

## Microbiological and molecular characterization of nosocomial and community *Staphylococcus aureus* isolates

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### SUMMARY

This study aimed to evaluate the incidence of *Staphylococcus aureus* infections in different departments of Belcolle Hospital in Viterbo and the surrounding area between January 2003 and June 2008. Isolates of methicillin-resistant *S. aureus* (MRSA) recovered in this time interval were characterized by microbiological and molecular methods to evaluate the reliability of simple criteria to distinguish between hospital-acquired and community-acquired isolates. MRSA accounted for 33% of all *S. aureus*, with a significantly higher prevalence in isolates from nosocomial infections. MRSA isolates were assayed by PCR for the presence of 13 genes associated with virulence, *agr* type and SCC<sub>mec</sub> type. Cumulative data were analysed by partial least square discriminant analysis and a clear correlation was demonstrated between genetic profiles and classification of isolates as hospital or community acquired according to simple temporal criteria. Nosocomial MRSA isolates from blood samples showed significantly higher genetic diversity than other nosocomial isolates. Our data confirm the existence of significant differences between community- and hospital-acquired MRSA isolates.

**Key words:** Hospital and community infections, methicillin resistance, *Staphylococcus aureus*.

### INTRODUCTION

*Staphylococcus aureus* is a leading cause of nosocomial and community infections around the world [1] and is associated with several different infections [2]. The emergence and spread of multiresistant strains, and particularly of methicillin-resistant *S. aureus* (MRSA) has made the treatment of staphylococcal infections a global challenge [3, 4].

In Italy, after an initial and continuous increase [3, 5, 6], the frequency of isolation of MRSA, from

2002 onwards, has undergone a constant, although small average increment, reaching values around 33% in 2007 and 2008 [European Antimicrobial Resistance Surveillance (EARSS) annual report 2008 at <http://www.earss.rivm.nl>]. Most MRSA are isolated from subjects aged >65 years, who are frequently hospitalized, in intensive care and/or following surgery [3, 6]. A recent study [7] suggests that, although indicative of general trends, data from the EARSS programme are only partially representative of the prevalence of nosocomial MRSA in Italy. In fact, MRSA is responsible for various infections and considerable variations exist between institutions and wards, even in the same geographical areas, probably due to local public health strategies and other variables.

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Complexity in the epidemiology of infections caused by *S. aureus* is also the result of significant genetic diversity and clonal evolution, which was undoubtedly accelerated by the strong selective pressure due to the widespread use of antibiotics that eventually broke interlineage barriers allowing the acquisition and/or loss of virulence genes transported by mobile genetic elements [8].

Resistance to methicillin is normally dependent on the presence of the *mecA* gene, encoding a penicillin binding protein (PBP) showing low-affinity for  $\beta$ -lactams [3]. The *mecA* gene is inserted into the staphylococcal chromosomal cassette (SCC*mec*), which is incorporated into the chromosome of *S. aureus* at a specific site [9, 10]. Currently, seven types of SCC*mec* (I–VII) and an increasing number of variants, have been described [11]. Besides differences in essential genetic components, the various SCC*mec* elements differ from each other in that they can host a variety of accessory resistance genes involved in resistance to non- $\beta$ -lactam antibiotics. In fact, in contrast with SCC*mec* types II and III that contain many antibiotic and heavy-metal resistance genes in integrated plasmids or transposons [4] other SCC*mec* types, with some exceptions, do not contain any antibiotic resistance genes other than *mecA*. In the Italian study cited previously [6], 54.6% of the isolates were resistant to >4 antibiotics, suggesting a large diffusion of SCC*mec* cassettes harbouring multi-drug resistance elements, although the presence of different determinants of resistance cannot be excluded.

MRSA strains emerged and diffused in the community (CA-MRSA) and were shown frequently to carry the smaller and more easily mobilized SCC*mec* cassettes and are generally less drug resistant than their counterparts acquired in hospitals (HA-MRSA) [4]. Nevertheless, CA-MRSA are becoming a serious public health concern since they generally produce a wider variety of pathogenicity and virulence factors, including the PVL toxin, and can cause different serious infections, including acute, invasive, and necrotizing syndromes, and may occasionally spread in hospitals [12]. The reasons why MRSA, after 40 years of fortuitous inability to colonize people outside the hospital setting, apparently acquired this crucial ability remain obscure [13].

As a consequence of several important differences in pathogenicity and resistance to antimicrobials existing between HA-MRSA and CA-MRSA, the availability of simple and reliable criteria to define the nature of the infection would be of considerable

importance. Currently there is some controversy in defining these two groups; distinction is generally based on temporal criteria in terms of time of MRSA isolation after hospital admission, history and time of recent hospitalization and exploitation of other risk factors, e.g. recent surgery, dialysis [14]. Therefore, making an absolute distinction between HA-MRSA and CA-MRSA based on these criteria is sometimes difficult to achieve. While the frequency of CA-MRSA isolation is apparently increasing, the careful evaluation of several CA infections often reveals a history of recent hospitalization, or of close contact with hospitalized or recently hospitalized people, or the existence of other predisposing factors. Nevertheless, most of these strains show resistance patterns typical of CA-MRSA, in which  $\beta$ -lactam resistance predominates [14] while the isolation of multidrug-resistant strains from infections arising in the community is not uncommon.

