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RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2014) 17:149-157
doi:10.2436/20.1501.01.217. ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in a university hospital in northwestern Spain

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Received 10 April 2014 · Accepted 30 September 2014

Summary. Continuous monitoring of methicillin-resistant *Staphylococcus aureus* (MRSA) is necessary to understand the clonal evolution of successful lineages. In this study, we identified the MRSA clones circulating in a Spanish hospital during a 2-year period, assessed their relationship with antimicrobial resistance profiles, and investigated the presence of the emerging community-associated and livestock-associated MRSA lineages (CA-MRSA, LA-MRSA). CC5-MRSA-IV isolates were the most frequently recovered, which supports the previously reported prevalence of this clone in Spanish hospitals. We observed ST125 isolates that harbored specific cassette chromosome recombinase (*ccr*) gene elements of the staphylococcal cassette chromosome *mec* (SCC*mec*) types IV and VI. That clone, which was first detected only recently, has increased resistance to erythromycin. Furthermore, 94% of the infections were caused by non-multiresistant isolates. Neither CA-MRSA nor LA-MRSA isolates were observed. These findings, along with related events over the last decade, suggest the establishment of a clonal endemic population in the Spanish clinical environment. [**Int Microbiol** 2014; 17(3):149-157]

Keywords: methicillin-resistant *Staphylococcus aureus* (MRSA) · clonal population · molecular epidemiology · multilocus sequence typing

Introduction

Staphylococcus aureus is an opportunistic pathogen that causes a wide range of diseases in humans, from mild skin infections to life-threatening diseases such as toxic shock syndrome, septicemia, endocarditis, and necrotizing pneumonia. Methicillin-resistant *Staphylococcus aureus* (MRSA) was first described in 1960, a year after the introduction of methicillin into clinical practice to treat infections caused by penicillin-resistant strains of *S. aureus* [11]. Methicillin resistance is mediated by the presence of the *mecA* gene, which encodes an additional penicillin-binding protein (PBP2a or PBP2′) with low affinity for β-lactam antibiotics [15]. More recently, a novel methicillin-resistance determinant, $mecA_{LGA251}$ or *mecC*, has been described that has 70% homology at the DNA level with *mecA* [9]. Those genes are harbored in a highly diverse mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), whose transmission to ecologically successful methicillin-susceptible *S. aureus* strains (MSSA) led to the emergence of the ancestral MRSA lineages [12]. Up to 11 SCC*mec* types, defined by combining the ge-

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netic structures of the cassette chromosome recombinase (*ccr*) gene complex and the *mec* gene complex, have been described [www.sccmec.org/Pages/SCC_TypesEN.html]. Thus, MRSA isolates are represented not only by their genetic backgrounds, but also by the genetic structure of their SCC*mec* elements [8].

MRSA lineages have become a truly global health concern over the last few decades, as their worldwide spread has occurred very quickly. The prevalence of MRSA greatly varies among countries, provinces and hospitals but it has increased significantly since its appearance in 1960. In the USA, MRSA infections increased from 4% in the 1980s to 50% of all *S. aureus* infections in the late 1990s [24]. In Spain, MRSA infections increased from 1.5% in 1986 to 31.2% in 2002 [34]. In the worst cases, such as in Taiwan, in 2007 MRSA accounted for 80% of all *S. aureus* isolates causing nosocomial infections [36]. However, the scenario has become even more complex. Until the mid 1990s the presence of MRSA was restricted to the clinical environment (hospital-acquired MRSA, HA-MRSA), but since then MRSA strains have been recovered increasingly from the community (community-acquired MRSA, CA-MRSA) [33]. In contrast to HA-MRSA, CA-MRSA strains are frequently isolated from children and young people without previous health-care contact and they show specific genetic and phenotypic traits: they have a different genetic background, harbor a smaller SCC*mec* type IV or V, which provides them with a selective advantage in terms of faster replication times, present more virulence mediated by factors such as the Panton-Valentine leukocidin cytotoxin (PVL), and usually have a non-multiresistant antimicrobial profile [26].

