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Spread of invasive Spanish *Staphylococcus aureus spa*-type t067 associated with a high prevalence of the aminoglycoside-modifying enzyme gene *ant(4′)-Ia* and the efflux pump genes *msrA*/*msrB*

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***Objectives*: We carried out a nationwide study aimed at the determination of the molecular epidemiology and antibiotic resistance mechanisms of invasive *Staphylococcus aureus* in 21 Spanish hospitals.**

***Methods*: The distributions of molecular markers, including antibiotic resistance genes, were investigated in 203 *S. aureus*, comprising 90 methicillin-resistant *S. aureus* (MRSA) and 113 methicillin-susceptible *S. aureus* (MSSA). Antimicrobial susceptibility was determined by standard methods. Panton–Valentine leucocidin (PVL) detection, staphylococcal cassette chromosome *mec* (SCC*mec*) types and *agr* types were performed/determined by PCR. All isolates were genotyped by PFGE after digestion of chromosomal DNA with *Sma*I. Multilocus sequence typing and *spa*-typing were also performed.**

***Results*: In MRSA isolates, 74.4% were *agr* allotype II and were positive for SCC*mec* IV. Sixty-nine *spa*-types were identified, 18 in MRSA and 57 in MSSA. Both MRSA and MSSA variants were detected in six *spa*-types (8.7%). The majority of *S. aureus* (51.2%) were grouped into four *spa*-types (t067, t002, t012 and t008). The *spa*-type t067 was detected in 18 of the 21 (85.7%) participating hospitals, including both MRSA and MSSA in six of them; in total, 25.9% of our isolates were *spa*-type t067 (49% in MRSA) in comparison with 0.6% in a central *spa*-typing database. The prevalence of the *ant(4′)-Ia* and *msrA/msrB* genes was significantly higher in the MRSA *spa*-type t067 than in the other MRSA *spa*-types. Association between *spa*-type t067 and ST125 is described here for the first time. A high prevalence (36.4%) of PVL-positive MSSA was detected.**

***Conclusions*: A higher than expected prevalence of *spa*-type t067 isolates was found among invasive MRSA in Spain. The oxacillin, tobramycin, erythromycin and ciprofloxacin resistance profile of *spa*-type t067 isolates was linked to the presence of *ant(4′)-Ia* and *msrA* or *msrB* genes.**

Keywords: invasive infections, molecular epidemiology, *spa*-typing, resistance mechanisms

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Introduction

Staphylococcus aureus is an important human pathogen responsible for a wide range of nosocomial and community-acquired infections.[1–4] These include invasive infections such as skin and soft tissue infections, pneumonia, bloodstream infections, osteomyelitis and endocarditis, as well as toxin-mediated syndromes such as toxic shock and food poisoning. The burden of disease imposed by *S. aureus* has increased over the last decade due to the spread of methicillin-resistant strains which frequently cause healthcare-associated infections, and this uncomfortable trend has been compounded by the recent emergence of community-acquired MRSA (CA-MRSA) caused by several clonal lineages that are genetically distinct from their nosocomial counterparts.[5,6]

For decades, various phenotypic and genotypic methods have been used for tracking the spread of methicillin-susceptible *S. aureus* (MSSA) and MRSA. PFGE is still the most widely used molecular typing method.[7] But this method, based on the position of anonymous bands, comes with some inconveniences, such as lack of biologically meaningful grouping[8] and difficulties in standardization and inter-laboratory comparison.[9] Moreover, PFGE is a time- and labour-intensive technique. Typing approaches based on DNA sequencing offer some advantages over PFGE. The sequencing of the polymorphic X region of the protein A gene (*spa*-typing) is fast, allows for a discrete grouping and generates portable data. Combining PFGE and *spa*-typing provides an extremely high discrimination and thus a very fine scale for the interpretation of the results of surveillance studies.[10]

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is based on the molecular characterization of the mobile genetic element carrying the methicillin resistance gene (*mecA*).[11] Six major types of SCC*mec* differing in sequence and size have been characterized according to the type of recombinase genes (*ccr*) and the class of *mec* complex they carry.[12,13] Panton–Valentine leucocidin (PVL) is an *S. aureus* specific exotoxin, encoded by two co-transcribed genes designated *lukF-PV* and *lukS-PV*, and is associated with skin and soft tissue infections and severe necrotizing pneumonia.[14,15] Both PVL and SCC*mec* type IV have been used as molecular markers for CA-MRSA infections.[16] In recent years, the *agr* ('accessory gene regulator') gene has received increasing attention, as the *agr* locus influences the expression of many virulence traits in *S. aureus* and allows the classification of *S. aureus* into four groups.[17,18]

The main mechanism of aminoglycoside resistance in staphylococcal isolates is drug inactivation by cellular aminoglycoside-modifying enzymes: resistance to gentamicin and concomitant resistance to tobramycin and kanamycin in staphylococci are mediated by the bifunctional enzyme AAC(6′)/APH(2″′) encoded by the *aac(6′)-Ie-aph(2″′)* gene; resistance to neomycin, kanamycin, tobramycin and amikacin is mediated by an ANT(4′)-I enzyme encoded by the *ant(4′)-Ia* gene; and resistance to neomycin and kanamycin is mediated by the APH(3′)-III enzyme encoded by the *aph(3′)-III* gene.[19] Ribosome modification confers MLS_B resistance, principally by a single base change in the 23S rRNA by methylases encoded by erythromycin ribosomal methylase (*erm*) genes *ermA* or *ermC*. Resistance to macrolides and streptogramin B (MS resistance) can also occur in staphylococcal isolates with active efflux by a membrane-bound transporter protein (*msrA* gene).[20] Quinolone resistance develops mainly as a result of chromosomal mutations in the target of quinolones, topoisomerase IV or DNA gyrase. The GrlA subunit of topoisomerase IV and the GyrA subunit of gyrase are the most common sites of resistance mutations; topoisomerase IV mutations are the most critical, since they are the primary target for many fluoroquinolones in *S. aureus*.[21]

