Estudio multicéntrico de cepas clínicas de SARM sensibles a antibióticos no-β-lactámicos: líneas genéticas y producción de la leucocidina de Panton-Valentine (LPV)

Multicenter study of clinical non-β-lactam-antibiotic susceptible MRSA strains: genetic lineages and Panton-Valentine leucocidin (PVL) production

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RESUMEN

Introducción: *Staphylococcus aureus* resistente a meticilina (SARM) es una de las principales causas de infecciones, **tanto relacionadas con la asistencia sanitaria como asociadas** a la comunidad (**AC**). Considerando la sensibilidad a antibióticos no β-lactámicos como marcador **potencial** de SARM-*mecC* y SARM-AC, el objetivo de este estudio multicéntrico fue determinar la frecuencia y líneas genéticas de cepas SARM sensibles a antibióticos no β-lactámicos (SARM-SNBL) en un estudio multicéntrico en España.

Métodos: Se analizaron 45 cepas SARM-SNBL procedentes de 12 hospitales obtenidas durante **enero-junio de** 2016. El tipado molecular se realizó mediante caracterización del *spa*-tipo, grupo *agr* y multi-locus-sequence typing. Mediante PCR/secuenciación se determinaron los genes: de resistencia a meticilina (*mecA* y *mecC*), del sistema de evasión inmune humano (***scn-chp-sak-sea-sep,* usando *scn* como marcador del sistema IEC**) y de la leucocidina de Panton-Valentine (LPV).

Resultados: El fenotipo SARM-SNBL fue infrecuente en los 12 hospitales analizados (frecuencia SARM-SNBL/SARM: 0,3%-7,7%). Todas las cepas fueron *mecA*-positivas (ninguna *mecC*). Se detectaron 22 *spa*-tipos diferentes, siendo el *spa*-t008/*agr*-I el prevalente (27%). Los principales complejos clonales fueron (**CC/%): CC8/42,2%, CC5/33,3% y CC30/4,4%**, **destacando las secuencias tipo ST8 y ST5 como mayoritarias**. El 38% de las cepas fue LPV-positiva (*spa*-tipos t008, t024, t019, t044, t068, t318 y t3060). El 78% de las cepas fue IEC-positivo: tipo-B (n=17), tipo-F (n=16), tipo-A (n=1) y tipo-E (n=1); 10 aislados fueron *scn*-negativos.

Conclusión: El fenotipo SARM-SNBL es poco frecuente en los hospitales analizados; aunque no se detectaron cepas *mecC*-positivas, este fenotipo puede ser un buen

marcador de aislados SARM LPV-positivos, frecuentemente asociados a infecciones por SARM-AC.

Palabras clave: SARM, sensible-no-betalactámico, *mecA*, *mecC*, LPV, CC8.

ABSTRACT

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of **healthcare-associated** (**HA**) and community-acquired (**CA**) infections. Considering non-β-lactam susceptibility as **potential** marker for *mecC*-MRSA and CA-MRSA, the aim of this multicenter study was to determine the frequency and the associated genetic lineages of non-beta-lactam-antibiotic susceptible MRSA (NBLS-MRSA) strains in a multicenter study in Spain.

Methods: A collection of 45 NBLS-MRSA strains recovered during **January-June of 2016** from 12 Spanish hospitals was analyzed. Molecular typing through *spa*-type characterization, *agr* group and multi-locus-sequence typing was performed. Methicillin resistance genes (*mecA* and *mecC*) as well as immune evasion cluster (***scn-chp-sak-sea-sep,* considering *scn* gene as the marker of IEC system**) and Panton-Valentine leucocidin (PVL) genes were determined with PCR/sequencing.

Results: The NBLS-MRSA phenotype was uncommon in the 12 hospitals analyzed (NBLS-MRSA/MRSA frequency: 0.3%-7.7%). All strains contained the *mecA* gene (and none *mecC*). Twenty-two different *spa*-types were detected among NBLS-MRSA strains, with *spa*-t008/*agr*-I as the most prevalent (27%). The main clonal complexes were (CC/%): **CC8/42.2%, CC5/33.3% and CC30/4.4%, with ST8 and ST5 as the main sequence types.** The PVL toxin was present in 38% of strains (with *spa*-types

t008, t024, t019, t044, t068, t318 and t3060). The IEC genes were detected in 78% of strains: IEC type-B (n=17), type-F (n=16), type-A (n=1) and type-E (n=1); 10 **MRSA** isolates were *scn*-negative.

