J Infect Chemother (2013) 19:233–242
DOI 10.1007/s10156-012-0486-4

ORIGINAL ARTICLE

Changes in genetic lineages, resistance, and virulence in clinical methicillin-resistant *Staphylococcus aureus* in a Spanish hospital

Carmen Lozano · Nerea Porres-Osante · Julien Crettaz · Beatriz Rojo-Bezares · Daniel Benito · Inés Olarte · Myriam Zarazaga · Yolanda Sáenz · Carmen Torres

Received: 26 July 2012 / Accepted: 13 September 2012 / Published online: 3 October 2012

Abstract A total of 204 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were isolated in a Spanish hospital in two different periods (2001 and 2009). The percentages of MRSA isolates detected in 2001 and 2009 were 29 and 27 %, respectively. Genetic lineages, resistance mechanisms, and virulence traits were determined in these isolates. The most frequent detected lineage in both periods was *S. aureus* protein A (*spa*)-type t067, assigned to clonal complex (CC) 5 (CC5-t067), being more prevalent in 2001 (93 %) than in 2009 (71 %). The remaining CCs and *spa*-types detected were (%2001/%2009): CC5-t002 (0/5), CC8-t008 (1/16), CC8-t024 (0/1), CC8-t190 (0/3), CC8-t2849 (0/2), CC22-t032 (0/2), CC30-t012 (1/0), CC228-t109 (1/0), CC228-t1318 (2/0), and CC247-t051 (2/0). Most of the MRSA were isolated from wounds, representing 39 % in 2001 and 63 % in 2009. The emergence of MRSA CC8 isolates, mainly from wounds, seemed to occur in the second period. Resistance to (%2001/%2009) quinolones (99/87), aminoglycosides (98/88), macrolides (32/30), lincosamides (30/17), and tetracycline (2/1) was found in isolates in both periods. Trimethoprim-sulfamethoxazole resistance was detected only in 2001 (1 %), and chloramphenicol (1 %) and mupirocin resistance (11 %) were detected only in 2009. An association between staphylococcal enterotoxin gene profiles and CCs was detected in most of the cases. The *egc*-cluster was related to CC5, CC22, CC30, and CC228 and most of the CC8 isolates presented the *sed*, *sej*, and *ser* genes. Four *tst-1*-positive (CC5 and CC30) isolates were detected in 2001 and two *lukS/F*-PV-positive isolates were detected in 2009. Therefore, there is still a predominance of CC5-t067 in our region, although an increase of lineage CC8 was observed.

Keywords MRSA · Spanish hospital · CC5-t067 · CC8 · PVL · TSST

C. Lozano · N. Porres-Osante · D. Benito · M. Zarazaga · C. Torres (✉)
Biochemistry and Molecular Biology, University of La Rioja, Madre de Dios 51, 26006 Logroño, Spain
e-mail: carmen.torres@unirioja.es

N. Porres-Osante · B. Rojo-Bezares · Y. Sáenz · C. Torres
Molecular Microbiology Area, Center of Biomedical Research of La Rioja (CIBIR), Logroño, Spain

J. Crettaz · I. Olarte
Laboratory of Microbiology, Hospital San Pedro, Logroño, Spain

Introduction

Staphylococcus aureus is an important pathogen which is able to develop resistance to multiple antimicrobial agents. This microorganism produces large amounts of virulence factors, and some of them can be classified as leukocidins [as Panton-Valentine leukocidin (PVL)], pyrogenic toxin superantigens (PTSAgs), haemolysins, or exfoliatins [1].

In 1961, an important public health problem arose when the first methicillin-resistant *S. aureus* (MRSA) strain emerged. The *mecA* gene, which encodes a modified penicillin-binding protein with low affinity to beta-lactams (PBP2a), confers resistance to methicillin but also to most other beta-lactams that leads to a serious therapeutic problem. Additionally, studies carried out in methicillin-susceptible *S. aureus* (MSSA) and MRSA have shown higher diversity of clonal lineages among MSSA in relation to MRSA. Thus, according to the results obtained with different molecular typing methods, it has been observed that some MRSA clones tend to occur more in certain countries or areas [2, 3] while other clones are disseminated worldwide, threatening public

health [4]. These typing methods allow us to know more about the epidemiology, evolution, and spread of this microorganism.

A differentiation between hospital-acquired (HA) and community-acquired (CA) MRSA was initially proposed. However, these distinctions have diminished and today it is often difficult to distinguish them. Recent studies have further shown that some HA-MRSA clonal lineages have been superseded by CA-MRSA clones [4]. In addition, new associated lineages in farm animals, known as livestock-associated (LA) MRSA, have also been found in healthcare settings [5].

Several surveillance studies have been conducted in European countries to determine the predominant MRSA clones. So far, the data obtained in Spain indicate that, while the Iberian clone (CC247-ST247-SCC*mec*I-*agr*I) was the dominant one in our country in the 1990s, a derived clone of pediatric origin (CC5-ST125-SCC*mec*IV-*agr*II) is the one the most commonly found in the 21st century [6–9]. The aim of our study was to identify circulating MRSA clones in a Spanish hospital, and to determine their changes, studying two periods distant in time. Besides the genetic lineage diversity, antibiotic resistance mechanisms and virulence traits were also analyzed to understand the evolution of MRSA in our country.

Materials and methods

Selection of isolates

A total of 204 MRSA isolates were included in this study. These isolates were recovered from different patients in San Pedro Hospital of La Rioja (Northern Spain) in two periods. One hundred and three of these isolates were obtained from January to June 2001, and the remaining 101 from January to April 2009. The criterion of choice was to study approximately the first 100 MRSA isolates causing any type of infection in each of these years. In the first period, 29 % of the obtained *S. aureus* were MRSA and in the second period the proportion was 27 %. The collection included isolates from different origins such as wounds, nasal fluid, urine, sputum, blood, bronchial and tracheal aspirates, abdominal and pleural fluids, ears, and catheters.

Confirmation of species and *mecA* gene detection

All MRSA isolates were typed by the Microscan system (Microscan, Alphen aan den Rijn, The Netherlands) in the hospital and were subsequently confirmed by a specific duplex polymerase chain reaction (PCR) of the *nuc* gene (*S. aureus* thermonuclease gene) and the *mecA* gene (methicillin-resistance gene) [10].

