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ARTICLE

Long-term persistence of a multi-resistant methicillin-susceptible *Staphylococcus aureus* (MR-MSSA) clone at a university hospital in southeast Sweden, without further transmission within the region

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Abstract The objective of this study was to characterise isolates of methicillin-susceptible *Staphylococcus aureus* (MSSA) with resistance to clindamycin and/or tobramycin in southeast Sweden, including the previously described ECT-R clone (t002) found in Östergötland County, focusing on clonal relatedness, virulence determinants and existence of staphylococcal cassette chromosome (SCC) *mec* remnants. MSSA isolates with resistance to clindamycin and/or tobramycin were collected from the three county councils in southeast Sweden and investigated with *spa* typing, polymerase chain reaction (PCR) targeting the SCC*mec* right extremity junction (MREJ) and DNA microarray technology. The 98 isolates were divided into 40 *spa* types, and by microarray clustered in 17 multi-locus sequence typing (MLST) clonal complexes (MLST-CCs). All isolates with combined resistance to clindamycin and tobramycin ($n=12$) from Östergötland County and two additional isolates (clindamycin-R) were designated as *spa* type t002, MREJ type ii and were clustered in CC5, together with a representative isolate of the ECT-R clone, indicating the clone's persistence. These isolates also carried several genes encoding exotoxins, *Q9XB68-dcs* and *qacC*. Of the isolates in CC15, 83 % (25/30) were tobramycin-resistant and were designated *spa* type t084. Of these, 68 % (17/25) were isolated from newborns in all three counties. The persistence of the ECT-R clone in Östergötland County, although not found in any other county in the region, carrying certain virulence factors that possibly enhance its survival in the hospital environment, highlights the fact that basic hygiene guidelines must be maintained even when MRSA prevalence is low.

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Introduction

In countries with a low prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA), such as Sweden, infections caused by methicillin-susceptible *S. aureus* (MSSA) constitute a larger problem than infections caused by MRSA. In a large multi-centre European study by Grundmann et al., MSSA *spa* types were more diverse and less regionally distributed than MRSA [1]. However, a few outbreaks caused by MSSA have recently been reported [2–5]. In 2005, an outbreak in Östergötland County, Sweden, of multi-resistant (MR) MSSA *spa* type t002 with concomitant resistance to erythromycin, clindamycin and tobramycin (ECT-R) was detected. By whole-genome sequencing (WGS), the ECT-R clone was shown to carry a pseudo-staphylococcal cassette chromosome

(SCC) (~12 kb in size), showing a resemblance of more than 99 % with the SCC*mec* type II element of MRSA strain N315 [multi-locus sequence typing (MLST) clonal complex (CC) 5], suggesting probable derivation from a highly successful MRSA strain, which had partially excised its SCC*mec*, including the *mec* gene complex as well as *ccr* genes [6, 7].

It has been shown that the degree to which patients are shared between hospitals crucially influences the rates of nosocomial infection [8–10]. Through regional cooperation within the framework "Southeast Sweden", the hospitals in Östergötland County are connected to hospitals in the neighbouring county councils and patient exchange occurs, especially those patients in need of advanced specialist care may be referred to a tertiary care hospital in Östergötland.

The objective of this study was to detect and characterise isolates of MSSA with resistance to clindamycin or tobramycin or a combination of these from patients within the region of southeast Sweden, in order to assess the presence of the previously characterised ECT-R clone and possibly detect other clones of MR-MSSA. Distinct virulence determinants and the existence of SCC*mec* remnants were investigated.

Materials and methods

Settings

Southeast Sweden is constituted of the county councils of Jönköping, Kalmar and Östergötland. The region has an estimated 1,006,000 inhabitants and includes one tertiary care hospital (Linköping University Hospital in Östergötland County) and eight secondary care hospitals (three in Jönköping County, three in Kalmar County, two in Östergötland County). Several primary care centres are also located in each county. Exchange occurs as Linköping University Hospital provides advanced specialist care for patients in the region. Approximately 4,600 and 3,700 patients from Jönköping and Kalmar counties, respectively, are referred annually to Linköping University Hospital (personal communication: Utdatagruppen för vårddatalagret, Östergötland County Council, July 5 2013).

