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ARTICLE

DNA microarray-based characterisation of Panton–Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus* from Italy

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Abstract Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates are widespread in many countries, with varying distribution and epidemiology. The aim of this study was to collect and characterise the CA-MRSA isolates circulating in Italy, since only some case reports have been published. Eighteen Panton–Valentine-positive CA-MRSA isolates were collected from different Italian hospitals during the period 2005–2009 from severe infections (skin and soft tissue infections, $n=10$; necrotising pneumonia, $n=7$; and sepsis, $n=1$). Accessory gene regulator (*agr*) typing, staphylococcal cassette chromosome (SCC) *mec* typing, *spa* typing, multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and DNA microarray were applied to categorise isolates into clones and to compare the relevant genetic features of each clone. Six different clones were identified, the most common (7 out of 18 isolates, 38.8%) being *agr*I/ST8/SCC*mec*IV, corresponding to the USA300 clone. Six out of the seven USA300 isolates did not harbour the arginine catabolic mobile element (ACME). Four strains (22.2%) were *agr*III/ST80/SCC*mec*IV, corresponding to the European clone. Two of the other clones, namely, *agr*III/ST88/SCC*mec*V and *agr*III/ST772/SCC*mec*V, corresponded to CA-MRSA clones rarely found in other countries and probably originating from Africa or the Indian subcontinent. The results of microarray hybridisations showed that the distribution of resistance genes and other virulence factors was specific to each clone. Some characteristics could be exploited as specific markers for a clone or a group of isolates, e.g. the *mer* operon, recovered only in ACME-negative USA300 strains. DNA microarray contributed to a more complete description of the variety of different CA-MRSA clones circulating in Italy.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) appeared and spread rapidly after the introduction of methicillin in clinical use in 1960, becoming one of the most prevalent pathogens in healthcare settings [1]. MRSA causes serious infections such as sepsis and pneumonia in hospitalised patients, with specific risk factors [2]. The emergence of MRSA causing community-acquired infec-

tions (CA-MRSA), particularly in healthy individuals without risk factors, has been reported since the end of the 1990s in the United States and in other countries [3]. Typical clinical presentations of CA-MRSA are skin and soft-tissue infections (SSTIs), including furuncles and skin abscesses, or deep-seated infections such as necrotising pneumonia, bone infections, sepsis and meningitis [2]. CA-MRSA strains appear phenotypically and genotypically different from hospital-acquired MRSA (HA-MRSA), although these differences have blurred over the recent years [2, 3]. CA-MRSA strains are generally susceptible to antibiotics other than beta-lactams and harbour staphylococcal cassette chromosome (SCC) *mec* type IV, V or VII [4]. On the other hand, HA-MRSA are generally multidrug-resistant and contain SCC*mec* type I, II or III, although in the last few years, some HA-MRSA clones containing SCC*mec* type IV have spread in Europe, e.g. EMRSA15 and the Lyon clone (sequence type [ST] 8) [5, 6]. Besides, antibiotic resistance within CA-MRSA is increasing [7]. Characteristically, CA-MRSA strains carry the genes encoding the Panton–Valentine leukocidin (PVL), a secreted virulence factor which causes polymorphonuclear leukocytes lysis and tissue necrosis [8].

Several different clones of CA-MRSA are spread worldwide. In the United States, the USA300 clone (ST8) is responsible for the major part of community-acquired SSTIs and for outbreaks in the community and in hospitals [3]. The presence of type I arginine catabolic mobile element (ACME) has been proposed to contribute to the fitness and transmissibility of the USA300 clone [9].

In Europe, CA-MRSA infections are less frequent than in the United States and are mainly associated with the European clone ST80 [10]. In Italy, some case reports or small studies have been published [11–15], but a more complete overview of CA-MRSA is lacking.

The aim of this study was: (i) to collect CA-MRSA strains from serious infections and characterise them by phenotypic and genotypic methods; (ii) to compare the results with those obtained applying a DNA microarray technique which permits to simultaneously identify a wide set of virulence factors, antibiotic resistance determinants and typing markers; and (iii) to recognise common or unique features among CA-MRSA isolates.

