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Phenotypic and genetic characteristics of fluoroquinolone- and methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Introduction: Fluoroquinolone resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) has increased in recent years. The objective of this study was to characterise two MRSA populations, one susceptible to fluoroquinolones and other resistant identifying the clonal types and the differential characteristics of both MRSA populations.
Methods: Molecular typing using PFGE, MLST, *spa* and SSC*mec* was performed on 192 MRSA strains isolated from 2009 to 2011, 49 only oxacillin-resistant (OX-R) and 143 oxacillin and levofloxacin-resistant (OX-R-LEV-R). Mutations that conferred resistance to fluoroquinolones, hypermutable phenotypes and the presence of eight microbial surface components recognising adhesive matrix molecules (MSCRAMMs) were also studied.
Results: A statistically significant increase in the OX-R-LEV-R phenotype was observed ($p < 0.05$). The most common clone of the OX-R isolates was sequence type (ST) 8 (32.6%), followed by ST72 (26.5%) and ST5 (26.5%). In the OX-R-LEV-R phenotype, the ST5 clone was the most common (65.7%), followed by ST72 (15.4%), and ST125 (12.6%). All isolates except the ST398 clone carried the SCC*mec*IVc. Clones ST5, ST72, ST125, and ST30 had hypermutable phenotypes. The ST72 clone and the ST30 clone in the OX-R phenotype harboured the highest number of MSCRAMMs.
Conclusion: ST5 and ST72 clones were the most frequent clones identified in OX-R-LEV-R phenotype. Both clones showed a hypermutable phenotype that favours their selection as the fluoroquinolone resistant clones. The genetic relationships identified indicate that OX-R-LEV-R clones have evolved from OX-R MRSA clones.

Características fenotípicas y genéticas de *Staphylococcus aureus* resistente a meticilina y a fluoroquinolonas

Palabras clave:
Staphylococcus aureus resistente a meticilina
Fluoroquinolonas
Resistencia
Componentes de la superficie microbiana que reconocen adhesinas de la matriz extracelular

RESUMEN

Introducción: La resistencia a fluoroquinolonas en *Staphylococcus aureus* resistente a meticilina (SARM) se ha incrementado en los últimos años. El objetivo de este estudio consistió en caracterizar 2 poblaciones de SARM, una sensible a fluoroquinolonas y otra resistente identificando los tipos clonales y las características diferenciales entre los mismos.
Métodos: En un total de 192 SARM aislados entre los años 2009-2011, 49 solo oxacilina resistentes (OX-R) y 143 oxacilina y levofloxacino resistentes (OX-R-LEV-R), se realizó el tipado molecular mediante PFGE, MLST, *spa* y SSC*mec*. Además se estudiaron las mutaciones que confieren resistencia a las fluoroquinolonas, los fenotipos hipermutadores y la presencia de 8 componentes de la superficie microbiana que reconocen adhesinas de la matriz extracelular.

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Resultados: En el periodo de estudio se detectó un incremento estadísticamente significativo del fenotipo OX-R-LEV-R (p < 0,05). Entre los OX-R el clon ST8 (32,6%) fue el más frecuente seguido de los clones ST72 (26,5%) y ST5 (26,5%). Entre los aislados del fenotipo OX-R-LEV-R, el clon ST5 fue el más frecuente (65,7%), seguido de los clones ST72 (15,4%) y ST125 (12,6%). Todos los aislamientos, excepto el clon ST398, portaban el SCC*mec*-IVc. Los clones ST5, ST30, ST72 y ST125 presentaron un fenotipo hipermutador. Los clones ST72 y ST30 OX-R son los que poseen una mayor dotación de componentes de la superficie microbiana que reconocen adhesinas de la matriz extracelular.

Conclusión: Los clones ST5 y ST72 fueron los más frecuentes en el fenotipo OX-R-LEV-R. Ambos clones poseían un fenotipo hipermutador. La estrecha relación genética entre los clones OX-R y OX-R-LEV-R pertenecientes al mismo ST sugiere que estos últimos han evolucionado a partir de una población OX-R preexistente.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes a large number of infections.[1] Its ability to adapt and acquire resistance to different antimicrobials has possibly favoured its spread both in hospitals and in the community.[1] Methicillin resistance in *S. aureus* is coded by the *mec* gene, included in a genomic island called the staphylococcal cassette chromosome *mec* (SCC*mec*). The fluoroquinolones are among the therapeutic agents whose activity is not affected by SCC*mec*. However, rates of fluoroquinolone resistance have increased,[2] mainly due to mutations in genes *gyrA* and *grlA*.[2]

S. aureus expresses surface proteins that are essential for its success both as a commensal and as a pathogen. The most important group of these proteins are the MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules).[3]

The objective of this study was to analyse the increase in MRSA resistant to fluoroquinolones and its possible relation with a clonal selection. In addition, the characteristics of the MRSA isolates studied were analysed including the phenotypic hypermutability of the identified clones and the MSCRAMMs content.