Molecular typing

Single-locus DNA sequencing of *S. aureus* protein A (*spa*) [11] was carried out following standard methodology and the obtained sequences were analyzed by Ridom Staph-Type software version 1.5.21 (Ridom, Würzburg, Germany). Determination of *agr* types was performed by two multiplex PCRs as described elsewhere [12]. SCC*mec*-typing was carried out by multiplex PCRs using sets of region-specific primers, as previously described [13]. Identification of the new recently reported variant of SCC*mec* IV (IVNv) was performed by PCR [14]. Multilocus sequence typing (MLST) was implemented by PCR and sequencing in 11 selected MRSA isolates (one isolate of each *spa*-type detected) (http://www.saureus.mlst.net). The clonal complex (CC) of the isolates was assigned according to the ST determined (in 11 isolates) or according to the *spa*-type detected (in the remaining 193 isolates) [15]. Pulsed field gel electrophoresis (PFGE) with the *Sma*I enzyme was performed in 18 of the MRSA t067 isolates and in one MRSA isolate of each of the other *spa*-types detected [16].

Antimicrobial susceptibility testing

Susceptibility testing for 17 antimicrobial agents was carried out by the disk-diffusion method [17]. The antibiotics tested were as follows (in μg/disk): penicillin (10 U), oxacillin (1), cefoxitin (30), erythromycin (15), clindamycin (2), lincomycin (15), gentamicin (10), kanamycin (30), tobramycin (10), tetracycline (30), ciprofloxacin (5), chloramphenicol (30), trimethoprim-sulfamethoxazole (1.25 + 23.75), vancomycin (30), teicoplanin (30), mupirocin (5 and 200), and linezolid (30). Clinical and Laboratory Standards Institute (CLSI) breakpoints were used for all antimicrobial agents, except for lincomycin, for which the breakpoints recommended by the Société Française de Microbiologie were considered (http://www.sfm.asso.fr).

Resistance genotypes

The presence of the *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(T), *msr*(A)/*msr*(B), *mph*(C), *lnu*(A), *vga*(A), *vga*(C), *lsa*(C), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *aac*(6′)-Ie–*aph*(2″)-Ia, *ant*(4′)-Ia, *aph*(3′)-IIIa, *dfrS1*, *dfrD*, *dfrG*, *dfrK*, *mupA*, *fexA*, *cfr*, *cat*(pC194), *cat*(pC221), and *cat*(pC223) resistance genes was tested by PCR [18, 19]. Mutations in quinolone targets were determined by sequence analysis of the *grlA* and *gyrA* genes in the 13 quinolone-resistant MRSA isolates from blood samples [10].

Virulence factors

The presence of the genes encoding PVL (*lukS/F*-PV), toxic shock syndrome toxin (TSST)-1 (*tst-1*), exfoliative

toxins A, B, and D (*eta*, *etb*, *etd*), and haemolysins alpha-, beta-, delta-, gamma- and gamma-variants (*hla*, *hlb*, *hld*, *hlg*, hlg_v) was determined by PCR [1, 10, 20]. The 18 genes that encode staphylococcal enterotoxins (SEs) (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, and *seu*) were investigated by three multiplex PCRs [21].

Results

Molecular typing of MRSA isolates

The occurrence of CC-*agr*-SCC*mec* and the *spa*-types detected in each period are shown in Fig. 1. The most frequent *spa*-type detected in both periods was t067, being more prevalent in 2001 (93 %) than in 2009 (71 %). Another detected *spa*-type in both periods was t008, although an important increase in its frequency was observed in 2009 in respect to 2001 (16 and 1 %, respectively) (Fig. 1). The *spa*-types detected only in the MRSA isolates of 2001 were t012, t051, t109, and t1318 (6 %) and in the MRSA isolates of 2009 they were t002, t024, t032 t190, and t2849 (13 %). MLST was implemented in 11 selected MRSA isolates and the following STs were detected (*spa*-types): ST8 (t008, t024, t190, and t2849), ST22 (t032), ST30 (t012), ST125 (t067), ST146 (t002), ST228 (t109 and t1318), and ST247 (t051). These STs belonged to six different CCs, and in the remaining isolates (in which MLST was not performed) the CC was assigned based on the *spa*-type detected. Thus, according to these criteria, the following CCs were found (%2001/%2009): CC5 (93/76), CC8 (1/22), CC22 (0/2), CC30 (1/0), CC228 (3/0), and CC247 (2/0) (Fig. 1). Therefore, most of the isolates presented *spa*-types associated with CC5, and a remarkably high percentage of MRSA isolates with *spa*-types related to CC8 was found in 2009 (Fig. 1).

The most frequently detected SCC*mec* type was SCC*mec* IVc (≥94 % in both periods) and it was associated with isolates of CC5, CC8, and CC22. Other subtypes of SCC*mec* type IV were detected in very low percentages: the SCC*mec* IVa was detected in two MRSA CC8 (t008 and t024) isolates and the new variant of SCC*mec* IVNv was detected in one MRSA CC5-t002 isolate. The MRSA CC30 isolate showed SCC*mec* III, and the three MRSA CC228 and the two MRSA CC247 isolates had SCC*mec* I. Three types of *agr* were detected (%2001/%2009): *agr* I (3/24), *agr* II (96/76), and *agr* III (1/0).

In addition, 28 MRSA isolates were studied by PFGE using the *Sma*I restriction enzyme (18 MRSA CC5-t067 isolates and one isolate of each of the other *spa*-types detected). Seven unrelated pulsotypes were identified (A-G); moreover, 5 subtypes were revealed in pulsotype A (A1-A5) and 2 subtypes were shown in pulsotype C (C1 and C2). The distribution of pulsotypes was as follows: A (A1-A5)/CC5-t067, B/CC5-t002, C1/CC8-t008-t190-t2849, C2/CC8-t024, D/CC22-t032, E/CC30-t012, F/CC228-t109-t1318, and G/CC247-t051.

Origin of MRSA isolates

Most of the MRSA were isolated from wounds, representing 39 % in 2001 and 63 % in 2009. Samples of other origins in which MRSA isolates were found were as follows (%2001/%2009): nasal (14/16), urine (20/7), sputum (10/8), blood (9/4), and others (8/2). The correlation between the origin of the isolates and the CC is shown in Fig. 2.