Materials and methods

Bacterial isolates, antimicrobial susceptibility and biofilm production testing

Eighteen CA-MRSA isolates were collected from April 2005 to October 2009. The isolates were referred by different Italian hospital laboratories all over the country on the basis of the type and the severity of the infections that appeared typical of CA-MRSA.

The isolates were tested for antimicrobial susceptibility by the disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [16]. When EUCAST breakpoints were not available for specific antibiotics, Clinical and Laboratory Standards Institute (CLSI) [17] or British Society for Antimicrobial Chemotherapy (BSAC) [18] breakpoints were applied. The agents tested included: cefoxitin, gentamicin, ciprofloxacin, erythromycin, kanamycin, clindamycin, tetracycline, rifampicin, trimethoprim–sulfamethoxazole, fusidic acid, fosfomycin and mupirocin. Minimum inhibitory concentrations of vancomycin, teicoplanin, linezolid, quinupristin–dalfopristin and daptomycin were performed using the E-test method (bioMérieux, Marcy-l'Etoile, France).

Biofilm production was determined spectrophotometrically as described elsewhere [15]. The isolates were categorised according to the optical density (OD) reading. OD values ≤ 0.12 corresponded to biofilm non-producers, OD=0.13–0.2 to weak producers, OD=0.2–0.4 to medium producers and OD>0.4 to strong producers [15].

Characterisation of the isolates by PCR

S. aureus genomic DNA was extracted with QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). *S. aureus* species and methicillin resistance were confirmed by polymerase chain reaction (PCR) assays detecting *nuc* and *mecA* genes [19]. PCR assays were performed to detect genes encoding PVL, *agr* alleles, ACME-specific *arcA* gene and genes encoding capsular types 5 and 8 [15, 19, 20]. Adhesin and toxin genes content was also evaluated as previously described [15]. SCC*mec* types were determined by combining the detection of *ccr* genes and *mec* complex genes as described elsewhere [19].

Molecular typing of the isolates

S. aureus protein A (*spa*) gene typing and multi-locus sequence typing (MLST) were performed on the isolates as described elsewhere [19]. Pulsed-field gel electrophoresis (PFGE) was applied to the seven CA-MRSA isolates belonging to ST8 to distinguish USA300 isolates [21]. USA300 strain FPR3757 was used as the control [20]. PFGE patterns were analysed following established criteria [21, 22].

DNA microarray hybridisation

DNA microarray hybridisation was performed at the French National Reference Center for Staphylococci (Lyon, France), on the ArrayTube platform (Identibac *S. aureus*

Genotyping, Alere, Sevres, France) [23, 24]. The array contains covalently immobilised probes specific for approximately 180 genes and 300 alleles of *S. aureus*, including typing targets, resistance genes, toxins and microbial surface components [23, 24]. The genes considered in this study and their function are listed in Supplementary Table S1.

Results and discussion

Out of 18 isolates, 8 (44.5%) were from males and 10 (55.5%) were from females (Table 1). The age of the patients ranged from 3 to 66 years (median 29.5 years). All patients lived in the community and reported no recent hospitalisation. The geographical distribution of the cases on the map of Italy is shown in Fig. 1. There was no apparent epidemiological relationship between cases, although in two areas, several cases were reported by the hospital laboratories, probably due to a better awareness of the problem.

All isolates were confirmed as PVL-positive MRSA by molecular methods. Only one strain (Sau65) harboured the ACME-specific *arcA* gene.

All isolates were resistant to at least one non-beta-lactam antibiotic among those tested: 12 isolates were resistant to fosfomycin (66.6%), ten to kanamycin (55.5%), six to tetracycline (33.3%), five to gentamicin (27.7%), four to erythromycin (22.2%), four to fusidic acid (22.2%), two to ciprofloxacin (11.1%), one to mupirocin (5.5%) and one to clindamycin (5.5%) (Table 1). All isolates were susceptible to rifampicin, trimethoprim–sulfamethoxazole, vancomycin, teicoplanin, quinupristin–dalfopristin, linezolid and daptomycin.

