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Staphylococcal cassette chromosome *mec* typing and *mec*A sequencing in methicillin-resistant staphylococci from Algeria: a highly diversified element with new mutations in *mec*A

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Genetic mechanisms of methicillin resistance are still relevant in staphylococci. The aims of this study are to assess the possible exchanges of staphylococcal cassette chromosome *mec* (SCC*mec*) among isolates of methicillin-resistant staphylococci (MRS) and to check for known or new mutations in *mec*A DNA. A total of 35 MRS non-repetitive isolates were recovered, including 20 *Staphylococcus haemolyticus*, 7 *Staphylococcus aureus*, 4 *Staphylococcus sciuri*, 2 *Staphylococcus saprophyticus* and 1 isolate each of *Staphylococcus xylosus* and *Staphylococcus lentus*. Only 16 of the 35 strains were assigned to known SCC*mec* types: 7 SCC*mec* VII, 6 SCC*mec* IV and 3 SCC*mec* III, with possible horizontal transfer of the SCC*mec* VII from methicillin-resistant *S. haemolyticus* to methicillin-susceptible *S. aureus*. *mec*A gene sequencing in ten selected isolates allowed description of nine punctual mutations, seven of which were reported for the first time. The most frequent mutation was G246E, identified in isolates of methicillin-resistant *S. aureus*, *S. sciuri*, *S. saprophyticus* and *S. lentus*. These results emphasized the high degree of genetic diversity of SCC*mec* element in MRS and describe new missense mutations in *mec*A, which might be important in understanding the evolution of methicillin and new β-lactam resistance.

INTRODUCTION

Coagulase-negative staphylococci (CNS) are normal inhabitants of human skin and mucous membranes, but their role as pathogens and their increasing impact have been recently recognized. CNS now represent one of the major nosocomial pathogens, with *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* being the most significant species (Becker *et al.*, 2014a). Since 1970, CNS have been recognized as aetiologic agents of a wide range of infections, including bacteraemia, central nervous system shunt infection, endocarditis, urinary tract infection, surgical site infections, endophthalmitis, foreign body infection and many other infections. Most frequently, patients with CNS infections are immunocompromised, with indwelling or implanted foreign bodies (Piette & Verschraegen, 2009; Becker *et al.*, 2014a).

A further alarming concern is about the spread of drug resistance within different species of staphylococci. Within resistance mechanism to β-lactam antibiotics, the *mec*A gene plays the keystone role, and confirming its presence has been established as a 'diagnostic dogma' to categorize an isolate as methicillin-resistant staphylococci (MRS). However, more recent studies have demonstrated that this approach does not take into account that phenotypic methicillin (and other β-lactam) resistance in *Staphylococcaceae* is conferred not only by *mec*A but also by the homologous genes *mec*B and *mec*C (Hiramatsu *et al.*, 2013; Becker *et al.*, 2014b). Even if the origin of *mec*A gene in methicillin-resistant *Staphylococcus aureus*

Abbreviations: CNS, coagulase-negative staphylococci; IWG-SCC, International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements; MGE, mobile genetic element; M-PCR, multiplex PCR; MR, methicillin-resistant; MRS, methicillin-resistant staphylococci; MRSA, methicillin-resistant *Staphylococcus aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*.

(MRSA) remains unknown, several studies have demonstrated its high similarity degrees with *mec*A of *Staphylococcus sciuri* and *Staphylococcus fleurettii*, suggesting that these might be sources of *mec*A precursor (Wu *et al.*, 1996; Tsubakishita *et al.*, 2010).

In MRS, *mec*A gene confers resistance to almost all β-lactam molecules by the synthesis of a new penicillin-binding protein, 'PBP2a'. However, ceftaroline and ceftobiprole, new cephalosporin molecules recently introduced and approved for the treatment of community-acquired pneumonia and complicated skin and soft tissue infections, are active against MRS. Ceftaroline causes an allosteric change of PBP2a so that a second molecule can bind to the newly exposed active site (Fishovitz *et al.*, 2014) and ceftobiprole can access the active site of PBP2a through its residue R2 (Chan *et al.*, 2015). In spite of these developments, *mec*A gene is still at the centre of interest for the scientific community because of recent reports on ceftaroline- and ceftobiprole-resistant MRSA isolates, mainly due to mutations in *mec*A (Chan *et al.*, 2015; Schaumburg *et al.*, 2016). This resistance is not due to a selective pressure following the introduction of these molecules but due to specific mutations in some *mec*A allotypes (Schaumburg *et al.*, 2016).

