Molecular epidemiology of community-associated methicillin-resistant
Staphylococcus aureus in Spain: 2004–12

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Objectives: In Spain, despite the high rates of healthcare-associated methicillin-resistant Staphylococcus aureus (MRSA), the incidence of community-associated (CA) MRSA seems to be low on the basis of a small number of studies. We analysed the evolution of CA-MRSA in Spain from 2004 to 2012, and identified the clonal lineages and population structure.

Methods: The study included 8326 MRSA strains. Susceptibility to 18 antimicrobials was determined. Isolates were tested for the presence of mecA, Panton–Valentine leucocidin (PVL) and the arginine catabolic mobile element (ACME) by PCR, and typed by staphylococcal cassette chromosome mec, PFGE, spa, multilocus sequence typing and agr.

Results: Among the 8326 isolates, 246 (2.9%) were CA-MRSA. We identified genotypically 226 PVL-positive CA-MRSA isolates (88% agr type I, 10.2% agr type III and 1.8% agr type II) and 20 PVL-negative CA-MRSA isolates (all agr type I) from children and adults (82.1% from wounds) from 13 different geographical areas. A significant increase in the rates of CA-MRSA was observed when comparing 2004–07 (0.43%) with 2008–12 (5.44%). Resistance rates were as follows: only β-lactams, 84.5%; erythromycin, 12.8%; tetracycline, 8.8%; clindamycin, 4.9%; ciprofloxacin, 3.1%; fusidic acid, 2.0%; others, 0.4%; and multiresistant, 6.2% (six isolates USA300). The strains belonged to the PVL-positive clones ST8-IVc (69.9%), ST8-IVa-ACME-positive (USA300, 8.9%), ST8-IVa-ACME-negative (0.8%), ST30-IVc (4.5%), ST80-IVc (2.0%), ST5-IVc (1.2%) and others (ST59, ST72, ST88, ST642, ST1472 and ST1829; 4.5%) and to the PVL-negative ST398-V (8.1%).

Conclusions: We confirm an increase in CA-MRSA in Spain, the predominance of the ST8-IVc clone, the emergence of the USA300 clone, a high genetic diversity among PVL-positive CA-MRSA isolates and the recent emergence of the pig-associated ST398-V clone.

Keywords: MRSA, community-associated MRSA, PVL, ST398, livestock-associated MRSA, skin and soft-tissue infections, linezolid

Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is a leading cause of hospital-acquired and healthcare-associated (HA) infections throughout the world.1,2 However, over recent decades, the epidemiology of MRSA has changed, and new MRSA strains are circulating in the community, causing infections in healthy patients without previous healthcare contact.3,4 In addition, MRSA has also emerged in animals, particularly in livestock. The predominant livestock-associated (LA) MRSA belongs to the clonal lineage ST398, a pig-associated clone that has also been found in calves, poultry and humans as a cause of infections.5,6

Methicillin resistance is mainly due to the presence of the mecA gene, which encodes an alternative penicillin-binding protein (PBP2a) that has low affinity for β-lactams. The mecA gene is carried within a mobile genetic element called the staphylococcal cassette chromosome mec (SCCmec). SCCmec elements have been classified into types I–XI and their variants.7–11 In general, HA-MRSA carry SCCmec types I–IV, whereas type IV is also frequently carried by community-associated (CA) MRSA, as well as types VI, VII and IX.11–13 Type V is carried by LA-MRSA ST398, and recently, the new SCCmecXI has been described in MRSA isolates from cattle and humans carrying the new mecC gene.9 Multilocus sequence typing (MLST) and other molecular typing
methods have also identified that the population of strains causing CA-MRSA differs from that causing HA-MRSA, with sequence types (STs) ST1, ST8, ST30, ST59, ST80, ST93, ST129, ST152 and ST398 being the most frequently isolated CA-MRSA globally.\(^{13–15}\) The majority of CA-MRSA carry specific virulence factors, such as the lukF-PV and lukS-PV determinants, which encode the Panton–Valentine leucocidin (PVL) toxin, the arginine catabolic mobile element (ACME) and other toxins that are expressed at high concentrations.\(^{13,16,17}\) Reports of PVL-positive isolates belonging to ST398 are currently rare, but ST398 isolates do carry a range of other Staphylococcus virulence genes.\(^{18}\)