Table 1 Clinical information and phenotypic and genotypic characteristics of the 18 community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains

Isolate	Sex	Age	Isolation year	Type of infection	*agr* allele	ST	*spa* type	SCC*mec* type	Resistance to non-beta-lactam antibiotics[a]	Capsular type	Biofilm[b]
Sau17	M	16	2006	Necrotising pneumonia, sepsis and meningitis	I	8	t008	IV	fos	5	MP
Sau18	F	21	2007	Necrotising pneumonia	I	8	t008	IV	fos	5	NP
Sau14	M	16	2005	Necrotising pneumonia	I	8	t008	IV	fos	5	WP
Sau16	M	3	2006	SSTI	I	8	t008	IV	fos	5	MP
Sau32	M	11	2008	SSTI	I	8	t008	IV	fos	5	SP
Sau25	F	30	2008	Sepsis	I	8	t008	IV	fos	5	SP
Sau65	F	28	2009	SSTI	I	8	t008	IV	ery, kan, mup, fos	5	ND
Sau19	F	66	2007	Necrotising pneumonia	III	80	t044	IV	kan, tet, fus	8	MP
Sau21	F	50	2008	Necrotising pneumonia	III	80	t2453	IV	kan, tet, fus	8	MP
Sau58	M	41	2009	SSTI	III	80	t044	IV	ery, cli, kan, tet, fus	8	ND
Sau57	M	65	2009	SSTI	III	80	t044	IV	kan, tet, fus	8	ND
Sau24	M	25	2008	Necrotising pneumonia	III	88	t2526	V	kan, gen	8	SP
Sau27	F	44	2008	SSTI	III	88	t002	V	kan, gen	8	SP
Sau30	F	54	2008	SSTI	II	772	t345	V	ery, kan, gen, cip, fos	5	SP
Sau33	M	58	2008	SSTI	II	772	t345	V	ery, kan, gen, cip, fos	5	SP
Sau22	F	29	2008	SSTI	II	5	t319	IV	tet, fos	5	MP
Sau31	F	3	2008	SSTI	II	5	t002	V	kan, gen, tet, fos	5	MP
Sau15	F	37	2008	Necrotising pneumonia	III	30	t755	IV	fos	8	NP

[a] EUCAST breakpoints were applied [16], except for kanamycin and mupirocin, for which CLSI [17] and BSAC [18] breakpoints were applied, respectively. fos, fosfomycin; ery: erythromycin; kan: kanamycin; mup: mupirocin; tet: tetracycline; fus: fusidic acid; cli: clindamycin; gen: gentamicin; cip: ciprofloxacin

[b] NP, non-producer; WP, weak producer; MP, medium producer; SP, strong producer (see text); ND, not determined; SSTI, skin and soft tissue infection; ST, sequence type; SCC*mec*, staphylococcal cassette chromosome *mec*

Fig. 1 Geographical distribution of the community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) cases. The numbers within each placemarker represent the number of cases

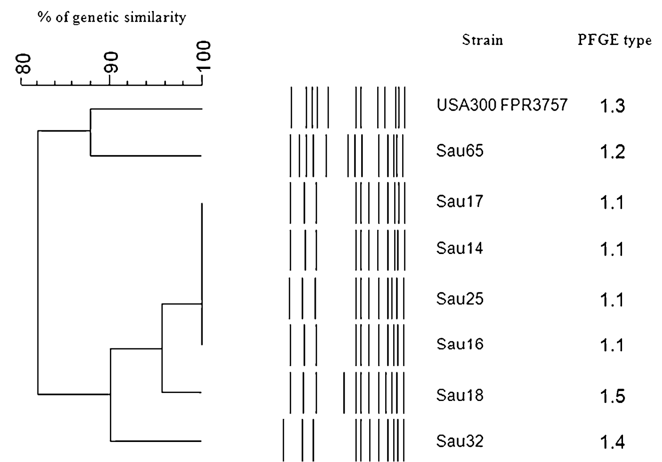

Fig. 2 Phylogenetic analysis of pulsed-field gel electrophoresis (PFGE) profiles obtained with the *agr*I/ST8/SCC*mec*IV isolates. The dendrogram was constructed with PFGE profiles by similarity and clustering analysis by the unweighted pair group method with arithmetic mean (UPGMA) and the Dice coefficient, by using a band tolerance of 1.5%. The percentage of genetic similarity is shown above the dendrogram. Isolates showed a percentage of similarity >80%

