J Antimicrob Chemother 2014; **69**: 45–50
doi:10.1093/jac/dkt327 Advance Access publication 24 August 2013

Journal of Antimicrobial Chemotherapy

Methicillin-resistant *Staphylococcus aureus* carrying the *mecC* gene: emergence in Spain and report of a fatal case of bacteraemia

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Received 26 June 2013; returned 8 July 2013; revised 15 July 2013; accepted 20 July 2013

Objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA) strains carrying the *mecC* gene have been reported from humans and animals from several European countries, but never from Spain. We describe the first isolates of *mecC*-positive MRSA of human origin collected in Spain and report a fatal case of bacteraemia.

Methods: Isolates were tested for phenotypic resistance using cefoxitin, tested for the *mecA*/*mecC* genes and toxin genes by PCR, and typed by staphylococcal cassette chromosome *mec* (SCC*mec*), PFGE, *spa*, multilocus sequence typing and *agr*.

Results: During 2008–13 five MRSA isolates showing resistance to cefoxitin and carrying the *mecC* gene were recovered at one hospital in Spain. In a review of 5505 *S. aureus* strains received at the Spanish National Reference Centre for Staphylococci from the same period, we found two additional *mecC*-positive isolates. The isolates were recovered from blood (two), wounds (two), joint fluid (one), urine (one) and a nasal swab (one). All MRSA were *mecA* negative, presented SCC*mec*XI, belonged to *agr* group III and to clonal complex 130, and were negative for the production of the toxin genes *tst1*, *eta*, *etb*, *etd* and Panton–Valentine leucocidin. Six isolates belonged to *spa* type t843 (ST130 and ST1945, where ST stands for sequence type) and one to *spa* type t6220 (ST1945). One patient with *mecC*-positive MRSA sepsis died in the emergency department.

Conclusions: We confirm the presence of MRSA carrying the *mecC* gene in Spain, the ability of this livestock-associated MRSA to cause severe infections in humans and the need to perform culture-based susceptibility testing methods in order to detect these emerging strains.

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

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of nosocomial infection, causing a variety of life-threatening syndromes, such as bacteraemia, endocarditis, wound infections and pneumonia.[1] For many years, MRSA infections were acquired in hospitals and other healthcare facilities; however, more recently new MRSA strains have emerged in the community, causing infections in patients without previous healthcare contact.[2–4] In addition, MRSA has also emerged in animals, particularly in livestock. The predominant livestock-associated MRSA (LA-MRSA) belongs to the clonal lineage ST398 (where ST stands for sequence type), a pig-associated clone that has also been found in calves, poultry and humans.[5,6]

Methicillin resistance is mainly due to the presence of the *mecA* gene, which encodes a modified 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* (SCC*mec*). SCC*mec* elements are highly diverse in their structural organization and genetic content, and have been classified into types and subtypes from type I to type XI.[7–11] In general, healthcare-associated MRSA carry SCC*mec* types I–IV, whereas type IV is also carried by community-acquired MRSA, as well as type VII. Type V is carried by LA-MRSA ST398, and recently the new SCC*mec*XI has been described in MRSA isolates from cattle and humans carrying a new *mecA* gene variant, $mecA_{LGA251}$, which has been renamed by the International Working Group on the Classification of Staphylococcal Cassette

Chromosome (SCC) Elements as *mecC*.[11–13] This novel LA-MRSA carrying the new *mecC* gene was first described in the UK and Denmark from cattle and, although very rarely, as a cause of infections in humans. Recently, the isolation of *mecC*-positive MRSA from animals and humans has also been described in Germany and in other countries in northern Europe, but never from southern Europe.[11,13–24] Moreover, to the best of our knowledge, this LA-MRSA has never been reported as a cause of fatal infections in humans and known virulence factors have been found to be absent in most of the *mecC* MRSA that have been described to date.

In this study, we report for the first time in Spain the presence of MRSA isolates causing human infections and carrying the *mecC* gene and describe a fatal case of bacteraemia.