Materials and methods

Selection of microorganisms and patients for inclusion in the study

The University Hospital Complex of Pontevedra has a catchment population of 296,463. We selected two groups of MRSA isolates from the years 2009 to 2011: (i) those that presented resistance only to beta-lactams (oxacillin, OX-R), being susceptible to all other groups of antimicrobials; and (ii) those with resistance to oxacillin and to levofloxacin OX-R-LEV-R, being susceptible to all other antimicrobial groups. Only one isolate per patient was analysed in the study. A total of 192 isolates were studied. The following patient data were recorded: sex, age, type of sample, underlying diseases and risk factors for MRSA infection. The criteria of the Centers for Disease Control and Prevention (CDC) were applied to determine the origin of the MRSA infection.[4,5]

Bacterial identification and susceptibility to antimicrobials

The biochemical characteristics and antibiotic susceptibility of the isolates were studied using the commercially available Wider system (Gram Positive MIC/ID Panel, Francisco Soria Melguizo, S.A.). The minimum inhibitory concentrations (MICs) for fluoroquinolones were determined using the gradient diffusion (epsilon-test) method. Resistance to methicillin was detected using a 30 μg cefoxitin disc. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were followed for the determination of breakpoints and resistance to methicillin.[6]

Molecular typing of the isolates

For the SCC*mec*, PFGE, and *spa* gene typing, the 192 strains included in the study were analysed.

Analysis of the staphylococcal cassette chromosome mec (SCCmec): a multiplex polymerase chain reaction (PCR) was employed to identify the *mecA* gene and SCC*mec*[7] type and subtype.

Pulsed-field gel electrophoresis (PFGE): cells were processed following the protocol proposed by Murchan et al.[8] Gels were analysed using the GelCompar II software (Applied Maths NV), applying the Dice similarity coefficient with an optimisation of 0.5% and tolerance 1%. Banding patterns were assigned to pulsotypes (using uppercase Latin letters) and subtypes (using numerical subindices). Pulsotypes were considered to be different if the coefficient of similarity was less than 80%. Subtypes were defined as pulsotypes with a coefficient of similarity between 80% and 95%.[9]

spa gene typing: amplification and sequencing of the X region of the *spa* gene were performed under the conditions described by Harmsen et al.[10] The *spa* type was established using Ridom StaphType software (Ridom GmbH, Würzburg, Germany).

Multilocus sequence typing (MLST): the Enright et al.[11] recommendations were followed. A total of 17 strains were studied including one isolate from each of the identified pulsotypes and *spa* types. The MLSTs of the strains not analysed by this method were deduced from the *spa* types.

Analysis of the frequency of mutation in the isolates

The frequency of mutations for rifampicin and streptomycin were performed under the conditions described by Trong et al.[12] One isolate from each clonal type and one from each phenotype were selected and processed in triplicate. In summary, five OX-R strains including one of each ST5, ST72, ST30, ST8, and ST398, and another five OX-R-LEV-R strains including one of each ST5, ST72, ST30, ST125, and ST22 were studied.

Analysis of mutations in genes coding for fluoroquinolone resistance

The amplification of genes *gyrA* and *grlA* was carried out under the conditions described by Schmitz et al.[13] A total of 30 strains belonging to the ST5 clone, 22 strains belonging to the ST72, 18 strains from the ST125, 8 strains from the ST22, and 1 strain from the ST30 were studied. Sequence analysis was performed using the BioEdit software, version 7.1.9 (Ibis Therapeutics, Isis Pharmaceuticals, Inc.).

Table 1
Staphylococcus aureus isolated between 2009 and 2011 at the University Hospital Complex of Pontevedra (%).

