Genotypic and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) clones with high-level mupirocin resistance

María González-Domínguez [a], Cristina Seral [a,b,*], Carmen Potel [c,d], Yolanda Sáenz [e], Maximiliano Álvarez [c,d], Carmen Torres [e,f], Francisco Javier Castillo [a,b]

[a] Servicio de Microbiología, Hospital Clínico Universitario Lozano Blesa, San Juan Bosco s/n, 50009, Zaragoza, Spain
[b] Departamento de Microbiología, Facultad de Medicina, Universidad de Zaragoza, C/Domingo Miral s/n, 50009, Zaragoza, Spain
[c] Servicio de Microbiología, Complexo Hospitalario Universitario de Vigo (CHUVI), C/Pizarro, 22, 36204, Vigo Pontevedra, Spain
[d] Unidad de Apoyo a la Investigación, Instituto de Investigación Biomédica de Vigo (IBIV), Spain, Complexo Hospitalario Universitario de Vigo (CHUVI), C/Pizarro, 22, 36204, Vigo Pontevedra, Spain
[e] Área de Microbiología Molecular, Centro de Investigación Biomédica de La Rioja (CIBIR), C/Piqueras, 98, 26006, Logroño La Rioja, Spain
[f] Área de Bioquímica y Biología Molecular, Universidad de La Rioja, Avda/Madre de Dios, 51, 26006, Logroño La Rioja, Spain

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ABSTRACT

A high proportion of methicillin-resistant *Staphylococcus aureus* isolates recovered in one year period showed high-level mupirocin-resistance (HLMUPR-MRSA) in our environment (27.2%). HLMUPR-MRSA isolates were mainly collected from skin and soft tissue samples, and diabetes was the main related comorbidity condition. These isolates were more frequently found in vascular surgery. HLMUPR-MRSA was more resistant to aminoglycosides than mupirocin-susceptible MRSA, linked to the presence of bifunctional and/or nucleotidyltransferase enzymes with/without macrolide resistance associated with the *msr*(A) gene. Most of HLMUPR-MRSA isolates belonged to ST125/t067. Nine IS*257-ileS2* amplification patterns (p3 was the most frequent) were observed in HLMUPR-MRSA isolates, suggesting the presence of several mupirocin-resistance-carrying plasmids in our environment and promoting the emergence of mupirocin resistance. The presence of the same IS*257-ileS2* amplification pattern p3 in 65% of HLMUPR-MRSA, all of them ST125/t067, suggests a clonal spread in our hospital and community environment which could explain the high prevalence of HLMUPR-MRSA during the study period. An outbreak situation or an increase in mupirocin consumption was not observed.

1. Introduction

Mupirocin, also known as pseudomonic acid A, is a topical antibiotic that was originally isolated from *Pseudomonas fluorescens*. It is used for decolonization of nasal carriers of methicillin-resistant *Staphylococcus aureus* (MRSA) (Sutherland et al., 1985). The increased use of this antibiotic has been accompanied by outbreaks of mupirocin-resistant MRSA (Schmitz and Jones, 1997; Simor et al., 2007).

Mupirocin is an analogue of isoleucine that inhibits protein synthesis by competitively binding to the enzyme isoleucyl-tRNA synthetase (Yanagisawa et al., 1994). The high-level mupirocin-resistant isolates show a MIC greater than 512 mg/L. This resistance is mediated by the acquisition of a plasmid containing the *ileS2* gene that encodes an alternative isoleucyl-tRNA synthetase enzyme and is generally flanked by copies of the insertion sequence IS*257* (Pérez-Roth et al., 2010; Woodford et al., 1998).

The objective of the study was to identify the prevalence, clonal lineages, resistance mechanisms and virulence genes of high-level mupirocin-resistant MRSA (HLMUPR-MRSA) isolates recovered from inpatients and outpatients in our institution during one year.