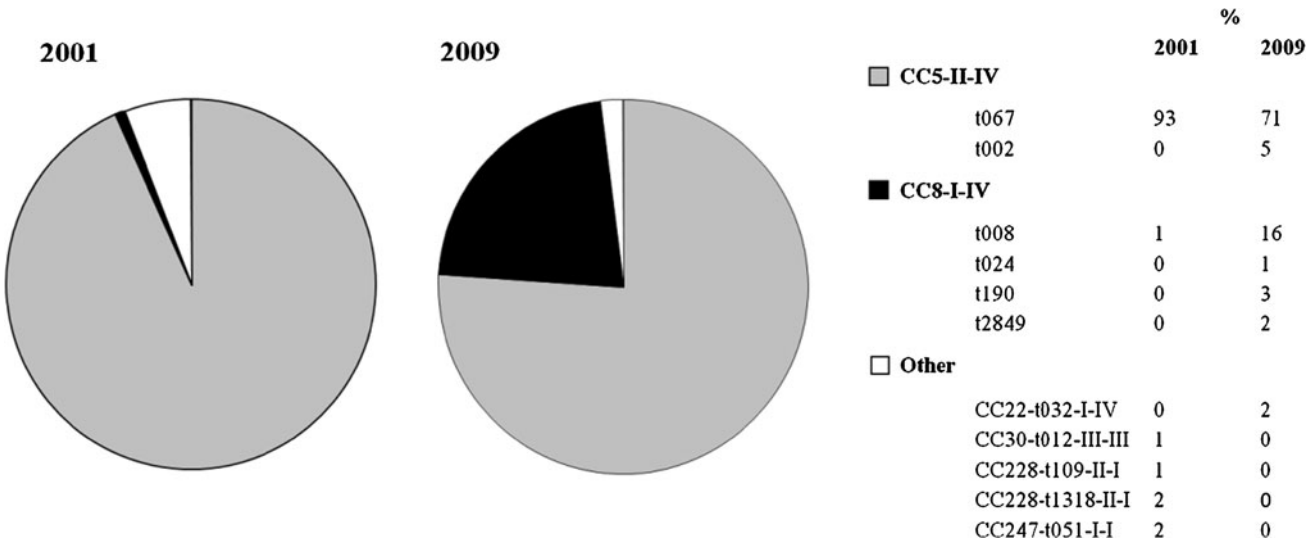

Fig. 1 Clonal complex (*CC*)-*agr*-SCC*mec* and *Staphylococcus aureus* protein A (*spa*)-types detected in each studied year

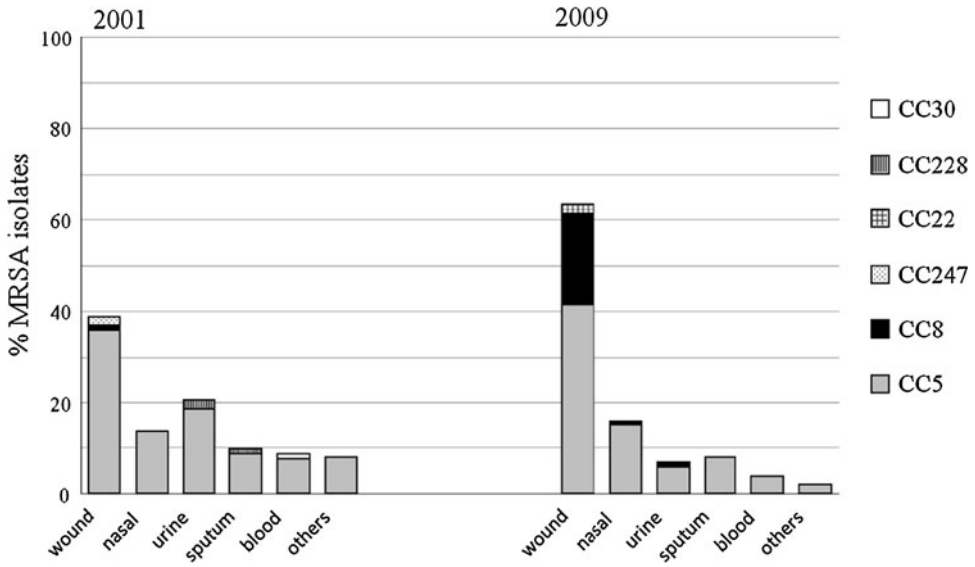

Fig. 2 Origins of the isolates and clonal lineages detected in 2001 and 2009. The category '*Others*' includes bronchial and tracheal aspirates, abdominal and pleural fluid, ears, and catheters. *MRSA* methicillin-resistant *Staphylococcus aureus*

Resistance phenotypes and genotypes

In general, the level of resistance to quinolones, aminoglycosides, macrolides, lincosamides, and tetracycline remained more or less constant in both periods, although it seemed that there could have been a slight downward trend in 2009 (Fig. 3). Resistance to trimethoprim-sulfamethoxazole was only detected in one MRSA isolate in 2001 and resistance to mupirocin and chloramphenicol was only detected in MRSA isolates in 2009. Moreover, in both years, some isolates (33 % in 2001 and 34 % in 2009) showed a multiresistant phenotype (resistance to three or more antibiotic families in addition to beta-lactams), with these isolates presenting a high number of resistance genes. Table 1 shows the different resistance patterns and the resistance genes detected in the 204 MRSA isolates.

More different resistance profiles were found in the isolates in 2001 than in isolates in 2009 (Table 1). Quinolone resistance was the one most frequently found among the 204 MRSA isolates. The 13 quinolone-resistant MRSA isolates from blood samples showed the amino acid changes Ser80Phe in GrlA and Ser84Leu in GyrA. Aminoglycoside resistance was also very common, the *ant*(4′)–Ia gene being the one most frequently found. The levels of resistance to tobramycin and kanamycin in 2001 and 2009 were very high, while the resistance to gentamicin was moderate. The level of macrolide resistance was similar in isolates from both periods, but the percentage of isolates showing clindamycin resistance was higher in 2001 (30 %) than in 2009 (17 %) (Fig. 3). So, it was not surprising that the *erm* genes were more commonly found in isolates from 2001 than in isolates from 2009.

