



# Enfermedades Infecciosas y Microbiología Clínica

www.elsevier.es/eimc



Original article

## Antimicrobial resistance, virulence factors and genetic lineages of hospital-onset methicillin-resistant *Staphylococcus aureus* isolates detected in a hospital in Zaragoza

1  
2  
3  
4 **María González-Domínguez<sup>a</sup>, Cristina Seral<sup>b,\*</sup>, Carmen Potel<sup>c</sup>, Lucía Constenla<sup>d</sup>,  
5 Sonia Algarate<sup>a</sup>, M. José Gude<sup>a</sup>, Maximiliano Álvarez<sup>c</sup>, Francisco Javier Castillo<sup>b</sup>**

6 <sup>a</sup> Servicio de Microbiología, Hospital Clínico Universitario Lozano Blesa, San Juan Bosco s/n, Zaragoza, Spain

7 <sup>b</sup> Servicio de Microbiología, Hospital Clínico Universitario Lozano Blesa y Departamento de Microbiología, Facultad de Medicina, Universidad de Zaragoza, San Juan Bosco s/n, Zaragoza, Spain

8 <sup>c</sup> Servicio de Microbiología y Unidad de Apoyo a la Investigación, Instituto de Investigación Biomédica de Vigo (IBIV), Complejo Hospitalario Universitario de Vigo (CHUVI), C/ Pizarro, 22, Vigo, Pontevedra, Spain

9 <sup>d</sup> Unidad de Apoyo a la Investigación, Instituto de Investigación Biomédica de Vigo (IBIV),

10 Complejo Hospitalario Universitario de Vigo (CHUVI), C/ Pizarro, 22, Vigo, Pontevedra, Spain

### ARTICLE INFO

#### Article history:

16 Received 6 November 2014

17 Accepted 27 January 2015

18 Available online xxx

#### Keywords:

19 Hospital-onset

20 MRSA

21 Clonal lineages

22 Resistance genes

23 Molecular typing

### ABSTRACT

**Introduction:** MRSA population dynamics is undergoing significant changes, and for this reason it is important to know which clones are circulating in our nosocomial environment.

**Materials and methods:** A total of 118 MRSA isolates were collected from clinical samples from patients with previous hospital or healthcare contact (named as hospital-onset MRSA (HO-MRSA)) during a one year period. Susceptibility testing was performed by disk diffusion and microdilution. The presence of resistance genes and virulence factors were tested by PCR. All isolates were typed by SCCmec, spa and agr typing. PFGE and MLST were applied to a selection of them.

**Results:** Eighty-three HO-MRSA isolates (70.3%) were resistant to any antibiotic included in the macrolide–lincosamide–streptogramin B group. Among these isolates, the M phenotype was the most frequent (73.5%). One hundred and seven of HO-MRSA isolates (90.7%) showed aminoglycoside resistance. The combination aac(6′)-Ie-aph(2′)-Ia + ant(4′)-Ia genes was the most frequent (22.4%). Tetracycline resistance rates in HO-MRSA isolates were low (3.4%), although a high level of mupirocin resistance was observed (25.4%). Most of the HO-MRSA isolates (approximately 90%) showed SCCmec type IVc and agr type II. Fifteen unrelated pulsotypes were identified. CC5 was the most prevalent (88.1%), followed by CC8 (5.9%), CC22 (2.5%), CC398 (2.5%) and CC1 (0.8%).

**Conclusion:** CC5/ST125/t067 lineage was the most frequent. This lineage was related to aminoglycoside resistance, and to a lesser extent, with macrolide resistance. The presence of international clones as EMRSA-15 (CC22/ST22), European clones as CC5/ST228, community clones related to CC1 or CC8 and livestock associated clones, as CC398, were observed in a low percentage.

© 2015 Published by Elsevier España, S.L.U. on behalf of Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica.

\* Corresponding author.

E-mail addresses: mariagdg@gmail.com (M. González-Domínguez), cseral@unizar.es (C. Seral), carmen.potel.alvarelllos@sergas.es (C. Potel), lucia.constenla.carames@sergas.es (L. Constenla), sonialgarate@gmail.com (S. Algarate), mjosegude@hotmail.com (M.J. Gude), maximiliano.alvarez.fernandez@sergas.es (M. Álvarez), fcastillo@salud.aragon.es (F.J. Castillo).

<http://dx.doi.org/10.1016/j.eimc.2015.01.015>

0213-005X/© 2015 Published by Elsevier España, S.L.U. on behalf of Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica.

Please cite this article in press as: González-Domínguez M, et al. Antimicrobial resistance, virulence factors and genetic lineages of hospital-onset methicillin-resistant *Staphylococcus aureus* isolates detected in a hospital in Zaragoza. *Enferm Infecc Microbiol Clin.* 2015. <http://dx.doi.org/10.1016/j.eimc.2015.01.015>

Palabras clave:  
Origen hospitalario  
SARM  
Líneas clonales  
Genes de resistencia  
Tipado molecular

## Resistencia a antibióticos, factores de virulencia y líneas clonales de *Staphylococcus aureus* resistente a la meticilina de origen hospitalario en un hospital de Zaragoza

### R E S U M E N

**Introducción:** Las dinámicas poblacionales de SARM están experimentando cambios significativos en los últimos tiempos. Por ello es importante conocer qué líneas clonales circulan en nuestro ambiente hospitalario.