The recurrent penetration of CA-MRSA into the clinical environment has blurred the boundary between HA-MRSA and CA-MRSA, making it more difficult to discriminate between them. In addition, a livestock-associated MRSA (LA-MRSA) has been described. LA-MRSA strains may act as genetic reservoirs of resistance and could play a major role in the adaptive evolution of MRSA. In fact, the recently described methicillin resistance gene *mecC* was originally detected in LA-MRSA strains but is now increasingly recovered from clinical settings [9,27]. Continuous molecular epidemiologic surveillance of MRSA is imperative to trace the evolution and spread of successful MRSA clones, both for therapeutic reasons and for the implementation of initiatives aimed at controlling and preventing MRSA infections. Here we describe a molecular epidemiology study of MRSA isolates recovered from the University Hospital of Leon from 2007 to 2008. The genetic backgrounds of these isolates were characterized by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), and the structures of the respective SCC*mec* elements were determined. Furthermore, we investigated both the antimicrobial resistance profiles of CA-MRSA and the presence of their PVL genes, as markers. By identifiying the MRSA clones circulating in the hospital, this study contributes to the knowledge of the MRSA lineages circulating in Spanish health-care setting.

Materials and methods

Clinical information and bacterial isolates. Seventy *Staphylococcus aureus* isolates recovered from patients at the University Hospital of Leon (Leon, northwestrn Spain), between 2007 and 2008 were determined to be MRSA based on antimicrobial susceptibility testing. The University Hospital of Leon is a 795-bed public teaching hospital. Forty-nine isolates were recovered from blood cultures (one of them was recovered also from bile); 14 from central venous catheter blood samples; 3 from peripheral vascular catheter blood samples; 1 from packed red blood cells; 1 from a drainage sample; 1 from synovial joint fluid; and 1 from ascites. The specimens were isolated and identified in conventional medium (mannitol salt agar, blood and chocolate agar plates) and commercial MicroScan microdilution panels (Siemens Diagnostics, Munich, Germany) and by real-time PCR [32] in a 7500 real-time PCR system platform (Applied Biosystems, Carlsbad, CA, USA). Methicillin resistance was confirmed in 68 of the 70 isolates by using internal amplification controls designed within the *mecA* gene [13,22]. Isolates SA13 and SA65, which were MRSA as revealed by antimicrobial susceptibility testing, tested negative for the presence of *mecA*. Repetition of the susceptibility tests confirmed both isolates as MSSA.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed by the microdilution method following the recommendations and minimal inhibitory concentration (MIC) breakpoints of the Clinical and Laboratory Standars Institute (CLSI) guidelines (2008). Susceptibility to the following 15 antimicrobial agents was tested: ampicillin, penicillin, oxacillin, amoxicillin/clavulanate, cefotaxime, erythromycin, clindamycin, teicoplanin, vancomycin, ciprofloxacin, imipenem, gentamicin, rifampicin, tetracycline, and cotrimoxazole. MRSA isolates were clustered in resistance profiles (RPs) according to their susceptibility to 10 antimicrobials: ampicillin, penicillin, erythromycin, teicoplanin, vancomycin, ciprofloxacin, imipenem, rifampicin, tetracycline, and cotrimoxazole. Isolates resistant to three or more antibiotics, in addition to β-lactams, were considered to be multiresistant.

Characterization of the genetic background. Genetic characterization of all *S. aureus* isolates was carried out by pulse-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). PFGE was performed following the method McDougal et al. [16] modified as follows: bacterial cells were suspended in PIV buffer (10 mM Tris-HCl, 1 M NaCl, pH 8) to an absorbance of 0.9–1.1 at 610 nm. Then, 200 µl of the adjusted culture was washed and resuspended in 300 µl of the same buffer. After the lysis step, the plugs were transferred into a new tube containing 1 ml ESP buffer (1% N-lauroyl sarcosine in 0.5 M EDTA pH 8, 100 µg proteinase K/ml) and incubated at 56ºC for 16–20 h. DNA fragment sizes were determined by comparing bands with a Lambda Ladder PFG Marker (New England Biolabs). PFGE profiles were analyzed using Bionumerics v.6.6 (Applied-Maths NV, Sint-Martens-Latem, Belgium) to describe genetic relationships among isolates.