Tetracycline resistance appeared in a very low percentage of isolates, and different combinations of *tet*(K), *tet*(L), and *tet*(M) genes were detected (Table 1). The only one trimethoprim-sulfamethoxazole-resistant MRSA isolate presented the *dfrS1* gene. Additionally, it is relevant to note the detection of 11 mupirocin-resistant isolates in 2009 which harbored the *mupA* gene. Resistance to

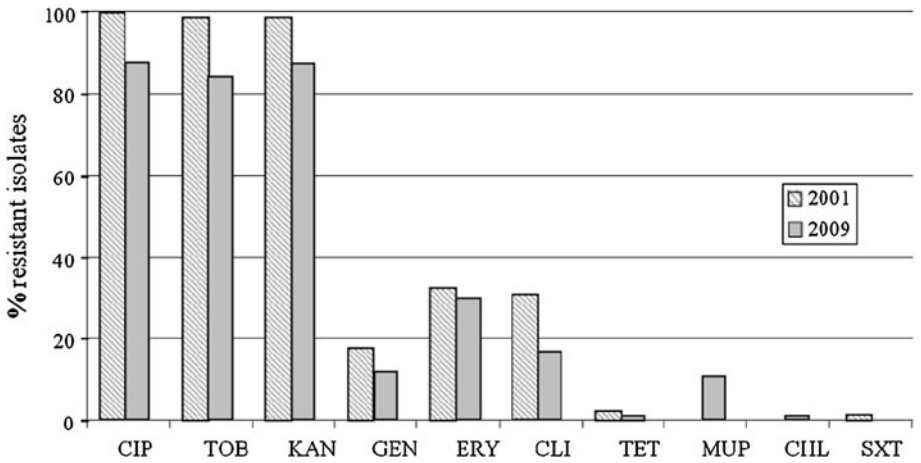

Fig. 3 Percentages of resistant isolates detected in the MRSA isolates in each year in this study. *CIP* ciprofloxacin, *TOB* tobramycin, *KAN* kanamycin, *GEN* gentamicin, *ERY* erythromycin, *CLI* clindamycin, *TET* tetracycline, *MUP* mupirocin, *CHL* chloramphenicol, *SXT* trimethoprim-sulfamethoxazole

Table 1 Resistance profiles, resistance genotypes, and CC-*spa* types of the studied MRSA isolates

CC[a] (*spa*-types)	Resistance profile	% of strains		Resistance genes (% of strains 2001/2009)
		2001	2009	
CC5 (t067/t002)	TOB-KAN-CIP[b]	55	38	*ant*(4′)-Ia (55/38), *aph*(3′)-IIIa (5/3)
	GEN-TOB-KAN-CIP[b]	8	3	*aac*(6′)-Ie–*aph*(2″)-Ia (8/3), *ant*(4′)-Ia (7/3), *aph*(3′)-IIIa (0/2)
	ERY-CLI-TOB-KAN-CIP[b]	22	11	*msr*(A)/*msr*(B) (9/0), *mph*(C) (2/0), *erm*(A) (1/0), *erm*(B) (4/1), *erm*(C) (18/11), *ant*(4′)-Ia (16/11)
	ERY-CLI-GEN-TOB-KAN-CIP[b]	4	1	*msr*(A)/*msr*(B) (1/0), *erm*(A) (4/0), *erm*(B) (1/0), *erm*(C) (2/1), *aac*(6′)-Ie–*aph*(2″)-Ia (3/1), *ant*(4′)-Ia (3/1)
	ERY-TOB-KAN-CIP[b]	1	4	*msr*(A)/*msr*(B) (1/3), *mph*(C) (1/3), *ant*(4′)-Ia (1/4), *aph*(3′)-IIIa (0/4)
	CIP[b]	1	1	
	ERY-GEN-TOB-KAN-CIP	1	0	*msr*(A)/*msr*(B) (1/0), *mph*(C) (1/0), *aac*(6′)-Ie–*aph*(2″)-Ia (1/0), *ant*(4′)-*Ia* (1/0)
	GEN-TOB-KAN-SXT	1	0	*aac*(6′)-Ie–*aph*(2″)-Ia (1/0), *ant*(4′)-Ia (1/0), *dfrS1* (1/0)
	ERY-KAN-CIP	0	6	*msr*(A)/*msr*(B) (0/6), *mph*(C) (0/4), *aph*(3′)-IIIa (0/6)
	GEN-TOB-KAN-MUP-CIP	0	2	*aac*(6′)-Ie–*aph*(2″)-Ia (0/2), *ant*(4′)-Ia (0/2), *mupA* (0/2)
	GEN-TOB-KAN-MUP	0	2	*aac*(6′)-Ie–*aph*(2″)-Ia (0/2), *ant*(4′)-Ia (0/2), *mupA* (0/2)
	ERY-CLI-KAN-CIP	0	1	*msr*(A)/*msr*(B) (0/1), *mph*(C) (0/1), *erm*(B) (0/1), *aph*(3′)-IIIa (0/1)
	Only oxacillin	0	1	
	TOB-KAN	0	1	*ant*(4′)-Ia (0/1)
	ERY-CLI	0	1	*erm*(C) (0/1)
	ERY-CLI-CIP	0	1	*msr*(A)/*msr*(B) (0/1), *mph*(C) (0/1), *erm*(C) (0/1)
	ERY-CIP	0	1	*msr*(A)/*msr*(B) (0/1), *mph*(C) (0/1)
	TOB-KAN-CIP-MUP	0	1	*ant*(4′)-*Ia* (0/1), *mupA* (0/1)
	ERY-TOB-KAN-CIP-MUP	0	1	*mph*(C) (0/1), *ant*(4′)-Ia (0/1), *mupA* (0/1)
	ERY-CLI-GEN-TOB-KAN	0	1	*erm*(A) (0/1), *erm*(C) (0/1), *aac*(6′)-Ie–*aph*(2″)-Ia (0/1)
CC8 (t008/t024/t190/t2849)	ERY-CLI-TOB-KAN-CIP	1	0	*erm*(C) (1/0), *ant*(4′)-Ia (1/0)
	TOB-KAN-CIP	0	7	*ant*(4′)-Ia (0/7)
	GEN-TOB-KAN-MUP	0	3	*aac*(6′)-Ie–*aph*(2″)-Ia (0/3), *ant*(4′)-Ia (0/3), *mupA* (0/3)
	CIP	0	2	
	GEN-TOB-KAN-CIP	0	2	*aac*(6′)-Ie–*aph*(2″)-Ia (0/2), *ant*(4′)-Ia (0/2), *aph*(3′)-IIIa (0/1)
	Only oxacillin	0	1	
	TET	0	1	*tet*(K) (0/1)
	GEN-TOB-KAN	0	1	*aac*(6′)-Ie–*aph*(2″)-Ia (0/1), *ant*(4′)-Ia (0/1)
	ERY-CLI-TOB-KAN	0	1	*erm*(C) (0/1), *ant*(4′)-Ia (0/1)
	TOB-KAN-CIP-MUP	0	1	*ant*(4′)-*Ia* (0/1), *mupA* (0/1)
	ERY-GEN-TOB-KAN-CIP-MUP	0	1	*msr*(A)/*msr*(B) (0/1), *mph*(C) (0/1), *aac*(6′)-Ie–*aph*(2″)-Ia (0/1), *ant*(4′)-Ia (0/1), *mupA* (0/1)
	GEN-TOB-KAN-MUP-CIP	0	1	*aac*(6′)-Ie–*aph*(2″)-Ia (0/1), *mupA* (0/1)
	TOB-KAN-CIP-CHL	0	1	*ant*(4′)-*Ia* (0/1), *cat*(pC221) (0/1)
CC22 (t032)	CIP	0	2	
CC30 (t012)	ERY-CLI-TOB-KAN-CIP	1	0	*msr*(A)/*msr*(B) (1/0), *erm*(A) (1/0), *erm*(B) (1/0), *erm*(C) (1/0), *ant*(4′)-Ia (1/0)
CC228 (t109/t1318)	GEN-TOB-KAN-CIP	2	0	*aac*(6′)-Ie–*aph*(2″)-Ia (2/0), *ant*(4′)-Ia (2/0)
	ERY-CLI-GEN-TOB-KAN-CIP	1	0	*msr*(A)/*msr*(B) (1/0), *erm*(A) (1/0), *aac*(6′)-Ie–*aph*(2″)-Ia (1/0), *ant*(4′)-Ia (1/0)