*mec*A is carried on a mobile genetic element (MGE), known as the 'staphylococcal cassette chromosome *mec*' or SCC*mec*. This MGE is inserted at the *att*B site downstream of *orf*X, a conserved gene among all staphylococci and described recently as ribosomal methyltransferase of the RlmH type (Boundy *et al.*, 2013). Various reports suggesting exchange of the SCC*mec* by horizontal transfer between different species of *Staphylococcaceae* have been published (Berglund & Söderquist, 2008; Bloemendaal *et al.*, 2010). According to the recent report of the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) (www.sccmec.org), 11 types (I–XI) of SCC*mec* have been assigned for *S. aureus*, which are defined by a combination of *mec* gene complex class and cassette chromosome recombinase gene (*ccr*) allotype. Probably, these SCC*mec* elements preceded MRSA and methicillin resistance as vectors for other genes in staphylococci. There are similar MGEs harbouring capsule group 1 factors of *S. aureus*, the fusidic acid resistance gene *fusB*/Q6GD50, several heavy metal resistance operons, enterotoxin H and the arginine catabolic mobile element, all of which can be found in different *Staphylococcaceae* members. This MGE is considered to confer better survival ability and increased virulence to the strain (Monecke *et al.*, 2012; Hiramatsu *et al.*, 2013).

In this study, we intended to assess the possible exchange of SCC*mec* elements among isolates of different methicillin-resistant (MR) *Staphylococcus* spp., which were recovered from patients periodically admitted at the same dialysis ward over several years. We proceeded by typing SCC*mec* elements and sequencing the *mec*A from different MRS isolates and subsequently by checking for new or recently described mutations.

METHODS

***Staphylococcus* spp., isolates and identification.** MRS were isolated by nasal swabbing from patients admitted to the Frantz-Fanon dialysis centre of Béjaia city (Algeria), during the first 6 months of 2012. They were admitted periodically, once to three times a week, to undergo dialysis sessions lasting 4 h. Swabs were cultured on mannitol salt agar plates for 24 h at 37 °C. A total of 105 patients have all been screened at the beginning of the study and then once a month only if the last culture was negative for MRS. Strains were identified by Gram staining, catalase test, coagulase test in rabbit plasma (Remel), agglutination test and API20Staph Gallery (bioMérieux). Then, identification was completed by a PCR-restriction fragment length polymorphism using the '*gap*' gene as previously recommended by Yugueros *et al.* (2001) and Karakulska *et al.* (2012).

Antimicrobial drugs tested. The agar dilution method described in the Clinical Laboratory Standards Institute guidelines was used. For CNS, oxacillin resistance is considered for MICs greater than or equal to 0.5 μg ml^{-1}. For *S. aureus* and *Staphylococcus lugdunensis*, MICs should be greater than or equal to 4 μg ml^{-1}. The antimicrobial drugs (Oxoid) included oxacillin (1 μg), cefoxitin (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), linezolid (30 μg), rifampicin (5 μg), tetracycline (30 μg), teicoplanin (30 μg), tobramycin (10 μg), trimethoprim–sulfamethoxazole (1.25/23.75 μg) and vancomycin (30 μg). *S. aureus* and MRSA ATCC 29213 and 43300, respectively, were used as quality control strains (CLSI, 2011).