Dendograms were constructed using the Dice similarity coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm. MLST was carried out as previously described [8]. DNA isolation was performed using the commercial kit QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. To guarantee analysis of identical genetic material by PFGE and MLST, DNA from the same culture was used for both methods. Allelic profiles obtained by MLST were assigned by comparing the consensus sequences with the information already published in the *S. aureus* MLST database hosted at [http://saureus.mlst.net/]. Thereby, genotypes were constituted by seven numbers representing the unique allelic profiles at seven housekeeping genes. Data regarding the 70 isolates analyzed, as well as the novel allelic profiles were submitted to the MLST database. Allelic profiles were used to compare the *S. aureus* isolates recovered from our hospital with those previously analyzed from other geographic areas. Simpson's index of diversity, which measures the probability that two unrelated strains sampled from the test population will be placed into different typing groups [10], was calculated to compare the discriminative power of PFGE typing versus MLST and for measuring genetic diversity among isolates using the comparing partitions website [http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Home]. The adjusted Wallace coefficient for quantification of the agreement between PFGE typing and MLST was calculated on that website.

Typing and subtyping of the SCC*mec* element. The genetic structure of the SCC*mec* element was determined by multiplex-PCR carried out on a Veriti 96 Well thermal cycler and GeneAmp PCR system 9700 (Applied Biosystems) as described elsewhere [13,22]. These two molecular methods allowed discrimination of SCC*mec* types I, II, III, IV, V and VI, as well as the variants IA & IIIA. SCC*mec* IV was further subtyped into eight different subtypes, from IVa to IVh, by multiplex-PCR as previously described [18].

Detection of Panton-Valentine leukocidin virulence factors. The presence of the PVL genes (*luk*S-PV & *luk*F-PV) was investigated by conventional PCR as previously described [14]. Reference strain ATCC 49775 was used as the positive control.

Results

Antimicrobial susceptibility testing. All 68 MRSA isolates were resistant to the β-lactam antibiotics tested (oxacillin, ampicillin, penicillin, cefotaxime and imipenem) as well as to amoxicillin/clavulanate and ciprofloxacin. Furthermore, 42.6% of the isolates were resistant to erythromycin, 21% were resistant to clindamycin, 15.9% were resistant to gentamicin, 4.4% to rifampicin and 1.5% to both tetracycline and cotrimoxazole. Of the isolates resistant to erythromycin, 34.8% had the M phenotype. All isolates were susceptible to the glycopeptide antimicrobial agents vancomycin and teicoplanin. The isolates were clustered into five RPs, with all the isolates resistant to the β-lactam antibiotics tested and to at least one more antimicrobial agent (Table 1). Notably, the percentage of MRSA resistant to gentamicin increased from 5.7% in 2007 to 28.6% in 2008. Resistance to erythromycin, rifampicin, tetracycline, and cotrimoxazole also increased during the study period albeit to a lesser extent, while resistance to clindamycin decreased from 23.5% in 2007 to 17.9% in 2008 (Table 2).

Genetic background. Genetic characterization of all 70 *S. aureus* isolates by PFGE with the restriction enzyme *Sma*I revealed 27 different pulsotypes (25 pulsotypes among the 68 MRSA isolates), resulting in a Simpson's index of diversity of 0.909, which indicated that, if two isolates were sampled randomly from the population, on 90.9% of the occasions they would fall into different types. Genotypes 19 and 26 accounted for 40% of the isolates (17.1% and 22.9%, respectively). A dendrogram constructed using the Dice similarity coefficient with the UPGMA clustering algorithm revealed four major clusters (arbitrarily designated from A to D) with a cutoff of 90% similarity. More than 81% of the isolates were grouped into these four genetic clusters. Genotypes that did not belong to these clusters were designed as "sporadic." Cluster A comprised 18 ST5-MRSA-IV isolates, two novel sequence types (STs) derived from ST-5, and one ST125-MRSA-IV. Cluster B comprised ten ST125-MRSA-IV and one ST5-MRSA-IV.

Table 1. Resistance profiles of 68 methicillin-resistant *Staphylococcus aureus* isolates at the University Hospital of Leon, 2007 and 2008

Resistance profile	Antimicrobial agent[a]	% MRSA clones	
RP0	Only to β-lactams	0	None
RP1	CIP	55.2	ST5-IV(10); ST8-IV(14); ST125-IV(12); ST125-IV/VI(2)
RP2	ERY, CIP	38.8	ST5-IV(10); ST8-IV(1); ST125-IV(4); ST125-IV/VI(8); ST228-I(1); ST2755-IV(1); ST2756-IV(1)
RP3	ERY, CIP, RIF	3	ST5-IV(2)
RP4	ERY, CIP, COT	1.5	ST8-IV(1)
RP5	CIP, RIF, TET	1.5	ST5-IV(1)

[a]All isolates were resistant to the β-lactam antibiotics tested: oxacillin, ampicillin, penicillin, cefotaxime and imipenem). CIP, ciprofloxacin; ERY, erythromycin; RIF, rifampicin; COT, cotrimoxazole; TET, tetracycline.