Table 1 continued

CC[a] (*spa*-types)	Resistance profile	% of strains		Resistance genes (% of strains 2001/2009)
		2001	2009	
CC247 (t051)	ERY-CLI-GEN-TOB-KAN-CIP-TET	2	0	*tet*(M) (2/0), *tet*(L) (1/0), *tet*(K) (1/0), *erm*(A) (2/0), *erm*(B) (1/0), *msr(A)/msr(B)* (2/0), *aac*(6′)-Ie–*aph*(2″)-Ia (2/0), *ant*(4′)-Ia (2/0)

MRSA methicillin-resistant *Staphylococcus aureus*, *CC-spa* clonal complex-*S. aureus* protein A, *TOB* tobramycin, *KAN* kanamycin, *CIP* ciprofloxacin, *GEN* gentamicin, *ERY* erythromycin, *CLI* clindamycin, *SXT* trimethoprim-sulfamethoxazole, *MUP* mupirocin, *CHL* chloramphenicol, *TET* tetracycline

[a] CC was assigned according to the determined sequence typing (ST) or the detected *spa*-type. The *spa*-types associated with these CCs are shown in parentheses

[b] The resistance profile present in strains in both periods

chloramphenicol was only detected in one isolate and was associated with the gene *cat*(pC221).

Virulence gene profiles

Different virulence gene profiles were detected in the 204 MRSA isolates (Table 2). The majority of the MRSA isolates (99 %) were positive for at least one staphylococcal enterotoxin (SE) gene, and all of them carried some haemolysin gene. An association between the SE gene profiles and CC was detected in most of the cases. The SE genes located in the *egc*-cluster (*seg*, *sei*, *sem*, *sen*, and *seo*) were found in isolates with *spa*-types associated with CC5, CC22, CC30, and CC228. In some cases, the *egc*-cluster appeared in isolates which also showed the *seu* (called *egc*-like cluster), *sea*, and/or *sep* genes. The *egc*-cluster and the *sed*, *sej*, and *ser* genes were identified in one isolate. Interestingly, the *egc*-like cluster was contained in a higher number of CC5-t067 isolates in 2001 (31 %) than in 2009 (4 %). The combination of the *sed*, *sej*, and *ser* genes and the *sea* gene was observed in several CC8 isolates. The *tst-1* gene was identified in four isolates (one CC30 and three CC5) from 2001. Two MRSA CC8 isolates were positive for *lukS/F*-PV. Both isolates were obtained in 2009 and were the only ones with SCC*mec* type IVa. Lastly, the two MRSA CC247 isolates showed the *sea* gene.

Discussion

Two hundred and four MRSA isolates obtained from a Spanish hospital in two different years (2001 and 2009) were included in this study. The prevalence of MRSA isolates was slightly higher in 2001 (29 %) than in 2009 (27 %). In some studies carried out in Spain a progressive increase in the oxacillin-resistance level was detected from 1986 (2 %) to 2002 (31 %) [22], and the level seemed to be stabilized in 2006 (30 %) [23]. It seems probable that the implementation of several control measures in the different Spanish hospitals has been able to avoid an increase in these values. In this regard, there have been several attempts to establish a consensus document [24, 25].

In Europe, according to the latest data of the European Antimicrobial Resistance Surveillance System (EARSS), seven countries reported decreasing trends for invasive MRSA, whereas an increasing trend was observed in four other countries [26]. In any case, the rates above 25 % detected in more than one-fourth of European countries, among them Spain [26], are worrisome, with the MRSA problem being a public health priority.

It has been observed that, in contrast to MSSA, MRSA *spa*-types principally have a regional distribution in European countries [3]. In our work, the t067 *spa*-type was the most prevalent one in both periods. This *spa*-type is the one most frequently found in Spain [9] and it is also quite common in Finland, as recently reported [27]; however, it has been found at a very low frequency in other European countries, in the United States, and in South America [3, 28] (http://spa.ridom.de/spa-t067.shtml). Additionally, the *spa*-type t067 is usually associated with ST125 and, in some cases, with ST5 (both included in CC5), SCC*mec* type IV, and *agr* type II. The most common subtypes of SCC*mec* IV are normally IVa and IVc, which have been detected in variable percentages according to different studies [6, 9]. All our MRSA CC5-t067 isolates presented the same subtype (SCC*mec* type IVc).

Of note, the association between MRSA CC5-t067 and ciprofloxacin, tobramycin/kanamycin and erythromycin resistance has been described. The resistance genes responsible for these tobramycin/kanamycin and erythromycin phenotypes are mainly the *ant*(4′)-Ia and *msr*(A)/*msr*(B) genes, respectively [9]. In our study, a high percentage of CC5-t067 isolates harbored the *ant*(4′)-Ia gene, and in regard to erythromycin, a moderate percentage of the CC5-t067 isolates showed resistance to this antibiotic and many of them contained the *msr*(A)/*msr*(B) genes. However, these resistance mechanisms, and mainly the

Table 2 Combinations of detected virulence genes in the 204 studied MRSA isolates according to clonal lineages and the year of isolation