SCC*mec* typing. All MRS isolates were subcultured on mannitol salt agar, and DNA was extracted from few colonies, using lysostaphin, proteinase K and RNase (Promega). For all PCRs, 1 μl of lysate was used. Methicillin resistance was confirmed by PCR amplification of *mec*A DNA (Milheiriço *et al.*, 2007a). Then, each isolate was submitted to SCC*mec* typing and SCC*mec* type IV subtyping methods. The first multiplex PCR (M-PCR) was performed to identify the SCC*mec* types I to VI (Milheiriço *et al.*, 2007a). The PCR products were then visualized on 3 % Tris/borate/EDTA-agarose gel. If not typeable by the first one, we used a second method based on a system of six M-PCRs to identify the *ccr* gene complex (*ccr*), the *mec* gene complex (*mec*) and specific structures in the junkyard (J) regions (Kondo *et al.*, 2007). The results were interpreted according to the guidelines of the IWG-SCC (www.sccmec.org). A further M-PCR was performed, as previously described by Milheiriço *et al.* (2007b), to distinguish between the different SCC*mec* IV subtypes. Identity of SCC*mec* VII was already confirmed in another study (Djoudi *et al.*, 2014). MLST was performed on MRSA strains (Enright *et al.*, 2000). Statistical analysis was performed to assess the association of the SCC*mec* types with different species isolated, using the chi-squared test or Wilks G^2 test, when necessary. $P<0.05$ was considered significant.

***mec*A DNA amplification and sequencing.** The *mec*A gene was amplified and sequenced in representative isolates, three *S. aureus*, two *S. haemolyticus*, two *S. sciuri* and one each of *Staphylococcus saprophyticus*, *Staphylococcus lentus* and *Staphylococcus xylosus*. DNA amplification was carried out as recommended using a previously published set of four pairs of primers (Eurofins), leading to four overlapping fragments (Malik *et al.*, 2006). *mec*A DNA fragments were purified with the CENTRI-SEP columns (Applied Biosystems) and sequenced using the ABI Prism Big-Dye Terminator v1.1 (Applied Biosystems). The conditions of amplification and sequencing were those previously described by Malik *et al.* (2006).

Sequence alignment. After partial sequencing, *mec*A genes were reconstructed, using the overlapping regions, by the BioEdit software. Sequence alignments were performed using CLUSTALW, as implemented in MEGA 6 (www.megasoftware.net), using as reference the *mec*A sequence of N315 (SA0038). Then, the aligned sequences and their respective coded proteins were analysed to check for any mutations.

RESULTS

MRS isolates and antimicrobial susceptibility

A total of 35 non-repetitive MRS isolates were recovered from 105 patients, including 20 isolates of *S. haemolyticus*, 7 *S. aureus*, 4 *S. sciuri* and 2 *S. saprophyticus*, and 1 isolate of *S. xylosus* and *S. lentus*, respectively (Table 1). Thirteen isolates were from patients under antibiotic treatment.

Resistance to tetracycline (14/35, 40 %), erythromycin (9/35, 25.7 %) and tobramycin (7/35, 20 %) was the most prevalent. Ten isolates (28.6 %) were susceptible to all antibiotics tested other than β-lactams. Epidemiological data and susceptibility testing results are shown in Table 1.

SCC*mec* typing

Only 16 (46 %) of the 35 strains could be assigned to known SCC*mec* types (Table 1). The first typing method characterized only nine strains, six with SCC*mec* IV in MRSA (four isolates) and *S. saprophyticus* (two isolates) and three SCC*mec* III in *S. sciuri.* The second method characterized a *ccr* complex type 5 (C1) and *mec* complex type (C1) in seven other strains, attributed to MRSA (three isolates) and MR *S. haemolyticus* (four isolates). According to the guidelines of the IWG-SCC (www.sccmec.org), the respective strains belong to the SCC*mec* VII. This result was confirmed by sequencing the SCC*mec* of MRSA isolate no. 15 in our last study (Djoudi *et al.*, 2014) and comparison with a sequence deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/AB462393.1) (Takano *et al.*, 2008) exhibiting 100 % nucleotide identity. Statistical analysis confirmed the association of type VII to MR *S. haemolyticus* and type IV to MRSA (P=0.027).