Year	*S. aureus* (no. isolates)	Total MRSA	Only (OX)-R MRSA	*p*-values	(OX-LEV)-R MRSA	*p*-values
2009	821	229 (27.9)	13 (5.7)		34 (14.9)	
2010	864	241 (27.9)	16 (6.6)	0.361	47 (19.5)	0.011
2011	902	258 (28.6)	20 (7.8)		62 (24)	

Abbreviations: LEV, levofloxacin; MRSA, methicillin-resistant *Staphylococcus aureus*; OX, oxacillin; R, resistant.

Detection of genes coding for surface proteins

Based on the relationship between the surface proteins and the *S. aureus* colonisation, we selected eight genes that coded for surface proteins belonging to the MSCRAMM group[3]: *clfA*,[14] *clfB*,[14] *fnbA*,[15] *fnbB*,[16] *cna*,[15] *sdrC*,[14] *ebpS*[14] and *efb*.[15] The presence of these genes was determined by PCR under the conditions described by the authors cited for each MSCRAMM studied. The 192 strains included in the study were analysed.

Statistical analysis

The chi square test for trend was used to analyse the years of the study and the OX-R-LEV-R increase. To analyse the mutation rate to streptomycin and rifampicin, the non-parametric test of Mann–Whitney was used to compare two groups of the studied strains. The non-parametric Kruskal–Wallis test was used for multiple comparisons. A *p* value less than or equal to 0.05 was considered significant. Statistical analysis was performed using the statistical package SPSS 22.0.

Results

Characteristics of the isolates and molecular typing of MRSA

Between 2009 and 2011, a total of 2587 *S. aureus* were isolated; 728 (28.1%) were MRSA. Of the 728 MRSA isolates, 49 (6.7%) were only resistant to beta-lactams (OX-R) and 143 (19.6%) were OX-R-LEV-R (Table 1). Throughout the three years of the study (2009–2011), an increase of 9.1% was identified in OX-R-LEV-R MRSA being statistically significant ($p < 0.05$), whereas OX-R MRSA isolates only increased by 2.1% (Table 1). A total of 60 MRSA were considered community-associated MRSA (CA-MRSA) strains (31.3%); this community origin was found in 44.9% ($n = 22$) of the OX-R phenotype and in 26.6% ($n = 38$) of the OX-R-LEV-R phenotype.

Ten pulsotypes (A–J) were identified (Fig. 1). The following subtypes were identified: four in pulsotype A, three in pulsotype E, and two in pulsotype G (Fig. 1). The *spa* and ST identified in the OX-R and OX-R-LEV-R phenotypes are shown in Table 2. All isolates carried a SCC*mec*IVc, except ST398 that harboured SCC*mec*V.

The yearly increase in the OX-R-LEV-R phenotype is shown in Table 2. The prevalence of ST5 decreased throughout the three years, from 88.2% in 2009 to 45.2% in 2011. Nevertheless, ST72 increased its prevalence from zero isolates in 2009 to 24.2% in 2011. In addition, ST125 increased its prevalence from 8.8% in 2009 to 20.9% in 2011.

Characteristics of the population studied

The 192 MRSA isolates corresponded to 192 patients. The median age of the patients was 77 years (range 10 days–94 years), and 50.0% were men. The most common underlying diseases associated with isolation of MRSA were cardiovascular diseases (54.2%),