Molecular typing

Thirteen isolates harboured SCC*mec* type IV and five harboured SCC*mec* type V. *agr* alleles I, II and III were found. The isolates belonged to six different STs: seven isolates belonged to *agr*I/ST8/SCC*mec*IV, four isolates to *agr*III/ST80/SCC*mec*IV, two isolates each belonged to *agr*III/ST88/SCC*mec*V, *agr*II/ST772/SCC*mec*V and *agr*II/ST5/SCC*mec*IV–V, and one isolate to *agr*III/ST30/SCC*mec*IV. Isolates of a given ST shared the same capsular type, *agr* allele, *spa* type (or correlated) and SCC*mec* type, except for ST5 isolates, which harboured two different SCC*mec* types (Table 1).

PFGE analyses performed on the ST8 isolates revealed that all of the isolates were related, since they had less than four bands different. Five subtypes (from 1.1 to 1.5) were recovered, which showed a percentage of similarity >80% (exactly, 82.38%) (Fig. 2).

Microarray analyses

The microarray results are synthesised in Figures 3, 4 and 5. With respect to antibiotic resistance, a strong correlation was found between phenotypical resistance and the presence of corresponding resistance genes by microarray. With respect to biofilm, although all isolates possessed the *ica* operon involved in biofilm formation, phenotypical tests showed different rates of biofilm production, likely reflecting differences in gene expression or in other characteristics among the isolates. The molecular characterisation obtained by PCR (*mecA*, PVL, *arcA*, capsular, ACME, *agr* SCC*mec* genes, other toxin and adhesion genes) was concordant with the microarray results, confirming the high fidelity of this latter approach.

Despite the genetic diversity of the isolates, some genes were homogeneously distributed in all strains, including genes encoding leukocidins (*lukS-F*, *hlgA*, *lukX*, *lukY*), haemolysins (*hla*, *hld*), proteases (*aur*, *sspA-B-P*), adhesion proteins (*clfA-B*, *ebh*, *eno*, *fib*, *fnbA-B*, *sdrC*, *vwb* and *sasG*) and immune-evasion factors (*mprF* and *isdA*). These genes have an almost ubiquitary distribution in *S. aureus*, as shown by Monecke et al. by the microarray hybridisation of 100 clinical strains, including methicillin-susceptible *S. aureus* (MSSA), CA-MRSA and HA-MRSA belonging to different genetic lineages [23].

In our collection, only two strains possessed an intact beta-haemolysin gene (*hlb*), while 16 others harboured *hlb* genes truncated after the insertion of phage-borne genes, such as *entA* (enterotoxin A), or immune-evasion genes, such as *sak* (staphylokinase), *chp* (chemotaxis inhibitory protein) or *scn* (staphylococcal complement inhibitor).

The overall presence of antibiotic resistance determinants and enterotoxin genes or clusters was different between, and sometimes within, the clones, in line with their location on mobile genetic elements.

The microarray results are analysed below according to the six different *agr*/ST/SCC*mec* combinations found:

1. *agr*I/ST8/SCC*mec*IV

   Isolates with these characteristics were the most prevalent in our collection. PFGE was performed in order to evaluate if the seven ST8 CA-MRSA isolates belonged or were related to the USA300 clone, since