This study was approved by the Regional Ethical Review Board in Linköping, Sweden (M164-09).

Bacterial isolates

From June 2009 to June 2010, consecutive clinical isolates of *S. aureus* from patients within Jönköping, Kalmar and Östergötland counties were prospectively collected at the clinical microbiology laboratories of Ryhov Hospital in Jönköping, Kalmar County Hospital and Linköping University Hospital, respectively. Isolates which had one of the three following resistance profiles were included: clindamycin (group C), tobramycin (group T) or both clindamycin and tobramycin (group CT). A maximum of 20 isolates per group and location (one isolate per patient) were included. Each isolate was named according to the county from which it was collected (J=Jönköping, K=Kalmar and Ö=Östergötland) and the antibiotic resistance profile it had (C=clindamycin resistance, T=tobramycin resistance and CT=clindamycin and tobramycin resistance). Information regarding each patient's year of birth, gender and sampling site was also noted. Isolate ECT-R2, representing the ECT-R outbreak clone, whose whole genome has been sequenced in a previous study [7], was included as a reference in the microarray analysis.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The Etest (bioMérieux, France) was used for erythromycin, clindamycin, tobramycin, gentamicin, fusidic acid, rifampicin, moxifloxacin and vancomycin. Constitutive or inducible resistance to clindamycin was determined with the D-shaped disc diffusion method (Oxoid AB, Sweden) [11]. The disc diffusion method was also used for cefoxitin.

Detection of the *nuc* and *mecA* genes

Genomic DNA extraction and preparation of the polymerase chain reaction (PCR) assay was followed by amplification of the genes in a real-time PCR by employing a Rotor-Gene 3000 thermal cycler (Corbett Robotics, Brisbane, Australia), using *nuc*- and *mecA*-specific primers, as previously described [7]. For each run, the *S. aureus* strains (CCUG 35601, ATCC 29213) and *S. saprophyticus* strain (CCUG 3706) were used as positive and negative controls.

spa typing

spa typing was performed as previously described [6].

Alternative *spa* primers 1084F: 5′-ACAACGTAACGGCT TCATCC and 1618R: 5′-TTAGCATCTGCATGGTTTGC (GenBank accession no. J01786: 1065–1084F and 1637–1618R) (Ridom GmbH, Germany) were used to amplify the *spa* fragment of isolate Ö-T8, which was *spa*-negative with the standard *spa* primers.

Microarray-based genotyping

All isolates, including isolate ECT-R2 (n=99), were characterised using the Alere StaphType DNA microarray (Alere Technologies, Jena, Germany), according to the manufacturer's instructions. Briefly, DNA was purified using the

DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), amplified and labelled before hybridisation to the microarray containing 333 probes targeting 221 distinct genes, including taxonomic, SCC*mec* typing, antimicrobial resistance, toxins and other virulence markers. The hybridisation profiles based on the 333 genetic markers were analysed using the software BioNumerics, version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium), with the Jaccard's coefficient and the unweighted pair group method with arithmetic mean (UPGMA) dendrogram type. *S. aureus* isolates were assigned to MLST-CCs by an automated comparison of hybridisation profiles to a collection of reference strains previously characterised by MLST [12, 13]. The microarray data were also used to identify the downstream conserved segment (*Q9XB68-dcs*), which is a marker of the SCC*mec* type II remnant in isolate ECT-R2. Macrolide and tetracycline resistance determinants were designated as per the nomenclature by M. C. Roberts: http://faculty.washington.edu/marilynr/.