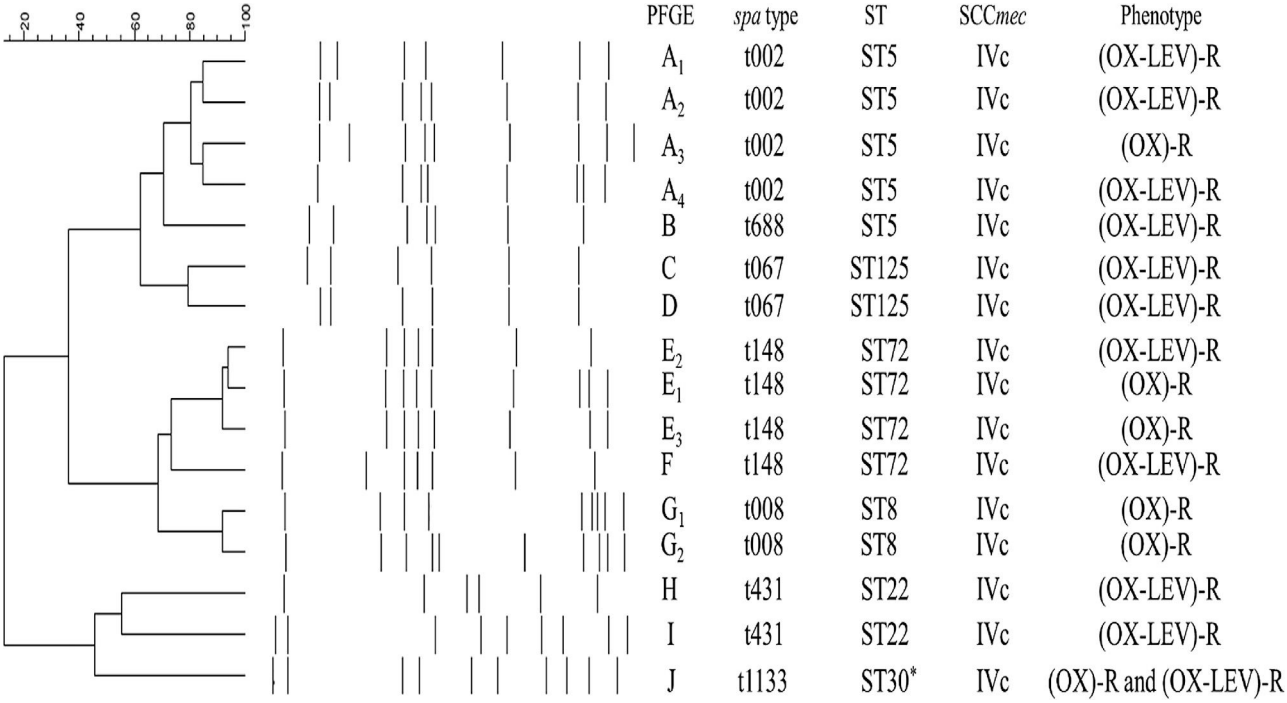

Fig. 1. MRSA clonal types identified in the study.
*ST30 clone showed the same PFGE profile (PFGE-J) in (OX)-R and (OX-LEV)-R phenotypes. *Abbreviations*: LEV, levofloxacin; MRSA, methicillin-resistant *Staphylococcus aureus*; OX, oxacillin; PFGE, pulsed-field gel electrophoresis; R, resistant; ST, sequence type.

Table 2
Yearly variation of clonal types, SARM (OX)-R and (OX-LEV)-R (%).

MLST/spa	2009		2010		2011		TOTAL	
	(OX)-R	(OX-LEV)-R	(OX)-R	(OX-LEV)-R	(OX)-R	(OX-LEV)-R	(OX)-R	(OX-LEV)-R
ST5/t002,[a] t688[b]	7(53.8)	30(88.2)	2(12.5)	36(76.7)	4(20)	28(45.2)	13(26.5)	94(65.7)
ST72/t148			6(37.5)	7(14.9)	7(35)	15(24.2)	13(26.5)	22(15.4)
ST125/t067		3(8.8)		2(4.2)		13(20.9)		18(12.6)
ST22/t431				2(4.2)		6(9.7)		8(5.6)
ST30/t1133	1(7.7)	1(3)	3(18.8)		1(5)		5(10.2)	1(0.7)
ST8/t008	5(38.5)		5(31.2)		6(30)		16(32.6)	
ST398/t011					2(10)		2(4.1)	
Total	13	34	16	47	20	62	49	143

[a] t002 ($n = 106$).
[b] t688 [$n = 1$, (OX-LEV)-R].

Table 3
Multiple comparison in the mutation rate for SARM (OX)-R and (OX-LEV)-R phenotypes.