In Spain, despite the high rates of HA-MRSA, the incidence of CA-MRSA seems to be low on the basis of a small number of studies published from a few institutions, and descriptions of sporadic cases and outbreaks. PVL-positive CA-MRSA isolates have frequently been associated with immigrants from South America, mainly from Ecuador,\(^{4,19–23}\) and CA-MRSA isolates of ST398 have been identified as a rare cause of infection in humans related to occupational contact with pigs,\(^{18}\) but have also been described as a cause of bacteraemia in patients with no clear contact with animals.\(^{18,24}\) However, studies regarding the molecular epidemiology of CA-MRSA in Spain are scarce and the present incidence of CA-MRSA in the whole country is not clearly understood.

In this study, we aim to determine the present situation of CA-MRSA in Spain and its evolution over the last 9 years, and to identify the main clonal lineages of CA-MRSA and its population structure by analysing all MRSA isolates received at the Spanish National Reference Centre for Staphylococci (SNRCS) from 2004 to 2012.

**Methods**

**Bacterial isolates**

From January 2004 to December 2012, we received at the SNRCS, from all geographical areas of Spain, a total of 8326 MRSA isolates for molecular characterization. The distribution of isolates by year was as follows: 2004, \(n = 1200; 2005, n = 1013; 2006, n = 1101; 2007, n = 822; 2008, n = 1214; 2009, n = 879; 2010, n = 815; 2011, n = 742;\) and 2012, \(n = 540.\) Only one isolate per patient was included in the analysis.

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility testing was performed using an automated broth microdilution method with the Pos Comba panel type 31 (MicroScan; Siemens, Sacramento, CA, USA), according to the manufacturer’s guidelines. The antimicrobials evaluated were: penicillin, oxacillin, cindamycin, erythromycin, gentamicin, tobramycin, rifampicin, trimethoprim/sulfamethoxazole, ciprofloxacin, fosfomycin, nitrofurantoin, vancomycin, fusidic acid, mupirocin, tetracycline, linezolid and daptomycin. Additional Etest susceptibility testing was performed for tigecycline. MIC breakpoints were determined according to the recommendations of EUCAST.\(^{29}\) Multiresistance was defined as resistance to three or more classes of antimicrobial agents.

**Methicillin resistance detection and SCCmec typing**

The meca gene was detected by PCR, as described by Geha et al.\(^{26}\) SCCmec types were determined by use of a multiplex PCR strategy that generated a specific amplification pattern for each SCCmec structural type, according to the method described by Oliveira and de Lencastre.\(^{27}\) Additional typing of the isolates was performed by two different PCR methods in order to detect SCCmec IV subtypes IVa, IVb, IVc, IVd and IVh\(^{28}\) and SCCmec type V.\(^{29}\)

**PFGE**

All MRSA isolates were genotyped by PFGE after Smal digestion of chromosomal DNA, prepared using a modification of the protocol described by Cookson et al.\(^{31}\) Analysis of the gels was performed according to the criteria of Tenover et al.\(^{52}\) and a dendrogram was constructed with Molecular Analyst software (Bio-Rad) using the Dice correlation coefficient and the unweighted pair-group method with averages with a tolerance position of 0.6%. Positive controls of different PFGE profiles were used as previously described by our group.\(^{10}\) In addition, a prototype USA300 strain kindly provided by Professor Herminio de Lencastre was also used as a PFGE control.