In the winter of 2006–07, the European Antimicrobial Resistance Surveillance System (http://www.rivm.nl/earss/) promoted a study aimed at the identification of the predominant *S. aureus* clones in European countries by *spa*-typing. Previously, we have shown that new prevalent clones of *S. aureus* have appeared in recent years in MRSA in Spain.[22,23] However, the prevalence of the genetically distinct strains defined by PFGE, *spa*-typing and multilocus sequence typing (MLST) and their association with other epidemiological markers such as *agr*, PVL and antibiotic resistance genes has never been investigated using a representative sample of invasive isolates in Spain or in other countries. We describe here the genomic diversity and antibiotic resistance mechanism of invasive isolates of *S. aureus* identified through a nationwide multicentre surveillance study.

Materials and methods

Test isolates

Twenty-one Spanish hospitals participated in this study. They were selected in order to provide a population snapshot that is demographically and geographically representative of the patient population treated in Spanish hospitals. Each hospital laboratory was asked to submit to the central reference laboratories the first five successive MSSA and the first five successive MRSA isolated from individual patients with invasive infections. If there were less than five MRSA isolates, successive MSSA isolates could be included to complete a set of 10 before submission. The duration of the sampling interval was 6 months, from 1 September 2006 to 28 February 2007. Each isolate was accompanied by epidemiological information detailing the date of isolation, origin of the specimen, demographic details (age and gender) and epidemiological context (such as healthcare-associated or outpatients). Infections were identified as community-acquired if a positive culture was obtained within the first 48 h of hospitalization.[24] All microbiological and molecular studies were carried out at the reference laboratories of the Centro Nacional de Microbiología, Madrid, Spain.

Susceptibility testing and antibiotic resistance gene detection

Antimicrobial susceptibility testing was determined by the disc diffusion and microdilution methods (BD Phoenix Automated Microbiology System; Becton–Dickinson Diagnosis, Sparks, MD, USA). The antimicrobial agents tested were cefoxitin, ciprofloxacin, vancomycin, linezolid, rifampicin, erythromycin, gentamicin, tobramycin, quinupristin/dalfopristin, co-trimoxazole and clindamycin. The CLSI guidelines for susceptibility testing and qualitative interpretation were used throughout.[25] Methicillin resistance was confirmed by detection of *mecA* using a PCR protocol as described previously.[26]

The macrolide–lincosamide resistance genes *ermA*, *ermB*, *ermC*, *msrA*, *msrB* and *linA/linA′* were detected by PCR as described previously;[20,27,28] *ermA*, *ermB* and *ermC* were tested in 47 isolates resistant to erythromycin and clindamycin, *msrA* and *msrB* were tested in 32 isolates resistant to erythromycin but susceptible to

clindamycin and *linA/linA′* were tested in two clindamycin-resistant but erythromycin-susceptible isolates.

Aminoglycoside-modifying enzyme genes *aac(6′)-Ie + aph(2′)*, *ant(4′)-Ia* and *aph(3′)-III* were screened by PCR as described previously.[19] The enzyme gene *aac(6′)-Ie + aph(2′)* was tested in 18 isolates resistant to gentamicin, tobramycin and kanamycin; *ant(4′)-Ia* was tested in 55 isolates that were tobramycin-resistant and gentamicin/kanamycin-susceptible and *aph(3′)-III* was tested in 7 isolates that were kanamycin-resistant but gentamicin/tobramycin-susceptible.

The amplification and sequence analysis of the quinolone resistance determining region of *grlA* and *gyrA* was performed as described in 40 ciprofloxacin-resistant isolates.[29]

Multiplex PCR SCCmec typing

SCC*mec* types were determined using a multiplex PCR strategy that generated a specific amplification pattern for SCC*mec* types I, Ia, II, III, IV and IVa SCC*mec* structural types.[11] This method does not identify the *ccrAB* alleles, but an excellent correlation has been demonstrated between the full characterization of SCC*mec* and this strategy.[11] Another two PCR methods were used to determine SCC*mec* IV subtypes (IVb, IVc, IVd and IVh) and SCCmec V.[30,31]

Determination of agr types

The determination of the four *agr* types was performed by the multiplex PCR method previously described by Shopsin *et al.*[18] The primers used were: a forward primer pan-*agr* (corresponding to conserved sequences from the *agrB* gene) and four reverse primers [*agr*I (in the *agrD* gene), *agr*II (in the *agrC* gene), *agr*III (in the *agrD* gene) and *agr*IV (in the *agrC* gene)].[18]