2. Material and methods

One hundred forty-seven MRSA isolates were collected from clinical samples in the University Teaching Hospital "Lozano Blesa" (Zaragoza, Spain) from July 2009 to July 2010. Only one isolate per patient was included. The study was conducted retrospectively. Clinical records of all patients were reviewed. For each patient the following data were collected: gender, age, medical service, source of the culture sample and comorbid conditions during the year prior to MRSA isolation (dialysis, diabetes, vascular disease, malignancy, hospitalization, MRSA isolation, medical devices, contact with healthcare settings and surgery). To establish the possible community origin of MRSA isolates, CDC criteria were followed, considering in this category the isolates from outpatients with no hospitalization history during the last year, or from patients within their first 48 h after admission, who presented no other

established risk factors for MRSA infection, such as a sojourn in a long-term care facility, dialysis, surgery, indwelling permanent catheters, or medical devices. Antibiotic consumption in the year prior to isolation was studied only in patients who carried HLMUPR-MRSA. Mupirocin consumption data in our hospital were obtained from pharmacy records.

Identification and susceptibility testing was carried out by WIDER®I System (Francisco Soria-Melguizo, Madrid, Spain) and disk diffusion method and interpreted according to the CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2014). High mupirocin MICs (mupirocin MICs ≥ 256 mg/L) were confirmed by E-test strip method.

The main genes that encode resistance to macrolides, lincosamide, streptogramin type B, aminoglycosides, tetracycline and mupirocin were investigated by PCR (Aarestrup et al., 2000; Aktas et al., 2007; Choi et al., 2003; Larsen et al., 2008; Lina et al., 1999; Udo et al., 2001). Clinical strains usually harbor a complex mixture of resistance plasmids, and laborious time consuming methodologies are required to associate HLMUPR-MRSA with a plasmid type (Leski et al., 1999; Pérez-Roth et al., 2006). To enable the monitoring of HLMUP resistance dissemination in staphylococci, we used a rapid typing method for strain characterization based on the heterogeneous IS*257-ileS2* spacer regions (amplification of up- and down- stream IS*257-ileS*2 spacer regions) found on *ileS2*-encoding plasmids. Four PCR reactions were performed, as previously described (Pérez-Roth et al., 2011), on all mupirocin-resistant isolates.

All isolates were typed by *spa* typing (Larsen et al., 2008). Only HLMUPR-MRSA isolates were typed by SCC*mec*, *agr* typing, PFGE using *Sma*I and MLST as previously described (González-Domínguez et al., 2012). PFGE profiles were analyzed with GelCompar II® software (Applied Maths, Kortrijk, Belgium). Dendrograms were generated by the unweighted pair-group method using arithmetic averages, based on the Dice similarity coefficient with a 1.0% band position tolerance. PFGE patterns were assigned into pulsotypes (named with capital letters) and subtypes (named with numbers in subscript). Different pulsotypes were considered if the similarity coefficient was <80%. Different subtypes were considered when the similarity coefficient oscillates in the 80–95% interval. HLMUPR-MRSA isolates were screened for virulence genes encoding Panton-Valentine leukocidin (PVL) (*lukS-PV* and *lukF-PV* genes), exfoliative toxins ETA and ETB (*eta* and *etb* genes) and toxic shock syndrome toxin (TSST) (*tst* gene) (Jarraud et al., 2002; Larsen et al., 2008).

Statistical significance was calculated for comparison of proportions using the chi-square test with Yates' correction. $P \leq 0.05$ was considered statistically significant (SPSS V15.0; SPSS, Chicago, IL).

3. Results and discussion

During the course of this study, MRSA prevalence in our hospital was 30.9%, consistent with other resistance rates found in different Spanish hospitals (Cuevas et al., 2008; González-Domínguez et al., 2015; Lozano et al., 2013). Forty MRSA isolates showed HLMUPR, representing 27.2% of the studied isolates (mupirocin MICs ≥ 256 mg/L). This percentage is higher than that found in other Spanish hospital (Daskalaki et al., 2009). Other isolates were mupirocin-susceptible (i.e. MIC, <8 mg/L).