**Detection of PVL and the ACME**

The PVL genes (lukS-PV and lukF-PV) were detected by PCR by the method described by Lina et al.\(^{16}\) S. aureus ATCC 49775 (a PVL-positive strain) was used as a positive amplification control. The detection of the ACME was performed by PCR as previously described.\(^{33}\)

**Determination of accessory gene regulator (agr) type**

A scheme of two PCRs, based on the method described by Shopsin et al.\(^{34}\) was used for the determination of the specific agr groups. PCR-positive controls were used.

**spa typing and BURP analysis**

The polymorphic X region of the protein A gene (spa) was amplified from all MRSA isolates, as described previously.\(^{35,36}\) The spa type was assigned using Ridom StaphType software.\(^{37}\) Clustering of the isolates was performed by the BURP (Based Upon Repeat Pattern) algorithm implemented in StaphType. Isolates with spa types with more than five repeats were clustered into different groups, with the calculated cost between the members of a group being \(\leq 6.\)

**MLST**

MLST was performed by the method described by Enright et al.\(^{38}\) Allelic profiles and ST types were assigned using the MLST database (http://www.mlst.net).

**Statistical analysis**

Proportions were compared using the \(x^2\) test. \(P\) values \(< 0.05\) were considered significant.

**Clinical information and ethical statement**

Isolates were obtained as part of routine testing at the SNRCS and were analysed anonymously. Isolates were accompanied by a protocol that included the sex and age of the patient, the origin of the isolate and information concerning any history of patient healthcare exposures. Isolates were considered as CA when they were obtained from persons with no history of the following: surgery; hospitalization or residence in a long-term care facility within the year before infection; the presence of a percutaneous device or indwelling catheter; dialysis within the previous year; hospitalization \(> 48\) h before MRSA culture; or previous MRSA infection or colonization (CDC criteria). Since only the isolates, and not the patients, were studied, ethical approval and informed consent were not required.
Community-associated MRSA in Spain

Results

Bacterial isolates and patient population
Among the 8326 MRSA received at the SNRCS over the study period, 246 (2.9%) were considered CA-MRSA isolates on the basis of the CDC definition for CA isolates, PFGE pattern, spa type and MLST. Among these, 226 were PVL-positive and 20 were PVL-negative. The isolates corresponded to 13 different geographical areas in Spain without any geographical variation in the distribution of the isolates. The number/percentage of CA-MRSA isolates per year was as follows: 3/0.25, 2004; 2/0.20, 2005; 4/0.36, 2006; 9/1.1, 2007; 51/4.2, 2008; 36/4.1, 2009; 53/6.5, 2010; 40/5.4, 2011; and 48/8.8, 2012. The first PVL-positive CA-MRSA was detected in 2004 and the first PVL-negative CA-MRSA was detected in 2008; 7 out of the 20 PVL-negative isolates were recovered in 2012. A significant increase in the percentage of CA-MRSA isolates was observed when comparing the period 2004–07 with the period 2008–12 (P<0.05).

The isolates corresponded to 147 males and 89 females; for 10 patients, no information was available regarding gender. The distribution of isolates according to the age of the patients was as follows: 76 isolates from patients aged 0–10 years; 26 isolates from patients aged 11–20 years; 29 isolates from patients aged 21–30 years; 28 isolates from patients aged 31–40 years; 28 isolates from patients aged 41–50 years; 8 isolates from patients aged 51–60 years; and 16 isolates from patients aged >60 years. No information was available regarding the age of 35 patients. The origins of the isolates were as follows: wounds (n=202), blood (n=13), lower respiratory tract (n=5), ear (n=4), sterile fluids (n=4), others (n=4) and unknown (n=14).