Table 2. Evolution of the resistance to antimicrobials by 68 methicillin-resistant *Staphylococcus aureus* isolates, University Hospital of Leon, 2007 and 2008

Antibiotics[a]	2007[b]	2008[b]	Δ(2008–2007)[c]
Erythromycin	41.7	43.8	+2.1
Clindamicyn	23.5	17.9	–5.6
Gentamycin	5.7	28.6	+22.9
Rifampicin	2.8	6.3	+3.5
Tetracicline	0	3.1	+3.1
Cotrimoxazole	0	3.1	+3.1

[a]All MRSA isolates were resistant to the β-lactam antibiotics tested: oxacillin, ampicillin, penicillin, cefotaxime, and imipenem. They were also resistant to amoxicillin/clavulanate and ciprofloxacin, and were susceptible to vancomycin and teicoplanin.
[b]Percentage of the mean values of antibiotic resistance.
[c]Differences between the percentages of the mean values of antibiotic resistance in 2007 and 2008.

Cluster C grouped nine ST125-MRSA-IV/VI isolates, and cluster D all sixteen ST8-MRSA-IV isolates (Fig. 1).

Overall, among the 68 MRSA isolates, six STs were determined by MLST analysis. Three of the six accounted for 95.6% of the isolates: ST-125 (38.2%), ST-5 (33.8%), and ST-8 (23.5 %). ST-228 and two novel STs were also detected, in one isolate each. Detected clones belonged to the clonal complexes CC5 (76.5 %) and CC8 (23.5 %). After submission to the *S. aureus* MLST database [http://saureus.mlst.net/], novel STs were designated as ST-2755 and ST-2756 (allelic profiles 314–4–1–4–12–1–10 and 1–4–1–4–12–1–320 at *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*, respectively). The novel STs were very similar to ST-5: ST-2755 had a unique difference of a single nucleotide polymorphism (SNP) in nucleotide 39 of *arcC*, showing 99% similarity with *arcC* allele 1. However, this difference at the DNA level was not translated into the functional level since both codons encoded the amino acid threonine. In addition, ST-2756 had a SNP in nucleotide 256 of allele 10 of *yqiL* (99% similarity), where thymine is replaced by guanine. This change leads to a non synonymous substitution in which cysteine is substituted by glycine in the encoded protein. MSSA isolates SA 13 and SA 65 belonged to ST-15 and ST-5, respectively. In 2007, the most frequently recovered isolates belonged to ST-5 and ST-125 (38.9% each one), followed by ST-8 (19.4%). ST-2756 was recovered just one isolate. In 2008, isolates showing ST-125 were the most frequently isolated (37.5%), followed by those of ST-5 and ST-8 (28.1% each). ST-228 and ST-2755 were also sporadically observed in one isolate.

Genetic structure of the SCC*mec* element. The genetic structure of the SCC*mec* element was investigated in all *S. aureus* isolates. During the 2-year study period, 67 out of 68 MRSA isolates (98.5%) harbored SCC*mec* type IV; the remaining isolate (SA16) harbored SCC*mec* type I (1.5%). Further subtyping of SCC*mec* IV revealed that all belonged to subtypes IVc/IVe. Note that, 38.5% of the ST125-MRSA-IV isolates (14.9% of all isolates harboring SCCmec IV) also had specific *ccr* gene elements of SCC*mec* type VI (*mecA* gene complex class B and *ccr* gene complexes type 2 and 4; A2B2 and A4B4, respectively) and consequently were designated as ST125-MRSA-IV/VI. Furthermore, 37 out of the 67 (55.2%) SCC*mec* type IV elements contained an additional 381-bp DNA fragment according to the system previously described by Oliveira and Lencastre [22], corresponding to the presence of the kanamycin/neomycin/bleomycin resistance plasmid pUB110. The evolution of the MRSA clones circulating at our hospital over the 2-year study period are shown in Fig. 2.