Analysis of the SCC*mec* right extremity junction

The SCC*mec* right extremity junction (MREJ) comprises the right extremity of SCC*mec*, the integration site sequence (ISS) and part of the *orfX* gene. Isolates were designated to different MREJ types according to Huletsky et al. [14].

Genomic DNA extraction, preparation of the PCR reaction, thermal cycling protocol, amplification and characterisation was performed as previously described [7]. Strains representing each SCC*mec* types I–V (type I: phenotype II 43.2, type II: 07.4/0237, type III: E0898, type IV: JCSC 4744 and type V: WIS) were included as positive controls [15].

Results

Bacterial isolates and antibiotic susceptibility testing

Between June 2009 and June 2010, a total of 100 clinical isolates of *S. aureus* with the defined antibiotic resistance profiles were collected from the three counties. Two MRSA isolates (cefoxitin-R and *mecA* gene-positive) were excluded from further analyses. Thus, 98 isolates were included; 42 isolates with clindamycin resistance (group C), 42 isolates with tobramycin resistance (group T) and 14 isolates with resistance to both clindamycin and tobramycin (group CT). All isolates in group CT were also erythromycin resistant, i.e. displaying an ECT-R phenotype. The distribution for each county and group is presented in Table 1.

All clindamycin-resistant isolates had minimum inhibitory concentration (MIC) values of >256 mg/L, except for one isolate (belonging to group C) with an MIC of 12 mg/L. Among tobramycin-resistant isolates, the MIC values ranged from 1.5 to 128 mg/L. In addition, 93 % (13/14) of the isolates in group CT showed resistance to moxifloxacin (MIC range 4–8 mg/L). Multi-resistance (i.e. resistance to two or more classes of non-beta-lactam antibiotics) was 21 % (9/42) in group C, 12 % (5/42) in group T and 100 % (14/14) in group CT. Antibiotic susceptibility in relation to microarray-derived MLST-CCs and *spa* type is presented in Table 2.

Table 1 Number of isolates per county and antibiotic resistance group. Clindamycin resistance (group C), tobramycin resistance (group T) and resistance to both clindamycin and tobramycin (group CT)

County	Antibiotic resistance groups		
	C	T	CT
Jönköping (J)	11	15	1
Kalmar (K)	13	8	1
Östergötland (Ö)	18	19	12
Total	42	42	14

spa typing

The isolates were divided into 40 different *spa* types. Of these, 34 types were represented by only one or two isolates. t084 (n=26) was the most common type and shared a common resistance profile as 25/26 isolates belonged to group T. The t084 isolates were found in all three counties (J: n=12, K: n=4, Ö: n=9). Fifteen isolates were t002 and included all isolates in group CT collected in Östergötland County Council (n=12). This *spa* type was also found among three additional isolates (K-C3, Ö-C3 and Ö-C12) in group C. *spa* types t089, t728, t005 and t034 were represented by six, six, three and three isolates, respectively. The antibiotic profile and microarray-derived MLST-CC for each *spa* type is presented in Table 2. Isolate Ö-T8, which was *spa*-negative with the standard *spa* primers, was designated a new *spa* type, t13024, using the alternative *spa* primers.

Microarray-based genotyping

The microarray analysis categorised the isolates into 17 different MLST-CCs, which were designated corresponding MLST-CC enumeration [12, 13, 16]. The results correlated well with the CC predictions based on *spa* typing, except for four isolates. Three of these were annotated as CC188 and one as CC15 by microarray, which, by the interpretation of *spa* types, belonged to CC1 (t189, n=2), CC45 (t065) and CC25 (t436), respectively. The derived dendrogram (Fig. 1) shows the branching of the various clades according to the content of genetic markers and confirms the four larger clades of isolates: CC5 (n=17), CC15 (n=30), CC30 (n=12) and CC45 (n=14). CC5 included all 15 isolates with *spa* type t002, isolate ECT-R2 and one

