec* carried these genes; no other isolates with type IV SCC*mec* were positive. Six of the 35 isolates that were SCC*mec* untypable were positive for the PVL toxin genes, with four of these isolates originating from Kazakhstan. Two additional isolates also carried the PVL genes, but there was no obvious

Table 4. Types of staphylococcal cassette chromosome *mec* (SCC*mec*) found in MRSA isolates collected in Örebro County, Sweden

No. of isolates (total *n* = 92)	SCC*mec* type	*ccr* gene complex			*mec* gene complex		Subtyping of SCC*mec* type IV				PVL+
		1	2	3	A	B	a	b	c	d	
5	I	+	−	−	−	+					0
3	II	−	+	−	+	−					0
8	III	−	−	+	+	−					0
41	IV	−	+	−	−	+					22 (54%)
10/41	IVa						+	−	−	−	0
0/41	IVb						−	+	−	−	0
27/41	IVc						−	−	+	−	22 (81%)
4/41	IV unknown						−	−	−	−	0
35	unknown	−	−	−	−	−					6

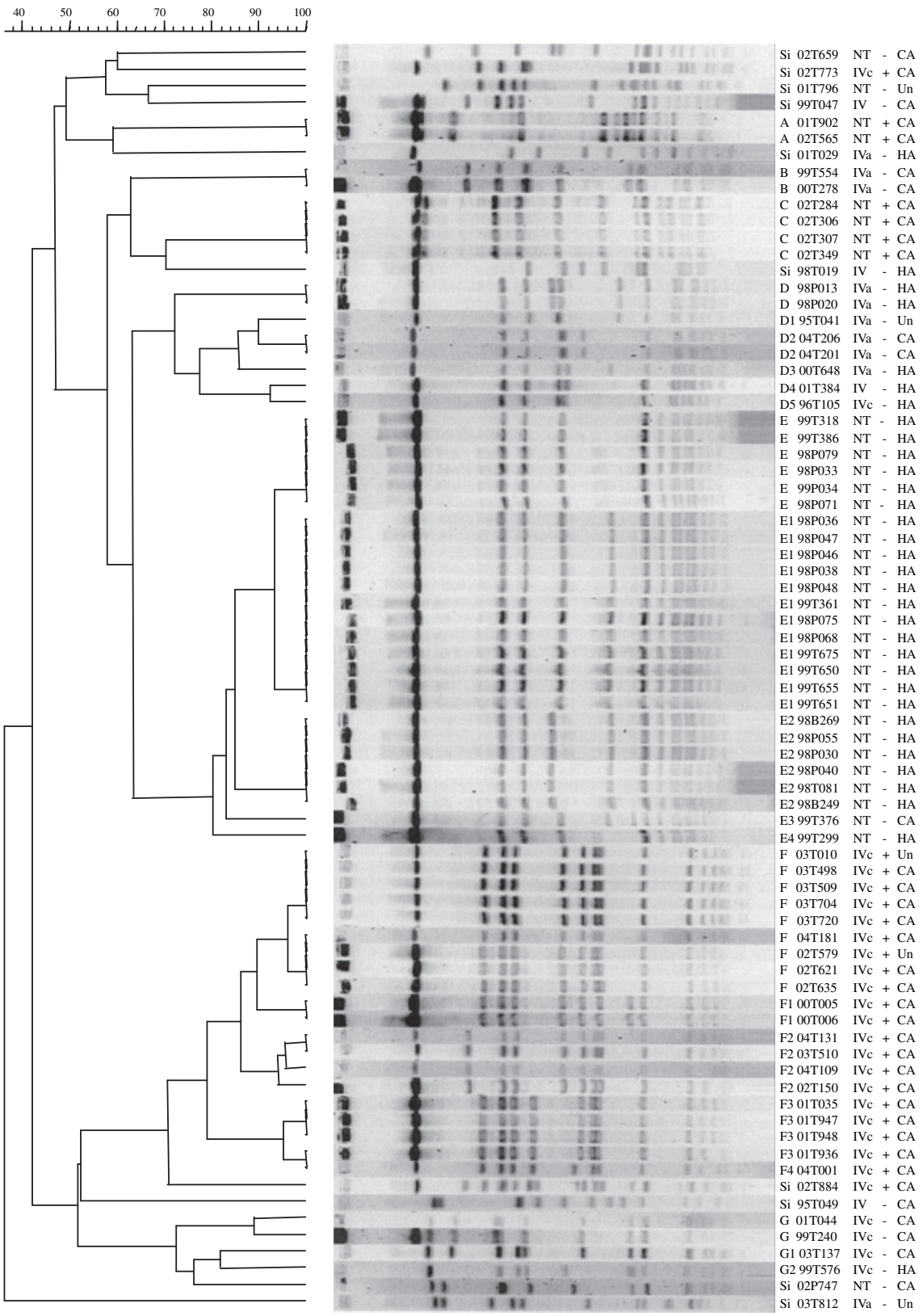

Fig. 1. Pulsed-field gel electrophoresis patterns of *Sma*I-digested total chromosomal DNA from MRSA isolates with type IV SCC*mec* and nontypable (NT) isolates for SCC*mec* types I–IV. Clone designation, SCC*mec*-type and the presence (+) or absence (–) of the genes for Panton-Valentine leukocidin is indicated to the right of the dendrogram. The origin of isolates is indicated by CA (community-acquired), HA (hospital-acquired) or Un (unknown).

epidemiological link between the two patients who were infected with these two isolates.

DISCUSSION

Most (45%) of the 92 MRSA isolates from Örebro County in this study contained type IV SCC*mec*, but only ten (24%) had SCC*mec* subtype IVa. In contrast to those findings, Okuma *et al.* [7] analysed 32 type IV SCC*mec* isolates from the USA and Australia, and found that 31 were subtype IVa and one was subtype IVb. Also, 27 of the 41 type IV SCC*mec* isolates in the present study were subtype IVc, a variant that was first identified in Japan [21] and was described recently as hospital-acquired in France [38]. Furthermore, the 41 type IV SCC*mec* isolates contained no isolates with subtype b, although four of the isolates could not be differentiated further and may therefore represent a novel subtype(s). Thirty-five of the 92 MRSA isolates studied did not carry any of the genetic structures that are known to exist in SCC*mec* types I–IV. Oliveira *et al.* [30] have reported an altered type IV SCC*mec* with a new type of *ccr* gene complex and a class B *mec* complex, but the untypable isolates investigated in the present study could not have belonged to that clone, since they did not contain a class B *mec* complex.