Congruence between PFGE-typing and MLST. PFGE better discriminated among the 70 *S. aureus* isolates than MLST (Simpson's index of diversity of 0.909 and. 0.701, respectively). The adjusted Wallace coefficient PFGE→MLVA was 0.883, with a 95% confidence interval (CI) of 0.765–1.000; the coefficient for MLVA→PFGE was 0.208 (CI 95%; 0.137–0.279). These values implied that if two isolates were in the same pulsotype, they had an 88.3% chance of having the same MLST genotype; conversely, sharing the same MLST type was associated with only a 20.8% chance of having the same pulsotype. Two discordant results (group violations) were obtained by PFGE typing. Isolate SA 45, belonging to ST-125, clustered closely with isolates showing ST-5. Conversely, isolate SA 29, which belonged to ST-5, was clustered in a branch closely related to other isolates from ST-125 (Fig. 1). The molecular typing results are summarized in Table 3.

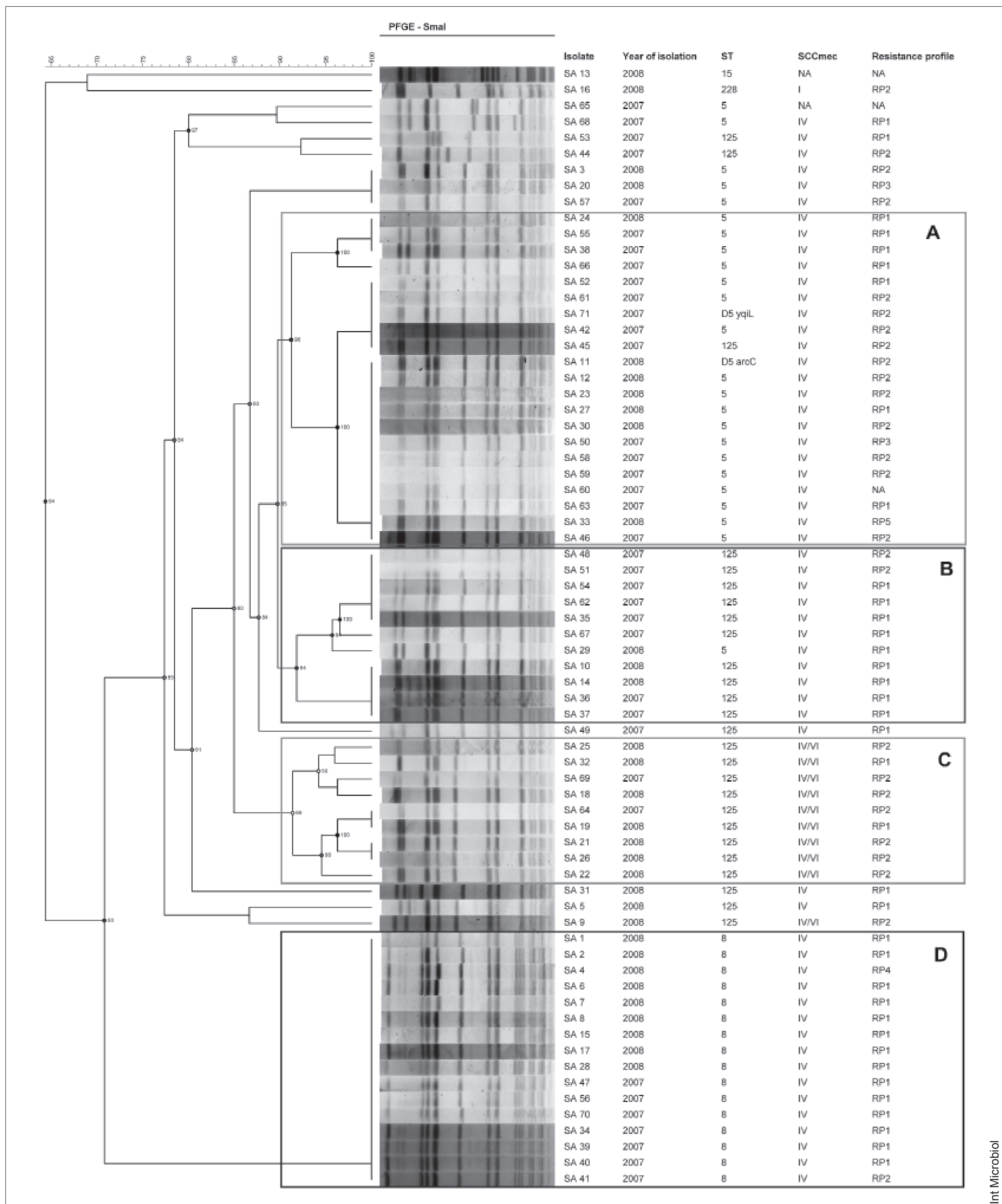

Fig. 1. Genetic relationships among 70 *Staphylococcus aureus* isolates based upon comparisons of the pulsed-field gel electrophoresis profiles obtained with the restriction enzyme *Sma*I. The dendrogram was produced using a Dice similarity coefficient matrix with the unweighted pair group method with arithmetic mean (UPGMA). Major clusters with a cutoff of 90 % similarity were arbitrarily designated from **A** to **D**. Scale bar indicates similarity values.

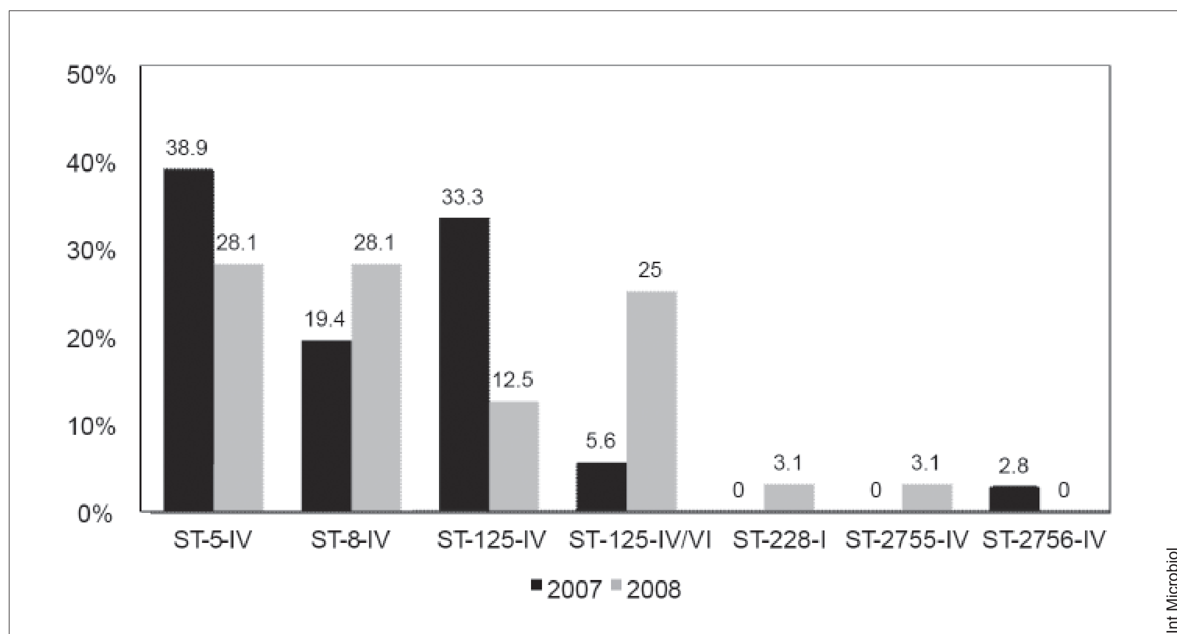

Fig. 2. Clonal evolution of 68 methicillin-resistant *Staphylococcus aureus* isolates recovered from 2007 to 2008 at the University Hospital of Leon.

Panton-Valentine leukocidin genes. All 70 *S. aureus* isolates tested negative for the presence of the PVL genes (*luk*S-PV and *luk*F-PV). Type strain ATCC 49775, included as a positive control, gave the expected 433-bp DNA fragment in all PCRs.