ST *vs* ST	Streptomycin				Rifampicin			
	(OX)-R		(OX-LEV)-R		(OX)-R		(OX-LEV)-R	
	CI 95%	*p* value	CI 95%	*p* value	CI 95%	*p* value	CI 95%	*p* value
ST5 ST8	3.07×10^{-5}, 3.46×10^{-5}	<0.001	NA		8.72×10^{-6}, 1.19×10^{-5}	<0.001	NA	
ST22	NA		3.10×10^{-5}, 3.45×10^{-5}	<0.001	NA		3.00×10^{-5}, 3.55×10^{-5}	<0.001
ST30	2.97×10^{-5}, 3.36×10^{-5}	<0.001	3.01×10^{-5}–3.36×10^{-5}	<0.001	7.61×10^{-6}, 1.08×10^{-5}	<0.001	2.87×10^{-5}, 3.43×10^{-5}	<0.001
ST125	NA		2.10×10^{-5}, 2.45×10^{-5}	<0.001	NA		-2.55×10^{-6}, 3.02×10^{-6}	0.999
ST398	3.14×10^{-5}, 3.52×10^{-5}	<0.001	NA		9.28×10^{-6}, 1.25×10^{-5}	<0.001	NA	
ST8 ST30	-2.97×10^{-6}, 8.98×10^{-7}	0.441	NA		-2.72×10^{6}, 5.11×10^{-7}	0.238	NA	
ST72	-6.63×10^{-5}, -6.24×10^{-5}	<0.001	NA		-3.52×10^{-5}, -3.2×10^{-5}	<0.001	NA	
ST398	-1.31×10^{-6}, $\times 2.56 \times 10^{-6}$	0.821	NA		-1.05×10^{-6}, 2.18×10^{-6}	0.777	NA	
ST125 ST30	NA		7.39×10^{-5}, 1.09×10^{-5}	<0.001	NA		2.85×10^{-5}, 3.41×10^{-5}	<0.001
ST72 ST125	NA		-1.12×10^{-6}, 2.38×10^{-5}	0.758	NA		-2.52×10^{-6}, 3.05×10^{-6}	0.997
ST22	NA		8.89×10^{-5}, 1.24×10^{-5}	<0.001	NA		3.00×10^{-5}, 3.56×10^{-5}	<0.001
ST5	2.96×10^{-5}, 3.35×10^{-5}	<0.001	2.03×10^{-5}, 2.38×10^{-5}	<0.001	2.17×10^{-5}, 2.49×10^{-5}	<0.001	-2.75×10^{-6}, 2.82×10^{-6}	<0.001
ST30	6.13×10^{-5}, 6.52×10^{-5}	<0.001	8.03×10^{-6}, 1.15×10^{-5}	<0.001	3.09×10^{-5}, 3.42×10^{-5}	<0.001	2.88×10^{-5}, 3.43×10^{-5}	<0.001
ST398	6.3×10^{-5}, 6.69×10^{-5}	<0.001	NA		3.26×10^{-5}, 3.58×10^{-5}	<0.001	NA	
ST30 ST22	NA		-8.95×10^{-7}, 2.61×10^{-6}	0.523	NA		-1.55×10^{-6}, 4.01×10^{-6}	0.610
ST398	-2.71×10^{-7}, 3.6×10^{-6}	0.102	NA		5.35×10^{-8}, 3.28×10^{-6}	0.042	NA	

NA: not applicable.

followed by diabetes mellitus (35.8%) and cancer (14.7%); two or more concurrent underlying diseases were identified in 30.3% of patients. In 28.4% of patients there was no detectable underlying disease. The source of the isolates was skin and soft tissue infection in 71.7% of cases, followed by bacteraemia (6.6%). Factors related to MRSA infection included hospitalisation in the 12 months prior to the isolation of the microorganism (42.1%) and a previous history of MRSA infection (19.2%).

Fluoroquinolone mutations and clonal hypermutants identified in OX-R and OX-R-LEV-R MRSA

All the OX-R-LEV-R strains showed a levofloxacin MIC of >32 mg/l and the mutations S84L, in *gyrA*, and S80F in *grlA*. In addition to mutation S84L, the isolates belonging to clone ST22 carried the mutation S85P in *gyrA*. Mutation frequency experiments revealed a higher frequency of mutations (10^{-5}–10^{-6}) for clonal types ST5, ST30, ST72, and ST125 compared with clonal types ST8, ST22 and ST398 (10^{-8}–10^{-7}). No statistically differences in mutation rates were detected when comparing the OX-R strains, or the OX-R-LEV-R strains. The mutation rate when comparing clones was statistically significant in the OX-R and OX-R-LEV-R phenotypes ($p < 0.05$) both for streptomycin and rifampicin. Analysing the mutation rate of the different pairs of clones (Table 3), the differences were statistically significant for all clones except ST8 *vs* ST30, ST8 *vs* ST398, and ST30 *vs* ST398 in the OX-R phenotype both for streptomycin and rifampicin. The mutation rate for streptomycin of the OX-R-LEV-R phenotype was statistically significant in all pair of clones except ST30 *vs* ST22 and ST72 *vs* ST125 (Table 3). The mutation rate for rifampicin of the OX-R-LEV-R phenotype was statistically significant in all pair of clones except ST5 *vs* ST125, ST72 *vs* ST125, and ST30 *vs* ST22 (Table 3).