It has been reported that SCC*mec* subtypes IVa and IVb do not carry additional genes for antibiotic resistance [7,18] whereas 20 (74%) of the isolates in the present study with subtype IVc were resistant to fusidic acid. Furthermore, strain 81/108 (referred to as MR108 in the present study) was the first isolate observed to carry SCC*mec* subtype IVc, and this element has been found to contain Tn*4001* and the *aacA* and *aphD* genes encoding resistance to aminoglycosides [21,39]. Only one SCC*mec* subtype IVc isolate from Örebro County was resistant to gentamicin. Type IV SCC*mec* was first recognised in 2002 [18], but has been found subsequently in *S. aureus* isolates from the early 1980s [40], and has been prevalent in *Staphylococcus epidermidis* isolates since the 1970s [40]. Therefore, it is plausible that type IV SCC*mec* has subsequently acquired additional genes following exposure to antibiotic selection pressures in hospitals. By adapting to a hospital environment and acquiring additional resistance genes, the type IV SCC*mec* may become so large that it loses its ability to transfer horizontally. Isolates (n = 35) that were SCC*mec* untypable, including the 25 isolates that were directly or indirectly linked to an MRSA outbreak at a neonatal ICU, generally had lower oxacillin MICs, with the exception of four isolates originating from Kazakhstan and one isolate that originated from China. All outbreak isolates and three additional isolates were resistant to gentamicin.

In contrast to the finding of SCC*mec* subtype IVc in HA-MRSA in France [38], most (78%) of the isolates with that subtype in Örebro County were CA-MRSA. In addition, most isolates with unknown types of SCC*mec* were CA-MRSA, with the exception of the isolates associated with the outbreak in the neonatal ICU. New variants of SCC*mec* have been described in methicillin-resistant coagulase-negative staphylococci (CoNS) in Norway [41], but only one MRSA isolate in that study was found to have an untypable SCC*mec*. It is tempting to suggest that the untypable strains in Örebro County carried novel types of SCC*mec*. A new SCC*mec* type, designated type V, consisting of a *ccrC* recombinase complex and the class C2 *mec* gene complex, has been described recently by Ito *et al.* [20].