Nineteen isolates (54 %) were not typeable by both methods, presenting unusual profiles. For instance, in *S. haemolyticus* strain no. 5, we only identified the *ccr* complex type 1 (A1B1), and in *S. sciuri* strain no. 22, only *mec* complex B was amplified. More surprisingly, *S. lentus* strain no. 8 proved to carry a *ccr* complex type C and a *mec* complex type B. These strains carried also the *mec*A gene. Moreover, in six isolates of *S. haemolyticus*, only *mec*A was amplified, and no further known component of SCC*mec* element was amplifiable.

*mec*A sequencing and alignment

Within the 10 *mec*A genes sequenced in MRS isolates, alignment by CLUSTALW (MEGA 6) showed a high homology with *mec*A gene of *S. aureus* reference strain N315. Nevertheless, nine mutations were revealed, including seven mutations reported for the first time, to the best of our knowledge (Table 2).

The *S. sciuri* strain no. 22 and *S. xylosus* strain no. 13 carried a *mec*A with 100 % homology to *S. aureus* N315 reference strain. However, their SCC*mec* elements have not been characterized. *S. haemolyticus* strain no. 4, carrying only *mec*A with no further component of the SCC*mec* element, shared an identical mutation with *S. sciuri* strain no. 31 (I154 N), carrying SCC*mec* III. The isolates of MRSA no. 6 (SCC*mec* IVh) and *S. lentus* no. 8 (SCC*mec* unidentified) shared a *mec*A gene, with three identical mutations: N71K, Y223D and G246E. *S. sciuri* strain no. 31 with SCC*mec* III displayed the highest number of mutations: I154N, F231I, G246E and I278F. The most frequent mutation was G246E, which was identified in two isolates of MRSA and one isolate each of *S. sciuri*, *S. saprophyticus* and *S. lentus*, respectively (Table 2).

DISCUSSION

High nasal carriage rates of staphylococci in patients under dialysis are well acknowledged around the world. MR *S. haemolyticus* is the more frequently isolated species. Indeed, according to De Mattos *et al.* (2003) and Barbier *et al.* (2010), 23 % and 12 % of isolates belonged to this species, respectively. Barros *et al.* (2011) have considered *S. haemolyticus* as an important hospital pathogen, as well as a carrier of methicillin resistance genes. Our results significantly differ to the findings by Al-Bakri *et al.* (2013), with 80 % isolates of MR *S. epidermidis* and only 2 % of MR *S. haemolyticus.* We also report species rarely isolated from human nasal specimens, like MR *S. lentus* and *S. sciuri*, already reported to be causative agents of endocarditis (Hedin & Widerström, 1998) and peritonitis (Rivera *et al.*, 2014), respectively. MRS isolates proved to be generally susceptible to antibiotics other than β-lactams. They exhibited significant resistance only toward tetracycline, erythromycin and tobramycin, according to reports from elsewhere (De Mattos *et al.*, 2003; Barros *et al.*, 2011; Al-Bakri *et al.*, 2013).

SCC*mec* IV was predominant in our MRSA strains, a common observation within MR staphylococci isolates in most studies (Ruppé *et al.*, 2009; Garza-González *et al.*, 2010; Al-Bakri *et al.*, 2013; Djoudi *et al.*, 2014). The two MR *S. saprophyticus* isolates carried SCC*mec* IVa; however, Söderquist & Berglund (2009) reported that, in this species, SCC*mec* III was predominant. In our study, SCC*mec* III was identified in three isolates of MR *S. sciuri*, according to literature (Machado *et al.*, 2007; Söderquist & Berglund, 2009). SCC*mec* IVa was recently described as the most prevalent type among MR *S. epidermidis* in clinical and nasal carriage isolates (Garza-González *et al.*, 2010; Al-Bakri *et al.*, 2013). SCC*mec* type VII, with *mec* complex type C1 and *ccr* complex type C1, was identified in four MR *S. haemolyticus* and three MRSA isolates. A study from four countries including Algeria showed that class C *mec* complexes were significantly associated to *S. haemolyticus* than to *S. epidermidis* (P=0.001) because classes C1 and C2 were found in 16/19 *S. haemolyticus* strains (Ruppé *et al.*, 2009). Furthermore, MR *S. haemolyticus* has been putatively identified as a reservoir of *mec* complex C and *ccr*C for the assembly of SCC*mec* elements (Bouchami *et al.*, 2012). Characterization of the SCC*mec* type VII in ST5-MRSA isolates can be considered as possible evidence for horizontal transfer of this MGE from MR *S. haemolyticus* to methicillin-susceptible *S. aureus*, maybe