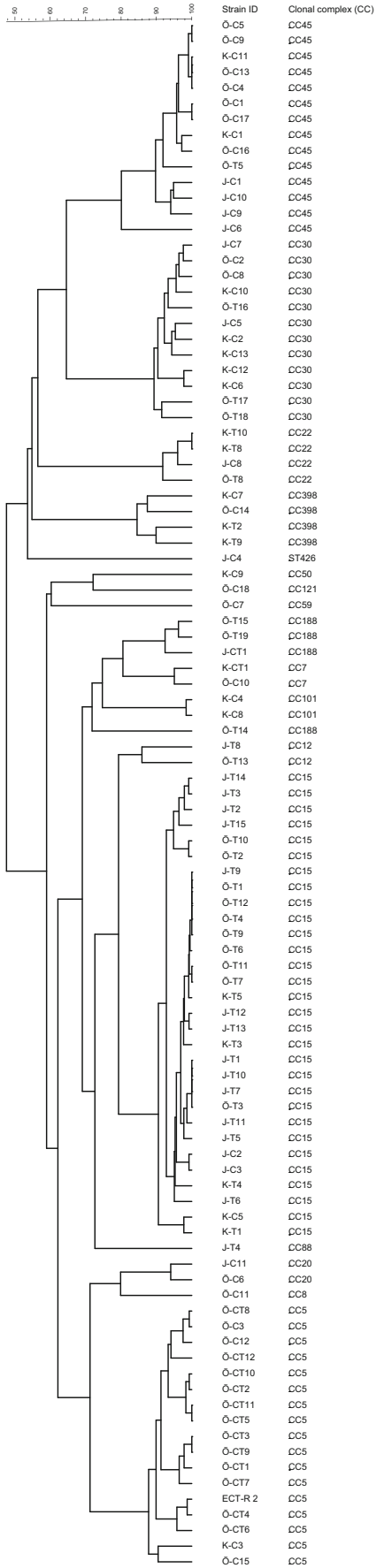
Table 2 Number of isolates with reduced susceptibility (I+R) in relation to microarray-derived multi-locus sequence typing clonal complexes (MLST-CCs) and *spa* type

CC	*spa* type	No. of isolates	EM	CM	TM	GM	FU	RI	MX	VA	FOX
CC5	t002	15	**15**	**15**	**12**	1	1	0	**14**	0	0
	t458	1	**1**	**1**	0	0	0	0	0	0	0
CC7	t091	1	**1**	**1**	0	0	0	0	0	0	0
	t796	1	**1**	**1**	**1**	**1**	0	0	0	0	0
CC8	t148	1	**1**	**1**	0	0	0	0	0	0	0
CC12	t160	2	0	0	**2**	0	0	0	0	0	0
CC15	t084	26	1	1	**25**	1	0	0	0	0	0
	t094	1	**1**	**1**	0	0	0	0	0	0	0
	t436	1	0	0	**1**	0	0	0	0	0	0
	t674	1	0	0	**1**	0	**1**	0	0	0	0
	t6684	1	**1**	**1**	0	0	0	0	0	0	0
CC20	t996	1	**1**	**1**	0	0	0	0	0	0	0
	t2919	1	**1**	**1**	0	0	**1**	0	0	0	0
CC22	t005	3	**3**	*1*	*2*	*2*	0	0	*2*	0	0
	t13024	1	0	0	**1**	**1**	0	0	0	0	0
CC30	t012	1	0	0	**1**	**1**	0	0	0	0	0
	t021	2	*1*	*1*	*1*	0	0	0	0	0	0
	t089	6	**6**	**6**	0	0	0	0	0	0	0
	t122	1	**1**	**1**	0	0	0	0	0	0	0
	t166	1	0	0	**1**	0	0	0	0	0	0
	t884	1	**1**	**1**	0	0	0	0	0	0	0
CC45	t073	2	**2**	**2**	0	0	0	0	0	0	0
	t230	1	**1**	**1**	0	0	0	0	0	0	0
	t302	1	0	0	**1**	0	0	0	0	0	0
	t722	1	**1**	**1**	0	0	0	0	0	0	0
	t728	6	**6**	**6**	0	0	0	0	**6**	0	0
	t2614	1	**1**	**1**	0	0	0	0	0	0	0
	t8560	1	**1**	**1**	0	0	0	0	0	0	0
	t8561	1	**1**	**1**	0	0	0	0	0	0	0
CC50	t705	1	**1**	**1**	0	0	0	0	0	0	0
CC59	t441	1	**1**	**1**	0	0	0	0	0	0	0
CC88	t4385	1	0	0	**1**	0	0	0	0	0	0
CC101	t524	2	**2**	**2**	0	0	0	0	0	0	0
CC121	t2092	1	**1**	**1**	0	0	0	0	0	0	0
CC188	t065	1	0	0	**1**	**1**	0	0	0	0	0
	t189	2	*1*	*1*	**2**	*1*	0	0	*1*	0	0
	t8562	1	0	0	**1**	0	0	0	0	0	0
CC398	t011	1	**1**	**1**	0	0	0	0	0	0	0
	t034	3	**3**	*1*	*2*	*2*	0	0	0	0	0
ST426	t1715	1	0	**1**	0	0	0	0	0	0	0