**Materiales y métodos:** Durante un año, se seleccionaron 118 SARM de muestras clínicas de pacientes con contacto previo con el ambiente hospitalario (SARM de origen hospitalario [SARM-OH]). Las pruebas de sensibilidad se realizaron mediante difusión con discos y microdilución. La presencia de genes de resistencia y factores de virulencia fueron estudiados mediante PCR. Se estableció el tipo de SCC-mec, spa y agr en todos los aislados, y en una selección se estudió su relación genética por PFGE y MLST.

**Resultados:** Ochenta y tres SARM-OH (70,3%) fueron resistentes a al menos un antibiótico del grupo de los macrólidos-lincosamidas-estreptograminas B. Entre estos, el fenotipo M fue el más frecuente (73,5%). Ciento siete aislamientos (90,7%) mostraron resistencia a aminoglucósidos. La combinación aac(6')-Ie-aph(2'')-Ia + ant(4')-Ia fue la más frecuente (22,4%). Las tasas de resistencia a tetraciclina detectadas fueron bajas (3,4%). Se observó un 25,4% de resistencia de alto nivel a mupirocina. Aproximadamente un 90% de SARM-OH mostraron SCC-mec tipo IVc y agr tipo II. Se identificaron 15 pulsotipos no relacionados. El CC5 fue el más prevalente (88,1%) seguido de CC8 (5,9%), CC22 (2,5%), CC398 (2,5%) y CC1 (0,8%).

**Conclusión:** La línea clonal CC5/ST125/t067 fue la más habitual. Esta línea se relacionó con resistencia a aminoglucósidos, y, en menor medida, con macrólidos. La presencia de clones internacionales como EMRSA-15 (CC22/ST22), clones europeos como CC5/ST228, clones comunitarios relacionados con CC1 o CC8 y clones asociados al ganado, como el CC398, se observaron en un bajo porcentaje.

© 2015 Publicado por Elsevier España, S.L.U. en nombre de Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica.

## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of human bacterial infections worldwide.<sup>1</sup> Currently, MRSA is now endemic in many hospitals and healthcare facilities in industrialized countries.<sup>2</sup> The prevalence of MRSA among European countries varies considerably being significantly lower in northern countries (approximately 1 or 2%) than in other European countries (up to 45%).<sup>3</sup>

In recent years, polyclonal emergence of new clones has been described. Pandemic spread of different hospital-associated MRSA (HA-MRSA) clones, as ST239/SCCmecIII-IV, EMRSA-16 or ST36/SCCmecII (CC30) and ST125/SCCmecIV (CC5) and community-associated MRSA (CA-MRSA) clones, as ST1/SCCmecIV (CC1), USA 300 or ST8/SCCmecIV (CC8), ST30/SCCmecIV (CC30) has been observed.<sup>4-6</sup> To date, livestock-associated MRSA (LA-MRSA) clones has not been frequently identified in hospitals or nursing homes in Europe, although the spread seems to be dependent on the region and the intensity of pig farming.<sup>7</sup>

In Spain in early twenties, the dominant clone was Iberian clone (ST247/SCCmecI) but over the years this clone has reduced its presence.<sup>8</sup> Currently, the most widespread HA-MRSA clone is ST125/SCCmecIV/t067 which is an allelic variant of ST5 pediatric clone.<sup>9</sup>

MRSA population dynamics is undergoing significant changes due to demographic variations such as immigration and the development of sophisticated and complex health systems (i.e. care of patients with severe underlying diseases and invasive devices in day hospitals, residency in a long-term care facility...<sup>4</sup> For this reason it is more difficult to establish the epidemiology of MRSA infections. The aim of the study was to analyze the molecular epidemiology, clonal lineages, resistance mechanisms and virulence traits of hospital and healthcare associated MRSA clones to understand the epidemiology of these infections.

## Materials and methods

### Setting

The study was performed in the University Teaching Hospital "Lozano Blesa" (Zaragoza, Spain), with 803 beds attending a population of 286,774 inhabitants with 29,506 annual admissions. The study was conducted between July 2009 and July 2010.

### Bacterial isolates and patient information

A total of 118 MRSA isolates were collected in our institution from clinical samples belonged to patients with previous hospital or healthcare contact (named as hospital or healthcare onset MRSA, HO-MRSA). HO-MRSA was defined as an isolate cultured from a clinical specimens obtained  $\geq 72$  h after patient's hospital admission or whose sources of isolation were associated with one of the following hospital or healthcare risk factors: history of previous hospitalization, surgeries or dialysis, residency in a long-term care facility, the presence of medical devices or previous MRSA infection or colonization.

One isolate per patient was included. For each patient the following data were collected: gender, age, nationality, source of the culture sample and underlying disease.

### Identification and antimicrobial susceptibility testing

The identification was performed using WIDER I System (Francisco Soria-Melguizo, Madrid, Spain). Susceptibility testing to cefoxitin, erythromycin, azithromycin, spiramycin, clindamycin, pristinamycin, chloramphenicol, tetracycline, minocycline, streptomycin, gentamicin, tobramycin, amikacin, kanamycin and netilmicin (Mast Diagnostics, Bootle, UK and Bio-Rad, Marnes La Coquette, France) was performed by disk diffusion and interpreted

according to CLSI guidelines<sup>10</sup> and the Société Française de Microbiologie breakpoints (<http://www.sfm-microbiologie.org>). Mupirocin susceptibility testing was confirmed by E-test strip method.