Isolate	Clonal Group	*blaZ-I-R*	*ermA*	*ermB*	*ermC*	*linA*	*msrA*	*mefA*	*mpbBM*	*vatA-B*	*vga*	*vgb*	*aacA-aphD*	*aadD*	*aphA-3*	*sat*	*dfrA*	*far1*	*mupR*	*tetK*	*tetM*	*cat*	*fexA*	*cfr*	*fosB*	*qacA*	*qacC*	*vanA-B-Z*	*mer operon*	ACME
		Antibiotic resistance genes																												
Sau17	*agr*I ST8 IV	•																							•				•	
Sau18	*agr*I ST8 IV	•																							•				•	
Sau14	*agr*I ST8 IV	•																							•				•	
Sau16	*agr*I ST8 IV	•																							•				•	
Sau32	*agr*I ST8 IV	•																							•				•	
Sau25	*agr*I ST8 IV	•																		•					•				•	
Sau65	*agr*I ST8 IV	•					•		•						•	•			•						•					•
Sau19	*agr*III ST80 IV	•													•	•		•		•										
Sau21	*agr*III ST80 IV	•													•	•		•		•										
Sau58	*agr*III ST80 IV	•			•										•	•		•		•										
Sau57	*agr*III ST80 IV	•													•	•		•		•										
Sau24	*agr*III ST88 V	•											•																	
Sau27	*agr*III ST88 V	•											•																	
Sau30	*agr*II ST772 V	•					•		•				•		•	•									•					
Sau33	*agr*II ST772 V	•					•		•				•		•	•									•					
Sau22	*agr*II ST5 IV																•				•				•		•			
Sau31	*agr*II ST5 V												•							•					•					
Sau15	*agr*III ST30 IV	•																							•					

Fig. 3 Presence of antibiotic-resistance genes and arginine catabolic mobile element (ACME) in the CA-MRSA strains. The dots indicate positive results for probe hybridization. The function of each gene is listed in Supplementary Table S1

Isolate	Clonal Group	*tsst-1*	*entA*	*entP*	*entB*	*entC-L*	*entD-J-R*	*entE*	*egc*	*entH*	*entK-Q*	*lukS-F*	*hlgA*	*lukF-PV P83*	*lukM*	*lukD*	*lukE*	*lukX*	*lukY var 1*	*lukY var 2*	*hla*	*hld*	*hlb intact*	*hlb truncated*	*sak*	*chp*	*scn*	*etA*	*etB*	*etD*	*edinA*	*edinB*	*edinC*
		Superantigenic toxins										Leukocidins									Haemolysins				Phagic genes			Exfoliative toxins			*edin* factors		
Sau17	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•	•	•									
Sau18	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•	•	•									
Sau14	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau16	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau32	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau25	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau65	*agr*I ST8 IV										•	•	•			•	•	•	•		•	•		•	•	•	•						
Sau19	*agr*III ST80 IV											•	•			•	•	•	•		•	•		•	•		•			•		•	
Sau21	*agr*III ST80 IV											•	•			•	•	•	•		•	•		•	•		•			•		•	
Sau58	*agr*III ST80 IV											•	•			•	•	•	•		•	•		•	•		•			•		•	
Sau57	*agr*III ST80 IV											•	•			•	•	•	•		•	•		•	•		•			•		•	
Sau24	*agr*III ST88 V			•								•	•			•	•	•	•		•	•		•	•	•	•						
Sau27	*agr*III ST88 V			•								•	•			•	•	•	•		•	•		•	•	•	•						
Sau30	*agr*II ST772 V		•			•			•			•	•					•	•		•	•					•						
Sau33	*agr*II ST772 V		•			•			•			•	•					•	•		•	•					•						
Sau22	*agr*II ST5 IV								•			•	•			•	•	•	•		•	•		•	•	•	•						
Sau31	*agr*II ST5 V			•			•		•			•	•			•	•	•	•		•	•		•	•		•						
Sau15	*agr*III ST30 IV								•			•	•					•		•	•	•		x	•	•	•						

Fig. 4 Presence of genes encoding miscellaneous virulence factors in the CA-MRSA strains. The dots indicate positive results for probe hybridization and the X indicates an ambiguous result. The function of each gene is listed in Supplementary Table S1