Discussion

In 2006, MRSA in Spain accounted for 30% of all *S. aureus* clinical isolates [5]. Until 1995, ST247-MRSA-I (classically known as the 'Iberian clone') had been the most frequently observed type in the clinical environment. However, this clone has been gradually replaced by others, and nowadays, ST5-MRSA-IV and ST125-MRSA-IV are the predominant clones in Spanish hospitals [28,34,35]. The results of our study are in agreement with that trend: in 2007–2008, the recovery rate of MRSA at the University Hospital of Leon reached 38.7%. Overall, ST5-MRSA-IV was the most frequently detected clone (33.8%), followed by ST125-MRSA-IV (23.5%), ST8-MRSA-IV (23.5%), and ST125-MRSA-IV/VI (14.7%). Moreover, ST228-MRSA-I and two novel STs were sporadically recovered, accounting for one isolate each (1.5%). ST125 isolates have rarely been recovered worldwide, according to the *S. aureus* MLST database [http://saureus.mlst.net]. In fact, only 7 out of the 5024 isolates available from that database belong to ST125 (0.14%, on 08/27/2013). Four of the isolates occurred in Spain, and the other three in Norway, Finland, and France, respectively. The reason for the apparent evolutionary success of clone ST125-MRSA-IV in Spanish hospitals is unclear, nor is it understood why that clone has not spread to other European countries.

Previous studies had reported the gradual replacement in the clinical setting of multiresistant SCC*mec* elements—classically found in HA-MRSA isolates—by SCC*mec* IV, which was initially detected in CA-MRSA isolates and harbors no further resistance elements [17,20,23,28,30,34,35]. The genetic structure of the SCC*mec* elements analyzed in this study supports that finding, since 98.5% of the isolates in this study harbored SCC*mec* IV (14.9% of them also harbored specific ccr gene elements of SCC*mec* type VI). The successful introduction and persistence of the genetically shorter SCC*mec* IV in clinical settings might have provided MRSA strains with an evolutionary advantage in terms of faster replication times, allowing them to outcompete the previously prevalent HA-MRSA clones harboring multiresistant SCCmec types [21]. Further subtyping of SCC*mec* IV revealed that all of them belonged to subtype IVc/IVe. Previous nationwide studies reported that around 70% of all MRSA isolates harboring SCC*mec* IV belong to subtype IVa [17,28,35]. However, our findings are in agreement with those published by Argudín et al. [2], in which 88.5% of MRSA isolates carry subtype IVc. Note that the study

Table 3. Correlation between the genotyping methods used in 70 *Staphylococcus aureus* isolates (68 MRSA), at the University of Leon Hospital, 2007 and 2008

CC(n)	ST(n)	Allelic profile	PFGE cluster(n)	SCC*mec* type(n)	MRSA clone(n)
CC5(54)	5(24)	1-4-1-4-12-1-10	A(18)	IV(18)	ST5-MRSA-IV(23); ST5-MSSA(1)
			B(1)	IV(1)	
			Sporadic[a](5)	IV(4); None[b](1)	
			A(1)	IV(1)	
			B(10)	IV(10)	
	125(26)	1-4-1-4-12-1-54	C(9)	IV/VI(9)	ST125-MRSA-IV(16); ST125-MRSA-IV/VI(10)
			Sporadic(6)	IV(5); IV/VI(1)	
	15(1)	13-13-1-1-12-11-13	Sporadic(1)	None[b](1)	ST15-MSSA(1)
	228(1)	1-4-1-4-12-24-29	Sporadic(1)	I(1)	ST228-MRSA-I(1)
	2755(1)[c]	314-4-1-4-12-1-10	A(1)	IV(1)	ST2755-MRSA-IV(1)
	2756(1)[c]	1-4-1-4-12-1-320	A(1)	IV(1)	ST2756-MRSA-IV(1)
CC8(16)	8(16)	3-3-1-1-4-4-3	D(16)	IV(16)	ST8-MRSA-IV(16)

CC, clonal complex. ST, sequence type. PFGE, pulse-field gel electrophoresis. SCCmec, staphylococcal cassette chromosome mec. MLST, multilocus sequence typing.
[a]Pulsotypes that did not belong to the main clusters were designed as “sporadic.”
[b]Methicillin-susceptible *S. aureus* did not harbor a SCCmec element.
[c]Novel genotype as determined by MLST.

of Argudín et al. was performed in a hospital located in Asturias, the neighbor region of León. Further research is necessary to clarify whether, in Spain, there is a correlation between SCC*mec* types and their geographical origin.