### Resistance genotypes and virulence factors

The presence of *mecA*, *aac(6′)-Ie-aph(2′′)-Ia*, *aph(3′)-IIIa*, *ant(4′)-Ia*, *erm(A)*, *erm(B)*, *erm(C)*, *msr(A)*, *vga(A)*, *vga(C)*, *lnu(A)*, *tet(K)*, *tet(M)*, *tet(L)* and *mupA* resistance genes was tested by PCR as previously described.<sup>11,12</sup> The presence of virulence genes encoding the Pantone Valentine leukocidin (PVL) (*lukF/lukS-PV*), TSST-1 (*tst*), Exfoliative Toxin A (ETA) (*eta*), B (ETB) (*etb*) was investigated by PCR.<sup>12</sup> Positive and negative controls were included in all PCRs.

### Molecular typing of isolates

All MRSA isolates were typed (SCC*mec*, *spa* and *agr* typing) as previously described.<sup>12</sup>

Pulsed field gel electrophoresis (PFGE) using *SmaI* and *EagI* restriction enzymes (New England Biolabs, Hertfordshire, United Kingdom) was performed in the isolates with different *spa* types, isolates with the same *spa* type but with different antibiotic resistance profile and mupirocin or tetracycline resistance.<sup>13</sup> *EagI* restriction enzyme was used in tetracycline resistant isolates because presumably could be related to the CC398 which are non-typeable by *SmaI*. Profiles were analyzed with BioNumerics fingerprinting software (Applied Maths, Kortrijk, Belgium). Dendrograms were generated by the unweighted pair-group method using arithmetic averages, based on the Dice similarity coefficient with a 1.0% band position tolerance.<sup>14</sup> PFGE patterns were assigned into pulsotypes (named with capital letters) and subtypes (named with numbers). Different pulsotypes were considered if the similarity coefficient was <80%. Different subtypes were considered when the similarity coefficient oscillates in the 80–95% interval.

Sixteen selected MRSA isolates (isolates represented of each PFGE type and the isolate with the new *spa* type (t10034)) were characterized by MLST amplifying fragments of *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* housekeeping loci.<sup>15</sup> All amplicons were sequenced and compared with the MLST database in order to know the sequence type (ST). The assignment of Clonal Complexes (CC) was achieved according to the ST determined (in 16 isolates). In those cases in which STs were not determined, CCs of the isolates was presumably assigned according to the *spa* type (in the remaining 102 isolates).

## Results

### Overview and epidemiological data

A total of 118 HO-MRSA isolates were collected from clinical samples during one year. These isolates belonged to 72 male (61.1%) and 46 female (38.9%). The median age and standard deviation of these patients was 75.27 ± 15.06 years old (range 1–96). These isolates were mostly recovered from skin lesions and wounds (60%). The remaining isolates were obtained from lower respiratory tract samples (20%), urine samples (8%), blood (5%), abscesses (4%) and upper respiratory samples (2%). These isolates were more frequently found in primary care (20.3%), internal medicine (18.6%), vascular surgery (14.4%), emergency department (8.5%), pulmonology (6.7%) and intensive care unit (5.9%).

The predominant risks factors during the year prior to MRSA isolation were hospitalization (71.9%), residency in a long-term care facility (38%), surgery (38%), medical devices (38%), dialysis (2.5%) or previous MRSA infection or colonization (2.5%).

Most common underlying diseases (often combined) were cardiovascular (80.5%), diabetes (25.3%), and neoplastic disease (27.1%). Other underlying diseases were found in 9.3% of the patients.

### Susceptibility testing, detection of antimicrobial resistance genes and virulence factors

All isolates showed resistance to betalactam antibiotics tested (cefoxitin, oxacillin, and amoxicillin–clavulanate) and *mecA* gene was amplified in all of them. Table 1 shows the characteristics of 118 HO-MRSA isolates including their phenotype and genotype resistance.

Eighty-three HO-MRSA isolates (70.3%) were resistant to any antibiotic included in the macrolide–lincosamide–streptogramin B (MLS<sub>B</sub>) group. The rates of erythromycin and azithromycin resistance were high (68.6% in both cases). Among erythromycin resistant isolates, the M phenotype was detected in 73.5% of them encoded by *msr(A)* (71.1%) and *msr(A)+erm(B)* genes (2.4%). Constitutive resistance (cMLS<sub>B</sub> phenotype) was observed in 16.9% of erythromycin resistant isolates encoded by *erm(C)* gene (7.2%), *erm(A)* gene (2.4%), *erm(A)+erm(B)* genes (3.6%) and *msr(A)+erm(C)* genes in 3.6% of the isolates. Inducible resistance (iMLS<sub>B</sub> phenotype) encoded by *erm(C)* was detected in 8.4% of resistant isolates. Finally, the L phenotype was observed in 1.2% of erythromycin resistant isolates encoded by *lnu(A)* gene.