Conclusion: The NBLS-MRSA phenotype is uncommon in the analyzed hospitals; although no *mecC*-positive strains were detected, it could be a good marker for MRSA PVL-positive isolates (38%), frequently associated to CA-MRSA infections.

Keywords: MRSA, Susceptible to non-beta-lactam antibiotics, *mecA*, *mecC*, PVL, CC8.

Introduction

Staphylococcus aureus can colonize the skin and nose of humans and animals, but it can also be an important opportunistic pathogen associated with a wide spectrum of diseases. This microorganism can acquire several antimicrobial resistance mechanisms, being methicillin resistance one of the most relevant. Methicillin-resistant *S. aureus* (MRSA) are capable of survive in presence of β-lactam antibiotics due to the acquisition of staphylococcal cassette chromosome *mec* elements (SCC*mec*) carrying the *mecA* gene.[1] In 2011, a new variant of the *mecA* gene, designated *mecC* or $mecA_{LGA251}$, which also confers methicillin resistance, was identified.[2] One of the most significant characteristics of *mecC*-MRSA is its usual susceptibility to non-β-lactam antibiotics.[3] The *mecC* gene was initially detected in human and bovine populations in Denmark and the UK,[2] but since then, this mechanism has been detected in MRSA isolates of humans and animals in many European countries.[3,4] In Spain, only a few cases of human infections have been reported,[5–7] although it has been detected in wild and livestock animals, as well as in water.[4,8–12] Additionally, other PBP2a-encoding gene named *mecB*, often found in a transposon *mec* complex (*Tn*6045) in *Macrococcus caseolyticus*, has also been found in *S. aureus*, but to date, only one case in humans has been reported in Germany.[13]

It is well known that MRSA is a major cause of **healthcare-associated** (HA-MRSA) infections, and also has a main role in community-acquired (CA-MRSA) infections. Since 1990s, MRSA infections in individuals without contact with health institutions have been reported, with USA300 strain as the CA-MRSA epidemic clone in the United States (ST8-IV clone).[14] There are some differences between HA-MRSA and CA-MRSA isolates, as the profile of antibiotic resistance. CA-MRSA usually carry smaller SCC*mec* elements containing less antimicrobial resistance genes and more virulence

93 factors. Consequently, CA-MRSA strains are more susceptible to antibiotics others than
94 β-lactams; moreover, they frequently carry the *lukF/lukS* genes encoding the Panton-
95 Valentine leucocidin (PVL), a two-component system with a toxin with cytolytic
96 activity. Meanwhile, HA-MRSA clones are usually multidrug-resistant and
97 unfrequently produce PVL toxin.[15]

98 Susceptibility for non-β-lactam antibiotics in MRSA isolates could be a marker for both
99 *mecC* mechanism and CA-MRSA variant. For this reason, we focused this study on
100 determining the frequency of non-β-lactam-susceptible MRSA (NBLS-MRSA)
101 throughout the analysis of the genetic lineages, methicillin resistance mechanism and
102 PVL gene detection in isolates recovered from 12 Spanish hospitals during a six-month
103 period.

104 **Methods**

105 *Selection of strains*

106 MRSA isolates recovered from clinical and epidemiological samples during a six-month
107 period (January-June 2016) were subjected to antimicrobial susceptibility testing. In
108 addition to β-lactams, other 14 agents were tested (erythromycin, clindamycin,
109 ciprofloxacin, levofloxacin, tetracycline, trimethoprim/sulfamethoxazole, vancomycin,
110 teicoplanin, linezolid, daptomycin, fusidic acid, mupirocin, gentamicin, and
111 tobramycin). All NBLS-MRSA isolates were included in this multicenter study, where
112 12 hospitals located in seven regions of Spain took part (full names of hospitals in Table
113 1). A final collection of 45 NBLS-MRSA isolates was obtained from the different
114 institutions, and they were transferred to the University of La Rioja (Logroño, Spain)
115 for further characterization. All strains were subcultured for 24 hours at 37ºC in brain-
116 heart infusion (BHI) agar and were stored frozen at -80ºC. The total number of *S.*

aureus and MRSA isolates of different patients recovered in the 12 hospitals in the six-month period was recorded for analysis.

Molecular typing

The 45 NBLS-MRSA isolates were subjected to *spa* (*S. aureus* protein A) characterization by PCR[16] and sequencing. The *spa* gene sequences were analyzed with Ridom® StaphType software[17] (version 2.2.1). Determination of the accessory gene regulator (*agr*) group was performed by multiplex PCRs.[18] The sequence type (ST) and clonal complex (CC) of selected isolates were determined by multilocus sequence typing (MLST),[19] and for the other isolates the ST/CC was assigned according to their *spa*-types.