It is assumed that SCC*mec* originated in CoNS [28,35], and three additional *mec* gene complexes (C1, C2, and D) have been identified in CoNS, while the class C2 *mec* complex occurs widely in strains of *Staphylococcus haemolyticus* [35]. In the present study, 20 of the HA-MRSA isolates with unknown SCC*mec* were involved in the ICU outbreak in 1998; it is notable that an endemic situation existed for several years with a specific methicillin-resistant genotype of *S. haemolyticus* in the neonatal ward, and that the MRSA outbreak took place during that period [42].

PFGE analysis showed that the ten isolates with SCC*mec* subtype IVa belonged to four different pulsotypes, one of which contained six of the isolates. SCC*mec* subtype IVc isolates clustered into five pulsotypes, of which two predominated.

Interestingly, no epidemiological or geographical correlation was found between isolates that belonged to the same pulsotype. The four isolates with an unknown subtype IV SCC*mec* were all unrelated and displayed no epidemiological linkage. PFGE analysis showed that there was substantial genetic diversity between isolates with type IV SCC*mec*, perhaps indicating that type IV SCC*mec* improves the capacity for transfer between different genetic clones of *S. aureus*. PFGE differentiated the group of 35 isolates with untypable SCC*mec* into six pulsotypes. Several CA-MRSA strains appear to be virulent [5] and possess multiple toxin genes that are not present in the genomes of HA-MRSA. This further suggests that CA-MRSA were generated independently of HA-MRSA by insertion of SCC*mec* into the genomes of virulent *S. aureus* strains, resulting in highly pathogenic CA-MRSA clones. PVL is expressed by *c*.2% of *S. aureus* isolates and is encoded by the *lukS*-PV and *lukF*-PV genes, which are carried on a bacteriophage [43]. Production of PVL has been described in CA-MRSA, albeit only in strains with a type IV SCC*mec* [7,44], and although *lukS*-PV and *lukF*-PV are not part of SCC*mec*, the presence of PVL among isolates with type IV SCC*mec* may facilitate spreading and colonisation by causing primary skin infections. It has been reported that PVL-producing strains cause lethal necrotising pneumonia in otherwise healthy young patients with no established risk factors for MRSA [38,45]. Furthermore, Liassine *et al.* [44] have described CA-MRSA strains in Europe which, in addition to PVL, express enterotoxins and exfoliative toxins.

The present analysis of the presence of the *lukF*-PV and *lukS*-PV genes in the MRSA isolates yielded three notable findings. First, five CA-MRSA clones in Örebro County carried the PVL genes, which can be compared with a study showing that CA-MRSA infections in France are caused by a single clone in which all isolates express PVL [38]. Second, most (22/28) isolates carrying the PVL genes in Örebro County carried SCC*mec* subtype IVc, and these isolates could be differentiated into three clusters by PFGE. No other subtype of SCC*mec* type IV carrying the PVL genes was identified. Overall, 70% of CA-MRSA and 66% of isolates with type IV SCC*mec* in this area carried the PVL genes. Third, some isolates in Örebro County with unknown types of SCC*mec* were positive for PVL. These isolates belonged to two clusters (A and C), with one cluster (A) exhibiting higher levels of oxacillin resistance (MIC >256 mg/L), while the other (C) was less resistant to oxacillin but was resistant to gentamicin. Thus far, only type IV SCC*mec* (i.e., not types I, II or III) has been associated with the PVL toxin [7,44], but it is possible that new MRSA clones are evolving and disseminating in the community, and that such clones may carry a novel SCC*mec* that offers a selective advantage for the colonisation of humans in the absence of antibiotic selection, e.g., by encoding the expression of toxins. None of the HA-MRSA isolates studied contained the PVL genes.

In conclusion, the epidemiology of MRSA in Sweden is changing, with an increasing proportion of CA-MRSA. The type IVc SCC*mec* is the predominant element among CA-MRSA, and many isolates may also carry novel types of SCC*mec*. The presence of PVL toxin may facilitate dissemination in a non-hospital environment. The origin of SCC*mec* has not been determined, but these elements are found commonly in CoNS, which may be a possible source.

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