One hundred and seven of HO-MRSA isolates (90.7%) showed resistance to any antibiotics included in the aminoglycoside family, mainly kanamycin (89%), tobramycin (72.9%) and amikacin (53.4%). *ant(4′)-Ia* gene was the most frequently detected (49.5%), followed by *aph(3′)-IIIa* (11.2%) and *aac(6′)-Ie-aph(2′′)-Ia* genes (3.7%). One-third of isolates (33.6%) showed combinations of several resistance genes. The combination *aac(6′)-Ie-aph(2′′)-Ia+ant(4′)-Ia* genes was the most frequent (22.4%). In two isolates, which showed only streptomycin resistance, we could not determine the resistance gene profile.

Tetracycline resistance rates in HO-MRSA isolates were low (3.4%). Four isolates showed tetracycline resistance encoded by *tet(K)* and *tet(M)+tet(K)* genes. One isolate exhibited tetracycline and minocycline resistance encoded by *tet(M)*.

Mupirocin MICs ≥256 mg/L were found in 30 isolates (25.4%) (the rest of them showed MICs < 8 mg/L). High mupirocin MICs were confirmed by E-test strip method showing high level mupirocin resistance (MIC > 512 mg/L). This resistance was encoded by *mupA* gene.

The genes encoding the PVL were detected in 3 of our 118 HO-MRSA isolates (2.5%). Only one isolate with *tst* gene was detected. This isolate, obtained from a respiratory specimen (sputum) from HIV patient, carry *erm(C)* and *ant(4′)-Ia* genes and was included in ST5/*spat9298/SCCmecI/agrII* profile. Genes encoding A and B exfoliatins were not detected in any isolate.

### Molecular typing of isolates

Most of the HO-MRSA isolates (90%) showed SCC*mec* type IVc. Other SCC*mec* found were: SCC*mec* type I (4.2%), SCC*mec* type IVh (2.5%), SCC*mec* type V (2.5%) and SCC*mec* type IVa (0.8%) (Table 1).

Highly diverse *spa* types were identified among HO-MRSA. The most frequently detected was *spa* type t067 (61%) followed by t2220 (7.6%), t2226 (6.7%), t002 (5.1%), t008 (4.2%), t041 (4.2%), t011 (2.5%) and t032 (1.7%). Sporadically *spa* types t068, t127, t1302, t190, t306, t4978 and t9298 were detected. A new *spa* type, *spa* t10034, was identified in this study which was subsequently included in Ridom StaphType<sup>®</sup> software. This new *spa* type belongs to ST5.

**Table 1**  
Antimicrobial resistance, virulence genes and genetic lineages of 118 HO-MRSA isolates.

CC	ST (n) number of isolates	spa type (n) number of isolates	SCCmec	agr	PVL/TSST	PGFE profile (n) number of isolates	Resistance profile* (n) number of isolates	Resistance genes
	ND					pA <sub>3</sub> (1)	ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pA <sub>4</sub> (1)	ERY, AZM, SPI, CLI, TOB, KAN	<i>erm(C)</i> , <i>ant(4')-Ia</i>
	ND					pA <sub>5</sub> (2)	ERY, AZM, SPI, CLI, TOB, AMI, KAN	<i>erm(C)</i> , <i>ant(4')-Ia</i>
	ND						TOB, KAN, CLO	<i>ant(4')-Ia</i>
	ND					pA <sub>7</sub> (2)	ERY, AZM, GEN, TOB, AMI, KAN	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i>
	ND						TOB, AMI, KAN	<i>ant(4')-Ia</i>
	ND					pA <sub>8</sub> (1)	ERY, AZM, SPI, CLI, TOB, AMI, KAN	<i>erm(C)</i> , <i>ant(4')-Ia</i>
	ND					pA <sub>9</sub> (2)	ERY, AZM, KAN	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND						GEN, TOB, AMI, KAN, MUP	<i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pA <sub>10</sub> (1)	TOB, AMI, KAN	<i>ant(4')-Ia</i>
	ND					pA <sub>11</sub> (1)	ERY, AZM, SPI, CLI, TOB, AMI, KAN, STR, MUP	<i>erm(C)</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND						ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pA <sub>12</sub> (2)	ERY, AZM, SPI, CLI, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>erm(C)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>aph(3')-IIIa</i> , <i>mupA</i>
	ND						ERY, AZM, GEN, TOB, AMI, KAN, STR, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pA <sub>13</sub> (6)	ERY, AZM, SPI, CLI, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>erm(C)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>aph(3')-IIIa</i> , <i>mupA</i>
	ND						ERY, AZM, GEN, TOB, AMI, KAN, MUP (2)	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND						ERY, AZM, GEN, TOB, AMI, KAN, NET, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND						GEN, TOB, AMI, KAN, STR, NET, MUP	<i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pA <sub>14</sub> (2)	ERY, AZM, TOB, AMI, KAN, STR	<i>msr(A)</i> , <i>ant(4')-Ia</i> , <i>aph(3')-IIIa</i>
	ND	067 (72)	IVc	II	-		ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pA <sub>17</sub> (1)	TOB, KAN	<i>ant(4')-Ia</i>
	ND					pA <sub>18</sub> (1)	-	-
	ND					pA <sub>19</sub> (2)	GEN, TOB, AMI, KAN, MUP	<i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND						ERY, AZM, GEN, TOB, AMI, KAN, STR, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>aph(3')-IIIa</i> , <i>mupA</i>
	ND					pA <sub>20</sub> (1)	ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>aph(3')-IIIa</i> , <i>mupA</i>
	ND					pA <sub>21</sub> (1)	ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND						ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pA <sub>22</sub> (3)	ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>aph(3')-IIIa</i> , <i>mupA</i>
	ND						ERY, AZM, GEN, TOB, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>mupA</i>
	125					pB <sub>1</sub> (1)	ERY, AZM, KAN, STR, CLO	<i>msr(A)</i> , <i>aph(3')-IIIa</i>
	ND						ERY, AZM, TOB, AMI, KAN, STR, CLO	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND					pC <sub>3</sub> (2)	ERY, AZM, TOB, KAN	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND						ERY, AZM, KAN (2)	<i>msr(A)</i> , <i>aph(3')-IIIa</i>
	ND					pC <sub>4</sub> (3)	ERY, AZM, TOB, AMI, KAN	<i>msr(A)</i> , <i>erm(B)</i> , <i>ant(4')-Ia</i>
	ND						GEN, TOB, KAN, MUP	<i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pC <sub>5</sub> (1)	ERY, AZM, TOB, AMI, KAN	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND					pC <sub>6</sub> (1)	ERY, AZM, TOB, AMI, KAN	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND					pC <sub>7</sub> (1)	ERY, AZM, TOB, AMI, KAN, CLO	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	125					pD <sub>1</sub> (1)	ERY, AZM, SPI, CLI, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>erm(C)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	125						ERY, SPI, STR, CLO	<i>erm(C)</i>
	ND					pE <sub>1</sub> (1)	ERY, AZM, GEN, TOB, AMI, KAN	<i>erm(C)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i>
	ND						ERY, AZM, GEN, TOB, AMI, KAN, MUP (2)	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND						ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>aph(3')-IIIa</i> , <i>mupA</i>
						ND (31)		