Table 1. Epidemiological data, drug susceptibility pattern and SCC*mec* typing of 35 MRS isolates, recovered from nasal carriage of patients under dialysis treatment in Algeria

Isolate number	Species	Patient age/ gender	Weekly dialysis frequency	Department attendance since	Antibiotic exposure	Resistance profile	*mec*A*	*ccr* complex	*mec* complex	SCC*mec*
1	*S. haemolyticus*	58/F	3	4 years	β-Lactams	TET	+*	C1	C1	VII
2	*S. haemolyticus*	35/M	2	9 years	–	TET, ERY	+	C1	C1	VII
3	*S. saprophyticus*	79/F	2	3 years	Macrolides	TET	+*	2 (A2B2)	B	IVa
4	*S. haemolyticus*	64/F	2	4 months	–	/	+*	/	/	*mec*A only
5	*S. haemolyticus*	62/F	1	1 month	–	TOB	+	1 (A1B1)	/	NT
6	*S. aureus*	32/F	1	3 years	Macrolides	TET, ERY, TOB	+*	2 (A2B2)	B	IVh
7	*S. haemolyticus*	46/F	2	5 years	β-Lactams	ERY, CLI	+	C1	C1	VII
8	*S. lentus*	28/F	2	4 years	–	ERY	+*	C1	B	NT
9	*S. haemolyticus*	68/M	1	9 months	–	TET, TOB	+	C1	/	NT
10	*S. haemolyticus*	64/F	3	5 years	Tetracyclines	/	+	/	B	NT
11	*S. haemolyticus*	46/F	2	3 years	–	/	+	/	/	*mec*A only
12	*S. haemolyticus*	63/M	2	3 years	β-Lactams	ERY, TOB, RIF	+	C1	C1	VII
13	*S. xylosus*	63/F	3	18 years	β-Lactams	/	+*	/	/	NT
14	*S. haemolyticus*	32/M	3	4 years	Macrolides	TET	+	/	/	*mec*A only
15	*S. aureus*	67/F	3	7 years	–	TET	+*	C1	C1	VII
16	*S. haemolyticus*	86/M	1	2 years	–	/	+	/	/	NT
17	*S. haemolyticus*	81/F	2	1 month	β-Lactams	/	+	/	/	*mec*A only
18	*S. haemolyticus*	37/F	1	1 month	–	TET	+	/	/	*mec*A only
19	*S. haemolyticus*	32/F	1	3 years	Macrolides	ERY, TOB	+	/	/	NT
20	*S. haemolyticus*	61/F	2	7 months	β-Lactams	RIF	+	/	/	NT
21	*S. haemolyticus*	74/M	1	11 months	–	/	+	/	/	NT
22	*S. sciuri*	50/F	1	6 months	–	RIF	+*	/	B	NT
23	*S. aureus*	67/F	3	7 years	–	TET	+	C1	C1	VII
24	*S. haemolyticus*	79/M	1	4 months	–	/	+	/	/	NT
25	*S. haemolyticus*	77/F	1	1 month	–	/	+	1 (A1B1)	/	*mec*A only
26	*S. haemolyticus*	64/F	2	3 years	–	/	+	/	/	NT
27	*S. aureus*	67/F	3	7 years	–	TET	+	C1	C1	VII
28	*S. aureus*	32/F	1	3 years	β-Lactams	ERY, TET, TOB	+	2 (A2B2)	B	IVh
29	*S. aureus*	66/M	2	2 years	–	TET, CIP	+*	2 (A2B2)	B	IVc
30	*S. sciuri*	33/F	2	1 year	–	RIF	+	3 (A3B3)	A	III

Table 1. cont.