We aimed to perform surveillance of *S. aureus*, with particular attention to MRSA and its distribution within the nosocomial and community district in the area of Viterbo (central Italy) and the surrounding areas. The genetic diversity of MRSA isolates was analysed by simple and inexpensive molecular methods to assess if classification of MRSA as HA and CA by simple temporal criteria correlates with the genetic profiles of isolates.

## METHODS

This study was conducted during the period January 2003 to June 2008 on different clinical samples submitted for routine analyses to the microbiology laboratory of Hospital ‘Belcolle’ in Viterbo, where isolation, identification and antimicrobial susceptibility testing were performed. MRSA isolates were then characterized by molecular methods at the Department of Public Health Sciences of the University of Rome ‘La Sapienza’.

### Microbiological analyses

Clinical samples were obtained from patients attending the hospital itself, withdrawal centres, clinics, day hospitals, and nursing services in Viterbo and district, and processed by standard methods [15]. Identification of antimicrobial susceptibility of *S. aureus* was determined by the Vitek 2 automated system (bioMérieux, Italy). Isolates were classified as MRSA on the basis of resistance to oxacillin ( $\geq 4 \mu\text{g/ml}$ ).

Table 1. Primers and conditions used in PCR assays

Primer	Concn ( $\mu\text{M}$ )	Primer sequence (5'–3')	Product length (bp)	Reference
sea F	0.1	TTGCAGGGAACAGCTTTAGGCAATC	252	[16]
sea R		TGGTGTACCACCCGCACATTGA		
seb F	0.1	GACATGATGCCTGCACCAGGAGA	355	[16]
seb R		AACAAATCGTTAAAAACGGCGACACAG		
sec F	0.1	CCCTACGCCAGATGAGTTGCACA	602	[16]
sec R		CGCCTGGTGCAGGCATCATATC		
tst F	0.1	AGCCCTGCTTTTACAAAAGGGGAAAA	306	[16]
tst R		CCAATAACCACCCGTTTTATCGCTTG		
eta F	0.1	CGCTGCGGACATTCTACATGG	676	[16]
eta R		TACATGCCCCGCACTTGCTTGT		
etb F	0.1	GAAGCAGCCAAAAACCCATCGAA	419	[16]
etb R		TGTTGTCCGCCTTTACCACTGTGAA		
PVL F	0.1	TGCTTTTGCTATCCAATACAGTTGATGC	572	This study, [16]
PVL R		TCTGCCATATGGTCCCCAACCA		
clfA F	0.1	ATTGGCGTGGCTTCAGTGCTTG	376	This study, [16]
clfA R		ATTCCTCCGCATTTGTATTGCTTGATTG		
cna F	0.1	GATAGAAGATGGTGGTAAAACGACAGTA	127	This study
cna R		ACTATAACCCTCTATCTTTACTGTACCG		
map/eap F	0.1	CACAGCAAACCTTAGTTCATGCGAAAGAT	862	[16]
map/eap R		TTATCCCTTGATCATTGCGCATTGCTGA		
sdrC F	0.1	CGCATGGCAGTGAATACTGTTGCAGC	731	[16]
sdrC R		GAAGTATCAGGGGTGAAACTATCCACAAATTG		
sdrD F	0.1	CCACTGGAAATAAAGTTGAAGTTTCAACTGCC	467	[16]
sdrD R		CCTGATTTAACTTTGTCATCAACTGTAATTTGTG		
sdrE F	0.1	GCAGCAGCGCATGACGGTAAAG	894	[16]
sdrE R		GTCGCCACCCGCCAGTGCATTA		
agr I F	0.25	CATGTATATAGTTATATATGCATATATCACC	447	This study
agr UN R		AAGATATTGACATAATCATGTCGGAAC		
agr II F	0.25	TTGATGAACCTAAAGTACCCGCT	802	This study
agr UN R		AAGATATTGACATAATCATGTCGGAAC		
agr III F	0.25	TTTGGTAGTGTATGTATCAATTTCTTATGC	382	This study
agr UN R		AAGATATTGACATAATCATGTCGGAAC		
agr IV F	0.25	AGCGTAAACCTTAATAACTAATACTATATTC	569	This study
agr UN R		AAGATATTGACATAATCATGTCGGAAC		

An infection was considered to be hospital acquired if it developed within 48 h after hospital admission, up to 3 days after discharge from hospital, or up to 30 days following surgery.