PFGE

All isolates were genotyped by PFGE following *Sma*I digestion of chromosomal DNA, prepared using a modification of the protocol described by Cookson *et al.*[32] The resulting agarose plugs were loaded on agarose 1% w/v gels, and PFGE was performed in a CHEF-DRII apparatus (Bio-Rad, Hemel Hempstead, UK) in 0.5× Tris–borate–EDTA buffer (1× TBE is 89 mM Tris, 89 mM boric acid and 1 mM EDTA), at 6 V/cm and 12–14°C. The total run time was 23 h as follows: 5–15 s for 10 h and 15–60 s for 13 h. The gels were stained with ethidium bromide, visualized under UV illumination and photographed. Following analysis according to the criteria of Tenover *et al.*,[33] a dendrogram was constructed with Molecular Analyst Software (Bio-Rad) using the Dice correlation coefficient[34] and the unweighted pair-group method with averages, with a tolerance position of 0.8%. PFGE profiles of the MRSA isolates were named according to a previous report;[23] PFGE types of MSSA isolates were assigned in this study following the Tenover criteria.[33]

DNA sequencing of the spa gene

To obtain a DNA preparation, a loopful of *S. aureus* cells were washed with distilled water and incubated with 200 μL of 6% InstaGene matrix solution (Bio-Rad, München, Germany) for 20 min at 56°C. The suspension was vortexed and heated for 8 min at 100°C and centrifuged at 8000 **g** for 2–3 min. DNA amplification was performed by PCR using the PuRe *Taq* Ready-ToGo PCR Beads (Amersham Biosciences), in a total volume of 25 μL containing LiChrosolv water (Merck), 5 μL of DNA template and 10 pmol of each primer.

The *spa* gene was amplified using primers spa-1113f and spa-1514r, and sequencing was performed as described previously.[35] Nucleotide sequences were analysed using the software Ridom StaphType™ (Ridom GmbH, Würzburg, Germany) as described by Harmsen *et al.*[36] BURP ('based upon repeat patterns'), as implemented in the Ridom StaphType software, was used to cluster (*spa*-CC) *spa*-types in MRSA and MSSA isolates.[37]

Detection of PVL genes

The PVL genes (*lukS-PV* and *lukF-PV*) were detected by PCR as described by Lina *et al.*[15] The PVL-positive *S. aureus* (PVL-SA) ATCC 49775 strain was used as a positive amplification control.

MLST

The sequence type (ST) of selected isolates of *S. aureus* was determined according to the method of Enright *et al.*[38] Analysis of the results was performed using the database available at www.mlst.net.

Results

Characteristics of participating hospitals

The 21 participating hospitals were located in 19 Spanish provinces covering all geographic regions of the country. The estimated catchment population of these hospitals was ∼6 000 000 persons, corresponding to ∼15% of the Spanish population. Three hospitals (14.3%) had more than 1000 beds, 8 (38.1%) had between 500 and 1000 and 10 (47.6%) had between 250 and 500.

Clinical isolates

A total of 203 unduplicated *S. aureus* were collected from blood cultures, of which 90 were MRSA and 113 were MSSA; all participating hospitals provided MRSA isolates (average 4.3).

One hundred and thirty-seven isolates (67.5%) were from males and 66 (32.5%) were from females. Four (2%) were from children ≤14 years of age, 74 (36.5%) were from patients between 15 and 65 years of age and 125 (61.6%) were from patients >65 years.

In total, 123 cases (60.6%) were considered nosocomial infections, 67 (33%) community-acquired infections, and in 13 (6.4%) isolates, this information was unknown.

Susceptibility testing and antibiotic genes detection

Resistance to ciprofloxacin, erythromycin, clindamycin, tobramycin and gentamicin was 96.7%, 81.1%, 31.1%, 77.8% and 17.8%, respectively, in MRSA isolates and 7.1%, 16.8%, 14.1%, 2.6% and 2.6%, respectively, in MSSA isolates. Resistance to rifampicin was observed in six isolates (6.7%), all of them MRSA; no resistance to linezolid, co-trimoxazole, quinupristin/dalfopristin or vancomycin was detected.

Multiresistance (defined as resistance to three antibiotics other than oxacillin) was observed in 58 MRSA isolates (64.4%) (Table 1) and in 4 MSSA isolates (3.5%). The most frequent resistance phenotypes found in MRSA were resistance to oxacillin, ciprofloxacin, tobramycin and erythromycin (19 isolates,

Table 1. Resistance phenotypes of invasive MRSA in relation to SCC*mec* type (Spain 2006–07)

Resistance phenotype	no. (%)	SCC*mec*				
		I	II	IVa	IVc	IVh
O	2 (2.2)	—	—	2	—	—
OC	11 (12.2)	1	—	5	3	2
OT	1 (1.1)	—	—	1	—	—
OCT	16 (17.8)	—	—	16	—	—
OCE	1 (1.1)	—	—	—	1	—
OCET	19 (21.1)	—	—	16	3	—
OCEK	5 (5.6)	—	—	3	2	—
OCECc	2 (2.2)	—	—	1	1	—
OCEGT	2 (2.2)	—	—	1	1	—
OCETCc	10 (11.1)	—	6	4	—	—
OCETCi	6 (6.7)	—	—	6	—	—
OCTCy	2 (2.2)	—	—	2	—	—
OCRET	1 (1.1)	—	—	1	—	—
OCEGTCc	6 (6.7)	4	—	2	—	—
OCEGTCi	1 (1.1)	—	—	1	—	—
OCRETCc	1 (1.1)	—	—	1	—	—
OCREGTCc	4 (4.4)	4	—	—	—	—
Total (%)	90 (100)	9 (10)	6 (6.7)	62 (68.9)	11 (12.2)	2 (2.2)

O, oxacillin; C, ciprofloxacin; G, gentamicin; T, tobramycin; K, kanamycin; E, erythromycin; Cc, constitutive clindamycin; Ci, inducible clindamycin; Cy, clindamycin intermediate and erythromycin susceptibility; R, rifampicin.