Isolate	Clonal Group	Capsule 5	Capsule 8	*aur*	*splA*	*splB*	*splE*	*sspA-B*	*sspP*	*icaA*	*icaC*	*icaD*	*bap*	*bbp*	*clfA*	*clfB*	*cna*	*ebh*	*ebpS*	*eno*	*fib*	*fib* MRSA25	*fnbA*	*fnbB*	*map*	*sdrC*	*vwb*	*sasG*	*mprF*	*isdA*	*isdA* 252	*hsdS1*	*hsdS2*	*hsdS3*
				Proteases						Biofilm				MSCRAMMs															Immune-evasion factors					
Sau17	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau18	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•			•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau14	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•			•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau16	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•			•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau32	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau25	*agr*I ST8 IV	•		•	•	•	•	•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau65	*agr*I ST8 IV	•		x	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•			•	•
Sau19	*agr*III ST80 IV		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•			•	
Sau21	*agr*III ST80 IV		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•			•	
Sau58	*agr*III ST80 IV		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•			x	
Sau57	*agr*III ST80 IV		•	x	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•				
Sau24	*agr*III ST88 V		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•		•	x	•
Sau27	*agr*III ST88 V		•	•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	•	•		•		•
Sau30	*agr*II ST772 V	•		•				•	•	•	•	•		•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•				
Sau33	*agr*II ST772 V	•		•				•	•	•	•	•		•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•				
Sau22	*agr*II ST5 IV	•		•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•			•	•
Sau31	*agr*II ST5 V	•		•	•	•		•	•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•	x	•				•
Sau15	*agr*III ST30 IV		•	•			•	•	•	•	•	•		•	•	•	•	•	•	•		•	•	•	•	•	•		x		•		•	•

Fig. 5 Presence of genes encoding miscellaneous virulence factors and adhesion factors in the CA-MRSA strains. The dots indicate positive results for probe hybridization and the Xs indicate ambiguous results. The function of each gene is listed in Supplementary Table S1 MSCRAMM, microbial surface components recognizing adhesive matrix molecules

the USA300 definition is based on a specific SmaI PFGE profile [21]. Although all USA300 strains initially shared this unique profile, some slightly divergent profiles were reported in the last few years, demonstrating the progressive diversification of this successful lineage [7]. All ST8 strains in our collection were related to the USA300 FPR3757 pulsotype, as assessed by a similarity percentage >80% (exactly, 82.38%). The pulsotype of Sau65 was the most closely related to the USA300 FPR3757 pulsotype (percentage of similarity=87.5%). Sau65 was also the only strain that harboured the ACME-specific *arcA* gene. The ACME element encodes proteins involved in an arginine deaminase pathway that converts L-arginine to carbon dioxide, ATP and ammonia. ACME has been proposed to play an important role in the pathogenesis of CA-MRSA skin infections, since arginine conversion raises the local pH, increasing the ability of USA300 to persist and spread on intact skin [9, 20]. However, some experimental data are not entirely consistent with this model [25]. The ACME element was rarely found in *S. aureus* clones other than USA300 [20]. On the basis of its characteristics, strain Sau65 can be considered to belong to the USA300 clone [9, 20, 21].

The other six ST8 strains that did not harbour the ACME-specific *arcA* gene shared all of the other characteristics of USA300 (ST, *spa*, PVL) that, according to Larsen et al., are even better markers than PFGE to assign a strain to the USA300 clone [26]. Therefore, the other six ST8 strains can be considered as an ACME-negative variant of the USA300 clone.

The antibiotic resistance profile of the USA300 strain was different from those of the ACME-negative variants. USA300 was multiantibiotic-resistant, since it harboured *msrA* and *mpbBM* (resistance to erythromycin), *aphA-3* (resistance to kanamycin), *sat* (resistance to streptothricin), *fosB* (resistance to fosfomycin) and, unique in our collection, also *mupR* (resistance to mupirocin). ACME-negative strains were more antibiotic-susceptible, bearing only the *fosB* gene. Strain Sau25 also harboured the *tetK* gene, but was phenotypically susceptible to tetracycline. Characteristically, ACME-negative strains harboured the *mer* operon (mercury resistance), which was not present in neither the USA300 isolate nor in the other CA-MRSA isolates examined. The *mer* operon is usually integrated within SCC*mec* type III, but it is likely that, in this case, it was carried by a plasmid, as previously described [27]. Both the USA300 and the ACME-