Bold: reduced susceptibility among 75–100 % of isolates/*spa* type; *italics*: reduced susceptibility among 25–75 % of isolates/*spa* type I=intermediate susceptibility, R=resistance. Antibiotic abbreviations: EM=erythromycin, CM=clindamycin, TM=tobramycin, GM=gentamicin, FU=fusidic acid, RI=rifampicin, MX=moxifloxacin, VA=vancomycin and FOX=cefoxitin

additional isolate (Ö-C15: t458). CC15 included all isolates with *spa* type t084 in group T (n=25) and five additional isolates from group CT (J-C2: t094, J-C3: t084, J-T15: t674, K-C5: t6684 and Ö-T10: t436).

The most common CCs, i.e. CC5 and CC15, were compared to all other isolates regarding a selection of resistance markers and virulence determinants (Table 3). The various resistance markers were well correlated with the genetic



◀ **Fig. 1** Unweighted pair group method with arithmetic mean (UPGMA) dendrogram showing clustering and genetic relatedness of *Staphylococcus aureus* isolates (n=99) based on 333 genetic markers. The scale indicates generic similarity using Jaccard's coefficient. *S. aureus* isolates were assigned to clonal complexes (CCs) by an automated comparison of hybridisation profiles to a collection of reference strains previously characterised by multi-locus sequence typing (MLST) [12, 13]

background of the strains. *ermA* was found in 21 isolates, 15 of these (e.g. including the ECT-R2 isolate) also shared other features (CC5, t002, all from Östergötland). In two of these 15 isolates (Ö-C3 and Ö-C12), the *aadD* gene was absent, as expected from their phenotype, but was found in the remaining 12 isolates. *aadD* was also detected in all 25 isolates in group T with t084 (CC15). The *Q9XB68-dcs* gene (an SCC marker) was present in 88 % (15/17) of the isolates belonging to CC5 (i.e. all isolates with t002 collected in Östergötland County and isolate ECT-R2) but was absent from the remaining 83 isolates. Similarly, the *qacC* gene was present in these 15 isolates (CC5, t002) but was absent in all isolates belonging to CC15 and present in only 8 % (4/52) of the isolates belonging to other CCs. Several genes encoding exotoxins were found in a majority of isolates belonging to CC5, but were absent in most isolates belonging to CC15, and present to varying degrees in other CCs (Table 3).

Analysis of the MREJ

A PCR product was found in 15 % (15/98) of the isolates. Fourteen isolates (all *spa* type t002) were MREJ type ii. The additional isolate (Ö-C10: *spa* type t091, group C) was MREJ type i.