#### Purification of genomic DNA

Genomic DNA was prepared from each MRSA isolate grown in LB broth overnight at 37 °C. The bacterial pellet was suspended at an OD 600 nm = 20 in 50 mM Tris–HCl, 145 mM NaCl (pH 7.5), containing sucrose 15% (w/v) and lysostaphin (40 U/ml) and the cell wall was digested at 37 °C for 1 h. Bacterial cells were then lysed by adding 4 vol of 50 mM NaCl containing *N*-lauroyl-sarcosine 1% (w/v) and DNA was extracted and purified by phenol and phenol/

chloroform treatments followed by precipitation with ethanol. DNA concentration was determined by the Qubit™ quantitation system (Invitrogen, Italy) and samples were stored at –20 °C until used.

#### PCR assays for genotyping

Seventeen genetic determinants were assayed by PCR; these included toxins (*sea*, *seb*, *sec*, *tst*, *eta*, *etb*, *PVL*), adhesins (*clfA*, *cna*, *map/eap*, *sdrC*, *sdrD*, *sdrE*), and *agr* groups I–IV. Primers and PCR conditions were essentially as described previously [16], with some modifications (Table 1). PCR amplifications were performed in a T personal thermal cycler (Biometra, Germany) with DreamTaq DNA polymerase (Fermentas, Italy). Genomic DNA (100 ng)

was added to each PCR mix containing 2.5 mM MgCl<sub>2</sub>, a 10 mM concentration of the deoxynucleoside triphosphates, and the appropriate concentration of each forward and reverse primer, and *Taq* polymerase. Detection of genes for adhesins and toxins was performed by single PCR reactions while detection of *agr* group-specific sequences was performed as a multiplex format using a universal reverse primer and four group-specific forward primers. PCR products were analysed by electrophoresis in 2% agarose gels.

### SCC*mec* typing

SCC*mec* PCR typing was based on three separate multiplex-PCR assays and a specific single PCR reaction according to published methods [17, 18]. The first multiplex-PCR assay contained nine pairs of specific primers for SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, and the primers for the *mecA* gene. The second and third multiplex-PCR assays were used for characterization of *mec* gene and *ccr* gene complexes, respectively, and contained four primers each. A single target PCR was used to determine type 5 *ccr* using *ccrC-F* and *ccrC-R* primers. All PCR assays were performed using 100 ng genomic DNA as the template in a PCR mix containing 3.0 mM MgCl<sub>2</sub>, a 10 mM concentration of the deoxynucleoside triphosphates, the appropriate concentration of each forward and reverse primer, and DreamTaq DNA polymerase under appropriate conditions [17].

### Partial least squares discriminant analysis (PLS-DA)

In order to evaluate if the presence/absence of specific genes correlated with the classification of isolates as HA-MRSA and CA-MRSA, or with the type of clinical sample in HA-MRSA [blood *vs.* various biological samples (VBS)] two 1/0 matrices were constructed based on results of PCR assays. Logistic PLS-DA, included in SIMCA-P+ software (Umetrics, Sweden), was performed to assess the model's predictability and gene importance in classifying strains. Data were automatically mean centred and unit variance scaled by the statistical software. Each gene was hierarchically classified based on a software-assigned variable importance (VIP) value. The variables with VIP value >1 were chosen as discriminatory for each strain belonging to class (i.e. 1 = HA-MRSA and 0 = CA-MRSA or 1 = blood and 0 = VBS). Data were

presented as a dendrogram, in which proximity of each gene variable to class centroid (1, 0) indicates the relevance for inclusion in that class.

Evaluation of significance of differences for positivity of PCR assays of single genes were performed by  $\chi^2$  test at significance levels of  $P \leq 0.05$  and  $P \leq 0.01$ .

## RESULTS

### Microbiological analyses

Five hundred and seventy-five *S. aureus* isolates were recovered from 55057 samples analysed during the study [27499 (49.9%) urine samples, 7152 (13%) blood cultures, and the remaining 20406 (37.1%) from various sources]. Two hundred and fifty-eight (44.9%) *S. aureus* isolates were from medical departments, 184 (32%) from surgical departments, and 133 (23.1%) from services in the surrounding area. Of the 575 *S. aureus* isolates, 211 (36.7%) were from blood cultures and 364 (63.3%) from other specimens. The distribution of *S. aureus* isolates from blood and VBS from either surgical or medical departments is illustrated in Figure 1. Most blood isolates were from intensive-care wards, although the highest number of isolates in the same sample type was from medical and infectious disease wards.

Table 2 shows the distribution of methicillin-susceptible (MSSA) and MRSA phenotypes in *S. aureus* isolates from both blood samples and VBS in HA and CA infections. Of the 575 isolates, 385 (67.0%) were MSSA, and 190 (33.0%) were MRSA. Of the 141 MRSA strains isolated from HA infections, 45 (31.9%) were from surgical, and 63 (44.7%) from medical departments (data not shown). The percentage of MRSA from blood cultures was higher in intensive-care wards (63.6% of 33 isolates) than in surgical wards (54.5% of 11 isolates) and the highest incidence of MRSA in VBS originated from intensive-care wards (54.1% of 37 strains) (Fig. 1).