Mupirocin resistance appears to emerge easily in health centers with unrestricted policies that allow widespread use of mupirocin for long periods (Lee et al., 2011). Between 2009 and 2010, mupirocin consumption in our hospital stayed in approximately 1250 units per year (the consumption in the community could not be established). This mupirocin consumption has remained stable over the years. A guideline for surveillance and control of MRSA in hospitals was published in Spain in 2008 (Rodríguez-Baño et al., 2008). Active surveillance cultures to detect asymptomatic MRSA colonization is recommended in all guidelines for control and prevention of MRSA. In general, the guidelines recommends screening the patients at high risk of colonization (previously colonized, patients with multiple hospital or healthcare facilities admissions with high prevalence of MRSA). In our hospital, mupirocin 2% nasal ointment is used for decolonization of nasal carriers of MRSA after positive MRSA screening.

No statistically significant differences were found between patients with HLMUPR-MRSA and mupirocin-susceptible MRSA isolates, regarding to age (median: 75 years versus 72 years; $P = 0.124$), male gender (50.0% versus 59.8%; $P = 0.285$), community onset (25.0% versus 17.7%; $P = 0.326$).

In relation to comorbidity conditions associated with patients included in our study, diabetes was more frequently found in patients with HLMUPR-MRSA than mupirocin-susceptible MRSA isolates (35.0% versus 18.7%; $P = 0.037$); while the presence of malignancy (10.0% versus 25.2%; $P = 0.044$) or medical devices (12.5% versus 30.8%; $P = 0.024$) were more related with patients who were infected with mupirocin-susceptible MRSA isolates.

HLMUPR-MRSA were more frequently collected from skin and soft tissue samples than mupirocin-susceptible MRSA isolates (72.5% versus 27.1%; $P < 0.001$). This association between HLMUPR-MRSA and skin and soft tissue infections has also been described by other authors (Daskalaki et al., 2009). HLMUPR-MRSA was more frequently found in vascular surgery service than mupirocin-susceptible MRSA (30.0% versus 7.5%; $P < 0.001$). No significant differences in the prevalence of other medical services were found. HLMUPR-MRSA were less frequently collected from lower respiratory tract samples than mupirocin-susceptible MRSA isolates (2.5% versus 25.2%; $P = 0.002$).

During the year prior to isolation, the antibiotics most frequently prescribed in patients who carried these HLMUPR-MRSA isolates were beta-lactams (57.5%) and mupirocin (30%). These results were not obtained in the group of mupirocin-susceptible MRSA isolates.