Methods

Bacterial isolates

We characterized five MRSA isolates, recovered over the period 2008–13, from patients at Hospital Lucus Augusti (Lugo, Spain) showing resistance to cefoxitin, but a negative PBP2a test. We also performed a retrospective search for the presence of the *mecC* gene among a total of 5505 *S. aureus* human isolates received over the same period at the Spanish National Reference Centre for Staphylococci, previously characterized by *spa* typing. The retrospective search strategy was performed on the basis of presenting *spa* types identical or similar to those previously reported in *mecC*-positive isolates (t742, t843, t978, t1535, t1736, t6220, t629, t6293, t6300, t6386, t7485, t7734, t7945, t7946 or t7947).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed for all *mecC*-positive isolates by an automated broth microdilution method with the Pos Combo panel type 37 (MicroScan; Siemens, Sacramento, CA, USA), according to the manufacturer's guidelines. In addition, oxacillin and cefoxitin susceptibility was also determined by the disc diffusion method. We followed the MIC breakpoints according to the recommendations of the CLSI.[25] The isolates were tested for growth on chromogenic selective agar plates (chromID™ MRSA agar, bioMérieux, Marcy l'Étoile, France) as follows. Colonies grown on blood agar plates after overnight incubation were suspended in 0.9% NaCl solution with a turbidity equivalent to that of a 0.5 McFarland standard. A swab dipped in the suspension and then expressed, was spread onto the surface of the agar plate and incubated at 35–37°C for 24 h. Any colony growth was considered positive.

Detection of PBP2a

A commercial system, PBP2a Clearview (Inverness Medical Innovations, Scarborough, ME, USA), was used to detect the presence of PBP2a following the recommendations given by the manufacturer.

Methicillin resistance detection

The *mecA* gene was detected by PCR, as described by Geha *et al*.[26] For the detection of the *mecC* gene by PCR we used the primers described by García-Alvarez *et al*.[11] as follows: 5′-GAA AAA AAG GCT TAG AAC GCC TC-3′ (forward) and 5′ GAA GAT CTT TTC CGT TTT CAG C-3′ (reverse). Positive PCR products were sequenced to confirm the identification of the *mecC* gene.

PCR to confirm the presence of the novel SCCmecXI

The primer pair *ccr*B3$_{M10/0061}$ F1/*ccr*A1$_{M10/0061}$ R1 was used to detect the presence of *ccr*A1B3$_{M10/0061}$ as described by Shore *et al*.:[13] 5′-CTA CTT GAA GTT ATC CAA TC-3′ and 5′-CAC ATT ACT CGC TGA TTT AG-3′.

PFGE

All *mecC*-positive MRSA isolates were genotyped by PFGE after SmaI digestion of chromosomal DNA, prepared by using a modification of the protocol described by Cookson *et al*.[27] Analysis of the gels was performed according to the criteria of Tenover *et al*.[28] and a dendrogram was constructed with Molecular Analyst software (Bio-Rad) by using the Dice correlation coefficient and the unweighted pair-group method with averages with a position tolerance of 0.8%.

Detection of Panton–Valentine leucocidin (PVL) and other toxin genes

The PVL genes (*lukS-PV* and *lukF-PV*) were detected by PCR using the method described by Lina *et al*.[29] *S. aureus* ATCC 49775 (a PVL-positive strain) was used as a positive amplification control. The toxin genes *tst1*, *eta*, *etb* and *etd* were detected by PCR as previously described.[30,31]

Determination of accessory gene regulator (agr) type

A scheme of two PCRs based on the method described by Shopsin *et al*.[32] was used for the determination of the specific *agr* groups.

spa typing and BURP analysis

The polymorphic X region of the protein A gene (*spa*) was amplified from all MRSA isolates, as described previously.[33,34] The *spa* type was assigned by using Ridom StaphType software.[35] 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.

Multilocus sequence typing (MLST)

MLST was performed by the method described by Enright *et al*.[36] Allelic profiles and STs were assigned by using the MLST database (http://www.mlst.net).

Clinical information

Clinical information from patients admitted to Hospital Lucus Augusti was retrieved from medical records.

Consent from the Ethics Committee of Hospital Lucus Augusti was obtained. Since no patient data relating to the isolates recovered at the National Reference Centre for Staphylococci were used, consent from the Ethics Committee was not required.