21.1%), and to oxacillin, ciprofloxacin and tobramycin (17 isolates, 18.9%) (Table 1). In MSSA, the phenotype characterized by erythromycin resistance plus inducible clindamycin resistance was the most prevalent (13 isolates, 11.5%).

The aminoglycoside-modifying enzyme gene *ant(4′)-Ia* was detected in all 55 tobramycin-resistant but gentamicin-susceptible isolates tested; 19 isolates resistant to both gentamicin and tobramycin had the bifunctional enzyme gene *aac(6′)-Ie + aph(2′)*. The remaining seven isolates resistant to kanamycin but susceptible to gentamicin and tobramycin contained the *aph(3′)-IIIa* gene only (Table 2).

In all 32 erythromycin-resistant but clindamycin-susceptible isolates, the efflux genes *msrA/B* were detected. The 47 isolates with the erythromycin-resistant and clindamycin-resistant phenotype had the methylase genes *ermA*, *ermB* and *ermC* as described in Table 2.

Most of the 40 ciprofloxacin-resistant isolates tested presented the 2614 (C→T) change encoding the Ser-80→Phe mutation in *grlA* in combination with the 2402 (C→T) change encoding the Ser-84→Leu mutation in *gyrA*. Only one isolate had the 2614 (C→T) change in *grlA* without mutations in *gyrA* (Table 2).

SCCmec typing

Seventy-five (83.3%) of the 90 MRSA isolates harboured SCC*mec* type IV, whereas 9 (10%) and 6 (6.7%) harboured SCC*mec* types I and II, respectively. SCC*mec* type III was not detected (Table 1). Of the isolates harbouring SCC*mec* type IV, 55 were subtype IVa (73.3%), 18 were subtype IVc (24%) and 2 were subtype IVh (2.6%).

Determination of agr type

The great majority of MRSA isolates, 78 of 90 (86.7%), had a*gr* type II, while only 7 (7.8%) and 5 (5.6%) isolates had *agr* types I and III, respectively. No *agr* type IV was detected in MRSA.

The distribution of *agr* types in MSSA was more diverse as 35 (31%), 40 (35.4%) and 35 (31%) isolates had *agr* types I, II and III, respectively, and 3 (2.7%) isolates had *agr* type IV.

Analysis of PFGE profiles

Of the 90 MRSA isolates, 28 distinct PFGE profiles were identified by PFGE, of which 63 isolates (70%) were grouped into eight clonal groups. The main groups were E7 (22 isolates, 24.4% of all MRSA) and E8 (17 isolates, 18.9%). The remaining 27 isolates (defined as sporadics) were assigned to 20 different PFGE profiles with 16 of them containing a single isolate (Table 3).

The 113 MSSA isolates studied were classified into 88 distinct PFGE profiles, 104 of which (92%) were grouped into 10 clusters (named clusters C1–C10 in this study). Four of the clusters were the most prevalent and contained 73 isolates (64.6%) distributed in clusters C2 (26 isolates), C5 (17 isolates), C6 (17 isolates) and C3 (13 isolates). Strains that were not included in any of these clusters were named as ‘not grouped’ (NG).

spa-type distribution

In 201 isolates successfully *spa*-typed, 18 types were detected among MRSA and 57 among MSSA. Overall, 52 (25.9%) isolates were t067, 28 (13.9%) isolates were t002 and 12 (6%) isolates were t012. The most common *spa*-type (t067) was

Table 2. Association between antibiotic resistance phenotypes and resistance genes in invasive *S. aureus* from Spain

Antibiotic class and resistance phenotype	Resistance genes	No. of strains with positive detection
Aminoglycosides		
TOB^r	*ant(4′)-Ia*	55
TOB^r–GEN^r	*aac(6′)-Ie + aph(2′)*	18
KAN^r	*aph(3′)-IIIa*	7
Macrolides		
ERY^r–CLI^r	*ermA*	13
	ermC	24
	ermA and *ermC*	7
	ermB and *ermA*	3
ERY^r	*msrA/B*	32
CLI^r	*linA/A′*	2
Quinolones		
CIP^r	*grlA* (Ser-80 to Phe)	1
	grlA (Ser-80 to Phe)/*gyrA* (Ser-84 to Leu)	37
	grlA (Ser-80 to Tyr)/*gyrA* (Ser-84 to Leu)	2

TOB, tobramycin; GEN, gentamicin; KAN, kanamycin; ERY, erythromycin; CLI, clindamycin; CIP, ciprofloxacin.