Table 1 (Continued)

CC	ST (n) number of isolates	spa type (n) number of isolates	SCCmec	agr	PVL/TSST	PGFE profile (n) number of isolates	Resistance profile* (n) number of isolates	Resistance genes
	ND						ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>erm(B)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>aph(3')-IIIa</i> , <i>mupA</i>
	ND						ERY, AZM, KAN (2)	<i>msr(A)</i> , <i>aph(3')-IIIa</i>
	ND						ERY, AZM, KAN (2)	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND						ERY, AZM, SPI, CLI, TOB, KAN	<i>erm(C)</i> , <i>ant(4')-Ia</i>
	ND						ERY, AZM, TOB, AMI, KAN (12)	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND						ERY, AZM, TOB, AMI, KAN	<i>msr(A)</i> , <i>ant(4')-Ia</i> , <i>aph(3')-IIIa</i>
	ND						ERY, AZM, TOB, AMI, KAN, CLO	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND						ERY, AZM, TOB, AMI, KAN, STR	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND						ERY, AZM, TOB, KAN (2)	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND						TOB, AMI, KAN (4)	<i>ant(4')-Ia</i>
	ND					pA <sub>16</sub> (1)	ERY, AZM, KAN, MUP	<i>msr(A)</i> , <i>aph(3')-IIIa</i> , <i>mupA</i>
	125					pC <sub>1</sub> (2)	ERY, AZM, KAN, STR	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND	2220 (9)	IVc	II	-		ERY, AZM, AMI, KAN	<i>msr(A)</i> , <i>ant(4')-Ia</i>
	ND					pC <sub>5</sub> (1)	ERY, AZM, KAN	<i>msr(A)</i> , <i>aph(3')-IIIa</i>
	ND					pC <sub>8</sub> (1)	ERY, AZM, KAN	<i>msr(A)</i> , <i>aph(3')-IIIa</i>
	ND					ND (4)	ERY, AZM, KAN	<i>msr(A)</i> , <i>aph(3')-IIIa</i>
	ND					pA <sub>4</sub> (1)	TOB, AMI, KAN, STR	<i>ant(4')-Ia</i>
	ND					pA <sub>5</sub> (1)	GEN, TOB, AMI, KAN, MUP	<i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pA <sub>6</sub> (2)	GEN, TOB, AMI, KAN, MUP	<i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND	2226 (8)	IVc	II	-	pA <sub>14</sub> (1)	ERY, AZM, GEN, TOB, AMI, KAN, MUP	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>mupA</i>
	ND					pA <sub>15</sub> (1)	TOB, KAN	<i>ant(4')-Ia</i>
	ND					ND (2)	TOB, KAN	<i>ant(4')-Ia</i>
	125	4978	IVc	II	-	pG <sub>1</sub> (1)	ERY, AZM, KAN, CLO	<i>msr(A)</i> , <i>aph(3')-IIIa</i>
	146 (1)					pA <sub>1</sub> (2)	ERY, AZM, TOB, AMI, KAN	<i>erm(C)</i> , <i>ant(4')-Ia</i>
	ND					pA <sub>17</sub> (1)	-	-
	ND	002 (6)	IVc	II	-	pA <sub>18</sub> (1)	-	-
	ND						-	-
	ND					ND (2)	TOB, AMI, KAN, STR	<i>ant(4')-Ia</i>
	228						ERY, AZM, SPI, CLI, GEN, TOB, KAN, STR	<i>erm(A)</i> , <i>erm(B)</i> , <i>aac(6')-Ie-aph(2'')</i>
	ND					pF <sub>1</sub> (3)	ERY, AZM, SPI, CLI, GEN, TOB, KAN, STR	<i>erm(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>aph(3')-IIIa</i>
	ND	041 (5)	I	II	-		ERY, AZM, SPI, CLI, GEN, TOB, KAN, STR	<i>erm(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i>
	ND					pF <sub>2</sub> (1)	ERY, AZM, SPI, CLI, GEN, TOB, KAN, STR	<i>erm(A)</i> , <i>erm(B)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>aph(3')-IIIa</i>
	ND		IVc			pF <sub>1</sub> (1)	ERY, AZM, SPI, CLI, GEN, TOB, KAN, STR	<i>erm(A)</i> , <i>erm(B)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>aph(3')-IIIa</i>
	ND		IVc			pA <sub>2</sub> (1)	TOB, KAN	<i>ant(4')-Ia</i>
	5	9298	I	II	TSST+	pH <sub>1</sub> (1)	ERY, AZM, TOB, KAN	<i>erm(C)</i> , <i>ant(4')-Ia</i>
	5	10,034	IVc		-	pC <sub>2</sub> (1)	ERY, AZM, GEN, TOB, AMI, KAN	<i>msr(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i>
	8 (1)						-	-
	8					PVL+	pI <sub>1</sub> (2)	-
	ND	008 (5)	IVc	I	-		TOB, AMI, KAN	<i>ant(4')-Ia</i>
	8						TOB, KAN	<i>ant(4')-Ia</i>
	8	68	IVc	I	-		-	-
	ND	190	IVc	I	-		TOB, KAN	<i>ant(4')-Ia</i>
	22 (1)	032 (2)	IVh	I	-		ERY, AZM, SPI, CLI	<i>erm(C)</i>
	ND						ERY, AZM	<i>erm(C)</i>
	ND	1302	IVh	I	-		-	-
	1	127	IVa	III	-		ERY, AZM, KAN, STR, TET	<i>erm(C)</i> , <i>ant(4')-Ia</i> , <i>tet(K)</i>
	398 (1)						-	-
	ND	011 (3)	V	I	-		STR, TET	<i>tet(K)</i> , <i>tet(M)</i>
	ND						GEN, TOB, KAN, TET, MIN	<i>aac(6')-Ie-aph(2'')</i> , <i>tet(M)</i>
	ND						CLI, GEN, TOB, KAN, STR, TET	<i>lnu(A)</i> , <i>aac(6')-Ie-aph(2'')</i> , <i>ant(4')-Ia</i> , <i>tet(M)</i>