MRSA represented 44.2% of the 319 *S. aureus* isolates from HA infections. As expected, these isolates were resistant to many of the tested antibiotics (Table 3). By contrast, MRSA represented only 19.1% of the 256 *S. aureus* isolates recovered from CA infections and these exhibited less antimicrobial resistance compared to HA-MRSA isolates (Table 3). Significant differences ( $P \leq 0.01$ ) were observed in the proportion of resistant strains between MRSA and MSSA for ciprofloxacin, clindamycin, erythromycin and gentamicin in the HA group and

Table 2. Distribution of the MRSA and MSSA phenotypes in 575 *S. aureus* isolates from blood samples and various biological samples (VBS) in hospital-acquired and community-acquired infections

Sample (no. of isolates)	Number (%) of isolates							
	Total		Hospital			Community		
	MRSA	MSSA	Total	MRSA	MSSA	Total	MRSA	MSSA
Blood (n=211)	40.3 (85)	59.7 (126)	91.0 (192)	44.3 (85)	55.7 (107)	9.0 (19)	0.0 (0)	100.0 (19)
VBS (n=364)	28.8 (105)	71.2 (259)	34.9 (127)	44.1 (56)	55.9 (71)	65.1 (237)	20.7 (49)	79.3 (188)
Overall (n=575)	33.0 (190)	67.0 (385)	55.5 (319)	44.2 (141)	55.8 (178)	44.5 (256)	19.1 (49)	80.9 (207)

Table 3. Resistance patterns of hospital-acquired (HA) and community-acquired (CA) MRSA and MSSA isolates

Class (no.)	Number of resistant strains (%)							
	CIP	CLI	ERY	GEN	QDA	RIF	TCY	SXT
HA-MRSA (141)	125 (88.6)**	60 (42.5)**	82 (58.2)**	89 (63.1)**	4 (2.8)	18 (12.8)*	4 (2.8)	16 (11.3)
HA-MSSA (178)	16 (9.0)	2 (1.1)	17 (9.5)	18 (10.1)	2 (1.1)	5 (2.8)	9 (5.1)	21 (11.8)
CA-MRSA (49)	6 (12.2)*	5 (10.2)*	14 (28.6)**	3 (6.1)	0	2 (4.1)	1 (2.0)	3 (6.1)
CA-MSSA (207)	6 (2.9)	5 (2.4)	18 (8.7)	6 (2.9)	0	0	2 (1.0)	14 (6.8)

CIP, Ciprofloxacin; CLI, clindamycin; ERY, erythromycin; GEN, gentamicin; QDA, quinopristin/dalfopristin; RIF, rifampicin; TCY, tetracycline; SXT, sulphamethoxazole/ trimethoprim.

\*  $P \leq 0.05$ , \*\*  $P \leq 0.01$  compared to the corresponding MSSA.

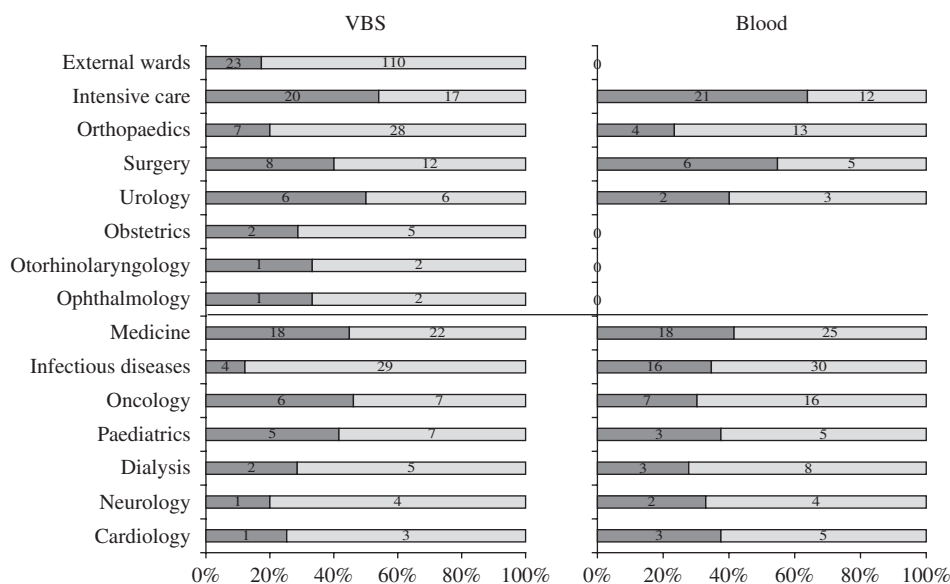


Fig. 1. Distribution of *S. aureus* isolates (%) in blood samples and various biological samples (VBS) from surgical and medical departments (separated by central black line). ■, MRSA.