Table 3. Association between five molecular markers (CCs/MLST lineages, SCC*mec*, *spa*-types/CCs, PFGE profiles and *agr* groups) in 90 invasive MRSA isolates (Spain 2006–07)

Presumptive, CCs/MLST lineages[a]	No. of strains	SCC*mec* (no. of strains)	*spa*-type/CC	PFGE (no. of strains)	*agr* (no. of strains)
CC5	40	IV (40)	t067/CC002	E7 (15), D[b] (9), E8 (6), E20 (6), E11 (4)	II (39), I (1)
	16	IV (15), I (1)	t002/CC002	E8 (7), E7 (5), D (2), E20 (1), E18 (1)	II (16)
	1	IV (1)	t1154/CC1154	E8 (1)	II (1)
	1	IV (1)	t2220/CC002	E8 (1)	II (1)
	1	IV (1)	t2226/CC002	E7 (1)	II (1)
	1	IV (1)	t2366/CC002	E8 (1)	II (1)
	1	IV (1)	t837/CC1154	E7 (1)	II (1)
	2	II (2)	t003/CC1154	D (2)	II (2)
	1	IV (1)	t062/CC1154	E3 (1)	II (1)
	1	IV (1)	t088/CC002	E10 (1)	II (1)
	4	IV (4)	t067/CC002	E3 (4)	II (4)
	4	I (4)	t109/CC109	E18 (4)	II (4)
	3	I (3)	t2222/CC109	E18 (3)	II (3)
	1	I (1)	t041/CC109	D (1)	II (1)
CC8	6[c]	IV (6)	t008/singleton	D (4), E19 (2)	I (5), II (1)
	1	IV (1)	t148/singleton	D (1)	II (1)
CC30	1	IV (1)	t012/no founder	D (1)	III (1)
	4	II (4)	t018/no founder	E12 (3), E13 (1)	III (4)
CC22	1	IV (1)	t032/singleton	D (1)	I (1)

[a]STs were determined in 9 selected isolates; in the remaining 81 isolates, STs and presumptive CCs were attributed according to Simor *et al.*[39], Wisplinghoff *et al.* [40] and the Ridom StaphType™ database (http://spa.ridom.de/index.shtml).
[b]D, sporadic PFGE profile.
[c]One isolate is PVL-MRSA, ST8, t008 and has a sporadic PFGE profile.

detected in 18 hospitals (85.7%) with both MRSA and MSSA variants found in 6.

The 90 MRSA isolates were grouped into four clusters and two singletons consisting of: cluster MRSA-1, *spa*-CC002 (64 isolates, 71.1%) with 6 *spa*-types and t067 as the predominant *spa*-type (44 isolates, 68.7% of all *spa*-CC002 isolates and 48.8% of all MRSA isolates); cluster MRSA-2, *spa*-CC1154 (4 *spa*-types, 5 isolates, 5.6%); cluster MRSA-3, *spa*-CC109 (3 *spa*-types, 8 isolates, 8.9%); and cluster MRSA-4, with no founder identified (2 *spa*-types, 5 isolates, 5.5%) (Table 3).

The 111 MSSA isolates were grouped into 7 clusters and 14 singletons consisting of: cluster MSSA-1, *spa*-CC012 (7 *spa*-types, 21 isolates, 18.9%); MSSA-2, *spa*-CC084 (7 *spa*-types, 12 isolates, 10.8%); MSSA-3, *spa*-CC002 (5 *spa*-types, 23 isolates, 20.7%); MSSA-4, *spa*-CC078 (4 *spa*-types, 6 isolates, 5.4%); MSSA-5, *spa*-CC116 (4 *spa*-types, 8 isolates, 7.2%); MSSA-6, with no founder identified (2 *spa*-types, 2 isolates, 1.8%); MSSA-7, with no founder identified (2 *spa*-types, 3 isolates, 2.7%); and 14 singletons (30 isolates, 27%). Six MSSA had less than five repeats and were excluded from the analysis.

Six *spa*-types common to MSSA and MRSA were detected. Two of them (t002 and t067) belonged to *spa*-CC002 and were distributed as follows: 12 MSSA and 16 MRSA isolates were *spa*-type t002; and 8 MSSA and 45 MRSA isolates were *spa*-type t067. Other common *spa*-types detected were: t012, found in 11 MSSA and in 1 MRSA; t018, found in 1 MSSA and in 3 MRSA; t008, found in 4 MSSA and in 6 MRSA; and t148, found in 4 MSSA and in 1 MRSA.

Presumptive CCs/MLST lineages in all MRSA and in 72 MSSA were assigned according to published data[39,40] and the data available at the Ridom StaphType™ database (http://spa.ridom.de/index.shtml). We found 4 clonal complexes in MRSA (CC5, CC8, CC30 and CC22; Table 3), and 11 clonal complexes in MSSA (CC5, CC8, CC30, CC45, CC15, CC25, CC1, CC7, CC12, CC22 and CC121; Table 4).

The ciprofloxacin/tobramycin/erythromycin resistance pattern was detected in 17 of 44 (38.6%) t067 MRSA and in 2 (4.3%) MRSA isolates of the remaining 46 isolates that were not t067 ($P < 0.001$; OR: 13.8, 95% CI: 2.9–64.7).

Among MRSA isolates, tobramycin resistance and gentamicin susceptibility [*ant(4′)-Ia* associated] was more prevalent in t067 isolates (33 of 44, 75%) than in isolates of other *spa*-types (22 of 46, 47.8%) ($P = 0.008$). Also, the erythromycin-resistant but clindamycin-susceptible pattern (linked to the efflux pump genes *msrA* or *msrB*) was more prevalent in *spa*-type t067 isolates (24 of 44, 54.5%) than in isolates with other *spa*-types (4 of 46, 8.7%) ($P < 0.0001$).

The *ant(4′)-Ia* gene and the efflux pump genes *msrA* or *msrB* were detected in 18 of 44 (40.9%) t067 MRSA and in 2 (4.3%) of the remaining 46 MRSA isolates that were not t067 ($P < 0.001$; OR: 15.2, 95% CI: 3.3–71).