ND: not determined; PVL: Pantón-Valentine leukocidin; TSST: toxic shock syndrome toxin.

\* ERY: erythromycin, AZM: azithromycin, SPI: spiramycin, CLI: clindamycin, GEN: gentamicin, TOB: tobramycin, AMI: amikacin, KAN: kanamycin, STR: streptomycin, NET: netilmicin, TET: tetracycline, MIN: minocycline, MUP: mupirocin.

Amplification of the hypervariable region of the *agr* locus showed that *agr* group II was predominant (88.1%), followed by *agr* group I (11.1%) and *agr* group III (0.8%).

In addition, 79 MRSA isolates were studied by PFGE. A high genetic variability was found, although some clusters were observed. Fifteen unrelated pulsotypes were identified (named pulsotypes pA-pO). Pulsotypes pA<sub>13</sub> (6 isolates), pA<sub>22</sub> (3 isolates) and pC<sub>4</sub> (3 isolates) were the most frequent (Table 1).

Sixteen isolates were further characterized by MLST being ST125 the predominant (5 isolates) followed by ST8 (4), ST5 (2), ST1, ST22, ST146, ST228 and ST398.

Distribution in CC was established according to the ST or presumably assigned according to the *spa* type. CC5 was the most prevalent (88.1%) followed by CC8 (5.9%), CC22 (2.5%), CC398 (2.5%) and CC1 (0.8%).

## Discussion

In Spain, MRSA prevalence has remained stable in recent years. During the course of this study, MRSA prevalence in our hospital was 30.9%, consistent with other resistance rates found in different Spanish hospitals.<sup>16,17</sup> It has been observed that some MRSA clones tend to occur more frequently in certain countries or areas<sup>18</sup> while other clones spread worldwide, threatening public health.<sup>19</sup> Controlling the infection in hospitals or healthcare centers could reduce the overall prevalence of MRSA, for this reason, it is important to know which clones are circulating in our nosocomial environment.

Studies conducted in Spain showed that in our country, a small number of international clones have spread.<sup>4</sup> The predominant Iberian clone before 1996 in Spain was replaced by CC5/ST228/SCCmecI clone. In our study, five strains belonged to CC5/ST228/*spat*041/SCCmecI/*agr*II profile (one of them possessed SCCmec type IVc instead of SCCmec type I). These strains showed high homology in PFGE patterns (pulsotypes pF<sub>1</sub> and pF<sub>2</sub>) and constant resistance pattern (*erm*(A) ± *erm*(B) + *aac*(6')-Ie-*aph*(2'')-Ia + *aph*(3')-IIIa). However, the presence of this clone in Spain has been steadily declining, being replaced by international EMRSA-16 clone (CC30/ST36/SCCmecII), very common in the UK.<sup>4,6</sup> Currently, this clone has an irregular representation in different institutions while CC5/ST125/SCCmecIV has become more prevalent.<sup>16</sup> High percentage of our isolates belonged to this CC5/ST125/SCCmecIV. This high proportion is mainly due to the large number of isolates belonging to *spa* type t067, although other *spa* types were found (Table 1). Approximately one-quarter (22.8%) of isolates included in *spa* type t067 were associated with tobramycin and erythromycin resistance mediated by *ant*(4')-Ia and *msr*(A) genes, respectively. Moreover, most of the high level mupirocin resistance isolates that are circulating in our hospital environment belonged to CC5/ST125/t067.