negative strains carried the enterotoxin cluster *entK-Q*. Sau17 and Sau18 were the only strains in our collection bearing an intact *hlb* (beta-haemolysin) gene; all of the other ST8 isolates harboured the truncated form due to the insertion of *sak*, *chp* and *scn* phage-borne genes. The profiles of the other virulence genes were similar in all ST8 strains, although *splE*, encoding the serine protease E, was absent in the USA300 strain and *bbp*, encoding the bone sialoprotein-binding protein, an adhesion factor, was variably present in the ST8 isolates. The microarray profile of the USA300 strain was similar to those previously reported for the USA300 lineage. Interestingly, the *mupR* gene has been rarely reported in USA300 isolates [23, 24, 28].

ST8 has been recognised as the major CA-MRSA clone also in other European countries, such as Austria, Bulgaria and Spain [10, 29]. In some studies, when additional typing was performed (e.g. ACME detection or PFGE), ST8 CA-MRSA isolates were identified as belonging to the USA300 clone [10].

2. *agr*III/ST80/SCC*mec*IV

   Four strains belonged to ST80, all of which carried the antibiotic resistance genes *aphA-3*, *sat*, *tetK* and *far* (resistance to fusidic acid). Strain Sau58 also carried *ermC*, encoding for erythromycin and clindamycin resistance. ST80 strains did not possess any enterotoxin genes or clusters. The gene *hlb* was truncated at the phage insertion site due to the presence of *sak* and *scn* genes. Microarray profiles were consistent with those previously published for ST80, showing that this clone is characterised by a specific antimicrobial resistance pattern and by specific virulence factors such as *etD* (encoding the exfoliative toxin D) and *edinB* (encoding for the epidermal cell differentiation inhibitor B), which are strong markers for the European ST80 clone [23, 24]. At variance with the vast majority of European countries, in Italy, ST80 appears not to be the most common CA-MRSA clone [10].

3. *agr*III/ST88/SCC*mec*V

   Two strains belonged to ST88. They possessed the bi-functional gene *aacA-aphD*, encoding gentamicin–kanamycin resistance. In this clone, enterotoxin P and the truncated *hlb* gene for the insertion of the *sak*, *scn* and *chp* genes were present. The *hsdS1* gene was found only in the two ST88 isolates. *hsdS1* encodes a site-specific deoxyribonuclease subunit type 1, involved in a restriction modification system responsible for DNA protection [30].

   CA-MRSA ST88 isolates are rather rare: they have been found only sporadically in Africa, Bangladesh, China and Europe [31, 32]. However, in Africa, the ST88 clone was found both in hospitals and in the community, both MSSA and MRSA [31]. Differently from the previously published microarray profile of this clone, ST88 isolates from this study did not bear *entA* or *tetK* genes [23].

4. *agr*II/ST772/SCC*mec*V

   Two strains belonged to ST772. They possessed the antibiotic resistance genes *msrA*, *mpbBM*, *aacA-aphD*, *aphA-3*, *sat* and *fosB*, and gave positive hybridisation for enterotoxins *entA*, *entK-Q* and *egc* (comprising *entG-I-M-N-O-U*) clusters and *scn*. The isolates lacked the leukocidins *lukD-lukE* and the serine proteases *splA*, *splB* and *splE*. Both isolates harboured the *cna* gene. This gene encodes a collagen-binding adhesin, a virulence factor that could have a role in necrotising pneumonia pathogenesis [33].

   ST772 isolates are rather rare: they have been found in Malaysia, Bangladesh, India and England [34, 35]. The toxin and virulence factor contents of the isolates were in accordance to those of the Bangladesh isolates [34], although our isolates did not carry *lukD* and *lukE* genes.

   ST772 is a single-locus variant of ST1. However, the microarray profile of ST772 is completely different from that of ST1, in terms of antibiotic resistance, toxin content, SCC*mec* elements and *agr* alleles [23, 24].