In MRSA, 37 of the 39 (94.9%) isolates of the PFGE clonal groups E7 and E8 belonged to *spa*-CC002 (Table 3). In MSSA strains, 17 of the 26 (65.4%) isolates of the PFGE cluster C2 were *spa*-CC012, 8 of the 13 isolates (61.5%) of the PFGE cluster C3 were *spa*-CC116, 13 of the 17 isolates (76.5%) of the PFGE cluster C5 were *spa* CC002, and, finally, 10 of the 17 isolates (58.8%) of the PFGE cluster C6 were *spa*-CC084. In 72 MSSA strains, it was possible to assign a presumptive CCs/MLST lineage; the molecular markers of these strains, including CCs/lineages, STs, *spa*-types, PFGE profiles and *agr* groups, are depicted in Table 4.

According to *spa*-typing data available at the Ridom StaphType™ database (http://spa.ridom.de/index.shtml; 19 July 2007, date last accessed), the most prevalent *spa*-types among 36 571 isolates studied worldwide were t003 (15.2%) and t032 (10.3%), but these two *spa*-types were very infrequent in our study (three isolates in total, 1.5%). In contrast, t002 and t067 *spa*-types were much more prevalent in our isolates as 28 of them (13.9%) were *spa*-type t002 in comparison with 1865 (5.1%) in the Ridom StaphType™ database (OR 2.03, 95% CI: 1.28–2.23; $P = 0.0037$); also, 52 of our isolates (25.9%) were *spa*-type t067 in comparison with 215 (0.6%) in the Ridom StaphType™ database (OR 58.2, 95% CI: 25.7–131.5; $P < 0.0001$).

PVL genes

PVL genes were detected in 42 (20.7%) isolates, 41 of them MSSA (36.3% of all MSSA) and 1 MRSA (1.1%) (Table 3). Eighteen of the 21 (85.7%) participating hospitals had at least one PVL-SA.

The unique PVL-positive MRSA strain (PVL-MRSA) (isolated from a 55-year-old outpatient) was resistant to oxacillin only, was ST8, carried SCC*mec* type IVa and belonged to *agr* group I and *spa*-type t008. This strain is similar to the CA-MRSA strain USA300 (ST8-MRSA IV) and had the same PFGE profile as CA-MRSA A1 described in Spain by Cercenado *et al.*[41]

Twenty (48.8%) of the PVL-positive MSSA (PVL-MSSA) isolates were considered to have arisen from community acquisition, while 15 (36.6%) were implicated in nosocomial infections (in 6 isolates, this information was not available).

The most prevalent *agr* types among PVL-MSSA were types II (20.4%) and I (13.3%). The most prevalent PFGE profiles among PVL-MSSA isolates were C5 (11 of 17; 64.7%), C6 (7 of 17; 41.2%) and C9 (5 of 6; 83.3%); cluster C2 had just one PVL-positive isolate of the 26 included in this cluster; and clusters C1 (5 isolates) and C3 (13 isolates) had no PVL-MSSA strains.

Of the 41 PVL-MSSA, 13 (31.7%) belonged to *spa*-CC002 (t002, 7 isolates; t067, 4 isolates; and t311 and t2225, one isolate each); 11 (26.8%) were singletons; 6 (14.6%) were *spa*-CC084 (t084, 3 isolates; t346, 2 isolates; and t2055, one isolate); and 4 (9.8%) were *spa*-CC078 (t078, 2 isolates; t258 and t1661, one isolate each). For the remaining PVL-MSSA, in three isolates (7.3%) the *spa* sequences were excluded because they were shorter than five repeats, two isolates (4.9%) had no founder, and in two isolates (4.9%) no *spa* sequences were amplified. The association between the molecular markers of invasive PVL-MSSA and presumptive CCs/MLST lineages is detailed in Table 4.

MLST

STs were determined in 52 isolates; 19 of them belonged to *agr* type II and *spa*-CC002 (t067 and t002): 12 were MSSA (9 of them PVL-MSSA) and 7 were MRSA. The remaining 33 (32 MSSA and 1 MRSA) isolates were PVL-SA. All PVL-SA isolates were typed by MLST.

The distribution of molecular markers in invasive *S. aureus* belonging to *agr*II and *spa*-CC002 is shown in Table 5. Of the

Table 4. Association between four molecular markers (CCs/MLST lineages, *spa*-types/CCs, PFGE profiles and *agr* groups) in 72 invasive MSSA isolates (Spain 2006–07)