An aminoglycoside resistance phenotype was more frequently observed among HLMUPR-MRSA than mupirocin-susceptible MRSA isolates (gentamicin: 85.0% versus 11.2%; $P < 0.001$, kanamycin: 97.5% versus 82.2%; $P = 0.016$, tobramycin: 92.5% versus 64.5%; $P = 0.001$, amikacin: 85.0% versus 42.0%; $P < 0.001$, streptomycin: 12.5% versus 19.6%; $P = 0.314$, netilmicin: 7.5% versus 0%; $P = 0.019$) (Fig. 1). Aminoglycoside resistance was encoded by *aac*(6′)-Ie-*aph*(2″)-Ia (85.0% versus 11.2%; $P < 0.001$) and *ant*(4′)-Ia (85.0% versus 64.5%; $P = 0.016$) genes. A high rate of macrolide resistance was observed, but no significant differences were found between high-level mupirocin-resistant and mupirocin-susceptible isolates (erythromycin: 75.0% versus 58.8%; $P = 0.07$, azithromycin: 75.0% versus 58.8%; $P = 0.07$, spiramycin: 17.5% versus 13.1%; $P = 0.496$, clindamycin: 17.5% versus 12.1%; $P = 0.4$). The difference in the prevalence of erythromycin and clindamycin resistance is due to the high percentage of the M phenotype that implicates erythromycin-resistant but clindamycin-susceptible pattern. Macrolide resistance by efflux due to the *msr*(A) gene is related with this phenotype. The main macrolide resistance gene found in our HLMUPR-MRSA isolates was *msr*(A) (67.5% versus 43.9%; $P = 0.011$). None of HLMUPR-MRSA isolates showed tetracycline resistance. Three predominant resistance gene profiles were found in HLMUPR-MRSA isolates compared with mupirocin-susceptible MRSA isolates. These profiles carried one or two aminoglycoside resistance genes with or without the macrolide resistance *msr*(A) gene. Profile 1: *msr*(A) + *aac*(6′)-Ie-*aph*(2″)-Ia + *ant*(4′)-Ia (30.0% versus 0.9%; $P < 0.001$), Profile 2: *aac*(6′)-Ie-*aph*(2″)-Ia + *ant*(4′)-Ia (25.0% versus 0%; $P < 0.001$) and Profile 3: *msr*(A) + *aac*(6′)-Ie-*aph*(2″)-Ia + *ant*(4′)-Ia + *aph*(3′)-IIIa (12.5% versus 0%; $P = 0.001$). However, mupirocin-susceptible MRSA isolates were more related to isolates harboring the combination of *msr*(A) + *ant*(4′)-Ia genes, only the *ant*(4′)-Ia gene or the susceptible phenotype.

All high-level resistant isolates carried the *ileS2* gene. No association between multidrug resistance and HLMUPR-MRSA were found as described by Pérez-Roth *et al.*, (Pérez-Roth et al., 2013). These isolates showed higher gentamicin and tobramycin resistance rates than mupirocin-susceptible MRSA isolates what could be related to the *aac*(6′)-Ie-*aph*(2″)-Ia presence. This fact has been previously demonstrated with the detection of the *aac*(6′)-Ie-*aph*(2″)-Ia and *ileS2* genes co-located on the same plasmid (McDougal et al., 2010).
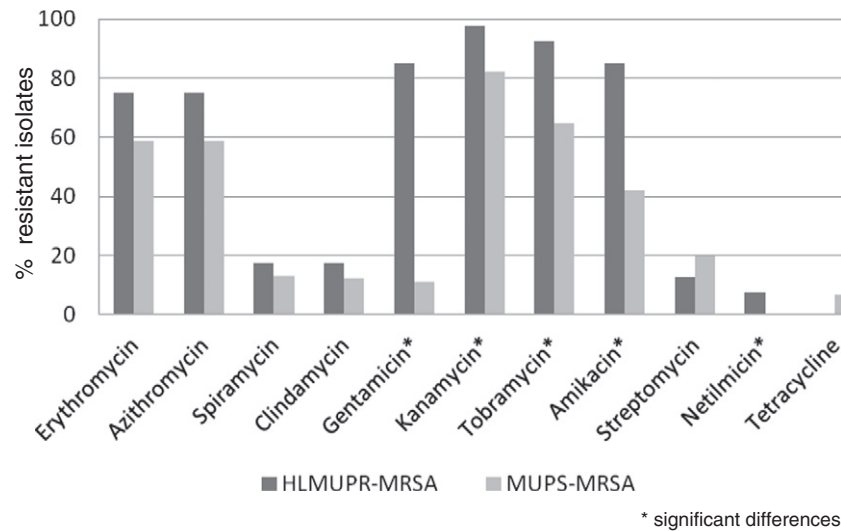
Fig. 1. Percentages of antibiotic resistance detected in high level mupirocin-resistant MRSA (HLMUPR-MRSA) and mupirocin-susceptible MRSA (MUPS-MRSA).