Detection of resistance genes

The presence of *mecA* and *mecC* methicillin-resistance genes penicillinase-encoding *blaZ* gene was studied by PCR.[12]

Detection of virulence factors (PVL and ACME) and the immune evasion cluster genes

All isolates were tested by PCR for the presence of *lukF/lukS* genes,[20] encoding the PVL leucocidin. The two loci (*arcA* and *opp3*) that compose the arginine catabolic mobile element (ACME) was analysed by PCR, as previously described,[21] on the CC8 strains or other PVL-positive strains belonging to different clonal complexes. For the detection of the immune evasion cluster (IEC), the presence of five genes (*scn*, *chp*, *sak*, *sea* and *sep*) was analyzed.[22] Attending to the **combination of genes,** the IEC could be ascribed to seven different groups (A-G).[22]

Results

Prevalence of MRSA and NBLS-MRSA in the 12 studied hospitals

The global rate of MRSA in the 12 hospitals included in the study in relation with *S. aureus* was of 30.4% (2190 MRSA out of 7198 *S. aureus*). Nevertheless, as it is shown in Table 1, important differences were found among hospitals (range: 12%-57%).

The phenotype NBLS-MRSA was very infrequent in the 12 hospitals included in the study (2.05% of total MRSA, and 0.63% of total *S. aureus* recovered in the six month-period), with differences among hospitals (range: 0.3%-7.7%) (Table 1).

Sample origin

Of the 45 NBLS-MRSA isolates included in this study, 80% were recovered from clinical samples and 20% of epidemiological surveillance (ES) samples. Within the group of clinical samples, 66.7 % belonged to SSTI (skin and soft tissue infections) and 13.8% to urinary tract infections (UTI).

Molecular typing of MRSA

The *spa*-typing results of the 45 NBLS-MRSA strains are shown in Table 2, as well as its relation per hospital in Table 1. Twenty-two different *spa*-types were detected, being *spa*-t008/*agr*-I the most prevalent with 12 isolates (27%), as well as the most geographically extended (eight out of 12 centers). A new *spa*-type was identified (t17233) with the repeat succession 26-23-17-16-23-17-16. The main clonal complexes to which isolates were ascribed were the following ones: CC8 (42.2%), CC5 (33.3%), CC30 (4.4%), CC80 (2.2%), CC1 (2.2%) and CC59 (2.2%), with ST8 and ST5 as the predominant sequence types. Two isolates were ascribed to ST72 (*spa*-type t148). None of the strains was ascribed to CC130 or other clonal complexes usually related to the *mecC* gene.

Genotypic characterization

All the 45 NBLS-MRSA strains harbored the *mecA* gene and were *mecC*-negative (Table 2). The penicillinase encoded by the *blaZ* gene was present in 51% of strains (23/45). The IEC system was identified in 78% of the studied strains, and the remaining 10 strains lacked the *scn* gene and, in consequence, were considered as IEC negative (Table 2). Seventeen isolates carried the genes of IEC type-B (*scn*, *chp*, *sak*), 16 were IEC type-F positive (*scn*, *chp*, *sak*, *sep*), one strain IEC type-A (*scn*, *chp*, *sak*, *sea*) and another one type-E (*scn*, *sak*). Regarding the PVL virulence factor, 38% of the strains carried the *lukF/S*-PV genes, corresponding to the following *spa*-types: t008, t024, t068, and t3060 (all CC8), t044 (CC80), t019 and t318 (both CC30). The PVL encoding-genes were not detected in the isolates with the remaining *spa*-types identified in the study (Table 2). All CC8 or other PVL-positive strains lacked the ACME locus, typical of the USA300 clone.

Discussion

According to this study, MRSA with *mecC* genotype seems to be very infrequent in the analyzed hospitals, at least when the NBLS-MRSA marker was used for *mecC*-MRSA detection. In fact, all NBLS-MRSA strains carried the *mecA* gene. There are not many studies reflecting the presence of the *mecC* mechanism in MRSA human infections in Spain,[5–7] and all of them report individual cases. The real prevalence of *mecC* is unknown in Europe, although in some countries *mecC*-MRSA isolates have increased (for instance, in Denmark from 1.8% in 2010 to 2.9% in 2011).[23] A meta-analysis study on the prevalence of *mecC*-MRSA based on previously published results obtained until April 2015,[24] suggested an estimated global *mecC* prevalence of 0.004% in humans and 0.1% in animals. In this way, our results confirm this low prevalence at hospital level.