Patient data

Of the patients, 52 % (51/98) were men and 48 % (47/98) were women, with a median age of 46 years (range: 0–95 years). The site of isolation was a wound for 90 % (88/98) of the isolates, an abscess for 3 % (3/98), blood for 2 % (2/98) and other for 5 % (5/98).

Patients with isolates belonging to CC5, designated *spa* type t002 and MREJ type ii (n=14, group CT: 12, group C: 2) were assembled from among patients in Östergötland County with a medium age of 79 years, with a wound as the site of isolation in 86 % (12/14) of the cases. In contrast, 83 % (25/30) of the isolates in CC15 were tobramycin-resistant and designated *spa* type t084. Of these, 68 % (17/25) were isolated from new-borns in all three counties.

Table 3 Comparison of selected genes, mainly exotoxins and antibiotic resistance markers, from the microarray analysis between the most commonly encountered clonal complexes (CCs) (CC5, CC15) and all other CCs (n=99). The previously characterised isolate ECT-R2 (CC5) is included in the analysis

Target	Description	No. (%) of positive isolates		
		CC5 (n=17)	CC15 (n=30)	Other CCs (n=52)
Regulatory genes				
agrII	Accessory gene regulator allele II	17 (100)	30 (100)	2 (4)
SCC*mec* typing				
Q9XB68-dcs	Hypothetical protein from SCC*mec* elements	15 (88)	0 (0)	0 (0)
Resistance genotype				
ermA	Macrolide/clindamycin resistance gene	15 (88)	0 (0)	6 (12)
ermB	Macrolide/clindamycin resistance gene	1 (6)	0 (0)	4 (8)
ermC	Macrolide/clindamycin resistance gene	2 (12)	3 (10)	33 (63)
aadD	Tobramycin resistance gene	12 (71)	27 (90)	9 (17)
aacA-aphD	Gentamicin/tobramycin resistance gene	0 (0)	0 (0)	9 (17)
tetK	Tetracycline resistance gene	1 (6)	26 (87)	10 (19)
qacC	Quaternary ammonium compound resistance gene protein C	15 (88)	0 (0)	4 (8)
Virulence				
tst1	Toxic shock syndrome toxin 1	1 (6)	1 (3)	8 (15)
sea (N315)	Enterotoxin A, allele from strain N315=enterotoxin P	15 (88)	0 (0)	6 (12)
sed	Enterotoxin D (= entD)	12 (71)	3 (10)	1 (2)
seg	Enterotoxin G (= entG)	17 (100)	0 (0)	35 (67)
sei	Enterotoxin I (= entI)	17 (100)	0 (0)	35 (67)
sej	Enterotoxin J (= entJ)	11 (65)	0 (0)	0 (0)
selm	Enterotoxin-like gene/protein M (= sem, entM)	17 (100)	0 (0)	36 (69)
seln	Enterotoxin-like gene/protein N (= sen, entN), consensus probe	17 (100)	0 (0)	35 (67)
selo	Enterotoxin-like gene/protein O (= seo, entO)	17 (100)	0 (0)	36 (69)
egc (enterotoxin gene cluster)		17 (100)	0 (0)	36 (69)
ser	Enterotoxin R (= entR)	11 (65)	0 (0)	0 (0)
selu	Enterotoxin-like gene/protein U (= seu, entU)	17 (100)	0 (0)	35 (67)
lukS-PV	Panton–Valentine leucocidin S component	0 (0)	0 (0)	10 (19)
sak	Staphylokinase	17 (100)	0 (0)	46 (88)
chp	Chemotaxis-inhibiting protein (CHIPS)	16 (94)	30 (100)	35 (67)
scn	Staphylococcal complement inhibitor	17 (100)	30 (100)	47 (90)
etA	Exfoliative toxin serotype A	0 (0)	0 (0)	2 (4)
etB	Exfoliative toxin serotype B	0 (0)	0 (0)	0 (0)
etD	Exfoliative toxin D	0 (0)	0 (0)	0 (0)