Four different *spa* types (t002, t067, t2220 and t2226) were observed among HLMUPR-MRSA isolates. The *spa* type t067 was the predominant one (82%), although this *spa* type was also frequent in mupirocin-susceptible group (52%) where 22 different *spa* types were found. *spa* type t067 is the most frequent *spa* type identified in Spain (Argudín et al., 2009; González-Domínguez et al., 2012, 2015; Lozano

Table 1
Characteristics of 40 HLMUPR-MRSA isolates.

IS257-ileS2 amplification patterns	PFGE profile	ST	spa type	SCCmec	agr	Antibiotic resistance genes	Date of culture	Onset
p1	C	125	t067	IVc	II	$msr(A) + erm(C) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	24/07/2009	Hospital
p2	A$_4$		t2226	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	28/07/2009	Hospital
	A$_5$		t2226	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	10/11/2009	Hospital
	A$_5$		t2226	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	24/12/2009	Hospital
	A$_6$		t067	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	25/01/2010	Community
	A$_{10}$		t2226	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	26/09/2009	Hospital
p3	A$_2$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	31/05/2010	Hospital
	A$_3$		t067	IVc	II	$erm(C) + ant(4')$-Ia $+ ileS2$	15/04/2010	Community
	A$_8$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	29/07/2009	Hospital
	A$_8$		t067	IVc	II	$msr(A) + erm(C) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ aph(3')$-IIIa $+ ileS2$	30/07/2009	Hospital
	A$_9$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	25/02/2010	Hospital
	A$_9$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	13/04/2010	Hospital
	A$_9$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	21/05/2010	Hospital
	A$_9$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	10/06/2010	Hospital
	A$_9$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	10/06/2010	Hospital
	A$_9$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	23/06/2010	Hospital
	A$_9$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ aph(3')$-IIIa $+ ileS2$	17/06/2010	Hospital
	A$_9$		t067	IVc	II	$msr(A) + erm(C) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ aph(3')$-IIIa $+ ileS2$	02/03/2010	Hospital
	A$_9$		t067	IVc	II	$msr(A) + erm(B) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ aph(3')$-IIIa $+ ileS2$	23/06/2010	Hospital
	A$_9$		t067	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	28/06/2010	Hospital
	A$_{10}$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	11/05/2010	Hospital
	A$_{11}$		t067	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	23/06/2010	Hospital
	B$_1$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ aph(3')$-IIIa $+ ileS2$	11/03/2010	Hospital
	B$_1$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ aph(3')$-IIIa $+ ileS2$	26/09/2009	Community
	B$_2$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ aph(3')$-IIIa $+ ileS2$	20/05/2010	Hospital
	B$_2$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ aph(3')$-IIIa $+ ileS2$	08/06/2010	Community
	B$_3$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	31/05/2010	Hospital
	B$_4$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ileS2$	05/07/2010	Hospital
	B$_4$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	19/04/2010	Hospital
	B$_4$		t067	IVc	II	$msr(A) + aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ aph(3')$-IIIa $+ ileS2$	21/04/2010	Hospital
	B$_6$		t067	V	II	$msr(A) + ant(4')$-Ia $+ ileS2$	03/11/2009	Community
	C		t067	IVc	II	$msr(A) + erm(C) + aac(6')$-le-$aph(2'')$-Ia $+ ileS2$	20/10/2009	Community
p4	A$_7$		t067	IVc	II	$erm(C) + ant(4')$-Ia $+ ileS2$	20/01/2010	Hospital
p5	B$_5$		t067	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	03/05/2010	Hospital
p6	A$_{11}$		t2220	IVc	II	$msr(A) + aph(3')$-IIIa $+ ileS2$	22/06/2010	Hospital
	A$_{11}$		t2220	IVc	II	$msr(A) + aph(3')$-IIIa $+ ileS2$	25/01/2010	Community
p7	B$_1$		t067	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	08/10/2009	Hospital
p8	A$_1$	5	t002	IVc	II	$erm(C) + ileS2$	25/05/2010	Community
p9	A$_6$	125	t067	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	31/05/2010	Community
	B$_1$		t067	IVc	II	$aac(6')$-le-$aph(2'')$-Ia $+ ant(4')$-Ia $+ ileS2$	12/06/2010	Community