Identification of MSCRAMMs in the different clones

Except for ST30, any single clonal type possessed certain specific MSCRAMMs independently of the resistance phenotype. The following MSCRAMMs were detected: ST72-(*clfA-clfB-fnbA-fnbB-sdrC-efb*), ST30-OX-R-(*clfA-clfB-fnbA-cna-sdrC-ebpS*), ST30-OX-R-LEV-R-(*clfA-clfB-fnbA-sdrC-ebpS*), ST5-(*clfA-clfB-fnbA-fnbB-ebpS*), ST8-(*clfA-clfB-fnbA-sdrC-efb*), ST22-(*clfA-clfB-fnbA-cna*), ST125-(*clfA-clfB-fnbA-ebpS*), ST398-(*clfA-fnbA*).

Discussion

MRSA infections are more common in older individuals with comorbidities.[17] In our study, CA-MRSA was the cause of 31.3% of the infections. This figure is higher than that previously reported in several nationwide studies in Spain[18] and in Europe.[19] However, the rates in the United States are much higher, above 50%.[20]

The increase in the prevalence of resistance to fluoroquinolones has been associated with different factors[21] including the persistent increase in quinolone prescriptions, as is the case of Spain.[22] The main mechanism of resistance to fluoroquinolones is through mutations in the genes coding for topoisomerase IV and DNA

gyrase, and the degree of resistance is proportional to the number of mutations accumulated.[23]

The international epidemic clone ST5 was the most successful in our study (Table 2). Over the first decade of this century, this clone has persisted in our healthcare setting carrying SCC*mec*II.[24] It currently carries SCC*mec*IVc, and is at present the main clone isolated.[9,25] The smaller size of SCC*mec*IVc may be an evolutionary advantage, making its spread more effective.[26] The fact that this clone circulated for a longer period in our setting,[24] and its hypermutable status may facilitate its selection as the predominant clone of the fluoroquinolone-resistant phenotype.

ST72 is also a hypermutable clone that emerged recently in our setting, and a significant increase in its prevalence has been reported.[25] Although this clone is uncommon in Europe, we must remain alert to its probable spread; in South Korea it is the primary cause of community-acquired MRSA infection, and it is also the main MRSA isolated from the meat of animals for consumption.[27] Its high number of MSCRAMMs may be related to its elevated capacity to colonise both humans and animals.

The close clonal relationship between most of the OX-R and OX-R-LEV-R strains in ST5, ST72, and ST30 clones suggests that quinolone resistance evolved from the OX-R population. Whereas ST30 is infrequent in our study, ST5 and ST72 were the two most common clones isolated. Interestingly, these were the only clones in which *fnbB-fnbA* were detected simultaneously. These two genes code for adhesins important in the adhesion and internalisation of *S. aureus* into mammalian epithelial cells and in the initiation of the biofilm.[3] This may have contributed to their epidemiological expansion.

The pandemic clone ST30 is highly prevalent in the Southwest Pacific, but is infrequent in Spain,[18] representing only 3.1% of the isolates in our study.

The international epidemic clones ST22 and ST125 have been isolated frequently in Spain, mainly clone ST125.[9,28] In our study, only strains belonging to the OX-R-LEV-R phenotype were detected belonging to these clones. Since no OX-R isolates were detected, we can speculate that these two clones were introduced in the MRSA population as OX-R-LEV-R and not evolved from a previous OX-R local population.

The ST8 clone has been selected as the major clonal type in the OX-R phenotype (32.6%). However, we did not identify ST8 OX-R-LEV-R strains in the present study, a finding that could be related to the fact that it is not a hypermutable clone.

The ST398 clone had the smallest number of MSCRAMMs and lacked the two adhesins (*clfB-sdrC*) with affinity for epithelial cells. This fact could limit its ability to colonise the nares in humans as ST398 is a clone typically associated with livestock.[29]

The ST5 and ST72 clones were the most frequently identified in the OX-R-LEV-R population. Both clones showed a hypermutable phenotype that favours their selection as the fluoroquinolone resistant clones. The genetic relationships identified between OX-R and OX-R-LEV-R clones that belong to the same ST indicate that OX-R MRSA clones have evolved from a pre-existing OX-R MRSA population. The richest MSCRAMMs environment in the most frequent clones could have played a role in their fitness to colonise the host.

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Conflict of interest

The authors declare that they have no conflict of interest.

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