Population structure of the CA-MRSA
The molecular characteristics of the isolates and the correlation between all typing methods are shown in Figure 1 and Table 1. Among the 246 CA-MRSA, 226 were PVL-positive and 20 were PVL-negative. The strains belonged to the PVL-positive clones ST8-IVc (69.9%), ST8-Iva-ACME-positive (USA300, 8.9%), ST8-Iva-ACME-negative (0.8%), ST30-IVc (4.5%), ST80-IVc (2.0%), ST5-IVc (1.2%) and other clones (ST59, ST72, ST88, ST642, ST1472 and ST1829; 4.5%), and to the PVL-negative ST398-V (8.1%). Among the 226 PVL-positive isolates, 199 (88%) harboured the agr type I, 23 (10.2%) the agr type III and 4 (1.8%) the agr type II. SCCmec type IV accounted for 99.1% (n=224) of the isolates and only two isolates carried SCCmec type II. Among the SCCmec type IV, the most frequent subtype was IVc, accounting for 86.2% (n=193) of the isolates, followed by subtype IVa (12.9%, 29 isolates) and subtype IVb (0.9%, 2 isolates). Among the 29 isolates carrying SCCmec type IVa, 22 were ACME-positive, representing the USA300 clone (9.7% of the isolates, see Figure 1). Genotyping by PFGE of the isolates divided ST8 into two major clonal lineages, clone A (with seven subtypes) and USA300, and five sporadic cases. Isolates of clones ST30 and ST80 (European clone) were detected by PFGE with a characteristic profile.

In general, we observed a high degree of concordance between the MLST clonal complexes (CCs) and the BURP groups. All isolates grouped as CC008 by spa typing belonged to ST8 with two exceptions. Isolates of spa group C2 belonged to ST30-IVc and isolates of spa group C3 belonged to ST80-IVc. All PVL-negative isolates belonged to spa type t011 and to agr group I, and were non-typeable by PFGE after SmaI digestion. The evolution of the most frequent clonal lineages over the study period is shown in Table 1.

Resistance profile
Among the 226 PVL-positive isolates, 84.5% were only resistant to β-lactams. The rates of resistance to other antimicrobials were 12.8% to erythromycin, 8.8% to tetracycline, 4.9% to clindamycin (63.6% inducible), 3.1% to ciprofloxacin, 2.0% to fusidic acid and 0.4% to gentamicin and tobramycin. Multiresistance occurred in 14 isolates (6.2%): in addition to β-lactams, 9 isolates were resistant to erythromycin and ciprofloxacin (6 corresponded to the USA300 clone, 1 to ST30 and 2 were sporadic cases), 4 isolates were resistant to erythromycin and tetracycline (2 corresponded to clone A and 2 were sporadic cases) and 1 isolate was resistant to tobramycin and tetracycline (sporadic case). Two isolates belonging to the ST8-Ivb clone were resistant to oxacillin and erythromycin. The 20 PVL-negative isolates were only resistant to tetracycline in addition to oxacillin. All CA-MRSA isolates were fully susceptible to trimethoprim/sulfamethoxazole, vancomycin, rifampicin, mupirocin, fosfomycin, nitrofurantoin, linezolid, daptomycin and tigecycline.

Discussion
In this study, we report the evolution of CA-MRSA in Spain and its present situation, demonstrating a significant increase in its incidence over the last 9 years from 0.25% in 2004 to 8.8% in 2012. In addition, we characterize the clonal lineages of CA-MRSA, and describe two predominant clones accounting for the majority of the isolates (ST8-IVc and USA300), but also an obvious level of genetic diversity among PVL-positive CA-MRSA, represented by different clones and sporadic cases. CA-MRSA infections began to emerge in patients without previous healthcare contact, predominantly children, in the 1990s, first in the USA and in Australia and then in Europe. The incidence of CA-MRSA infections increased rapidly during the late 1990s and the early twenty-first century, mainly in the USA, where some studies reported increases from 25% to 67% over a 5 year period in some institutions as well as an increase in the number of outbreaks affecting populations of healthy persons. At present, CA-MRSA infections are highly prevalent worldwide and, in some countries, CA-MRSA has also been introduced into hospitals, thus blurring the borders between CA and HA strains.