Isolate number	Species	Patient age/ gender	Weekly dialysis frequency	Department attendance since	Antibiotic exposure	Resistance profile	*mec*A*	*ccr* complex	*mec* complex	SCC*mec*
31	*S. sciuri*	63/F	3	3 years	–	CLI	+*	3 (A3B3)	A	III
32	*S. sciuri*	33/F	3	4 years	–	TOB	+	3 (A3B3)	A	III
33	*S. aureus*	34/M	1	4 years	–	TET, ERY	+	2 (A2B2)	B	IVc
34	*S. saprophyticus*	79/F	2	3 years	β-Lactams	TET	+	2 (A2B2)	B	IVa
35	*S. haemolyticus*	71/F	2	3 months	–	ERY	+	/	/	NT

F, female; M, male; NT, not typeable, *mec*A positive with unknown structures; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; RIF, rifampicin; TET, tetracycline; TOB, tobramycin.
**mec*A sequenced.

inside our dialysis ward, and this hypothesis is supported by statistical analysis (P=0.027). The same exchange has already been observed, with horizontal transfer of SCC*mec* from MR *S. haemolyticus* to methicillin-susceptible *S. aureus* strains, resulting in a new MRSA clone with an epidemic potential (Berglund & Söderquist, 2008). These findings, in accordance with other studies, confirm that *S. haemolyticus* can be considered as a reservoir of *ccr*C and a source for generating SCC*mec* VII (Ruppé *et al.*, 2009; Urushibara *et al.*, 2011; Bouchami *et al.*, 2012). In a similar way, MR *S. epidermidis* appears to be a reservoir of *ccr*B and SCC*mec* IV (Ruppé *et al.*, 2009; Barbier *et al.*, 2010; Bloemendaal *et al.*, 2010). With these observations, we suggest that the type of SCC*mec* is defined by species where it was generated. Accordingly, it could be stated that SCC*mec* VII comes from MR *S. haemolyticus* and SCC*mec* IV comes from MR *S. epidermidis*.

We also detected an unusual combination of *ccr*C complex with *mec*B complex in an MR *S. lentus* isolate. This is a rare finding but previously reported in two isolates of MR *S. epidermidis* and *Staphylococcus hominis* (Ruppé *et al.*, 2009). However, 19 of 35 isolates were untypeable, a common observation within MRS, underlying the high degree of variability in genetic background of this genus and the great capacity of generating new SCC*mec* types (Ruppé *et al.*, 2009; Garza-González *et al.*, 2010; Urushibara *et al.*, 2011; Al-Bakri *et al.*, 2013). Such findings underline the importance of studying prevalence and composition of SCC*mec* in MRS, in order to understand and predict the evolution of methicillin

Table 2. Missense mutations in *mec*A DNA from 10 MRS isolates, recovered from nasal carriage of patients under dialysis treatment in Algeria

Isolate number	Identification	ST	*ccr* complex	*mec* complex	SCC*mec*	Mutations in *mec*A DNA	Mutation in PBP2a protein/domain of mutation (Kelley *et al.*, 2015)
1	*S. haemolyticus*	ND	C1	C1	VII	675T>A	S225R/nPBD
3	*S. saprophyticus*	ND	2 (A2B2)	B	IVa	737G>A	G246E/nPBD
4	*S. haemolyticus*	ND	/	/	*mec*A only	461T>A, 1830T>A	I154N/nPBD
6	*S. aureus*	80	2 (A2B2)	B	IVh	737G>A, 1645T>A	G246E/nPBD, L549I/TD
8	*S. lentus*	ND	C1	B	NT	213T>A, 423C>T, 667T>G, 737G>A	N71K/nPBD, Y223D/nPBD, G246E/nPBD
13	*S. xylosus*	ND	/	/	NT	None	None
15	*S. aureus*	5	C1	C1	VII	675T>A, 1768T>C	S225R/nPBD, S590P/TD
22	*S. sciuri*	ND	/	B	NT	None	None
29	*S. aureus*	80	2 (A2B2)	B	IVc	213T>A, 423C>T, 667T>G, 737G>A	N71K/nPBD, Y223D/nPBD, G246E/nPBD
31	*S. sciuri*	ND	3 (A3B3)	A	III	461T>A, 691T>A, 737G>A, 832A>T	I154N/nPBD, F231I/nPBD, G246E/nPBD, I278F/nPBD