Presumptive CCs/MLST lineages[a]	No. of strains	*spa*-type/CC	PFGE (no. of strains)	*agr*	No. of PVL-MSSA
CC5	12	t002/CC002	C5 (10), C6 (2)	II	7
	1	t2225/CC002	C5 (1)	II	1
	1	t311/CC002	C6 (1)	II	1
	1	t653/singleton	C5 (1)	II	1
	1	ND[b]	C5 (1)	I	1
	2	t067/CC002	C5 (1), NG[c] (1)	II	2
CC8	4	t008/singleton	C8 (2), C9 (2)	I	3
	1	t1892/singleton	C9 (1)	I	1
CC30	11	t012/CC012	C2 (9), C1 (2)	II	—
	4	t018/CC012	C2 (3), C1 (1)	II	—
CC45	5	t015/CC116	C3 (5)	I	—
	2	t230/no founder	C3 (2)	I	—
	1	t026/excluded[d]	C3 (1)	I	—
CC15	1	t067/CC002	C7 (1)	II	1
	1	t085/CC084	C6 (1)	II	—
	1	t2055/CC084	C6 (1)	II	1
	2	t346/CC084	C6 (2)	II	2
	1	t605/excluded	C6 (1)	II	1
	4	t084/CC084	C6 (3), C2 (1)	II	3
CC25	1	t067/CC002	C8 (1)	IV	1
	1	t227/excluded	NG (1)	I	1
	1	t643/excluded	C4 (1)	I	1
	1	ND	C4 (1)	I	1
	1	t258/CC078	C4 (1)	I	1
	2	t078/CC078	C4 (2)	I	2
CC1	3	t127/singleton	C7 (2), C8 (1)	III	1
	1	t2219/singleton	NG (1)	II	1
CC7	1	t091/singleton	C7 (1)	I	1
	1	t1051/CC012	C2 (1)	III	—
CC12	1	t160/singleton	C8 (1)	IV	1
CC22	1	t005/singleton	NG (1)	I	—
CC121	1	t272/no founder	C10 (1)	II	1
	1	t645/no founder	C10 (1)	II	1

[a]STs were determined in 41 selected isolates; in the remaining 31, STs and presumptive CCs were attributed according to the Ridom StaphType™ database (http://spa.ridom.de/index.shtml).
[b]ND, not determined.
[c]NG, strains not grouped in the main 10 clusters.
[d]*spa*-types shorter than five repeats excluded in the BURP analysis.

7 MRSA isolates, 5 (71.4%) were ST125 and 2 (28.6%) were ST5; of the 12 MSSA isolates, 1 PVL-SA isolate was ST125 and 11 (8 of them PVL-SA) were ST5 (Table 5).

The molecular features of the PVL-MSSA isolates are shown in Table 4. The most common MLST types found were ST5 (13 isolates), ST15 (8 isolates) and ST25 (4 isolates). Of the 13 isolates belonging to *spa*-CC002, 7 shared all the molecular markers studied. The six isolates of *spa*-CC084 were ST15 (Table 4). The unique PVL-MRSA strain was ST8.

Discussion

In this study, 203 blood *S. aureus* isolates (90 MRSA and 113 MSSA) from 21 Spanish hospitals were typed using several molecular methods. After analysis of the data, several findings were found to be of clinical and epidemiological interest. First, *spa*-type t067 (ST5-MRSA-IV) was dominant in the Spanish population, a situation not described in other countries. Second, in MRSA isolates, *spa*-type t067 was strongly associated with multiresistance to ciprofloxacin, tobramycin and erythromycin and to the expression of the enzyme gene *ant(4′)-Ia* and the efflux pump genes *msrA/msrB*. Third, the prevalence of PVL-MSSA was unexpectedly high. Fourth, an important proportion of MRSA and MSSA blood isolates had similar epidemiological markers in common, including *agr*, *spa* and MLST, suggesting that they may share common epidemic clones.

The resistance phenotypes encountered were heterogeneous (17 patterns), with a high percentage (35.6%) of MRSA isolates presenting resistance to only one or two antibiotics besides

Table 5. Distribution of molecular markers in invasive *S. aureus* belonging to *agr*II and *spa*-CC002 (Spain 2006–07)

MRSA ($n = 76$)		MSSA ($n = 40$)	
molecular marker pattern[a]	no. (%)	molecular marker pattern[a]	no. (%)
II/E7/t067/ST125 (1)	15 (19.8)	II/C5/t002/ST5 (9)	10 (25.0)
II/E8/t067/ST125 (1)	7 (9.2)	II/C6/t002/ST5 (1)	2 (5.0)
II/E20/t067/ST125 (2)	7 (9.2)	II/C5/t067/ST5 (1)	1 (2.5)
II/E8/t002/ST5 (1)	7 (9.2)	II/not grouped/t067/ST125 (1)	1 (2.5)
II/E7/t002/ST5 (1)	6 (7.9)		
II/E11/t067/ST125 (1)	4 (5.2)		
Total	46 (60.5)		14 (35.0)

[a]*agr*/PFGE type/*spa* type/MLST type (no. studied by MLST).

oxacillin. This finding is of clinical relevance. Most MRSA isolates exhibit multiresistance phenotypes,[42] but a narrowing of resistance patterns in invasive and epidemic MRSA strains has been reported.[43,44] This reduction may be the result of the emergence and dissemination of the SCC*mec* type IV cassette, which was also the most frequent in this study (85.5%), and in other previous studies.[22,23] A decrease in MRSA isolates harbouring SCC*mec* type I, classically associated with most extensive multiresistance patterns, has been noticed in other European countries.[45,46]

In this study, the great majority of the MRSA isolates (84.4%) belonged to *agr* type II. In MSSA isolates, we detected similar percentages of *agr* types I, II or III, although 35.4% of them were also type II. These results differ from those obtained by Hallin *et al.*[47] in 614 Belgian isolates from all body sites (8% blood isolates) in which *agr* type I was the most frequent among both MSSA and MRSA. In Spain, the high number of MRSA strains with *agr* type II could be due to the replacement of the Iberian PFGE-clone (*agr* type I, SCC*mec* I) by E7 and E8 PFGE clonal groups (*agr* type II, SCC*mec* IV).