Table 4. Frequency of putative virulence genes and SCCmec types detected by PCR in 190 MRSA isolates from hospital-acquired (HA) and community-acquired (CA) infections, and 141 HA-MRSA from blood and various biological samples (VBS). Percentages and number of PCR-positive assays for each gene tested

	% PCR positives (no.)					P value*
	CA (n=49)	HA (n=141)		HA VBS (n=56)	HA blood (n=85)	
<i>sea</i>	34.7 (17)	36.9 (52)	0.9176	51.8 (29)	27.1 (23)	<b>0.0052</b>
<i>seb</i>	18.4 (9)	24.1 (34)	0.5331	23.2 (13)	24.7 (21)	0.9980
<i>sec</i>	75.5 (37)	53.2 (75)	<b>0.0103</b>	23.2 (13)	72.9 (62)	<b>0.0001</b>
<i>tst</i>	8.2 (4)	27.7 (39)	<b>0.0090</b>	37.5 (21)	21.2 (18)	0.0543
<i>eta</i>	36.7 (18)	36.9 (52)	0.8832	89.3 (50)	2.4 (2)	<b>0.0001</b>
<i>etb</i>	46.9 (23)	2.1 (3)	<b>0.0001</b>	3.6 (2)	1.2 (1)	0.7130
<i>PVL</i>	65.3 (32)	68.8 (97)	0.7837	67.9 (38)	69.4 (59)	0.9981
<i>clfA</i>	55.1 (27)	99.3 (140)	<b>0.0001</b>	100 (56)	98.8 (84)	0.8472
<i>cna</i>	28.6 (14)	2.8 (4)	<b>0.0001</b>	3.6 (2)	2.4 (2)	0.9222
<i>eap</i>	42.9 (21)	49.6 (70)	0.5204	10.7 (6)	75.3 (64)	<b>0.0001</b>
<i>sdrC</i>	81.6 (40)	100 (141)	<b>0.0001</b>	100 (56)	100 (85)	n.a.
<i>sdrD</i>	79.6 (39)	99.3 (140)	<b>0.0001</b>	98.2 (55)	100 (85)	0.8259
<i>sdrE</i>	30.6 (15)	56.7 (80)	<b>0.0029</b>	33.9 (19)	71.8 (61)	<b>0.0001</b>
SCCmec I	14.3 (7)	15.6 (22)	0.9900	37.5 (21)	1.2 (1)	<b>0.0001</b>
SCCmec II	14.3 (7)	10.6 (15)	0.6610	25.0 (14)	1.2 (1)	<b>0.0001</b>
SCCmec III	0.0 (0)	0.7 (1)	0.5712	0.0 (0)	1.2 (1)	0.8472
SCCmec IVa	10.2 (5)	0.7 (1)	<b>0.0050</b>	1.8 (1)	0.0 (0)	0.8259
SCCmec IVb	6.1 (3)	0.0 (0)	<b>0.0220</b>	0.0 (0)	0.0 (0)	n.a.
SCCmec IVc	42.9 (21)	80 (56.7)	<b>0.0001</b>	35.7 (20)	70.6 (60)	<b>0.0001</b>
SCCmec IVd	0.0 (0)	1.4 (2)	0.9881	0.0 (0)	2.4 (2)	0.6548
SCCmec IVND†	12.2 (6)	14.2 (20)	0.9127	0.0 (0)	23.5 (20)	<b>0.0002</b>
SCCmec V	0.0 (0)	0.0 (0)	n.a.	0.0 (0)	0.0 (0)	n.a.

n.a., Not applicable.

\* A two-tailed  $\chi^2$  test with Yates' correction for continuity was applied.

† IV ND: strain showing PCR positivity for more than one type IV SCCmec cassette.

for erythromycin in the CA group (Table 3). All MRSA strains were susceptible to linezolid, teicoplanin, and vancomycin (data not shown).

#### agr typing of MRSA strains

Of the 190 MRSA strains isolated during the study, 103 (54.2%) were *agrI*, 52 (27.4%) *agrII*, 29 (15.3%) *agrIII*, and six (3.2%) were *agrIV*. There were no major differences in the distribution of HA-MRSA and CA-MRSA or origin of sample according to *agr* type.