Results

The detection at the hospital in Lugo (northern Spain) of a fatal case of bacteraemia produced by an MRSA isolate showing resistance to cefoxitin, but a negative PBP2a test, prompted a retrospective investigation of other isolates in this hospital with similar phenotypic characteristics. These isolates were sent to the reference laboratory for PCR detection of the *mecA* and *mecC* genes. A total of seven MRSA isolates harbouring the *mecC* gene were characterized

in this study: five isolates recovered at the hospital in Lugo and two additional isolates recovered in a retrospective search at the Spanish National Reference Centre for Staphylococci. The characteristics of the patients and the isolates are shown in Table 1. Among the five isolates recovered from patients admitted to the hospital in Lugo (northern Spain), four were from adults and one was from a healthy 3-year-old child, who was a carrier of MRSA in his nares. Origins of the four isolates obtained from adults were: blood (two isolates), wound (one isolate) and urine (one isolate). One of the patients with bacteraemia died in the emergency department despite adequate antimicrobial treatment, and his grandson was colonized in a household transmission. The remaining *mecC*-positive MRSA isolated from blood was probably colonizing the skin of the patient and was considered a contaminant of the blood culture (Case 6). The isolate recovered from the urine of another patient was also considered a colonizer since the patient did not present urinary symptoms (Case 7). Finally, in Case 3, the patient had cellulitis and recovered after treatment with co-trimoxazole. The isolates were recovered in 2008 (one isolate), 2009 (one isolate), 2012 (two isolates) and 2013 (one isolate, family transmission). The two additional isolates recovered in the search at the Spanish National Reference Centre for Staphylococci were obtained from a 63-year-old man (joint fluid) and a 50-year-old woman (infected wound), and were recovered in 2009 and 2012 from Andalucía (southern Spain) and Madrid (central Spain), respectively.

By using the MicroScan automated microdilution method, all isolates were resistant to cefoxitin (MIC >4 mg/L), but susceptible to oxacillin (MIC ≤0.25 mg/L), as well as susceptible to all other antimicrobials tested: gentamicin, tobramycin, amikacin, erythromycin, clindamycin, vancomycin, teicoplanin, ciprofloxacin, levofloxacin, tetracycline, fosfomycin, mupirocin, fusidic acid, nitrofurantoin, rifampicin, co-trimoxazole, linezolid and daptomycin. By using the disc diffusion method, all isolates were resistant to cefoxitin (inhibition zone <21 mm), but intermediate to oxacillin (inhibition zone 11–12 mm). All isolates grew on selective chromogenic agar plates, but gave a negative result when subjected to phenotypical test detection of PBP2a by means of the PBP2a test.

Figure 1 shows the molecular characteristics of the isolates. All isolates gave a PCR-negative result for the *mecA* gene, but were positive for the presence of the *mecC* gene and SCC*mec*XI, and all belonged to *agr* group III. None of the isolates carried any of the toxin genes tested: *tst1*, *eta*, *etb*, *etd* and PVL.

The five isolates from Hospital Lucus Augusti showed *spa* type t843, belonged to ST130 and were assigned to clonal complex (CC) 130. The two additional isolates recovered at the Reference Centre showed *spa* types t843 and t6220, belonged to ST1945 and were also assigned to CC130. PFGE analysis revealed that all isolates were closely related. Among the isolates from the hospital, two of them showed the same PFGE pattern, demonstrating household transmission between grandfather and grandson, and the remaining three were very closely related. The PFGE profile of the two isolates recovered at the Reference Centre were also closely related among themselves, but with a slightly different PFGE pattern (Figure 1).

Epidemiological investigation

Since our study was retrospective, except for Case 2, we were not able to obtain epidemiological data from the patients and therefore it was not possible to investigate any animal contact. In Case 2, the patient did not have any contact with cattle or other animals; however, we investigated if there was a household transmission, and 2 months after the isolation of the MRSA from the blood cultures of the patient nasal swabs were collected from all family members for culture; *mecC*-positive MRSA was isolated from the nares of the grandson of the patient, showing identical PFGE results (Figure 1). Both isolates showed indistinguishable SmaI PFGE patterns, confirming their genetic relationship. These results corroborate the household transmission of MRSA carrying the *mecC* gene. None of the other family contacts carried *mecC*-positive MRSA.