5. *agr*II/ST5/SCC*mec*IV–V

   Two strains shared this ST, but they harboured different SCC*mec* elements (IV and V). Uniquely among the isolates under study, the two ST5 isolates did not possess the *bla* genes encoding the staphylococcal penicillinase. The content of the other antibiotic resistance genes and the toxin genes was quite different in the two isolates. Sau22 possessed *fosB*, *dfrA* (trimethoprim resistance), *tetM* (tetracycline resistance) and *qacA*, responsible for quaternary ammonium compounds resistance tolerance. The enterotoxin cluster *egc* and the phage-borne genes *sak*, *scn* and *chp* were present. Sau31 harboured *aacA-aphD*, *tetK* and *fosB*, the enterotoxin gene *entP*, the enterotoxin clusters *entD-JR* and *egc*, and the phage-borne genes *sak* and *scn*. The two strains shared the same MSCRAMMs and proteases genes.

   The *agr*II/ST5 clone is widely disseminated and can present different gene profiles, as microarray results have previously described [23, 24].

6. *agr*III/ST30/SCC*mec*IV

   Only one strain belonged to ST30. This strain was relatively antibiotic-susceptible, since it possessed only the *fosB* gene. It harboured the enterotoxin *egc* cluster, *sak*, *scn*, *chp* and *cna* genes. As expected, in this strain, the allelic variants of some genes were found: for instance, the variant 2 of the leukocidin *lukY*, the alleles MRSA252 of *fib*, encoding a fibronectin-binding protein and of *isdA*, an immune-evasion factor. The

microarray profile is in general accordance with that of the ST30 strain MRSA252, with the exception that Sau15 lacks *aadD* and *ermA* [23]. ST30 corresponds to the South-West Pacific clone, a pandemic clone spread in Oceania, East-Asia, the United States, South America and some European countries [3].

Conclusions

Our data provide evidence of the diversity of the clones circulating in Italy. The presence of six distinct community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) clones probably reflects the introduction into the country of different clones by international travellers or immigrants. Among a limited number of Panton–Valentine leukocidin (PVL)-positive CA-MRSA isolates, we found isolates belonging to uncommon clones, such as *agr*III/ST88/SCC*mec*V and *agr*III/ST772/SCC*mec*V, which are only sporadically reported in other countries [31, 32, 34, 35].

Isolates belonging to ST8 were the most common. In previously published case reports [12, 13], some of the isolates were already assigned to the USA300 clone. In fact, with the exception of one strain that presented all of the USA300 characteristics, the other ST8 isolates should be defined as arginine catabolic mobile element (ACME)-negative USA300 strains. ACME-negative strains were identified inside the USA300 clone in the USA [7], Australia [28], Austria [10], Spain [29], Latin America [36] and Italy [37], but, often, they represented only a minority of the isolates. On the contrary, the majority of the ST8 isolates found in Italy are ACME-negative.

Microarray hybridisation has the great potentiality of investigating simultaneously the presence of a large number of genomic loci. Hence, it allows the detection of unexpected characteristics in a particular isolate that would not be searched for by more labourious methods (e.g. by polymerase chain reaction [PCR] assays). This applies to less common antibiotic resistance genes and to complex patterns of virulence genes. Some of the peculiarities found, if confirmed, have the potentiality to represent new epidemiological markers for the clones. One example is the *mer* operon, which, in our study, was recovered only in the ACME-negative USA300 strains and not in the ACME-positive USA300 or in the other CA-MRSA isolates. In the study by Monecke et al. [28], the *mer* operon was also present in the USA300 ACME-negative strains from Australia and not in the USA300 ACME-positive strains. Although the microarray has allowed to find characteristics that are unique to clones and strains, no specific patterns of toxin or virulence factors genes have been identified that characterise CA-MRSA or CA-MRSA strains causing specific diseases, such as necrotising pneumonia, as already pointed out by previous studies [23, 24, 28].

In conclusion, this study confirms that microarray hybridisation represents a valid alternative approach to the conventional molecular typing techniques, providing additional features that are complementary to the characterisation of the strains.

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