In a previous study conducted by our group, we observed that isolates belonged to CC5/ST125/t067 were predominant in community areas.<sup>12</sup> This finding suggests a great exchange of genetic lineages from hospital or healthcare services to the community environment.

Other HA-MRSA clones were detected in our hospital environment. Three strains included in CC5/ST125/*spat*2226/SCCmecIVc/*agr*II profile belonged to patients admitted to the same nursing home. Isolates belonging to ST5 were sporadic in our study. Three strains were included in CC22 (EMRSA-15), a British clone first detected in 1999 in Spain, always detected in a very low percentage (<2%).<sup>6</sup>

The *tst* gene was detected in one isolate (belonging to CC5/ST5/*spat*9298/SCCmecI/*agr*II). This strain seems to be related with TSST-1 positive ST5 Geraldine clone

(ST5/*spat*002/SCCmecI/*agr*II) frequently found in hospital and community environment in France.<sup>20</sup>

However, we not only observed HA-MRSA clones circulating by our hospital or healthcare environment. The presence of typically community clones (CC8 and CC1) and livestock associated clones (CC398) was observed. Most of the CA-MRSA strains circulating in our country were included in USA300 clone or its Latin American variant mainly due to the large population exchanges with American continent in recent years.<sup>24,25</sup> The presence of this clone was low (five isolates) in our hospital environment. Three isolates, belonging to Bolivian patients, contained the *lukF/lukS*-PV genes. Two of them were recovered from a patient with a breast abscess and her daughter that showed cellulite in her right inframandibular area. CC1 includes several strains of CA-MRSA,<sup>26</sup> but this CC also has been detected in animals.<sup>27</sup> One of our isolates, obtained from a Romanian patient with a previous history of animal contact, belonged to CC1 (ST1/*spat*127/SCCmecIVa PVL negative).

In our healthcare environment, we observed the presence of CC-related to animals as CC398. These strains were recovered from different specimens (sputum, urine and surgical wound) and belonged to patients with no history of previous contact with animals (one of them was living in a rural area with a high density of farms, but we could not confirm animal contact because the patient died). The relationship between tetracycline resistance and CC398 strains was already suggested.<sup>28</sup> Resistance is mainly related to *tet*(M) gene, but not excluded other genes as *tet*(K) or *tet*(L), or even a combination of both, as we have found in one of our isolates.<sup>7</sup> An unusual resistance profile (clindamycin resistance but erythromycin susceptibility) has been described in ST398 isolates associated with the presence of *vga*(A) and *vga*(C) or *lnu*(A) genes.<sup>29,30</sup> This unusual profile was found in our study encoded by *lnu*(A) gene.

In conclusion, in our healthcare environment, most of the strains belong to CC5, where ST125/t067 lineage was the most important. This lineage was related to aminoglycoside resistance, and to a lesser extent, with macrolide resistance. The presence of international clones as EMRSA-15 (CC22/ST22), European clones as CC5/ST228, community clones related to CC1 or CC8 (mainly carried by nonnative patients) and livestock associated clones, as CC398, were observed in a low percentage.

## Funding

This work was supported by the Department of Science and Technology and Universities of the Regional Government of Aragón, Spain (Project DGA-FSE/Consolidated groups, B24-211130), MGD received a grant from the S.E.I.M.C. (Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica).

## Conflict of interest

The authors declare that they have no conflict of interest.

## Uncited references

Refs. 21-23.

## Acknowledgments

We are grateful to Dr. J. Pintado for his help with the BioNumerics fingerprinting software, Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas-IIM-CSIC, c/. Eduardo Cabello 6, Vigo, Spain.