Discussion

In this study, we report for the first time in different geographical areas of Spain the presence of *mecC*-carrying MRSA strains isolated from humans. In addition we report a fatal case of bacteraemia due to this apparently low-virulence new LA-MRSA. The presence of *mecC*-positive MRSA has been previously reported from animals and humans from several northern European countries, including the UK,[11] Ireland,[13] Denmark,[17] France,[14] Germany,[15] Switzerland,[16] the Netherlands,[18] Sweden,[19,20] Norway,[21] Belgium[22] and Finland,[23] showing a wide geographical spread of these isolates. However, these strains have never been reported from southern Europe. Several studies have demonstrated that *mecC*-positive MRSA is relatively frequent in dairy cattle, suggesting that cows might provide a reservoir of infection, and farmers in contact with dairy cattle could be at risk of acquisition of these isolates.[11,13,17,37] Moreover, by the use of whole-genome sequencing, evidence of

Table 1. Characteristics of patients with *mecC*-positive MRSA isolates

Year of isolation	Region	Patient age (years)/sex	Site(s) of isolation	MLST	*spa* type[a]	Case no.	Comments
2013	Lugo	3/male	nasal swab (taken ambulatory)	ST130	t843	1	household transmission, colonization
2012	Lugo	64/male	blood, wound, urine	ST130	t843	2	fatal sepsis, cellulitis
2012	Lugo	85/male	wound	ST130	t843	3	cellulitis
2012	Madrid	50/female	wound	ST1945	t6220	4	infection
2009	Andalucía	63/male	joint fluid	ST1945	t843	5	arthritis
2009	Lugo	76/male	blood	ST130	t843	6	colonization
2008	Lugo	80/male	urine	ST130	t843	7	colonization

[a]All isolates belonged to CC130.

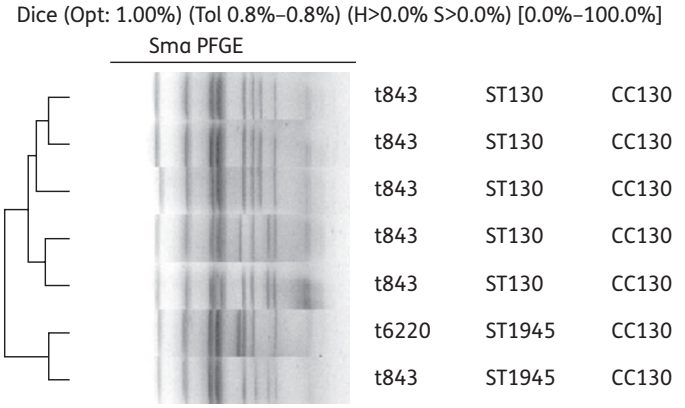

Figure 1. Molecular characteristics of the *mecC*-positive MRSA isolates. PFGE lanes 1–5, isolates recovered at Hospital Lucus Augusti; lanes 1 and 2, household transmission; lanes 6 and 7, isolates recovered at the Reference Centre.

the zoonotic transmission of *mecC*-positive MRSA from cattle to humans has been demonstrated.[38] In addition, *mecC*-carrying MRSA has also been found in sheep, which could also provide a reservoir.[11,17]

Most of the patients were elderly, six of the seven were males, and three out of the four patients attending our hospital with infection or colonization with this new MRSA were farmers living in rural areas. Since our study was retrospective, except for Case 2, we were unable to find epidemiological data to establish a relationship or contact between cattle or ovine livestock and the patients. Among the four patients attending our hospital, two were infected with *mecC*-carrying MRSA and the other two were probably colonized. The third colonized patient was the child (Case 1). The two additional cases recovered at the Reference Centre corresponded to patients with clinical infections. In general, skin and soft-tissue infections were the most frequent, as previously reported in other studies.[13,17] In Case 2, the patient had bacteraemia and a severe skin and soft-tissue infection, and despite adequate treatment he died in the emergency department. To our knowledge there are no previous reports of fatal bacteraemia due to *mecC*-positive MRSA. As previously described, *mecC*-positive MRSA are well adapted as human colonizers.[11,17] In fact, in Case 1 of our study, we demonstrate household transmission and the colonization of one family member (Case 2's grandson) who was a healthy carrier of the same strain 2 months later, as demonstrated by PFGE analysis. Children tend to have higher colonization rates, which may be transient or persistent and can last for years.[1]