In Spain, despite the high prevalence of HA-MRSA (30.5%), the rates of CA-MRSA have been low, as demonstrated in several studies performed over the last decade. However, since the first description in 2003 of a PVL-positive CA-MRSA in this country, its incidence has increased. Two nationwide surveys performed in 2006 and in 2010 in Spain demonstrated that the prevalence of PVL-positive CA-MRSA increased significantly from 0.7% in 2006 to 7.2% in 2010. In this study we report that in 2012 this figure was even higher, at 8.8%. The emergence of PVL-negative CA-MRSA is more recent in Spain as well as in the rest of Europe. The ST398-V pig-associated clone was reported first in the Netherlands in 2006, and several years later in Spain with descriptions of sporadic cases, although...
Table 1. Evolution of PVL-positive CA-MRSA isolates over the study period (only isolates belonging to the most frequent clonal lineages are included)

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>ST8 with PFGE profile A*</th>
<th>no. (percentage of isolates)</th>
<th>ST30, no. (percentage of isolates)</th>
<th>ST8-USA300, no. (percentage of isolates)</th>
<th>ST398, no. (percentage of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>3 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2005</td>
<td>1 (50)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2006</td>
<td>3 (75)</td>
<td>1 (25)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2007</td>
<td>8 (88.8)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2008</td>
<td>41 (80.4)</td>
<td>1 (1.9)</td>
<td>1 (1.9)</td>
<td>4 (7.8)</td>
<td>1 (1.9)</td>
</tr>
<tr>
<td>2009</td>
<td>20 (55.5)</td>
<td>1 (2.8)</td>
<td>1 (2.8)</td>
<td>6 (16.6)</td>
<td>5 (13.8)</td>
</tr>
<tr>
<td>2010</td>
<td>38 (71.7)</td>
<td>1 (1.9)</td>
<td>1 (1.9)</td>
<td>3 (5.7)</td>
<td>5 (9.4)</td>
</tr>
<tr>
<td>2011</td>
<td>26 (65)</td>
<td>0</td>
<td>5 (12.5)</td>
<td>3 (7.5)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>2012</td>
<td>29 (60.4)</td>
<td>1 (2.1)</td>
<td>2 (4.2)</td>
<td>6 (12.5)</td>
<td>7 (14.6)</td>
</tr>
</tbody>
</table>

*PFGE profile A: see Figure 1.
retrospective studies have shown the presence of this clone in Spain since 2003.\textsuperscript{18,24,42} In this study, at the SNRCS we detected this LA clone for the first time in 2008, and its incidence seems to be increasing, since 35\% of the isolates were detected in 2012. It is interesting to note that the majority of the cases did not have any livestock contact, and only three of the ST398 isolates (15\%) were from areas with high levels of livestock production. Concerning the characteristics of the patients colonized or infected with CA-MRSA, the strains were more frequently isolated from males and from patients < 10 years old. The majority of children were immigrants from South America, or children born in Spain from immigrant South American mothers, a population with high rates of colonization with CA-MRSA, as we have described in another study.\textsuperscript{10} More than 50\% of the isolates were recovered from patients aged ≤ 30 years, as described in other studies.\textsuperscript{3,13,14} However, there was a distribution of isolates in all age groups. The majority of strains were isolated from wounds, as previously described, indicating that CA-MRSA is a frequent cause of skin and soft-tissue infections.\textsuperscript{3,13}

Resistance to other antimicrobials in addition to β-lactams was not frequent, and only 15.5\% of the isolates showed additional resistance to other antimicrobial groups. In general, resistance to erythromycin was associated with the ST8-IVc clone, the one most frequently found in this study, resistance to tetracycline was associated with the ST398-V clone and resistance to fusidic acid was associated with the European ST80-IVc clone, as described in other European studies.\textsuperscript{14} In Spain, previous studies performed in areas with high levels of livestock production have shown a multiresistance phenotype associated with the ST398-V clone.\textsuperscript{24,42} However, in our study the isolates belonging to this clone were only resistant to β-lactams and tetracycline, a fact probably due to the origin of the isolates in this study, with only three isolates coming from areas with high levels of livestock production.