## References

1. Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, et al. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. *Clin Infect Dis*. 2001;32:S114–32.
2. DeLeo FR, Otto M, Kreiswirth BN, Chambers HF. Community-associated methicillin-resistant *Staphylococcus aureus*. *Lancet*. 2010;375:1557–68.
3. European Antimicrobial Resistance Surveillance System (EARSS). Antimicrobial resistance surveillance in Europe. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net); 2013. Available from: <http://www.ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-surveillance-europe-2013.pdf> [accessed 01.05.15].
4. Coque TM, Martínez JL. Grampositive pathogen clonal diversification and evolution: new perspectives in the XXI century. *Enferm Infecc Microbiol Clin*. 2010;28:333–5.
5. Deurenberg RH, Vink C, Kalenic S, Friedrich AW, Bruggeman CA, Stobberingh EE. The molecular evolution of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect*. 2007;13:222–35.
6. Pérez-Roth E, Lorenzo-Díaz F, Batista N, Moreno A, Méndez-Álvarez S. Tracking methicillin-resistant *Staphylococcus aureus* clones during a 5-year period (1998 to 2002) in a Spanish hospital. *J Clin Microbiol*. 2004;42:4649–56.
7. Lozano C, Rezusta A, Gómez P, Gómez-Sanz E, Báez N, Martín-Saco G, et al. High prevalence of *spa* types associated with the clonal lineage CC398 among tetracycline-resistant methicillin-resistant *Staphylococcus aureus* strains in a Spanish hospital. *J Antimicrob Chemother*. 2012;67:330–4.
8. Cuevas O, Cercenado E, Bouza E, Castellares C, Trincado P, Cabrera R, et al. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Spain: a multicentre prevalence study (2002). *Clin Microbiol Infect*. 2007;13:250–6.
9. Pérez-Vázquez M, Vindel A, Marcos C, Oteo J, Cuevas O, Trincado P, et al. 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*. *J Antimicrob Chemother*. 2009;63:21–31.
10. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. Twenty informational supplement, CLSI document M100-S20; 2010.
11. Udo EE, Jacob LE, Mathew B. Genetic analysis of methicillin-resistant *Staphylococcus aureus* expressing high- and low-level mupirocin resistance. *J Med Microbiol*. 2001;50:909–15.
12. González-Domínguez M, Seral C, Sáenz Y, Salvo S, Gude MJ, Porres-Osante N, et al. Epidemiological features, resistance genes, and clones among community-onset methicillin-resistant *Staphylococcus aureus* (CO-MRSA) isolates detected in northern Spain. *Int J Med Microbiol*. 2012;302:320–6.
13. Murchan S, Kaufmann ME, Deplano A, de Ryck R, Struelens M, Zinn CE, et al. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. *J Clin Microbiol*. 2003;41:1574–85.
14. Lamaro-Cardoso J, de Lencastre H, Kipnis A, Pimenta FC, Oliveira LS, Oliveira RM, et al. Molecular epidemiology and risk factors for nasal carriage of *Staphylococcus aureus* and methicillin-resistant *S. aureus* in infants attending day care centers in Brazil. *J Clin Microbiol*. 2009;47:3991–7.
15. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol*. 2000;38:1008–15.
16. Lozano C, Porres-Osante N, Cretzaz J, Rojo-Bezares B, Benito D, Olarte I, et al. Changes in genetic lineages, resistance, and virulence in clinical methicillin-resistant *Staphylococcus aureus* in a Spanish hospital. *J Infect Chemother*. 2013;19:233–42.
17. Cuevas O, Cercenado E, Goyanes MJ, Vindel A, Trincado P, Boquete T, et al. *Staphylococcus* spp in Spain: present situation and evolution of antimicrobial resistance (1986–2006). *Enferm Infecc Microbiol Clin*. 2008;26:269–77.
18. Grundmann H, Aanensen DM, van den Wijngaard CC, Spratt BG, Harmsen D, Friedrich AW, et al. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. *PLoS Med*. 2010;7:e1000215.
19. Stefani S, Chung DR, Lindsay JA, Friedrich AW, Kearns AM, Westh H, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. *Int J Antimicrob Agents*. 2012;39:273–82.
20. Robert J, Tristán A, Cavalie L, Decusser JW, Bes M, Etienne J, et al. Pantón-valentine leukocidin-positive and toxic shock syndrome toxin 1-positive methicillin-resistant *Staphylococcus aureus*: a French multicenter prospective study in 2008. *Antimicrob Agents Chemother*. 2011;55:1734–9.
21. Cercenado E, Cuevas O, Marín M, Bouza E, Trincado P, Boquete T, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* in Madrid, Spain: transcontinental importation and polyclonal emergence of Pantón-Valentine leukocidin-positive isolates. *Diagn Microbiol Infect Dis*. 2008;61:143–9.
22. Manzur A, Domínguez AM, Pujol M, González MP, Limón E, Hornero A, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* infections: an emerging threat in Spain. *Clin Microbiol Infect*. 2008;14:377–80.
23. Otter JA, Havill NL, Boyce JM, French GL. Comparison of community-associated methicillin-resistant *Staphylococcus aureus* from teaching hospitals in London and the USA, 2004–2006: where is USA300 in the UK. *Eur J Clin Microbiol Infect Dis*. 2009;28:835–9.
24. Battisti A, Franco A, Merialdi G, Hasman H, Iurescia M, Lorenzetti R, et al. Heterogeneity among methicillin-resistant *Staphylococcus aureus* from Italian pig finishing holdings. *Vet Microbiol*. 2010;142:361–6.
25. Potel C, Álvarez-Fernández M, Constenla L, Álvarez P, Pérez S. First human isolates of methicillin-resistant *Staphylococcus aureus* sequence type 398 in Spain. *Eur J Clin Microbiol Infect Dis*. 2010;29:351–2.
26. Kadlec K, Pomba CF, Couto N, Schwarz S. Small plasmids carrying *vga(A)* or *vga(C)* genes mediate resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in methicillin-resistant *Staphylococcus aureus* ST398 from swine. *J Antimicrob Chemother*. 2010;65:2692–3.
27. Lozano C, Aspiroz C, Sáenz Y, Ruiz-García M, Royo-García G, Gómez-Sanz E, et al. Genetic environment and location of the *lnu(A)* and *lnu(B)* genes in methicillin-resistant *Staphylococcus aureus* and other staphylococci of animal and human origin. *J Antimicrob Chemother*. 2012;67:2804–8.