The successful establishment of SCC*mec* IV in the clinical environment has led to a global change in MRSA resistance profiles. Many studies have reported the gradual replacement of the classical multiresistant HA-MRSA clones by non-multiresistant isolates harboring SCC*mec* IV [1,2,6,25,31,33]. Accordingly, in this work 94% of MRSA isolates were non-multiresistant, as they were resistant to only one or two antibiotics in addition to β-lactams (RP1 and RP2) (Table 1). This scenario is in agreement with the presence of the prevalent lineage CC5 harboring SCC*me*c IV, which carries no further resistance elements. Moreover, all the isolates were susceptible to glycopeptides. The increase in gentamicin resistance—from 5.7% in 2007 to 28.6% in 2008 (Table 2)—was not associated with any particular genotype.

The presence of CC5-MRSA strains harboring multiple or composite SCCmec elements has been previously reported [19]. In this study, we found ST125 isolates that carried specific *ccr* gene elements of both SCC*mec* types IV and VI (38.5% of ST125 isolates and 14.9% of all isolates harboring SCC*mec* IV). This clone was recently detected in a third-level hospital in Valladolid, a neighbor city of León [17]. Nine out of the ten isolates were grouped into cluster C by PFGE, and the remaining one yielded a sporadic pulsotype (Fig. 1). The recovery rate of ST125-MRSA-IV/VI increased from 5.6% in 2007 to 25% in 2008, while the presence of ST125-MRSA-IV decreased from 33.3% in 2007 to 12.4% in 2008 (Fig. 2). As previously observed by Menegotto et al. [17], in our study, 80% of ST125-MRSA-IV/VI isolates but just 25% of ST125-MRSA-IV isolates were resistant to erythromycin (RP2). These findings suggest that the clonal replacement observed might have occurred because of antibiotic selective pressure.

Two novel STs, designated ST-2755 and ST-2756, were detected in this study. Both genotypes were single-locus variants of ST5 belonging to CC5. ST-2755 had one SNP in nucleotide 39 of allele 1 of arcC, and ST-2756 one SNP in nucleotide 256 of allele 10 of *yqiL*. These STs might have emerged as punctual mutations from ST5-MRSA isolates since both are single-locus variants of ST5 and, to our knowledge, this genotype has not been observed in MSSA strains (or at least there are no *S. aureus* strains with those genotypes in the MLST database). Both strains were recovered from blood culture samples, were RP2, and were grouped by PFGE into cluster A, along with ST5 isolates. Moreover, neither CA-MRSA nor LA-MRSA strains were detected in this study, and

all 70 isolates tested negative for the presence of PVL genes. According to previous studies carried out in Spanish clinical settings, it seems that, despite worldwide emergence of CA-MRSA strains harboring PVL genes [33], this is still an atypical situation in Spain and is mainly restricted to the introduction of exotic strains from South America [3,4,29].

As expected, molecular typing by PFGE provided better discrimination than MLST (Simpson's index of diversity of 0.909 vs. 0.701, respectively). We observed two group violations by PFGE (Fig. 1), which has been previously reported since these two typing methods index very different types of variations. PFGE-typing can detect genomic variations with a wide range of causes, and thus identify minor variations among isolates collected within a restricted geographic area (e.g., in one hospital). However, such rapidly evolving sequences are susceptible to homoplasy, which can lead to incorrect phylogenetic inferences. MLST indexes variations in seven housekeeping genes under stabilizing evolution, and is therefore more suitable for population studies.

Continuous monitoring of MRSA clones circulating in clinical settings is necessary to better understand the clonal evolution of successful MRSA lineages and to apply appropriate treatment, control, and prevention strategies aimed at hindering the dissemination of MRSA infections. Our molecular epidemiology study of MRSA isolates in a Spanish health-care institution identified CC5-MRSA-IV strains (ST5-MRSA-IV and ST125-MRSA-IV clones) as the most frequently recovered strains, which confirms the previously reported prevalence of these clones in the Spanish clinical environment.

Acknowledgments. This work was supported by EU 7th Framework Programme under the project PROMISE (project.265877). We thank Hortensia Rodríguez Pollán for her technical assistance, and Dr. Bruno González-Zorn for kindly providing MRSA strain ATCC 49775, used as a positive control for the presence of the PVL genes.

Competing interests. None declared.

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