Concerns have been raised about the virulence potential of *mecC*-positive MRSA; however, most studies have demonstrated an absence of pyrogenic toxin superantigen (PTSAg)-encoding genes, staphylococcal enterotoxin genes and exfoliative toxin genes (with the exception of the *edinB* gene) for isolates associated with the CC130 clonal lineage, the most frequent among *mecC*-positive MRSA.[11,17] However, in a recent study, other MRSA genotypes containing SCC*mec*XI (t5930 and t978) showed several PTSAg-encoding genes, including *tst1*.[18] The presence of PVL, an exotoxin associated with severe necrotizing pneumonia and skin and soft-tissue infections, has been associated with several lineages of community-acquired MRSA.[3–5,39] We investigated the presence of the PVL genes, *tst1* and exfoliative toxin genes (*eta*, *etb* and *etd*) in the seven strains isolated, but none of them presented any of these virulence factors. Although *mecC*-positive MRSA is a community-acquired MRSA, the absence of the PVL toxins seems to be another feature of this new MRSA. Other reports that have evaluated the presence of the PVL toxin in a large number of *mecC*-carrying MRSA strains found that none of them was PVL positive.[11,17] Our report of a fatal case of *mecC*-positive MRSA bacteraemia provides evidence that, even in the absence of these toxins, skin and soft-tissue colonization can progress to potentially fatal illness, and other virulence factors could be involved. Moreover, the detection of these MRSA isolates in infected wound sites and joint fluid suggests that they are adapted to humans and are capable of causing severe clinical disease.

Another cause of concern is the difficulty of detecting *mecC*-positive MRSA in a routine clinical microbiology laboratory by using automated methods. In our study, all isolates showed resistance to cefoxitin, but were fully susceptible to oxacillin by the MicroScan method and showed intermediate susceptibility to oxacillin by the disc diffusion method. Laboratories performing only automated susceptibility tests should be aware of this issue. In addition, all isolates tested negative for the expression of PBP2a, and were *mecA* negative, as expected, but grew on commercial selective chromogenic medium. However, other authors have reported low sensitivity of chromogenic media for the recovery of *mecC*-positive MRSA.[15] As previously reported for *mecC*-carrying MRSA, all isolates were fully susceptible to all antibiotics tested, except for β-lactams, although additional resistance to ciprofloxacin in some isolates has been reported.[15]

The molecular characterization of our seven MRSA isolates showed that all carried the *mecC* gene and SCC*mec*XI. Six isolates exhibited *spa* type t843 (ST130 and ST1945) and one exhibited *spa* type t6220 (ST1945), both *spa* types being assigned to CC130. As already described, *mecC*-positive MRSA strains have been found to belong to different lineages typically reported in cattle (i.e. CC130, CC1943, CC2361, CC705 and ST425, among others), CC130 being the most frequently reported.[11,13–24] Previous studies have indicated that *spa* type 843 has been identified in 60% of bovine isolates[11] and is the most frequent CC reported in the UK and Denmark,[11,17] as it was in our study. On the other hand, ST1945, a single-locus variant of ST130, has been reported as *mecC*-carrying MRSA in humans in the UK and in France.[11,14] Geographical variation in the frequency and host distribution of *mecC*-positive MRSA lineages is found in Europe, since in another report from Sweden none of the bovine isolates of *mecC*-positive MRSA showed *spa* type t843.[20] In our study, all isolates belonged to *agr* type III, as reported in other series.[15]

In conclusion, our report confirms the presence and potential virulence of this new MRSA CC130 lineage containing the *mecC* gene in Spain, at least from 2008. Due to the wide geographical distribution of novel MRSA strains and their rapid spread into different lineages, measures to detect and prevent dissemination of these strains are needed. Clinicians should be aware that patients living in rural areas could be colonized or infected with these strains, and clinical microbiology laboratories should perform traditional culture-based techniques in order to detect them and, if appropriate, include routine PCR analysis for the detection of the novel *mecC* gene.

Funding

This study was supported by internal funding.

Transparency declarations

None to declare.

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