Resistance to ciprofloxacin was not frequent (3.1\%), indicating that ciprofloxacin resistance is, in general, a marker of HA-MRSA.\textsuperscript{21} The rate of antimicrobial multiresistance was low and occurred in 14 isolates; however, it is of interest that, although the presence of the USA300 clone in our study was low, the majority of the multiresistant isolates belonged to this clone and also to the south-west Pacific ST30-IVc clone. USA300 is at present the predominant cause of MRSA infection in the USA, and it is replacing other strains as the main cause of HA-MRSA infection. In addition, multiresistance is frequent in this highly epidemic clone, as seen in a number of the isolates reported here.\textsuperscript{5,3} However, it is important to note that all isolates analysed in this study were fully susceptible to several oral and intravenous agents frequently used for the treatment of staphylococcal infections, including trimethoprim/sulfamethoxazole, linezolid, vancomycin, daptomycin and tigecycline.

Concerning the population structure of PVL-positive CA-MRSA, the most prevalent clone was ST8-IVc showing the PFGE profile A (73.9\%). This clone has been previously described as the most frequent in several studies performed in Spain, and has been associated with immigrants from South America, mainly from Ecuador.\textsuperscript{6,2} However, at present, ST8-IVc seems to be disseminated across the country. Due to the high genetic diversity found among isolates belonging to ST8, typing by PFGE is necessary in order to differentiate the circulating strains. We also detected other successful clones described worldwide, including the European clone ST80-IVc, the pandemic south-west Pacific ST30-IVc clone, the ST59-II clone (related to the Taiwan clone), the clones ST88-Iva and ST5-IVc (frequently found in Africa and sporadically in other parts of the world) and USA300, as well as a variety of sporadic cases, showing a high genetic diversity among the isolates.\textsuperscript{3,12–14,44} These results are similar to those described in a recent multicentre European study, in which a high level of genetic diversity in all countries was found, and as many as 10 different CA-MRSA clones from a single country.\textsuperscript{13} This situation found in Europe and in our study in Spain is in contrast to that described in the USA, where a single USA300 epidemic clone is the cause of the majority of CA-MRSA infections. Although the prevalence of the USA300 clone in our study was low, it was the second most frequent among the strains analysed (9.7\%). This clone appeared in 2008 and was uniformly distributed over the second period of the study. This emerging clone in Spain is a cause of concern since it shows multiresistance, is highly virulent and well adapted to the community, and has a high capacity for dissemination. In the European study mentioned above, it was the most frequent clone detected. In contrast, the European clone ST80-IVc was very infrequent, as it was in our study, represented by only five isolates (2.2\%).\textsuperscript{13} The pandemic south-west Pacific ST30-IVc clone has been previously detected in some European countries\textsuperscript{13,17} and in our study represented 4.8\% of the PVL-positive CA-MRSA isolates.

In relation to the population structure of PVL-negative CA-MRSA, in this study we only found the pig-associated ST398-V clone. The low rates of this clonal type (8.1\%) are in contrast to those found in other northern European countries, where ST398-V is among the most frequent CA-MRSA.\textsuperscript{5} In Spain, other studies from areas with high livestock production have also described high rates of this clonal lineage.\textsuperscript{12}

Finally, concerning the agr types, although the most frequent was agr I (88\%), the highest genetic diversity was found among agr II; agr type II was infrequently found, but it is the most frequent type in our country among HA-MRSA isolates, as we described in previous studies.\textsuperscript{21,30}

In conclusion, our report confirms a recent increase in the rates of CA-MRSA in Spain, the predominance of the PVL-positive ST8-IVc clone, the emergence of the USA300 clone showing multiresistance and a high level of genetic diversity among PVL-positive CA-MRSA isolates, probably reflecting the introduction of different clones by international travellers. In addition, we also confirm the recent emergence of and increase in the PVL-negative pig-associated ST398-V clone among humans. These figures underline the importance of performing continuous surveillance in order to determine the incidence and geographical distribution of CA-MRSA in Spain and to establish measures for preventing the dissemination of these strains in the community.

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Transparency declarations
None to declare.

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