Nevertheless, we cannot discard the existence of *mecC* strains with resistance to non-β-lactam antimicrobials, very unusual at the present moment, but that has been occasionally described (for instance: two isolates of human origin resistant to ciprofloxacin,[21] and one isolate recovered from wastewater resistant to erythromycin[10]). Moreover, *mecC* strains sometimes show borderline susceptibility results for oxacillin or cefoxitin, and could appear phenotypically as MSSA (methicillin-susceptible *Staphylococcus aureus*).[25] So, future studies could be focused in determining the presence of the *mecC* gene in *S. aureus*, with independence of the antimicrobial resistance phenotype (including β-lactams).

The zoonotic origin of *mecC*-MRSA is hypothesized since its origin in 2011 in cattle. Although the detection of *mecC* in humans is unusual, it presents a wide distribution in all animal species (livestock, companion or wildlife animals).[3,4,8–11,26] Contact with animals might be a zoonotic risk,[12] as *mecC*-MRSA can be easily transmitted between species.[27]

The NBLS-MRSA phenotype seems to be a good marker for PVL detection in MRSA isolates, considering that more than 1/3 of these strains were PVL-positive, while this factor is infrequent among non-selected MRSA isolates. The detected association of PVL production with the *spa*-type t008 (ST8/CC8), a classical CA-MRSA lineage[28] and the most disseminated NBLS-MRSA in our country,[29] is of relevance. In a previous study carried out in Spain, CA-MRSA corresponded to 2.9% of all studied MRSA obtained during the period 2004-2012 in the Spanish National Reference Centre of Staphylococci, and most of them showed susceptibility for non-β-lactams (84.5%), being most of them PVL-producers (91.9%).[29]

Observing the clonal complexes detected among NBLS-MRSA isolates, 42.2% belonged to CC8, which is strongly associated to the CA-MRSA USA300 clone,[14,28] and **74%** of them were PVL producers. On the other hand, none of the tested strains contained the ACME island. The other major clonal complex was CC5 (**33.3%**), a typical HA-MRSA, and none of these strains carried the genes for the PVL toxin, as expected. CC30 and CC80, both well-known CA-MRSA lineages,[14,28] were present with 100% of PVL-positive strains. Two isolates with the *spa*-type t148, belonging to the CA-MRSA ST72, were detected. This clone is the most prevalent CA-MRSA in Korea causing infections, it was spread into hospital settings and it is also present in pigs and cattle carcasses.[30] ST72 is not so frequent in Europe, although it has been detected more and more often in Spanish hospitals.[21,29] Overall, at least 50% of CCs are associated to CA-MRSA, and the vast majority of strains with CCs related to CA-MRSA were PVL-producers.

Another important point is that NBLS-MRSA phenotype is very infrequent in the hospitals tested (0.3%-7.7%, media 2.05%). Therefore, it could be important to test the presence of the PVL genes when this phenotype is detected, mostly if strains are recovered from SSTI infections, due to the clinical relevance of this toxin. In our study, more than 75% of the NBLS-MRSA isolates that harbored the *lukF/S*-PV genes **were** isolated from SSTI.

The presence of the *scn* gene (marker of IEC system) in most NBLS-MRSA strains is expected, since its frequent detection among human isolates.[31] Nevertheless, 22% of the studied strains were *scn*-negative, common feature of animal isolates. These *scn*-negative isolates belonged to many different STs and *spa*-types. Moreover, no relation between the production of PVL and the presence of the IEC system was observed: 11 out of the 17 PVL-producer strains had IEC type-B, two IEC type-F and four lacked the

IEC genes. In the future it would be important to have epidemiological data of patients carrying *scn*-negative MRSA isolates to analyze the variables that could be associated to their acquisition.

Altogether, we can conclude that *mecC*-MRSA is very uncommon in human infections in the reported hospitals, but NBLS phenotype can be a valuable marker for PVL-producer strains (usually related to CA-MRSA), especially for the t008/*agr*I clone. It is important to maintain an active surveillance for these clones, not only for epidemiological control, but also for the right and early treatment in virulent PVL-infections.