CC[a]-*spa* (% 2001/2009)	Leukocidin and haemolysin genes	PTSAg[b]	Percentage of positive strains	
			2001	2009
CC5-t067 (93/71)	*hla*, *hlb*, *hld*, hlg_v	*egc*[c]	24	22
	hla, *hlb*, *hld*, hlg_v	*egc*, *sep*	26	39
	hla, *hlb*, *hld*, hlg_v	*egc*-like[d]	17	1
	hla, *hlb*, *hld*, hlg_v	*egc*-like, *sep*	11	2
	hla, *hlb*, *hld*	*egc*	4	0
	hla, *hlb*, *hld*	*egc*, *sep*	5	1
	hla, *hld*, *hlg*	*egc*, *sep*	1	0
	hla, *hlb*, *hld*, *hlg*	*egc*, *sep*	1	0
	hla, *hlb*, *hld*	*egc*-like, *sep*	2	0
	hla, *hlb*, *hld*, hlg_v	*egc*, *tst-1*	1	0
	hla, *hlb*, *hld*, hlg_v	*egc*, *sep*, *tst-1*	2	0
	hla, *hlb*, *hld*, hlg_v		0	1
	hla, *hld*, hlg_v	*egc*	0	1
	hla, *hlb*, *hld*, hlg_v	*sep*	0	1
	hla, *hld*, hlg_v	*egc*, *sep*	0	4
CC5-t002 (0/5)	*hla*, *hld*, hlg_v	*egc*	0	1
	hla, *hlb*, *hld*	*egc*, *sep*	0	1
	hla, *hlb*, *hld*, hlg_v	*egc*, *sep*	0	1
	hla, *hlb*, *hld*, hlg_v	*egc*, *sed-sej-ser*	0	2
CC8-t008 (1/16)	*hla*, *hlb*, *hld*, hlg_v	*sea*, *sed-sej-ser*	0	13
	hla, *hlb*, *hld*	*sea*, *sed-sej-ser*	0	1
	hla, *hlb*, *hld*, hlg_v	*sea*	1	1
	hla, *hlb*, *hld*, hlg_v, *lukS/F*-PV[e]		0	1
CC8-t024 (0/1)	*hla*, *hlb*, *hld*, hlg_v, *lukS/F*-PV[e]		0	1
CC8-t190 (0/3)	*hla*, *hlb*, *hld*, hlg_v	*sea*	0	3
CC8-t2849 (0/2)	*hla*, *hlb*, *hld*, hlg_v	*sep*	0	1
	hla, *hlb*, *hld*, hlg_v	*sed-sej-ser*	0	1
CC22-t032 (0/2)	*hla*, *hlb*, *hld*, hlg_v	*egc*	0	2
CC30-t012 (1/0)	*hlb*, *hld*, *hlg*	*egc*-like, *tst-1*	1	0
CC228-t109 (1/0)	*hla*, *hlb*, *hld*, *hlg*	*egc*, *sea*	1	0
CC228-t1318 (2/0)	*hla*, *hld*, hlg_v	*egc*, *sea*	1	0
	hla, *hlb*, *hld*, *hlg*	*egc*, *sea*	1	0
CC247-t051 (2/0)	*hla*, *hlb*, *hld*, hlg_v	*sea*	2	0

[a] CC was assigned according to the determined ST or the detected *spa*-type. The percentages of isolates that presented the corresponding CC in each year are shown in parentheses

[b] *PTSAg*, pyrogenic toxin superantigen

[c] *egc*, enterotoxin gene cluster (*seg*, *sei*, *sem*, *sen*, and *seo*)

[d] *egc*-like, enterotoxin gene cluster (*seg*, *sei*, *sem*, *sen*, *seo*, and *seu*)

[e] The SCC*mec*-type IVa was associated with the *lukS/F*-PV gene

ant(4′)-Ia gene, were also found in most of the MRSA non-CC5-t067 isolates in our study.

Another *spa*-type (t002) related to CC5 was identified in our isolates from 2009. This *spa*-type has already been detected in other studies carried out in Spanish hospitals [6, 9, 29]. It is interesting to remark that one MRSA CC5-t002 isolate presented the new variant of SCC*mec* type IV, which had been previously described in isolates with the same *spa*-type in Argentina [14].

The higher prevalence of MRSA CC8 in 2009 with respect to 2001 is outstanding. While in 2001 only one CC8 isolate was identified (1 %), 22 % of the isolates were of this lineage in 2009. Some STs belonging to CC8 have been considered predominantly as CA-MRSA [2, 30]. Most

of our MRSA CC8 isolates were obtained from wound samples, and CA-MRSA usually causes skin and soft-tissue infections. The substitution of HA-MRSA isolates by CA-MRSA isolates has already been reported by others [4, 31–33].

Interestingly, differences between HA-MRSA and CA-MRSA are less clear with time [31]. Thus, most of our MRSA CC8 isolates showed resistance, in addition to beta-lactams, also to aminoglycosides, quinolones, mupirocin, macrolides, or lincosamides. Moreover, only two isolates harbored the PVL genes.

Interestingly, some of our CC8 and CC5 isolates of 2009 showed mupirocin resistance. The emergence of this resistance has already been described in other hospitals [34]; this resistance could be produced by the use of mupirocin for treating nasal MRSA carriers.

Other detected CCs (CC22, CC30, CC228, and CC247) in our study have been described previously in studies carried out in our country [6, 8, 9, 29]. CC22 is a British clone (EMRSA-15) which was detected in Spain in 1999, and it has always been found at a very low percentage (<2 %) [8]. MRSA CC30 (EMRSA-16) has usually been described as showing SCC*mec* II or IV [9, 29]. However, our MRSA CC30 isolate presented SCC*mec* III and, in the only description of this type of SCC*mec* in CC30, an association with Tn*6072* was established [35]. CC228 (Southern German clone) is, in addition to CC5 (pediatric clone), one of the major HA-MRSA clones widely disseminated around the world [2]. Our two MRSA CC247 isolates were the only tetracycline-resistant ones in 2001 and this CC is known as the Iberian clone. In several studies performed in Spanish hospitals this CC was found to be the dominant one in the 1990s. However, its presence has been decreasing in subsequent studied years [7, 8]. Interestingly, in our study, CC247 was only detected in two isolates from 2001, and it was not identified in any isolates from 2009.

We note that some *spa*-types associated with CC5 (t003, t041, t062, t088, t837, t1154, t2222, t2226), CC8 (t148), and CC30 (t018), which have been detected previously by others in Spain [9], were not identified in the present study. Moreover, other major HA-MRSA and CA-MRSA clones, such as CC45, CC59, and CC80 [2], which are common in other countries, were not detected in our hospital.