In this study, we have detected a high prevalence of *spa*-CC002 (CC5/SCC*mec*IV) in MRSA isolates, mainly t067 (50% of all MRSA isolates), that was distributed in almost all participant hospitals. However, according to the Ridom StaphType™ database, the geographical distribution of the t067 *spa*-type is very limited and mainly reported in northern European countries such as Sweden, Norway, Denmark and Germany. The second most prevalent MRSA *spa*-type in our study was t002 (13.9%), which has been described in Germany,[36] Austria[48] and the USA.[49]

The predominant MRSA *spa*-type t067 was gentamicin-susceptible and presented the *ant4′* gene that confers resistance to kanamycin, tobramycin and amikacin. The spread of this clone may help to explain why gentamicin resistance has decreased in recent years in Spain.[23,44]

Recently, an increase in the number of circulating MRSA clones has been described,[50] in comparison with the few epidemic strains described previously.[51] The predominant MRSA clonal groups found in this study were ST5/125-MRSA IV which belonged to the PFGE clonal groups (E7, E8 and the closely related E20). These strains were isolated from blood infections and are very similar to the MRSA epidemic strains implicated in other clinical scenarios in this country.[23] A high correlation between *spa*-CC002 (*spa*-types t067 and t002) and the previously described E7, E8 and E20 PFGE clonal groups[23] has been observed in this study, as all isolates belonging to these PFGE clonal groups except two were *spa*-CC002; the two remaining isolates were *spa*-CC1154, closely related to *spa*-CC002 (http://spa.ridom.de/index.shtml).

In accordance with previous studies,[22,23] we have shown that the E7, E8 and E20 PFGE clonal groups belonged to ST125 and harboured SCC*mec* IV; in addition, these same isolates mainly belonged to *spa*-type t067 (Table 3). This association between t067 and ST125 is described here for the first time, to our knowledge. The geographical distribution of *spa*-type t067 has not been reported previously. So far, ST125 has been detected in Spain (Tenerife, Canary Islands), Norway and Finland.[23,52–54] Our data suggest that an endemic circulating clone is present among MRSA isolates in Spain characterized by being *agr* type II, very similar to the PFGE profile, ST125 and *spa*-type t067.

The t002 *spa*-type has been associated with ST5, closely related to ST125, and ST231 (http://spa.ridom.de/index.shtml). ST5 and ST125 are highly related as ST125 differs from ST5 in just one nucleotide in the amplified region of the *yqiL* gene, while the remaining six MLST alleles that define the ST are identical. We found that *spa*-type t002 and ST5 were associated in both MRSA and MSSA isolates (Tables 3 and 4). We also found that 14 MSSA and 46 MRSA isolates shared the same *agr* type II and the same CC002; since they also had the same MLST type ST5 or the closely related ST125, this may suggest that both MRSA and MSSA invasive isolates in Spain have common circulating clones. Moreover, nine MSSA isolates of this clone were PVL-MSSA, indicating that the eventual acquisition of the *mecA* gene by this clone could increase the PVL-positive prevalence in MRSA isolates.

Issartel *et al.*[55] found that the most frequent discrepancy observed between PFGE and *spa*-typing methods was mainly due to MRSA isolates characterized as sporadics by PFGE but that belonged to the same *spa*-types, *spa*-CC008 and *spa*-CC002. This could reflect genetic events such as the insertion/deletion of different mobile genetic elements that may be involved in the epidemic behaviour of *S. aureus* leading to different PFGE patterns, while *spa* and MLST types remain indistinguishable.[55]

A remarkable finding from this study was that 36.4% of the MSSA isolates studied had PVL genes; since in a recent study in Spain[56] this percentage was 1.6%, it seems that the prevalence of PVL-SA isolates is rapidly progressing in this country. In Argentina, 19 of 31 MSSA isolates (61.3%) were PVL-positive.[57] Our PVL-MSSA isolates were distributed among different PFGE clusters, *agr* types, *spa*-types and MLST types, although most of them were associated with the prevalent *spa*-CC002 and the CC5/MLST lineage. Due to the predominance of CC5 and *spa*-CC002 among Spanish blood isolates, principally in MRSA, the potential increase in PVL prevalence in both MSSA and MRSA is a subject of concern.

The classical definition of CA-MRSA infection used in this and other studies needs a revision on the basis of the data provided by molecular markers. Morin and Hadler[58] proposed a new category called 'healthcare associated' for a better epidemiological interpretation. In our study, only one isolate of the 90 MRSA studied had the typical characteristics of CA-MRSA (PVL-positive, single oxacillin resistance, SCC*mec* type IV, *agr* group I and a PFGE profile closely related to CA-MRSA type A1[41]), but up to 21 (23.3%) may have been considered community-acquired according to the classical definition.

In summary, we carried out a multicentre study aimed at the molecular characterization of *S. aureus* causing invasive infections; 203 blood isolates (90 MRSA and 113 MSSA) from 21 Spanish hospitals were typed using several molecular methods. We found that most MRSA isolates were of *agr* type II and harboured SCC*mec* type IV; also, *spa*-type t067 was highly predominant in this bacterial population. A high prevalence (36.4%) of PVL-MSSA was detected. Some MRSA and MSSA blood isolates of *agr* type II also shared other molecular markers, including *spa*-CC002, CC5/MLST 5 and its closely related ST125. Moreover, in MRSA isolates, *spa*-type t067 was found to be associated with simultaneous resistance to ciprofloxacin, tobramycin and erythromycin, and to the detection of the enzyme gene *ant(4′)-Ia* and the efflux pump genes *msrA/msrB*.

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None to declare.

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