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Table 1. Distribution of isolates (number of *S. aureus*, MRSA and NBLS-MRSA), prevalence and NBLS-MRSA *spa*-types per hospital

Hospital (H.), location	Total *S. aureus*	Total MRSA	NBLS-MRSA	% MRSA/ *S. aureus*	% NBLS-MRSA/ MRSA	*spa*-types NBLS-MRSA (No of isolates)
H. Universitario de Donostia, San Sebastián	1009	130	10	12.9%	7.7%	t002 (4), t008 (4), t024 (1), t067 (1)
H. Virgen Macarena, Sevilla	250	84	5	33.6%	6%	t008 (1), t019 (1), t502 (1), t648 (1) , t3060 (1)
H. San Pedro, Logroño	368	112	4	30.4%	3.6%	t008 (2), t010 (1), t068 (1)
H. Miguel Servet, Zaragoza	1024	251	7	24.5%	2.8%	t002 (1), t008 (2), t127 (1), t437 (1), t4450 (1), t17233 (1)
H. de Alcañiz, Alcañiz, Teruel	99	36	1	36.4%	2.8%	t008 (1)
H. Universitario de Burgos	666	220	6	33%	2.7%	t008 (1), t024 (2), t148 (1), t179 (1), t3682 (1)
H. Ernest Lluch Martin, Calatayud, Zaragoza	126	42	1	33.3%	2.4%	t002 (1)
H. Marqués de Valdecilla, Santander	1124	371	6	33%	1.6%	t008(1), t010 (1), t044 (1), t148 (1), t12908 (2)
H. Royo Villanova, Zaragoza	180	76	1	42.2%	1.3%	t318 (1)
Complejo Hospitalario de Navarra, Pamplona	799	206	2	25.8%	1%	t002 (1), t008 (1)
H. San Jorge, Huesca	575	328	1	57%	0.3%	t002 (1)
H. Universitario de Álava, Vitoria	978	334	1	34.2%	0.3%	t4955 (1)

Table 2. Molecular typing, samples origin and genotypic characterization of the 45 NBLS-MRSA isolates

spa-type	**ST/CC**[a]	No of strains	Sample origin (No of strains)	*agr*	IEC type (No strains)	Resistance genes[b]	Virulence genes[b]
t008	**ST8/CC8**	12	SSTI[d] (7), ES[e] (2), UTI[f] (1), biopsy (1), drain (1)	I	A (1), B (7), F (1), *scn*-negative (3)	*mecA*, *blaZ*[7]	*lukF/S*-PV[9]
t002	**ST5/CC5**	8	SSTI (6), UTI (1), SSI[g] (1)	II	B (2), F (5), *scn*-negative (1)	*mecA*, *blaZ*[3]	-
t024	**(ST8/CC8)**	3	SSTI (1), ES (1), blood (1)	I	B (2), *scn*-negative (1)	*mecA*, *blaZ*	*lukF/S* -PV
t010	**(ST5/CC5)**	2	ES (1), SSI (1)	II	F (2)	*mecA*, *blaZ*	-
t148	**(ST72)**	2	ES (1), UTI (1)	I	B (1), F (1)	*mecA*, *blaZ*[1]	-
t12908	**ST5/CC5**	2	ES (1), RTI[h] (1)	II	F (2)	*mecA*	-
t019	**(ST30/CC30)**	1	SSTI	III	F	*mecA*	*lukF/S* -PV
t044	**(ST80/CC80)**	1	SSTI	III	B	*mecA*	*lukF/S* -PV
t068	**(ST8/CC8)**	1	SSTI	I	B	*mecA*, *blaZ*	*lukF/S* -PV
t318	**(CC30)**	1	SSTI	III	B	*mecA*, *blaZ*	*lukF/S* -PV
t3060	**ST8/CC8**	1	SSTI	I	*scn*-negative	*mecA*	*lukF/S* -PV
t127	**(ST1/CC1)**	1	SSTI	III	E	*mecA*	-
t437	**(ST59/CC59)**	1	ES	I	*scn*-negative	*mecA*	-
t3682	**(CC8)**	1	ES	I	B	*mecA*, *blaZ*	-
t067	**(ST125/CC5)**	1	SSTI	II	*scn*-negative	*mecA*	-
t648	**(ST8/CC8)**	1	biopsy	I	*scn*-negative	*mecA*	-
t179	**(ST5/CC5)**	1	UTI	II	F	*mecA*, *blaZ*	-
t502	**(ST5/CC5)**	1	ES	II	F	*mecA*	-
t4450, t4955	-	2	SSTI (1), UTI (1)	I	B (1), F (1)	*mecA*, *blaZ*	-
t088, t17233[c]	-	2	SSTI	II	F (1), *scn*-negative (1)	*mecA*, *blaZ*[1]	-

[a]Determined **by MLST,** or presumptive assumed according to the *spa*-type (in parentheses in the last case); [b]In superscript: number of strains in cases not all strains have the same characteristic; [c]New *spa*-type; [d]SSTI: skin and soft tissue infections; [e]ES: epidemiological surveillance; [f]UTI: urinary tract infections; [g]SSI: surgical site infections; [h]RTI: respiratory tract infections.