PFGE profile: Capital letters (A, B and C) mean strains with different PFGE profiles. The numbers in subscript $_{(1-11)}$ indicate that those strains show closely related PFGE profiles.

et al., 2013; Menegotto et al., 2012), so this fact could be the reason for its high prevalence in both groups. Among the HLMUPR-MRSA strains, 97.5% of them belonged to ST125 and 2.5% to ST5. Both ST are included in CC5, widely distributed in hospital and community settings in Spain (Argudín et al., 2009; González-Domínguez et al., 2012, 2015; Lozano et al., 2013). An association between MRSA CC5/ST125/t067 and erythromycin resistance (encoded by msr(A)/msr(B) genes) has been observed. This relation was previously described by other authors (Daskalaki et al., 2009; González-Domínguez et al., 2015; Lozano et al., 2013; Pérez-Roth et al., 2013). All HLMUPR-MRSA strains carried SCCmec type IVc and agr type II, with the exception of one strain included in SCCmec type V that belonged to ST125. None of the studied virulence genes were identified among HLMUPR-MRSA isolates. Pulsed field gel electrophoresis grouped the HLMUPR-MRSA strains in three different PFGE pulsotypes (Table 1). The most prevalent was pulsotype A that included 65% of the strains. This pulsotype had 11 subtypes where subtype A9 was the most frequent. Thirty per cent of the strains were included in pulsotype B, and subtype B1 was the most frequent. Finally, 5% of strains were included in pulsotype C. These results showed that the strains were closely related.

Analyzing the HLMUPR-MRSA strains selected from vascular surgery we observed the presence of different clones (genetically unrelated).

Considering clinical, microbiological and molecular data, seventy-five percent of HLMUPR-MRSA strains were classified as healthcare-associated MRSA (HA-MRSA) clones (Table 1). The remaining ones were community-onset MRSA (CO-MRSA) isolates related to HA-MRSA clones (CO-HA-MRSA). HLMUPR-MRSA was not observed in community-associated MRSA (CA-MRSA) clones.

In HLMUPR-MRSA isolates, distinct IS257-ileS2 spacer arrangements (characterized by a different location of direct repeated copies of ileS2-flanking IS257) allowed us to distinguish 9 IS257-ileS2 amplification patterns, named p1 to p9. Transfer of ileS2-encoding plasmids is important in the spread of HLMUPR-MRSA as evidenced by the recovery of distinct plasmid configuration types in the same MRSA clone (Pérez-Roth et al., 2013). We know that the IS257-ileS2 PCR might have exceptional limitations (Pérez-Roth et al., 2006, 2010, 2011, 2013) but we used this PCR as a first and simple approximation to address the molecular epidemiology of plasmid-mediated HLMUPR-MRSA isolates in our clinical setting. Only plasmid sequencing could definitely clarify whether ileS2 plasmids are the same or not. The p3 pattern was the most frequent in our hospital and community MRSA isolates (65%) (Table 1). All of them belonged to the same clonal lineage ST125/t067 widely distributed in hospital and community settings in Spain. This suggests a clonal spread in our environment which could explain the high prevalence of HLMUPR-MRSA during the study period.

4. Conclusions

Most of HLMUPR-MRSA isolates that are circulating in our environment belonged to ST125/t067. This specific lineage is predominant in our area and it is associated with resistance to aminoglycosides, and to a lesser extent, to macrolides. The presence of the same IS257-ileS2 amplification pattern p3 in 65% of HLMUPR-MRSA, all of them ST125/t067, suggests a clonal spread in our hospital and community environment which could explain the high prevalence of HLMUPR-MRSA during the study period. An outbreak situation or an increase in mupirocin consumption was not observed.

Conflict of interest

None.

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