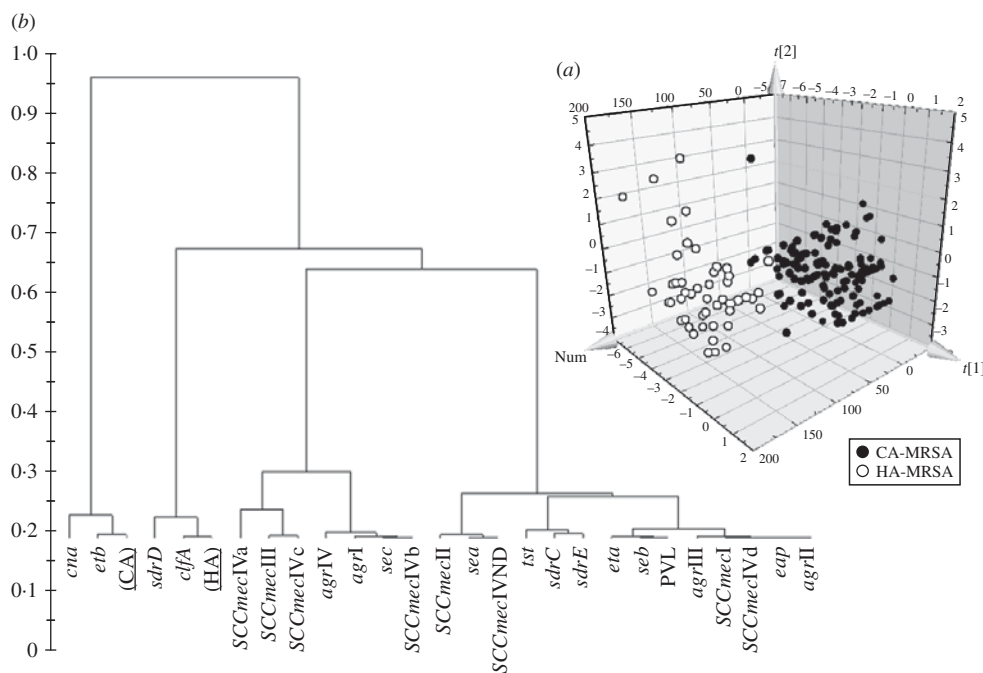
#### Presence of putative virulence determinants in MRSA strains

The distribution of virulence genes in the 190 MRSA strains is reported in Table 4. PCR assays for several genes, including *sdrC*, *sdrD*, *sdrE*, *clfA*, *lukS-F* PV, and *sec*, were overall positive in > 50% of the strains

but *cna*, *seb*, and *tst* were less frequent. Table 4 shows significant differences between HA-MRSA and CA-MRSA isolates for the presence of adhesin (*clfA* and *cna*) and toxin genes (*etb*). There were also significant differences in the gene profiles of isolates from blood and VBS (*eap*, *sec*, *eta*).

#### SCCmec typing of MRSA strains

The distribution of SCCmec types in the 190 MRSA strains is reported in Table 4; 138 (72.6%) were SCCmec type IV, with subtype IVc being the most represented (101/190, 53.2%). Twenty of 85 (23.5%) isolates from blood samples harboured SCCmec cassettes that gave specific PCR products for more than one type IV subtype; these strains were classified as not defined type IV (IV ND). Minor, although significant differences in the distribution of SCCmec types between HA-MRSA and CA-MRSA were evident only for the scarcely represented subtypes IVa



**Fig. 2.** (a) 3D plot of distribution of the 190 MRSA strains according to presence of specific genes and correlation with the classification of isolates as HA-MRSA and CA-MRSA according to simple temporal criteria, calculated by PLS-DA analysis. (b) Dendrogram of the hierarchical classification of genes for discriminating between groups.

and IVb (six and three strains respectively). Positivity for subtype IVc and IV ND cassettes was significantly higher in the 85 isolates from blood samples ( $P < 0.01$ ), while types I and II were 21 (37.5%) and 14 (25.0%), respectively, of the 56 isolates from VBS in the HA infections group (Table 4).

### PLS-DA analysis

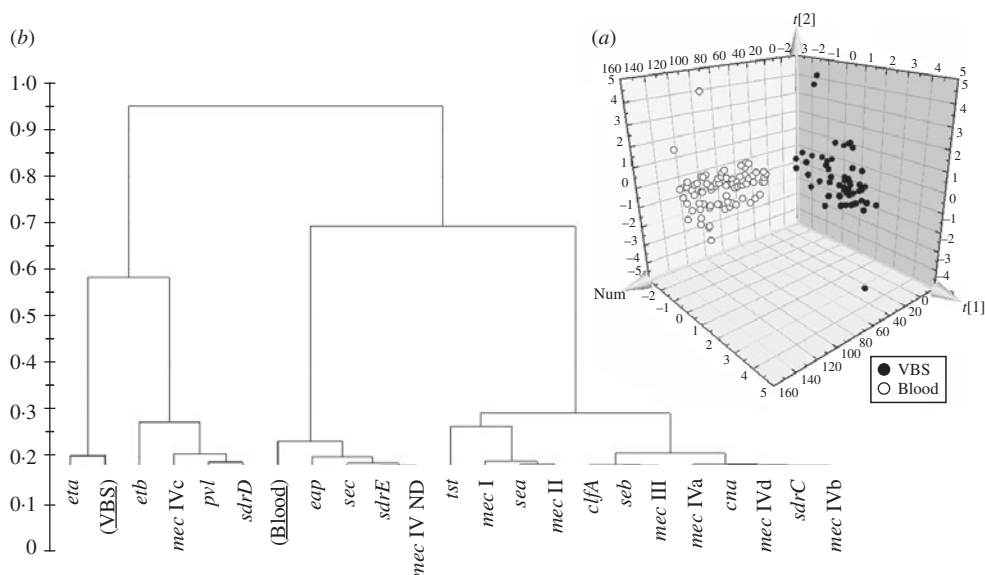
Significant differences in the patterns of selected genes of MRSA isolates classified as HA and CA according to simple temporal criteria, were shown by PLS-DA, resulting in an overall predictability of 93.7%, with a Fisher probability of  $1 \times 10^{-28}$ , and an ANOVA probability of  $6.4 \times 10^{-29}$  (Fig. 2a). The PLS-DA model was generated with an overall variance of 71.6% and required the reallocation of 3/141 HA-MRSA (2.13%) as CA-MRSA, and 9/49 CA-MRSA (18.37%) as HA-MRSA. Construction of a dendrogram plotting the hierarchical classification of genes according to variable importance in discriminating HA-MRSA from CA-MRSA showed that genes *sdrD* and *clfA* were highly discriminatory for HA-MRSA, and genes *cna* and *etb* were highly discriminatory for CA-MRSA (Fig. 2b).