Macrolide and tetracycline resistance determinants were designated as per the nomenclature by M. C. Roberts: http://faculty.washington.edu/marilynr/

Discussion

In this study, MSSA isolates with resistance to erythromycin or tobramycin or a combination of these were analysed. Combined resistance to clindamycin and tobramycin (i.e. the ECT-resistance profile) was shown to be rare, except in Östergötland County, where all of these isolates (n=12) were of *spa* type t002. This *spa* type was also found among two additional isolates in group C from Östergötland County, which seemed to have lost the *aadD* gene, but were otherwise similar to the isolates of t002 from Östergötland County. All of these 14 isolates were designated MREJ type ii, belonged to CC5 and carried the *Q9XB68-dcs* gene according to the microarray results. Comparison with a representative isolate of the ECT-R clone showed close relatedness between these isolates and the ECT-R clone. WGS in a previous study suggested probable derivation of the ECT-R clone from a highly successful MRSA strain (New York/Japan, CC5), with existence of transposon Tn*554* carrying *ermA* outside the SCC but with the excision of a 41-kb fragment of the SCC*mec* type II element [7]. The *qacC* gene was present in 88 % (15/17) of the isolates belonging to CC5, including all 14 isolates with t002

collected in Östergötland County and isolate ECT-R2, but was only detected in four other isolates. This gene is most often found in hospital-associated (HA) MRSA and encodes an efflux pump, which confers tolerance to disinfectants. Interestingly enough, as it has been discussed that, as *qacC* expression is induced by sub-inhibitory levels of biocides (chlorhexidine etc.), resulting in increased tolerance, the presence of such genes might give isolates a selective advantage in the hospital environment [17].

All isolates of the ECT-R clone included in the present study were collected from elderly patients with a wound as the site of isolation in 86 % (12/14) of the cases. This is in accordance with the patient profile found in a previous study [6]. One might speculate that these patients are not necessarily cared for at the same departments as patients referred for tertiary care from the neighbouring counties, which is a possible explanation for why the ECT-R clone has not been transmitted outside Östergötland County.

spa type t084 was the most commonly found *spa* type, mainly among the isolates with tobramycin resistance (group T) in all three counties. These isolates were highly similar according to the microarray analysis and belonged to CC15. Of the isolates, 68 % (17/25) were isolated from new-borns in all three counties. This observation calls for further study, as the design of the present study does not allow a thorough analysis of transmission routes or a discussion of host–pathogen-specific issues.

Grundmann et al. found, in their large European multicentre study, that MSSA *spa* types were more diverse and less regionally distributed than MRSA, and concluded that this finding indicated spread of a limited number of MRSA clones within the health care network [1]. As in the present study, they found the *spa* types t002 and t084 to be the most common types of MSSA, accounting for 4.8 and 4.6 %, respectively. However, our findings suggest that there might be outbreaks and nosocomial spread within these common types. An early warning system for outbreaks should be established at the local level. Monitoring antibiotic resistance patterns might be one way to do so.

In conclusion, this study demonstrated that the MR-MSSA ECT-R clone (t002) has persisted in Östergötland County, but was limited to this county despite a frequent exchange of patients with the neighbouring hospitals in the region. However, the persistence of the ECT-R clone in Östergötland, and the observation that it carried genes encoding certain virulence determinants which might have enhanced its survival in the hospital environment, highlights the fact that basic hygiene guidelines must be maintained even when MRSA prevalence is low.

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Conflict of interest The authors have no conflicts of interest to be declared.

Ethical statement This study was approved by the Regional Ethical Review Board in Linköping, Sweden (M164-09), who judged that there was no need for informed consent to be obtained is this study.

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