We detected several isolates that presented a multiresistant phenotype; infections caused by this microorganism are difficult to treat. Remarkably, in some cases, multiple genes encoding the same resistance were identified. However, about 65 % of the isolates were resistant to fewer than three antimicrobials, besides oxacillin, and this finding could be explained by the high prevalence of SCC*mec* IV, as suggested by others [9]. We note that there appeared to have been a slight downward trend in the level of resistance in 2009. A significant decrease in the levels of resistance to gentamicin and clindamycin had already been observed [9, 23].

The *tst-1* gene was detected in four isolates from 2001; three of them belonged to CC5-t067 and the other one to CC30-t012. CC5-t067 isolates harboring the *tst-1* gene have been detected in Spain [6] and a TSST-1 CC5 clone has recently been described in France [36]. Moreover, an association between TSST-1 and CC30 has been reported previously [37].

There seems to be a linkage between SE genes and the clonal background [38]. In our study, the *egc* cluster was detected in isolates belonging to CC5, CC22, CC30, and CC228. This is in accordance with the results obtained by others, where *egc* was detected in CC5, CC22, and CC30 isolates, but not in CC8 isolates [10, 38]. The combination of the *sed*, *sej*, and *ser* genes was identified in 15 CC8 isolates (t008 and t2849, *spa*-types) and in two CC5-t002 isolates. These genes are located on the plasmid pIB485 [39] and their presence in CC8 and CC5 isolates has been described previously [40]. Moreover, a strong linkage between the plasmid-borne SE genes (*sed*, *sej*, and *ser*) and CC8 has been suggested [38]. Other SE genes identified were *sea* and *sep*, both located on bacteriophages [41, 42]. In the present study, the *sea* gene was mainly found in CC8, CC228, and CC247 isolates and the *sep* gene in CC5 (but also in one CC8 isolate). The detection of the *sea* gene in several CCs is explained by the broad distribution of this gene [38]. Moreover, it has been observed that the *sep* gene is more frequent in MRSA *agr* II isolates than in MSSA isolates [6].

In conclusion, in the present study, the most commonly found *spa*-type in both periods was t067 associated with CC5. This CC5-t067 lineage is still predominant in our country and it is associated with resistance to quinolones, aminoglycosides, and to a lesser extent, with resistance to macrolides. The possible emergence of CC8 was identified in the second period with two *lukS/F*-PV-positive MRSA isolates. The presence of four *tst-1*-positive isolates in the first period was also remarkable. The level of resistance among MRSA isolates remained more or less constant in both periods, although it seemed that there could have been a slight downward trend in 2009. This fact would be very positive and requires further research. The surveillance of genetic lineages, antibiotic resistance mechanisms, and virulence traits of MRSA isolates in our hospitals is highly important to better understand the behavior of this microorganism.

Acknowledgments This work was supported by Project SAF2009-08570 from the new Ministerio de Economía y Competitividad of Spain. C. Lozano has a fellowship from the Ministerio de Economía y Competitividad of Spain, and N. Porres-Osante has a fellowship from the Instituto de Salud Carlos III, Ministerio de Economía y

Competitividad of Spain. Part of these data was presented at the XIV Congreso Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica that was held in Barcelona, Spain, on 19–22 May 2010.

Conflict of interest None.

References

1. Jarraud S, Mougel C, Thioulouse J, Lina G, Meugnier H, Forey F, et al. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infect Immun. 2002;70:631–41.
2. Deurenberg RH, Stobberingh EE. The evolution of *Staphylococcus aureus*. Infect Genet Evol. 2008;8:747–63.
3. Grundmann H, Aanensen DM, van den Wijngaard CC, Spratt BG, Harmsen D, Friedrich AW, et al. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med. 2010;7:e1000215.
4. Stefani S, Chung DR, Lindsay JA, Friedrich AW, Kearns AM, Westh H, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonization of typing methods. Int J Antimicrob Agents. 2012;39:273–82.
5. Wulf MW, Markestein A, van der Linden FT, Voss A, Klaassen C, Verduin CM. First outbreak of methicillin-resistant *Staphylococcus aureus* ST398 in a Dutch hospital, June 2007. Euro Surveill. 2008;13:pii:8051.
6. Argudín MA, Mendoza MC, Méndez FJ, Martín MC, Guerra B, Rodicio MR. Clonal complexes and diversity of exotoxin gene profiles in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from patients in a Spanish hospital. J Clin Microbiol. 2009;47:2097–105.
7. Gasch O, Ayats J, Domínguez MA, Tubau F, Liñares J, Peña C, et al. Epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) bloodstream infection: secular trends over 19 years at a university hospital. Medicine (Baltimore). 2011;90:319–27.
8. Pérez-Roth E, Lorenzo-Díaz F, Batista N, Moreno A, Méndez-Alvarez S. Tracking methicillin-resistant *Staphylococcus aureus* clones during a 5-year period (1998 to 2002) in a Spanish hospital. J Clin Microbiol. 2004;42:4649–56.
9. Pérez-Vázquez M, Vindel A, Marcos C, Oteo J, Cuevas O, Trincado P, et al. Spread of invasive Spanish *Staphylococcus aureus spa*-type t067 associated with a high prevalence of the aminoglycoside-modifying enzyme gene *ant*(4′)-Ia and the efflux pump genes *msrA/msrB*. J Antimicrob Chemother. 2009;63:21–31.
10. Lozano C, Gómez-Sanz E, Benito D, Aspiroz C, Zarazaga M, Torres C. *Staphylococcus aureus* nasal carriage, virulence traits, antibiotic resistance mechanisms, and genetic lineages in healthy humans in Spain, with detection of CC398 and CC97 strains. Int J Med Microbiol. 2011;301:500–5.
11. Harmsen D, Claus H, Witte W, Rothgänger J, Claus H, Turnwald D, et al. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J Clin Microbiol. 2003;41:5442–8.
12. Shopsin B, Mathema B, Alcabes P, Said-Salim B, Lina G, Matsuka A, et al. Prevalence of *agr* specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. J Clin Microbiol. 2003;41:456–9.
13. Zhang K, McClure JA, Elsayed S, Louie T, Conly JM. Novel multiplex PCR assay for characterization and concomitant subtyping of Staphylococcal Cassette Chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol. 2005;43:5026–33.
14. Sola C, Paganini H, Egea AL, Moyano AJ, Garnero A, Kevric I, et al. Spread of epidemic MRSA-ST5-IV clone encoding PVL as a major cause of community onset staphylococcal infections in Argentinean children. PLoS ONE. 2012;7:e30487.
15. Lozano C, Rezusta A, Gómez P, Gómez-Sanz E, Báez N, Martin-Saco G, et al. High prevalence of *spa* types associated with the clonal lineage CC398 among tetracycline-resistant methicillin-resistant *Staphylococcus aureus* strains in a Spanish hospital. J Antimicrob Chemother. 2012;67:330–4.
16. Murchan S, Kaufmann ME, Deplano A, de Ryck R, Struelens M, Zinn CE, et al. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J Clin Microbiol. 2003;41:1574–85.
17. Clinical Laboratory Standards Institute (CLSI), 2011. Performance standards for antimicrobial susceptibility testing. Twenty first informational supplement. M100-S21. Wayne, PA: National Committee for Clinical Laboratory Standards.
18. Gómez-Sanz E, Torres C, Lozano C, Fernández-Pérez R, Aspiroz C, Ruiz-Larrea F, et al. Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in Spanish slaughter pigs of different age groups. Foodborne Pathog Dis. 2010;7:1269–77.
19. Schnellmann C, Gerber V, Rossano A, Jaquier V, Panchaud Y, Doherr MG, et al. Presence of new *mecA* and *mph*(C) variants conferring antibiotic resistance in *Staphylococcus* spp. isolated from the skin of horses before and after clinic admission. J Clin Microbiol. 2006;44:4444–54.
20. Lina G, Piémont F, Godail-Gamot F, Bes M, Peter MO, Gauduchon V, et al. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis. 1999;29:1128–32.
21. Hwang SY, Kim SH, Jang EJ, Kwon NH, Park YK, Koo HC, et al. Novel multiplex PCR for the detection of the *Staphylococcus aureus* superantigen and its application to raw meat isolates in Korea. Int J Food Microbiol. 2007;117:99–105.
22. Cuevas O, Cercenado E, Vindel A, Guinea J, Sánchez-Conde M, Sánchez-Somolinos M, et al. Evolution of the antimicrobial resistance of Staphylococcus spp. in Spain: five nationwide prevalence studies, 1986 to 2002. Antimicrob Agents Chemother. 2004;48:4240–5.
23. Cuevas O, Cercenado E, Goyanes MJ, Vindel A, Trincado P, Boquete T, et al. *Staphylococcus* spp. in Spain: present situation and evolution of antimicrobial resistance (1986–2006). Enferm Infecc Microbiol Clin. 2008;26:269–77.
24. Rodríguez-Baño J, Bischofberger C, Álvarez-Lerma F, Asensio A, Delgado T, García-Arcal D, et al. Surveillance and control of methicillin-resistant *Staphylococcus aureus* in Spanish hospitals. A GEIH-SEIMC and SEMPSPH consensus document. Enferm Infecc Microbiol Clin. 2008;26:285–98.
25. Freixas N, Sopena N, Limón E, Bella F, Matas L, Almirante B, et al. Surveillance of methicillin-resistant *Staphylococcus aureus* (MRSA) in acute care hospitals. Results of the VINCat Program (2008–2010). Enferm Infecc Microbiol Clin. 2012;30:39–42.
26. European Antimicrobial Resistance Surveillance System (EARSS), 2010. Summary of latest data on antibiotic resistance in the European Union 2010. Available online: http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx.
27. Vainio A, Koskela S, Virolainen A, Vuopio J, Salmenlinna S. Adapting *spa* typing for national laboratory-based surveillance of methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis. 2011;30:789–97.