In order to evaluate if the presence/absence of selected genes in HA-MRSA correlated with the

clinical source of isolates, the PLS-DA model was performed and gave an overall predictability of 96.4%, with a Fisher probability of  $1.1 \times 10^{-32}$ , and an ANOVA probability of  $2.2 \times 10^{-35}$  (Fig. 3a). The PLS-DA model was generated with an overall variance of 82.9% and required the reallocation of 2/56 VBS (3.57%) isolates into the blood class, and 3/85 blood isolates (3.52%) into the VBS class. Construction of a dendrogram plotting the hierarchical classification of genes according to their variable importance in discriminating blood from VBS isolates, showed that genes *eta* and, to a lesser extent, *etb*, *lukS-F*, *sdrD* and *mec IVc* cassette, were highly discriminatory for VBS isolates, whilst genes *eap*, *sec*, *sdrE*, and *mec IV ND* cassette were highly discriminatory for blood isolates (Fig. 3b).

### DISCUSSION

In this paper we report data on *S. aureus* isolations of both HA and CA infections in patients attending Viterbo Hospital and the surrounding areas in the period January 2003 to June 2008. During this period *S. aureus* was isolated from 1.04% of samples tested. Our analysis was particularly focused on MRSA, because during the last few years they have become a great public health concern, due to increasing



**Fig. 3.** (a) 3D plot of distribution of the 141 HA-MRSA strains according to presence of specific genes and correlation with the isolation of strains from blood or various biological samples (VBS), calculated by PLS-DA analysis. (b) Dendrogram of the hierarchical classification of genes for discriminating between groups.

morbidity and mortality rates and longer hospitalization times. Recent Italian data investigating the incidence of MRSA and characterizing isolates at the molecular level [5–7] revealed a constantly evolving situation making it necessary for surveillance to be conducted at both national and local levels. The prevalence of MRSA in the district of Viterbo during the period of this study was aligned to national data.

We defined HA infection as one that developed within 48 h after admission, up to 3 days after discharge, or up to 30 days following surgery. As expected, HA-MRSA were frequently resistant to several antibiotics, while CA-MRSA were generally resistant to  $\beta$ -lactams alone. The PLS-DA analysis evaluating the distribution of specific genes in the isolates confirmed that this simple classification method is reliable and divided them into two distinct and well separated groups, one of which contained all but three of the HA-MRSA and nine strains originally classified as CA-MRSA. Only 5/12 reclassifications (all CA to HA) of the strains showed a prevalence of genetic characteristics of the other group >70%, while the remaining seven strains showed intermediate characteristics. These observations may be indicative of a limited but relevant spread of HA-MRSA into the community, which could prove to be a serious problem by allowing the transfer of resistance genes from HA-MRSA to strains circulating in the community, potentially

arming them with increased virulence determinants. This hypothesis is supported by the observation that in a limited but significant number of cases, strains that were originally classified as CA-MRSA, in spite of a further and careful re-evaluation that confirmed classification, were resistant to most of the tested antibiotics. Each of the nine CA-MRSA that were reclassified as HA-MRSA showed atypical resistance patterns; all were resistant to erythromycin and sulphamethoxazole/trimethoprim, and there was variable susceptibility to ciprofloxacin, gentamicin, clindamycin, tetracycline, and rifampicin. Their genetic similarity also correlated with antibiotic resistance patterns as three strains that were genetically most related to HA-MRSA proved to be resistant to all antibiotics tested with the exception of rifampicin susceptibility in a single strain.

SCC*mec IVc* was more prevalent in HA-MRSA while all other subtypes were more prevalent in CA-MRSA. These data suggest that strains harbouring SCC*mec IVc* cassettes might host other resistance genes responsible for the multi-resistant phenotype.

Moreover, the distribution of virulence genes was not typical in the MRSA isolates in the study. CA-MRSA are generally regarded as having more virulence genes than HA-MRSA and production of the PVL toxin is normally much more frequent in these strains [12]. In our sample, the majority of MRSA isolates were PVL positive and were equally



distributed in HA-MRSA and CA-MRSA although significant differences were found between the groups for genes encoding adhesins and toxins. Several recent reports suggest that this situation could be a consequence of the strong selective pressure generated by overuse of antibiotics that accelerated the evolution of *S. aureus* and eventually broke interlineage barriers allowing the frequent acquisition and/or loss of virulence genes carried by mobile genetic elements [8, 19]. The widespread diffusion of virulence genes to all *agr* types in our strain seems to be in accord with this hypothesis.