ND, Not done; nPBD, non-penicillin-binding domain; NT, not typeable, *mec*A positive with unknown structures; ST, sequence type; TD, transpeptidase domain.

resistance in *S. aureus*. These 19 untypeable isolates include six MR *S. haemolyticus* with only *mec*A DNA amplified, without any known fragment of staphylococcal chromosomal cassette elements. Non-*mec*A-containing staphylococcal cassette chromosomes were described in other studies (Mongkolrattanothai *et al.*, 2004; Hanssen & Sollid, 2006). However, to our knowledge, no reports on only-*mec*A-containing isolates were reported. However, whole-genome sequencing is needed to confirm or deny this result.

Alignment and comparison of *mec*A DNA sequences to the reference strain revealed infrequent mutations, confirming the high stability of this gene in different species of *Staphylococcus* Malik *et al.* (2006) reported that *mec*A genes from animal isolates were identical to those found in human MRS strains, presenting 100 % homology. A further study identified 32 *mec*A allelic variants sharing $\geq$95 % nucleotide similarity (Monecke *et al.*, 2012). In sight of these findings, consistently, two hypotheses can be considered. The first one is that evolution of *mec*A inside the SCC*mec* element, due to antibiotic exposure, can explain these mutations. The second one, even if never reported, we suggest that a *mec*A allele can be integrated, independently in any type of SCC*mec* element and an SCC*mec* element can carry any one of different *mec*A alleles, without any specificity.

A total of nine missense mutations were confirmed; two had been previously described. The first one is G246E, reported in Greece (Mendes *et al.*, 2012), Thailand (Alm *et al.*, 2014) and Switzerland (Kelley *et al.*, 2015). A limited number of mutations on *mec*A DNA can be of utmost importance; recently, Schaumburg *et al.* (2016) reported that the high rates of resistance to the new cephalosporins, ceftaroline and ceftobiprole, in isolates belonging to ST241, were associated with a combination of three missense mutations of PBP2a (N146K–N204K–G246E) in Africa. In this study, we confirm the presence of this G246E missense mutation in the European clone ST80-MRSA-IV and also in MR *S. haemolyticus, S. lentus* and *S. sciuri*. The second previously described mutation is S225R, which seems to be present in West and Central Africa, but with no effect on ceftobiprole and ceftaroline resistance (Schaumburg *et al.*, 2016). We confirm here that this mutation is also present in ST5-MRSA-VII and MR *S. haemolyticus*.

In addition to these known mutations, we report, to the best of our knowledge, new missense mutations in many species of MRS that have not been already described. It would be interesting to study the effect of such changes in the PBP2a on resistance to fifth-generation cephalosporins, especially because some of them are located in the non-penicillin-binding protein domain. Mutations in this part of PBP2a have already been reported as causing allosteric changes, conferring resistance to ceftaroline through an alteration of the salt bridge network at the allosteric site, thereby deteriorating the access of a second ceftaroline molecule to the active site (Fishovitz *et al.*, 2014).

Our results, together with previous reports, emphasize the high degree of genetic diversity in *mec* gene complex and *ccr* genes in MRS. Observing the inability to characterize the different SCC*mec* types by the two methods used, an alternative strategy to identify increasing new types of SCC*mec*, e.g. by next-generation sequencing, will likely be needed in the near future. More widespread analysis and data accumulation would help to understand the mechanisms of horizontal transfer of SCC*mec* among staphylococci and to discover from which species different elements originate. However, even if our results describe new mutations in *mec*A gene and show that its allotypes seem unrelated to the type of SCC*mec* element, different missense mutations herein described might certainly contribute to understanding of the evolution of methicillin and new β-lactam resistance by studying the correlation between these mutations and the resistance levels.

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