28. Fossum AE, Bukholm G. Increased incidence of methicillin resistant *Staphylococcus aureus* ST80, novel ST125 and SCC*mec* IV in the south-eastern part of Norway during a 12-year period. Clin Microbiol Infect. 2006;12:627–33.
29. Vindel A, Cuevas O, Cercenado E, Marcos C, Bautista V, Castellares C, et al. Methicillin-resistant *Staphylococcus aureus* in Spain: molecular epidemiology and utility of different typing methods. J Clin Microbiol. 2009;47:1620–7.
30. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis. 2003;9:978–84.
31. David MZ, Daum RS. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev. 2010;23:616–87.
32. Otter JA, French GL. Community-associated methicillin-resistant *Staphylococcus aureus* strains as a cause of healthcare-associated infection. J Hosp Infect. 2011;79:189–93.
33. Campanile F, Bongiorno D, Falcone M, Vailati F, Pasticci MB, Perez M, et al. Changing Italian nosocomial-community trends and heteroresistance in *Staphylococcus aureus* from bacteremia and endocarditis. Eur J Clin Microbiol Infect Dis. 2012;31:739–45.
34. Rossney A, O'Connell S. Emerging high-level mupirocin resistance among MRSA isolates in Ireland. Euro Surveill 2008;13:pii:8084.
35. Chen L, Mediavilla JR, Smyth DS, Chavda KD, Ionescu R, Roberts RB, et al. Identification of a novel transposon (Tn*6072*) and a truncated staphylococcal cassette chromosome *mec* element in methicillin-resistant *Staphylococcus aureus* ST239. Antimicrob Agents Chemother. 2010;54:3347–54.
36. Robert J, Tristan A, Cavalié L, Decousser JW, Bes M, Etienne J, et al. Panton-valentine leukocidin-positive and toxic shock syndrome toxin 1-positive methicillin-resistant *Staphylococcus aureus*: a French multicenter prospective study in 2008. Antimicrob Agents Chemother. 2011;55:1734–9.
37. Deurenberg RH, Rijnders MI, Sebastian S, Welling MA, Beisser PS, Stobberingh EE. The *Staphylococcus aureus* lineage-specific markers collagen adhesin and toxic shock syndrome toxin 1 distinguish multilocus sequence typing clonal complexes within *spa* clonal complexes. Diagn Microbiol Infect Dis. 2009;65:116–22.
38. Holtfreter S, Grumann D, Schmudde M, Nguyen HT, Eichler P, Strommenger B, et al. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. J Clin Microbiol. 2007;45:2669–80.
39. Omoe K, Hu DL, Takahashi-Omoe H, Nakane A, Shinagawa K. Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. Infect Immun. 2003;71:6088–94.
40. Monecke S, Coombs G, Shore AC, Coleman DC, Akpaka P, Borg M, et al. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS ONE. 2011;6:e17936.
41. Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, et al. Genome and virulence determinants of high virulence community-acquired MRSA. Lancet. 2002;359:1819–27.
42. Chiang YC, Liao WW, Fan CM, Pai WY, Chiou CS, Tsen HY. PCR detection of staphylococcal enterotoxins (SEs) N, O, P, Q, R, U, and survey of SE types in *Staphylococcus aureus* isolates from food-poisoning cases in Taiwan. Int J Food Microbiol. 2008;121:66–73.