Our data serve to show the existence of significant genetic diversity between HA-MRSA strains with respect to the site of isolation as a small number of PCR assays could separate with high predictivity isolates from blood and VBS. The non-homogeneous distribution of virulence and resistance genes in isolates from different samples might possibly indicate that interruption of interlineage barriers enables *S. aureus* strains to rapidly enhance their fitness to different hosts and tissues. Such events could be facilitated by the enhanced circulation of strains of different origin (HA or CA) in the same environments and hosts, as a consequence of public health policy measures directed at reducing costs by shortening periods of hospitalization.

Overall the data presented underlines the fact that the clinical relevance of distinction between HA-MRSA and CA-MRSA is becoming less evident for treatment plans and clinical outcomes. Moreover, the data show that simple criteria that have been developed in the past remain a valid approach for the classification of the origin of an MRSA infection. The findings of our study support the importance of coordinated national and local molecular monitoring of MRSA isolates as a fundamental instrument to inform public health measures designed to reduce the risks associated with the diffusion of infections caused by MRSA both in hospitals and the community.

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#### DECLARATION OF INTEREST

None.

#### REFERENCES

1. **Lowy FD.** *Staphylococcus aureus* infection. *New England Journal of Medicine* 1998; **339**: 520–532.
2. **Diekema DJ, et al.** SENTRY Participants Group. Survey of infections due to *Staphylococcus* species: frequency of occurrence, antimicrobial susceptibility and resistance trends of isolates collected in the United States, Canada, Latin America and the Western Pacific for the Sentry antimicrobial surveillance program 1997–1999. *Clinical Infectious Diseases* 2001; **32**: 114–132.
3. **Fuda CC, Fisher JF, Mobashery S.** Beta lactam resistance in *Staphylococcus aureus*: the adaptive resistance of a plastic genome. *Cellular and Molecular Life Science* 2005; **62**: 2617–2633.
4. **Grundmann H, et al.** Emergence and resurgence of methicillin-resistant *Staphylococcus aureus* as a public-health threat. *Lancet* 2006; **368**: 874–885.
5. **Stefani S, Varaldo PE.** Epidemiology of methicillin-resistant staphylococci in Europe. *Clinical Microbiology and Infection* 2003; **9**: 1179–1186.
6. **D’Ancona FP, et al.** Surveillance of antibiotic resistance in Italy for the years 2003, 2004, 2005 [in Italian]. *Bollettino Epidemiologico Nazionale* 2006; **19**, no. 6.
7. **Campanile F, et al.** Hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) in Italy. *Annals of Clinical Microbiology and Antimicrobials* 2009; **8**: 22.
8. **Argudin MA et al.** Clonal complexes and diversity of exotoxin gene profiles in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from patients in a Spanish hospital. *Journal of Clinical Microbiology* 2009; **47**: 2097–2105.
9. **Katayama Y, Ito T, Hiramatsu K.** A new class f genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* 2000; **44**: 1549–1555.
10. **Kondo Y, et al.** Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. *Antimicrobial Agents and Chemotherapy* 2007; **51**: 264–274.
11. **Higuchi W, et al.** Structure and specific detection of staphylococcal cassette chromosome *mec* type VII. *Biochemical and Biophysical Research Communications* 2008; **377**: 752–756.
12. **Boyle-Vavra S, Daum RS.** Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Pantone-Valentine leukocidin. *Laboratory Investigation* 2007; **87**: 3–9.
13. **Gorwitz RJ.** Understanding the success of methicillin-resistant *Staphylococcus aureus* strains causing epidemic disease in the community. *Journal of Infectious Diseases* 2008; **197**: 179–182.
14. **Maltezou HC, Giamarellou H.** Community-acquired methicillin-resistant *Staphylococcus aureus* infections. *International Journal of Antimicrobial Agents* 2006; **27**: 87–96.

15. **Kloos WE, Jorgensen JH.** Staphylococci. In: Lennette EH, Balows A, Hausler Jr. WJ, Shadomy JH, eds. *Manual of Clinical Microbiology*, 4th edn. Washington, DC: American Society for Microbiology, 1985, pp. 143–153.
16. **Campbell SJ, et al.** Genotypic characteristics of *Staphylococcus aureus* isolates from a multinational trial of complicated skin and skin structure infections. *Journal of Clinical Microbiology* 2008; **46**: 678–684.
17. **Zhang K, et al.** Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology* 2005; **43**: 5026–5033.
18. **Ito T, et al.** Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* 2001; **45**: 1323–1336.
19. **Waldron DE, Lindsay JA.** *SauI*: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* of different lineages. *Journal of Bacteriology* 2006; **188**: 5578–5585.